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REVIEW / ARTÍCULO DE REVISIÓN

Control of vibriosis in shrimp through the management of the microbiota and the immune system

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Abstract: Shrimp aquaculture is constantly threatened by recurrent outbreaks of diseases caused by pathogenic bacteria of the genus *Vibrio*. Acute hepatopancreatic necrosis disease (AHPND) is one of the most aggressive vibriosis reported to date in the shrimp industry. AHPND provokes massive mortalities, causing economic losses with strong social impacts. Control of vibriosis requires the application of multifactorial strategies. This includes vibrio exclusion, shrimp microbiota, particularly in the digestive tract, and shrimp health management through immune stimulation. This paper reviews these two strategies for the prophylactic control of vibriosis. First, we describe the devastating effects of AHPND and the cellular and humoral effectors of the shrimp immune system to cope with this pathology. Secondly, the mechanisms of action of probiotics and their positive impacts are highlighted, including their immunostimulant effects and their role in the balance of the shrimp microbiota. Finally, we reviewed immunostimulants and prebiotics polysaccharides that together with probiotics act benefiting growth, feed efficiency and the microbiota of the digestive tract of farmed shrimp.

Key words: Penaeus vannamei, Vibrios, probiotics, immunostimulant, prebiotics.

Introduction

Penaeus vannamei shrimp aquaculture is one of the fastest-growing aquaculture sectors worldwide[1]. In Latin America, the productions of Ecuador, Mexico, Honduras and Brazil stand out[2]. However, outbreaks of infectious diseases caused by pathogenic agents such as viruses and bacteria[3–5] limit the industrial development of shrimp farming. Among the most aggressive viral diseases that have attacked farmed shrimp are white spot syndrome (WSSV)[6–8] and Taura syndrome (TSV)[9,10]. Unlike viruses, bacteria, can remain indefinitely in culture systems because of their ability to produce biofilms5. Vibrios spp are the most aggressive bacteria for farmed shrimp, causing recurrent and emerging diseases[11–13].

Strains of Vibrio parahaemolyticus and other vibrio species carrying the toxin, PirA and PirB are highly virulent for penaeid shrimp[14–16] because they can cause acute hepatopancreatic necrosis disease (AHPND)[17–19]. AHPND causes significant losses in the shrimp industry globally and socioeconomic impacts20 that are estimated at more than $1 billion per year[21]. *V. parahaemolyticus* is a marine microorganism, which can grow in estuarine waters with tolerance to high temperature, high pH and high salinities with an affinity for marine plankton[22,23]. The success of vibrios is based on their ability to grow in shrimp culture systems characterized by high temperatures, high salinity[24] abundant presence of exuviae and high organic matter load. Under these conditions, vibrios thrive and modulate the microbiome at their advantage[25].

Among the aspects that should be considered in closed systems designed to prevent the entry of vibrios and other potential pathogens are adequate nutrition with functional additives to improve the immune response[26,27] and biosecurity controls[28]. In semi-extensive cultures, which involve earthen ponds in estuarine environments biosecurity is not sufficient and other alternatives must be considered to protect shrimp against vibriosis[28]. Among these alternatives, there are anti-virulence therapy[29,30], probiotics[31,32] and immunomodulation[33,34].

The main objective of probiotics is to exclude pathogenic bacteria from culture systems. In addition, probiotics enhance host growth and health by improving stress resistance[35,36] and promoting better nutrient utilization in shrimp by improving feed digestibility[37,38]. Despite their effectiveness, the application of living organisms faces several challenges due to the preparation, bioactivity, stability, and success in different aquaculture environments. Another promising strategy is the enhancing of immune defenses. Immuno-stimulation seeks to activate the shrimp immune system based on the use of pathogen-associated molecular patterns (PAMPs) or molecules that resemble them and the recognition by their receptors in the host[39,40], thus increasing immune resistance. Different polysaccharides fall in this category, among these, β-glucans and peptidoglycans[41]. The synergistic use of probiotics and immunostimulants[26,42] or the use of polysaccharides as prebiotics that help the establishment and aptitude of probiotics[27,43] are also explored to face vibriosis. In this review, we present the devastating effects of AHPND and the cellular and humoral effectors of the shrimp

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immune system to cope with this pathology. We also report the strategies to control vibriosis in farmed shrimp, based on probiotics and immunostimulants.

Vibriosis in shrimp farming focusing on AHPND

Diseases produced by species of the genus Vibrio have caused significant economic losses in the shrimp industry worldwide[20,44]. Belonging to the phylum Proteobacteria, class Gammaproteobacteria, the genus Vibrio of the family Vibrionaceae includes 65 well-documented species of heterotrophic bacteria[45]. Vibrio harveyi and V. parahaemolyticus belonging to the clade Vibrio harveyi are among the most critical pathogens for shrimp46. Vibriosis cause massive mortality rates and can affect 100% of production[8,12,47]. Vibrios cause tail necrosis, loose shell syndrome, white gut, septic hepatopancreatis, systemic and wound vibriosis, causing hypoxia and death of shrimp from hatchery tanks to growing ponds[12].

AHPND, the most aggressive vibriosis recorded to date, was detected in China in 2009 and other Southeast Asian countries[48]. In 2013, this disease was reported in Mexico 18 and spread to South America[49,50]. PirA and PirB toxin genes were detected in other vibrio species of the *V. harveyi clade*[51], *Vibrio owensi*i[52], *Vibrio Campbelli*[53] and recently in a *Vibrio* not belonging to this clade, *Vibrio punensis*[50]. The observed damage to shrimp health by AHPND has been devastating. *Vibrios* initiate their attack by colonizing the stomach14 of infected shrimp, originating dysbiosis[25,54] and generating toxins that subsequently cause hepatopancreatic cell damage[13] and eventually hepatopancreatic septicemia[5,17]. Shrimp affected by AHPND show severe necrosis in the hepatopancreas with massive detachment of epithelial cells and total loss of tissue structure. Pale atrophied hepatopancreas, intestinal epithelial necrosis and massive infiltration of hemocytes are observed[55]. It has also been reported that *V. parahaemolyticus* strains carrying PirA and PirB toxin genes alter the microbiome of infected shrimp, decreasing bacterial diversity and increasing the fitness of vibrios to proliferate in culture environments[25,56].

Shrimp immune system

Shrimp, like all crustaceans, possess an immune system that effectively protects them from harmful microorganisms[57]. In penaeid shrimp, two lines of immune defense can be distinguished. The first line is a physical barrier formed by the cuticle which is mainly composed of polysaccharides, chitin, and mineral deposits[58]. Internally, contact with the medium is very limited due to the presence of cuticle in the stomach[59], the constantly growing peritrophic membrane lining the epithelium of the midgut and hindgut[60] and the gastric sieve that limits passage into the hepatopancreas to particles smaller than 1 µm. The second line of defense is its innate immune system. It is based on humoral and cellular effectors, being hemocytes the main effectors of immunity. These effectors act together to eliminate infectious microorganisms[61].

Innate immunity with specificity or immune priming has also been described in shrimp and other arthropods. This is based on the Down syndrome cell adhesion molecule (Dscam), a hypervariable recognition protein produced by alternative splicing. Dscam may be involved in both innate immunity and pathogen-specific immune response[62]. Shrimp cells innately recognize invading microbes through pattern-recognition proteins (PRRs) that act as pathogen-associated molecular pattern receptors (PAMPs)[63]. Innate immune cells respond rapidly to this activation through intracellular signaling cascades leading to humoral and cellular immunity activation[64].

Humoral mechanisms

Humoral effectors involve the synthesis and secretion of immune proteins from hemocytes into the circulatory system or hemocele[65] and the release of enzymes of the prophenoloxidase (proPO) cascade leading to the generation of oxidative metabolites and deposition of toxic melanin[66]. Pathogens are recognized by pattern recognition proteins that recognize lipopolysaccharide (LPS) and β-1,3-glucan (BGBP)[67–69], initiating the chain of proPO activation, culminating in melanization of the wound or intruding organisms[70]. Another mechanism of humoral response is the clotting system. The clotting cascade involves both hemocytes, which release transglutaminase[71], and the coagulation factor present in the plasma[72]. Released transglutaminase polymerize the plasmatic clotting factor[72].

Upon microbial challenge, hemocytes release antimicrobial peptides (AMPs)[73]. AMPs are cationic or anionic proteins that attack invading microorganisms[65,74]. AMPs are small, from 15 to 100 amino acids and amphipathic in structure[75]. They can rapidly diffuse to the point of infection and destabilize the membrane of microorganisms, acting as antibiotics against gram-negative and gram-positive bacteria[76] and against yeasts and filamentous fungi[77]. It has also been reported that AMPs modulate immunity[74,78]. In Crustacean the main AMPs are represented by the three families of cationic peptides: penaeidins, crustins and antilipopolysaccharide factor (ALF)[79,80]. Shrimp AMPs can be classified as cationic and anionic, the former being more abundant. Only stylicin, a peptide derived from hemocyanin and some ALFs are anionic AMPs65. Each family of shrimp AMPs shows sequence diversity and constitute multiple classes, isoforms, or subgroups[75].

Penaeidins have affinity for shrimp chitin, being detected in the cuticle after microbial challenge[73,81]. Penaeidin mRNAs are present in all larval stages from nauplius onwards but vary in expression levels[82]. In penaeid shrimp, crustin homologs have been primarily identified by expressed sequence tag (EST) analysis of cDNA libraries from hemocytes of *P. vannamei*, *P. setiferus*[83], and *P. monodon*[84,85]. Crustin is the largest AMP family reported in crustaceans[86]. In shrimp, most crustins are constitutively expressed in hemocytes but not in the hepatopancreas[80-88]. Some *P. monodon* crustin isoforms are expressed primarily in tissues other than hemocytes. ALFs are 10 to 14 kDa molecules. In shrimp, they were first identified in hemocytes of *P. setiferus*[87] and *P. monodon*[84,89]. ALFs exhibit a potent minimum inhibitory concentration (MIC) (<6.25 µM) and spectrum of antimicrobial activity, covering a large number of gram-positive and gram-negative bacteria, filamentous fungi and even enveloped viruses[90,91].

Cellular mechanisms

Cellular response includes all those reactions directly mediated by hemocytes in processes such as phagocytosis[92], and nodule formation[93]. The classification and identification of hemocytes is based on morphological, antigenic, and functional aspects[93,94]. The three types of hemocytes are: (1) hyaline hemocytes which do not possess granules, which adhere and spread quickly, have phagocytic capacity and are in charge of clotting[95]; (2) small granular hemocytes possess abundant small granule, (Figure 1A) are involved

in phagocytosis, encapsulation and melanization and synthesize and release AMPs; (3) large granular hemocytes (Figure 1A), which are loaded with large granules that store recognition proteins, adhesion proteins, regulatory enzymes, AMPs[81] and proPO system[96].

A lower-than-normal number of circulating hemocytes in crustaceans correlates with reduced resistance to pathogens[97], while an increase in the number of hemocytes enhances the immune response during periods of stress, increasing resistance to diseases[98,99]. In addition, wound repair processes in shrimp are influenced by the concentration of hemocytes and penaeidins released by them in the wound area to eliminate invasive pathogens and improve hemocyte adhesion[100]. The open circulatory system of shrimp[101] allows hemocytes to infiltrate and adhere to many tissues through the interstitial spaces of different organs and tissues without being limited to the vascular system[102].

Hemocytic nodules, detected in connective tissue (Figure 1B), gills and hepatopancreas, are formed by numerous hemocytes that act synergistically to trap microorganisms or large antigens that cannot be eliminated by phagocytosis. These nodules undergo subsequent activation of the proPO system, with consequent melanization and destruction of microbes[103], such as the hemocytic nodules observed in tubules of the hepatopancreas of shrimp infected by vibrios[18,104]. If the lesions are very large, they will form hemocytic capsules like those observed around hepatopancreatic tubules injured by vibrios[105]. In shrimp, typical inflammatory response reactions with hemocyte infiltration and melanin deposition in nodules and capsules are observed during bacterial infections[106] (Figure 2A). The respiratory burst, the mechanism that leads to the generation of reactive oxygen intermediates, is another microbicidal process used by shrimp hemocytes in phagocytosis and encapsulation[107].

Other immune tissues

In penaeids shrimp, some of the defense functions are performed in the lymphoid organ (LO), which actively participates in capturing, isolating, and eliminating pathogens by phagocytosis[108,109]. The LO is a bilobed structure located in the anteroventral region of the hepatopancreas and posterior to the antennal gland, being an integral part of the distal portion of the subgastric artery[110,111]. The LO is considered an integral part of the circulatory system of penaeid shrimp and is believed to work as a hemolymph filter[110,112]. In histological sections it is seen as a vascularized tissue surrounded by hemal sinuses. The LO tubules have an inner layer of endothelial cells, surrounded by stromal tissue, bound by connective tissue[109,110,113]. In the presence of natural or experimental infections (mainly of viral etiology), nodular hyperplasia known as lymphoid organ spheroids (LOS) form (Figure 2B). These structures have also been detected in the connective tissues of other organs of shrimp and are called ectopic spheroids[114,115]. It has been reported that the LO filters viral particles of TSV[114] and WSSV[116]. In late stages of infection, viral particles are detected in the spheroids of the lymphoid organ, whose cells enter apoptosis[114,116]. In addition to LOS, apoptosis has been reported in viral infections as a control mechanism of viral replication[114,116]. Other tissues involved in hemolymph clearance mechanisms are the heart reserve phagocytes, podocytes of the gills, and antennal gland[117]. The antennal gland is an important excretory organ in shrimp that is functionally similar to the kidney in vertebrates[118,119], and therefore exposed to external medium.

Prophylactic methods to control vibriosis in shrimp culture

Vibriosis is the leading bacterial disease-causing mortality of cultured shrimp[120,31]. Antibiotics and chemotherapeutic agents were the first tools to control vibriosis. However, antibiotics have led to the development of resistant microorganisms[121] with risk to human health due to the horizontal transfer of resistance genes from shrimp vibrios to human pathogenic bacteria[122,123]. The use of antibiotics also carries risks to the environment, due to the accumulation of environmental residues[124]. In addition, excessive levels of antibiotics can deteriorate the general condition of shrimp[125] and cause rejection of the final product in international markets. Therefore, implementing effective and environmentally

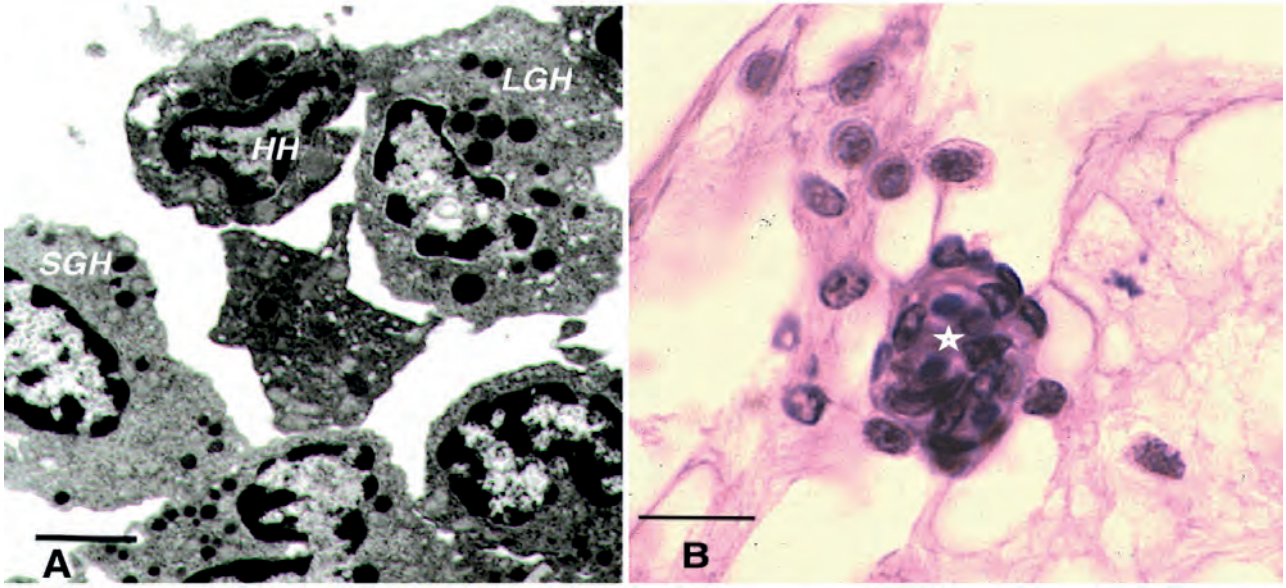


Figure 1. Shrimp hemocytes. (A) Transmission electron micrograph of shrimp hemocytes, bar = 2 mm. (B) H&E staining of hemocytic nodule (star) in connective tissue of shrimp, bar 12 mm. HH: Hyaline hemocytes, SGH: Small granular hemocytes, LGH: Large granular hemocytes.

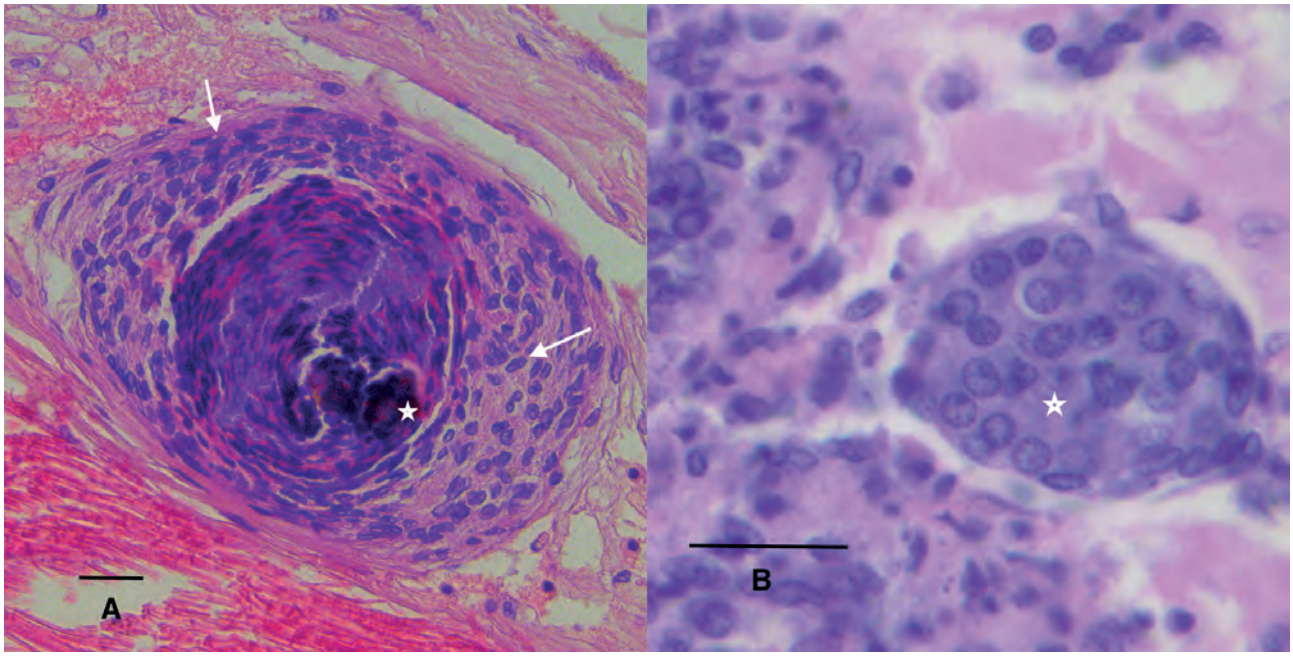


Figure 2. Histopathological signs related to bacterial and viral infections in shrimp. (A) Hemocytic nodule encapsulated in connective tissue. Note melanin deposition (star) and massive hemocyte infiltration (arrows), bar = 20 mm. Source Marisol Morales-Covarrubias. (B) Spheroid (star) in the lymphoid organ of shrimp infected with WSSV, bar = 20 mm.

friendly multifactorial strategies for the control of vibrios in shrimp production systems is to be considered a priority[126]. These include the anti-virulence strategy[127,128], which is based on blocking bacterial communication known as quorum sensing (QS), to inhibit the expression of virulence genes. The QS mechanism uses small chemical signaling molecules called autoinducers (AIs)[129,130]. This strategy has been applied in aquaculture and proposed by several authors as a promising tool for the control of pathogenic bacteria[128]. Other alternatives to control vibriosis are the management of the microbiota, through probiotics[121,131,132] and immunostimulation[31,133](Figure 3).

Probiotic

The term probiotic was defined by Fuller (1989)[134] as "a live microbial food supplement that improves bacterial balance, benefiting the host's health"[135]. Probiotics emerge as a promising alternative to decrease the aquaculture industry's dependence on antibiotics[136]. Probiotics have been applied in fish and invertebrate cultures[137], especially in South America, Ecuador, and Asia, particularly China and India. Probiotics for aquaculture belong mainly to bacteria of the genera Bacillus, Lactobacillus. Pseudomonas, and vibrio, in addition to yeasts[31,138,139].

In the field of aquaculture, the concept of probiotic is extended to “a live microbial supplement that has a beneficial effect on the host by improving the environmental microbial community, ensuring better use of the feed or its nutritional value, and improving the response to disease or the quality of culture environment”[121]. In addition probiotics can be applied directly to the culture water as bio remediators[140,141], the modes of action of probiotics are diverse[142] (Table 1). Applied in feed, probiotics improve digestion, enhance immune response, and inhibit the growth of pathogenic bacteria[143–145]. Probiotics competitively exclude vibrios[26,146,147] and enhance shrimp immune indicators, phagocytosis, nodule formation, activation of the proPO system and generation of AMPs[148]. Probiotics release several antibacterial agents with bactericidal or bacteriostatic properties that inhibit the colonization of pathogenic organisms in the shrimp gut[149–151]. Probiotics can modulate the expression of immune genes and gut microbiota during stress induced by high stocking density[152].

Bacteria of the Bacillus genus are the most widely used in shrimp aquaculture as probiotics because they form spores, which facilitates conservation and commercial distribution. In addition, Bacillus bacteria are well perceived by the consumer because they are gram-positive bacteria. Among other advantages of Bacillus spp are their ability to grow using different carbon and nitrogen sources[153]. Bacillus spp can grow in the marine environment and produce a variety of bioactive compounds that act on growth, health, disease resistance[154,155] and culture water quality[156,157]. It has been further reported that *Bacillus spp.* produce a wide range of antimicrobial peptides158,159. *Bacillus spp.* supplementation decreases mortality in shrimp exposed to pathogenic *Vibrios*[160,161]. The most used species are Bacillus subtilis and Bacillus lechiniformis. Other probiotics successfully used in aquaculture belong to other bacterial genera, including *Vibrios*[162] or marine symbionts such as Pseudovibrio denitrificans[163]. Vibrio diabolicus is a very effective probiotic controlling vibriosis affecting *P. Vannamei* in larval stages[164] and juveniles[56]. Because it is a vibrio, this bacterium has the advantage of thriving in the culture conditions of shrimp. *V. diabolicus* can compete effectively with pathogenic vibrios[56], thus having positive effects on the immune system of shrimp[133,164].

Considerations for the selection and use of probiotics in shrimp aquaculture

Several aspects must be considered for selecting and using probiotics in shrimp. Among these are the ability to grow in marine environments, exclude vibrios, and compete with them for colonization of shrimp and surfaces of culture systems to which vibrios are well adapted. Bacterial antagonism is a common phenomenon in nature; therefore, microbial interactions play an important role in balancing

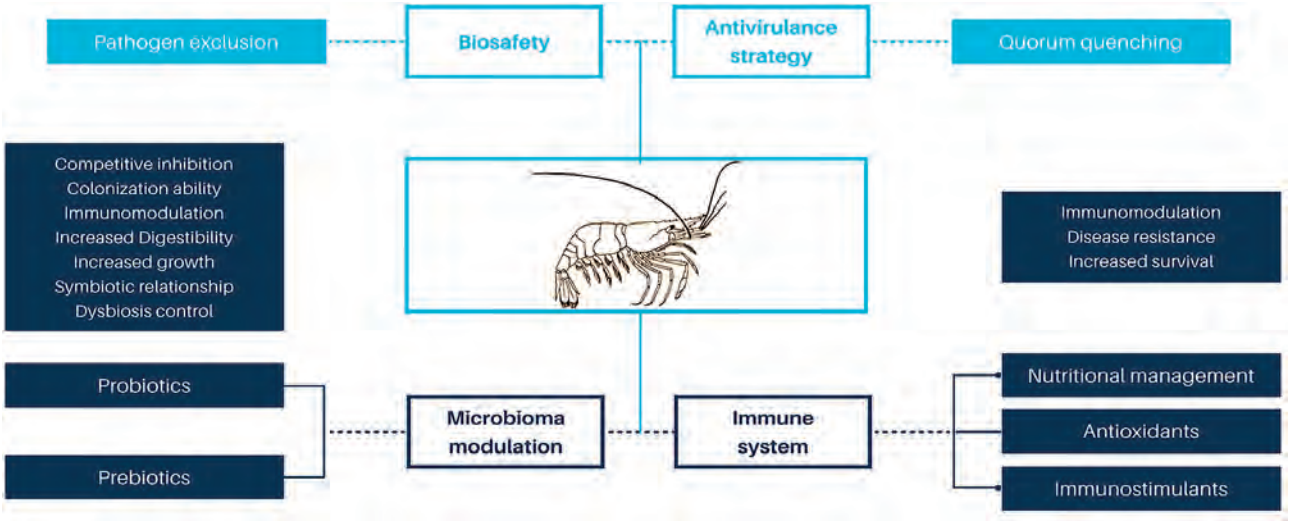


Figure 3. Approaches to control vibriosis in shrimp farming.

beneficial competitors and potentially pathogenic microorganisms[31]. In this sense the ability to compete and colonize is one of the most desirable properties of probiotics. There are several aspects behind the ability to compete and colonize one of them is competitive exclusion, which occurs when an established microflora prevents or reduces the colonization of a bacterial competitor for the same location[140]. For pathogenic bacteria the ability to adhere to host substrates is related to virulence and is considered as the first step for an infection[178].

Probiotics must have the ability to compete for mucosal attachment sites and competition for nutrients[140]. Therefore, the attachment ability of probiotics is another important criterion for their selection and application in aquaculture[179,180]. Colonization ability further depends on migration in a coordinated manner across organism surfaces[181]. For these processes, bacteria use cell appendages such as fimbriae and flagella, structures important for colonization and the creation of biofilms[182–184]. By producing inhibitory substances, probiotics prevent pathogen replication and reduce pathogen colonization[132,140]. Among the extracellular products that inhibit or kill other potentially pathogenic bacteria have been found: antimicrobial substances[185,186], organic acids[187], bacteriocins[188], or hydrogen peroxide. Another important aspect is the morphophysiology of shrimp. Indeed, the shrimp has an external and internal cuticle covering the stomach, which is lost during molting, and the peritrophic membrane in continuous growth, covering the midgut and hindgut[60]. Therefore, probiotics with affinity for the cuticle and chitin, should be applied continuously.

New approaches have emerged to identify and isolate novel probiotics in recent years. One of them is the use of marine symbionts, which are perfectly adapted to marine environments. *P. denitrificans* is a symbiont that generates antimicrobial substances. It is metabolically versatile, exhibits competitive inhibition against several shrimp pathogenic Vibrio spp and increases survival against vibrio challenges[163]. Another aspect that is gaining relevance is the ability of probiotics to recover or maintain healthy microbiota, curbing dysbiosis. The probiotic *V. diabolicus* can control the bacterial populations of the gastrointestinal tract of shrimp, restoring the microbial balance[56].

Immuno-stimulation and Immunostimulants

The immune system can recognize and react to PAMPs through different PRRs[189]. Different PAMPs, such as LPS, peptidoglycans and β-glucans can activate the shrimp immune system, acting as immunostimulants. Immunostimulants are an effective alternative to prevent diseases in shrimp. There are several sources of these molecules in nature, including gram-negative bacteria (lipopolysaccharides), gram-positive bacteria (peptidoglycans), fungi, yeasts, and algae (β-glucans). The most used immunostimulants are β-glucans from different marine and terrestrial sources[190]. The β-glucans are polysaccharides other than starch; they are present in the cell wall of bacteria, fungi, and plants. They are used to increase the resistance of shrimp against bacterial and viral infections[26,191]. The β-glucans extracted from different sources exhibit various immunomodulatory functions, acting as PAMPs, they interact with PRRs to activate the innate immune response[192]. In shrimp β-glucans extracted from yeast Candida parapsilosis has an immunomodulatory effect against WSSV, obtaining high survivals[193]. In addition to their effects on the immune system, β-glucans from bacteria improve the nutritional and antioxidative status of shrimp[194]. β-glucans from marine yeasts improve respiratory burst and phenoloxidase activity[195]. The beneficial effect of β-glucans can be enhanced by degradation to obtain oligo β-glucans with improvements in their immunostimulatory and growth, promoting effect in shrimp[191]. The benefits of using β-glucans have been evidenced by successful results in survival and performance in shrimp ponds[26]. Immunomodulatory effects on penaeid shrimp have been also reported for other polysaccharides that exhibit repeating molecular patterns, such as arabinose and galactose heteropolysaccharides from Angelica sinensis, and cocoa pectin[196].

As previously described, innate immunity with specificity or immune priming has been reported in shrimp and other arthropods[197]. From the perspective of a possible pathogen-specific immune response, vaccination trials have been performed in shrimp. Thus in *P. monodon* the VP28 protein (WSSV) has been used as antigen, observing immunomodulatory effects on different immune effectors:caspase, penaeidin, crustin, astakine, syntenin, PmRACK, Rab7, STAT and C-type lectin[198].

Prebiotics

Prebiotics are indigestible fibers for the host, but are digested by beneficial bacteria, which improve the health

Identity of the probiotic	Applied	Method of application	Beneficial effects	Reference
***Bacillus* spp.**	*Penaeus monodon*	Feed additive	Increased survivability in challenge against *Vibrio harvery*.	Rengpipat *et al*., 1998 [165]
***Vibrio hepatarius*, Vibrio P63, Bacillus P64 (*Bacillus cereus*), *Vibrio alginolyticus* (*Vibrio diabolicus*)**	*Penaeus vannamei*	Addition to culture water	Highest average weights, immunostimulatory effects.	(Gullian *et al*., 2004) [164]
Bacillus subtilis	*Penaeus vannamei*	Feed additive	Antimicrobial activity.	Balcázar & Rojas-Luna 2007 [166]
Lactobacillum plantarum	*Penaeus vannamei*	Feed additive	Enhanced immunity, Increased resistance to vibrios.	Chiu *et al*., 2007 [167]
V.alginoliticus, Bacillus subtilis,, Roseobacter gallaeciensis, Pseudomona aestumarina	*Penaeus vannamei*	Feed additive	Reduction of vibriosis disease.	Balcázar *et al*., 2007 [168]
Bacillus subtilis	*Penaeus vannamei*	Feed additive	Improves survival in challenge against vibrios.	Tseng et al., 2009 [169]
Lactobacillus acidophillus, Rhodopseudomonas palustri, Bacillus coagulans	*Penaeus vannamei*	Addition to culture water	Improved growth, enhanced immune response.	Wang &Gu 2010 [170]
Bacillus subtilis	*Penaeus vannamei*	Feed additive	Improved growth, increased activity of digestive enzymes, and decreased mortality after challenge with *V. harvery*.	Zokaeifar *et al*., 2012 [122]
Bacillus cereus	*Penaeus monodon*	Feed additive	Increase in immunological parameters and growth.	Navin Chandran et al., 2014 [171]
Bacillus subtilis	*Penaeus vannamei*	Feed additive	High survival rate, enhanced immune response.	Liu *et al*., 2014 [172]
Shewanella haliotis*, *Bacillus cereus* and *Aeromonas bivalvium	*Penaeus vannamei*	Feed additive	Increased weight, improved immune parameters and survival in challenge with *Vibrio harveyi*	Hao *et al*., 2014 [37]
Bacillus licheniformis, Bacillus subtilis, B. subtilis* subsp. *Subtilis	*Penaeus vannamei*	Feed additive	Enhanced immune-related gene expression in WSSV and IHHNV infected shrimp	Sanchez-Ortiz *et al*., 2016 [173]
Pseudoalteromonas sp	*Penaeus vannamei*	Feed additive	Reduced mortality after challenge with AHPND infection	Wang et al., 2018 [174]
Bacillus subtilis	*Penaeus vannamei*	Feed additive	Improved growth, enhanced immune response, increased resistance to AHPND.	Kewcharoen, W., & Srisapoome, P. 2019 [153]
Pseudovibrio denitrificans	*Penaeus vannamei (larvae and juveniles)*	Feed additive	Increase survival in challenge with *Vibrio campbellii* and *Vibrio parahaemolyticus*	Dominguez-Borbor *et al*., 2019 [163]
Bacillus subtilis, Bacillus. licheniformis	*Penaeus monodon*	Feed additive	Increased production of digestive enzymes	Wang *et al*., 2020 [176]
***Bacillus* sp**	*Penaeus indicus*	Feed additive	Enhanced growth	Kim *et al*., 2020 [177]
Bacillus probiotics consortium.	*Penaeus vannamei*	Addition to culture water	Increase in immunological parameters Improved growth.	Fernandes *et al*., 2021 [133]

Table 1. Several probiotic bacteria applied in shrimp cultures.

of the intestine[199,200] and can be referred to as functional saccharides[201]. Moreover, prebiotics are associated with improvements in growth, feed efficiency, digestive enzyme activities, proper gut morphology, improved immune status and disease resistance[136,202,203]. Prebiotics such as fructooligosaccharides (FOS), galactooligosaccharide, mannan oligosaccharides, and inuline are applied in shrimp culture[204]. The application of short-chain fructooligosaccharides (ScFOs) in P. vannamei diet increased the weight and specific growth rate of shrimp205. ScFOS supplemented at concentrations of 0.025 to 0.800 wt% increase the respiratory burst hemocytes206. More details about prebiotics in farmed shrimp are shown in table 2.

Conclusions

The survival rate, yield, size, color, and taste of shrimps are critical for its market. With the increased loss of worldwide production due to vibriosis and the risks inherent in the use of antibiotics, new products to control vibriosis must consider the need for “organic” shrimps without antibiotics. Among the most promising alternatives are probiotics. The modes of action of probiotics are diverse. They improve digestion, enhance immune response, and inhibit the growth of pathogenic *Vibrio spp*. Probiotics release several antibacterial agents with bactericidal or bacteriostatic properties that inhibit the colonization of pathogenic Vibrio spp in the

Prebiotic	Used on	Method of application	Result	References
Short- chain Fructooligosaccharides (ScFOS)	*Penaeus vannamei*	Feed additive	Increased hemocyte countd and hemocyte respiratory burst and microbial community modification	Li *et al*., 2007[206]
ScFOS	*Penaeus vannamei*	Feed additive	Improved weight and increased growth rate.	Zhou *et al*., 2007[205]
Mannan oligosaccharides (MOS)	*Penaeus vannamei (larvae)*	*Artemia* sp. additive	High survival in challenge with *Vibrio. harveryi*, enhanced immune response, high growth, improved digestive enzyme activities.	Widanarni *et al*., 2019[207]
Astragalus polysaccharide (APS), chlorogenic acid (CGA), and berberine (BBR)	*Penaeus vannamei)*	Feed additive	Enhanced immune response.	Ding *et al*., 2020[208]
Inulin	*Penaeus vannamei)*	Feed additive	Increased final weight, improved oxidative status, improved digestive enzyme activity.	Zhou *et al*., 2020[204]
***Caulerpa racemosa* polisacáridos (galactose, glucose, mannose)**	*Penaeus vannamei)*	Feed additive	Enhanced immune response.	Lee *et al*., 2020[209]

Table 2. Several prebiotic polysaccharides applied in shrimp cultures.

shrimp. During the last years, new approaches have emerged to identify and isolate novel probiotics. One of them is the use of marine symbionts, but even if first results are highly encouraging, a deeper understanding of the biological mechanisms is still needed. Another aspect that is gaining relevance is the ability of probiotics to recover or maintain healthy microbiota, curbing dysbiosis, and restoring microbial balance. The application of living organisms faces several challenges, due to their differences in bio activity, and effectiveness in marine aquaculture environments. Another promising option is the use of polysaccharides that increase shrimp resistance to pathogenic Vibrio spp. by immune stimulation. Polysaccharides can also act as prebiotics, being used as energy sources by beneficial bacteria, which improve the health of the host intestine. The significant advances in combating the AHPND in the shrimp industry have evidenced several strategies to solve this critical challenge, but it is not yet clear which is the best of the previously mentioned in this review. It is likely that while each has its advantages and disadvantages, a possible solution could be a combination of them.

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ARTICLE / INVESTIGACIÓN

Cost Analysis of Cacao (*Theobroma cacao L.*) Plant Propagation through the Somatic Embryogenesis Method

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Abstract: In vitro cacao (*Theobroma cacao L.*) production via somatic embryogenesis (SE) is being implemented to mass propagate clonal plant material with the donor material's prominent characteristics. Though it is an advanced technology, it is con-sidered expensive compared to other propagation techniques. This work focused on identifying the critical financial feasi-bility factors for the SE productive process. The process's costs were estimated, identifying factors influencing each la-boratory's standardized ES process. A Monte Carlo Simulation (MCS) was performed to evaluate different variables upon increasing productive scale in a biofactory (commercial-scale production). The projected lot volume was 100,000 plantlets in solid media, considering the process flow from in vitro introduction to acclimation. A biofactory operational model was projected, establishing time and operator movements and identifying direct and indirect costs.
Costs were defined by the standardized or integral method, with estimated and budgeted calculations to set the cost per plantlet. The identified cost components were culture medium (CM), indirect manufacturing costs (IMC), labor (direct and indirect) and operating expenses. Labor had the most significant share of the costs, at 53%, followed by operating expenses, at 30%, CM, at 12%, and IMC, at 5%. The MCS helped define that the variables with the highest impact on unit price were the embryos' response in the germination-acclimation stage and the proliferation coefficient during the maturation stage. This projection yielded a figure of US $0.73 per plantlet. However, strategies to reduce this cost have been proposed. These strategies are mainly conducive to optimizing labor and implementing practices that increase multiplication.

Key words: Plant Tissue culture, Cost analysis, Large-scale production, Biofactory, Monte Carlo Simulation (MCS).

Introduction

Cacao (*Theobroma cacao L.*) production as a raw material for the chocolate industry has grown in many countries worldwide. A concentration can be observed in tropical countries, led by African countries, such as Ivory Coast, Ghana, Cameroon and Nigeria, with 63.2% global production[1]. In Indonesia and Papua New Guinea, the continent of Asia holds 17.4% of all production, and Latin America, in the countries of Brazil, Ecuador, Peru, the Dominican Republic, and Co-lombia, contributes 19.4% of the global output[2,3]. Colombia is the ninth-largest producer worldwide, with a minimum participation of 1.3% despite having significant production potential[4]. Colombia's geographic characteristics, such as its topography, climate, hydrography, and relative humidity, are ideal for developing this crop. These conditions can be found in a significant percentage of the territory[5]. Production is currently performed in 29 of the country's 32 departments, in which Santander, Antioquia, Arauca, Huila, and Tolima stand out, representing 70% of national production[6].

Developing mass propagation systems that facilitate plants' high availability to establish new crops and renew existing old crops is required to increase national production competitively. Asexual or vegetative propagation plays an essential role in reliably reproducing desirable characteristics, and, in the case of vegetative propagation, it is traditionally performed through grafting and root cuttings. However, both techniques have low production levels, the reason why extensive clonal gardens are required to produce enough material, limiting automation and scaling. Moreover, due to genetic improvement programs performed in several research centers worldwide for cacao, there are a considerable amount of improved genotypes[7–10]. Nevertheless, one of the largest limiting factors to harnessing this germplasm is the lack of mass cloning methods for the selected financially and agriculturally efficient plants.

In this sense, developing in vitro plant tissue culture techniques allows obtaining plants from one same tissue by taking advantage of plant cells' totipotence[11]. The main advantage of in vitro cultures as a propagation method is the uniform production of plants that conserve the original plant's characteristics. Besides, the processes are amenable to being scaled up to an industrial level[12]. In addition, it allows mass propagating elite plant material in any season, independent of natural environmental conditions, conserving the plants' genetic potential[13]. There is a possibility of obtaining pathogen-free plants (fungi, bacteria, viruses)[14]. This technology facilitates optimizing the use of controlled environmental and nutritional factors, optimizing culture areas with large numbers of plants per unit of area, and

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conserving genotypes selected for prolonged periods. Furthermore, genetically modified plants can be obtained using in vitro regeneration technology, shortening plant breeding program times.

There are various in vitro asexual propagation methods. Among them is multiplication via SE, which, by Mendéz *et al.* 2019[15], is defined as a process in which a bipolar structure with radial and apical axes similar to a zygotic embryo is developed from a somatic cell without a vascular connection to the original tissue and is capable of growing and creating typical plants. Studies on SE in cacao have been performed for over 40 years[16]. Esan 1977[17] was the first to report the formation of somatic embryos, and, since then, numerous studies have been performed to optimize the number of embryos formed by explant[18–21]. Subsequently, the work performed by Maximova *et al.* 2002[22] and Fontanel *et al.* 2002[23] stands out, in which secondary embryogenesis was proven to allow obtaining a more significant number of regenerants synchronously. In the last five years, the work performed by Garcia *et al.* 2018[24] has stood out, which reports efficiently obtaining plants via direct SE and in liquid and solid media.

On the other hand, it has been demonstrated that cacao plants resulting from this propagation system have an agronomic behavior without significant differences concerning plants propagated by conventional methods, such as grafting[25–27]. Companies, such as Nestlé, have currently standardized the process to establish their crops in countries such as Ecuador, Indonesia, Puerto Rico, Brazil, Ghana, and Ivory Coast[28]. There are no reports of large-scale cacao plant material production in Colombia by biotechnological methods, such as SE. SE propagation results have been obtained in solid media for universal genotypes CCN51, TSH565, EET8, ICS1, ICS39, ICS60, ICS95 and IMC67[29] and regional genotypes CNCh12, CNCh13, CNCh16, CNCh24 and CNCh4[30,31].

The financial analysis's important to ensure the production process's feasibility via SE has been mentioned in various studies. It is currently recognized that one of its most challenging aspects is reducing the process's cost[32–34]. There has been increased interest in the problems related to large-scale plant production in the last two decades[35–38] and in reducing commercial production costs. However, the accurate details of calculating costs of producing plantlets by SE are seldom discussed or reported[12,39–41]. A series of cost reduction strategies have been developed to overcome this limitation in the entire production process, but the cost would largely depend on the effectiveness and efficiency of the SE, which depends on the cacao genotype of interest. It usually has been argued that high propagated material costs, specifically by SE, can be justified by increasing the crop's productivity and uniformity[42]. Even though this is plausible, it will not always be achieved until they can prove it at the corresponding production scale and know how much of a return on investment can be obtained and, even more importantly, when it will be obtained[38].

The time factor and frequency of productive lots are essential considerations for a ligneous species like cacao since it requires a long time to finish the entire cycle. In addition, the species is recalcitrant to in vitro cultures and has different responses depending on the genotype[43]. Various researchers have estimated the cost of producing plants propagated via SE for lig-neous species and have observed that approximately over 50% of the total cost per plantlet corresponds to labor, clarifying that they are studies performed in developed economies, such as the United States[44–46]. Labor is highly competitive in countries like Colombia, circling the US $229 as of October 2020[47], representing an opportunity to develop these technologies.

Nowadays, risk management is an integral part of evaluating any project. Therefore, techniques, such as the Monte Carlo Simulation (MCS), a computerized mathematical approach that allows taking risk into account in quantitative analyses and decision-making, become relevant[48]. The study considers repeating a process that generates many random samples linked to specific variables of interest n times. Professionals from fields as disparate as finance, project management, energy, manufacturing, engineering, research and development, insurance, oil and gas, transportation, and the environment using this technique[49]. The MCS offers the responsible person or organization the option to make decisions from a series of possible results and the probability that they will occur according to the measures taken. It shows extreme possibilities, the results of taking the riskiest and most conservative estimates, and all the possible consequences of intermediate decisions, which are pertinent for this case study because production on an industrial scale is being discussed.

In this context, and with the Laboratory of Plant Physiology and Tissue Culture of Universidad de Antioquia's experience with SE in cacao, this study's objective is to analyze the productive process of cacao plantlets by SE from a financial perspective. The process's costs were estimated to identify each standardized stage in the laboratory, and a projection of the production process was performed on an industrial production scale-like biofactory (commercial laboratories used for propagation). The analysis allows planning a pilot for biofactory installations, defining and estimating the factors that in-fluence the cost per obtained plant the most and the least through an MCS. This information helps identify low-cost strategies to apply during technological development.

Materials and methods

Location

The study was carried out on the cacao plants' productive process via standardized SE at the Laboratory of Plant Physiology and Tissue Culture of Universidad de Antioquia, located in the city of Medellín, in the department of Antioquia - Colombia. The work team has a significant track record researching this topic from 2008 to (13,16,30,31,50,51). The infrastructure of the Universidad de Antioquia biofactory was used to project costs on an industrial scale, located in the municipality of Carmen de Viboral in the department of Antioquia, Colombia. Universidad de Antioquia's biofactory is the only installation specializing in vitro plant material in Colombia. It has a strategic location 20 minutes from José María Córdoba interna-tional airport. It is a 1,227 m^2 building capable of producing up to 7 million plants each year via organogenesis and SE. The installations allow automating and scaling propagation protocols and have an architectural design that harnesses sunlight efficiently all day in growth chambers, a natural water source according to the regional environmental authority and its water purification system. They also have a modular design that facilitates expanding specific areas, an automated 276 m^2 greenhouse for plantlet acclimation, and 1,101 m^2 of manual operation

greenhouses. These structural characteristics allow efficiently using sunlight and water at a low cost. Other authors have reported these factors to be significant within process costs[52].

Stages of Cacao Plant Production via SE

The productive concepts proposed by Egertsdotter *et al.* 2019[38] for producing ligneous plants were adapted to define the stages of cacao plant production by SE.

Cost Components

The systems engineering methodology for in vitro tissue cultures proposed by Chen 2016 53 was used to identify and select the factors that influence the production process. Three main components were included in the cost structure: direct and indirect labor, the cost of materials and supplies required for plant propagation, which extends to some indirect manufac-turing costs, and, lastly, operating expenses, which include the infrastructure, depreciation, and administrative expenses required to launch the business. All parameters and their composition are listed in detail in supplement 1.

Assumptions

The following premises were considered to project cacao propagation costs:

- Losses due to contamination and necrosis depend on the quality of the initial tissue and the planting process.
- Multiplication coefficients and percentage of explants' response depend on controlling subculture times and environ-mental factors, such as light, temperature, and relative humidity.
- Work performed by employees is calculated under-skilled labor's operational efficiencies with at least one year of ex-perience handling explants in each stage.
- Labor standards can change depending on the hired technical personnel's training time and experience.
- Thirty percent of the total is regarded to cover the biofactory's administrative expenses, in which operating expenses and utility are considered.
- The practical exercise was performed to produce 100,000 plants, equal to 1.4% of the biofactory's installed capacity.
- The performed analyses are a projection of data obtained in the laboratory over different years. They allow establishing a baseline of work by simulating productive factors.

The analysis was performed based on the whole plant produced by lot to begin identifying cost components, considering the number of explants, vessels, and quantity of culture media for each process stage through the following variables and formulas listed in table 1.

Culture Medium (CM)

Each productive stage has different culture media formulations following the developmental stage of SE to be induced: induction - INDI, multiplication (an expression I - INDIexp, expression II - CM2, expression III - EM2), maturation EM2, germination - MM6 (Table 1 S1: Composition culture medium), and the cost may differ (Sheet 4 S2: Culture medium). The culture medium is calculated with the formula (1) (Table 2). On the other hand, it is essential to remember that most culture media supplies are imported, and costs are affected by the Representative Market Rate (RMR).

Indirect Manufacturing Costs (IMC)

Indirect manufacturing costs are part of the production but cannot be allocated to a production plant or lot because they also have a staggering behavior. Indirect manufacturing costs are calculated using formula (2) (Table 2).

Labor

Direct labor includes the hours required to collect the plant material used as ex-plants (flower buds), which extends to packaging and shipping them. It also in-cludes work in the laminar flow cabinet (LFC) and works performed in support activities, such as preparing, dispensing, and replacing culture media; preparing and disposing of materials; washing vessels; monitoring and selecting.

A supervisor also provides indirect labor with functions related to coordinating direct work, calculating the number of necessary dishes and media, managing chemical products and media stock, equipment, and maintenance for the critical areas. Moreover, they are responsible for ensuring all explants begin production without contamination, on time, and in the correct amounts. They are responsible for maintaining productivity, and, most importantly, they will decide whether or not crop production must be continued, harvested, or discarded at their discretion. The supervisor must coordinate decisions on what product, how many units, and when and where to ship with the administrative department during packaging and shipping.

Direct labor is calculated with the formula (3), and indirect labor is calculated using formula (4) (Table 2).

Operating Expenses

The biofactory's operating expenses include management, sales, equipment depreciation (caster racks, laminar flow, pH meter, balance, magnetic stirrer, auto-clave, distillation unit, minor Equips, mist chamber, fridge, etc.), leasing and public utilities (water, light, Internet, telephone), among others. A historical average of the biofactory's years was considered to calculate operating expenses. Operating expenses are calculated using formula (5) (Table 2).

Finally, the total costs associated with production are calculated following formula (6) (Table 2).

Monte Carlo Simulation (MCS)

The RMR, loss percentage, percentage of explants with responses, multiplication coefficient, and standard productivity variables are defined in each of the production stages and phases of SE as variables of interest to establish their impact on costs when modifications are made to the productive process. Each one of the variables is assigned a triangular distribution, which is traditionally used for cost analyses. Three model estimation points are defined, placing the laboratory results as the central value, and a 10% variation range is established above and below each variable. A triangle-shaped distribution takes form from the above, where the highest probability is around the middle.

A significant number of simulations can be performed using the MCS analysis, modifying the established ranges for each variable. Having all variables in a similar range allows identifying the most strategic variables on which the team's work should focus to obtain the best results. In this study, the MCS was performed with @risk 8.0.1 54 software, with 100,000 iterations for each variable and 100 simula-tions of the same process. The response objective was cost per plant.

Productive Stage	Phase	Period	Loss Rate	Multiplication Rate	% Explant Response	No. of Explants per Stage	No. of Vessels	Quantity of Media
Initiation	Introduction	t_{15}	L_0	1	B_0	$Ni=N_0/((1-L_0)*B_0)$	$V_0=Ni/C_0$	V_0*R_0
	Induction	t_{45}	L_1	1	B_1	$N_0=N_1/((1-L_1)*B_1)$	$V_1=N_0/C_1$	V_1*R_1
Multiplication	Expression I	t_{75}	L_2	M_1	B_2	$N_1=N_2/((1-L_2)*B_2*M_1)$	$V_2=N_1/C_1$	V_2*R_2
	Expression II	t_{95}	L_3	M_2	B_3	$N_2=N_3/((1-L_3)*B_3*M_2)$	$V_3=N_2/C_2$	V_3*R_3
	Expression III	t_{115}	L_4	M_3	B_4	$N_3=N_4/((1-L_4)*B_4*M_3)$	$V_4=N_3/C_2$	V_4*R_4
Maturation	Maturation	t_{135}	L_5	M_4	B_5	$N_4=N_5/((1-L_5)*B_5*M_4)$	$V_5=N_4/C_2$	V_5*R_5
Germination	Conversion	t_{155}	L_6	1	B_6	$N_5=N_6/((1-L_6)*B_6)$	$V_6=N_5/C_2$	V_6*R_6
	Plantlet Development	t_{215}	L_7	1	B_7	$N_6=N_7/((1-L_7)*B_7)$	$V_7=N_6/C_3$	V_7*R_7
Acclimation		t_{335}	L_8	1	B_8	$N_7=N_8/((1-L_8)*B_8)$	$V_8=N_7/C_4$	V_8*R_8

Table 1. Variables and formulas for calculating the number of explants, number of vessels and quantity of culture media by SE productive stage.
t (Time): The period each stage of the production process requires, measured in days; L (Loss rate): Measured as a percentage, this is each productive stage's losses due to contamination or necrosis; M (Multiplication rate): The multiplication coefficient of plant material during the multiplication and maturation stages. It is produced at different moments during embryo development. Five times the initial number was obtained in M1 and M2, and it doubled in M3 and M4 (ten); B (Explant response): Measured as a percentage, it is equal to the rate of explant response in each stage; N (No. of explants per stage): This is the number of explants processed in each production stage. The model projects labor requirements and materials in accordance with the desired plants at the end of the process. For this reason, N8 was calculated as the number of cacao plants desired at the end of the process, N8 = 100,000. It is added, for the calculation, that Ni (initial) represents the flower buds entering the process, which have 5 staminodes, for which reason it increases by 5 from Ni to N0; V (No. of Vessels): This is the number of vessels required in each production stage. The calculation is performed based on the number of processed explants and between each type of vessel's capacity; C (Vessel Capacity): This refers to the number of explants in accordance with the type of vessel used in each stage. The vessels are conical tubes, Petri dishes, magenta vessels and germination trays. The assigned variables are: C0 is a conical tube with a capacity for 350 flower buds, C1 is a Petri dish with a capacity for 25 calli, C2 is a Petri dish with a capacity for 49 embryos, C3 is a magenta vessel with a capacity for 20 plantlets, and C4 represents trays with a capacity for 50 plantlets; R (Culture medium): Equal to the volume of culture media required by the type of vessel in a 50 ml conical tube, 30 ml Petri dish, 150 ml magenta vessel and 100 g germination trays; Q (Quantity of Media): This is the result of the number of vessels required in each productive stage by the necessary media volume per vessel.

Results

Stages of Plant Production via SE

The production process for the in vitro propagation of cacao plants by SE was identified to have five stages: initiation, multiplication, maturation, germination, and acclimation (Figure 1). Various processes, such as the explant disinfection process, called introduction, and callogenesis induction, are included in the initia-tion stage. In the multiplication stage, undifferentiated tissues are transferred to the primary embryo expression medium with a first multiplication coefficient of 5 for the phase called an expression I. An average of 5 embryos are produced for each callus. Once primary somatic embryos are obtained, they are divided using a blade. The pieces are placed in the repetitive or secondary embryogenesis induction media with a second multiplication coefficient of 5. This phase is called expression II. Therefore, somatic embryos develop asynchronously and are harvested from the globular and early cotyledonary stage to prevent embryonic axes from fusing during development. The embryos are subsequently transferred to a hormone-free medium where repetitive embryogenesis occurs again, with a multiplication coef-ficient of 20. This is called the expression III phase. Up until this point, both the initiation and multiplication stages are performed in a dark room at an average temperature of 26 – 27 ± 2 °C. In the maturation phase, somatic embryos develop with clear differentiation between their apical and radicular meristematic poles. Embryo maturation is set off by a change in the culture medium's composition, where the source of carbon and growth regulators are vital components. Subsequently, the germination stage is composed of the conversion phase to plantlets and their development in terms of increased

Culture Medium (CM)	Indirect Manufacturing Costs (IMC)	Direct Labor	Indirect Labor	Operating Expenses	Total Cost (TC)
(1) MC= (V * C) * (P1*I1+P2*I2+… +Pi*Ii)	(2) CIF = MH / U + CT + DWV	(3) W_f= (V/Wc + Wa) * (SMLMV + FP)	(4) W_f= (Ta * D * MOI) * (SMLMV + FP) * (r)	(5) GO: (MC + CIF + MOD + MOI) *(1/(1-%Admon) – 1)	(6) CT = MC + CIF + MOD + MOI + GO
- P (Price): Each input's price. - I (Input): The inputs required to produce one liter of culture medium. - V (No. of Vessels) - C (Vessel Capacity)	- MH (Materials and Tools): The materials and tools that get partially or fully worn out in producing the productive lot. - U (Uses): The number of times a partially worn out material or tool is used. - CT (Transportation cost): The cost of transporting the plant material to the greenhouse. - DWV (Daily staffing): Daily staffing required for the productive lot's production.	- V (No. of Vessels): This is the number of vessels required in each productive stage. - Wc (LCF work standard): The days an employee requires to process the volume of vessels in a workday. - Wa (support process work standard): The days an employee requires to provide support in each productive stage, measured in days. - MOD (Direct labor): The labor required for total production. The obtained result is given in work days multiplied by cost. - SMLMV (Current Legal Monthly Minimum Wage): The minimum wage established by the Colombian national government. - FP (Social benefit factor): The additional payment due to employee benefits, legal and extralegal benefits, subsidies and all additional payments enforced by the Colombian national government for employees.	- Ta (Accumulated time): The time of each productive stage is calculated in months from when the induction phase begins until it ends with acclimation. - D (% Time allocation): Refers to the supervisor's time. - MOI (Indirect labor): The labor required for supervision. - SMLMV (Current Legal Monthly Minimum Wage) - FP (Social benefit factor)	- CM (Culture medium) IMC (Indirect Manufacturing Costs) - MOD (Direct labor) - MOI (Indirect labor) - %ADMON: The percentage of administration designated by the Biofactory's management.	- CM (Culture medium) - IMC (Indirect Manufacturing Costs) - MOD (Direct labor) - MOI (Indirect labor) - GO (Operating Expenses)

Table 2. Formulas and variables for calculating the components of the productive process' cost.

stem length, secondary root formation, and leaf formation. They are transferred to 500 ml culture vessels when they reach a certain height. Plantlets are kept under natural lighting in growth rooms during the maturation and germination stages. Finally, plantlets are transferred to greenhouse conditions for growth and elongation during the acclimation phase. Plantlets that are 3 - 5 cm high, with 1 - 3 leaves and both primary and secondary roots, are se-lected, removed from their culture vessels, and washed with topwater to eliminate culture medium surpluses. They are then transferred to 50 alveoli germination trays with a substrate made of a mixture of sand and unenriched basic coconut coir. The tray is hermetically sealed for one day, and holes are subsequently opened to allow gas exchange, keeping the substrate hydrated with a Hoagland solution[55]. After this time, the tray is opened and kept in the ideal growth conditions for cacao in this stage, with a 50% shade percentage, the temperature of 24 – 30 ºC, and relative humidity over 60%.

Cost Components

It was determined that Ni 4190 flower buds are required during plant material introduction, and N0 9429 staminodes must be processed to induce callogenesis to produce a batch of 100,000 plantlets (Sheet 2 S2: Stages of production). For the multiplication stage, N1 + N2 + N3, a total of 27,827 embryos were processed. 101,189 embryos were processed in the N4 maturation stage, and 506,803 plantlets were processed in germination stages N5, N6, and N7 (Table 3).

Considering the following amounts and sufficient work times is required for labor payments in Colombia, as presented in the Substantive Labor Code (Sheet 3 S2: Direct and indirect labor). The base salary in Colombia is US $250.8, transportation allowance US $29.4, health US $21.3, pension US $30.1, occupational risks US $1.3, severance payments US $22.6, premium US $23.3, losses US $23.3, interest on severance pay US $2.8, vacation US $10.5 and endowment US $12.5. The sum of the total cost of a salary in Colombia is US $428. To calculate effective work time, it was decided that, of the year's 365 days, 52 are Sundays, 18 are holidays, 15 are vacation days, and an average of 3 are taken off due to incapacity. Therefore, there are 277 effective workdays a year and 23 effective workdays a month. Fol-lowing the above, taking a salary into account with the social benefit factor and effective monthly workdays, yields US $18.53/effective workday.

Once the daily labor cost was identified, the time personnel used to perform work in each process's phases was calculated. Three main activities were identified: (1) collecting flower buds from the genotypes of interest in the field, (2) processing material in an LFC, and (3) support processes, among which are preparing, dispensing, and replacing culture media, preparing materials, reviewing to discard material, cleaning functions and vessel and tool disinfection (Table 4).

A total of 28 days, or 168 hours of work (approximately), were required to collect flower buds in the field. Personnel working in the LFC involves 285 days or 1,710 hours to execute all the productive process' stages, and support personnel requires 1,547 days of work or 9,286 hours. Therefore, the production process requires 334 days from flows bud introduction to plantlet attainment, and 7 employees are re-quired to execute the process.

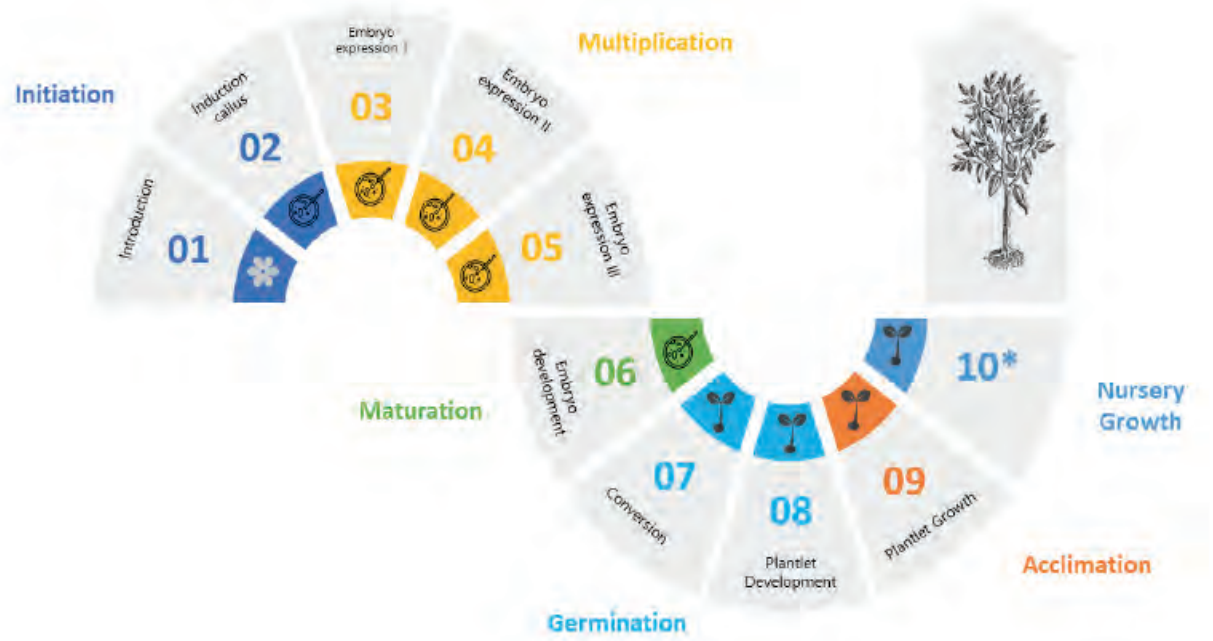


Figure 1. Stages of cacao plant production via somatic embryogenesis (SE).
* Plantlets growth in greenhouses is not considered in this study.

Formula	Calculation Details	No. of Total Explants
$N_1=N_0/((1-L_0)*B_0)$	= 9427/((1-10%)*50%) =	4190 (Flower buds)
$N_0=N_1/((1-L_1)*B_1)$	= 8776/((1-2%)*95%) =	9427 (Staminodes)
$N_1=N_2/((1-L_2)*B_2*m_2)$	= 4300/((1-2%)*10%*500%) =	8776 (Primary embryos)
$N_2=N_3/((1-L_3)*B_3*m_3)$	= 14751/((1-2%)*70%*500%) =	4300 (Secondary embryos)
$N_3=N_4/((1-L_4)*B_4*m_4)$	= 101189/((1-2%)*70%*1000%) =	14751 (Secondary embryos)
$N_4=N_5/((1-L_5)*B_5*m_5)$	= 694155/((1-2%)*70%*1000%) =	101189 (Cotyledonary embryos)
$N_5=N_6/((1-L_6)*B_6)$	= 340136/((1-2%)*50%) =	694155 (Plantlets)
$N_6=N_7/((1-L_7)*B_7)$	= 166667/((1-2%)*50%) =	340136 (Plantlets)
$N_7=N_8/((1-L_8)*B_8)$	= 100.000/((1-0%)*60%) =	166667 (Plantlets)
$N_8=$		100000 (Plantlets)

Table 3. Total number of explants processed for each productive stages.

Workdays	Collection in the Field	*In vitro* Introduction	Induction	Expression I	Expression II	Expression III	Maturation	Conversion	Plantlet Development	Plantlet Growth	Total Days
In the Field	28										28
In LFC		6	4	1	4	13	86	96	47	28	285
In Support Processes		23	24	27	17	21	54	555	612	216	1.547

Table 4. Personnel days allotted to propagation activities and support processes in each one of somatic embryogenic phases.

The cost of personnel was obtained by multiplying the 1861 required days for batch production by the cost of one effective monthly workday at US $18.53/day, for US $34,500. Supervision does not need to be project-exclusive once standardized processes are achieved, for which reason a 25%-time allotment was established during the production year. A monthly price with a social benefit factor of US $1,284 was obtained for the supervisor, 12 months. Therefore, the total cost of indirect labor was US $3,852.

It was observed that the germination stage contributes significantly to the total cost for culture media since it requires more culture media than other stages (Sheet 4 S2: Culture medium). The amount goes from 30 ml in a Petri dish to 150 ml in a higher-volume vessel (Table 5). For IMC, transportation resulted in US $543, personnel endowment US $282, and materials and tools US $3,049, which adds up to US $3,874. It was previously clarified that operational expenses are calculated as 30% of total production costs. Therefore, they correspond to US $21,895 (Sheet 5 S2: IMC).

Using this estimation methodology, the total cost of US $72,986 was obtained for a production batch with 100,000 plants, with a cost per plantlet of US $0.73/unit.

Monte Carlo Simulation Analysis (MCS)

After structuring costs, the most influential cost component was direct labor, representing 53% of the total cost. The cost of culture media was 12% of the total, IMC repre-

sented 5%, and operating expenses, including administrative expenses and infrastructure, were 30% (Figure 2).

Plants propagated by SE. Figure 3 shows that the cost of plants per unit can be inferred between USD $0.6835 and USD $0.7786, with an average USD $0.7290 (Sheet 1 S2: Total cost), due to the production process's cost structure. If strict control is maintained over the variables while executing the productive lot as es-tablished in this study, the average price per plantlet has been proposed to be USD $0.7290, with a 95% reliability. However, as one may observe, there is a certain asymmetry towards the right, which indicates that the process could increase in cost. That is to say, the cost may have deviated over the average or over USD $0.7290 after moving the lot.

On the other hand, a regression was performed on the correlation coefficients of each of the cost model's variables to identify which ones influence the variable response, which is to say cost the most. This analysis showed that the most significant variables are in the productive process' last stages, corresponding to ger-mination and acclimation, followed by the maturation stage. It was specifically found that the plantlet growth (-0.69) and plantlet development (-0.60) phases, as well as the plantlet conversion phase (0.35) had more significant effects on cost, with 95% reliability. Therefore, when these variables, which are expressed as the percentage of explants' response, rise above 60%, 50%, and 50%, respectively, the variable of cost per plantlet decreases. During the embryos' development phase, both the multiplication coefficient (-0.07) and percentage of explants' response (-0.07) tend to decrease cost per plantlet as their prices increase. They are currently at 10% and 70%, respectively. Besides, it was observed that the RMR (0.15) has a positive effect on cost. Cost per plantlet will increase as RMR increases (Figure 4).

Discussion

In Colombia, approximately 176,000 cultivated hectares benefit 52,000 families in 422 municipalities of 30 departments, with biannual growth of 4% in planted area. It is established that approximately 7000 hectares biannually is required in the short term and some 10,000 hectares for renovation. Having an average amount of 1,100 plants per

Stage		Amount (USD)	Unit	Liters Required	Lot Cost in USD
Initiation	Introduction	$ 1,74	cost/liter	0,47	$ 0,84
	Induction	$ 1,74	cost/liter	11,31	$ 19,74
Multiplication	Expression I	$ 1,83	cost/liter	10,53	$ 19,22
	Expression II	$ 1,73	cost/liter	2,63	$ 4,55
	Expression II	$ 1,61	cost/liter	9,03	$ 14,52
Maturation	Embryo development	$ 1,61	cost/liter	61,95	$ 99,59
Germination	Conversion	$ 2,05	cost/liter	2.124,96	$ 4.361,81
	Plantlet Development	$ 2,05	cost/liter	2.551,02	$ 5.236,33
Acclimation	Plantlet Growth	$ 0,01	cost/kilogram	11.666,67	$ 133,33

duction.

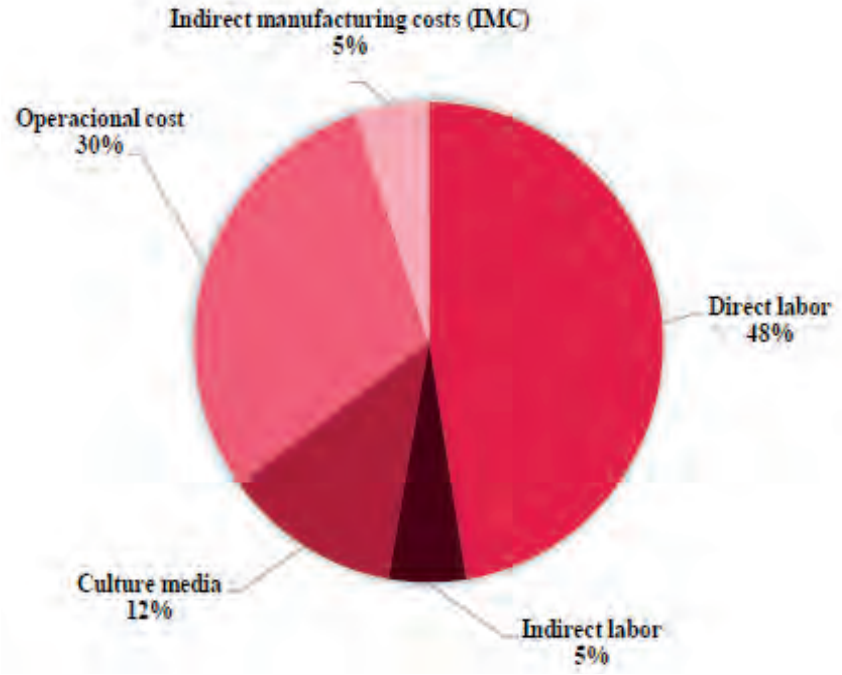


Figure 2. Components of production cost share in the total cost of producing cacao plantlets.

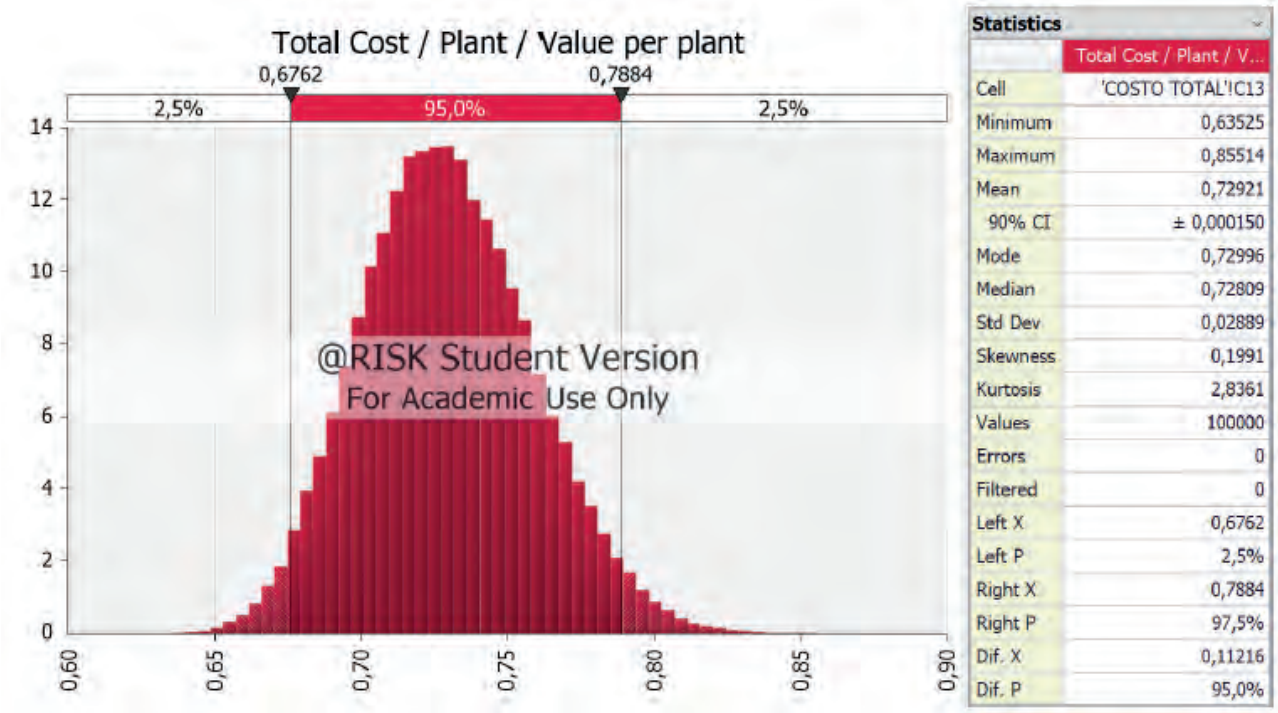


Figure 3. Frequency distribution for the cost of producing one cacao plantlet by somatic embryogenesis.

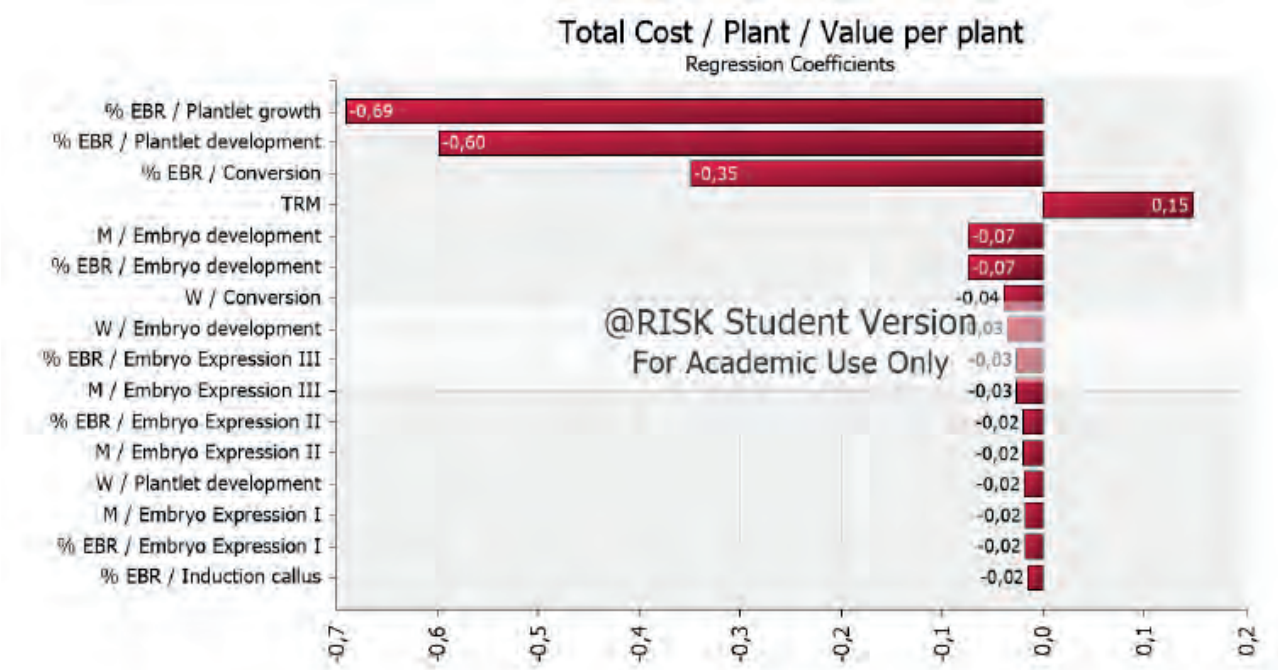


Figure 4. Regression coefficients for the variables associated with the productive process of cacao plants propagated by somatic embryogenesis over the production cost per plant. Variables: % EBR (% explants with response), RMR (Representative Market Rate), M (Multiplication rate), W (Labor standard).

hectare, with 5% additional plants to replace those that die at the time of being transplanted, it is estimated that the potential demand is 19.6 million plants biannually for 2021[56]. In the last two decades, there has been a growing demand for high-quality, high-performance, and pathogen-free planting material, increasing demand for agricultural, forest, and horticul-tural products. Within this trend, cultivating plant tissue in vitro has become an important, commercially feasible tool for generating high-quality, high-performance, and pathogen-free planting material regardless of climate fluctuations. Most notably, SE's potential as a propagation method has not only been described in the last years for cacao[24,57,58] but also other plant species of in-terest[59–61]. Various authors agree that the main factor limiting SE's deployment on a commercial scale is the plants' relatively high cost compared to those cultivated in greenhouses[62]. Therefore, financial cost analyses for these processes are vital, and more so is examining strategies to reduce costs without affecting production efficiency and quality[41].

In the analysis performed for cacao, the component that contributed most to cost was direct labor, at 53%. This result coincides with what other authors reported for plants

obtained by SE other than cacao. For example, Cervelli and Senaratna (1995)[63] reported a 70% contribution to cost for labor and Chu (1995)[64] reported a 63.8% contribution. However, other authors recommend decreasing direct labor's con-tribution percentage as much as possible, by between 30 and 40%[36,52]. Following these results, it is evident that cacao plantlet production via SE is a labor-intensive system, and labor is the main production cost component. Maintaining high labor efficiency is essential to make the process feasible. Therefore, one of the method-ological strategies to achieve this objective is optimizing personnel's actions in LFCs. For example, unnecessary existing times can be eliminated. Despite having adequate efficiencies in processing material, such as the number of transfer opera-tions per day, this study has estimated 6,000 explants/ workday for the initiation stage, a work standard similar to that which Ahloowalia & Savangikar (2004)[36] reported for this same operation, at 5,000 explants/ workday.

On the other hand, following Suárez-Castellá (2006)[65], the productivity of 4,595 explants/ workday can be achieved by providing training on handling and pro-cessing tissue. This figure is higher than the work standards for the cacao multiplication stage, which oscillates at 1,176 explants/ workday on average. However, lack of skilled labor is always the main problem in countries such as Colombia. Implementing training programs is an alternative to promote operating personnel's work capacity and overcome this limiting factor.

The second component contributing most to cost is operating expenses, at 30% of the total cost. This percentage is very close to the ranges reported when prop-agating other species, such as Phalaenopsis *sp.*, where operating expenses were 32.7%[66]. However, lower figures are reported for Paulownia tomentosa produc-tion, at 19%[45] and Saccharum officinarum, 24.4%[67]. It must be clarified that both are productive processes performed by organogenesis. In this respect, it is essential to indicate that one of the highest infrastructure costs is electricity associated with air conditioning systems used to regulate culture room temperatures. According to Tomar (2010)[52], this factor can consume 85% of the electricity in vitro propagation installations. Therefore, if the process does not require exact control over temper-ature, it is recommended facilities avoid using this resource if the area's climate conditions allow it. Another aspect of electricity consumption is associated with expenses during the water distillation process, which is the main component of culture media. Distilled, double-distilled or deionized water is generally used in in vitro tissue cultures. However, on a productive scale, according to Sahu (2013)[68], using alternative water sources is recommended to reduce the cost of sterilizing tap water in an autoclave, as long as the water has proper conductivity, pH, is free of heavy metal or pollutants[69].

The third most important component within the cost structure is culture medium, which corresponds to 12% of the total cost and can also be improved to reduce costs. Most plant tissue culture media's main components are mineral salts and sugars as sources of carbon and water. Other components can include organic supplements, growth regulators, and gelling agents[70]. According to Prakash *et al.,* (2004)[71], the chemical reagents of culture media cost less than 15% of the total production cost of the vitroplant. In some cases, the cost can lower down to 5%. Of culture media components, gelling agents, such as agar, contribute to 70% of the total cost, followed by sources of carbon and, finally, growth regulators, which minimally influence the cost of production, since they are reasonably inexpensive due to their minimal concentration within culture media[12]. Carbon sources are commonly used, such as glucose, fructose, and maltose. However, they are reagents with high purity levels, making them expensive. Sucrose is the most widely used carbon source in the in vitro propagation of plants. Table sugar has also been used as a strategy to reduce culture media costs. Regular sugar has a high enough quality for micropropagation, and using it reduces media culture costs between 78 and 87%[41]. In Colombia, the cost of local sugar is USD $0.86 / kg compared to USD $8.13 / kg for imported sucrose. Another strategy for decreasing costs is associated with the mineral formulation. Many companies sell media prepared as a liquid or powder. However, the benefits and disadvantages of formulations prepared in laboratories must be considered concerning commercially prepared media. Although prefabricated media save time, their relative cost is high. Pre-packaged media preparations are usually helpful when the required amounts are small - generally on a scale for research - and they have less errors. It is much more cost-effective for large-scale use to prepare media by combining the basic in-gredients. Nevertheless, if errors occur, losses are more significant depending on the size of the lot. Moreover, the time the hired technician takes preparing the formulations must be considered, for which reason it is recommended for stock solution concentrations to be up to 1,000X.

Indirect manufacturing costs are included in the fourth component, with 5% cor-responding to transportation, culture vessels, materials, supplies, tools, etc. Some authors recommend pre-sterilized, reusable plastic Petri dishes because the material is more inexpensive than glass[71]. However, our organization encourages mini-mizing its ecological impact by substituting single-use supplies or tools for lasting and reusable materials due to environmental, social responsibility policies. Fur-thermore, following Datta (2017)[41], using supplies, such as aluminum, is not recommended for wrapping tools before sterilization. Instead, sterilizable stainless steel containers can be used in autoclaves as substitutes with significantly long valuable lives. Also, PVC or polyethylene plastic film is used as lids in common practice and for large-scale production. These lids have been replaced with stainless steel or polypropylene screw caps sterilizable in an autoclave.

The sensitivity analysis showed that the most significant variables on cost per plantlet are in the productive process' last stages, corresponding to plantlet ger-mination and acclimation. This specifically applies to the percentage's response during the plantlet conversion, development, and growth stages. These results are consistent given the accumulated time, effort, and energy invested up to this point in the productive process. Following Von Aderkas *et al.* (2016)[72], practices per-formed during the entire production process via SE, especially managing embry-ogenic structures and somatic embryos in cotyledonary states, significantly affect the performance and quality of the embryo conversion, as well as production costs. One of the most crucial stages is when plantlets are transferred from in vitro to ex vitro conditions since approximately 8 months have transpired in the process' previous stages. It is well-known that plantlet survival can be improved by optimizing somatic embryos' maturation and germination conditions in cotyledonary states. These conditions can be optimized by, for example, making adjustments to components, such as abscisic acid and gibberellic acid and modifying the cultu-

re medium's water potential and nitrogen source, among others[73–75]. In this process stage, the production cost can also be reduced if time in hardening facilities is shortened without reducing plantlet survival rates. Plants require relatively more minor time to harden if natural light is previously used during plantlet development[41] and if microorganisms that promote growth, such as fungi and bacteria, are used[76].

The variables associated with the maturation stage, which is to say the development phase of embryos, such as multiplication coefficient and percentage of explants' response, were also significant on cost in the sensitivity analysis. Under other au-thors and the obtained results, improving embryo multiplication rate is one of the most efficient ways to reduce costs[66]. Some of the alternatives for improving the multiplication rate in the case of SE include selecting the most suitable develop-ment stage of primary embryos or primary embryonic tissues as a source for pro-ducing repetitive embryos. That is to say, the more control there is over the exact point in which tissues answer to recurring embryo formation, the more embryos will be produced. The same applies to using substances to promote embryo formation, such as demethylating agents like 5-Azacytidine (5-azaC). In this sense, in terms of cacao, it has been demonstrated that decreases in embryogenic potential seem to be related to DNA methylation[77]. In addition, environmental conditions like micro-climate, at least while controlling temperature between 27 and 28 °C and total darkness in the culture room, are definitive in the cacao multiplication stage. In this context, all these stages add up cumulatively, reducing production costs.

A price per plantlet of USD $0.73 was obtained based on the analysis performed on the cacao production process, with a final sales price to customers of USD $0.88 and grafted of USD $1.16. Upon reviewing the market prices of traditionally grafted cacao plants, an average of USD $0.71 was obtained for Colombia and, in countries like Ecuador, USD $0.70. Also, the cost in Colombia is above the prices offered in Latin America. For example, cacao plantlets propagated by SE in a biofactory in Brazil cost USD $0.70[78]. Despite there being a significant difference concerning plantlets propagated by the traditional method, there is a significant opportunity to be considered by developing the technology on a biofactory scale with obvious cost reduction objectives. In this sense, to validate technology for cacao plant production by SE and for it to be a cost-effective product on the national market, the factors that contribute most to cost, such as percentage of explants response, must continue to be optimized, and other options that do not compromise quality must be considered. Adopting flawed low-cost strategies can make the production process prone to failure since it must be highlighted that this is a productive process inherent to a biological process. These processes may vary significantly after changing some culture conditions. Low-cost techniques will only be successful if they scrupulously comply with tissue culture's primary require-ments, thus maintaining plantlet quality. According to Datta (2017)[41], procedure standardization is more critical for ensuring cultivated plants' quality than proce-dure sophistication. It can be said that continuing to develop a low-cost productive process signifies an advanced generation technology, improving the process' effi-ciency and use of resources. Therefore, different strategies can be intelligently adopted, such as simplifying various technological operations to reduce specialized facility costs, such as a biofactory's costs.

Conclusions

A calculation matrix that estimates production costs by defining the number of plants required by productive batch and estimating cost developing volume was created in this study. Labor costs contributed to 53% of the total cost, followed by operating expenses at 30%, culture media at 12%, and IMC at 5%. The proposed costing and analysis approach can be adapted and applied to other cultures propagated by SE.

The percentage of explants' response variable in the germination and acclimation stages is considered to affect cost per unit most significantly for cacao plantlet production by SE and the multiplication coefficient during the maturation stage.

The applicability of the MCS was proven, showing that it is possible to consider different strategies for reducing costs. In this sense, nowadays, deciding what productivity indicator should be used to consider these scenarios is practiced. It represents an opportunity for developing technology and business. Finally, it is essential to mention that the presented cost reduction strategies must be tested on a short-term pilot batch to validate the product's performance.

Supplementary Materials

The following are available in this PDF, Table S1: Composition culture medium, Sheet 1 S2: Total cost, Sheet 2 S2: Stages of production, Sheet 3 S2: Direct and indirect labor, Sheet 4 S2: Culture medium, Sheet 5 S2: IMC, Sheet 6 S2: 6. Assumptions.

Author Contributions

Conceptualization, Ana María Henao Ramírez and Aura Inés Urrea Trujillo; methodology and software, Hernando David Palacio Hajduck and Ana María Henao Ramírez; validation anf formal analysis, Ana María Henao Ramírez; investigation, resources, data curation, writing—original draft prepara-tion, Ana María Henao Ramírez; writing—review and editing and supervision, Aura Inés Urrea Trujillo. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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REVIEW / ARTÍCULO DE REVISIÓN

Cambios en la concentración de ácido fítico, fósforo libre y hierro soluble durante la fermentación de repollo blanco y repollo chino

Changes in phytic acid concentration, free phosphorus and soluble iron during fermentation of white cabbage and Chinese cabbage

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Resumen: Se estudió la evolución de la concentración de ácido fítico, fósforo libre y hierro soluble durante la fermentación de repollo blanco y repollo chino. En ambos casos, la máxima población de bacterias ácido lácticas se logró a los cinco días del proceso y luego disminuyó continuamente hasta el final. El pH inicial del repollo blanco y repollo chino fue de 6,1 y durante los primeros cinco días disminuyó a 3,7 y 4,3 respectivamente, luego permanecieron estables hasta los 30 días. En el repollo blanco, la concentración de ácido fítico disminuyó y el fósforo libre se incrementó durante los primeros cinco días, después no se detectaron cambios significativos. En el repollo chino, la degradación del ácido fítico se observó durante los primeros 15 días, mientras que el fósforo libre aumentó hasta el final del proceso. Ambos vegetales mostraron una concentración inicial de hierro comparable, luego, se observó un incremento hasta el final del proceso, siendo este fenómeno más notable en el repollo chino. Los resultados obtenidos sugieren que la degradación del ácido fítico producida durante la fermentación por la actividad de fitasas vegetales y bacterianas, no sólo origina la liberación de fósforo libre, sino que mejora la bioaccesibilidad del hierro.

Palabras clave: Biodisponibilidad de nutrientes, fermentación espontánea, *Brassica*.

Abstract: The evolution of phytic acid concentration, free phosphorus, and soluble iron was studied through the fermentation of white cabbage and Chinese cabbage. In both cases, the maximum population of lactic acid bacteria was achieved at five days of process and then decreased continuously until the end. The initial pH in white cabbage and Chinese cabbage was 6.1, decreased to 3.7, and 4.3, respectively, at five days and remained stable until fermentation. The decrease of phytic acid concentration and the increase of free phosphorous could be observed in white cabbage during the first five days of fermentation; then, both parameters did not show significant changes. In Chinese cabbage, the degradation of phytic acid could be observed during the first 15 days of fermentation, while free phosphorus increased continuously through 30 days. At the beginning of fermentation, both vegetables displayed comparable iron concentration, then the increase could be observed steadily until the end of the process; however, this phenomenon was more remarkable in Chinese cabbage. The results obtained suggest that phytic acid degradation produced during fermentation by the activity of vegetables and bacterial phytases originates the release of free phosphorus and enhances iron bioaccessibility.

Key words: Nutrient bioavailability, spontaneous fermentation, *Brassica*.

Introducción

Las fermentaciones han sido utilizadas históricamente por el hombre con el propósito de aumentar la vida útil de los alimentos y eliminar o inhibir bacterias patógenas o deteriorantes. En el caso específico de la flora epifítica de los vegetales, especialmente las bacterias ácido lácticas (BAL), utilizan los hidratos de carbono presentes en el tejido de las plantas transformándolos en ácidos orgánicos, logrando de esta manera un marcado descenso del pH y, en consecuencia la inhibición del desarrollo de bacterias indeseadas[1]. Además de este fenómeno, se producen distintas reacciones metabólicas que influyen por ejemplo, en la capacidad antibiótica mediante la formación de peróxido de hidrógeno, diacetilo y bacteriocinas[2]; aumento de la capacidad antioxidantes como consecuencia de la hidrólisis de polifenoles y flavonoides[3,4]; incremento de moléculas bioactivas como la producción de tioglicolatos a partir de glucosinolatos por la acción de mirosinasas[5,6], entre otras.

Un cambio importante que se produce durante la fermentación es la disminución o eliminación de factores antinutricionales dentro de los que tenemos que considerar a los inhibidores de enzimas degradativas del tracto gastrointestinal, especialmente la tripsina, o moléculas que interfieren en la biodisponibilidad de nutrientes[7]. En este grupo de moléculas uno de los más importantes lo constituye el ácido fítico y sus derivados. Aproximadamente el 80 % del total del fósforo contenido en las células vegetales está presente

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como fitato y no solo su biodisponibilidad resulta prácticamente inaccesible[8,9] sino que la liberación al medio ambiente podría provocar efectos negativos, como sucede con la eutrofización de los cuerpos de agua en las zonas donde se practica la ganadería y agricultura intensiva[10].

El ácido fítico es definido desde el punto de vista químico como ácido mioinositol (1,2,3,4,5,6) hexafosfórico. Su estructura le permite formar quelatos estables e insolubles con cationes divalentes como Ca^{++}, Mg^{++}, Zn^{++}, Cu^{++} y Fe^{++}, fenómeno que trae como consecuencia una disminución en la absorción y digestión de estos minerales en el tracto intestinal[11]. También tienen la capacidad de formar complejos con las proteínas disminuyendo su solubilidad e interfiriendo su digestibilidad[11].

En consecuencia, la degradación del ácido fítico o sus derivados durante el procesamiento de alimentos de origen vegetal es esencial para aumentar la disponibilidad de fósforo y oligoelementos. En el caso específico del hierro, algunos autores afirman que para mejorar sustancialmente su absorción la degradación del ácido fítico deber ser casi completa[12].

Los tratamientos térmicos, la cocción, el malteado y la fermentación son procesos que logran disminuir la concentración de estas moléculas quelantes. En el caso específico de la fermentación, la acción conjunta de las fitasas vegetales y microbianas logran hidrolizar y liberar fósforo soluble a partir de los fitatos[1,13,14].

Durante los últimos años, ha cobrado un significativo interés el estudio de la fermentación de brasicáceas debido a su alto consumo en distintas regiones del mundo y el alto valor nutritivo que poseen[15]. Estos vegetales son ricos en carbohidratos, fibras, vitaminas y minerales. Además, su capacidad antioxidante tiene influencia positiva en la lucha contra patologías humanas tales como enfermedades cardiovasculares, cáncer, diabetes, etc[4]. Las brasicáceas son cultivadas con relativa facilidad gracias a su adaptación a diferentes climas y calidad de suelos y, en algunos países constituyen la base de su alimentación[15].

El objetivo de este trabajo fue determinar la evolución del contenido de ácido fítico, fósforo y hierro soluble durante la fermentación espontánea de repollo chino (*Brassica rapa L. ssp. glabra*, Regel) y el repollo blanco (*B. oleracea L. ssp. capitata*, (L) Metzg) cultivados en el Valle Inferior del Río Chubut (Patagonia, Argentina).

Materiales y métodos

Proceso de fermentación

El repollo chino (*Brassica rapa L. ssp. glabra*, Regel) y el repollo blanco (B. oleracea L. ssp. capitata, (L) Metzg) fueron obtenidos de establecimientos localizados en el Valle Inferior del Río Chubut, Patagonia, Argentina (latitud -43,14; longitud -65,19; 11 metros sobre el nivel del mar).

Con el objeto de preparar el material a fermentar se eliminaron las hojas exteriores, los bulbos limpios fueron cortados en tiras de 2 mm de espesor y se colocaron en frascos. Se adicionó NaCl en una concentración final del 3 % (m/m). Ambos vegetales se fermentaron a 18 °C durante 30 días. Las pruebas se realizaron por duplicado.

Parámetros de fermentación

La toma de muestras para determinar el pH y el recuento de BAL se realizó a los días 0, 1, 2, 3, 4, 5, 10, 15, 20, 25 y 30 del proceso de fermentación. La determinación del pH se realizó con un pH-metro calibrado modelo Orion 410. El recuento se realizó en agar Man Rogosa Sharpe (MRS) (Laboratorios Britania, Buenos Aires, Argentina) a 30 °C durante 24-48 h. Los resultados se expresaron en Unidades Formadoras de Colonia por mililitro (UFC/ml).

Determinación de fosfato libre

Las muestras se centrifugaron a 2000 rpm durante 5 min en una centrífuga Rolco CP 2036 (Buenos Aires, Argentina). El fosfato libre se determinó utilizando 100 µl de sobrenadante y 900 µl de una solución que contenía 0,6 M de H_2SO_4, 2 % (m/v) de ácido ascórbico y 0,5 % (m/v) de molibdato de amonio. Luego de una incubación a 50 ºC durante 30 min se realizó la lectura de la densidad óptica a 820 nm. Para la curva de calibración se utilizó una solución patrón de K_2HPO_4. Los resultados fueron expresados en mg de fósforo/100 ml[16].

Determinación de ácido fítico

La concentración de ácido fítico en los sobrenadantes se determinó utilizando el kit comercial Megazime Internacional (Wicklow, Irlanda) siguiendo las instrucciones del fabricante. El ensayo se basa en la hidrólisis del ácido fítico por la actividad de la fitasa en mio-inisitol-fosfato y fósforo libre, y la posterior actividad de la fosfatasa alcalina que libera el resto de los fosfatos de la molécula de mio-inositol[17]. El fosfato libre se determinó según se describió anteriormente y los resultados se expresaron en mg ácido fítico/100 ml, utilizando la siguiente ecuación:

$$\text{Ácido fítico} = \frac{\text{Fósforo } \frac{\text{g}}{100\ \text{ml}}}{0.282} \qquad (1)$$

Determinación de hierro libre

Se trataron 200 µl de sobrenadante con 1 ml de una solución 30 mM de ácido ascórbico en buffer citrato (0,05 M, pH 4,5). Luego de una incubación a 37 °C durante 5 min se agregó 200 µl de una solución de ferrozime (5 mM). Se incubó a 37 °C durante 40 min y se midió la densidad óptica a 562 nm en un espectrofotómetro Jenway modelo 6405 (Londres, Reino Unido). Se realizó una curva patrón con $Fe(NO_3)_3$. Los resultados se expresaron en mg hierro/100 ml[18].

Resultados y discusión

Parámetros de fermentación

El descenso de pH y el recuento de BAL constituyen los parámetros que permiten determinar la evolución del proceso de fermentación. La población inicial de BAL en ambos vegetales fue: 2,1 y 2,5 log UFC/mL para repollo blanco y repollo chino, respectivamente, estos valores coinciden con reportes previos[1]. Los vegetales mostraron el máximo de población luego de 5 días de proceso, determinándose un recuento de 9,2 log UFC/mL para repollo blanco y 9,5 log UFC/mL para repollo chino. Posteriormente, la población de BAL comenzó a descender en ambos vegetales, alcanzando un valor próximo a 6,0 log UFC/mL después de 30 días (Figura 1). El repollo blanco exhibió un pH inicial de 6,0 y descendió hasta lograr un valor de 3,7 en el día 10, manteniéndose estable hasta el final del proceso. En el caso del repollo chino, el pH inicial fue de 6,1 y descendió a 4,3 en

el día 5, valor que se mantuvo estable hasta los 30 días de ensayo (Figura 1). La marcada diferencia en los valores de pH alcanzados luego de 30 días de experiencia, es atribuible a la mayor concentración de carbohidratos que exhibe el repollo blanco con respecto al repollo chino[19], fenómeno que permite una síntesis mayor de ácidos orgánicos y el consecuente aumento de la acidez titulable.

El descenso de la población de BAL observado, luego de alcanzar el valor máximo, se debe a las limitantes nutricionales de los vegetales estudiados. En el caso particular de las brasicáceas, la baja concentración de proteínas no permite la duplicación celular una vez agotado este recurso. Las BAL, no obstante adaptarse y duplicarse con velocidad en sustratos vegetales, presentan numerosas auxotrofías que las hace dependientes de una constante provisión de proteínas, péptidos y aminoácidos[20].

Determinación de fosfato libre y ácido fítico

El ácido fítico y sus derivados constituyen la mayor reserva de fósforo de las plantas, sin embargo, la mayoría de los animales monogástricos no logran degradarlo por carecer de fitasas o exhibir actividades enzimáticas insuficientes en su mucosa intestinal[8]. La degradación de fitatos en los alimentos es de vital importancia para aumentar la biodisponibilidad de nutrientes, ya que evita que estos formen quelatos insolubles en el tracto intestinal[11,21].

Durante la fermentación espontánea, las fitasas vegetales son liberadas al medio degradando el ácido fítico y aumentando la concentración de fósforo soluble. En forma paralela, las BAL aumentan su población y contribuyen a la degradación mediante su actividad fitásica extracelular[22]. En los casos estudiados, puede observase que en el repollo blanco la concentración inicial de ácido fítico fue de

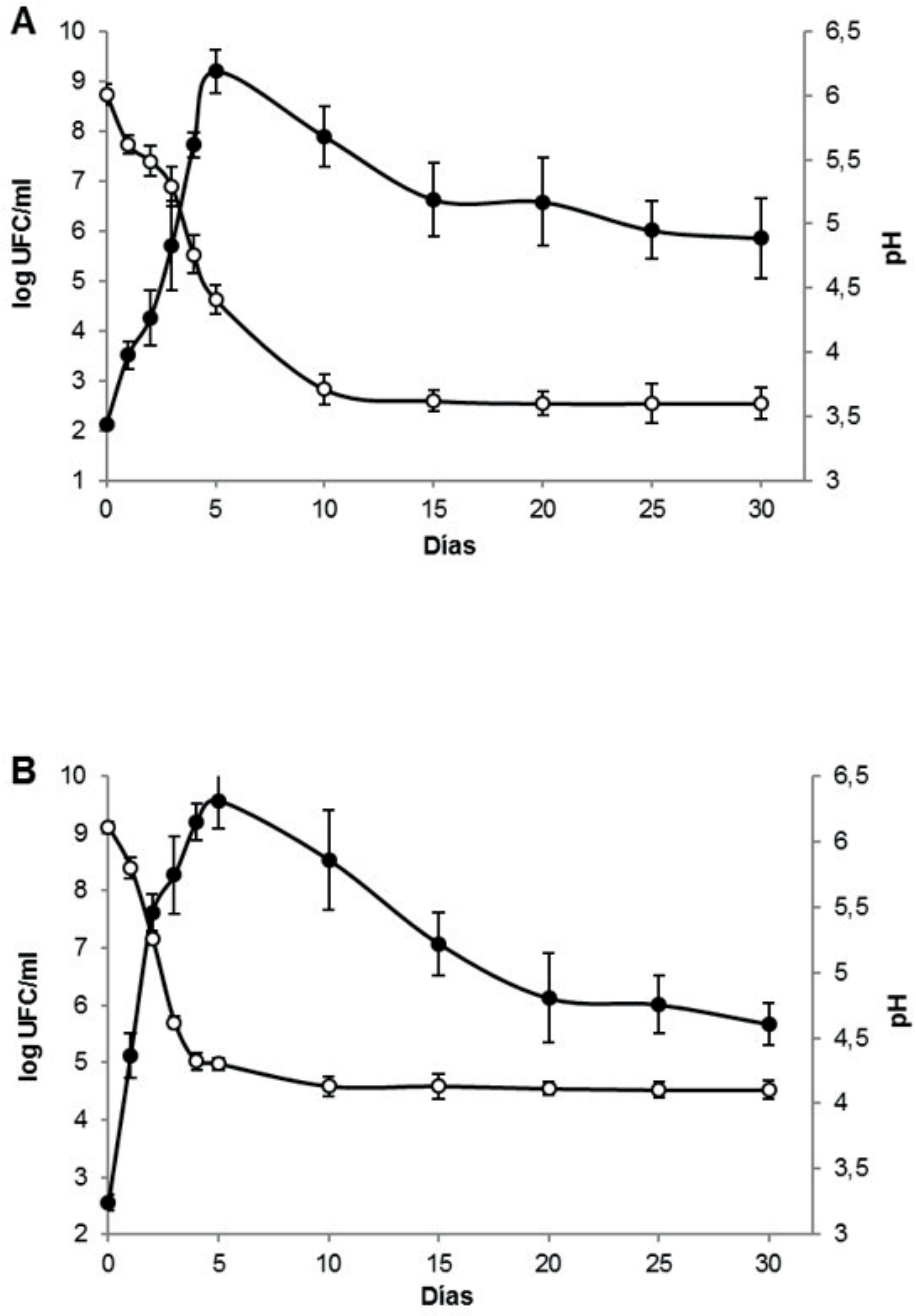


Figura 1. Evolución del pH (○) y población de bacterias ácido lácticas (log UFC/ml) (●) durante la fermentación del repollo blanco (a) y del repollo chino (b).

145,9 mg/100 ml, descendiendo hasta 101,1 mg/100 ml en el día 5 y manteniéndose estable hasta el final del proceso fermentativo. En forma inversa, la concentración de fósforo soluble aumentó desde una concentración inicial de 1,1 mg/100 ml hasta estabilizarse en el día 5 en 7,4 mg/ 100 ml manteniéndose sin modificaciones hasta los 30 días de experiencia. Mientras que, en el repollo chino se determinó una concentración inicial de ácido fítico de 119 mg/100 ml pero a diferencia del repollo blanco, el descenso se observó hasta el día 15, alcanzado una concentración de 25 mg/100 ml al final de la experiencia. En cuanto a la concentración de fósforo soluble, el repollo chino presentó una mayor concentración inicial (19,4 mg/100 ml) que en el repollo blanco; y el incremento se observó de manera continua durante todo el proceso alcanzando un valor final de 29,6 mg/ml (Figura 2).

Los diferentes comportamientos en la evolución de los parámetros estudiados se deben a las condiciones originadas durante la fermentación que influyen en la actividad de las fitasas vegetales y bacterianas. Las fitasas vegetales actúan principalmente durante la germinación con el objetivo de aprovechar la reserva de fósforo contenida en los fitatos y solo degradan 2 grupos fosfatos de los 6 que posee la molécula original[8].

Las fitasas de las BAL en cambio, son enzimas extracelulares y tienen un pH óptimo de actividad que varía entre 5,5 y 7,0. Valores de pH por debajo de 4,5 reducen sensiblemente la capacidad degradativa y a valores menores a 4,0 la actividad es casi nula[16,22,23]. En los casos estudiados, el repollo blanco, como se señaló anteriormente, posee una mayor concentración de azúcares fermentables y en consecuencia mayor producción de ácidos orgánicos, en especial láctico. Luego del día 5, el pH continuó descendiendo registrándose en el día 10, un pH por debajo de 4,0. En forma paralela se puede observar que luego del día 5, no se registra un descenso de la concentración de ácido fítico ni un aumento en la de fósforo.

En el caso del repollo chino, el descenso del pH fue menos abrupto y no descendió a valores menores a 4,3 durante los 30 días de ensayo. El descenso marcado de la concentración de ácido fítico se produjo durante los primeros 10 días, sin embargo, el proceso degradativo continuó, en forma menos marcada, hasta el final de la experiencia. Como consecuencia de este fenómeno se observó que la concentración de fósforo aumentó en forma continua durante los 30 días.

En ambos casos se puede concluir que la actividad enzimática depende de las condiciones establecidas en la fermentación y del aumento poblacional de las BAL. La capacidad nutricional de los repollos permite una rápida duplicación celular y mayor oferta de fitasa extracelular, sin embargo, el metabolismo de carbohidratos trae como resultado un descenso marcado de pH y una menor actividad enzimática[14,16].

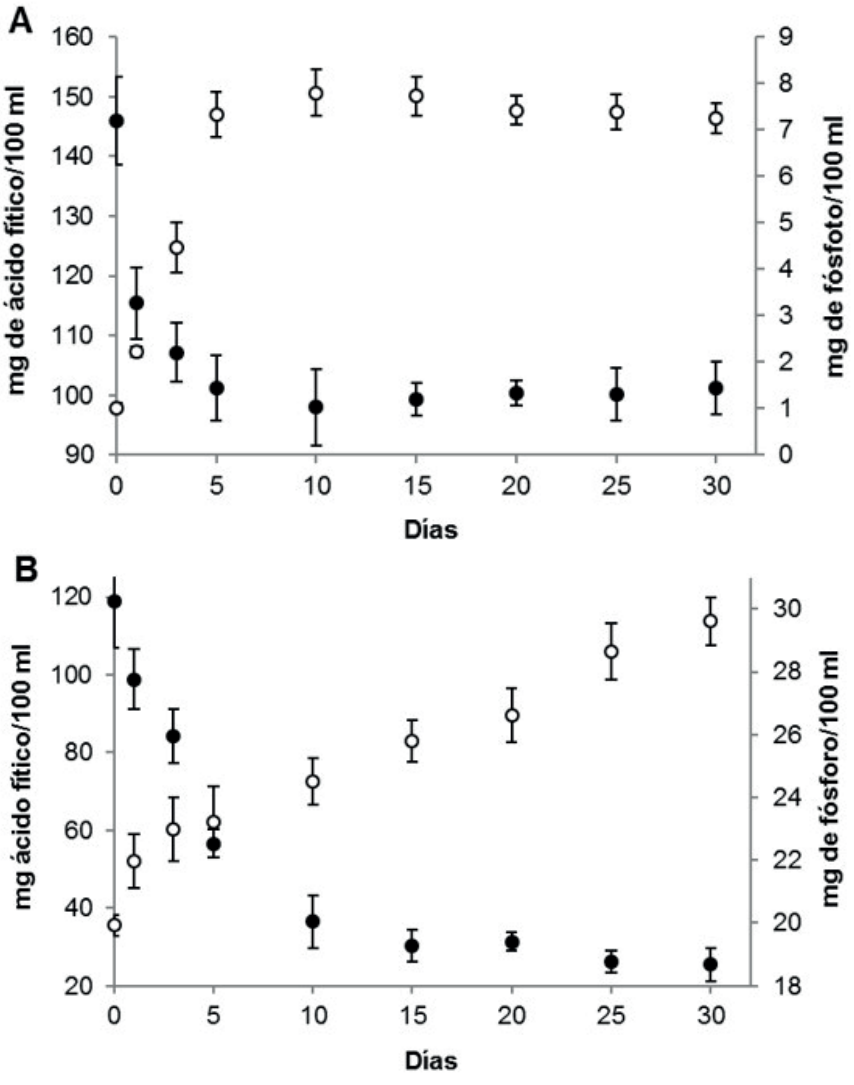


Figura 2. Evolución de la concentración de ácido fítico (●) y fósforo libre (○) durante la fermentación del repollo blanco (a) y del repollo chino (b).

Determinación de hierro libre

En la Figura 3 se puede observar la evolución de la concentración de hierro libre en los vegetales estudiados durante la fermentación. Las concentraciones iniciales son comparables y estuvieron en valores menores a 0,05 mg/100 ml. Durante el desarrollo del proceso se observa un constante aumento de la concentración, sin embargo, el fenómeno es más marcado en el caso del repollo chino que alcanzó a los 30 días 0,29 mg/100 ml mientras que el repollo blanco exhibió una concentración de 0,16 mg/100 ml.

Estos distintos comportamientos tienen correlación con la variación de la concentración de ácido fítico, su degradación implica la liberación de hierro que, en forma de quelatos, resulta inaccesible para la absorción intestinal[11]. El hierro liberado de la molécula de los fitatos tiene gran afinidad por el ácido ascórbico con el que forma quelatos estables y solubles facilitando su biodisponibilidad. Las brasicáceas contienen concentraciones de ácido ascórbico que no solo permiten esta función, sino que además su alto poder reductor transforma el hierro trivalente en bivalente, condición necesaria para ser transportado a través de la membrana del enterocito[12].

Conclusiones

Las fermentaciones en general y de las crucíferas en particular constituyen una forma fácil y económica para conservar vegetales por largo tiempo y en forma segura. Las BAL, el grupo más relevante en este proceso, no solo logran descender el pH a valores incompatibles para bacterias patógenas o deteriorantes sino que su metabolismo produce otros cambios favorables a la salud y/o nutrición. En este trabajo se determinó que, en un proceso de 30 días de fermentación, ambas brasicáceas exhibieron marcados descensos en las concentraciones de ácido fítico y, como consecuencia un aumento del fósforo soluble y de hierro libre. El efecto se observó en forma más marcada en el repollo chino, su menor oferta de carbohidratos no permite un descenso de pH tan abrupto como en el repollo blanco y, en consecuencia, se mantiene en valores compatibles con la actividad enzimática de las fitasas.

Contribuciones de los autores

Conceptualización, Parada R., Vallejo M. y Marguet E.; metodología, Marguet E.; software, Parada R..; validación, Parada R., Sosa F., Marguet E. y Vallejo M.; análisis formal, Parada R y Marguet E.; investigación, Parada R., Sosa F., Marguet E. y Vallejo M.; curación de datos, Parada R. y Marguet E.; redacción — preparación del borrador original, Parada R. y Marguet E..; redacción — revisión y edición, Parada R., Sosa F., Marguet E., Vallejo M.; visualización, Parada R., Sosa F. ; supervisión, Parada R, Marguet E., Vallejo M..; administración de proyectos, Vallejo M. y Marguet E..; adquisición de financiación, Vallejo M. y Marguet E. Todos los autores han leído y aceptado la versión publicada del manuscrito

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Conflictos de Interés

Los autores declaran no tener conflictos de interés.

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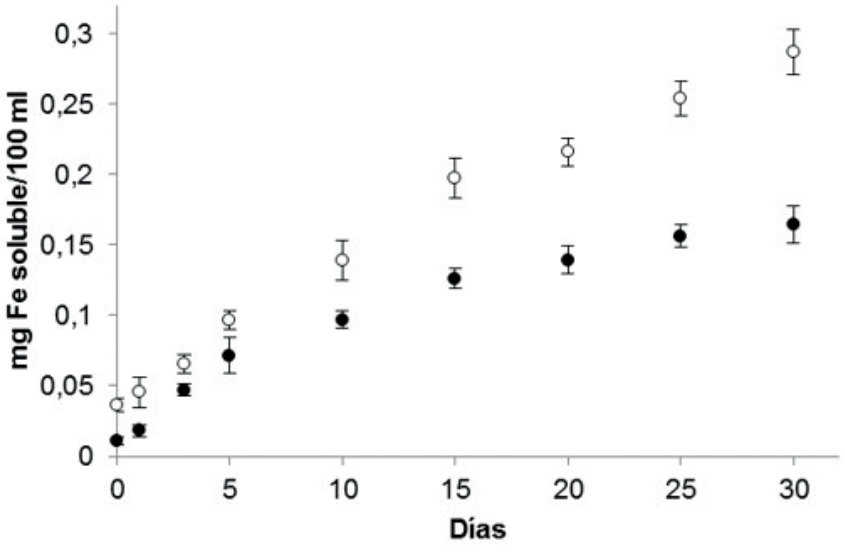


Figura 3. Evolución de la concentración de hierro soluble durante la fermentación del repollo blanco (●) y repollo chino (○).

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ARTICLE / INVESTIGACIÓN

Evaluation of Heavy Metal Pollution in the Blood Serum of Industrial Workers, Mosul, Iraq

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Abstract: The study was conducted in the two main industrial areas in Mosul, northern Iraq, where the study included measuring the number of heavy metals (cadmium, aluminum, mercury, chromium) in the blood of workers in those areas. For 20 workers, in contrast, blood samples were drawn from Mosul University employees. They were considered as a control group for being far from industrial activity and for 20 employees. An atomic absorption device was used to analyze blood samples after diluting. In addition, workers were divided into groups according to (age, smoking status, and period of occupational exposure). The results revealed statistically significant differences when comparing the samples of workers to the control group, while the results did not show significant differences between groups of workers.

Key words: Pollution, Blood Serum, Industrial Area.

Introduction

Heavy elements are characterized by their toxic behavior when they exceed the permissible limit[1], in addition to the fact that their effect on humans does not appear directly but after a period of time, and this is what gives them a cumulative character in the organs of the human body, causing many carcinogenic and mutagenic diseases and chronic infections such as lead, cadmium[2,3].

These elements are released from two natural sources, represented by natural disasters, and the other source is human, represented by multiple human activities, primarily industrial, which results from the release of these elements into the environment, causing an imbalance in the ecological balance[4,5]. These chemical elements have been linked to several health issues faced the human. For example, in a person with liver disease, the continued exposure to these elements raises the harm to this organ[6]. The effect of the heavy metals is to interfere with the conformation of the protein in the body and with enzymes functions[7].

The element mercury is characterized by its high toxicity, especially when inhaled, as it causes scratches and ulcers in the respiratory tract and is mainly produced from industrial processes[8,9].

Cadmium is highly toxic and directly affects humans through smoking, as it enters as a central component of cigarettes and products by industrial plant emissions and contaminated food and water[10,11]. Cd has a half-life period of 15-30 years and accumulates in the liver as the primary target tissue[12].

Studies have shown that Cd damage to cells is caused mainly by reactive oxygen species (ROS) production[13]. resulting in single-strand damage and destruction of nucleic acid[10]. This toxic trace metal can cause liver or renal cell apoptosis in a low dose of exposure[14].

Aluminum is one of the most abundant elements on the earth's surface, especially in the soil. Because of its high importance, researchers pay great attention to it, especially considering that humans are mainly exposed to Al through drinking water, household goods, cosmetics, and drugs[15,16].

Then, the (Al) ability to cross the blood-brain barrier features its tropism for the central nervous system[17,18]. The Al exposure is one of the predisposing factors for changes in nervous tissue, promoting the degeneration of motor neurons, affecting learning, inducing excitotoxicity[19,20]. modulating cell biochemistry and tissue integrity, and may drive behavioral dysfunctions[21,22].

Researchers such as (23) They confirmed in their study about the measurement of heavy metals in human blood for those in the industrial areas, corresponding to the measurement of heavy metals in the blood of Mosul University employees as a control group, where the results showed that there are significant impairment and an apparent accumulation in the blood of industrial workers of heavy elements.

(24) also indicated in their study about measuring the concentrations of heavy metals (Pb, Co, Hg) in blood Serum that a highly significant increase of $P<0.001$ in the serum of the industrial areas compared with the control group.

Materials and methods

Study Area

The two main industrial areas of the city of Mosul are the manufacture of the Wadi Okab on the right side (the western part) and the Karama on the left side (the eastern part) (Figure 1).

Study Design

The blood samples were taken from (20) industrial agents randomly in addition to taking blood samples from

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Figure 1. Location of Study Area.

(20) people as a control group working at Mosul University without a history of industrial activity. The control group is not different from the group of workers in terms of gender and age groups.

Blood samples

Samples were collected on 14-10-2019 from 10 a.m. to 4 p.m. samples were taken from 20 workers in (plastic recycling plant, mechanics, car thighs). 20 samples were taken from those working at Mosul University as a control group. 5 ml of the blood of industrial workers was taken and delivered to the laboratory for examinations by means of an automatic absorption device after separating it and isolating the serum from it.

Statistical analysis

The data was processed using the statistical program spss.

Results

The results showed significant differences for heavy metals (Al, Cr, Cd, Hg) elements. There is an apparent increase in the mix of elements measured in the industrial workers' serum from the control group (Table 1).

The group of industrial workers has been classified according to (age groups, smokers and non-smokers, occupational exposure period). Age groups (1-29) years and (30-57) showed the results to lack significant moral differences. (Table 2).

For the smokers and non-smokers category of the industrial workers' group, the results showed no significant differences between the two categories for the measured elements (table 3).

Regarding the occupational exposure period category

Elements	Grouping	N	Mean ± SD (ppm)	p- Value
Al	Workers	20	1.4050 ± 0.66872	p<0.001
	Control	20	0.7095 ± 0.71500	
Cr	Workers	20	0.9890 ± 0.49871	p<0.001
	Control	20	0.5545 ± 0.50351	
Cd	Workers	20	0.5590 ± 0.69120	p<0.001
	Control	20	0.3835 ± 0.20709	
Hg	Workers	20	2.1800 ± 0.90082	p<0.001
	Control	20	2.7400 ± 0.98131	

Significant P<0.05 P> 0.05 No significant

Table 1. Heavy metals concentrations in industrial workers and control group.

Elements	Age grouping	N	Mean ± SD (ppm)	p- Value
Al	(1-15)	10	0.6500 ± 0.46542	p>0.05
	(16-35)	10	1.3000 ± 0.40662	
Cr	(1-15)	10	0.6430 ± 0.32406	p>0.05
	(16-35)	10	1.100 ± 0.29264	
Cd	(1-15)	10	0.7910 ± 0.24854	p>0.05
	(16-35)	10	1.4400 ± 0.39710	
Hg	(1-15)	10	2.3300 ± 0.38646	p>0.05
	(16-35)	10	2.2000 ± 0.52689	

Significant P<0.05 P> 0.05 No significant

Table 2. Determination of heavy metals between two ageing group of workers.

(1-19) and (20-39) years for the industrial group and all measured elements, the results showed that there were no significant differences. (Table 4).

Discussion

Because of the severe and cumulative toxicity of heavy metals, they pose a tremendous and real threat to all living organisms, including humans, as many diseases and epidemics occur to them and accumulate inside human organs for extended periods according to the occupational exposure of people, which results in cancers that infect and damage organs[25,26].

Conclusions

Industrial workers face an imminent danger of death due to the high concentrations of heavy metals to which they are exposed, and this requires rapid and urgent intervention from all health and environmental sides to protect them.

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Conflicts of Interest

There is no conflict.

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Elements	Smoking	N	Mean ± SD (ppm)	p- Value
Al	Smokers	10	0.7800 ± 0.59889	P> 0.05
	Non -Smokers	10	1.6100 ± 0.67667	
Cr	Smokers	10	0.5500 ± 0.42725	P> 0.05
	Non -Smokers	10	1.5600 ± 0.43166	
Cd	Smokers	10	0.1360 ± 0.18202	P> 0.05
	Non -Smokers	10	0.6370 ± 0.43603	
Hg	Smokers	10	1.4500 ± 0.26726	P> 0.05
	Non -Smokers	10	1.9050 ± 0.52113	

Significant P<0.05 P> 0.05 No significant

Table 3. Determination of heavy metals between smokers & non-smokers of workers.

Elements	Period of Working (year)	N	Mean ± SD (ppm)	p- Value
Al	(1-15)	10	0.6800 ± 0.49889	P> 0.05
	(16-35)	10	1.2500 ± 0.42281	
Cr	(1-15)	10	1.0200 ± 0.32725	P> 0.05
	(16-35)	10	1.4500 ± 0.25355	
Cd	(1-15)	10	1.600 ± 0.39602	P> 0.05
	(16-35)	10	1.5000 ± 0.13594	
Hg	(1-15)	10	1.1500 ± 0.36575	P> 0.05
	(16-35)	10	1.3500 ± 0.24334	

Significant P<0.05 P> 0.05 No significant

Table 4. Determination of heavy metals between workers depending on the work period.

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ARTICLE / INVESTIGACIÓN

Identification and phylogenetic characterization based on DNA sequences from RNA ribosomal genes of thermophilic microorganisms in a high elevation Andean tropical geothermal spring

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Abstract: Several microorganisms can survive in harsh acid environments in geothermal springs at high temperatures across the Equatorial Andes Mountains. However, little is known about their physiological features and phylogenetic composition. Here we identify thermophilic microorganisms (bacteria, fungi, and microalgae) hosted in an almost unexplored geothermal spring (known as “Aguas Hediondas”). The phylogeny of the cultures was determined by analyzing physiological features and DNA sequences of PCR products for 16S rRNA, ITS, and 23S rRNA genes. Twenty pure cultures were isolated from the samples, including 17 for bacteria, one for cyanobacterium, one for eukaryotic microalgae, and one for fungus. Most bacterial strains were gram-positive, spore-forming, and bacilli (*Bacillus*). Cyanobacterium strain belonged to *Chroococcidiopsis* and the eukaryotic microalgae to *Chlorophyta*. The unique fungal strain isolated was closely related to *T. duponti*. Through our study, isolated thermophilic bacteria, microalgae and fungi from the “Aguas Hediondas” geothermal spring were characterized and identified. This study represents one of the first extensive molecular characterizations of extremophile microbes in the Tropical Equatorial Andes.

Key words: Microbial diversity, DNA markers, extremophiles, phylogenetics.

Introduction

There has been a growing interest in studying extreme ecological niches and microorganisms living under extreme environmental conditions in the last decades. The increasing socio-economic and scientific relevance in discovering the biodiversity of extremophile microorganisms is primarily due to the direct and indirect (by-products) use in biotechnology and industries. Historically, a great diversity of microorganisms has been found in hostile conditions of geothermal environments[1–5]. Microorganisms living in geothermal springs have developed several adaptations to survive hostile environments[6]. For instance, these organisms can synthesize enzymes that work under high temperatures, high salt concentrations, high alkalinity or acidity conditions, and under high pressure[7,8]. Their capacity to maintain their metabolic activity under these extreme conditions makes these enzymes (synthesized by thermophilic organisms) incredibly attractive for developing various human applications in diverse fields such as medicine, cosmetics, and the food industry[7,8]. The culture-dependent method has played a crucial role in isolating microbes and preserving them for further research on biotechnological applications and developing new bioproducts[9].

The biodiversity in geothermal springs is an adaptable response to the environmental conditions correlated with the mineral composition, pH, gases, salinity, redox potential, nutrients availability, and temperature variables[10,11]. Additionally, climate change and anthropogenic activities can cause significant changes in the water chemistry of lentic and lotic ecosystems, which may affect the microbial biodiversity of these unique ecosystems. Biodiversity in tropical mountains is wealthy and high, particularly across the Andean mountains around Ecuador[12]. Around 25 % of all terrestrial areas on Earth are mountain regions hosting more than 85% of the world’s species of amphibians, birds, and mammals, many exclusively restricted to mountains[13]. Moreover, it has been well documented that hot spots in the tropical Andean mountains, including water reservoirs, can hold higher diversity than wet lowlands[14]. The biodiversity of the different mountain ecosystems reflects the great importance of the evolutionary and ecological processes in these regions, a history worth understanding, preserving, and protecting.

Our study emphasizes microbial diversity in high-elevation Andean geothermal spring waters within these ecosys-

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tems. The survival of thermophiles depends on temperature regulation processes, activities, and behavior[15–17]. It has been proposed that microorganisms shape ecosystems to make the environment more suitable for life[18–20]. Evidence for symbiotic assemblages has been reported for diverse microbial communities in the microbe mats co-existing in hot springs[21,22]. Main groups of microorganisms found in thermophilic environments, like archaea, bacteria, microalgae, and fungi, have adaptations to adverse conditions[15,23,24].

Only a few studies have addressed the microbial and physicochemical characterization of "Aguas Hediondas" geothermal spring; however, they revealed only the diversity of microalgae communities without considering other important groups of microorganisms[25,26]. Most existing reports have focused exclusively on tourism and geothermal energy production applications[27–30]. It is believed by the local people that water from the geothermal spring has medicinal and healing properties[31,32]. We find these proposed properties interesting to investigate, therefore relevant to characterizing the microbial communities in "Aguas Hediondas."

This study aimed to identify thermophilic microorganisms in a little-explored hot spring in the Equatorial Andes using culture-dependent methods and classification based on DNA sequences from RNA ribosomal genes.

Materials and methods

Site characteristics and sample collection

Sediment and water samples were collected from the "Aguas Hediondas" geothermal spring located in Carchi province in northern Ecuador (00º 48.587'N & 77º 54.362'W) at 3428 m.a.s.l. The samples were taken from three different places in the same geothermal spring in April 2016. Three replicates were taken at each sampling point. Each replicate consisted of 50 ml of water and a portion of sediment from the bottom of the spring. The samples were maintained at 50 ºC during transportation and storage until the culture using a portable incubator (BIOBASE). The average temperature recorded in the three geothermal water points was 54.67±1.63 ºC. Environmental water and sediment samples were collected under permit # MAE-DNB-CM-2017-0071 granted by the Ministry of Environment of Ecuador. Water and sediment samples were sent for physicochemical analysis to a commercial service provider.

Media and culture conditions

Samples were cultured within six hours after collection. Sub-samples of 100 µl were spread on specific media for each group of microorganisms. The pour plate culturing method was used to get pure isolations. Bacteria were cultured on agar M9 (Difco) between 51 and 56 ºC for 72 hours[33]. Cultivation of fungi was performed on PDA (Difco) supplemented with 50 mg l^{-1} of chloramphenicol (Sigma) at 55 ºC for 2 weeks[34]. Microalgae were cultured on BG11 (Sigma) solidified with 1.5% Difco Bacto agar at room temperature (25 ºC) with illumination between 1000-2000 lux with 12 hours light and 12 hours dark photoperiod[35]. Pure microalgae cultures were obtained by transferring part of each algal colony in 3 ml of liquid BG11 (Sigma). All the culture media were adjusted to the pH of the geothermal spring, around 5. Individual bacterial and fungal colonies were isolated by transferring to a fresh plate and incubated as indicated before prior to phenotypic characterization. Phenotypic features were examined according to colony pigmentation, texture, appearance, shape, and edge. After identification, bacterial and microalgal strains were cryopreserved in broth-glycerol (8:2) at -20ºC. Fungal isolates were preserved by culturing on PDA (Difco) dishes and then coated with mineral oil and stored at 4ºC.

Optical microscopy

All isolates were examined using a 100X magnification with a CX21 Olympus® microscope. Each isolate was characterized based on colony pigmentation, texture, appearance, shape, and edge (Supplemental Material). Mycelium structure was also observed in fungal isolates[36]. Bacterial isolates were Gram-stained for easier identification.

DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA from bacteria, fungi, and microalgae was extracted by different protocols established by Moore *et al.*[37], Weising *et al.*[38], and Cai & Wolk[39], respectively. NanoDrop 8000 UV-Vis quantified DNA purity and concentration. Fragments of 16S rRNA, ITS, and 23S rRNA genes were amplified with specific primers (Table 1) through PCR. A 25 µl PCR reaction was carried out with the kit GoTaq® Green Master Mix from Promega. The PCR reaction mix was composed of 7 µl of ultrapure water, 12.5 µl of GoTaq® Green Master Mix (2X), 1.5 µl of each primer (10 µM) and 2.5 µl of DNA template. The protocol used for PCR amplification was 94°C for 5 min, 35 cycles of denaturation at 94°C for 30-sec primer annealing at 50°C for 1 min and extension at 72°C for 7 min and 30 sec with a final extension at 4°C for 10 min. The PCR products were visualized on 1 % agarose gels using GelStar™ dye. Amplified DNA fragments were sent for sequencing to a commercial sequencing service provider (Macrogen, South Korea).

Gen	Sequence (5'-3')	Target	Size (pb)	Reference
16S rRNA	27F: AGATTTGATYMTGGCTCAG	Bacteria	~1500	37
	1492R: ACGGYTACCTTGTTACGACTT			
23S rRNA	p23srV_f1: GGACAGAAA-GACCCTATGAA	Microal-gae	~410	39
	p23SrV_r1: TCAGCCTGTTATCCCTA-GAG			
ITS	ITS1F-Bt1: CTTGGTCATTTAGAG-GAAGTAA	Fungi	~650	38
	ITS4Rbt: TCCTCCGCTTATTGATATGC			

Table 1. PCR primer sequences used in this study.

Blast, sequence alignment, and phylogenetic analyses

DNA sequences used for this analysis are available under GenBank accession numbers MT765288-MT757927, MT757926, and MT764950-MT764966. To search for the similarity of the DNA sequences, the GenBank database (NCBI, National Centre for Biotechnology Information) was applied using the Basic Local Alignment Search Tool (BLAST) based on the most similar matches (>99% similarity). The obtained sequences were aligned using Geneious Prime® 2020.2.2 software through MUSCLE and ClustalW alignment methods and the online tool (https://mafft.cbrc.jp/alignment/server/index.html) for the MAFFT 7 alignment method. Phylogenetic reconstruction was performed with the Markov Chain Monte Carlo (MCMC) Bayesian approach implemented in BEAST version 1.10.4. Phylogenetic analysis was carried out with the GTR+Gamma as the best substitution model suggested by the software jModelTest for DNA sequences alignments[40]. A non-parametric Bayesian Skyline (Piecewise-constant) coalescent model was used with a Strict molecular clock method. MCMC was developed with 20 million generations, subsampled every 1000 generations by applying a Random as the starting tree. Moreover, a Marginal Likelihood Estimation (MLE) using path sampling (PS) / stepping-stone sampling (SS), which performs an additional analysis after the standard MCMC chain has finished, was implemented. The MCMC/MLE analysis output was summarized using TreeAnnotator software included in the Beast package (.log files are provided in the supplemental material as a support of the analysis). The maximum Clade Credibility tree was produced after discarding 10 % of burn-in. The final tree was visualized through FigTree version 1.4.4.

Results

The mean water pH measured was 4.8, while the sediment means pH was 5.8. Water and sediment samples showed distinct compounds and concentrations. Water contained 0.054 mg l^{-1} of arsenic, 83.1 mg l^{-1} of chlorides, 0.53 mg l^{-1} of iron, 2.5 mg l^{-1} of manganese 225 mg l^{-1} of sodium, 45.4 mg l^{-1} of potassium, 168.44 mg l^{-1} of magnesium, 72 mg l^{-1} of calcium, and 744.8 mg l^{-1} of sulphates. Water samples composition included volatile and non-volatile solids with 120 and 1 290 mg l^{-1} respectively. The sediment portion had the following compounds 1.3 mg kg^{-1} of cadmium, 29.3 mg kg^{-1} of copper, 312 mg kg^{-1} of potassium, 12 500 mg kg^{-1} of iron, 79 mg kg^{-1} manganese, and 1 860 mg kg^{-1} of magnesium. Sediments also contained organic matter, which represents approximately 60.59 % mean for all the samples. Electrical conductivity (EC), an indicator of water quality and inorganic constituents' presence, was 0.1725 Sm^{-1} in water and 0.0164 Sm^{-1} in sediments. Higher EC levels denote higher total solids dissolved concentrations (TSD). EC is an indicator of dissolved minerals.

A total of 20 pure cultures were isolated, including 17 bacterial isolates, one cyanobacterium, one eukaryotic microalga, and one fungus. Most bacterial isolates were gram-positive spore-forming bacilli. The unique fungal strain isolated produced a grey dusty mycelium. Microalgae colonies were light green (eukaryotic) and bluish-green (cyanobacterium) (Figure 1).

Table 2 shows the relationships of isolated geothermal strains with the most similar sequences from the Genbank database.

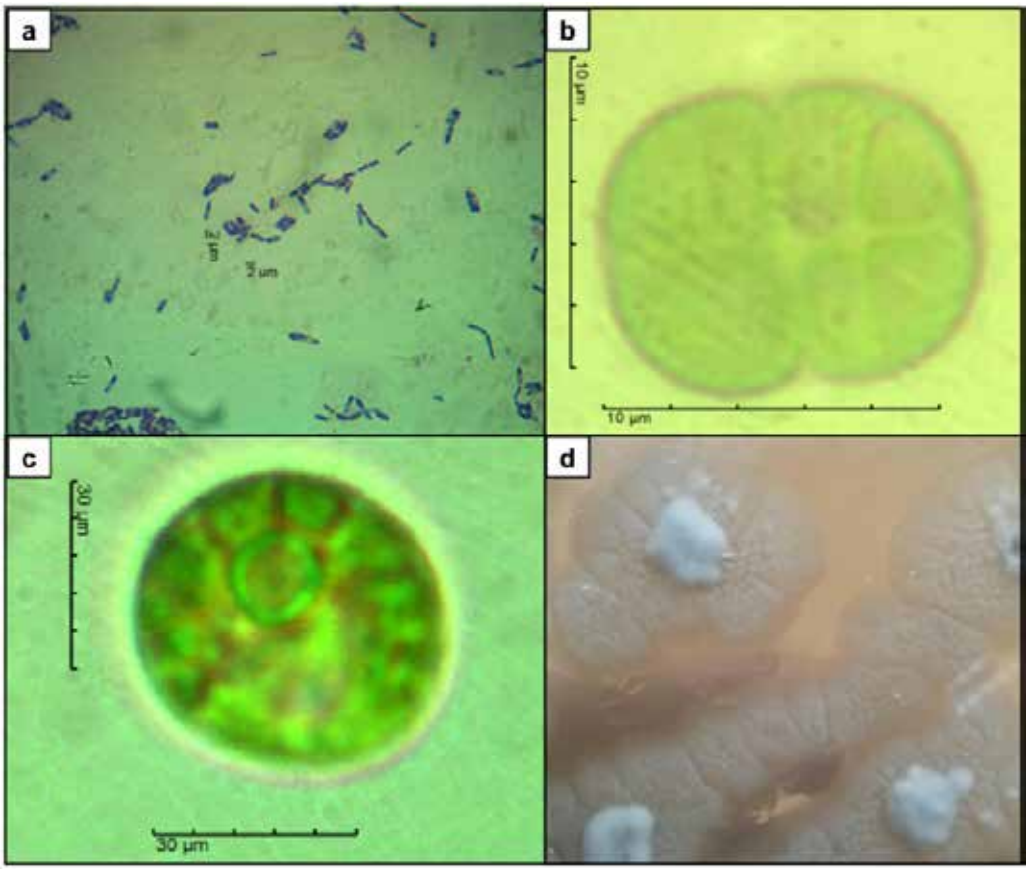


Figure 1. Microbial strains isolated. (a) Bacteria (*B. licheniformis*). (b) Cyanobacterium (*Chroococcidiopsis*). (c) Eukaryotic microalgae (*C. vulgaris*). (d) Fungi (*T. thermophilus*).

strain	BLAST	Accession no.	Coverage (%)	Identity (%)
1R	*Bacillus licheniformis*	NR_118996	100	97.3
2R	*Bacillus licheniformis*	NR_118996	98	96.1
3R	*Bacillus licheniformis*	NR_118996	97	94.7
6R	*Bacillus licheniformis*	NR_118996	100	97.2
7R	*Bacillus licheniformis*	NR_165685	100	99.1
10R	*Bacillus licheniformis*	NR_165685	100	99.0
18R	*Bacillus licheniformis*	NR_118996	100	96.1
20R	*Bacillus licheniformis*	NR_118996	99.93	99.4
21R	*Bacillus licheniformis*	NR_118996	100	98.1
24R	*Bacillus licheniformis*	NR_165685	81.29	97.7
25R	*Bacillus licheniformis*	NR_118996	100	98.6
30R	*Bacillus licheniformis*	NR_118996	100	95.3
32R	*Bacillus thermoamylovorans*	NR_117028	99.93	99.5
34R	*Bacillus licheniformis*	NR_118996	100	99.5
35R	*Bacillus licheniformis*	NR_165685	100	98.7
36R	*Bacillus thermoamylovorans*	NR_144578	99	98.47
37R	*Bacillus licheniformis*	NR_118996	99	97.4
R1A	*Chroococcidiopsis thermalis*	NR_102537.1	100	95.1
R2A	*Chlorella mirabilis*	KM462865.1	99	94.1
RR	*Thermomyces duponti*	NR_163517.1	99	99.8

Table 2. Sequences from GenBank producing significant alignments.

Figure 2 presents the phylogenetic tree of 17 isolated bacterial DNA strains. Two clades are marked, where the distance was compared by measures based on rRNA sequence divergence. All the bacteria (17) were contained within the genus *Bacillus* in the phylum *Firmicutes*. The nucleotide similarity in the ribosomal RNA gene sequences reveals that the bacterial strains were separated into two main groups: a branch closely related to *Bacillus licheniformis* (Figure 2a), and a group closely related to strains of *Bacillus thermoamylovorans* (Figure 2b).

On the other hand, the BLASTn analysis exhibits that the only fungal isolate recovered from the hot spring was classified as closely related to *Thermomyces duponti* (Table 2).

Finally, the prokaryote microalgal isolate was a cyanobacterium classified as closely related to *Chroococcidiopsis thermalis*, and the eukaryote microalgal isolate was categorized as closely related to *Chlorella mirabilis* within *Chlorophyta* (Table 2).

Discussion

The predominant presence of bacteria compared to fungi and microalgae may be due to the intrinsic geochemical properties of hot springs[41]. However, the culture process must also be considered a restrictive issue for the determination of abundance. It is well established that approximately 1% of microorganisms can be readily cultivated in vitro[42,43]. Because most microorganisms remain unculturable, the diversity in our study is inevitably underestimated using culture-dependent methods. For instance, M9, PDA, and BG11 are media that might not be the best for the recovery of 'unculturable' microorganisms from environmental samples[44]. Methods, including the use of dilute nutrient media, could be a great approach to the recovery of 'unculturable' microorganisms adapted to oligotrophic conditions[45].

Seventeen bacterial strains isolated in our study belong to the genus *Bacillus*, gram-positive and spore-forming. Similar findings were reported by Darland and collaborators[46], who observed that the genus *Bacillus* predominates in geothermal springs at Yellowstone National Park, especially those gram-positive with a pronounced tendency to form endospores. Bacterial strains isolated were classified as closely related to two species: *B. licheniformis*, and *B. thermoamylovorans* (Fig. 2). There are several reports of same species growing at similar conditions of temperature and pH in other geothermal springs around the world[47–54]. *B. licheniformis* has been found in different geothermal springs in Ecuador, including Papallacta, Chachimbiro, Guapán, and Baños de Agua Santa[55–57].

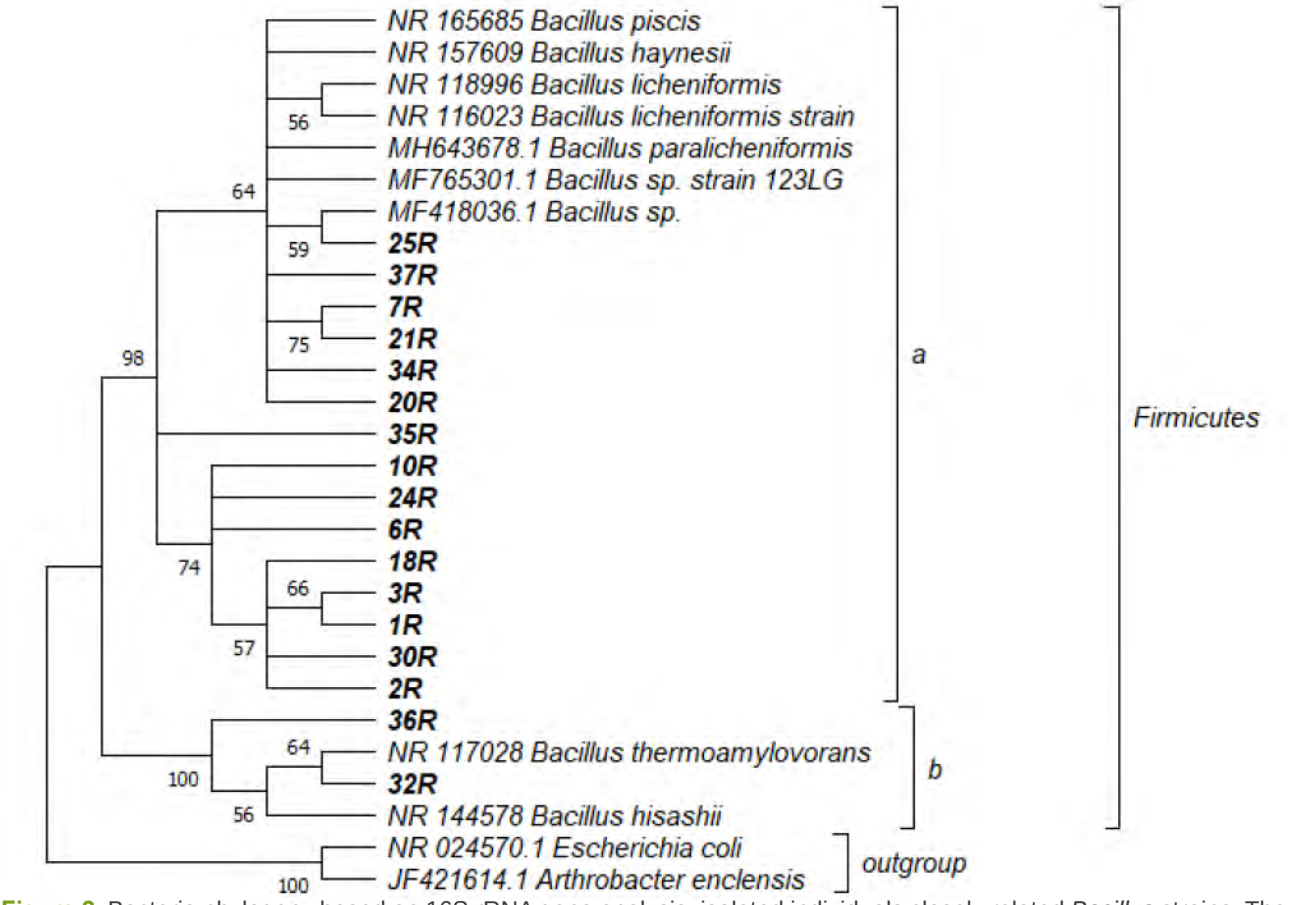


Figure 2. Bacteria phylogeny based on 16S rRNA gene analysis: isolated individuals closely related *Bacillus* strains. The phylogenetic tree was constructed using 1456 bp and maximum likelihood analyses under a GTR+Gamma Sites model. Numbers at nodes are support values generated from 1000 bootstrap replicates. GenBank accession no. MT764950-MT764966.

In the phylogenetic tree, *E. coli* and *A. enclensis* are located outside of the branch close to the genus *Bacillus*, as it should be expected, because these organisms belong to different phyla: *Proteobacteria*, *Actinobacteria*, and *Firmicutes* respectively[58–62]. Wei Wang[63], also confirmed the position of *B. thermoamylovorans* concerning *B. licheniformis*. Furthermore, Yang Liu *et al.*[64] and Christopher Dunlap *et al.*[65] described that *B. licheniformis* and *B. paralicheniformis* are sister taxa sharing a common ancestor.

The Fungal ITS sequence analysis exhibited an association between *T. duponti* and the isolated strain (Table 2). *T. duponti* has been previously isolated from sediment samples in China's Tengchong Rehai National Park hot springs[66]. Optimal growth conditions are similar with these found in “Aguas Hediondas” spring. Pan *et al.*[66] established an optimal growth temperature for this fungus of 45-50°C and tolerable pH ranges of 4-12. *Thermocyces dupontii* belong to the order *Eurotiales* (Phylum *Ascomycota*)[67,68].

The examined samples in “Aguas Hediondas” geothermal spring contained prokaryotic and eukaryotic microalgaes. According to our analysis, isolated algae strains were classified as closely related to two species: *Chroococcidiopsis thermalis*, and *C. mirabilis* (Table 2). *C. thermalis* are part of the phylum *Cyanobacteria*[69]. Moreover, *C. mirabilis* is an eukaryotic algae[69]. Both species have been previously isolated from a similar environment. The genus *Chroococcidiopsis* has mainly been isolated from the interior and exterior of rocks in hot and cold deserts of the planet[66,70]. It appears to be highly tolerant to desiccation[66,70]. Other studies have identified strains of the genus *Chroococcidiopsis* in geothermal springs in northern Thailand with an optimal growth temperature of 50°C and pH 8[71]. The eukaryotic microalgae *C. mirabilis*, which belongs to *Chlorophyta*, has been established as a phylum with an important role in algal communities from geothermal springs of Bulgaria[72]. The data showed that 75 of the 200 species surveyed correspond to the phylum *Chlorophyta*, which can grow at temperatures in between 30 to 101ºC, and pH between 1-10[72]. Our study is significantly different from local previously algal characterizations. Morales *et al.*[25] reported the predominant presence of *Closterium sp.*, *Maougeotia sp.*, *Navícula sp.*, *Dictyosphaerium sp.*, and *Ulothrix sp.* in the Aguas Hediondas geothermal spring.

Ribosomal RNA barcodes were used for the identification and phylogenetic analysis of isolates because these kinds of markers are universal and composed, at the same time, of highly conserved as well as variable domains[73]. Even if some researchers consider that ribosomal RNA is the best target for studying phylogenetic relationships, choosing the most suitable barcode could contribute to the resolution enhancement of the analysis presented in this study[74]. Due to the increasing amount of information, 16S and 18S rRNA barcodes could improve the characterization of prokaryotic and eukaryotic microalgal isolates.

Because of their potential to produce thermostable extracellular enzymes with essential biotechnological uses, thermophilic and thermotolerant microorganisms are of significant economic value[75–77]. The benefits of using ther-

mostable enzymes for biotechnological processes at elevated temperatures include reduced risk of contamination by mesophilic microorganisms[78], decreased reaction-medium viscosity, increased bioavailability and solubility of organic compounds, and increased substrate and product diffusion coefficients, resulting in faster reaction rates[79]. The species of microorganisms isolated in this study have been previously reported as producers of thermostable lipases[51], cellulases[80], xylanases[81], and pectinases[82]. There are several reports of the use of these kinds of enzymes in biotechnological processes such as biobleaching of paper pulp[83], animal feed production[83], fermentation of sugars to obtain biofuel from cellulosic wastes[84,85], fruit juice extraction and clarification[84,85], refinement of vegetable fibers[86], degumming of natural fibers[86], curing of coffee[86], cocoa[86], and tobacco[86], and waste-water treatment[86]. Further research is needed to elucidate the biotechnological applications of the isolated microorganisms in this study.

Conclusions

Through our study, isolated thermophilic bacteria, microalgae, and fungi from the “Aguas Hediondas” geothermal spring were characterized and identified. These results are confirmed by previous studies in the phylogeny and characterization of other geothermal waters. Our results represent an initial contribution to the study of thermophilic microorganisms in the geothermal spring “Aguas Hediondas”.

Author Contributions

Conceptualization, R.R.-P. and A.I.; methodology, R.R.-P., A.A.-N. and A.I.; software, R.R.-P. and A.A-N.; validation, R.R.-P.; formal analysis, R.R.-P., A.A.-N.; investigation, R.R.-P.; resources, A.I.; data curation, R.R.-P.; writing—original draft preparation, R.R.-P, K.S., D. B.-G.; writing—review and editing, R.R.-P, K.S., D. B.-G., A.A.-N., A.I.; visualization, R.R.-P.; supervision, A.I.; project administration, A.I.; funding acquisition, A.I. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement

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Data Availability Statement

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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ARTICLE / INVESTIGACIÓN

Morphological and histological study of respiratory system of rabbits (*Oryctolagus cuniculus*)

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Abstract: The rabbits are considered small mammals that had, in comparison to big animals, they have a high metabolic rate and oxygen requirement. Because the larynx is located at a high angle to the oropharynx, it may immediately access the nasopharynx of rabbits categorized as nose breathers. The lungs were symmetrical in size, with the right lung being more prominent than the left and situated at the trachea's bottom. The trachea is located between the left and right bronchus. Twenty rabbits were transported to the laboratory, and by the appropriate laboratory, the instrument opened the thoracic cavity, and also the organs were filmed while the respiratory organs were removed. Due to the heart interposition and its left deviation, the left lung in rabbits is smaller than the right lung, which has just three lobes. The lungs also lack septa. The trachea is separated into two bronchi, the last of which is branched into tiny bronchioles. Achieve a histological and morphological description of rabbits' respiratory tract because the rabbits are valuable models in respiratory research due to their respiratory hyperresponsiveness, which resembles asthma in humans. The larynx is located lower than the oropharynx, where it may directly access the nasopharynx, and the rabbits are nasal breathers. The primary respiratory organs of rabbits are the left and right lung, trachea and bronchi, as in humans and rats. Still, it is supported by double cartilage in the conducting portion of the respiratory system.

Key words: Respiratory system, *Oryctolagus cuniculus*.

Introduction

The respiratory system of the laboratory animals is a benefit to discovering unique species variations; therefore, it is used as animal models for direct scientific studies[1]. The lungs are the essential part of the respiratory system; they are characterized by compact soft tissue and a spongy mass of tissue covered by the pleura, which is extended from the thoracic cavity within the thorax. The lung is lobulated and has different lobes from side to side.

The right part of the lung consists of four lobes, while the left part of the lung includes three-part (lobes); the lung's organs are associated with the trachea, which branched into the bronchi of the right-left sides inside the lung tissue. The trachea is tubular that exchanges the air in and out of the lung lobes. Subsequently, the bronchi enter the lungs at the hilus region called intrapulmonary bronchus. It branched off into smaller and even smaller tubes called primary and secondary bronchioles and ended with clusters of tiny air sacs called alveoli[2,3]. Alveoli are looked like little grapes, branches or very tiny balloons. The bubble shape of alveoli is given the lungs a surprising amount of surface area[4]. In particular, Rabbits' respiratory system comprises anatomical features of the thoracic cavity and its components and the diaphragm, which serves as the significant respiratory muscles[5]. The diaphragm is a large dome-shaped muscle that contracts rhythmically and involuntarily most of the time[6].

The trachea, bronchi, and bronchioles are part of the lower respiratory system, the branching structure of the airways that supply the lungs with oxygen. Interested in studying the histological and morphological description of the respiratory tract of rabbits because the rabbits are valuable animal models; the respiratory system is considered hyper-responsive to diseases that could be closer to asthma disease in the human.

Materials and methods

Preparations of the Rabbit

Twenty male rabbits were used for this study. The rabbits were killed under good health conditions and followed the appropriate instructions of the animal ethics of the University of Kufa in Iraq. Animals were dissected directly for gross study and collected specimens for the histology study. Skin, muscles and ribs were removed to study the respiratory system. The anatomical study identified the larynx, trachea, bronchus and lungs, and the length and width of the bronchi and lungs were measured.

Histology staining of specimens

The organs are individualized and photographed, and

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the width and length of each lung and the size of the trachea and bronchi are measured; after that, the specimens of organs are kept in special containers with 10% formalin to prevent lysis of tissue for the histological study[7].

Hematoxylin and eosin stain

Take 1 gram of Hematoxylin stain powder melting in 10 milliliters of ethyl alcohol, then 20 gram of potassium alum which dissolved in 200ml of distilling water, heat the mix reach to boil, added the Hematoxylin into solution 35 boiled for 1/2 minutes finally 0.5 gram of mercuric oxide[7].

After 15 minutes in an alum hematoxylin stain jar, the slides were washed with running tap water for 5 minutes. After that, dying slides with eosin working liquid for 10 minutes. They were then transferred directly to 70% ethanol for 1 minute. The slides were placed twice in a jar containing 80% and 90% ethanol for 1 minute. Specimens were incubated 2x in absolute ethanol, then a minute in xylene. Finally, a neutral mounting media was used to attach the slides. All steps were carried out at room temperature[7].

Results

Anatomical studies and histological study

In the current study, the result showed that the thoracic cavity of rabbits is smaller than the abdominal cavity and the respiratory of rabbits consists of the upper and lower respiratory tract. The upper respiratory tract refers to the section of the respiratory system that lies above the sternal angle (on the exterior of the thorax), upper the folds of the voice, or upper the cartilage of the cricoid figure 1. The larynx was located partially between the part of the upper and lower airway of the respiratory system.The upper respiratory system consists of the nasal cavity and paranasal sinuses, the pharynx (nasopharynx, oropharynx, and laryngopharynx), and a part of the larynx. Each lung was covered by a costal surface, which was adjacent to the ribcage; a diaphragmatic surface, which was facing downward toward the diaphragm; and a mediastinal surface, which was facing toward the medium of the chest, against the heart, great vessels, and the carina has two mainstem bronchi branching off from the base of the carina. Although, The larynx and trachea, bronchi (primary, secondary, and tertiary), bronchioles (including terminal and respiratory), and lungs make up the lower respiratory system (including alveoli). The trachea was divided into two bronchi which are extended inside the parenchyma of the lung and branched into small primary and secondary bronchioles.

The left lung rabbits are smaller than the right lung, consisting of three lobes resulting in the left deviation and its heart interposition, and there are no septa in the lungs. Our study is clarified the different lengths and widths between the left and right of the lung in the table (1). Rabbits' left lungs were smaller than their right lungs and had three lobes: cranial (lobuscanialis), middle (Lobos Medius), and caudal (Lobos Cauda) (lobuscaudalis). The cranial, middle, caudal, and accessory lobes of the right lung were the biggest.

This table shows the significant (p≤0.05) length of the lung left and right and clarifies no significant (p≤0.05) between the width of the lung left and right.

The trachea

The ciliated pseudostratified epithelial tissue lines the wall of the trachea. It contains the unicellular mucous cell called goblet cell; this epithelial seated on the basement membrane, and below it is the lamina propria, which is a loose connective tissue, and then the submucosa dense irregular connective tissue, which contains seromucous glands and opens into the lumen of the trachea by a duct, and under the submucosa, its cartilage-like c-shaped and the tow ends found smooth muscle. Finally, the layer is adventitia, its loose connective tissue.

	left lung mean ± SD	Right lung R means ± SD	t.	Sig.
Length of the lung	4.4 ± 0.0725	0.420 ± 0.1642*	22.060	0.001
Width of the lung	3.216 ± 0.1553	3.290± 0.21174	1.146	0.266

Table 1. Shows the length and width Left lung and Right lung.

	left bronchus mean ± SD	R mean ± SD Right bronchus	t.	Sig.
Length of the bronchus	0.865 ± 0.0489	0.520 ± 0.0696*	13.464	0.007

Table 2. Shows the length and width Left lung and Right bronchus.
This table shows the significant (p≤0.05) length of the lung left and right and clarifies no significant (p≤0.05) between the width of the lung left and right.

	left bronchus mean ± SD	R mean ± SD right bronchus	t.	Sig.
Length of the bronchus	0.865 ± 0.0489	0.520 ± 0.0696*	13.464	0.007

Table 3. Show the Length of the Left and Right bronchus trachea 10 +0.918.

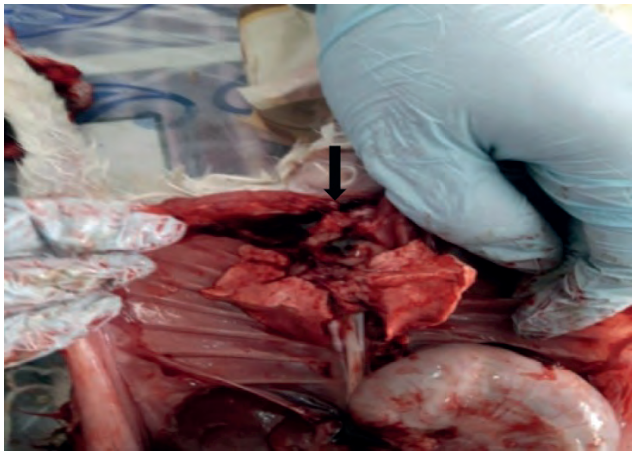


Figure 1. A photograph of the lung of the respiratory organs(black arrowhead) In the thoracic cavity

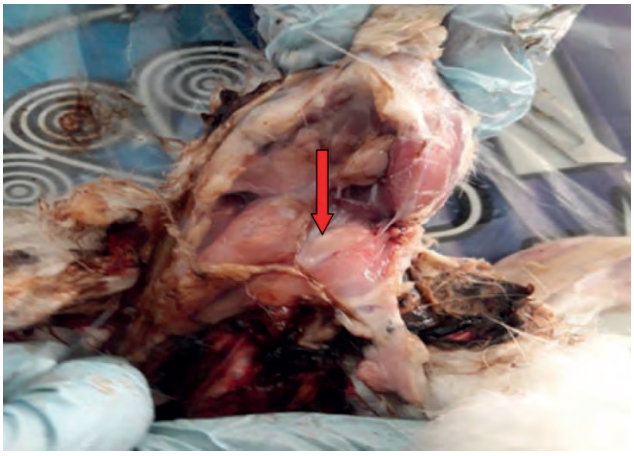


Figure 2. A photograph of the esophagus of a rabbit (red arrowhead).



Figure 3. Histological sections of the trachea, the wall of the bronchus. The pseudostratified epithelial (red arrowhead) is taken pink color. The cartilage (black arrowhead) is taken purple color. H&E stain X400.

The left and right bronchus

The lobar bronchus, segmental bronchus, subsegmental bronchus, conducting bronchiole, terminal bronchiole, respiratory bronchiole, alveolar duct, the alveolar sac, and alveolus are all found in the left and right bronchus, according to this study. Also, the trachea is the biggest tubular in the respiratory tract and is made up of hyaline cartilage tracheal rings. It divides into two major bronchial tubes, one on the left and one on the right. The bronchi are divided into smaller portions inside the lungs as tiny bronchioles. These bronchioles give birth to the alveoli, the air sacs in the lungs. The lung, which was suspended inside the pleural cavity of the thoracic cavity, was the biggest organ in the lower respiratory tract. Histologically the wall of the bronchus was lined by pseudostratified epithelial tissue with unicellular mucus cell (goblet cell) underlying loose connective tissue (lamina propria) based on the layers of the smooth muscle and submucosa consisting of denes irregular connective tissue richly with blood vessels and ducts of the seromucous gland, under it plates of hyaline cartilage that is showing in figure 4. The pulmonary artery was supplied to the bronchus, and it is branched into the left and right arteries of the bronchi. The bronchus was surrounded by a high amount of adipose tissue which was reduced with the lungs.

The wall of the intrapulmonary bronchus

In represent study that exposed the double layers of cartilage found in the wall of pulmonary branches is a unique character found only in rabbits which is present until the small bronchi.the little amount of adipose tissue around intrapulmonary bronchus comparative with bronchus. Draws attention the layer of hyaline cartilage that formed many layers, its considerably unique feature in branches in the rabbit that is showing in figure 8.

Bronchioles

The intrapulmonary bronchus is bronchus into secondary tertiary, then become much change in the wall, the pseudostratified are converted into simple columnar epithelial and absent the mucous gland and cartilage. The bronchioles were branched in many times to reach the respiratory duct. These wall features were minor in diameter, are many changes in the wall. The epithelial are binding from simple columnar with cilia and tiny goblet cells to low columnar and leak cilia and goblet cells. The respiratory system's epithelial tissue is pseudostratified, primarily epithelial in the upper respiratory tract, the trachea and pulmonary bronchus, and intrapulmonary bronchus inside the lung. It begins gradually with a tall, simple columnar epithelial transformation. It

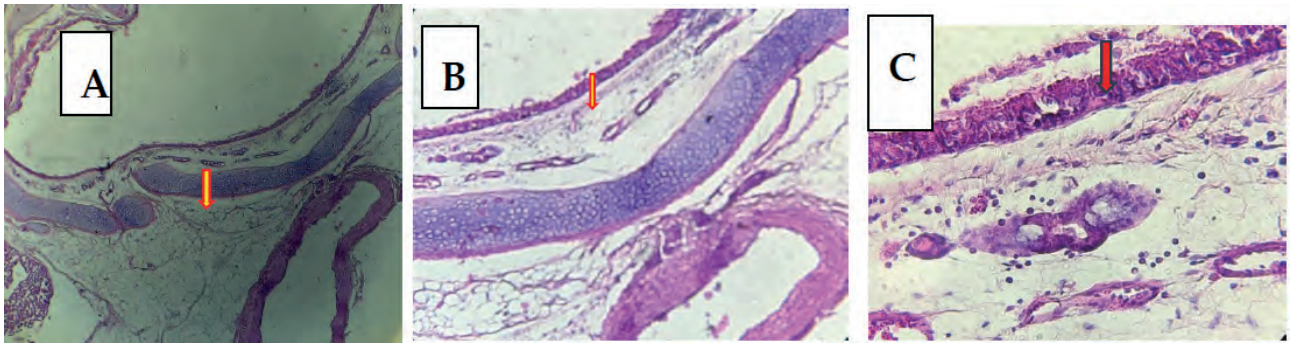


Figure 4. Histological sections of left bronchus A) Layers of the bronchus wall (red arrowhead)H&E stain. X100. B) the plate of the cartilage(red arrowhead)H&E stain. X100. C) lining by pseudostratified epithelial with goblet cells (red arrowhead) and the seromucous glands (blue arrowhead) H&E stain. H&E stain. X400.

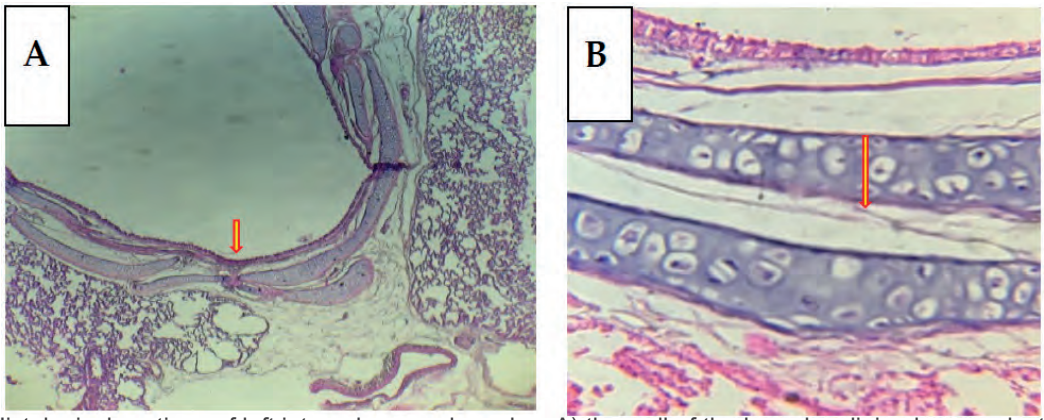


Figure 5. Histological sections of left intrapulmonary bronchus A) the wall of the bronchus lining by pseudostratified epithelial with goblet cells(red arrowhead). stain X100. B) The bronchus wall contains two layers of cartilage(red arrowhead). H&E stain. X400.

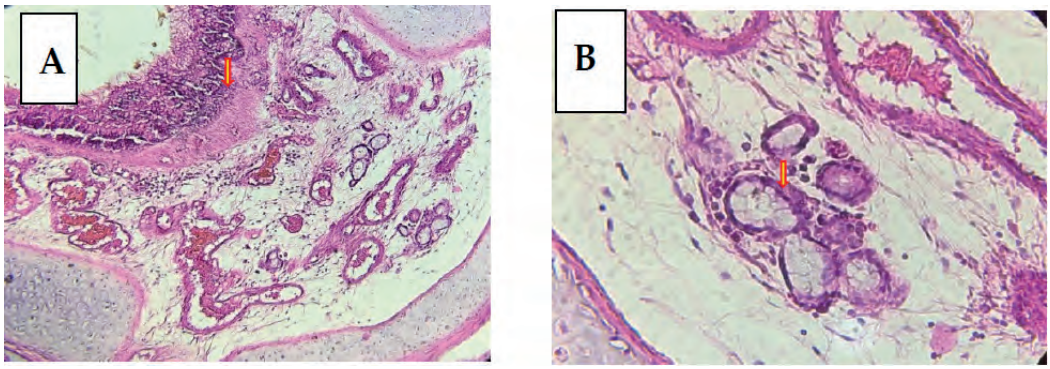


Figure 6. Histological sections of left intrapulmonary bronchus A) the bronchus wall contains blood vessels in the submucosa(red arrowhead). H&E stain. X100. B) The bronchus wall contains the seromucous glands in the submucosa (red arrowhead). H&E stain.X400.

starts with the loss of cilia gradually in bronchioles, then turns into a superficial cuboidal epithelial tissue at the terminal bronchioles. It turns into a simple squamous epithelial tissue when inter the respiratory portion.

The alveoli of the lung

The alveoli have appeared as tiny airbags cutting-edge the parenchyma of the lungs. Also, continuous diaphragm contracts generated a negative pressure cutting-edge the thorax and air dailies into the full hollow. However, the sacs were fully air, creation the lung expanded more. Alveoli were very high through alveolar capillaries. At this time, red blood cells transport the oxygen from the air by oxyhemoglobin process toward nurture cells of the body. Then red blood cells likewise are carried carbon dioxide (CO_2), missing as cells in the form of carboxy-hemoglobin, and released into the external environment by expiration. In addition, the diaphragm would be generated positive pressure cutting-edge thoracic hollow besides air dailies available of the alveoli ejecting carbon dioxide.

Discussion

In our study, the respiratory system of local rabbits (domestic) is smaller than the respiratory system of human[8,9]. also we clarified different lengths and widths between the left and right of the lung, this anatomical separate left and right of the lung of humans and many animals such as rats and guinea pig[9,10].

The tracheal tube consists of semi half-ring of hyaline

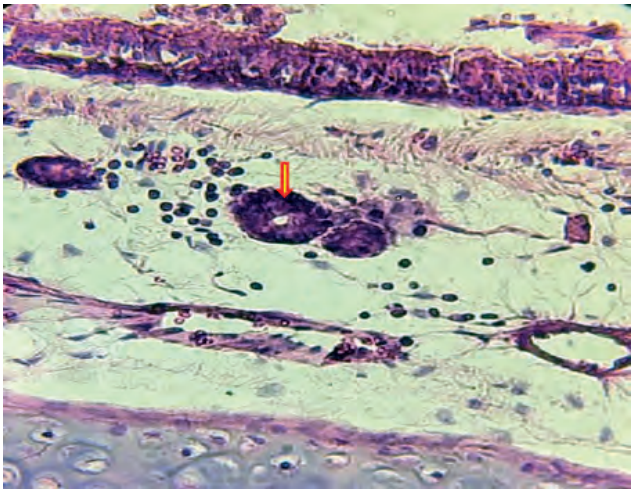


Figure 7. Histological sections of the left intrapulmonary bronchus contain ducts of the seromucous glands in the submucosa(red arrowhead). H&E stainX400.

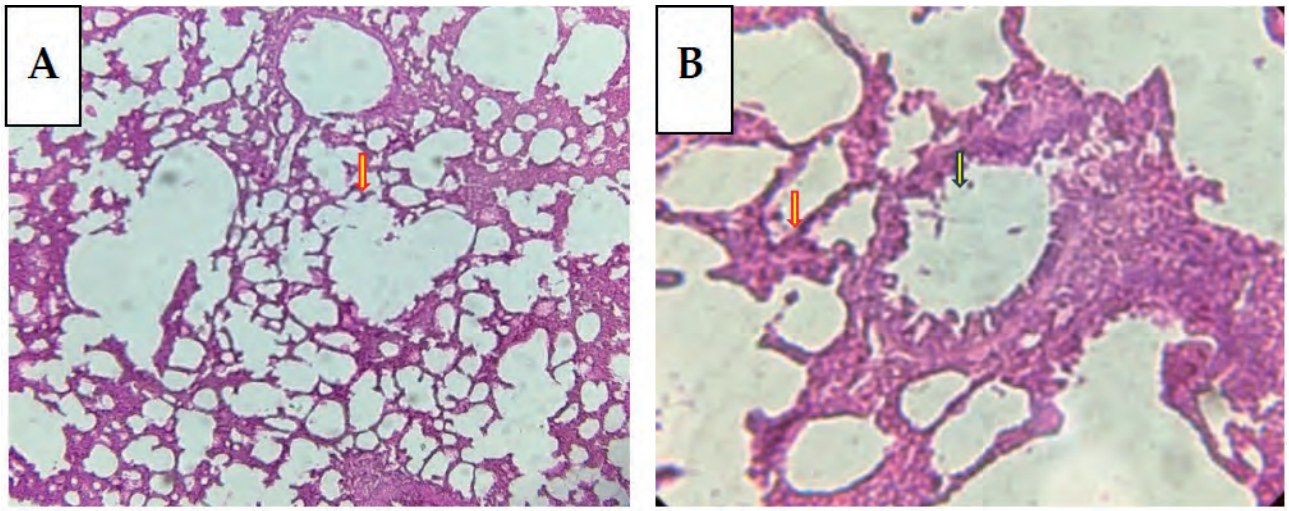


Figure 8. Histological sections of left lung A) the terminal bronchioles and respiratory ducts (blue arrowhead) and alveoli(red arrowhead). H&E stain. X100. B(the terminal bronchioles(red arrowhead). H&E stain. X400.

N=20	Mean ±SD
bronchioles	140.00±23.508
Width of bronchioles	135.00±41.675
respiratory bronchioles	66.25±12.234
Width of respiratory bronchioles	40.10±9.188

Table 4. Shows the length and width of the Left lung, Right bronchioles, and respiratory bronchioles.

cartilage. However, divisions into two bronchi, left and right, become inter into a left and right lungs named bronchioles, found in other respiratory systems in other animals[11]. But we show in this research that the rabbit's lungs are significant concerning the lower respiratory tract, which is different from other animals[12]. It may need to prepare a large amount of air for its fast and muscular movement.

Histologically the wall of the bronchus was lined by pseudostratified epithelial tissue with unicellular mucus cell (goblet cell) underlying loose connective tissue (lamina propria) based on the layers of the smooth muscle and submucosa consisting of denes irregular connective tissue richly with blood vessels and ducts of the seromucous gland, under it plates of hyaline cartilage that was showing in figure 4, this agrees in other research in different animals and humans[9,14].

Conclusions

This research shows that the alveoli appeared as minute airbags in the parenchyma of the lungs. Also, the continuous diaphragm contracts generated a passive pressure cutting-edge thorax besides air dailies into the hollow plug. This, like the other reference[13,14]. The epithelial tissue that lines the respiratory system is pseudostratified, primarily epithelial in the upper respiratory tract and the trachea and pulmonary bronchus and intrapulmonary bronchus inside the lung. It begins gradually with a tall, simple columnar epi-

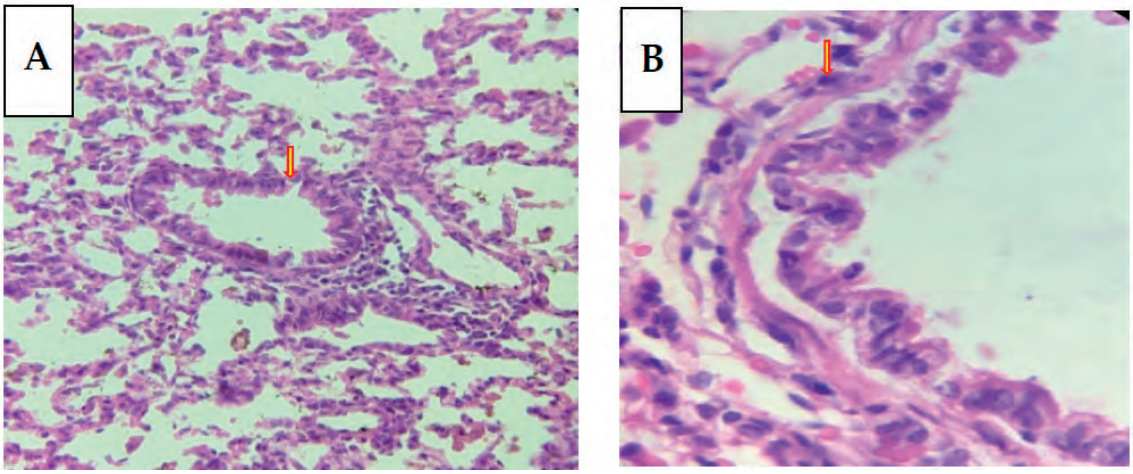


Figure 9. Histological sections of lung bronchioles of lung A) the lung's bronchioles (red arrowhead). H&E stain. X100. B) The epithelial bronchioles of the lung(red arrowhead). H&E stain. X1000.

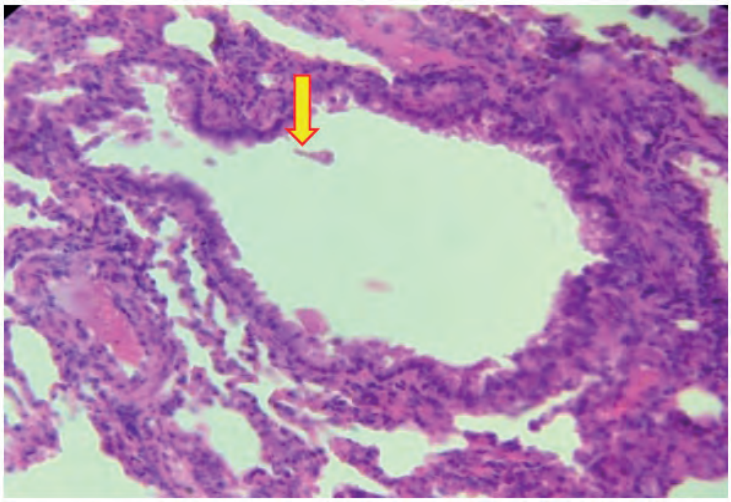


Figure 10. Histological sections terminal bronchioles of the lung(red arrowhead).H&E stain. X1000.

N=20	Mean ±SD
Long of alveoli	41.90±14.788
Width of alveoli	23.881±6.4844
Thick of the wall of alveoli	2.786±.2535

Table 5. Shows length and width of Left lung and Right alveoli

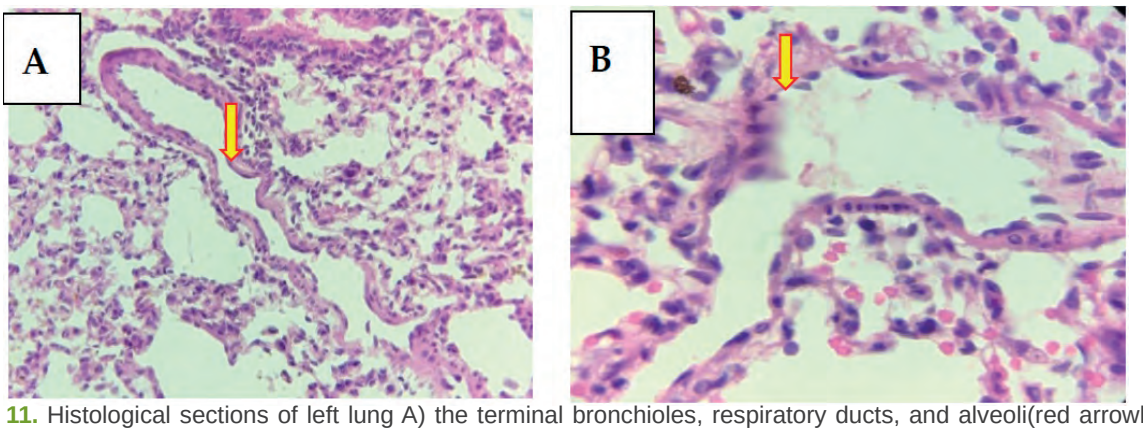


Figure 11. Histological sections of left lung A) the terminal bronchioles, respiratory ducts, and alveoli(red arrowhead). H&E stain. X400. B the terminal bronchioles with Clara cell (red arrowhead). H&E stain. X400.

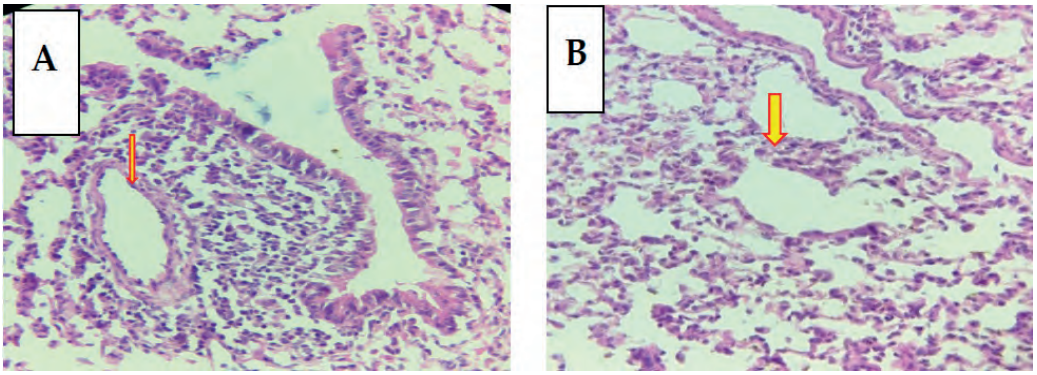


Figure 12. Histological sections of left lung A) the respiratory ducts and alveoli (red arrowhead). H&E stain. X100. B) the terminal bronchioles(red arrowhead). H&E stain. X100.

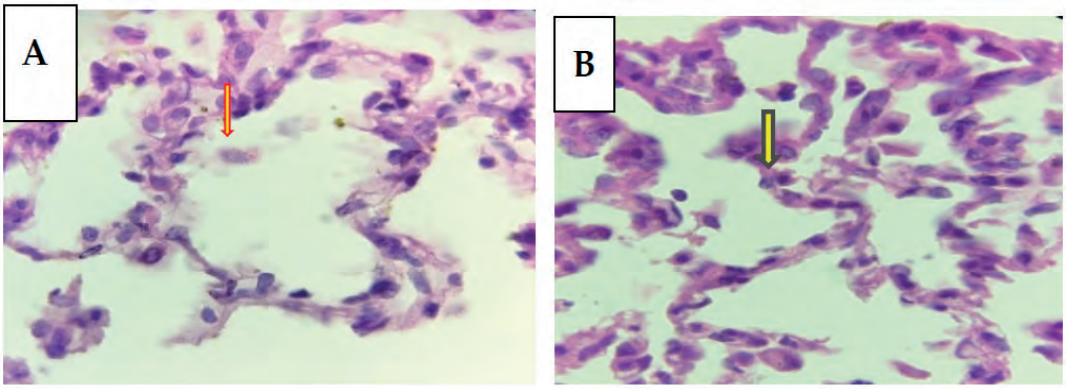


Figure 13. Histological sections of left lung A) the wall of alveoli (red arrowhead). H&E stain. X1000. B) partial cells (green arrowhead). H&E stain. X400.

thelial transformation. It begins with gradually losing cilia in bronchioles, then turns into a simple cuboidal epithelial tissue at the terminal bronchioles. It turns into a simple squamous epithelial tissue when inter the respiratory portion. Similar energies aime that goblet cells even though there are a few in the early bronchioles[15].

In represent study that exposed the double layers of cartilage found in the wall of pulmonary branches is a unique character found in local rabbits, which is currently the minor bronchi; this unique feature we found in the local rabbit. The researcher[9] performed the cartilage of pulmonary branches in humans, not double layers of cartilage.

Funding

"Self-funded this research."

Acknowledgments

I would like to extend my thanks and gratitude to all who support us.

Conflicts of Interest

None

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LETTER TO EDITOR / CARTA AL EDITOR

EndoCovid: Thyroid and adrenal hormonal alterations in a cohort of critically ill patients with COVID-19. A preliminary analysis

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Coronavirus disease 2019 (COVID-19) infection was primarily described as atypical pneumonia with severe respiratory disorders[1], although the reported evidence revealed a condition of multisystemic impact over time. It maintains a variable respiratory severity that significantly affects other organs and systems, such as the vascular endothelium, the central nervous system, the heart, and many endocrine glands such as the thyroid, the adrenals, and the pancreas[2]. The impact of SARS-Cov-2 on the endocrine system is so frequent that specialists have coined the term "EndoCovid" to refer to the endocrinological disorders that occur during this disease and require special attention in post-COVID 19 patients[3]. The universal distribution of the angiotensin 2 receptor (ACE-2), which is the proven gateway for the virus to enter our cells, makes the viral presence in glandular tissue possible and causes damage by three fundamental mechanisms: direct damage immunological mechanism and functional alteration[4,5]. Specifically, it has already been demonstrated with SARS-Cov-1 and currently with SARS-Cov-2, damage at the pituitary level[6], with hypophysitis reported in autopsies[7]. This alteration leads to secondary hypopituitarism that must be considered for its diagnosis and treatment, emphasizing cortisol deficiency, which often debuts hyponatremia, a condition associated with a poor prognosis[6]. The virus can also cause direct damage to the thyroid (COVID-19-associated thyroiditis and post-COVID-19 or post-vaccination autoimmune thyroid pathology) and the adrenal glands, which is associated with severe forms of the disease although the impact of this disorder in outcomes such as mortality is not clear[8]. Several of the critically ill patients that we attended with a diagnosis of severe COVID-19 infection, who had overcome the hypoxemia, were noticed to worsen and died after day 10 of hospitalization. A non-pharmacological distributive shock with negative bacterial cultures was determined as their cause of death. Under this premise, we requested the adrenal and thyroid hormones dosage, observing a significant hormonal deficit. The subsequent compensation of the endocrine disorder meant a significant clinical improvement and even survival of patients whose clinical characteristics and severity scales pointed to a high probability of death. We present a series of 66 patients with severe COVID-19 infection, where 28 (42.4%) died, and 38 (57.6%) survived; the patient's average age was 47.74 years, with differences being observed when comparing by discharge condition (p-value 0.024, 51.79 years in non-survivors vs. 44.76 years in survivors); male predominance was observed 81.82%. (Table 1).

Thyroid and adrenal alterations were observed in 59.09% and 68.18% of the patients. In addition, when comparing mortality between the presence or absence of thyroid and adrenal alterations, no significant differences were observed, which can be explained by the indicated hormonal replacement (adrenal and thyroid replacement were 63.64% and 62.12%, respectively). This would also explain the absence of difference in electrolyte disturbances, fever, eosinophil count and shock. Methylprednisolone was the primary corticosteroid used in adrenal hormone replacement (68.18%), and levothyroxine in thyroid replacement. The median number of days of mechanical ventilation was 8, while hospitalization stays was10 days. The hospital stay presented significant differences by discharge condition (p-value 0.049, 9 days in non-survivors vs. 12 days in survivors). (Table 2).

When comparing the median cortisol levels of COVID-19 patients at admission and 6 days later, significant differences were observed (p-value <0.001, 2.24 mcg/dL at admission vs 17.88 mcg/dL.at day 6). The data indicate that hormone replacement was effective in normalizing the adre-

Demographic characteristics	Total	Discharge condition		p-value
		Non survivor	Survivor	
Age (mean (SD))[1/]	47,74 (12,55)	51,79 (12,36)	44,76 (11,99)	0,024*
Sex (n (%))[2/]				
Male	54 (81,82)	24 (44,44)	30 (55,56)	0,481
Female	12 (18,18)	4 (33,33)	8 (66,67)	

Note: SD=standard deviation; * significance, 1/ t test, 2/Chi-square test

Table 1. Demographic characteristics by discharge condition.

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nal profile. (Figure 1).

When comparing the TSH values between patients with and without thyroid alteration, significant differences were observed (p-value <0.001, 0.26 µIU/mL for those with alteration vs. 1.11 µIU/mL for those without alteration) (Figure 2), but no significant differences were observed for T4 levels (Figure 3), which indicates that thyroid involvement is predominantly central in this series. Median cortisol values showed significant differences in the presence or absence of adrenal alteration (p-value <0.001, 2 mcg/dL in the presence of alteration vs. 17 mcg/dL in the absence). (Figure 4)

Conclusions

Based on the above, we can infer that endocrinological alterations associated with COVID-19 infection are frequent and probably more prevalent in patients who progress to severe forms and develop distributive shock with unknown infectious or pharmacological causes.

- The use of corticosteroids in patients with severe symptoms would be helpful for the management of the hyperinflammatory condition and control the adrenal insufficiency in these patients; however, further evidence is needed.
- The data regarding thyroid hormone alterations are consistent with evidence reported worldwide, where the alteration of TSH is a distinctive characteristic of subacute thyroiditis due to COVID-19 infection.

We are waiting for the new publications to be written on this exciting topic.

Clinical characteristics	Total	Discharge condition		p-value
		Non survivor	Survivor	
Thyroid disorder (n (%))[1/]				
Yes	39 (59,09)	18 (46,15)	21 (53,85)	0,461
No	27 (40,91)	10 (37,04)	17 (62,96)	
Adrenal alteration (n (%))[1/]				
Yes	45 (68,18)	19 (42,22)	26 (57,78)	0,961
No	21 (31,82)	9 (42,86)	12 (57,14)	
Vasoactive requirement (n (%))[1/]				
Yes	42 (63,64)	20 (47,62)	22 (52,38)	0,259
No	24 (36,36)	8 (33,33)	16 (66,67)	
Hypoglycemia (n (%))[1/]				
Yes	15 (22,73)	7 (46,67)	8 (53,33)	0,705
No	51 (77,27)	21 (41,18)	30 (58,82)	
Fever (n (%))[1/]				
Yes	16 (24,24)	5 (31,25)	11 (68,75)	0,299
No	50 (75,76)	23 (46)	27 (54)	
Days with corticosteroids (median (IQR))[2/]	9 (5-10)	7 (4-10)	10 (6-10)	0,084
Corticosteroid type (n (%))[1/]				
Dexamethasone	21 (31,82)	7 (33,33)	14 (66,67)	0,307
Methylprednisolone	45 (68,18)	21 (46,67)	24 (53,33)	
Hyperkalemia (n (%))[1/]				
Yes	7 (10,61)	3 (42,86)	4 (57,14)	1,000
No	59 (89,39)	25 (42,37)	34 (57,63)	
Sodium alteration (n (%))[1/]				
Hyponatremia	29 (43,94)	14 (48,28)	15 (51,72)	0,560
Normal	31 (46,97)	11 (35,48)	20 (64,52)	
Hypernatremia	6 (9,09)	3 (50)	3 (50)	
MV days (mediana (IQR))[2/]	8 (5-17)	9 (6-13)	8 (4-20)	0,840
Hospitalization days (median (IQR))[2/]	10 (7-19)	9 (6-13)	12 (8-30)	0,049*

Note: SD= standard deviation; IQR= interquartile range; * significance, 1/ Chi-square test, 2/ Mann Whitney test, MV: mechanical ventilation

Table 2. Clinical characteristics by discharge condition.

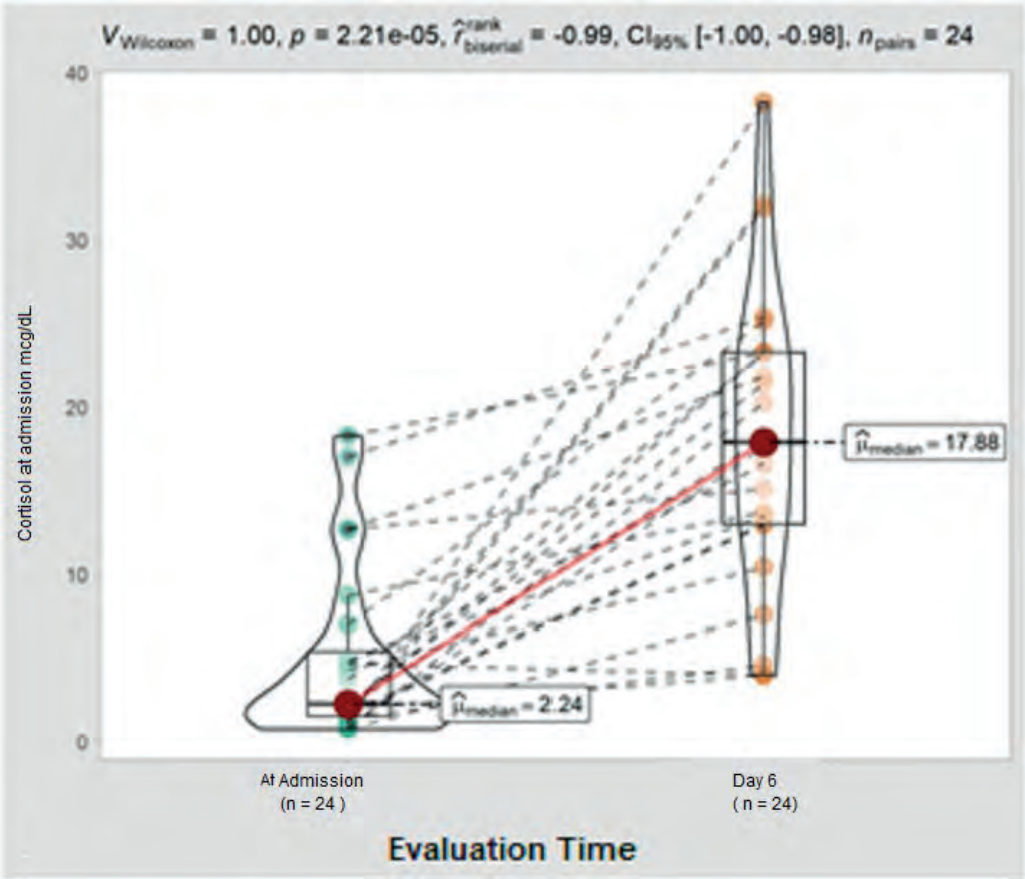


Figure 1. Comparison of cortisol at admission and six days later.

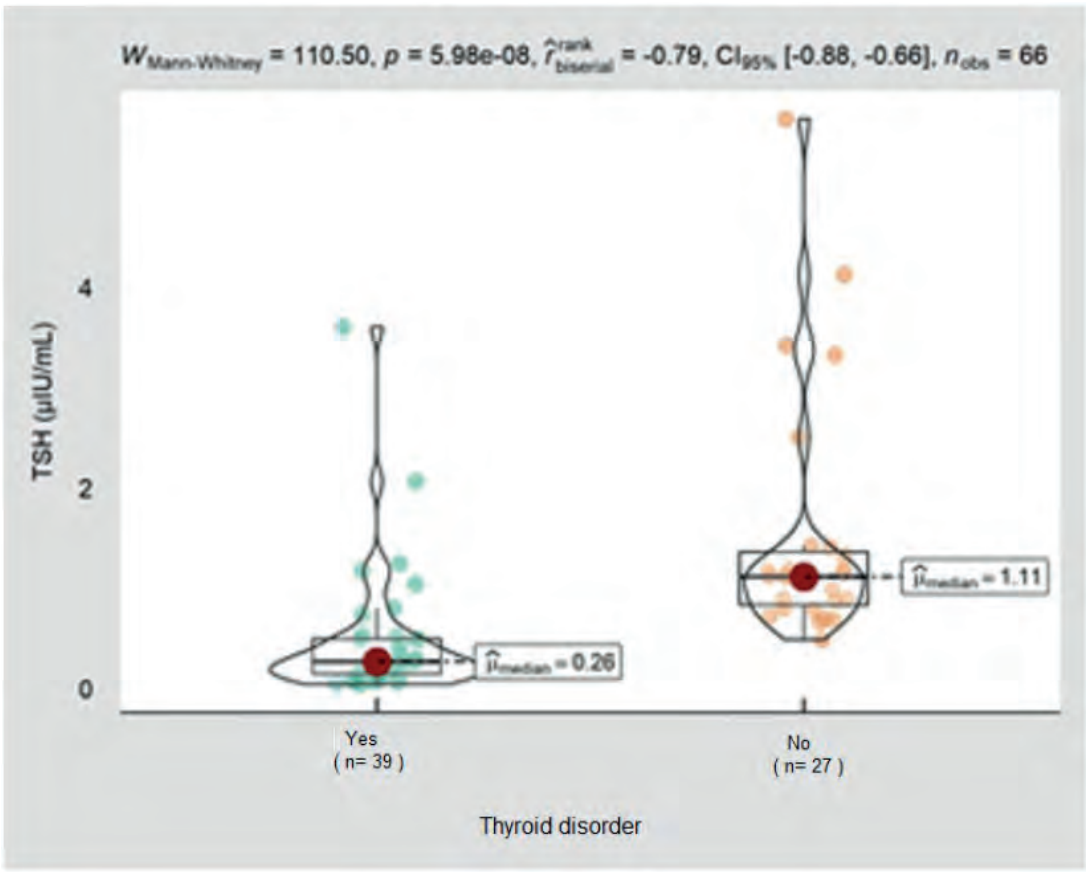


Figure 2. Comparison of TSH by presence or absence of thyroid alteration.

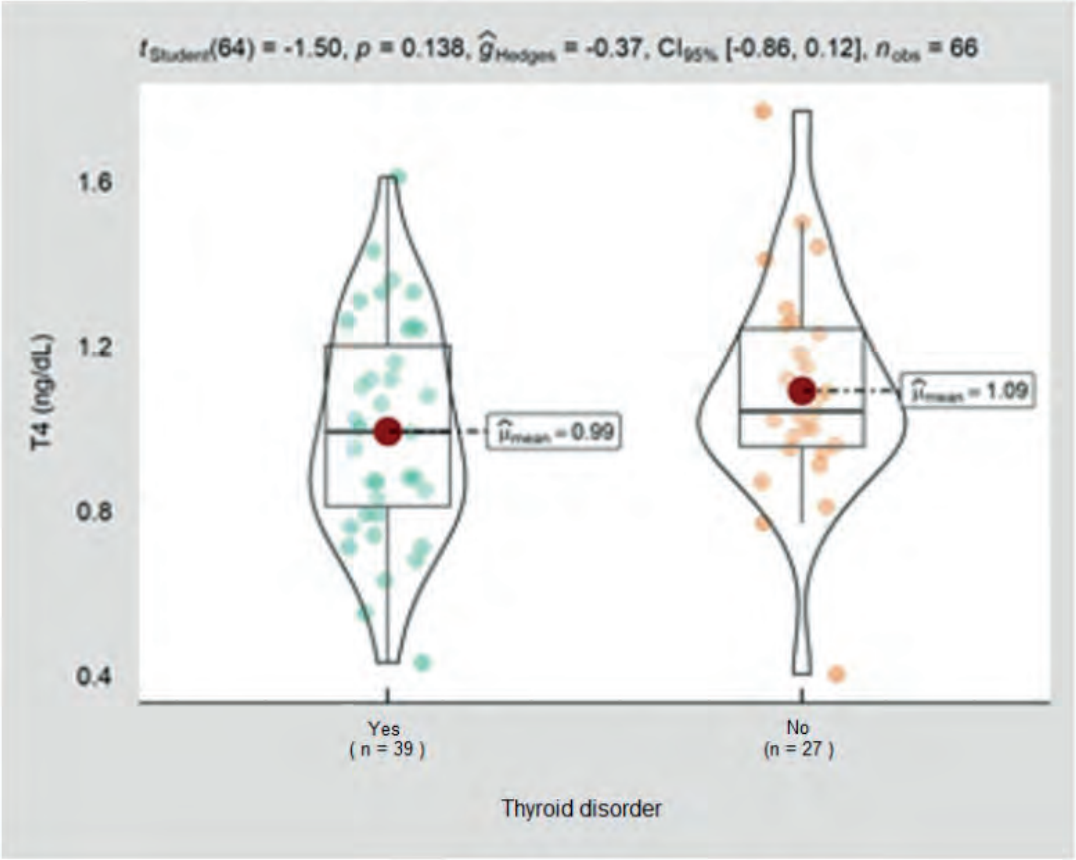


Figure 3. Comparison of T4 values in the presence or absence of thyroid alteration.

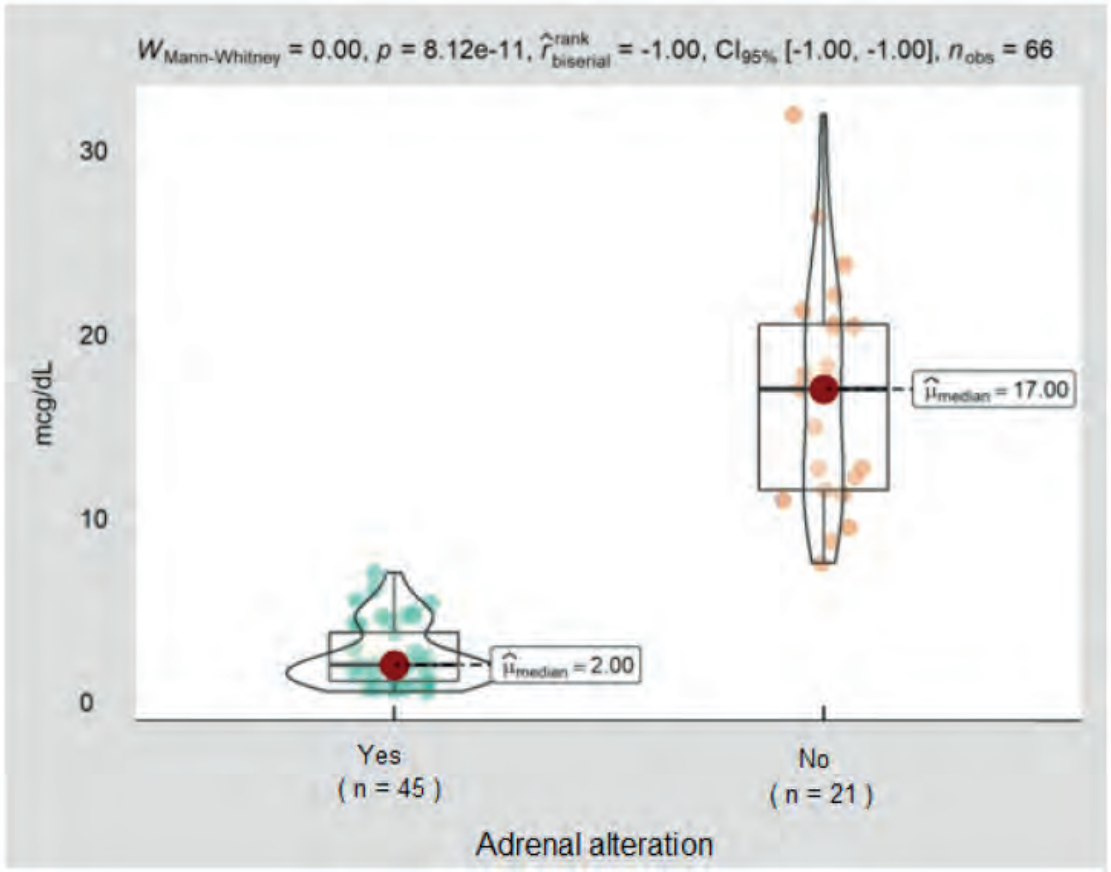


Figure 4. Comparison of cortisol values in the presence or absence of adrenal alteration.

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REVIEW / ARTÍCULO DE REVISIÓN

Probe on Various Experimental Cigarette Smoke Subjection Structure

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Abstract: Different methods of subjection to smoke from experimental cigarettes are essential for understanding tobacco smoke. The major toxicants found in tobacco are acetaldehyde, acetone, acrolein, acrylonitrile, ammonia, benzene, cadmium, catechol, chromium, cyanide hydrogen, arsenic, nickel, nitric oxide, nicotine last but not least, mono-oxide gases. While experts say, cigarette smoke contains more than 4000 different compounds. These are substantially toxic and can destroy cells, and many of them are carcinogenic. Various smoke-exposure devices are used for in-vitro tobacco smoke generation, dilution, and distribution.

Such devices are used widely by well-known manufacturers or can be tailor-made setups. We can set up different in-vitro models to better treat smoke-related diseases using these subjection structures. The fundamental goal will be to build a tobacco-free society of available subjection systems. Some have been identified and established as biological endpoints in some published scientific literature. In the scientific field, many new technologies are coming out and showing their presence. There are many systems of exposure to cigarette smoke in vitro which offer a more flexible approach to the challenges of exposure to tobacco smoke. This review covers some topics such as the description of available new subjection structures and reviews their work, setting up and application for Scenarios of in-vitro treatment. The benefits and disadvantages of both subjection mechanisms and the similarities between the setups and the data extracted from these structures. Measuring the smoke dose is also discussed here as an important field of research, particularly in the preclinical phase.

Key words: Cigarette smoke, Cigarette Subjection Structures, Cigarette Subjection Mechanisms, Cigarette Subjection Advantages, Cigarette Subjection Use, Cigarette Subjection Modern advancements.

Introduction

Most findings of respiratory disease relate specifically to tobacco consumption. Comprehension of the complex dynamics of tobacco smoke is fundamental, which can allow the precursors and mechanisms responsible for adverse health effects[1]. The smoke from tobacco kills up to half its users. More than 8 million people are killed by cigarettes each year. About 7 million of those deaths result from direct use of cigarettes, while about 1.2 million results from second-hand smoke exposure by non-smokers[1,4,6]. Tobacco causes severe injuries to the health because of hazardous chemical compounds such as nicotine, cadmium, lead, polonium-210, benzene, acrylonitrile, various aldehydes, aromatic amines, aromatic polycyclic hydrocarbons[2,6]. The compositions are related to COPD, lung toxicity, and multiple cancers.

In-vitro tobacco smoke measurement is conducted on the particulate process collected on a Cambridge filter pad and eluted in DMSO or bubbled through the media or PBS to cell culture[3]. The cell cultures are then exposed to the particulate phase under submerged conditions. Sadly, particulate-based exposures do not consider the period of cigarette smoke vapor or the related interactions between the level of particulate matter and vapor[3,4]. Aquatic agricultural environments and particulate-based concentrations do not match the human lung's primary stream exposure to tobacco smoke. Separating fractions of smoke in this manner could also contribute to alterations[5]. The chemical changes may not indicate the smoke aerosol as a whole. Whole smoke exposure systems were developed to overcome the problems[6]. Different whole-smoke exposure systems are available worldwide, and most of them in Germany is very popular. There must be some drawbacks, but they could play an important role and bridge the gap between technologies, not only in calculating the actual cell dosage but also in characterizing and validating these systems[5,7]. A review article covers recent advances in various subjection systems for cigarettes, their mechanisms of action and their application in different fields[3,7]. These are very useful for understanding the processes of the cell damage and disease mechanisms caused by tobacco[8]. This review describing tobacco smoke and disease, elucidating disease processes and defining smoke toxicants responsible for adverse health effects will be critical in-vitro approaches using dose instruments will add strength to the in-vitro data resulting from this[9].

Whole Smoke Exposure Systems

Traditional techniques of exposure to smoke are based on the particulate phase of cigarette smoke and omit any subsequent analysis of the vapor and semi-volatile phase. Whole smoke exposure systems allow evaluating all stages of smoke together or separately, depending on the experi-

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mental setups[5,7,10]. This has enabled researchers to conduct their experiments to investigate tobacco smoke phases, yielding valuable information. Some of the systems on the market include Bespoke[11], Borgwaldt[12], Burghart[13], Vitrocell[14], and Cultex[15]. Among these is the more advanced Vitrocell system. A summary of different whole smoke exposure systems can be found in table 1.

Vitrocell Systems

A brief detailing with advancements of Vitrocell systems where now a days this is the advanced in-vitro exposure systems. The system's goal is to assist in attaining the research goal by working hand in hand with the researchers. This integral commitment to one's research means one can count on the system to propose optimally tailored components[40], provide the latest installations[41], an international training and a world-class service precisely to the requirements of the researchers.

Vitrocell Inhalational Toxicology

They advanced device systems for in vitro characterization of airborne substances at the air / liquid interface, such as gases, complex mixtures, nanoparticles and fibers. The device design considers the type of aerosol, the insert sizes of the cell culture, the mammalian cell system and the dosimetry. The details of this type are illustrated in table 2, table 3 and table 4, respectively[45-47].

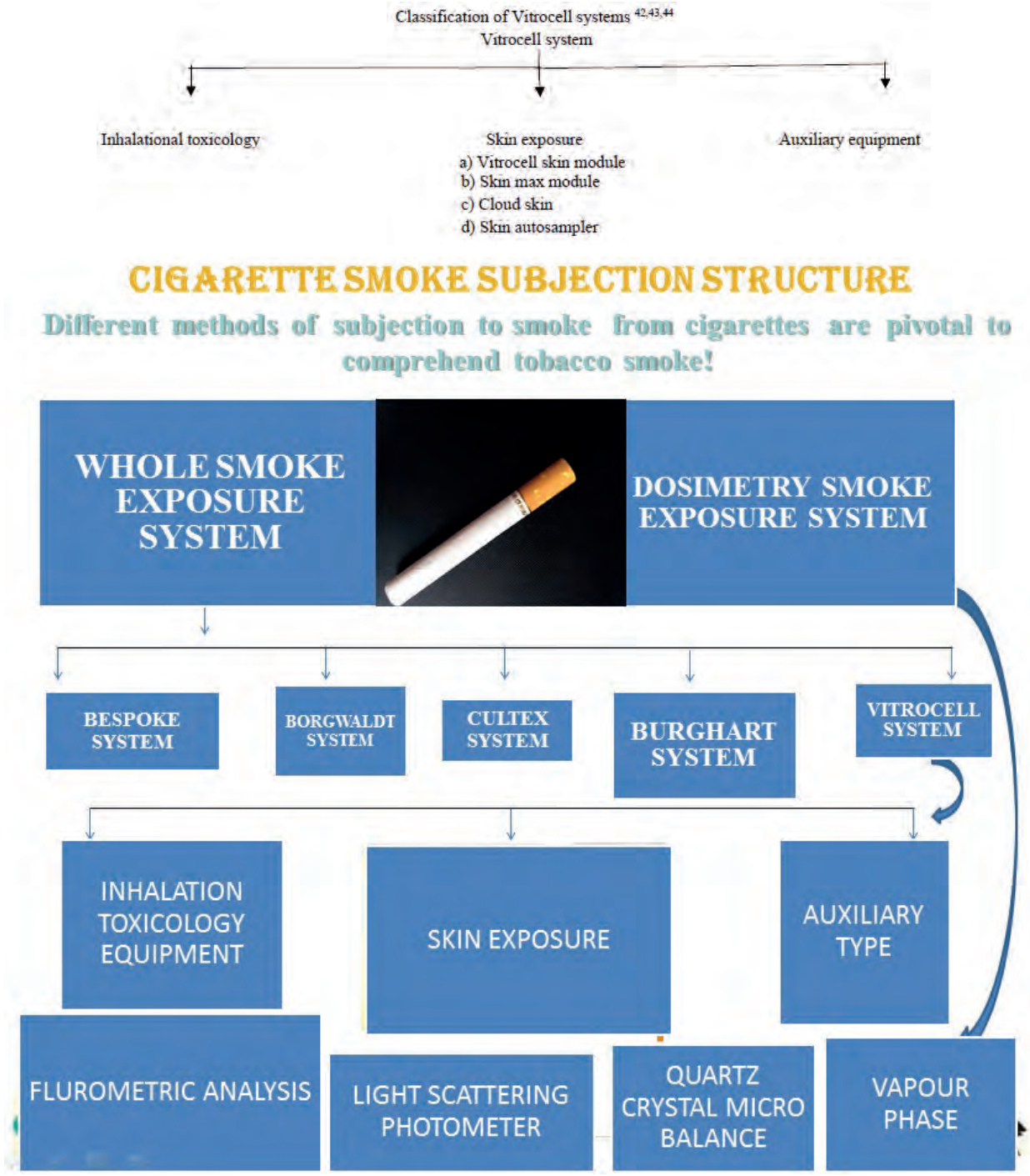


Figure 1. Graphical abstract on various cigarette smoke subjection structures.

	2.1 Bespoke System	2.2 Borgwaldt System	2.3 Burghart System	2.4 Cultex System
Another Name	-	Borgwaldt RM 20S [16] (Borgwaldt KC Gmbtt, Hamburg, Germany.	The Burghart Mimic Smoker MSB-01 (Burghart Tabaktechnik, Wedel, Germany) [16,17]	Cultex Laboratories (Hannover, Germany) [18]
Parts of the System	a) Hermetic chamber b) Two large ventilation holes c) Cell culture plate d) A small fan [19].	a)8 syringes b)Cigarette smoke generator c)A syringe based dilution system d)Additional 4 syringe unit e)Airflow controller f)Cell culture media g)Chamber house h)Smoke diluter [19,20]	a)Integrated multi format b)96 micro well format c)Simplistic chamber d) An independent exposure module [28].	a)Hyp-hydraulic press b)Cultex dust generator c) Integrated elutriator [31,32].
Mechanism	Exposure of cells to smoke for two days followed by assessment of cell supernatant by enzyme linked immunosorbent assay. Then comparison of results [21].	Smoke generation by syringe followed by a puff of filtered air for dilution. Exposure of diluted smoke into the chamber followed by dilution of smoke and cellular exposure between 12 and 24s depending on solution [22,23].	Doses of cigarettes will be assessed by independent syringes, followed by the dilution capability up to 1:150. A consistent exposure is provided across the multiwall plate. At last, the disclosures of cigarette puffs are taken for approx. 6s [29,30].	a)Particle deposition assessment by fluorescence spectrophotometer b) This module is useful in response to mainstream cigarette smoke and deducing a possible mechanism for losing tight junction stability in the respiratory epithelium in smokers [33,34].
Uses	-	a)Product assessments b)Fundamental research [24]	-	a)In-vitro toxicological substances of air-bone substances b)Research related inhalational toxicology c) Access a multitude of smoke-related research [35,36].
Advantages	a)Non-expensive b)Easy maintenance c)Simple structure d)Sophisticated design e) Unique nature of smoke generation, dilution and exposure characteristics [24].	a) Rotary style structure b)Capable of smoking up to 8 cigarettes simultaneously c) No cross cigarette contamination [26].	a)High throughput in-vitro experimentation [30].	a)Precisely control system b)Provide homogeneous distribution c)Trumpet technology facilitates aerosol delivery directly onto the cell monolayer d) Very useful for Ames test [37].
Advancement	Set up maintaining 3D arrangement of the human mucosa and allow a quasi-replication model of the inhalation/exhalation cycle [24,25].	The interchangeable system, an essential system of in-vitro development and machine characterization papers [27].	-	a)Easily understand a disease mechanism b)Useful tool to assess therapeutic efficacies in-vitro c) Latest development is the radical flow system [38,39].

Table 1. Comparison between different types of whole smoke subjection structures.

Method	Exposure system	Aerosol and gas supply	Aerosol generator	Smoking machine	Dilution	Humidification	Dose monitoring
Air/liquid interface	a)Modules for 6 well inserts b)Modules for 12 well inserts c)Modules for 24 well inserts d)Modules for 35mm Petri-dishes e)Modules for suspension cells f)Modules for 96 well inserts g)Vitrocell cloud system h)Vitrocell powder chamber i)Automated exposure system	a)Aerosol generator b)Smoking machine	1)Bioaerosol nebulizing generator 2)VAGF nebulizing generator 3)VAGK nebulizing generator 4)VRGB generator	1)VC1 type 2)VC1S type 3)VC1/7 4)VC10 5)VC10 S type 6)Chem control feature 7)Human puff profiles 8)Vitrocell vapestrater	a)Gas distribution system b)Only dilution system c)HD distribution system d)Sidestream chamber	a)Humidification station b)Washing bottle c)Inline humidifiers	a)Microbalance sensor and software b)Photometer and software c)Filter housing d)Co-monitoring e)Vitrocell FT-IR f)Vitrocell photon ion

Table 2. Details of vitrocell inhalational toxicology system.

Bio-aerosol nebulizing generator	VAGF nebulizing generator	VAGK nebulizing generator	VRGF generator
Nebulizes clean liquids, solutions and suspensions.	Available and reproducible particle size (<2 micron) by cyclone.	Nebulizes clean liquids and different concentrated solutions.	Dispersion of non-cohesive powders and dusts of particle sizes <0.1 micron to 100 micron.
Ideal for proteins, bacteria and micro-organisms.	Nebulizes clean liquids, suspensions and solutions.	Long dosing time	Optional cyclone for particle sizes <5 micron.
Approx. particle diameter is 0.7 to 2.5µm. Low flow rate with optional precision pump.	Long dosing time. Distribution system designed for vitrocell modules.	HD distribution system designed for Vitrocell modules.	Very constant output and good reproducibility. Variable mass flow and HD distribution system designed for Vitrocell modules.

Table 3. Some features of the aerosol generator systems used in Vitrocell systems[48,49].

Vitrocell Skin Exposure Systems

High tech equipment solutions for the exposure of skin tissue to solid, liquid or gaseous substances. VITROCELL Skin modules are a powerful alternative to glass-made Franz cells as they offer improved handling, higher throughput and options for automation[53-55].

Vitrocell Auxiliary Equipment

A range of durable components for a successful and long-lasting operation of our exposure systems. Own equipment solutions were developed when commercial solutions are not available. VITROCELL approved auxiliary equipment fits perfectly into our turnkey installations[56].

Name of the Section	Types	Uses
Aerosol and gas supply	Aerosol generator Smoking machine	For liquids, suspensions, solutions and dry powders Well suited for e-cigarettes
Smoking machine	VC 1 type VC 1 S type VC 1/7 type VC 10 type VC 10 S type Chem control feature Human puff profiles Vitrocell Vapestarter	For conventional and electronic cigarettes with high technical features For manual smoking For linier smoking For fully automated smoking robot For high end smoking robot For fully automated smoke lung robot Option for Vitrocell smoking machine Innovative solution for the button activation of e-cigarettes
Dilution	Gas distribution system Only dilution systems HD distribution systems Side stream chamber	For clean air and gases For dilution of gases, mixtures and conventional exhaust For testing and dilution of atmospheric substances
Humidification	Washing bottle Inline humidifiers	For simple solution of initial tests For humidification of the test atmosphere directly before exposure
Dose Monitoring	Microbalance sensor and software Photometer and software Filter housings Co-monitoring Vitrocell FT-IR Vitrocell photon ion	For high precision mass determination in ng resolution For inline assessment of particle concentration without aerosol loss For gravimetric methods For monitoring of Co in the gas phase. For online gas analysis of e-cigarettes For fast online measuring system of chemical gas analysis

Table 4. Different parts and their uses of Vitrocell Inhalational Toxicology[50-52].

Sr No.	Type	Parts of the Machine	Features
1	Vitrocell skin module	a)Base module with the cover unit and exposure top b)Controller for magnetic stirrer c)Skin module illustration d)Tissue holder with an extraction tool e)Holder compartments	i)Durable and long-lasting design ii)Unique tissue holder iii)Permanent or static medium supply iv)Integrated water bath heating circuit v)Suitable for diverse tissue thicknesses up to 2000µm vi)Exposure top for the analysis of volatile compounds
2	Vitrocell skin max module	a)Skin max module compartment b)Skin max module cover fixation bracket	i)Durable and long lasting system with integrated water bath heating circuit ii)Suitable for the tissue diameter of 50mm iii)Tissue exposure surface diameter is 25mm
3	Vitrocell cloud skin	a)Tissue holder with extraction tool b)Full set of holder counter parts	i)Exposure system for liquid aerosol ii)High droplet output rate with cloud dynamics iii)No external air flow required iv)No humidity control required v)Easy handling of unique tissue holder vi)Small residual volume in nebulizer reservoir
4	Vitrocell skin auto sampler	a)8 chamber capacitors b)Water bath heating circuit c)Magnetic stirrer d)8-stopper motor controlled individual syringes e)Magazines	i)Ideal tool for toxicokinetic study ii)High reproducibility results iii)Programmable sample volume of 0.1 to 1.0ml iv)Bubble free automated delivery of fresh receptor fluid with high reproducibility v)Possibility to integrate impedance measurements

Table 5. The various types, their parts and some essential features.

Dosimetry for smoke exposure systems

This system is essential for understanding exactly which components of smoke are exposed to the culture of cells. Tobacco smoke has two phases that can cause injury to the lung and damage to cells[60]. The dosimetry system is thus helpful in understanding the characteristics and interactions of both steps[61]. The different tools are used for particle deposition assessment under dosimetry are-fluorometric analysis[62], light scattering photometers[63], quartz crystal microbalance[64], and vapor phase[65].

Recent findings on different issues of the whole smoke exposure system

These subjection structures may be too unique in their evolution to be widely used for a consistent approach. No commercially available or otherwise available exposure system has yet been fully characterized or validated, and each system offers unique advantages and inconveniences[17]. Interestingly, an assessment by the Cooperation Authority for Scientific Research Relative to Tobacco (CORESTA) on various whole smoke exposure technologies – a tobacco-related task force in vitro, found remarkably consistent results, indicating the similar performance of these systems (CORESTA air-liquid interface report). Entire smoke exposure systems are an important development for producing an in vitro physiologically relevant test smoke aerosol[18]. In support of this, the Committee on Mutagenicity in the UK reviewed the field of 'chemicals in food, consumer goods, and the environment' in June 2009 and commented that the implementation of whole-smoke exposure protocols was likely to provide more specific data on tobacco smoke's mutagenic effect, but noted that none of the test systems

Sr. No.	Equipment parts	Components
I)	Flow measurement and control [57]	Vacuum flow calibration, Mass flow meter, Mass flow controller
II)	Clean air purification station [58]	Pressure regulator, Pre-filters with condensate trapping, Membrane dryer, Activated control filter, Fine filter
III)	Temperature control [59]	Water bath, High-performance water bath
IV)	Vacuum pump	-
V)	Precision pump system	-
VI)	Vitrocell epithelix nanopress	-
VII)	Aero list	-

Table 6. The details of the equipment are given below.

Sr No.	Name	Mechanism	Uses	Advantages
3.1	**Fluorometric analysis (Wet chemistry technique)**[64,66]	Pre-wet cell cultures are kept within the exposure chamber followed by the whole main stream smoke. Then deposited particulate materials are exerted by HPLC and analyzed with fluorescence detection along with graph generation (Fig-2a)	Quantification of particulate deposition	a)Simplistic approach b)Used in a variety of studies c)It can be applied to any subjection structure format d)Results are relatively consistent
3.2	**Light scattering photometers** [67]	Inline measurement of the particle droplet suspended in the gas via a light scattering optical sensor (Fig-2b)	Useful for in-vitro and in-vivo studies	a)Measurement of optical density at low flow rates b)No loss of particle mass c)Acts as a real time monitoring tool d)Results are always consistent and reproducible
3.3	**Quartz crystal microbalance** [68,69]	Works via piezoelectric effect and measures and detects changes in mass in the nanogram range	Investigates deposition data from a variety of cigarettes	a)It can control exposure based on particulate dose b)Modern technique consists of a gravimetric unit of deposited mass per surface area for more accuracy
3.4	**Vapour phase** [70]	Vapour phase quantification by infrared gas analyzers. The markers can be quantified inline or remotely.	Analysis of vapor phase components using chemistry trapping setups that measures phase dilution	It can quantify carbonyls in tobacco smoke easily but has many limitations.

Table 7. Several dosage tools available for particulates and vapors are explored in more detail.

had been 'successfully validated'[32]. Validation remains a prominent area for improvement in this research field, and no one has yet carried out a multi-laboratory or multi-system study. Several models, techniques, findings, limitations, and further suggestions are explored in more detail in Table 8. Quartz crystal microbalance takes advantage of the piezoelectric character of quartz to characterize the formation and structural properties of thin films in real-time. This is done by simultaneously measuring the changes in the resonant frequency related to the mass/thickness of the film in contact with the surface and energy dissipation associated with the rheological properties of the adsorbed film, induced by adsorption/desorption processes or by structural changes produced within the thin film[68]. The 4-sensor chambers of the Q-Sense E4 enable the performance of four simultaneous measurements under controlled experimental conditions (temperature, flow rate), increasing reproducibility and decreasing experiment duration[69]. Also, different coatings (gold, silicon dioxide, titanium, hydroxyapatite) can be used, broadening the application field of the technique.

The scientific literature covering whole smoke structures in vitro has provided a variety of related biological endpoints, illness, and toxicology. For instance, in vitro tobacco smoke has been shown to induce various cellular effects potentially associated with disease processes, including the up-regulation of a series of factors related to lung damage and inflammation[40]. It has also been shown that tobacco smoke produces high levels of reactive oxygen species and oxidative stress that may cause cellular damage to lipids, proteins and DNA Moreover; it has been shown that cigarette smoking has several effects on gene expression in the human airways. Studies of bronchial epithelial cells obtained by bronchial brushing from smokers 'and non-smokers' airways have shown that cigarette smoke induces metabolizing and redox genes, tumor suppressor genes, and oncogenes alongside inflammatory process control[68].

Discussion

Different smoke exposure systems have already been published in various investigative journals. With respiratory conditions such as asthma and COPD symptoms increasing worldwide, many new up-gradations associated with these subjection structures are launching in the commercial markets. Such severe respiratory diseases caused by in-

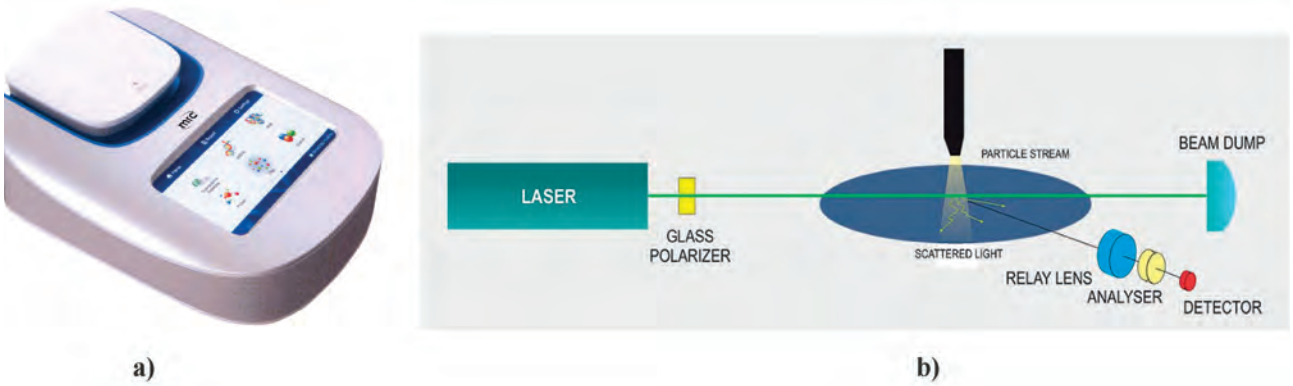


Figure 2. (a) Fluorometric analysis (Wet chemistry technique): A susceptible instrument, the wet-chemistry-based fluorimeter, is a reproducible instrument used for all fluorometric applications. The key advantages are precision and versatility[64]—four fluorescent channels in common nucleic acids and protein assays. Fluorimeters based on wet chemistry can ultimately personalize the measuring applications[66]. This instrument has powerful software and full network integration to handle the data seamlessly. This figure is obtained from MRC Laboratory-Instruments, (Available: https://www.mrclab.com/portable-fluorometer-uv-blue-channels); (b) Light scattering photometers: Light scattering photometer is essential to determine absolute molar masses from cigarette ignition. (Molar mass range-200 to million g/mole and 10nm to 50nm roots means square radii). It is an excellent method for characterizing polymer and protein in different inquiries, or product quality controls[67]. This figure is obtained from Holmarc Opto-Mechatronics PVT Ltd, Available: https://www.holmarc.com/scattering_photometer_-_single_channel_light_detection.php).

halable pollutants, such as tobacco smoke, diesel exhaust, or other sources of pollution[70,71]. Therefore, a substantial effect on national health systems is expected, indicating the long-term treatment, with routine tests accompanied by high costs[4,71]. In this context, in vitro approaches can help develop more efficient protocols, such as evaluating new compounds' toxicity during the research and development process[56]. This research will aim to better select substances suited to further development and a final reduction in the number of animal experiments[67]. Hence, researchers have developed an in vitro exposure device that allows the examination of inhalable compounds for their pharmacological and toxicological effects. There are various options for using the different smoke exposure systems to produce a steady supply of cigarette smoke with slight variations over an extended period[71]. A unique feature of the smoking systems is the conditioning and mixing chamber, which sets the primary concentration and provides a steady amount of "feed smoke" for the exposure components. Measuring and monitoring the flow rates to the exposure chambers is essential for controlling smoke levels. Any process or a mixture of measurements in the exposure portion is highly desirable for the continuous smoke concentration measurement[71]. Carbon monoxide measurement can be an anchor measurement and can be reported as a record of exposure concentration over time. During treatment, measurement of different concentrations of smoke components should be carried out on a regular schedule[70,71]. In an experiment involving the generation of smoke over prolonged periods, well-defined and documented exposure must be created. Smoke exposures are manpower-intensive and require continuous monitoring throughout the exposure cycle to maintain a steady supply of smoke[71].

Cleaning them regularly also is essential to prevent tar buildup. The smoking machine crashes and gets out of balance when pieces of tobacco and paper hit the sliding parts, and tar builds up[71]. The efficiency will improve considerably as the user becomes more familiar with aligning and keeping the system clean. If carried out correctly, an experiment with a known exposure level will be conducted with good documentation of the smoke level, which is monitored very well from day[67]. A great deal of advanced subjection structure for tobacco smoke is now used for a few days. Scientific knowledge has been published in various journals on these systems, and biological endpoints have been described. They are new to the research field and continue developing their presence[8,59].

Conclusions

Subjection structures for in vitro smoke occupy a role in fundamental and mechanical science. Various scientific literature showed the wealth of associated biological endpoints, disease, and toxicological data. Using such structures, multiple advances in vitro test methods are a promising sign and suggest that these devices can support and supplement several potential exposure scenarios. Depending on the equipment used, the ratio of smoke to air, the flow rate of mixing air added to the smoke diluter, and the fraction of smoke, in-vitro exposure systems can be viewed in many ways. Dosimetry is the most common and influential of all the methods for evaluating in-vitro tobacco smoke. The dosimetry method can bridge the gap and play an essential role in calculating and validating the actual cellular dosage. Different in-vitro models were developed based on exposure systems useful for evaluating tobacco smoke's biological activity. To understand tobacco smoke and disease and the mechanisms of disease and classify smoke toxicants responsible for severe medical conditions, in-vitro approaches will be necessary, ensuring the related exposure system is appropriately characterized.

Author Contributions

Moulima Das: Literature Review & Investigation, Software, Data Curator, Visualization, Graphs and Table preparation, Writing – Original Draft Preparation & corresponding author. Anupam Saha: Writing – Reviewing and Editing, Acquiring Permissions, Reformatting, Correction, Software and other essentials.

Serial No	System Name	Author/Year	Model Name	Technique Used	Key Findings	Limitations Suggested by the Author	Further Works Suggested by the Author
1) a.	Bespoke system	St.Laurent et al.,(2009).	Isolated rat bronchial epithelial cell model	"Harmetic" chamber used to accommodate cell culture plate following by the assessment of ELISA technique	I.MCP-1 release was inhibited II.IL-10 production was reduced.	VEGF showed no difference in production	Different models can influence the data and modulated bronchial epithelial cell mediator production differently Precautions required for choosing the models
1) b.		Gualerzi et al.,(2012)	3D *In-Vitro* set up	3D arrangement of human mucosa and quasi replication process of the inhalation/exhalation cycle	The keratin protein(K14) Was over expressed as early as after smoke exposure	Only *In-Vitro* work is done here so *In-Vivo* work is needed	
2) a.	Borgwaldt system	Phillips et al., (2005).	Bronchial epithelial NCI-H292 cell model	Cells exposed to cigarette smoke using RM 20S and BAT exposure chamber	Study demonstrates cytotoxicity, particulate deposition m-RNA and protein expression in response to tobacco smoke		-
2) b.		Maunders et al., (2007).	Bronchial epithelium cell model	Affymetrix and microarray technology	Gene expression changes	The down-regulated responses in cell adhesion may provide a possible mechanism for the permeability induced by smoke and may entail the development of various diseases caused by tobacco smoke.	British American Tobacco has used the method primarily making clear comparisons with other studies difficult.
3	Burghart system	Adamson et al., (2011b). Scian et al., (2009).	High throughput *In-Vitro* experimentation	96-micro well format of high throughput screening	Checking of cellular viabilities and smoke loss measurement	At the air-liquid interface, the cells are not supported and some implication is set up, such as smoke collection chamber, mixing bag etc.	This is simplistic cellular study so higher research related studies are required
4	Vitrocell system	Okuwa et al., (2010).	Chinese hamster lung model	Induction of micro-nuclei in hamster lung following exposure to mainstream cigarette smoke using multiple smoking regimens.	Okuwa and colleagues noted differences in micronuclei in smoking induction schemes as well as differences in the particulate and vapor phases of mainstream cigarette smoke and found robust and repeatable data	Vitrocell 10 has several variables that can be altered to establish the correct exposure setup and this is changing a recent study has evaluated these variables in the exposure module on particle deposition. An increased module flow rate in the exposure module had an inverse effect on particulate deposition	-
5	Cultex system	Aufderhlide et al., (2011).	Human bronchial epithelial 16HBE cell model	"Trumpet" technology where the human cells are exposed to cigarette smoke	Cytotoxic responses were noticed here	-	-

Table 8. Models with techniques, findings, limitations, and further suggestions.

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Conflicts of Interest

Authors declare that there is no conflict of interest.

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ARTICLE / INVESTIGACIÓN

Soluble (PD-1) and Autophagy protein (ATG-5) in patients with tuberculosis

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Abstract: Immune checkpoints such as programmed cell death protein (PD-1) and autophagy-related protein 5 (ATG5) can directly affect the immune response and thus, interfere with infection with Mycobacterium tuberculosis (Mtb). This study aimed to determine the role of soluble ATG5 and soluble PD-1 proteins in tuberculosis (TB) patients. The study included a collection of 90 blood samples (47 samples from patients with confirmed tuberculosis and 43 samples from age and gender-matched healthy subjects to estimate the concentration of soluble autophagy (ATG-5) and programmed cell death protein (PD-1). Enzyme-linked immunosorbent assay was used to estimate the serum levels of these markers. The median value of ATG5 level in patients was 2.08 µg/ml, which is higher than the level of the control group (median = 0.93 µg/ml) with a highly significant difference. Similarly, the median value of PD-1 level in patients was 259.4 ng/ml (range 107.76-924.25 ng/ml), which was higher than that of the control (median = 146.4 ng/ml, range = 50.32-1117.1 ng/ml) and with a highly significant difference. It can be concluded that both soluble ATG5 and PD-1 increased significantly in patients with TB compared with controls.

Key words: Tuberculosis, M. tuberculosis, Autophagy, Programmed cell death protein-1.

Introduction

Tuberculosis (TB) is considered one of the essential epidemic diseases, and it is caused by *Mycobacterium tuberculosis* (Mtb) which commonly attacks the lungs and other organs such as bones, lymph and joints[1]. Patients often present with pulmonary symptoms such as a bloody cough (hemoptysis), chest pain and shortness of breath (SOB), and general clinical symptoms such as fever, poor appetite, weight loss and night sweats[2].

According to World Health Organization (WHO) statistics in 2018, one-third of the world's population is infected with MTB, 90% of whom have latent infection. With most severe cases are in developing countries. The prevalence is associated with poor treatment, drug resistance and limited vaccines[3].

Understanding the host response to Mtb infection is a critical factor in efforts to eradicate TB by developing effective vaccines and immune therapeutics. The ability of Mtb to survive and replicate in host macrophages is an essential step for its pathogenesis, with intracellular growth being often associated with virulence[4,5].

Autophagy is an intracellular self-digestion process whereby cytoplasmic constituents are delivered to and degraded by lysosomes[6]. Besides being crucial for quality control and energy supply, autophagy plays critical roles in immune defenses against invading bacterial and viral pathogens, such as regulation of inflammation, antigen presentation and microorganism capture and degradation[7-9].

Programmed cell death protein 1 (PD-1) is a 288 amino acid cell surface molecule that has been designated as a membrane protein of the immunoglobulin superfamily in humans. This protein is expressed on T cells, pro-B cells, and myeloid-derived dendritic cells, leading to negative regulation of the proliferation and activity of these cells[10].

Increasing evidence suggests PD-1 expression 5and some autophagy proteins such as ATG5 are upregulated in patients with active TB[11-13]. This study aimed to measure the serum levels of soluble PD-1 and ATG5 in patients with TB.

Materials and methods

The Study Population

The study involved 47 patients with TB attending the TB control center/ Baghdad during the period from 1st January 2019 to 31st October 2020. The TB diagnosis was made first with acid-fast stain (figure 1), then confirmed with culturing on Löwenstein–Jensen (LJ) culture and biochemical test specific for Mtb.

Other age- and gender-matched 43 apparently healthy subjects where selected to represent controls. Blood samples were obtained from patients and controls. The sera were separated and stored at -20ºC for immunological studies. Demographic data (age, gender, socioeconomic status, smoking habit) were collected from each participant through direct interview. The ethical committee approved the study of the College of Science, Tikrit University.

Serum Level of Soluble PD-1 and ATG5

Ready commercial kits (SunLong Biotech Co., LTD/ China) were used to measure serum levels of soluble ATG5 and PD-1 through enzyme-linked immunosorbent assay (ELISA). This assay is based on the presence of antibodies

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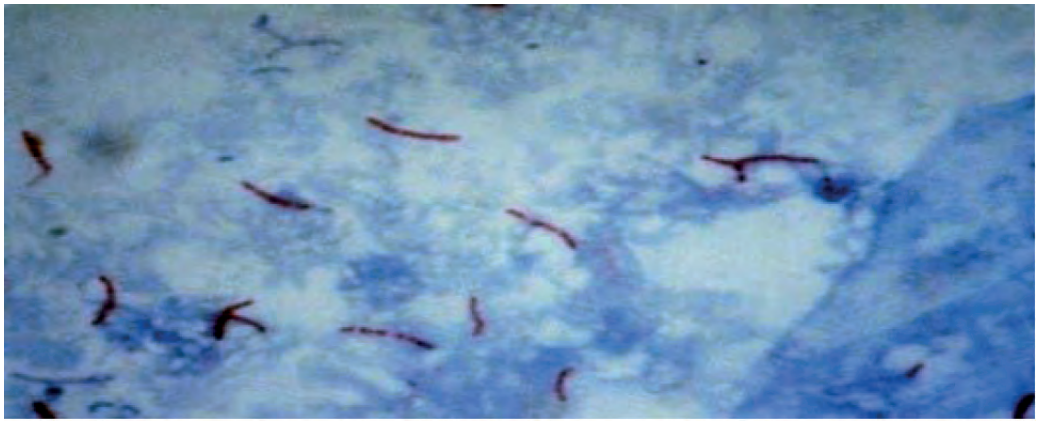


Figure 1. Acid fast bacilli of *M. tuberculosis* 100X.

(for PD-1 orATG-5) attached to microliter wells. Soluble PD-1 or ATG-5 reacts with these antibodies. An enzyme-conjugated anti- PD-1 orATG-5 (horseradish peroxidase (HRP) is then added to the reaction. The enzyme acts as an amplifier of the detection signal by converting a substrate that results in color changes in the wells. The density of color is proportional to the serum concentration of PD-1 or ATG-5. The manufacturer's instructions were followed precisely. A fully automated apparatus read the results. These assays were performed at the Medical Research Unit, College of Medicine, Al-Nahrain University/ Baghdad.

Statistical Analysis

Statistical analyses were performed using SPSS software version 25.0 (SPSS, Chicago). Continuous data were subjected to a normality test (Shapiro Wilk test); Data with normal distribution were presented as mean and standard deviation and analyzed with a Student t-test. Data with non-normal distribution were presented as median and range and analyzed with the Mann Whitney U test. Categorical variables were expressed as numbers and percentages and analyzed with the Chi-square test. The receiver operating characteristic curve (ROC) was used to evaluate the diagnostic value of PD-1 and ATG5 in the discrimination TB patients and controls. A p-value less than 0.05 was considered to indicate a statistically significant difference.

Results

Demographic characteristics of the study population

The mean age of the patients was 40.94 ± 16.79 years, which did not differ significantly from the control group (39.0 ± 16.21 years). Similarly, the distribution of females and males, the socioeconomic status and the percentage of smokers were similar between the two groups, with no significant differences. On the other hand, 25.53% of patients without comorbidities compared to 58.14% of the control group with a significant difference. In particular, the incidence of diabetes was higher in patients versus the control group (29.79% versus 13.95%)(Table 1).

Serum level of soluble ATG5 and PD-1

Data regarding soluble ATG5 and PD-1 levels were found to be non-normally distributed. Accordingly, they expressed as median and range and analyzed with Mann Whitney U test. The median value of ATG5 level in patients was 2.08 µg/ml (range 1.1-4.06 µg/ml), which was higher than that of the control group (median = 0.93 µg/ml, range = 0.45-5.26 µg/ml) with a highly significant difference (Figure 2).

Similarly, the median value of PD-1 level in patients was 259.4 ng/ml (range 107.76-924.25 ng/ml) which is higher than that of the control (median = 146.4 ng/ml, range = 50.32-1117.1 ng/ml) and with a highly significant difference (Figure 3).

Diagnostic value of ATG5 and PD-1

The receiver operating characteristic curve was used to determine the diagnostic value of ATG5 and PD-1 in discriminating between patients and control. For ATG5, the area under the curve was 0.921, 95% CI = 0.984-0.857, p < 0.001. The sensitivity and specificity of the test at the cut-off value for ATG5 1.32 µg/mL were 89% and 84%, respectively. For PD-1, the area under the curve was 0.797, 95% confidence interval = 0.898-0.695, P < 0.001 The sensitivity and specificity of the test at the cut-off value of PD-1 = 205.4 ng/ml were 80% and 74 % respectively (Figure 4).

Discussion

When the host exposes to Mtb, and autophagy mechanism is induced. This results in fusion of lysosomal compartments with mycobacterial phagosome and triggers the generation of neo-antimicrobial peptides, essential for mycobacterial killing[14]. Moreover, autophagy may play an indirect role in Mtb phagocytosis through the regulation of scavenger receptors expression[15]. *In vivo* experiments have documented that autophagy protein ATG5 in macrophages is required to restrain Mtb infection in laboratory animals[16,17]. Moreover, using autophagy inhibitors enhances mycobacterial load in zebrafish embryo model[18]. This reflects the importance of autophagy proteins, particularly ATG5, in host defense against TB. Thus, it is reasonable to elevate serum levels of ATG5 protein in TB patients because this protein may reflect the gene expression of membranous ATG5.

In laboratory animals, PD-1 expression was found to be upregulated in severe TB disease infection[19]. Mtb infection of PD-1−/− mice leads to enhanced susceptibility to TB disease characterized by increased bacterial loads, increased inflammatory and necrotic responses in the lungs, and reduced survival[20,21]. These studies in PD-1-deficient mice demonstrate an essential role for PD-1 in limiting excessive IFN-γ production by CD4 T cells, which has been asso-

Variables	control group (43)	patients group (47)	P-value
Age (year)			
Mean ± SD	39.0±16.21	40.94±16.79	0.580
Range	12-65	12-66	
Gender			
Male	24(55.81%)	26(55.32%)	0.962
Female	19(44.19%)	21) 44.68%)	
Socioeconomic status			
low	9(20.93%)	19(40.43%)	0.057
Middle	27(62.79%)	18(38.3%)	
High	7(16.8%)	10(21.28%)	
comorbidities			
None	25(58.14%)	12(25.53%)	
Diabetes	6(13.95%)	14(29.79%)	**0.011**
Hypertension	5(11.63%)	11(23.4%)	
kidney stones	5(11.63%)	3(6.38%)	
Other	2(4.65%)	4(8.51%)	
Smoking			
Never	26(60.47%)	26(55.32%)	0.622
Ex/current	17(39.53%)	21(44.69%)	

Table 1. Demographic characteristics of the study population.

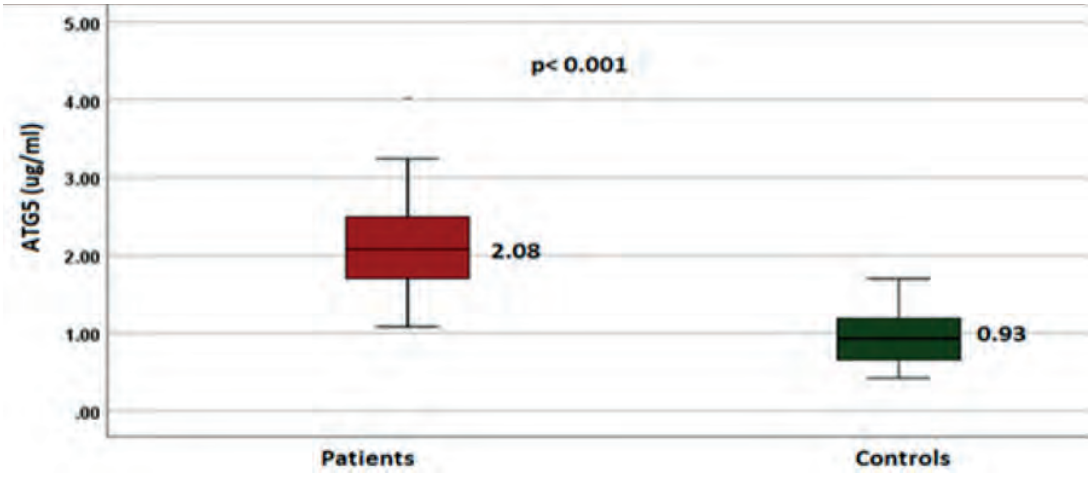


Figure 2. The Median serum level of soluble ATG5 in patients and controls.

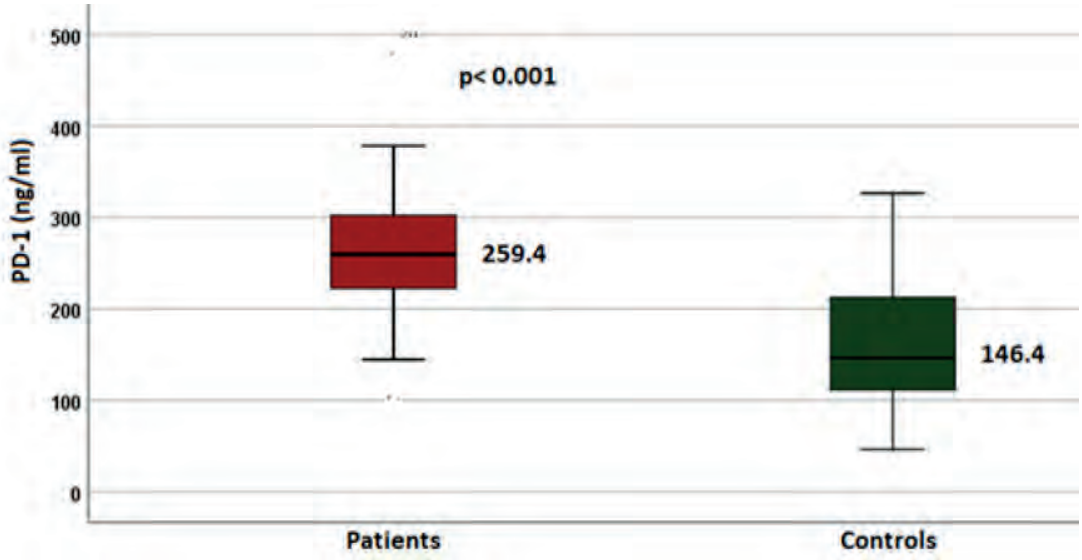


Figure 3. The Median serum level of soluble ATG5 in patients and controls.

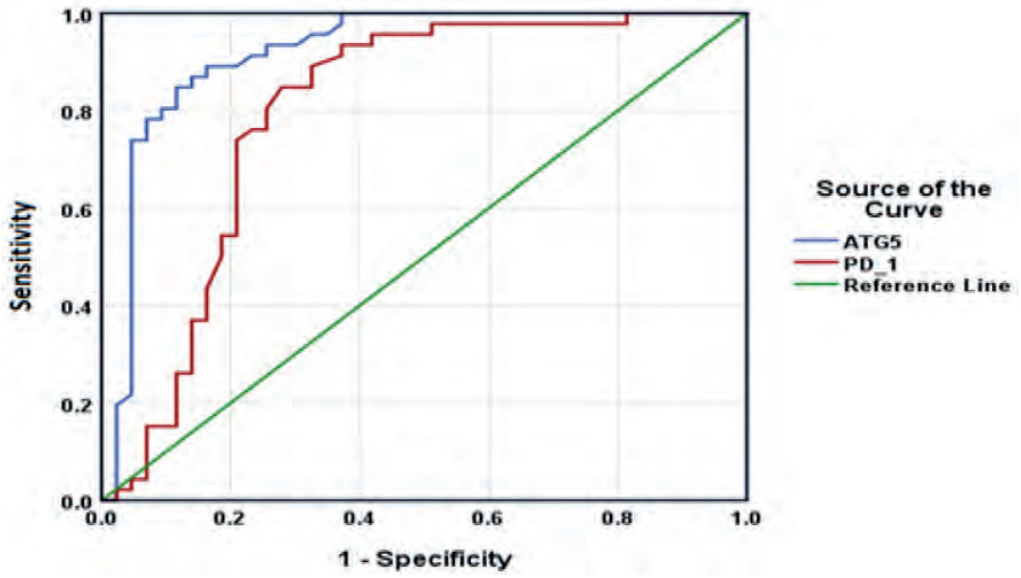


Figure 4. Receptor operating characteristic curve for ATG5 and PD-1 to distinguish between TB patients and controls.

ciated with exacerbated disease in murine models of TB[22]. Increasing evidence suggests PD-1 expression is upregulated on innate and adaptive immune cells in the setting of Mtb infection and disease in humans[23]. Although all these studies focused on membranous PD-1, soluble PD-1 may also reflect the host's immune status.

In a Chinese study, Li *et al.*[24] showed that the concentration of soluble PD-1 in the serum of patients with cystic echinococcosis was elevated compared with the healthy control subjects or patients that received treatment. The authors explained this elevation in terms of an increase in soluble PD-1 expression inhibits the PD-1/PD-L1 signaling pathway in T cells through negative feedback, which reduces the inhibition of T cell activation and increases the activity of the immune system in managing the infection. This supports the findings of Wang et al., which suggest that sPD-1 blocks the membrane PD-1 binding site on activated T-cells, thereby attenuating the PD-1 signaling pathway and increasing the immune response[25].

Conclusions

Serum levels of soluble PD-1 and ATG5 protein are increased in tuberculosis patients compared with controls. The soluble ATG5 protein has an outstanding diagnostic value in discrimination between patients with TB and controls and could be used as additional diagnostic tools in suspicious cases of TB.

Author Contributions

Suhaib Waad Oklah Musa: sample and data collection; manuscript writing; Reyam F. Saleha: statistical analysis and manuscript editing.

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ARTICLE / INVESTIGACIÓN

Effect of *Enterobacter cloacae* toxin on immune cells isolated from Leukemia patients

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Abstract: Toxin extract affects Neutrophils and macrophages, which are phagocytic cells. A total of (180) samples, (50) urine and (130) peripheral blood have been obtained from leukemia patients referred to Medical city/ Baghdad Teaching Hospital/ Hematology center; all patients enrolled in this study were diagnosed by the physician according to clinical presentation and laboratory findings as leukemic patients. Investigate the role of *Enterobacter cloacae* toxins on phagocytic activity/THP-1cells and epithelial line/HBL-100 cells. THP-1 and HBL-100 cells have been kept in RPMI-1640 that have been supplemented by (10%) of the fetal bovine serum (100µg/mL) of the streptomycin and 100units/mL of the penicillin. The cells have been passaged with the use of the Trypsin-EDTA that has been re-seeded at a confluence of 80% twice every week, and then it has been incubated afterward at a temperature of (37C°). The results of toxin extract from *Enterobacter cloacae* revealed that (22) fraction/toxins were taken from (11) bacterial samples; these toxins were separated by gel electrophoresis for protein. (18) samples were positive results for gel electrophoresis of protein according to molecular weight: (13.3 KD for α-hemolysin), (70.5 KD for thiol), (75 KD for enterotoxin). This study demonstrates the cytotoxic activity of E. cloacae toxin by using tissue culture through their effect on suppressing the growth of "THP-1 cells" and HBL-100 cells".

Key words: Tissue culture, Toxin, THP-1 cells, HBL-100 cells, *Enterobacter cloacae*, Leukemia patients.

Introduction

Enterobacteriaceae can be defined as a large, heterogeneous set of the Gram (–ve) rods whose natural habitat represents the gastro-intestinal tract of the animals and humans and enteric bacteria, which are referred to as coliforms as well, have a complicated antigenic structure, and create various toxins as well as other factors of virulence. *E. cloacae* and *E. aerogenes* took on clinical importance as opportunistic bacteria and appeared as nosocomial pathogens in intensive care patients pathogenic, particularly those on the mechanical ventilations[1].

Even though *E. cloaca* is the major prevalent *Enterobacter* species responsible for nosocomial infections; not much is identified regarding the factors that influence its virulence and pathogenicity. The pathogenesis may be multifaceted and complex, with the involvement of various putative virulence factors whose significance in disease progression remains unknown[1]. There are three types of toxin that is produced from *Enterobacter* species. So, following the adhesion to epithelial cells, in vitro strains of the *Enterobacter spp.* Create several possible virulence factors, including hemolysin, enterotoxins, and pore-forming thiol-activated cytotoxins similar to the Shiga-like toxin II[2]. This toxin represents hexamer protein with a (70.5kDa) molecular weight and includes A & B units[3]. The Thiol-activated cytotoxic toxin created by those bacteria could suppress innate host immune defenses[4]. Enterotoxin can be defined as a protein exotoxin released by a micro-organism targeting the intestines with a (75kDa) molecular weight. Cytotoxic enterotoxin has been detected through the rounding and the shrinkage of the epithelial cells. The enterotoxins have been often cytotoxic and kill the cells by altering apical membrane permeability of mucosal (i.e., epithelial) cells of the intestinal wall[5]. Hemolysins are lipids and proteins that cause red blood cell (RBCs) lysis through the cell membrane's disruption. Many hemolysins produced by pathogens cause no considerable destruction of the RBCs during infection[6]. Cytotoxic activities of the *E. cloacae* strains have been related to the toxin of (13.3kDa) molecular weight[7]. The toxin showed stability to the acid treatments at a (2 pH); however, not higher than (7). The hemolysin resulted in the accumulation of the hemorrhagic fluids and the severe histological changes[8]. Neutrophils and macrophages are phagocytic cells contributing to the primary line related to innate defense against bacterial pathogens through removing and destroying them at the epithelial barrier. To escape the host's innate immune protection, several bacterial pathogens have developed unique ways to decrease the efficient antimicrobial immune response of the macrophages[9].

The present study aimed to investigate the role of *E. cloacae* and their toxins (crude and partially purified toxin) on phagocytic activity (epithelial cell/HBL-100 and macrophage "monocytic cell line"/THP-1).

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Materials and methods

Patients

The sample was taken from leukemia patients between (the 14 - > 54) age group in Medical city/ Baghdad Teaching Hospital/ Hematology center, Iraq, from April 2020 to April 2012. The identification of bacterial isolates was to the level of species using the traditional morphological and biochemical tests, according to the methods of McFadden. Confirmed identification with 16S rRNA & type three secretion system. This study showed that *E. cloacae* had a higher percentage in ALL patients (42.7%) than AML patients (36.6%), and a lesser percent was in CLL patients (11.6%) and CML patients as (8.8%). Furthermore, *E. cloacae* are one of the opportunistic pathogens[11]. Comparative efficacy of tissue culture for bacteria and their toxin for detecting *Enterobacter cloacae* infection.

Bacterial Isolation from blood of Leukemia patients

Leukemia patient's blood of (2-3) ml was taken in test tubes that contained sterile Brain-Heart Infusion medium for blood culturing to ensure the existence of bacteria in the blood samples; it was then left in the incubator at (37C°) for (24h.) for enhancing bacterial growth[12]. Therefore, all media have been prepared based on the manufacturer's instructions; Trypticase soy agar or MacConky agar were sterilized through autoclaving for (15 minutes), after cooling the media to (56 C°), after incubation on Brain-Heart Infusion for a period of 24 hr in 37C° the suspected positive samples were subculturing on MacConky agar. Otherwise, the medium was sterilized by incubation for (24) hours at (37C°). Pure strains suitable for characterization, the bacterial strains found in positive blood cultures were purified on a Nutrient agar medium. Furthermore, single bacterial strains were recognized using various techniques, including biochemical testing and molecular methods[13].

Preparation of cell-free culture filtrate (toxin extraction)

To produce crude toxin, the strains have been propagated in (50ml) of the tryptone soy broth that has been supplemented by (0.6%) extract of the yeast. They were incubated at a temperature of (37C°) with agitation at (120 rpm) in an incubator shaker for (24hour). Cultures have been centrifuged after that in the sterile centrifugation tubes at (10,000rpm) for (30min) at a temperature of (4C°). The resulting supernatant fluids have been filtered via sterile (0.45µm) membrane filters. The cell-free filtrates have been stored at a temperature of (4C°) for not less than (48hour) to be used in the toxin bio-assay[14].

Determination of protein concentration

The concentration of the protein has been characterized based on Bradford, with the use of the bovine serum albumin standard curve by the following steps: various concentration values (0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) have been made from the (BSA) stock solution (1mg/1ml), after that, (2.50ml) of the prepared Coomassie brilliant blue G250 dye has been added, and then mixed and left to sit for (2min) at the temperature of the room. The absorbance has been measured at (595nm); a blank has been made from (0.45ml) of (0.05 M) phosphate buffer pH7 and (2.5ml) of dye reagent. The standard curve has been plotted between concentrations of the BSA towards corresponding bovine serum albumin absorbance at (595nm).

The concentration of the protein has been calculated through the mixing of (0.05 ml) of the testing sample, (0.45 mL) of the phosphate buffers and (2.5mL) of the Coomassie brilliant blue G250, which has been left to sit for (2min) at the temperature of the room before the measurement of absorbance at (595nm).

Purifications through the chromatography of gel filtration (1x30cm)

Sephadex G1500 has been produced as Pharmacia Fine Chemicals Co has recommended it. A Sephadex G150 quantity has been suspended in (0.05M) of the phosphate buffer pH 7, which has been subjected to the heating at a temperature of (90 C°) for (5h.) to ensure swelling of beads, packed and degassed in a glass column (1x30cm), equilibrated after that with the same buffer. The concentrated sample obtained from the preceding step has been applied to the column. The elution has been accomplished at a (30 ml/h) flow rate, and this same buffer has been utilized for the equilibration. The absorbance of every one of the fractions was assessed at (280 nm). The peak fraction showed toxin activity was collected and concentrated by dialysis against polyethylene glycol for further tests[14].

Protein activity has been determined in every one of the fractions, and protein concentration has been determined using Bradford.

Sample preparation and identification

A sample of the protein has been made from the mixing of the protein (6volume) with (1 volume) 0.002% of the BPB (i.e., Bromophenol Blue). All the steps that have been involved in preparing the native protein samples have been carried out at (4 C°). Protein samples for the SDS-polyacrylamide have been substituted in (0.0625M) Tris-HCl (pH 6.8) is brought after that to 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 0.002% (w/v) BPB and 10% (v/v) glycerol utilizing the concentrated stock solution. The samples for the SDS-PAGE have been heated in a bath of boiling water for (3 min), which ensures protein denaturation post-heating. The sample has been left to cool down to the room's temperature (25C°) and centrifuged at (10000 rpm for 5min)[16].

Sample loading and electrophoresis

The approach of the liquid sample loading has been summarized for the native PAGE as well as the SDS-PAGE. Gels have been fixed on the electrophoresis set; the reservoir buffer has been added to the upper line. The sample has been loaded carefully on the surface of the gel with the use of micropipette or a micro syringe. The electrophoresis device was connected to a power pack, and the gel was subjected to (100V) for (2hour)[16].

Gel staining

The Coomassie brilliant blue G250 solution has been made through the dissolution of (100 mg) coomassie blue in (50 ml) a mixture of the Methanol: Water: Glacial acetic acid (5:5:2vol.) with the stirring and its volume has been filled to (1 L) with the distilled water and filtered via Whatman No1 filter paper and kept in the dark bottle at a temperature of (4C°) to be used later.

Maintenance of cell cultures

THP-1 and HBL-100 cells were kept in RPMI-1640 supplemented by (10%) of the fetal bovine serum (100µg/

No.	Items	Company	Country
1	DMSO	Santacruz Biotechnology	USA
2	Fetal bovine serum	Capricorn	Germany
3	MTT stain	Bio-World	USA
4	RPMI 1640	Capricorn	Germany
5	Trypsin/EDTA	Capricorn	Germany

Table 1. Chemicals and reagents.

mL) of the streptomycin 100units/mL of the penicillin. The cells have been passaged using the Trypsin-EDTA that has been re-seeded at a confluence of 80% twice every week; then, it has been incubated afterward at a temperature of (37C°)[17,18].

Cytotoxicity assays

To determine the cytotoxic effects of (Toxin), the assay of the MTT has been done using the (96-well) plates[17,18]. The cell lines have been seeded at (1x10^{4}cells/well), (24 h.) later, or after achieving a confluent monolayer, the cells have been treated by the tested compounds at a variety of the concentrations. The viability of the cells was measured (72 h.) after the treatment with the removal of the medium, addition of (28.0µL of 2mg/mL) solution of the (MTT) and cell incubation for (2.5 h.) at a temperature of (37C°).

Following the removal of (MTT) solution, crystals that remain in wells have been solubilized by adding (130µL) of the (DMSO) then (37oC) incubation for (15min) with the shaking[19]. Absorbency has been specified on the micro-plate reader at 492nm; the assay has been carried out in triplicates. The cell growth inhibition rate (i.e., the cytotoxicity percentage) has been computed based on the following formula[20,21]:

$$\text{Rate of inhibition} = A - B/A * 100$$

(A) represents the control's optical density, and (B) illustrates the samples' optical density[21,22].

To visualize the shape of the cell under an inverted microscope, cells were seeded in (24-well) micro-titration plates at (1x10^5) cells/mL density and incubated for (24 h.) at a temperature of (37C°). After that, the cells were exposed to (Toxin) at a concentration (IC50) for (24 hours). After the exposure, plates were stained with the crystal violet stain and then incubated at (37C°) for (10 –15 min)[22,23]. This stain has been gently washed off using the tap water to the point where the dye has been removed entirely. Cells have been examined using the inverted microscope at a magnification of (40x), and images have been taken using a digital camera attached to a microscope[18].

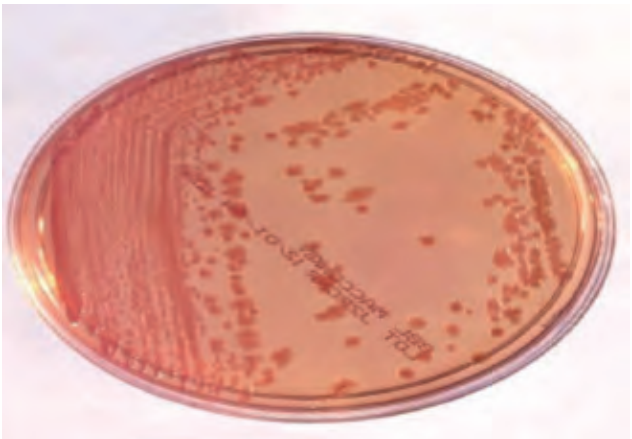


Figure 1. *Enterobacter cloacae* culture on MacConky agar.

Statistical analysis

The data that were obtained have been analyzed statically with the use of unpaired t-tests by Graph-Pad Prism 6[17]. The values have been given in the mean ± SD of the triplicated measurements[24].

Results

Sample preparation and identification

The 30 (23.07%) blood samples were positively cultured using MacConky agar medium as shown in fig.1; isolates were further identified by biochemical tests (table 2). All the isolates were found in smooth form by biochemical tests. Oxidase, H_2S, urease and indol were negative, citrate and catalase were positive, and all were found motile. All (30) isolates were found positive by biochemical tests, as shown in Figure 1 and Table 1.

Toxin extraction and protein electrophoresis for Enterobacter cloacae

The isolates were reconfirmed by molecular technique, including 16SS rRNA and TTSS from (11) bacterial samples of *E. cloacae* we screened for toxin after extraction it, so the result showed that 22 fractions (crude toxin, determination of protein concentration and partially purified toxin) as Figure 2, Figure 3, Fig.4 and Table 3.

Tissue culture for Enterobacter cloacae toxin

According to toxin extract from Enterobacter cloacae, our results revealed that (22) fraction/toxin were taken from (11) bacterial samples; these toxins were separated by gel electrophoresis for protein. (18) samples were the positive result for gel electrophoresis of protein according to molecular weight: (13.3 KD for α-hemolysin), (70.5 KD for thiol) and (75 KD for enterotoxin). The cytotoxic activity related to E. cloacae strains has been associated with the toxin of α-hemolysin toxin (13.3 kDa) around 6 (33.33%) toxin samples of the isolates26. After isolates of E. cloacae from the blood of leukemia patients and it is culturing on blood agar, it showed that E. cloacae produce α-hemolysin exerted an effect on blood agar through partial lysis regarding red blood cells the disruption of cell membrane for producing a brownish or greenish-grey discoloration around colony as Figure 5.

The cytotoxic effect of Enterobacter cloacae toxin against THP-1, and HBL-100 cells was studied, as shown in Figures 6 - 13.

No. of Test	Name of Tests	Test
1.	Indole test	-
2.	M R test	-
3.	V-P test	+
4.	Citrate test	+
5.	Catalase test	+
6.	Oxidase test	-
7.	Urease test	-
8.	Motility	+
9.	**Production of H_2S**	-

+ = Positive, - = Negative, MR test, V-P test

Table 2. Biochemical characters of *Enterobacter cloacae* isolates.

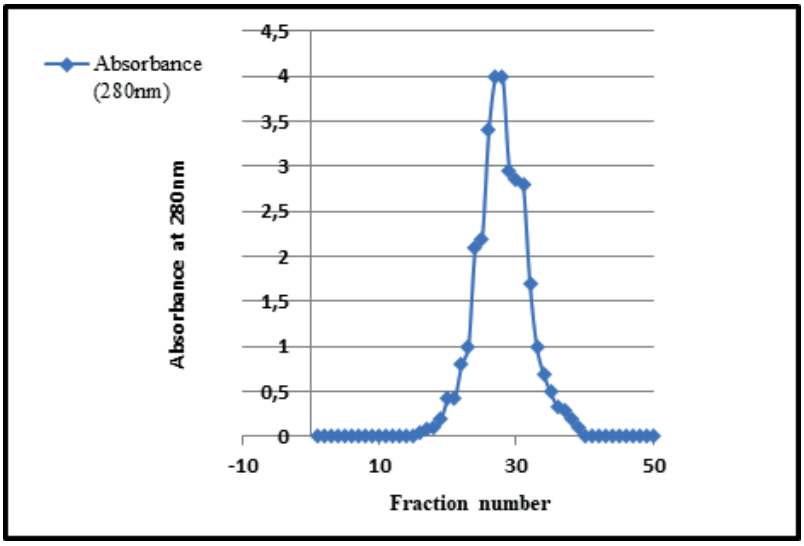


Figure 2. Gel filtration chromatography used Sephadex G 1500 column equilibrated with (0.05 M) sodium phosphate buffer (pH 7.0) at flow rate of (30 ml / hour).

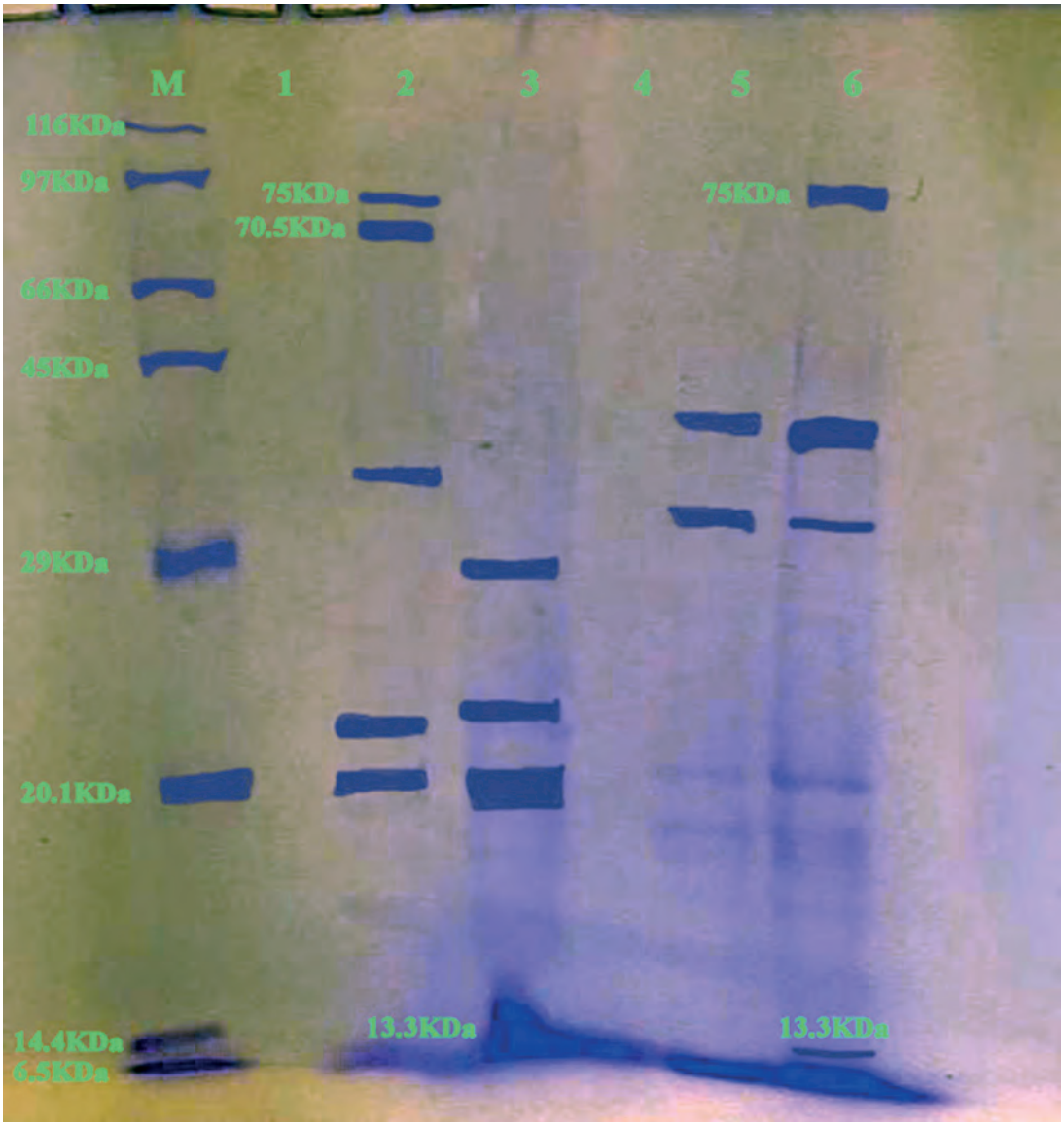


Figure 3. SDS-PAGE gel electrophoresis (1.4gram SDS-PAGE, 100 V/Amp) and Bromophenol Blue dye staining to detect 244 protein "toxin" size product (band 13.3KDa, 70.5 KDa & 75 KDa). Line 2&6 were positive samples and other have negative. M 245 lane: molecular weight confirmation of protein (Marker).

Toxins	Number	Percentages
Enterotoxin	7	38.88
Thiol toxin	5	27.77
α-hemolysin toxin	6	33.33
Total	18	100
Chi-square χ^2 value	--	4.91 *
* (P≤0.05)		

* (P<0.05) = significant, P= probability

Table 3. Detection of types of toxins for *E. cloacae* depends on molecular weight using SDS PAGE.

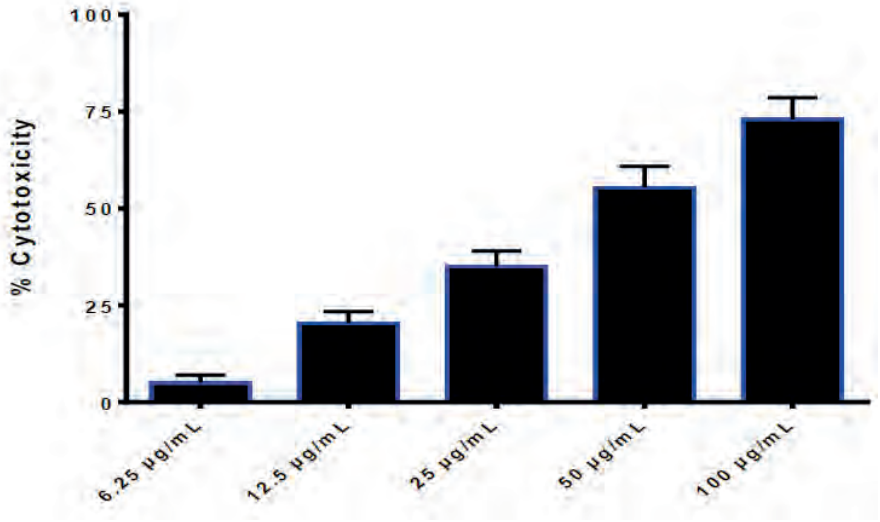


Figure 4. Growth inhibition of HBL-100 cell line effected by thiol. IC50=35.44 µg/ml.

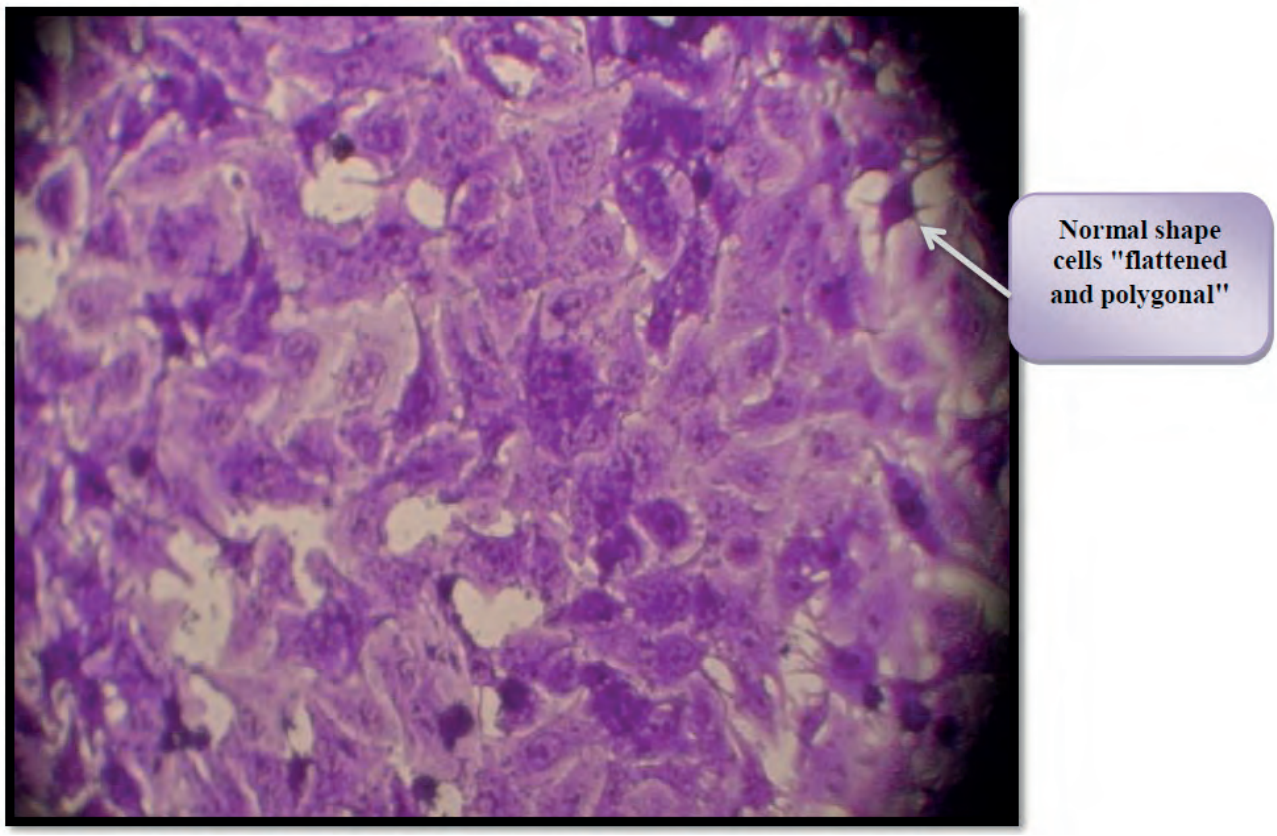


Figure 5. Control untreated HBL-100 cell. Cells.

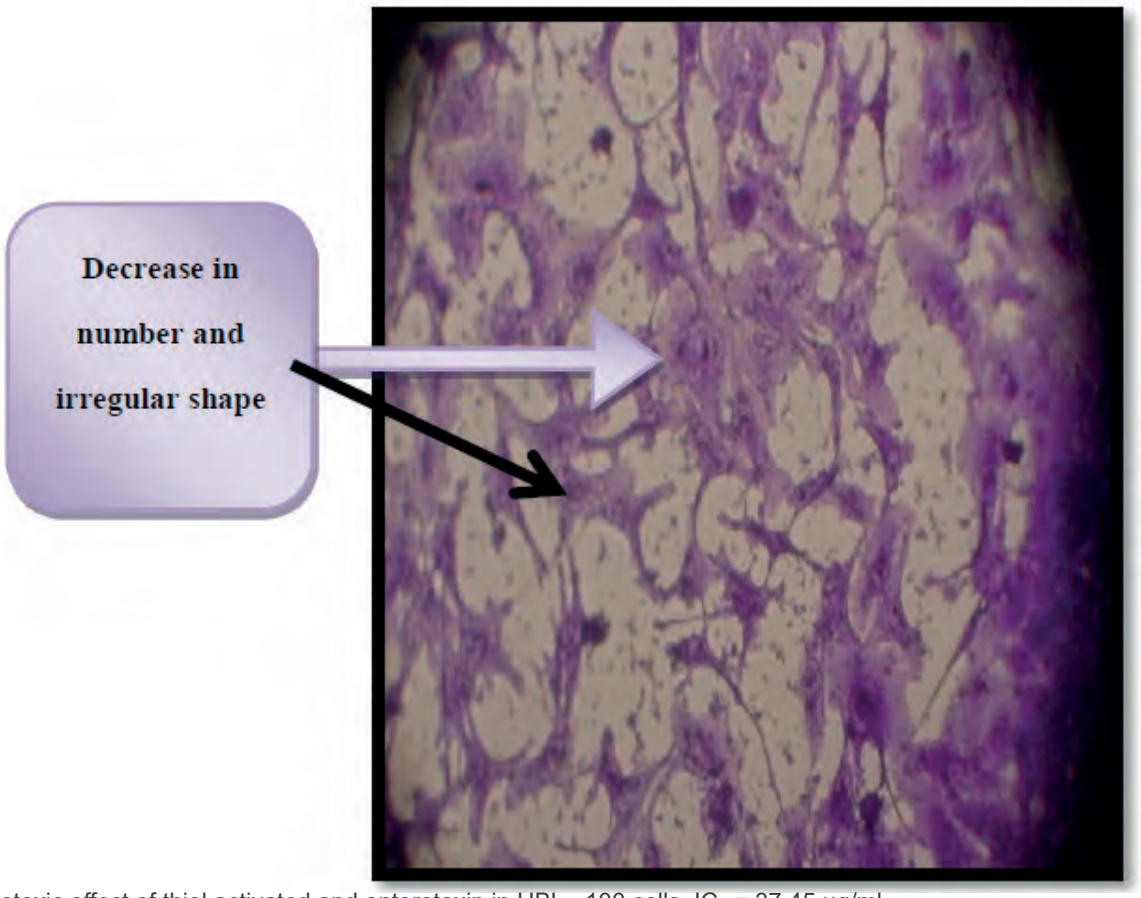


Figure 6. Cytotoxic effect of thiol activated and enterotoxin in HBL - 100 cells. IC_{50}= 37.45 µg/ml.

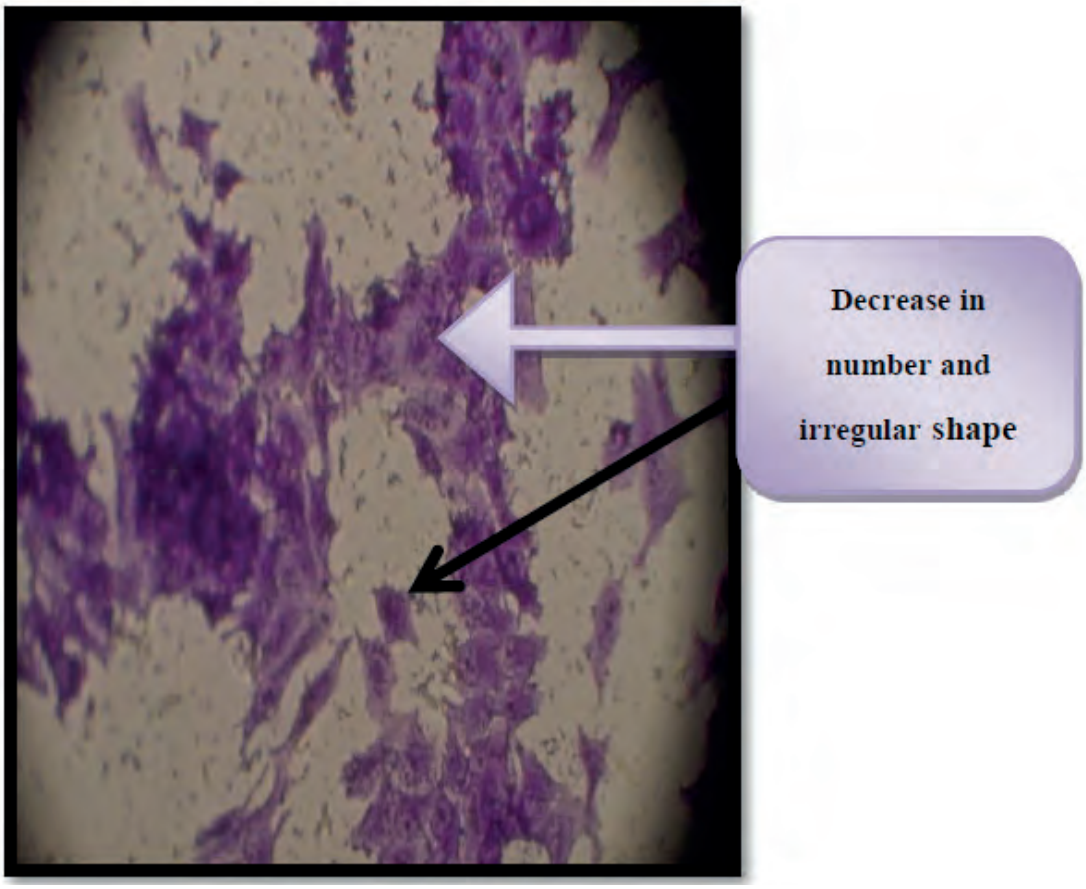


Figure 7. Control untreated HBL-100 cell.

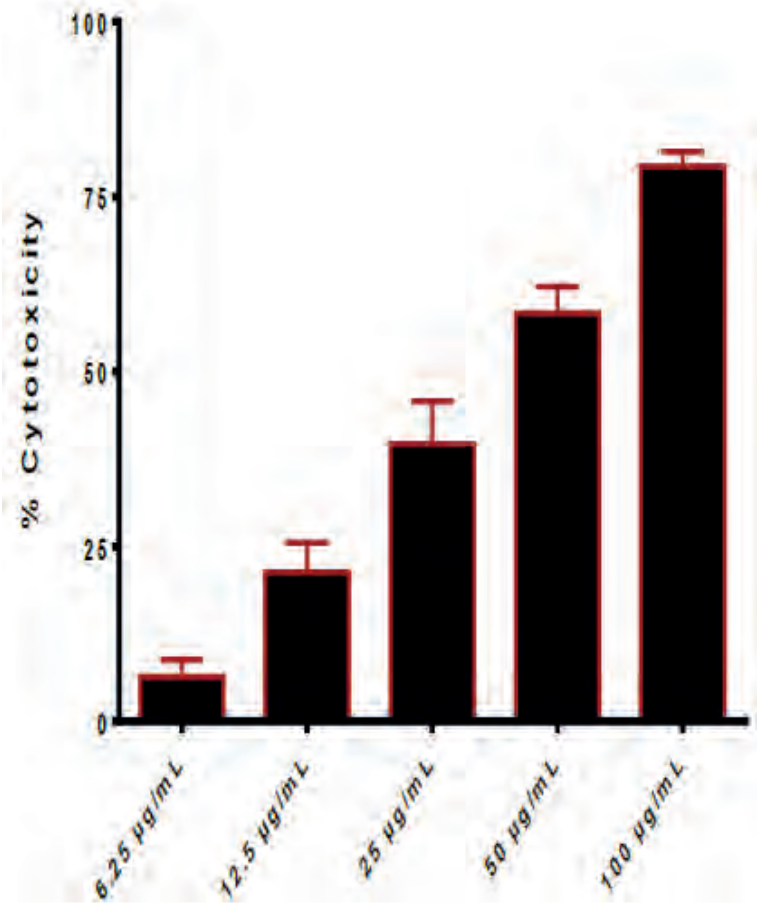


Figure 8. Cytotoxic effect of thiol-activated and enterotoxin in THP-1 cells. IC50= 30.02 µg/ml.

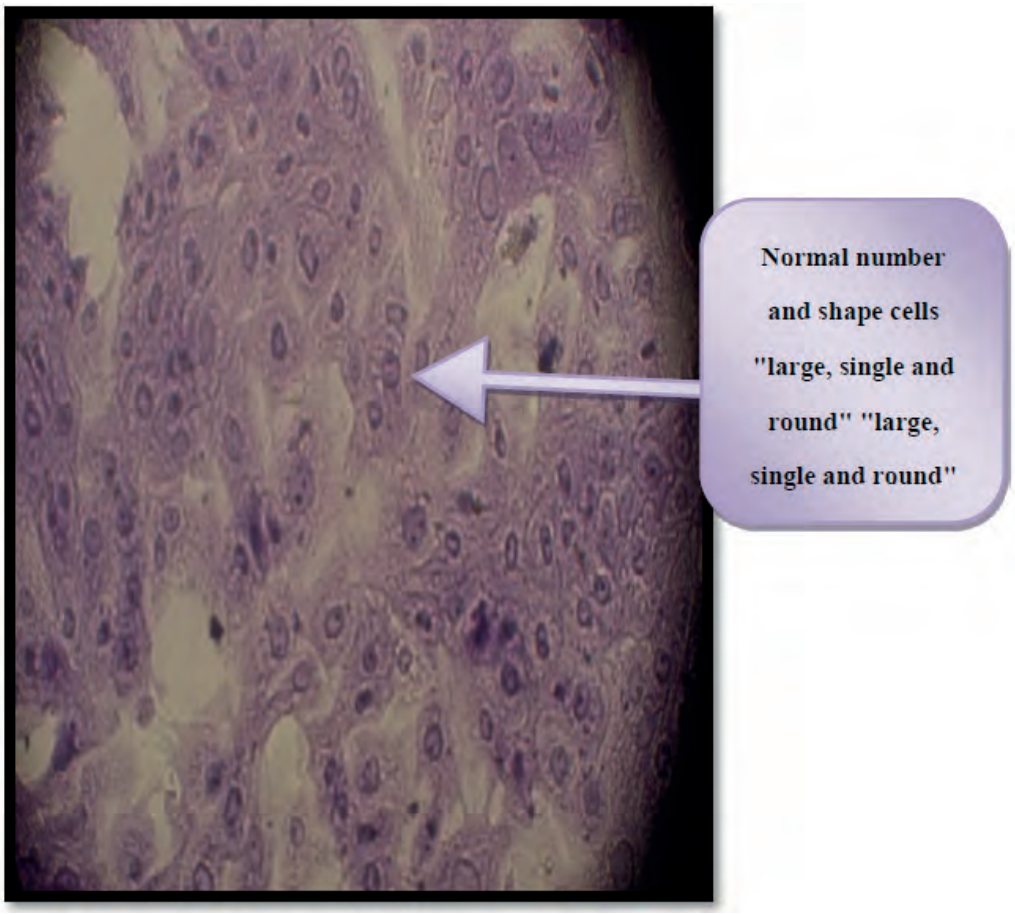


Figure 9. Control untreated THP-1 cells.

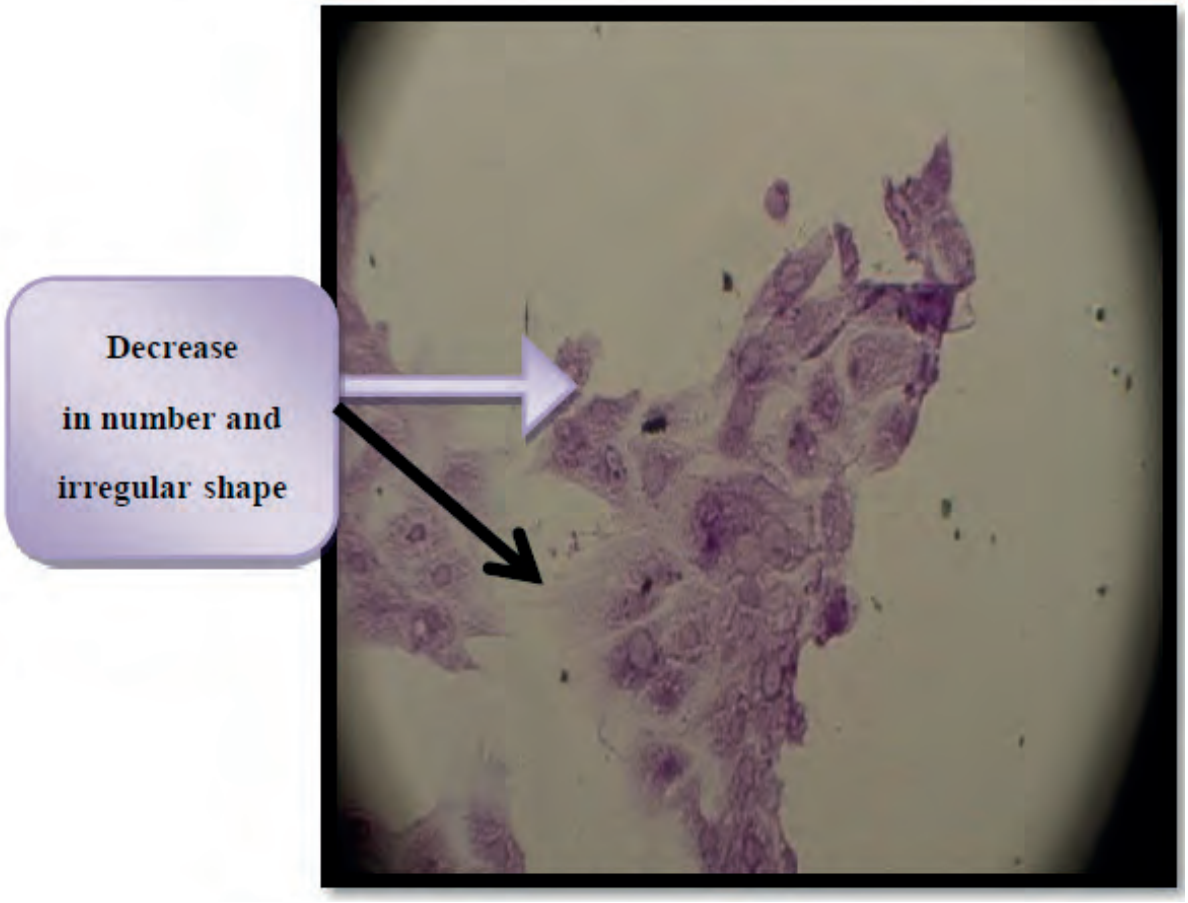


Figure 10. Morphological changes in THP-1 cells affected by thiol toxin activity IC50 for 24 hr.

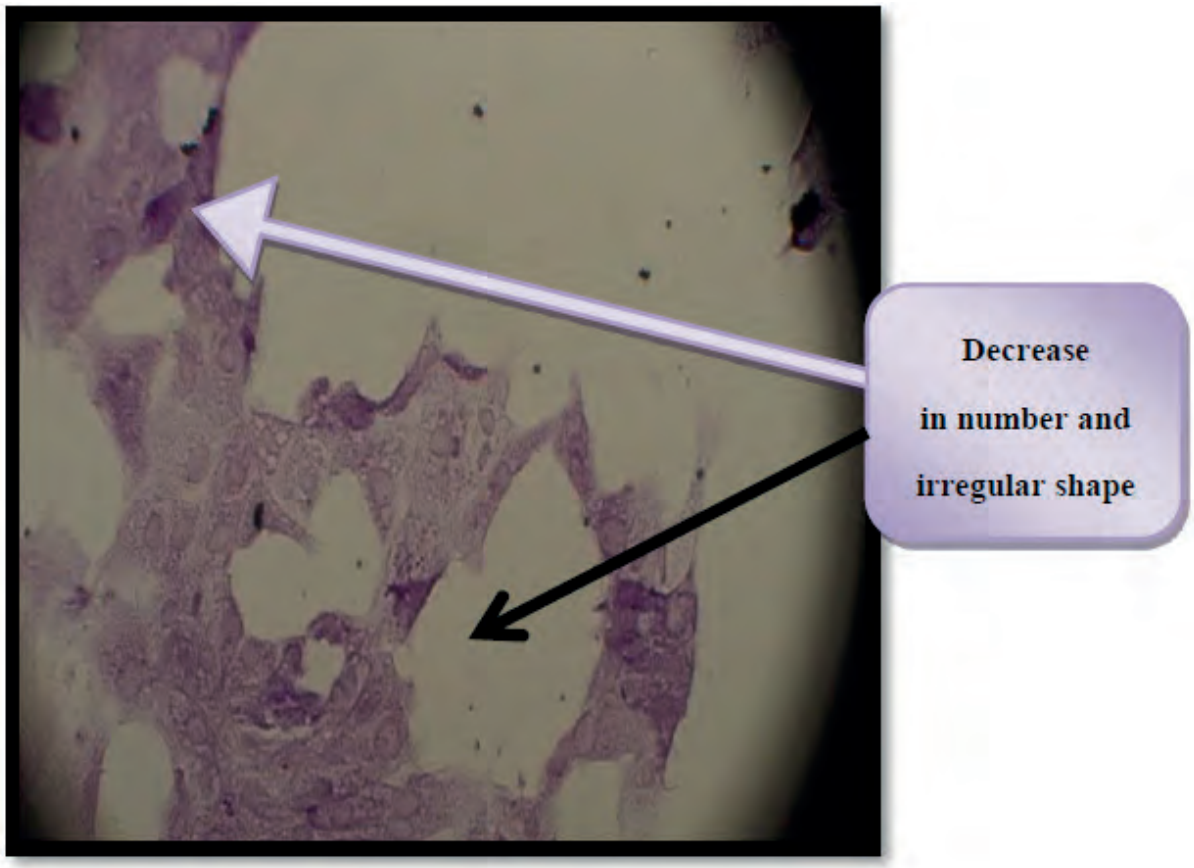


Figure 11. Morphological changes in THP-1 cells affected by enterotoxin activity IC50 for 24 hr.

Discussion

The analyses of incidence that are related to the markers of virulence in the isolates of *E. cloacae* indicated that 18 (81.81%) produced cytotoxic toxins and the absorbency of toxin has been determined on a microplate reader at (595 nm) for crude was (0.8 nm) and for toxin was (0.2 nm). The activity was observed on macrophage and epithelial cells. Cytotoxic strains were detected from blood leukemia patients. After that used gel electrophoresis of protein, showed 18 (81.81%) samples contain bacterial toxin out of (22) fraction and it was separated by molecular weight.

The cytotoxic activity related to *E. cloacae* strains has been related to the toxin of α-hemolysin toxin (13.3 kDa)[25]. After isolates of *E. cloacae* from blood of leukemia patients and it culturing on the blood agar, showed *E. cloacae* produce α-hemolysin exerted an effect on blood agar through partial lysis regarding red blood cells through the disruption of cell membrane for producing a brownish or greenish-grey discoloration around colony. Those monomers are diffused to target cells and attached to them through certain receivers. After that, they oligomerize, resulting in ring-shaped heptamer complexes[26]. Rapid discharges of essential molecules like the ATP, the dissipation of ion gradients and membrane potential, and irreversible osmotic swelling resulting in cell wall rupture (i.e. lysis) might all contribute to hosting cell death[27].

In this study, we have 5 (27.77%) toxin samples of that were cytotoxic to THP-1 and HBL-100. The findings imply that the bacteria's thiol-activated (70.5 kDa) cytotoxic toxin might impair the host's innate immune response. *E. cloacae* strains[28], identified a novel thiol-activated toxin that had an impact on leukocytes and erythrocytes. A high dose of toxin produced leukocyte lysis prior to apoptosis was triggered[29]. Also, in this work, we have 7 (38.88%) enterotoxin samples of the isolates that cytotoxic to THP-1 and HBL-100.

Enterotoxin (75 kDa) is produced and secreted from *E. cloacae*. Enterotoxins are typically cytotoxic, killing cells via affecting the permeability of epithelial cells in the intestinal wall's apical membrane. Pore-forming toxins (mainly chloride pores) are bacteria-secreted toxins that combine to produce pores in cell membranes[30]. The cytotoxic effect of toxin extraction against cell lines (THP-1 and HBL-100) was studied. The capacity of thiol activated and enterotoxin to decrease the proliferation of cell lines was used to investigate their antitumor efficacy (HBL-100 and THP-1). The results of this work revealed significantly considerable cytotoxic activity against human cancer cell lines (HBL-100 and THP-1). The findings reveal that thiol activated and enterotoxin can inhibit the growth of cell lines (HBL-100 and THP-1), and that this impact is concentration-dependent.

In this work, we looked at the virulence factor (toxin) production and associated aspects of the human isolates of the *E. cloacae*. The cytotoxic effect of bacteria and its toxin on macrophages (THP-1) and epithelial cells (HBL-100) was investigated. Another work found that *E. cloacae* human isolates produced virulence factor (toxin) related features in Vero, CHO (i.e., Chinese hamster ovary), and murine macrophage J-774 cells and the existence of TTSS[31].

Conclusions

This study focused upon the explanation of contributions of cytotoxic activities, culture and also focused on tissue culture for *E. cloacae* pathogenesis toxin extraction, and discovered during our study that the tissue culture observed bacterial toxin causes inhibition and suppress to monocytic cell "THP-1 cells" and epithelial cells "HBL-100 cells".

Funding

Self funded this research.

Informed Consent Statement

Written informed consent has been obtained from consultants of hematology center referred to Medical city/ Baghdad Teaching Hospital/ Hematology center.

Data Availability Statement

The results might be achieved using an evaluation software or assessment table, which could be available at www.diagnostics.sk/idmicro.

Acknowledgments

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Conflicts of Interest

None

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REVIEW / ARTÍCULO DE REVISIÓN

Obsessive-Compulsive disorder is a severe threat to society

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Abstract: To give an overview of Obsessive-Compulsive Disorder (OCD). In this review, the vital information about OCD and the possibility of linking suicide through this mental issue, getting to provide references for cover. OCD is a chronicle real unbalanced form of mental disorder that leads to an unreasonable configuration of obsession and compulsion. Obsessive-compulsive disorder (OCD) is usually believed to follow a persistent, repetitive course. The onset of illness has a bimodal peak – in early adolescence and adulthood. Obsessions are the elevation of thoughts and feelings irrespective of common or genuine sentiments, infuriating and repetitive effects on mental beings. Compulsion is a short part tempo result after the obsession to repeat a particular activity. This anomalous situation helps us understand that it turns out to be critical and last for a short period; it gets back as before when done. Obsessive-compulsive Disorder (OCD) is a psycho-neurological issue that sets out to be impulsive and cause distress and uneasiness in a person's life. OCD has the potential to trigger suicide. An obsessive-compulsive disorder is outlined by the two proximity of continuous impulse and compulsions, and recent studies have also identified neuroimaging and neuropsychological correlations of a natural outcome, but these need further replication. After a comprehensive study, the evidence provided in this article presents that OCD may be a grueling mental issue and a significant threat to society.

Key words: OCD, Obsession, Compulsion, Neuropsychiatric, Treatment, Survey.

Introduction

Obsessive-compulsive disorder is a neurological disorder that distinguishes the appearance of obsession and/or compulsions for a prolonged time zone and makes the sufferer uncomfortable and distressed. OCD can occur in any seniority or age group regardless of race or sexuality, or religion. OCD has been evaluated with approximately 8 billion dollars per year as per U.S. data. Despite being frequent and impaired, according to studies, almost 60% of patients suffering from OCD wait for a long time to get treated, or some do not get proper treatment scarcity of health professionals to recognize the disorder of OCD[1].

Obsessive-compulsive disorder (OCD) is an anxiety disorder distinguished by the existence of two different phenomena: obsession and compulsions. The prevalence rate of OCD is 2 to 3 percent, and in India, it is lower than 0.6 %. OCD is common in both males and females, although males are more prone to this disorder. The existence of OCD in various studies ranges from 0.4-to 2.5 %. Occurrence in elder patients ranges from 0.6% to 2% of patients. According to epidemiologic studies, India has found a lifespan existence of 0.6%. Males are more significant than that of females. Sexual obsession was characterized in the early age of onset, and getting more beneath those continuously leads to compulsion. Females have compulsive washing (66%) and avoidance (26%). More percentage of divorce is one reason reported in OCD sufferers in contrast with married people. It has also been reported divorce rate in men is 40% and in women is 39.6%[2].

In addition to high acceptance of clinical OCD, symptoms of OCD have been experienced without actual disorder of obsessive-compulsive disorder. Episodes of OCD have been experienced by one-fourth of the population once in their lifetime. According to reports, sub-threshold OCD has an acceptance rate of 12.6%. Reported community studies in OCD have a slight female majority, and sub-threshold OCD has been reported among males. Epidemiological studies and phenomenological studies have chief benefits. OCD has been associated with other psychological and anxious symptoms. In the group of people with OCD, sufferers consume a high rate of powders and liquors. Other issues related to OCD are hyperactivity issues (ADHD) to adolescence OCD. Various groups of OCD have clashed with other reports of co-event running from 0% to 60%. Barely any examinations likewise report high rates of sexual maltreatment in OCD[3].

Obsessions are the repetition of intense occurrence of thoughts/emotions or feeling (e.g., fear of something); compulsions are recurrent physical/mental that are amenable to obsession and very acute episodes of own coincide (e.g., abnormal washing of hands etiquette). OCD currently has a compound genetic pattern and combination with psychosocial factors on an inheritance basis. In the studies, X chromosomes have an obsessive-compulsive disorder in family members' background[4].

Obsessive-compulsive disorder occurs throughout the lifetime, and youngsters as young as 6 or 7 present with

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the characteristic impairing symptoms. At the opposite end of the age range, patients may present for the primary time in adulthood. Maximum adults with this disorder report onset in childhood or adolescence. The condition may result in considerable disability; for instance, children may drop out of education, and adults can become housebound. The planet Health Organization rates obsessive-compulsive disorder as one of the highest 20 most disabling diseases. It generally persists and evidence-based psychological and drug treatments are available if untreated. Recent epidemiological study results report prevalence rates of 0.8% in adults and 0.25% in 5-15-year-old children, although earlier studies suggested rates as high as 1-3% in adults and 1-2% in children and adolescents[5–8].

Criteria for Obsessive-Compulsive Disorder

Either obsessions or compulsions where Obsessions as well-defined by recurrent and persistent thoughts, images or impulses that are felt, at certain times during the commotion, as intrusive inappropriate and that cause marked anxiety, nervousness or distress. It also can be said that the thoughts, thoughts or impulses that aren't, without a doubt, are immoderate concerns causing approximately real-existence problems. The character tries to disregard or suppress such thoughts and impulses to neutralize them with a few different notions or actions. The character acknowledges that the obsessions with thoughts, and impulses, are made of their thoughts and now no longer imposed from without as in notion insertion.

Compulsions as described via way of means of repetitive behaviors like hand washing, ordering, checking or intellectual acts like praying, counting, repeating phrases silently that the man or woman feels pushed to carry out in reaction to an obsession or in keeping with regulations that have to be carried out rigidly. It is likewise the behaviors or intellectual acts are geared toward stopping or decreasing misery or stopping a few dreaded occasions or situations; however, those behaviors or intellectual acts both aren't linked in a practical manner with what they're designed to neutralize or save you or are truly excessive.

During the disorder, the individual has been diagnosed with obsessions or compulsions that are immoderate or unreasonable. The obsessions or compulsions purpose marked distress, are time-ingesting, take greater than 1 hour a day, or drastically intervene with the individual's ordinary routine, occupational or educational functioning, or ordinary social sports or relationships. If every other Axis I disease is present, the content material of the obsessions or compulsions isn't confined to it. The disturbance isn't because of the direct physiological outcomes of a substance like a drug of abuse, a medicinal drug or a fashionable clinical condition.

Source

The above three paragraphs are obtained from the works of Professor Dan J. Stein, the University of Cape Town[9], where form it is adapted from the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision; DSM-IV-TR (i.e., American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR). Washington, DC: American Psychiatric Association, 2000). For educational purposes only and omitted from the DSM-V. This has been changed in the DSM-V (American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition. Washington, DC: American Psychiatric Association, 2013). The symptoms of another mental disorder[9] do not better explain the disturbance.

The Symptoms Associated With OCD

Sufferers/patients were necessary to be rated for suicide threat and proximity of subjects over the lap of their sickness. The cure is signified when OCD reflection impairs the victim and leads to a big disaster. Good cure aim is working through which an hour irrespective of the whole day over the most necessary work and leads to uncertain opposition with everyday conditions. Fig 1A (Obtained from Foss K, California OCD and Anxiety Treatment Center[10], Available: https://www.calocd.com/obsessive-compulsive-disorder/scrupulosity/) and Fig 1B(Obtained from Soule K, Soule Therapy[11], Available: https://www.souletherapy.com/post/how-to-tell-if-you-have-ocd-and-what-to-do-about-it) showing mark of rhythm of OCD. The psychological condition leads to patients with the worsened condition of OCD[12–14].

Some Common OCD Patterns

Common Obsessive Compulsive Disorder patterns are counting and repeating, protecting against contamination, checking, hoarding, strange movements, and scrupulous[13]. Fanatical considerations are not possible to support; they naturally occur. Obsessions cause shivering, which resembles the inclination to get in the abdomen or when making emotional prospective like seems like a stranger. The restlessness and tension are terrifying, and teenagers have episodes of obsession which results in continuous compulsions. However, compulsion can never reshape or add up until the obsession becomes enlarge[14].

The psychological etiquette that have the massive rate of commandment in anyone's with OCD involvements such as depression which leads to lifetime occurrence range from 45.9 percent to 68.4 percent. Commonly uneasiness or anxiety disorders that lead to whole life range from 31.9 percent to 34.6 percent. Social phobias, which lead to lifetime occurrence, range from 17.3 percent to 36.1 percent. Specific phobias which lead to lifetime occurrence range from 15.1 percent to 33.0. Panic disorders that estimate lifetime co-occurrence range from 12.8 percent to 20.2 percent (panic disorder and/or agoraphobia). Post-traumatic stress disorder leads to lifetime occurrence ranging from 11.6 percent to 19.2 percent[15].

Behavioral Treatment & Dysfunctional Beliefs Associated With OCD

Obsessive-compulsive disorder (OCD) is explained by the proximity of unachievable, probing to others' matter and movement (depressingly dull and bleak). OCD in teenagers has been cleanup up by the change in research since the 1980s. Focus in the USA and the U.K. have proven that OCD in teenagers can be built under guidance; the cognitive conduct treatment I.e., Cognitive-Behavioral Therapy (CBT), becomes the primary treatment. The supper psychological cure for OCD turns on gaining hypothesis. The idea behind their chief use was straight; the practice was being driven and secured by declining in difficulty caused by fixation. Regularly, the fixation is commune as a few kinds' of physical misbehaviors going to the particular patient. Though it happens to be gradual amorphous, some children are unwell to show frustration. The conduct treatment in shapes consists of admiring to break the network within the decli-

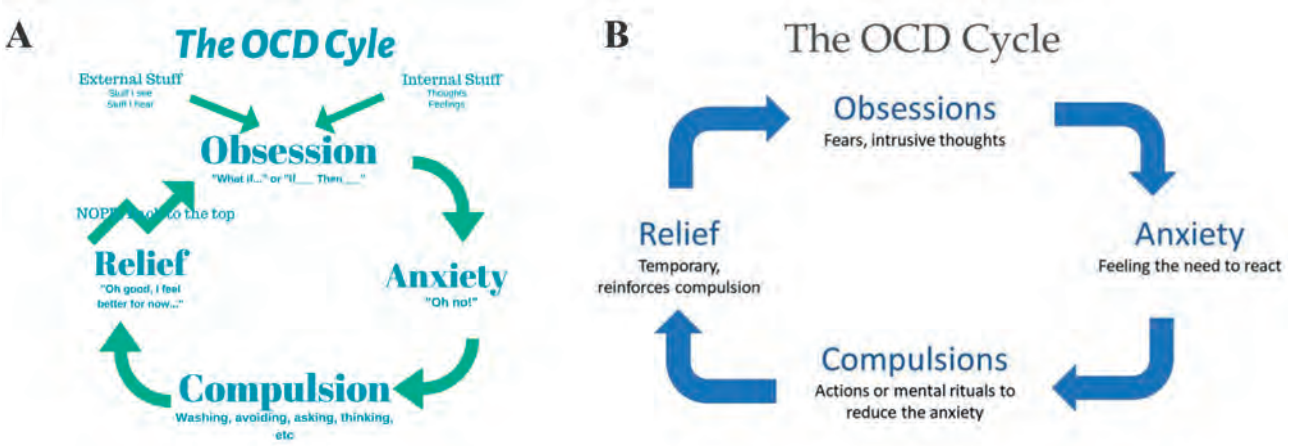


Figure 1. A and B represent The different patterns and cycles of OCD.

ning of difficulty by allowing self to consider the pressure and will not start the impulse. A notable contemporary intellectual model is designed by the obsessive-compulsive awareness active breed. Such a massive congress of more than 40 members showed their interest in getting to know the job of mental means in obsessive-compulsive disorder by attaining a document concerning those most severe basis sufferers' of OCD, and they classified them according to thought and thought space which was said to provide the similar screening. Two own-result estimates of the Obsession reliance on personality inventory and the self believes of intrusions examination –were designed to screen different zones[16,17].

Treatment of OCD

Clomipramine hydrochloride (Anafranil) was the chief FDA-initial therapeutic medication for OCD. They know that it is a powerful serotonin reuptake blocker by a molecular structure having tricyclic stimulant properties. It is slightly more specific than the SRIs and leads to increasing antagonistic complications. The more particular operators have many results and give the image of being vigorous as c*lomipramine hydrochloride* in scanning, and the same gets into SRI in fewer days upon day dosages of 25mg to 50mg; if a higher amount of *chlorpromazine hydrochloride* can lead to risk due to hindrance. Dimensions of tricyclic depressants in OCD patients' blood samples will be examined habitably to determine whether the two therapeutic medications are correct. For elevated amounts of tension (I.e., for those patients who are disabled with the last motto), benzodiazepines are of great use in treating dominion. On other thought, antipsychotic drugs are also recommended with SRI[18].

Haloperidol dosage of 0.5mg to 5mg is a customarily used therapeutic drug. In some screening, the atypical antipsychotics (e.g., Olanzapine, Risperidone) have some effects. *Buspirone hydrochloride* is added to SRI for use. Jotted that is barely utilized, psychosurgery (e.g., sub caudate tractotomy, foremost capsulotomy, or limbic leucotomy) coincides with the efferent pathway, frontal cortex, and basal ganglia with the extreme of 67% cases. By having all the desired proof and confirmation, therapeutic drug and cognitive-behavioral therapy variants take Obsessive Compulsive Disorder efficiently, some OCD patients sake from their merger. The data do not clearly explain the superiority of merger therapy over the most effective mental therapy. Initially, psychotherapy is the first step to reducing the unwanted price and danger of therapeutic drug[19].

Anatomical Effect on Brain

Mutual differences from the normal with the white issue volumes have been detected in OCD patients and dependence from the starting with the few issue changes in OCD. Duran, Hoexter, Valente, Miguel, Busatto (2009) stated that an increase in white issue volume in the two ways appendages of the inner ear is affiliated with symptomatic OCD severity. A meta-analysis later concluded that OCD sufferers had a specified white issue promoting the corpus callosum. Similarly, a VBM ponder said white issue promotes OCD in cerebrum areas, such as the prerogative dorsolateral prefrontal cortex, center frontal gyrus, precuneus, and substandard parietal lobe[20].

Observation from a spread of research sources has made it possible to think about the pathogenesis of obsessive-compulsive disorder (OCD) in terms of brain anatomy. Neurological patients showing obsessions and compulsions generally have diseases involving the basal ganglia or the inferior frontal area. Neuropsychological alterations in patients with OCD include deficits within the executive functions of the frontal lobes. Neurosurgical procedures to treat OCD symptoms directly or indirectly disconnect frontal subcortical loops. Functional brain imaging reveals orbitofrontal and basal ganglia hyper-metabolism in patients with OCD at rest and through symptom provocation that normalizes with response to treatment[21–23].

Mental Disorder Leading to Suicide

It is reported in a study that maximum suicide victim has suffered from mental disorders[24,25] and it can be a strong predictor of suicidal behavior[26], anxiety, personality, eating factors, and trauma-related disorders; mental disorders are the contributing factors of suicide[27]. Frustration, depression, shame and loneliness can also be the main factor for being a victim of suicide. Any obsession leads to depression, and depression leads to death. In this generation, mental health is equally important as physical health. Women with OCD are at more risk of suicide than men with OCD[28].

OCD is associated with an inflated risk for suicidal tendency, and with depression and hopelessness, it correlates with suicidal behavior. Treatment of depression may be subjected to alter the risk for suicide, and hence patients with OCD must undergo tests for suicide risk associated depression[29–31]. Obsessive-Compulsive Disorder (OCD) was delegated as an anxiety issue, and like other minor mental issues, OCD is additionally a mental incapacity related to extreme trouble and suffering[32]. 15% of undiagnosed de-

pressed patients may commit suicide, but the highest risk of suicide occurs in combinations of affective or psychotic disorders with abuse of alcohol or drugs[33].

90% of people who commit suicide are considered to be suffered from a psychiatric disorder[34], and about 6.7% of suicidal patients received a diagnosis of OCD as per a study made by Hollander and team[33,35]. Obsessive-compulsive symptoms are related to suicidal outcomes independently of depressive symptoms or mood instability, and thus OCD is a threat to suicide as in own rights[28,36].

There are several studies on OCD reported cases concerning years. For the best finding and understanding, a data-sheet prepared upon a literature survey is observed the percentage of cases reported in a research paper against previous reports. Although the graph is decreasing, if we consider the percentage 2.3%, it is enormous in the annual population, i.e., the of people is enormous against non-OCD people concerning time. The hike in 2020 is likely due to the COVID 19 pandemic, which took the world by surprise. This changed people's lifestyles and habits, causing fear and anxiety, which resulted in OCD.

There can be no exact value upon the number of reported cases against all the years as not all data gets published. Lack of information helps the data sheet to be incomplete. The value obtained is solely based on the available information gathered. The authors do not claim the numbers or figures are accurate as it is impossible to quantify as per the author's perspective. Different sets of data are reported for different countries yet not complete.

Conclusions

All or few of us have always wondered about our manifold tendency and views and have it in rituals through which we bought up like having a desire cent or having a good path for different ways but the one with extreme persistence with strand ford practice take a different shape leads to pressure on every day. Fanatical urgent turmoil, the Obsessive-Compulsive Disorder (OCD) is a savior, and neurological, a psychiatric disorder that grazes with the appeal for hard three percent of the people, the scheme of its illustration it's challenging to pigeon disseminated the officer in the media. Persistently like one, is suggested to the radically different approach.

OCD is explained by critical, continuously unwanted happenings, aspiration or simple image that makes other perfection or wishes. Addiction is hazardous and irritating leading to provocative action in daily basis activities. Feelings or emotion with a different state of mindset and physiological activities which are being taken out over and over again. The occurrence of OCD leads to a pragmatic input cycle where daily basis activities create the add-on pressure and load with a feeling of uncomfortableness. The person may feel restlessness and quickly suffer from any contagious substance resulting in getting ill, or might want to wash their hands to avoid such illness frequently. This habit of frequently washing hands or cleaning things can make him feel satisfied but only for some time, but still, if he gets ill makes him again unsatisfied and irritated.

OCD patients expose to two types of therapeutic drugs. For example, SSRIs are used in OCD patients. Haloperidol, Buspirone hydrochloride, Olanzapine, and Risperidone are some therapeutic drugs used in OCD. The most widely used treatment for OCD is CBT. CBT is a brief objective having mental therapy/ psychotherapy that is used to guide patients on how their thoughts and emotions make their work/ task more unsatisfied. A comprehensive study and the investigation provided in this article present that OCD may be a challenging mental issue and a significant threat to society hence more research is required.

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Ermission

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REVIEW / ARTÍCULO DE REVISIÓN

Propuesta de modelo integral de intervención terapéutica paliativa en salud

Proposal of an integrated care model for a palliative therapeutic intervention in health care

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Resumen: Los Cuidados Paliativos (CP) constituyen un valor y una responsabilidad de los sistemas sanitarios y sociales. En vista de la alta demanda de servicios para la atención integral y humanizada del paciente con enfermedad crónica, degenerativa e irreversible, es necesario crear un modelo de atención integral paliativa con el fin de ejecutarlo en una población requiriente del mismo. Los CP son un derecho de la persona, reconocido internacionalmente, que ha de prestarse en 3 niveles de atención: primario (básico), secundario (atención especializada - AE) y terciario (Unidades de Cuidados Paliativos - UCP). El primer nivel de atención es responsable de los cuidados paliativos domiciliarios y debe garantizarlos cuando la complejidad no es elevada. La presente revisión bibliográfica propone un modelo integral, integrado e integrador de cuidados paliativos CP que permita justificar las intervenciones terapéuticas con el objetivo de obtener bienestar o atenuación del sufrimiento en pacientes que acuden al primer nivel de atención.

Palabras clave: Cuidados paliativos, evaluación integral, evaluación de necesidades multidimensionales, derivación, complejidad.

Abstract: Palliative Care (PC) is a value and a responsibility of the health and social systems. Because of the high demand for services for the comprehensive and humanized care of the patient with a chronic, degenerative and irreversible diseases, it is necessary to create a model of comprehensive PC to execute it in a population that requires it. PCs are an internationally recognized right of the person that must be provided at three levels of care: primary (basic), secondary (specialized care - SC), and tertiary (Palliative Care Units - PCU). Primary care (PC) is responsible for palliative home care and must guarantee it when there is no crucial complexity. The present bibliographic review proposes a comprehensive palliative care model that justifies therapeutic interventions to obtain well-being or alleviation of suffering in patients who attend primary health care.

Key words: Palliative care, comprehensive assessment, multidimensional needs assessment, referral, complexity.

Introducción

De acuerdo a lo publicado por la OPS en la región de las Américas, las enfermedades no transmisibles (ENT) muestran una incidencia con tendencia creciente en los últimos años[1]. Cuando a prevención y el control de las ENT no son efectivos y no se consigue frenar su desarrollo, se generan múltiples complicaciones y necesidades de soporte y CP. Independientemente del alcance de la prevención y el control, un porcentaje importante de la población con estas enfermedades requerirá cuidados integrados de soporte en etapas iniciales y CP en etapas avanzadas así como al final de la vida.El presente modelo integral de CP permitirá demostrar las intervenciones sanitarias que tiene como finalidad atenuar el sufrimiento de los pacientes enfatizando los 3 componentes de la situación paliativa: el enfermo en situación terminal, sus familiares y los miembros del equipo sanitario. El manejo y cuidado de pacientes con tales condiciones no solo es físicamente exigente sino también monetariamente agotador. A esto se agrega el hecho de que los sistemas de atención médica contemporáneos enfrentan dificultades para resolver estos desafíos, ya que originalmente fueron diseñados para resolver enfermedades únicas, agudas y principalmente a corto plazo[2].

Los CP adoptan una actitud positiva empeñada en ofrecer la mejor calidad de vida posible hasta el final. Esta etapa, tan compleja, sólo puede ser entendida desde una visión multidisciplinaria y así definir la actitud humanitaria frente al enfermo en la etapa terminal, la aptitud científica en el control del dolor y el tiempo disponible para atender las necesidades y considerar los deseos de estos pacientes y sus familias[3]. El modelo que se presenta en esta publicación es una propuesta teórica que tiene como objetivos definir la situación de CP en el Ecuador, dar a conocer los servicios de soporte y de CP como derechos fundamentales, exponer un plan de atención que incluya su nivel de complejidad actual y nivel de atención de salud, describir los criterios de ingreso a la Unidad de Cuidados Paliativos

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(UCP), detallar las prácticas esenciales y los indicadores de CP.

El presente documento va dirigido a todos los actores vinculados y preocupados por brindar soporte y CP a los pacientes. La presente revisión bibliográfica propone un modelo integral de cuidados paliativos que permita justificar las intervenciones terapéuticas con el objetivo de obtener bienestar o atenuación del sufrimiento en pacientes que acuden al primer nivel de atención.

Situación de los Cuidados Paliativos en Ecuador

La Constitución de la República del Ecuador del año 2008 obliga y la sociedad demanda la atención de Cuidado Paliativo (CP) en el Sistema de Salud público. Es preciso que los médicos se capaciten, se incluya este servicio en los diferentes niveles de atención y se asegure la disponibilidad y acceso a medicamentos especializados. Así, se podrá brindar calidad de vida a las personas que requieran esta atención; acompañándoles a ellos y sus familiares durante el proceso de enfermedad hasta el final de la vida y posteriormente durante el duelo, contribuyendo, de esta forma, a crear una sociedad más justa y solidaria[4]. Uno de los objetivos del Ministerio de Salud Pública (MSP) es priorizar la calidad de vida de los ciudadanos con necesidad de atención paliativa y sus familias, el cual se expone en el Acuerdo Ministerial número 101, el 9 de febrero de 2011 concerniente a CP, el mismo que establece que es su responsabilidad, organizar en el marco del Modelo de Atención Integral de Salud - MAIS, la conformación y funcionamiento de servicios de CP integrales con enfoque intercultural. Por lo que, ha decidido elaborar el Plan Nacional de CP, que es de carácter obligatorio, y debe utilizarse para organizar los representantes de los CP, tanto públicos como privados[5].

Aunque existen muchas limitaciones en las comunidades rurales y remotas, el MAIS tiene fortalezas en su capacidad para proporcionar atención de alta calidad al capitalizar profesionales comunitarios comprometidos en CP. Por lo tanto, adapta modelos de CP, utilizando métodos de entrega novedosos culturalmente apropiados y brindando soporte y ayuda a los proveedores comunitarios al identificar a los pacientes que se encuentran al final de la vida atendiéndolos de forma humana, integral, eficiente, con un equipo multidisciplinario[6]. Estas gestiones resultan fundamentales, ya que el prohibir al paciente con enfermedad terminal el derecho legal de elegir sus preferencias sociales y deseos al final de la vida va en contra de la libertad del paciente[7].

Aspectos generales de los CP

La revolución diagnóstica y terapéutica de los últimos 50 años junto a la mejoría progresiva de las condiciones sociales y económicas han logrado aumentar la sobrevida de la población de poco más de 60 años a casi 80, incluido en países en desarrollo[8]. Dicho escenario causa un aumento considerable de pacientes con padecimientos crónicos incurable. Estos son los pacientes que despertaron las raíces compasivas de la medicina a mediados de los sesenta en el siglo pasado en Inglaterra, donde la preocupación central se puso en el control de síntomas, control total del dolor[9] y mejoría de la calidad de vida, incluyendo los aspectos familiares, sociales, psicológicos y espirituales de los pacientes en situación de fin de vida[10].

Es notable que la mayoría de los pacientes recién diagnosticados con cáncer morirán en última instancia por su neoplasia maligna. De hecho, según GLOBOCAN, 18.1 millones de pacientes fueron diagnosticados recientemente con neoplasia maligna (excluyendo el cáncer de piel no melanoma) en 2018, y aproximadamente 9,5 millones de personas murieron con neoplasia maligna en ese mismo año[11]. En Ecuador la incidencia de cáncer es de 157,2 casos por 100.000 habitantes, según se desprende del informe dado a conocer por la Agencia Internacional para la Investigación en Cáncer (IARC), perteneciente a la Organización Mundial de la Salud (OMS), que reveló el aumento de los casos de cáncer a nivel mundial en el año 2018[12].

Se considera que fallecen 56 millones de personas al año, el 85 % de ellas en países en vías de desarrollo como Ecuador. Se asume que cada muerte también afecta a por lo menos otras 5 personas en términos de relación informal y asistencia al duelo de la familia y los amigos. El total de personas que se encuentran afectadas cada año en el mundo por problemas relacionados con la atención de los pacientes moribundos bordea los 300 millones es decir, el 5 % de la población mundial. Wetteland y col. sugieren que 3 a 6 meses de atención en CP se requiere para lograr una atención de alta calidad al final de la vida. Ellos indican que en la actualidad la derivación a los servicios especializados de CP son considerablemente más cortos que esto, lo que probablemente limitará la provisión de cuidados de alta calidad al final de la vida[13]. Esto hace que la calidad de la atención al final de la vida sea uno de los problemas de salud pública global más importantes que se enfrentan hoy en el mundo[14].

Un estudio realizado por la Base de datos Cochrane en Norteamérica en el año 2016 en el cual se realizaron revisiones sistemáticas, mostró que en pacientes con neoplasias avanzadas, los CP tempranos pueden mejorar ligeramente la calidad de vida. También puede disminuir la intensidad de los síntomas sobre todo el dolor[14]. El alivio del dolor para los CP se considera una necesidad humanitaria urgente. Es notable resaltar que el dolor moderado a intenso al final de la vida se experimenta en una gran proporción de pacientes adultos con enfermedad cardiovascular (64%), cáncer (84%), enfermedades pulmonares obstructivas crónicas (64%), SIDA (80%), diabetes (64%), enfermedad renal (50%), cirrosis hepatica (34%), Enfermedad de Alzheimer y otras demencias (47%), tuberculosis farmacorresistente (90%), Enfermedad de Parkinson (82%), artritis reumatoide (89%) y esclerosis múltiple (SM) (43%)[15].

El papel de la participación temprana en los CP para pacientes con cáncer se reconoce como un acto viable y eficaz. Los pacientes consiguen control de los síntomas, de la toma de decisiones con apoyo y una mejor calidad de vida. Se ha demostrado que es eficaz el CP junto con el tratamiento modificador de la enfermedad por medio del uso de la atención multidisciplinaria. También es una forma factible de reducir el tratamiento innecesario y excesivamente agresivo al final de la vida[16].

Modelo de soporte integral y CP al enfermo crónico

Son el conjunto de servicios dirigidos a las personas con enfermedad crónica a lo largo de la trayectoria de la enfermedad, con el fin de aliviar los síntomas producidos por la enfermedad y por el tratamiento, las comorbilidades preexistentes, complicaciones agudas, estrés psicológico, problemas sociales, así como el estrés en los cuidadores (Figura 1)[17]. Los beneficios de estos cuidados dependen de cómo los profesionales de la salud evalúan las necesidades de los pacientes en el o los encuentro(s) inicial(es). Esto resulta crucial para el diseño de un plan terapéutico y de

acompañamiento personalizado orientado a cubrir dichas necesidades, sean físicas, sicológicas, espirituales y/o sociales del paciente y la familia[18].

El modelo de atención de CP inicia con la identificación de las personas con necesidades paliativas: 1.-Pacientes con patologías crónicas, evolutivas, avanzadas acorde a los registros del Sistema Nacional de Salud (SNS) y 2.-Pacientes con enfermedades crónicas, avanzadas, con pronóstico de vida limitado, con pobre o nula respuesta al tratamiento curativo. Estos pacientes son evaluados mediante el uso del instrumento NECPAL (Necesidades Paliativas) para lo cual se requieren seis pasos[20]: 1) Identificar las necesidades multidimensionales de la persona y familia y/o cuidadores; 2) Practicar un modelo de atención impecable; 3)Elaborar un plan terapéutico multidimensional y sistemático; 4) Identificar valores y preferencias de la persona: ética, clínica y planificación de decisiones anticipadas; 5) Involucrar a la familia y al cuidador/a principal y 6) Realizar gestión de caso, seguimiento, coordinación y cuidados continuos.

En las últimas décadas se han realizado esfuerzos para brindar CP utilizando enfoques basados en el hogar o en la comunidad. Sin embargo, las experiencias son pocas y se limitan solo a ciertas geografías. A menos que la prestación de CP esté arraigada en el sistema de atención médica del país, los beneficios de tales esfuerzos no se evidenciarán[21]. El éxito y el crecimiento del desarrollo del CP son validados por la importancia que la sociedad civil (familiares, cuidadores, voluntarios) le da a los cuidados de alta calidad al final de la vida[22]. El objetivo del modelo centrado en el paciente es “promover, restaurar y mantener el nivel máximo de comodidad en todos los niveles de atención de CP, con énfasis en la atención primaria y atención comunitaria”, a continuación se detallan cada uno de estos aspectos.

Modelo de Atención Domiciliaria de CP a pacientes en fase Terminal

Consiste en proporcionar CP especializados a pacientes derivados de los diferentes niveles de atención, que se encuentran en su domicilio durante la fase terminal de su patología crónica. Estos equipos deben apoyar a los familiares y cuidadores en la casa del paciente. También prestan asesoramiento especializado a los médicos generales, médicos de familia y enfermeros que atienden al paciente en casa[23]. Un programa de atención domiciliaria siempre debe incluir el acceso diurno y nocturno a asesoramiento médico paliativo especializado y apoyo de enfermería paliativa especializada, aunque a veces esto puede implicar la forma de un asesoramiento especializado telefónico que respalde la atención de cabecera de los equipos locales, a los servicios universales[24]. Las alteraciones en la organización de la atención primaria afectada por la comunicación deficiente y la falta de capacitación exacerban la incompleta implementación de esta atención en los pacientes con necesidades paliativas[25].

El desarrollo de las prácticas esenciales de CP en la comunidad deben incluir capacitación, seguimiento y apoyo al cuidador. Estas deben realizarse de manera transversal en todos los niveles de complejidad existentes y ser incluidas dentro de la red de atención. En la tabla 1 se muestran las prácticas esenciales de CP primarios[26]. Es importante enfatizar que para poder ofrecer un adecuado servicio de primer nivel en CP se requiere formación adecuada de los profesionales sanitarios. La ausencia de ésta ha demostrado ser una de las principales causas del pobre control sintomático de muchos pacientes[27]. Es importante tener en cuenta la visión del proceso para implementar los CP dentro de un país, los cuales deben ser ejecutados integralmente en los niveles de la sociedad desde el nivel comunitario hacia arriba y del experto en atención paliativa en el sistema de atención médica hacia el nivel bajo[28]. (Figura 2).

Modelo de Atención Ambulatoria de CP a pacientes en fase Terminal

Prestan atención y soporte en consultorios especializados a los pacientes, familiares y a los profesionales de otras especialidades durante el seguimiento de la enfermedad. Evalúan síntomas específicos no controlados con el fin de disminuir el número de consultas por urgencias. El objetivo es suministrar medicamentos con titulación para tratamiento del dolor y manejo de intolerancia a la vía oral y otros síntomas. Se atienden a personas vulnerables, es

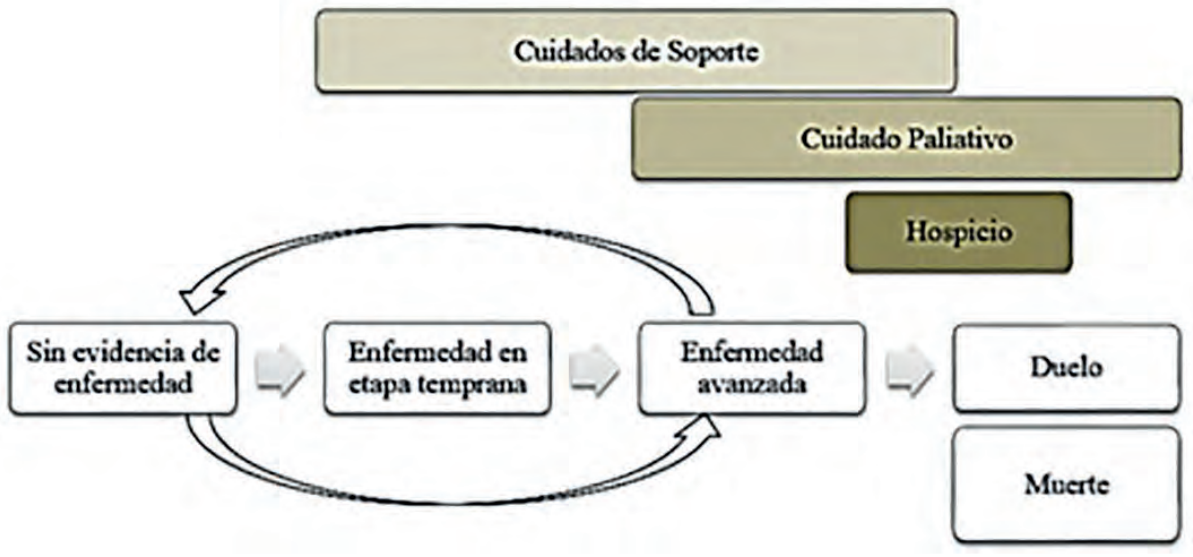


Figura 1. Modelo de integración entre cuidados de soporte y paliativos. Evolución del modelo clásico de Cuidados Paliativos al Nuevo Modelo de Atención Integrada en la Enfermedad Crónica Avanzada y en Situación Terminal (New Health Foundation; 2017)[19].

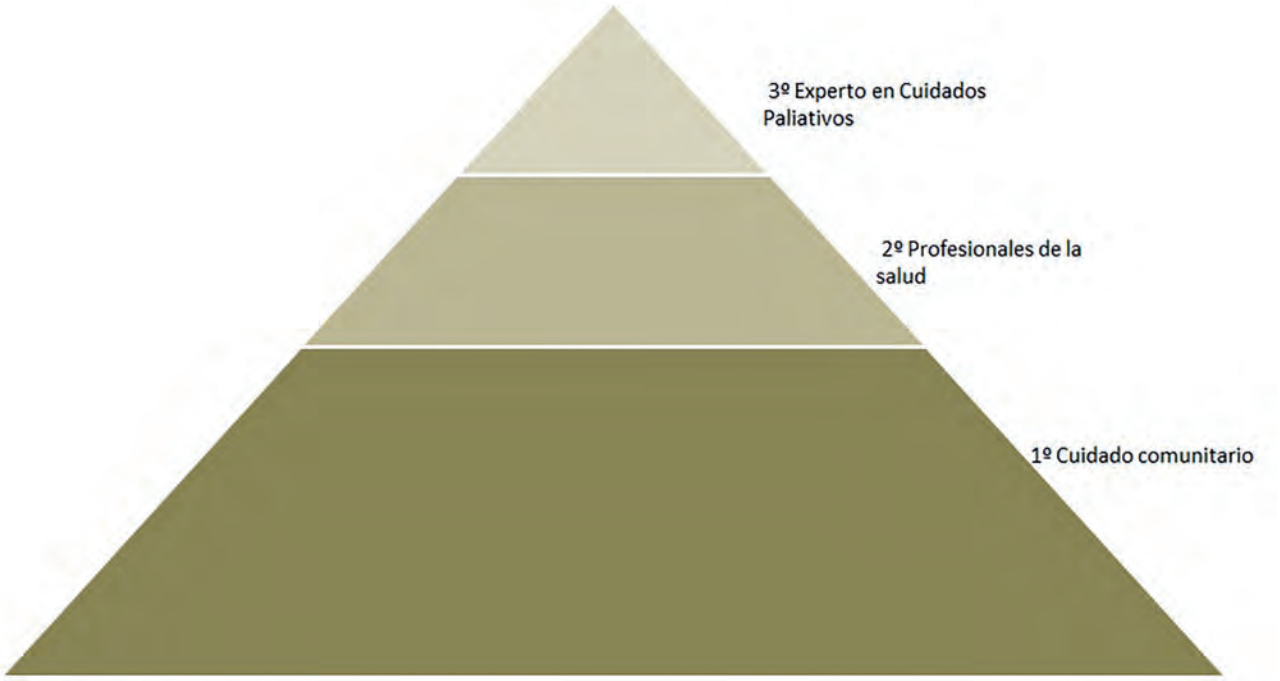


Figura 2. Cuidados Paliativos para todos. Stjernsward J, M. Foley K, D. Ferris. La Estrategia de Salud Pública para cuidados paliativos. Revista de Manejo del Dolor y Síntomas. 2017.

decir aquellas que están en riesgo de tener peores resultados de salud y a menudo se refiere a las características de una persona, como edad avanzada, afecciones crónicas de salud, y problemas nutricionales[29]. Por ejemplo, un estudio realizado en el Instituto Nacional del Cáncer en Brasil respaldó que el estado nutricional deteriorado se asoció con una baja calidad de vida en pacientes con cáncer incurable[30]. Los CP se asociaron consistentemente con mejoras en la planificación previa del cuidado, la satisfacción del paciente y del cuidador[31].

Modelo de Atención Hospitalaria de CP a pacientes en fase Terminal: Equipos de CP hospitalarios o Unidades de CP (UCP)

Las UCP son áreas delimitadas, con asignación específica de camas, pueden o no tener todos los otros recursos asistenciales como hospicio, internación parcial de corta y mediana estancia. La atención la proporciona un equipo interdisciplinario con formación especializada en CP, conformado por médico especialista en CP, enfermera, psicólogo, trabajador social, guía espiritual, grupo de voluntariado[32]. Los profesionales especializados en CP asumen la atención y la gestión clínica de los pacientes admitidos en la unidad. Pueden diferenciarse entre UCP ubicadas en hospitales para pacientes agudos de alta complejidad o UCP de instituciones de complejidad intermedia o de atención para paciente crónico. Proporcionan atención, asesoramiento y apoyo especializado a otros profesionales sanitarios, pacientes, familiares y cuidadores en el entorno hospitalario. Son reconocidos y prestan atención en una entidad hospitalaria (al menos al 30% de los casos valorados en hospital)[33]. Los criterios de ingreso a una UCP son: 1) Dificultad para control de síntomas y necesidades en el nivel intermedio; 2) Necesidad de manejo quirúrgico o intervencionista Avanzado; 3) Deseo del paciente y la familia de fallecer en una unidad hospitalaria; 4) Requerimiento de toracentesis o paracentesis guiada por imágenes; 5) Requerimiento de transfusión sanguínea determinada en reunión de equipo; 6) Dolor complejo, delirium hiperactivo refractario; 7) Requerimiento de sedación paliativa profunda prolongada y 8) Requerimiento de manejo integral de casos con otras especialidades médicas.

Los servicios de CP ofrecen atención a aquellos pacientes cuyas necesidades exceden la capacidad y los recursos del proveedor del primer nivel de atención. La derivación a un servicio de CP especializado, en la mayoría de los casos será a través del proveedor del primer nivel de atención. Los CP especializados se pueden proporcionar por medio de la consulta y el apoyo de los proveedores del primer nivel de atención o mediante la prestación de atención continua a pacientes, cuidadores primarios y familias con necesidades complejas. En una UCP los aspectos a valorar son[34] : a) Evaluación específica de la enfermedad de base y del pronóstico de evolución del paciente, b) Valoración sobre la situación funcional del paciente, c) Evaluación del estado cognitivo del paciente, d) Valoración psicosocial, e) Utilización de una escala de evaluación de múltiples síntomas, f) Valoración y tratamiento de úlceras, g) Seguridad en el manejo de fármacos, h) Prevención de caídas, i) Reunión del equipo de CP con el paciente y su familia, j) Planificación de otras actividades en pacientes ingresados, k) Planificación del alta, l) Evaluación del duelo.

La disponibilidad de servicios especializados de CP proporciona beneficios al sistema de atención de salud en general, aunque los servicios especializados pueden no proporcionar atención directamente a todas las personas que componen la población objetivo. Los especialistas en CP resuelven las demandas de pacientes con necesidades complejas que están más allá de la experiencia o exceden la capacidad de los proveedores de los otros niveles de atención. Los CP especializados generalmente se describen como proporcionados a través de consultas individuales basadas en pacientes o administración de casos comunes con proveedores del primer nivel de atención.

Los servicios especializados contribuyen a mejorar la atención en todo el sistema de atención médica, además proporcionan apoyo indirecto a los proveedores de aten-

Identificar, evaluar, diagnosticar, tratar, aplicar medidas de solución para:	**Identificar, evaluar, brindar apoyo, y referir cuando sea necesario para:**
Necesidades de cuidado físico	
Dolor (todos los tipos) Problemas respiratorios (disnea, tos) Problemas gastrointestinales (estreñimiento, náuseas, vómitos, boca seca, mucositis, diarrea) Heridas, úlceras y lesiones cutáneas. Delirio e Insomnio	Fatiga Anorexia Anemia Somnolencia o sedación Sudoración
Necesidades de cuidado psicológico / emocional / espiritual	
Trastorno Psicológico Ansiedad Sufrimiento de familiares o cuidadores	Necesidades espirituales y angustia existencial Depresión Apoyo de duelo para familiares / cuidadores
Considere y gestione	
Planificación y coordinación de la atención. Identificar el apoyo y los recursos disponibles; desarrollar e implementar un plan de atención basado en las necesidades del paciente Brindar atención en las últimas semanas / días de vida. Facilitar la disponibilidad y el acceso a medicamentos (especialmente opioides) Identificar las necesidades psicosociales / espirituales de los profesionales que brindan atención	
Problemas de comunicación Comunicarse con el paciente, la familia y los cuidadores sobre el diagnóstico, el pronóstico, el tratamiento, los síntomas y su manejo, y cuestiones relacionadas con la atención en los últimos días. Identificar y establecer prioridades con pacientes y familiares / cuidadores Proporcionar información y orientación a pacientes y cuidadores de acuerdo con los recursos disponibles.	

Fuente: Chestnov , Kieny MP. Planificación e implementación de servicios de cuidados paliativos: una guía para gerentes de programas. Organización Mundial de la Salud.2016; 9-10. [26]

Tabla 1. Prácticas esenciales para los CP primarios.

Provincia / Año	**2018**	**2019**
Guayas	18.984	20.335
Pichincha	11.857	12.134
Azuay	3.592	7.737
Manabí	6.777	7.115
Los Ríos	3.978	4.234
El Oro	2.967	3.138
Tungurahua	1.976	2.852
Chimborazo	1.854	2.526
Loja	1.765	2.426
Imbabura	1.843	2.174
Fuente: Anuario de Defunciones Generales 2018 -2019. Instituto Nacional de Estadística y Censos. INEC.[37]		

Tabla 2. Principales provincias con mayor frecuencia de defunciones.

Políticas Gubernamentales			
Indicador/Denominación	**Definición/Unidad de medida**	**Fuente de datos**	**Periodicidad**
Existencia de un programa nacional de cuidados paliativos vigentes.	Se entiende como el programa específico de cuidado paliativo de cada EAPB del SGSSS. Medición: dicotómico (Si/No)	Coordinador del programa	Anual
Educación			
Proporción de facultades de Medicina que ofrecen formación en CP en pregrado	Número de escuelas de Medicina que ofrecen CP en pregrado, expresado como porcentaje del número total de Facultades de Medicina en cada departamento. Medida: porcentaje	Facultades de Medicina	Anual
Proporción de Facultades de Enfermería que ofrecen formación en CP en pregrado.	Número de escuelas de Enfermería que ofrecen CP en pregrado, expresado como porcentaje del número total de Facultades de Enfermería en cada departamento. Medida: porcentaje	Facultades de Enfermería	Anual
Número de programas de formación especializada en CP para médicos.	Número de programas acreditados de formación especializada en CP para médicos por año. Medida: número absoluto	Facultades de Medicina	Anual
Medicamentos			
Consumo de opioides fuertes per cápita.	Consumo anual de opioides fuertes en equivalencia a morfina, en una población definida, per cápita.	Base de datos	Anual
Prestación de servicios			
Razón de servicios de CP por millón de habitantes	Razón de servicios de CP por millón de habitantes. Medida: Número	Base de datos	Anual

Fuente: Instituto Nacional de Cancerologia-Colombia. Modelo de Soporte Integral y cuidado paliativo al enfermo cronico. Primera Edicion ed. Bogota DC: Instituto Nacional de Cancerologia-Colombia; 2017. [39]
EAPB: Empresas Administradoras de Planes de Beneficios; SGSSS: Sistema General de Seguridad Social en Salud.

Tabla 3. Indicadores de Cuidados Paliativos.

ción primaria a través de iniciativas tales como el desarrollo de protocolos estándar o directrices para la gestión de problemas comunes, y para el aprendizaje domiciliario y la impartición de educación continua en el mismo[35].

Bases para la implementación del modelo

La implementación de los componentes descritos para los cuidados de soporte y CP debe considerar los contextos, las rutas y los procesos. De acuerdo con la política de atención en salud, es importante tener en cuenta las diferencias que se presentan en los grandes centros urbanos, las zonas rurales y las áreas de baja densidad poblacional. En dichas zonas, la demanda de servicios varía según las características de la población, la disponibilidad de recursos, los perfiles de morbimortalidad, la disponibilidad de medicamentos, de tecnologías y de talento humano.

En Ecuador, el riesgo de desarrollar cáncer antes de los 75 años es de aproximadamente un 20%, y constituye un importante problema de salud pública, con una incidencia creciente. Por lo cual, según el INEC, ocupa la segunda causa de mortalidad general después de las enfermedades cardiovasculares[36]. El mayor número de defunciones en el año 2019 se registra en la región costa con 37.787 representando el 51,5% del total de defunciones[37]. En la tabla 2 se registran las diez principales provincias con mayor frecuencia de defunciones. Por consiguiente, esto daría la pauta de las provincias con mayor demanda de CP.

La implementación de este Modelo de soporte integral y CP al enfermo crónico se relaciona conceptualmente con el Modelo de Cuidado del Paciente con Cáncer con la organización de prestación de servicios cuya característica central es el servicio integrado de cuidados de soporte que incluye los CP. Se proponen otros escenarios de práctica en las redes de servicios de CP y las unidades de soporte y CP en diferentes grados de complejidad.

Indicador/Denominación	Definicion/Unidad de medida	Fuente de datos	Periodicidad
Estructura			
Población objetivo susceptible de ser atendida por CP, por patología oncológica y no oncológica.	N.º de pacientes susceptibles de recibir CP/total de personas fallecidas	Base de datos	Anual
Mortalidad	(N.º de personas fallecidas/total de la población) x 100 000	Base de datos	Anual
Razón de equipos de CP	N.º de equipos de CP/población fallecida por causa susceptible de CP	Base de datos	Anual
Razón de profesionales	N.º de profesionales de CP/población fallecida por causa susceptible de CP	Base de datos	Anual
Razón de camas	N.º de camas/población fallecida por causa susceptible de CP	Base de datos	Anual
Procesos			
Pacientes en programa	N.º de pacientes en programa de CP		
Supervivencia	Días de estancia en programa hasta fallecimiento	Base de datos	Anual
Actividades asistenciales	• N.º de visitas domiciliarias • N.º de visitas hospitalarias • N.º de consultas externas • N.º de asesorías	Base de datos	Anual
Resultados			
Costo de atención	Costo medio aproximado y por paciente atendido en CP	Base de datos	Anual

Tabla 4. Indicadores para evaluar un programa de CP

Fuente: Instituto Nacional de Cancerologia-Colombia. Modelo de Soporte Integral y cuidado paliativo al enfermo cronico. Primera Edicion ed. Bogota DC: Instituto Nacional de Cancerologia-Colombia; 2017.[39]

Evaluación y seguimiento

El seguimiento del modelo integral paliativo se plantea con base en los indicadores de CP definidos por la Asociación Latinoamericana de Cuidados Paliativos (ALCP) en 2015, los cuales se encuentran detallados en las tabla 3 y 4. Es necesaria la revisión periódica de los indicadores y de los estándares, así como la adecuación de los mecanismos y actuaciones establecidos para la verificación del grado de cumplimiento real de actividades y objetivos. Las conclusiones extraídas de la evaluación periódica deberán guiar las reestructuraciones necesarias para asegurar el alcance de los estándares y objetivos del modelo[38].

Se puede considerar la incorporación del seguimiento y evaluación de los programas nacionales en función de inspección, vigilancia y control. Por ejemplo, con un informe del resumen de las principales acciones llevadas a cabo anualmente para lograr los objetivos del programa y el porcentaje de cumplimiento de los mismos. Cada institución aseguradora proporcionará los datos de actividad atendiendo los indicadores nacionales seleccionados por su pertinencia y relevancia para evaluar la calidad de los servicios prestados en CP. Se recomienda progresivamente asumir otros indicadores que muestren con mayor profundidad cómo se está atendiendo a estos pacientes (por ejemplo, grado de atención psicológica, control sintomático, etcétera)[40].

Conclusiones

La atención a enfermos terminales no es un servicio exclusivo del primer nivel de atención o AE, domicilio, hospital o residencia: todos los niveles asistenciales deben estar implicados.

Los CP de un paciente con enfermedad terminal que desea permanecer en la comunidad y si el nivel de complejidad no requiere derivarlo al nivel especializado, consisten en que el primer nivel de atención lo atienda y controle los síntomas físicos y emocionales según la correcta evaluación mediante escalas validadas cuando la complejidad lo requiera.

Todo profesional de AP debe tener conocimiento del modelo integral básico en CP, que le permita garantizar a

sus pacientes y los familiares de éstos el derecho a que se les atienda con dignidad al final de la vida. Pese a que los modelos son diferentes, la finalidad de todos ellos es la misma, atender con calidad a los pacientes, favoreciendo la atención en su hogar.

Es necesaria la revisión periódica de los indicadores de CP, así como la adecuación de los mecanismos y actuaciones establecidas para la verificación del grado de cumplimiento real de actividades y objetivos.

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Las autoras declaran no tener conflicto de interés.

Declaración de la autoría

MV diseñó el artículo. MV, EFT y JB escribieron y revisaron el artículo. MV y EFT validaron la versión final del manuscrito.

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ARTICLE / INVESTIGACIÓN

The Effects of Sex Hormones and some Respiratory Diseases on the Severity of Corona Virus Infection

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Abstract: Sex hormones and chronic respiratory diseases play a role in the pathogenesis and the management of Covid-19 infection. Present research tries to shed light on the role of sex hormones, bronchial asthma and lung cancer on the severity of Covid-19 infection and the resulting mortality rate. This study included a follow-up of the health status of 85 patients infected with Covid-19, and all the patients previously diagnosed had hereditary respiratory diseases (bronchial asthma (64) and lung cancer (21)). The serum level of progesterone and testosterone and the stages of lung cancer development were measured in the laboratories of Diwaniyah Teaching Hospital. After conducting medical examinations, computed tomography and x-rays, the severity of Covid-19 infection was classified according to the WHO to moderate, severe and critical infection. The current results showed that most of the hospitalized cases were males (65%) with a death rate (18%), so bronchial asthma was associated with the death rate in males (70% of dead males), followed by postmenopausal (POM) women (66.7% of the dead woman), while pre-menopausal (PRM) women had the highest cure rate (100%). The results of the study showed that the rise in the mean of progesterone in PRM (13.55 ng/ml) was associated with moderate symptoms of Covid-19, while the increased testosterone in males and POM (1018 ng/dL and 67.3 ng/dL, respectively) was associated with an increase in the severity of the infection. In conclusion, asthma and high testosterone directly affected the increase in the severity of Covid-19 and the high mortality rate among the Corona sufferers. While advanced lung cancer had a clear role in that, at the same time, progesterone appeared as a protective factor in young women.

Key words: Covid-19, bronchial asthma, lung cancer, testosterone, progesterone.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was recognized as the reason for the Covid-19 sickness (Coronavirus) flare-up in Wuhan, China, on 9 January, 2020[1,8,18]. Since the World Wellbeing Association proclaimed Coronavirus a pandemic, the quantity of cases has expanded at a dramatic rate. To 2020, there have been 19,369,210 confirmed cases around the world with more than 732,499 deaths[2,3]. The novel coronavirus disease 2019 shows a wide range of clinical outcomes as severity and fatality rates. Older populations and health-compromised groups may be at higher risk of getting very sick with Covid-19. Specifically, persons with respiratory diseases, such as lung cancer and bronchial asthma, and individuals in active cancer treatments may be susceptible to a more serious form of the infection[4,5]. Accessible information affirm that patients with innate respiratory illnesses as hypersensitivity and malignancy are potentially bound to be tainted by SARS-CoV-2 and, all the more critically, absolutely inclined to foster extreme difficulties characterized here as confirmation in an emergency unit[5], the requirement for mechanical ventilation (MV) or demise because of Coronavirus. As of late, a few investigations have proposed conceivable non-hurtful impacts of asthma on the clinical results of Coronavirus yet a review concentrate by Zhao *et al.*, assessed the danger factors among 548 Coronavirus inpatients in Wuhan and announced that there were not many patients with asthma and the danger was steady with severe Coronavirus cases[6,7].

On the other hand, Disease patients and malignancy survivors address a weak populace for Coronavirus. Generally, the pervasiveness of Coronavirus in malignancy patients is assessed at 6% and is higher than in the non-disease populace. Besides, malignancy patients have more awful results from Coronavirus, upheld by past reports from China[8,9,10], Italy[11], and all the more as of late New York[12]. Hazard of solemn occasions characterized as admission to the ICU, the requirement for MV or demise is accounted for up to 39% of patients with the disease, and death rate could be just about as high as 30%[9].

Patients with the cellular breakdown in the lungs and hematological malignancies appear to be at the most elevated danger of death from SARS-CoV-2 infection[6] as opposed to hematologic malignancies, significantly immunosuppressive treatment isn't regular in the cellular breakdown in the lungs[13]. Sex hormones and associated comorbidities can play a role in the pathogenesis and the management of Covid-19 infection. Sex hormones, such as estradiol, progesterone, and testosterone, might concern the age-dependent and sex-specific severity of Covid-19. According to several studies, men have higher Covid-19-related morbidity and mortality rates than women[14,15]. The severity of Covid-19 is associated with various comorbidities. However, not ade-

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quate studies have yet demonstrated the potential risk of respiratory failure and mortality in Covid-19 patients with pre-existing asthma or lung cancer; therefore, the current study aimed to detect the role of inherent bronchial asthma and lung cancer in the severity of Covid-19 infection and the resulting mortality rate. Additionally, this article investigates the possible master/calming impacts of progesterone and testosterone during Coronavirus disease, with specific consideration regarding the expected restorative job of chemical substitution treatment in people with Coronavirus.

Materials and methods

The current study included a follow-up of the health status of 85 hospitalized cases infected with Coronavirus in Al- Diwaniyah Teaching Hospital during the period from 6/8/2020 to 5/2/2021. In addition, the present study was in agreement with the ethics of Al- Diwaniyah Teaching Hospital and verbal informed consent were obtained from all participants. The patients' ages ranged from 18-65 years, and the inclusion criteria as all patients were positive for Covid-19, the patients had the respiratory disease (bronchial asthma or lung cancer) and were not smokers, patients had a positive family history of bronchial asthma or lung cancer, age range from 18-65 year and determined the menstruation of females.

Hormone level measurement: after blood collection, centrifuging preform for serum preparation. serum concentration of progesterone and testosterone measured by routine protocol of hormone assay analyzer in chemistry lab.

Diagnosis of respiratory disorders: the present study included only Covid-19 infected patients who have bronchial asthma or lung cancer. Identification of Covid-19 infection detected by enzyme-linked immunosorbent assay and real-time polymerase chain reaction in a virology lab. After conducting medical examinations, computed tomography and x-rays, the severity of Covid-19 infection was classified according to the World Healthcare Organization (WHO) as moderate, severe and critical infection[16]. Asthmatics were previously diagnosed with chronic inherent respiratory disorder by respiratory disease specialists. Moreover, Lung cancer patients are pre-diagnosed and receive treatment, and the stages of cancer are known to some of them, while this has been determined for others in the Histopathology lab according to American Cancer Society[17].

Statistical analysis: Statistical Package for Social Sciences version 20 (SSPS20) computer software and Microsoft Dominate 2010 were dependent on measurable Investigation and just outcomes that had a P esteem <0.05 were considered statically huge[25,35].

Results

The current study aims to monitor the health status of MERS-CoV patients with bronchial asthma or lung cancer as a model for chronic respiratory diseases that could play a role in infection severity, response rate to treatment, or death. Patients' ages ranged from 18-65 years; younger patients were excluded, while we did not reach older patients during the current study period. In addition, the results showed that more males than females stayed in the hospital (55 and 30, respectively) with a mean age ± standard deviation of 38.20±3.68 years and 34.13 ± 5.61 years for males and females, respectively, as in table (1) so no significant differences were found when comparing the average ages of both sexes (P= 0.471). The studied samples showed that Covid-19 infected elderly people increase the rate of their stay in the intensive care unit, especially in males when the highest percentage of infected males and females appeared within the age group of 47-65 years (45% and 40% respectively) followed by the age group from 31-46 years (31% and 37% respectively) However, there were no statistical differences in the distribution of females and males according to the mentioned age groups (P>0.05) as explained in the table (1).

On the other hand, females were divided into two groups according to the menstruation to determine the extent of the impact of hormonal changes on the severity of infection. The percentage of pre-menopause (PRM) women appeared to be 60%, and post-menopause (POM) by 40% of the total number of women (figure 1).

The current study included knowledge of the relationship between the effect of bronchial asthma and lung cancer on the severity of infection with the Coronavirus, as 64 Covid-19 patients (75%) had bronchial asthma of these, 42%, 36% and 22% have severe, moderate and critical infection respectively as in table 2 and figure 2. On the other hand, the percentage of Covid-19 patients and those with lung cancer accounted for 25% (21 patients), and a large percentage of them suffered from severe infection (43%), and 33% had a moderate infection, while 24% have an acute infection.

The mortality rate during the present survey was 10 males (7 asthmatics and 3 have lung cancer), and 3 women (2

Age /year	Female	Male	P value
Age range	18-65	18-65	
Mean+ SD	34.13 ± 5.61	38.20 ± 3.68	0.471
SE	0.106	0.123	
Age groups	N (%)	N (%)	
18-30	7 (23)	13 (24)	0.782
31- 46	11 (37)	17 (31)	0.560
47-65	12 (40)	25 (45)	0.708
Total N=85	30 (35)	55 (65)	0.0131 *

SD=Standard Deviation; SE = Standard Error; * = enormous quantifiable differentiation (P<0.05)

Table 1. Distribution of Covid-19 patients with bronchial asthma or lung cancer over gender and age.

asthmatics and 1 lung cancer) are dead, while all the young women recovered (Table 3). Moreover, the results in figure (3) also showed that the severity of the infection increases with the progression of cancer, as 75% of cancer patients in the IV stage were distressed from critical Covid-19 infection, and this was reflected in the death of 2 males and 1 female infected with Covid-19 in this cancer stage so 50% of patient in III cancer stage have severed Covid-19 infection. However, the number of deaths among males (18%) was more than that of females (10%), and the death rate mainly appeared among POM women and older males within the age group of 47-65 years (25% and 28% for POM woman and males respectively) as shown in the table (4).

Table (5) showed the effect of gender and maturity on the severity of Covid-19, as a straightforward percentage of males and POM women suffered from severe infection (42% and 58%, respectively), while most PRM women have a moderate infection (61%). While high rates of acute infections were 27%, 25% and 6% for males, POM and PRM women, respectively, this was accompanied by significant differences in the moderate and severe infections among the studied groups (P<0.05).

The current results also showed an expansion in the Coronavirus severity and symptoms with an increase in the male hormone testosterone, especially in males and POM women, compared to PRM women, where the mean testosterone in the Covid-19 infection was 871 ng/dL, 22.0 ng/dL and 20.5 ng/dL for males, POM and PRM women respectively (P=0.041). On the other hand, in severe infections was 995 ng/dL, 36.7 ng/dL and 27.72 ng/dL for males, POM and PRM women, respectively (P=0.048), while the serum means of testosterone in acute infections was 1018 ng/dL, 67.3 ng/dL and 50.16 ng/dL for males, POM and PRM women respectively (P=0.039)as shown in table (6). While the effects were different in the case of progesterone hormone in females, whose high concentration in the blood was accompanied by moderate symptoms and signs of Coronavirus in females (especially PRM women) as shown in table (7) when the mean progesterone in the moderate Covid-19 infection was 0.03ng/ml, 0.037ng/ml and 13.55ng/ml for males, POM and PRM women respectively (P=0.036). Moreover, in severe infections was 0.015ng/ml, 0.022ng/ml and 10.14 ng/ml for males, POM and PRM women respectively (P=0.022), while the serum mean of progesterone in critical infections was 0.012ng/ml, 0.021ng/ml and 8.36ng/ml for males, POM and PRM women respectively (P=0.009).

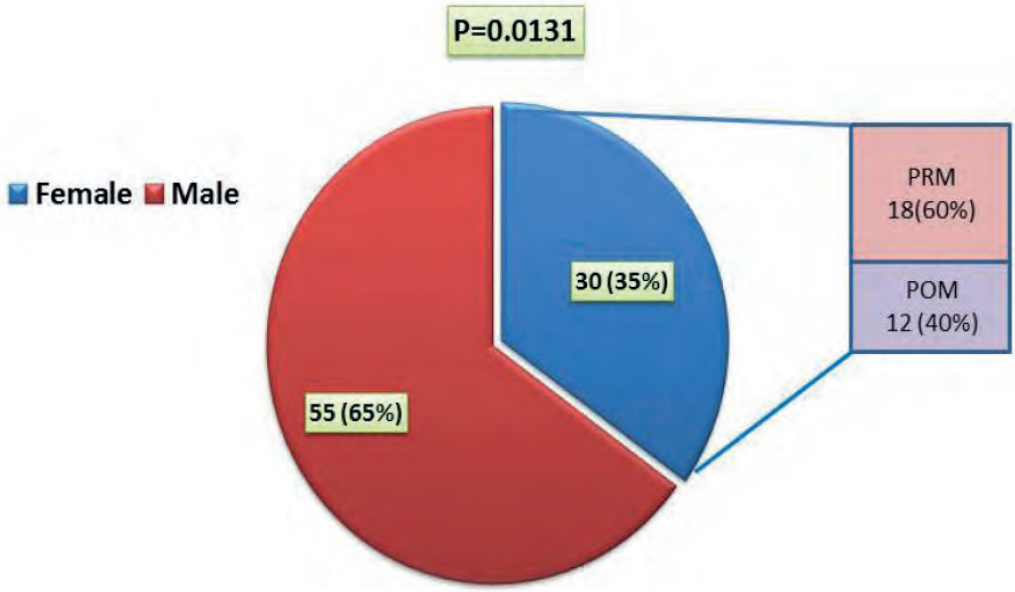


Figure 1. Distribution of Covid-19 infected according to gender. PRM=Pre menopause, POM=Post menopause.

Severity of Covid-19	Bronchial asthma	Lung cancer	P value
	N (%)	N (%)	
Moderate infection (N=30)	23 (36)	7 (33)	0.637
Sever infection (N=36)	27 (42)	9 (43)	0.840
Critical infection (N=19)	14 (22)	5 (24)	0.777
Total Number = 85	64 (75)	21 (25)	0.008 *

* = enormous quantifiable differentiation (P<0.05)

Table 2. The effect of bronchial asthma and lung cancer on the severity of Covid-19 infection.

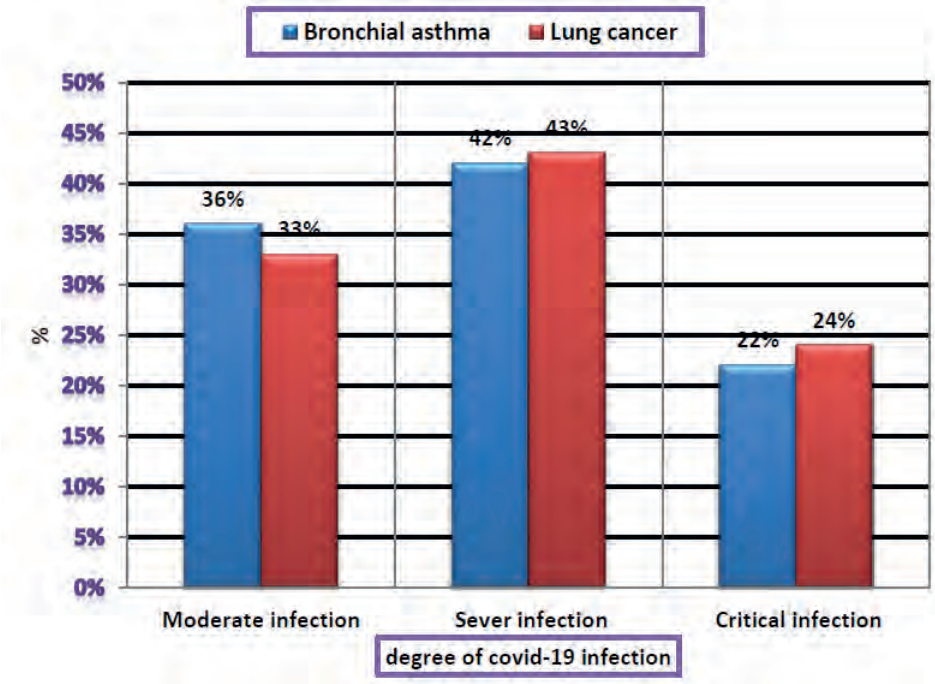


Figure 2. Frequency of Covid-19 infection among asthmatic and lung cancer patients.

Respiratory diseases	Recovery N (%)			P value	Mortality N (%)			P value
	PRM female	POM female	Male		PRM female	POM female	Male	
Asthma	10 (55.6)	9 (100)	36 (80)	0.015*	0 (0)	2 (66.7)	7 (70)	0.009*
Lung cancer stages								
I	3(16.6)	0 (0)	5 (11.11)	0.0477*	0 (0)	0 (0)	0 (0)	1.00
II	4(22.2)	0 (0)	0 (0)	0.052	0 (0)	0 (0)	0 (0)	1.00
III	1(5.6)	0 (0)	2 (4.44)	0.355	0 (0)	0 (0)	1 (10)	0.083
IV	0 (0)	0 (0)	2 (4.44)	0.639	0 (0)	1 (33.3)	2 (20)	0.026*
Total N. (%)	**18**	**9 (100)**	**45 (82)**		**0 (0)**	**3 (10)**	**10 (18)**	

*** = enormous quantifiable differentiation (P<0.05), PRM=Pre menopause, POM=Post menopause**

Table 3. The effect of bronchial asthma and lung cancer on the mortality and recovery rate among patients with Covid-19.

Discussion

The growing statistics support the notion that gender and older age are associated with a significantly higher risk of severe health deterioration and death from covid-19[18-20]. The current study showed that the severity of infection with the Coronavirus is more among the males and POM females than in PRM females, and the severity of the infection and the number of deaths increases with age, and this result is similar to many previous studies of different countries. Of the 1591 patients admitted to ICU in the Lombardy region, Italy, 82% were male[21]. Another Canadian study analyzed over 200,000 residents tested for SARS-CoV-2; although only 36% of individuals tested were males, compared with women, they showed higher rates of laboratory-confirmed infection (13.5% vs. 9.8%), hospitalization (15.6% vs. 10.4%), ICU admission (4.1% vs. 1.7%), and death (8.7% vs. 7.6%)[22]. Several other reasons have also been suggested to elucidate the gender disparity in Covid-19, like the differential habit of smoking and drinking, sociological, psychological factors, and therefore the differential profile of comorbidities among sexes[23]. In contrast to our study, studies conducted in South Korea showed that Covid-19 was the foremost prevalent in women within the 20-39 years age bracket. This finding was also observed in another study using data reported to the Korea Centers for Disease Control and Prevention[24,25].

Our study showed that the most common hereditary respiratory disease among people with Coronavirus is bronchial asthma (75%), and its coincidence with the Covid-19 increased the severity of infection and incidence of death. Two studies about asthmatic patients with Covid-19

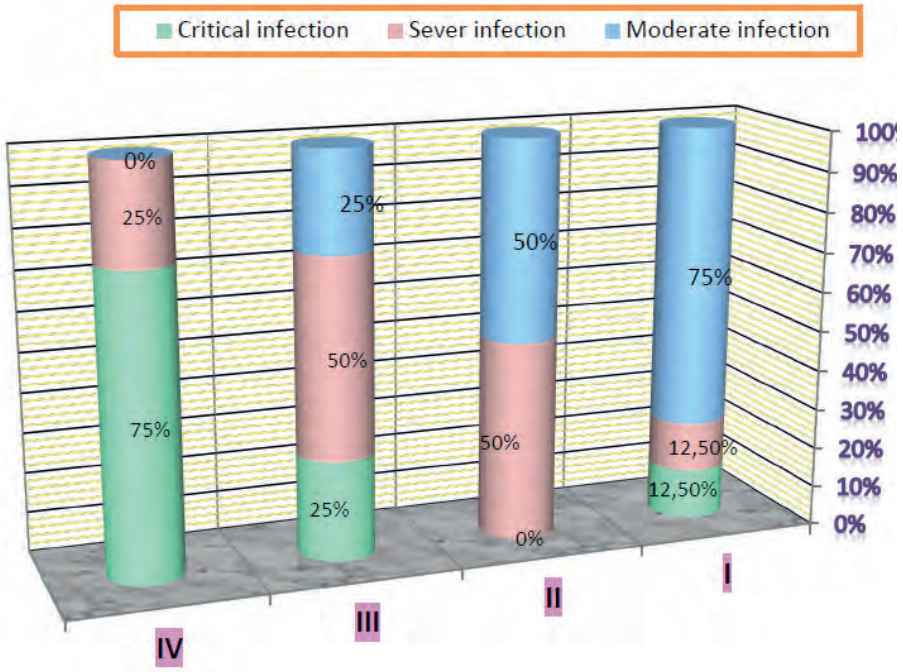


Figure 3. Distribution of lung cancer stages according to severity of Covid-19 infection.

Age groups/ years	Females (N = 30)				Males (N = 55)			
	Recovery rate	Death rate	$X2$	P value	Recovery rate	Death rate	X^2	P value
	N (%)	N (%)			N (%)	N (%)		
18-30	7 (100)	0 (0)	90.04	< 0.0001*	12 (92)	1 (8)	88.864	0.0008*
31- 46	11 (100)	0 (0)	89.98	< 0.0001*	15 (88)	2 (12)	54.61	0.0031*
47-65	9 (0.75)	3 (25)	69.43	0.002*	18 (64)	7 (28)	32.14	0.0056*
Total N(%)	27 (90)	3 (10)	78.65	0.00083	45 (82)	10 (18)	44.10	0.0037*

*** = enormous quantifiable differentiation (P<0.05)**

Table 4. The effect of patients 'age on the rates of cure and mortality.

Severity of Covid-19 infection	Males	PRM females	POM females	P value
	N (%)	N (%)	N (%)	
Moderate infection (N=30)	17 (31)	11 (61)	2 (17)	0.0131*
Sever infection (N=36)	23 (42)	6 (33)	7 (58)	0.0374*
Critical infection (N=19)	15 (27)	1 (6)	3 (25)	0.0512
Total N.	55	18	12	

*** = enormous quantifiable differentiation (P<0.05), PRM=Pre menopause, POM=Post menopause**

Table 5. The effect of gender and menstruation on the severity of Covid-19 infection.

Severity of Covid- 19	Testosterone ng/dL Mean ± SD			X^2	P value
	Male	PRM women	POM women		
Moderate infection	871 ± 115	20.5 ± 4.52	22.0 ± 5.73	1.32	0.041*
Sever infection	995 ± 208	27.72 ± 3.88	36.7 ± 9.62	3.62	0.048*
Critical infection	1018 ± 482	50.16 ± 12.40	67.3 ± 10.57	5.66	0.039*

SD=Standard Deviation; * = enormous quantifiable differentiation (P<0.05); PRM=Pre menopause, POM=Post menopause

Table 6. Mean serum testosterone level according to Severity of Covid-19 among patients with respiratory disorders.

Severity of Covid-19	Progesterone (ng/ml) Mean ± SD			X^2	P value
	Male	PRM female	POM female		
Moderate infection	0.03 ± 0.005	13.55 ± 2.81	0.037 ± 0.011	4.52	0.036*
Sever infection	0.015 ± 0.006	10.14 ± 2.03	0.022 ± 0.013	7.64	0.022*
Critical infection	0.012 ± 0.002	8.36 ± 1.34	0.021 ± 0.009	11.81	0.009*

SD=Standard Deviation; * = enormous quantifiable differentiation (P<0.05), PRM=Pre menopause, POM=Post menopause

Table 7. Mean of serum Progesterone level according to Severity of Covid-19 among patients with respiratory disorders.

in South Korea using insurance Review and Assessment database were recently published. The first study by Yang *et al.* analyzed the entire cohort who underwent Covid-19 testing and reported that non-allergic asthma patients showed a greater risk of susceptibility to Covid-19 infection and severe clinical outcomes of Covid-19, like ICU admission, application of mechanical ventilation, or death[26]. The second study of Choi *et al.* also reported higher mortality in asthmatic patients than in non-asthmatic patients among Covid-19 patients[27,49].

Present data in same line with several previous studies that recorded cancer patients, mainly lung cancers, experience disproportionately severe illness and deaths from Covid-19[28,29]. Lung cancer is characterized by a clinical scenario of increased risk of pulmonary complications, acute lung injury, and high incidence of Covid-19 infection due to pathophysiological, clinical and treatment-related risk factors. So, in the current study, the death rate for lung cancer patients with Coronavirus was 19% (4/21), and most of them had advanced stages (IV or III stages) of cancer. In contrast, in other studies, the mortality rates in patients with cancer have been recorded between 25%-30%[30,31], which has mentioned a high interaction between causes frequently associated with lung cancer, including smoking-related lung damage or disorder, major cardiovascular and respiratory diseases, and elderly age which is associated with increased severity of SARS-CoV-2 infection[31,32]. Another study from Memorial Sloan Kettering Cancer Center was done on a cohort of 423 cancer patients with Covid-19 (including 8% lung cancer patients) and detected that 20% had severe respiratory disorders (involving 9% with mechanical ventilation) 12% died during 30 days. Furthermore, the researchers determined that given immuno-therapy was correlated with more risk of health problems. Newly published data from current work and previous research showed that stage IV non-small cell lung cancer patients are at greater risk of health complications and death if infected with SARS-CoV-2[33,34].

In the existing study, changes in the level of sex hormones in the blood were linked to changes in the severity of infection. According to the current study, high progesterone may have a role in the recovery of all PRM women. At the same time, the decline of this hormone and the rise of the hormone testosterone may play a role in the severity of Covid-19 and death among men and women after menopause. In previous mice models, female hormones had a protective effect on mortality related to SARS-CoV infection[15]. McCoy et al. suggested that androgens, which may play an essential role in SARS-CoV-2 entry into the host cell, were implicated in Covid-19 mortality[34]. A study in Korea showed that male sex was not an independent risk factor for mortality, and there was no significant association between hormone therapy use and the clinical outcomes of peri- and postmenopausal women. Cattrini et al Showed that high testosterone levels could upregulate transmembrane serine protease 2, facilitating the entry of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) into host cells via angiotensin-converting enzyme 2 (ACE2)[35]. Information from patients with prostate disease treated with androgen-hardship treatment appears to affirm this theory.

Androgens, for the most part, smother the provocative reactions by diminishing the action of the fringe blood mononuclear cells, just as the arrival of incendiary components and cytokines, for example, interleukin-1β, interleukin-2, cancer rot factor-α, inducible nitric oxide synthase, and nitric oxide[36-38]. They can likewise advance the creation of suppressive cytokines, for example, interleukin-10 and the changing development factor-β through the androgen receptor flagging[38-40]. Furthermore, androgens are remembered for the thymic involution after adolescence[41]; thymic involution decreases the fare of lymphocytes towards the fringe and lessens the capacity of fringe immune system

microorganisms and the collection of white blood cells receptors. It isn't shocking that high testosterone levels have been related to a helpless infection-killing movement after flu inoculation[42]. These immunosuppressive impacts of androgens may advance SARS-CoV-2 contamination; however, they may likewise hinder the cytokine storm that describes the most extreme instances of Coronavirus.

An Italian review of 31 male patients conceded to ICU found that lower testosterone plasma levels anticipated more terrible results, supporting a good job of androgens during SARS-CoV-2 contamination[43]. A German review revealed that fundamentally sick male patients with Coronavirus experienced serious testosterone, and dihydrotestosterone lacks[44]. Another Chinese review showed that the proportion of testosterone to luteinizing hormones was altogether diminished in patients with Coronavirus[45]. This information supports the thought that decreased testosterone level in prior or conceivably infection instigated hypogonadism may be related to unfavorable anticipation in Coronavirus[46]. In outline, it remains hazy right now whether androgens may be great or horrible during SARS-CoV2 disease[35].

As recently revealed, estrogens were observed to be urgent to shield female mice from SARS-CoV disease[47]. The perception that ladies with Coronavirus show better results than men and that postmenopausal ladies are those at a greater danger of extreme Coronavirus is steady with the likelihood that progesterone advances mitigating reactions and can support the slanting of CD4+ White blood cell from TH1-type towards TH2-type reactions[14,48]. It has been suggested that an insusceptible framework inclined to a TH2-type reaction, for example, in patients with asthma, may ensure against serious Coronavirus[49]. Likewise, progesterone includes an insufficient glucocorticoid and mineralocorticoid action. Considering that steroidal treatment is valuable and is utilized in clinical practice for a couple of cases of serious Coronavirus[50], the enactment of the steroid receptor by progesterone may add to relieve extreme insusceptible reactions[51,52].

Conclusions

The current study supported the results of previous studies on the increase in the severity of Covid-19 infection with age, especially among males and postmenopausal women who show high testosterone levels accompanied by an increase in disease symptoms, while a therapeutic role for progesterone has also appeared in reducing the risk of the Covid-19 in PRM women. The current study also showed that Covid-19 poses a challenge for people who suffer from respiratory diseases related to the family, as the proportion of hereditary bronchial asthma increases among males and has played a clear role in raising the death rate of Covid-19 patients. On the other hand, the advanced stages of lung cancer were similar to that. Moreover, We hope that there will be subsequent studies on a greater number of participants for a more extended period to clarify the effect of sex hormones on the extent of individuals' susceptibility to infection with the Coronavirus, in addition to studying the effect of these hormones as a successful treatment to reduce the risk of infection. Also we recommend, through the current study, patients with bronchial asthma and lung cancer to take all health precautions to avoid the occurrence of Covid-19 infection.

Author Contributions

Meraim A. Kazaal: Conceptualization, Methodology, Software, Data curation, Writing- Original draft preparation, Investigation. Ghanim A. Abbas: Supervision. Masar J. Jari: Visualization, Validation, Writing- Reviewing and Editing . All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Ethical Clearance: The ethical research committee at scientific research by ethical approval of both environment and health and higher education and scientific research ministries in Iraq. CONFLICT OF INTEREST the authors declare that they have no conflict of interest.

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Conflicts of Interest

The authors declare no conflict of interest.

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ARTICLE / INVESTIGACIÓN

Assessing Heavy Metals Emission and Workers' Health Risks at the Eastern Industrial Region, Mosul, Iraq

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Abstract: This study was executed to investigate the heavy metal (Ni, Cu, Zn) pollution of the Eastern Industrial Region in Mosul city in Nineveh province of Iraq. Heavy metals are measured In the blood serum of 40 workers and compared with the control group of (40) people from Mosul university. Elements were measured by using an atomic absorption (flame) spectrometer. The statistical analysis results revealed that investigated Blood serum samples are seriously polluted with toxic heavy metals.

Key words: Heavy Metals Emission, Workers' Health Risks, Blood Serum, Eastern industrial Region, Pollution.

Introduction

The pollution of air, soil, ground, and surface waters with toxic heavy metals and other pollutants is a growing threat to human and aquatic life, even in small doses[1]. Heavy metal pollution has detrimental effects on humans and other ecosystems. Therefore, heavy metal pollution is the main problem in Iraq as in many developing countries[2].

The knowledge of heavy metal concentrations in the environment is fundamental to evaluating the potential impacts of industrial activities on the environment and human health. Recent studies have focused on investigating heavy metal pollution of accumulation in the human body[3,4].

Many workers in industrial areas exposed to the risks of heavy metals. The level of the concentration and other elements reflects the chemical condition of the body[5].

Heavy metals are frequently and regularly produced in different industries, like paint and ceramics, metallurgy, electroplating, heavy manufacturing industries, civil construction, etc., which are discharged through industrial wastewater. These metals are non-biodegradable, stable, toxic and carcinogenic to living organisms[6,7].

Despite the high toxicity of heavy metals, they enter into many industrial processes, either directly or as a by-product, causing many environmental problems that are difficult to solve and thus affect the ecosystem and what it contains for humans, plants, etc. animals[8-11].

Nickel absorption is considered weak by the digestive system. The daily intake should be 50 - 100 micrograms and not more than 500 micrograms. In children, the amount must not exceed 200 micrograms between 1-3 years and 300 micrograms between 4-8 years of age[12].

Exposure to the high concentration of Copper causes Wilson's disease (bloody dyes) and Alzheimer's disease[13].

The risk of zinc infection is often associated with symptoms of leg pain. Zinc concentration in serum can detect poisoning by it (14) .(15) concluded in their study about the toxicity of heavy metals and their effect on industrial workers. The results showed statistical differences when comparing the concentrations of heavy metals in the workers' blood compared to a group far from industrial activity.

In addition (16) to a study on heavy metals in the industrial scale, and the results that industrial workers are exposed to the dangers of these elements directly through inhalation or skin.

Materials and methods

Study Area

The study area is the city of Mosul, which is located in the Eastern part of Iraq.

Sampling and Analysis

The study was conducted on 40 workers in industrial areas, and they were randomly collected representing the region. In contrast, 40 blood samples were drawn from people far from industrial activity. The quantity withdrawn for both groups was 5 ml and was transferred to the laboratory and isolated, and the flame spectrometer measured heavy metals.

Statistical analysis

Processing was carried out on the data obtained for the concentrations of heavy metals by the statistical program spss and the use of many tools to express the statistical differences.

Results

The results show that there is a significant of ($P<0.001$) for elements (Ni, Cu, Zn) element. (Table 1).

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Conclusions

Industrial areas today pose a significant danger to their workers and visitors, as the results showed that there is a cumulative increase in the blood of industrial workers as a result of their direct exposure to the danger of these elements either through skin or inhalation and that these areas are in dire need of treatment as they are an economical and vital resource that cannot be dispensed with.

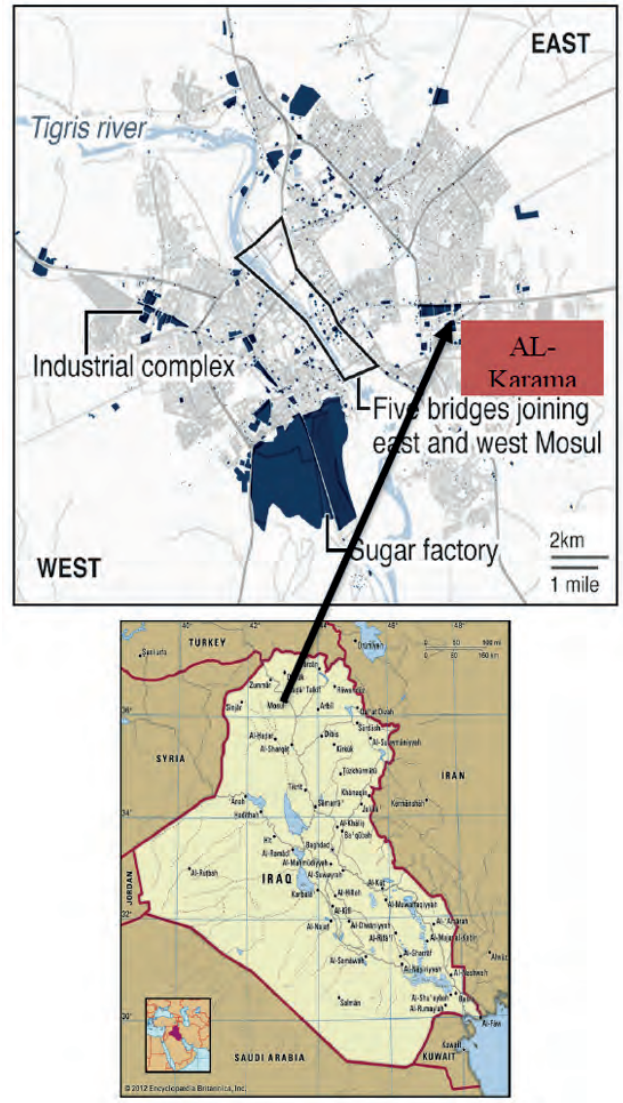


Figure 1. Location of Study Area.

Funding

Self-funding

Conflicts of Interest

There is no conflict

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Elements	Grouping	N	Mean ± SD (ppm)	p-Value
Ni	Workers	40	2.8050 ± 2.0100	p<0.001
	Control	40	0.8095 ± 0.81500	
Cu	Workers	40	1.5890 ± 1.12871	p<0.001
	Control	40	0.6545 ± 0.60351	
Zn	Workers	40	0.7590 ± 0.61120	p<0.001
	Control	40	0.4835 ± 0.30709	

P< 0.05 Significant P> 0.05 No significant

Table 1. Heavy metals in blood serum of workers & controls groups.

ARTICLE / INVESTIGACIÓN

Determination of cumulative glucose levels HbA1C and some biochemical variables in the serum of Diabetic nephropathy patients

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Abstract: The study included measuring the concentrations of each of the cumulative sugar HbA1C Glycosylated hemoglobin, urea, creatinine, uric acid, glucose, glucose uric acid, albumin, total protein and calcium, in addition to identifying the concentrations of some electrolytes (sodium, potassium, chloride, calcium) in (35) blood samples from patients with diabetic nephropathy (14 males, 12 females) aged (16-62) years who came to some laboratories in the city of Mosul and compared them to control samples (26) samples. The results showed a significant increase in cumulative sugar, uric acid, glucose and potassium concentrations in the serum of patients of both sexes. An insignificant increase in urea and creatinine concentrations and a significant decrease in albumin, sodium and chloride concentrations, while calcium and total protein did not show a significant difference compared to control samples. When comparing cases of infection between males and females, the results showed a significant increase in the cumulative sugar level for males and an insignificant increase in glucose concentration. In contrast, creatinine, uric acid, total protein and albumin did not significantly differ for males compared to infected females.

Key words: Cumulative glucose, biochemical variables, diabetic.

Introduction

Diabetic neuropathy is one of the most common complications of diabetes, which affects a third of patients with type 2 diabetes[1], and is characterized by the occurrence of many pathological severe changes that may lead to death, and about 40% of these patients eventually develop kidney disease[2] compared to other causes of kidney failure.

The increase in the concentration of sugar in the blood is the main factor for diabetic nephropathy because it affects the physiological functions of the kidneys and leads to their damage. In addition to other factors that contribute to the infection, including genetic predisposition, high blood pressure and atherosclerosis[3] Capillary damage occur in diabetic nephropathy, which leads to protein loss through diuresis, a decrease in the filtering activity of the renal glomerulus, and an increased risk of death from heart and blood vessels disease[4,5].

The illness affects the efficiency of the kidneys in removing toxins and excreting excess fluids from the body and thus their accumulation in the blood[6] and symptoms of morbidity appear after ten years in diabetics (type 1). However, symptoms appear quickly in diabetics (type 2) and are represented by high blood pressure, increased cholesterol concentration, swelling of the feet, loss of appetite, fatigue and nausea[1,7] Research has indicated that patients with type 1 diabetes develop symptoms similar to those of the end-stage kidney deficit early compared to patients with type 2 diabetes[8,9]. Many tests are considered guides to indicate kidney function and detect morbidity in diabetic patients, including measuring the concentration of urea, creatinine, uric acid, total protein, albumin and sugar, and cumulative sugar[10].

Hemoglobin glucose (cumulative sugar) HbAIC is one of the essential proteins associated with red blood cells[11] as its level increases with the increase in the concentration of glucose in the blood, and this measurement is used to determine the concentration of glucose in the blood for the previous three months[9], which helps to maintain blood sugar. Blood glucose level is used to assess metabolic changes during treatment[3].

Urea is synthesized in the liver and is one of the pathways for protein breakdown into amino acids. The level of urea represents the amount of protein that is consumed and excreted by the kidneys[12] A defect in the filtering activity of the renal glomerulus leads to a change in the concentration of urea in blood, as well as the condition in cardiovascular diseases and liver diseases[13]. As for creatinine test, it is more accurate, and its concentration is affected by age and weight, this changes the efficiency of the glomerular filtration and increases its concentration in cases of renal failure and urinary retention[3]. Another test that falls within the determination of kidney functions is uric acid, whose concentration is controlled by many factors such as heredity, high blood pressure, diabetes and bone diseases, which affect the efficiency of the kidneys, and consequently to the formation of stones and possibly to renal failure[14].

Tests for protein are indicators of kidney, liver, and bowel disease[3]. In addition to the seventh variable, electrolytes are among the essential elements inside and outside cells, including fluids and blood. Calcium is a necessary element for the body, and the potassium test helps diagnose diseases of the bones, heart, nerves and kidneys. At the same time, magnesium has an essential role in diagno-

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sing diseases. Disorders of the nervous system, muscles and kidney efficiency, as well as its role in the transport of calcium and potassium, as well as sodium, have a role in maintaining osmotic pressure and are considered a guide in diagnosing kidney diseases and estimating chloride concentrations has a role in determining renal diseases and adrenal gland functions[13,15].

To determine the effect of cumulative sugar level and biochemical variables on diabetic patients, this study discovered the serum concentrations in diabetics patients of both types who suffer from renal impairment.

Materials and methods

The blood samples were collected from (35) patients (14 males, 12 females) of different ages ranging from (16-62) years who arrived at some laboratories in the city of Mosul, as well as (26) blood samples were collected from healthy people of the same age groups. The following tests were performed on the blood serum: Measuring the cumulative glucose level HbA1C Multimeter (AFIAS) Korea-Boditech Corporation. Using an AFIAS device and measuring urea concentration Urea in Switzerland-AGAPPE and Measuring creatinine concentration creatinine, total protein, albumin, Elements (CI-, K+, Na+) by France-BIOLABO kit and Concentration measurement Uric Acid, Glucose, Calcium Ca+ in several sizes Spain-Linear.

Statistical analysis

The results were statistically analyzed, using a test t-test to show the difference between two groups at the level of significance ($\alpha = 0.05$), as well as finding the linear correlation coefficient to find the relationship between biochemical variables, HbA1C and Creatinine at the level of significance ($\alpha = 0.05$, $\alpha = 0.01$), with finding the coefficient of determination to show the effect of age on the change in HbA1C, in addition to finding the significance of the relationship between them through the ANOVA table[16].

Results and discussion

The results of table (1) showed a significant increase in the concentrations of cumulative sugar, HbA1c, uric acid and glucose in the patients' serum compared to the control groups and a non-significant decrease in the concentrations of urea and creatinine; the reason for the increase in the cumulative sugar level may be due to damage to the blood vessels[17]. When glucose concentrations rise, glucose molecules stick to molecules of proteins and platelets, and this leads to disruption of their function as a result of protein molecules sticking together; this narrows the diameter of the arteries and thus reduces the amount of oxygen that reaches the tissues, so high levels of HbAIC lead to great risks of cardiovascular disease Hematologic and stroke[18,19].

The increase in urea and creatinine levels is due to the rise in sugar levels as a result of insulin secretion disturbance[20], nephropathy that leads to arterial hypertension and a defect in its physiology, and the cause of high creatinine concentration may be urinary retention that leads to renal failure as a result of a defect in the filtering activity of the renal glomeruli[10], which leads to an increase in it in the blood, and is accompanied by the deposition of uric acid forming kidney stones in case it is higher than the normal level[15]. The high concentration of glucose in the blood affects the nerves of the kidney nephrons over time[10].

The results of table (1) showed a significant decrease in albumin and electrolytes (sodium and chloride) in patients of both sexes compared to the control groups. This is due to the large quantities of albumin excreted daily in this type of patient and for both types (first and second), which causes kidney damage and then cardiovascular complications[3]. And the reason for the decrease in chloride and sodium is due to the significant correlation between these two elements and the fact that the volume of extracellular water decreases, the state of respiratory acidosis and the slowness of metabolic processes, and this causes renal impairment[6,21].

Calcium and total protein did not significantly differ in both sexes compared to the control groups, unlike potassium, as it showed a significant increase. The reason may be its effect on the concentration of hormones, including insulin and thyroid hormones[17,22].

To show the effect of the sex factor on the variable's concentrations, the results of table (2) showed a significant increase in the concentration of cumulative sugar and glucose and an insignificant rise in both the concentration of urea and creatinine in the serum of both males and females compared to the control groups, but it was found that there was a significant increase in the concentration of uric acid in females than in males and this is as a result of metabolic disorder, especially after menopause and the change in the level of hormones[17,19], and a significant decrease in the concentration of total protein and chloride in the blood serum of both sexes was shown. The reason is mainly due to the presence of chloride in extracellular fluids, formation of respiratory acidosis and impaired renal function in regulating water balance[23].

As for a reason for the decrease in total protein, it is due to the high glucose, which helps to get infections in the renal tissue and its damage, leading to the loss of large amounts of protein through urine. Accordingly, the concentration of protein in the serum is lower than the normal limits[24].

The results of table (2) showed an insignificant decrease in the albumin concentration in males and a significant reduction in females compared to the control groups. The reason is attributed to the complications of diabetes, which leads to a defect in the function of the kidneys and the occurrence of cardiovascular complications, and thus the excretion of large amounts of protein through Urine[15].

It is noteworthy from the same table that the calcium concentration did not show a significant difference in males, while it showed an insignificant decrease in females compared to the control groups. This is attributed to a defect in kidney function that leads to glomerular filtration disorder and vitamin D deficiency, which causes osteoporosis and osteoporosis[17,25].

A significant decrease in sodium concentration was also observed in the serum of males and an increase in the serum of females compared to the control groups. The reason is fluid loss due to diarrhea and frequent urination of diabetic patients and the effect of renal impairment associated with diabetes mellitus. The increase in potassium concentration in both females and males is due to the disorder of the kidneys' functioning, its low filtering capacity, the acid-base imbalance that affects the concentration and the accompanying nephropathy and heart disease[22].

To find the relationship between the concentration of the cumulative sugar level and the rest of the biochemical variables in diabetic nephropathy patients, according to the

linear correlation coefficient, the results of table (3) showed a significant direct relationship between the cumulative sugar level and each of the concentrations of urea, creatinine, uric acid and glucose, and this is due to the direct relationship between glucose concentration and cumulative sugar[26].

The table results also showed an insignificant inverse relationship between the cumulative sugar concentration and each of the total protein, albumin and elements (calcium, chloride, potassium, sodium).

The results of table (4) showed that there was a significant positive relationship between creatinine concentration and each of (HbA1C, urea, uric acid, glucose), the cause is due to diabetic nephropathy, and this is the main cause of chronic renal failure, so both urea and creatinine are good indicators that help determine kidney functions during nephropathy in patients with diabetes[27] There was a significant inverse relationship between the concentration of creatinine and each of the total protein, albumin and elements (calcium, chloride, potassium and sodium).

The results of table (5) showed that there is a direct relationship between the cumulative sugar level HbA1C and age; according to the correlation coefficient and determination, the reason is due to increasing age, many physiological variables occur, such as a decrease in the function of the pancreatic islets, the sensitivity of tissues to insulin, the activity of insulin receptors, and in muscle tissues, glucose consumption gradually decreases in general, and the level of glucose increases with age[28,29].

Through a table of variance analysis ANOVA The results of table (6) showed a significant effect of age on the level of HbA1C, which led to a decrease in the diagnostic efficiency of the level of HbA1C, as with age the number of RBCs decreases physiologically[30] This leads to a decrease in bone marrow production, especially in the elderly, and a decrease in kidney function[31].

Conclusions

We conclude that there is an increase in the cumulative sugar level of the affected males compared to the control samples. With no significant difference for the other indicators, values for females, no significant difference was recorded.

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Self-funding

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In this section, we acknowledge any person who supports us to complete this project.

Patients	Control	Biochemical variables
9.10* ± 0.39	3.97 ± 0.18	HbA1C (%)
39.05 ± 2.05	34.11±1.34	S.Urea (mg/dl)
0.92 ± 0.04	0.88 ± 0.04	S.Creatinine (mg/dl)
6.66* ± 0.28	5.63 ± 0.26	S.Uric Acid (mg/dl)
242.11* ± 11.11	86.23 ± 2.50	Glucose (mg/dl)
6.26* ± 0.12	6.98 ± 0.12	T.Protein (mg/dl)
3.90* ± 0.80	4.54 ± 0.10	Albumin (mg/dl)
119* ± 3.06	145 ± 1.06	Na + (MEq/L)
5.60* ± 0.08	4.60 ± 0.11	K+ (MEq/L)
90.7* ± 2.63	102 ± 0.93	Cl- (MEq/L)
9.04* ± 0.11	9.22 ± 0.11	Ca++ (MEq/L)

* Significant at the level of significance (0.05 -α).

Table 1. Concentrations of variables in the blood serum of diabetic nephropathy patients compared to control groups Variable concentrations standard deviation ± average.

Female patients	Female control	Male patients	Male control	Biochemical variables
8.87* ± 0.55	4.31 ± 0.22	9.10* ± 0.37	3.97 ± 0.18	HbA1C (%)
39.14 ± 2.91	33.91 ± 1.98	39.00 ± 2.87	34.28 ± 1.89	S.Urea (mg/dl)
0.92 ± 0.06	0.87 ± 0.05	0.93 ± 0.06	0.89 ± 0.06	S.Creatinine (mg/dl)
6.60* ±0.33	5.01 ± 0.25	6.70* ± 0.43	6.15 ± 0.37	S.Uric Acid (mg/dl)
235.85* ± 18.89	87.66 ± 3.84	246* ± 13.88	85.00 ± 3.37	Glucose (mg/dl)
6.20* ± 0.20	7.00 ± 0.17	6.30* ± 0.16	6.97 ± 0.18	T.Protein (mg/dl)
3.80* ± 0.23	4.65 ± 0.13	3.98* ± 0.16	4.45 ± 0.14	Albumin (mg/dl)
118.35* ± 5.59	145.33 ± 1.38	120* ± 3.59	145 ± 1.63	Na + (MEq/L)
5.62* ± 0.10	4.70 ± 0.18	5.58* ± 0.12	4.51 ± 0.15	K+ (MEq/L)
92.7* ± 1.61	102 ± 1.32	89.45* ± 4.28	103.00 ± 1.35	Cl- (MEq/L)
8.76* ± 0.11	9.20 ± 0.18	9.25* ± 0.15	9.25 ± 0.15	Ca++ (MEq/L)

* Significant at the level of significance (-0.05).α).

Table 2. Concentrations of biochemical variables in the blood serum of male and female patients compared to control groups standard deviation ± average.

Patients	Biochemical variables
0.416*	S.Urea (mg/dl)
0.374*	S.Creatinine (mg/dl)
0.392*	S.Uric Acid (mg/dl)
0.957*	Glucose (mg/dl)
-0.300	T.Protein (mg/dl)
-0.270	Albumin (mg/dl)
-0.207	Na + (MEq/L)
-0.120	K+ (MEq/L)
-0.157	Cl- (MEq/L)
-0.115	Ca++ (MEq/L)

* Significant correlation at the level of significance (-0.05). α).

(-) Insignificant correlation.

Table 3. The relationship between HbA1C and other biochemical parameters in patients with diabetic nephropathy.

Patients	Biochemical variables
0.374*	HbA1C (%)
0.880*	S.Urea (mg/dl)
0.563*	S.Uric Acid (mg/dl)
0.335*	Glucose (mg/dl)
-0.684**	T.Protein (mg/dl)
-0.402	Albumin (mg/dl)
-0.397*	Na + (MEq/L)
0.162	K+ (MEq/L)
-0.351*	Cl- (MEq/L)
-0.491*	Ca++ (MEq/L)

* Significant correlation at the level of significance (-0.05). α).

(-) Insignificant correlation.

Table 4. The relationship between concentration creatinine and other biochemical parameters in patients with diabetic nephropathy

correlation coefficient R	The coefficient of determination R.Square
0.428	0.183

Table 5. Correlation coefficient and determination to show the relationship between age and the value of HbA1C in patients

R: correlation coefficient.

R.Square: coefficient of determination.

Table 6. Shows the variance ANOVA to show the effect of age on HbA1C in patients

	Sum of Squares	df	Mean Square	F	Sig
Regression	21.321	2	10.660	3.595	0.039
Residual	94,889	32	2.965		
Total	116.210	34			

The independent variable is age (0.05) (0.039).≤

Conflicts of Interest

There is no conflict.

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ARTICLE / INVESTIGACIÓN

Isolation and molecular identification of *Exiguobacterium profundum* from drain water and study of some physiological properties

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Abstract: Some microorganisms' stress tolerance and adaptability through the ability to live in new environments previously considered hostile to dense microbial growth. The aim of the study: In this work, the isolation, molecular identification, and analysis of some physiological properties of *Exiguobacterium profundum* isolated from washing machine drain water were carried out. Fifteen samples were collected from different washing machines' drain water, and 42 isolates were characterized and identified. Based on the morphological assay and 16S rRNA gene sequencing, one isolate was designated as *Exiguobacterium profundum* Kh-Am2 and was deposited in the Gene bank database under the accession number MW447498.1. The physiological properties assay showed that *E. profundum* could not decompose red blood cells while proving their ability to decompose crude and coconut oil, producing lipopeptide biosurfactants. It has also demonstrated its ability to form biofilm using the tube method, which helps it to live in harsh environments such as the environment of washing machines. The results showed that *E. profundum* is halotolerant bacteria with its ability to grow at a concentration of (75) g/l NaCl and the best growth was at(25) g/l NaCl concentration. The results also showed the effect of different concentrations of NaCl on *E. profundum*, where the best movement rate was at a concentration of (25)g/l NaCl, while the movement was completely inhibited at a concentration of (75)g/l NaCl. We have concluded that *E. profundum* possesses traits that enable it to live in harsh environments.

Key words: *Exiguobacterium profundum*, drain water, biosurfactant.

Introduction

Exiguobacterium is a bacterial genus with a wide range of species obtained from various habitats. *Exiguobacterium* first was recognized as a genus about three decades ago, and there are presently Seventeen species in this genus. *Exiguobacterium* is a halotolerant bacteria that has piqued the interest of many researchers as a crucial agent in salinity stress reduction, capable of thriving in a wide range of temperatures and pH[1].

Exiguobacterium isolates have been employed in biotechnology and industrial applications such as enzyme synthesis, bioremediation, and the breakdown of toxic materials released into the environment. Other isolates can promote plant development, and they are currently being investigated to enhance agricultural production. In the genomic sequences of diverse strains of this species, many genes encoding products crucial to agriculture and the environment have been found. In the genomic sequences of various strains of this species, many genes encoding products important to agriculture and the environment have been found. Many strains also feature stress-response genes, allowing them to colonize and thrive in a wide range of ecological settings[1,2]. *Exiguobacterium* species such as "*E. sibiricum* , *E. alakliphilum*, , *E. undae*, *E. oxidotolerans*, *E. mexicanum* , *E. antarkticum*, *E. profundum*, and *E. indicum*" have been discovered in a variety of environments[3] .

E. profundum is a species of *Exiguobacterium*, Gram-positive, nonsporulating rods that can be found mono, pairs or in short motile chains with peritrichous flagella. Colonies are spherical (1–2 mm) and creamy or orange in color under anaerobic or aerobic conditions. Chemo-organ trophic and facultatively anaerobic. Positive for catalase and negative for oxidase. It is slightly thermophilic (growing within 12 - 49 °C, optimum at 45 °C) and halotolerant (growth in 11 percent NaCl, optimal 0–2 percent NaCl). Its pH range for growth is 5.5–9.5. (Optimum pH 7.0). Dextrose, lactose, maltose, mannose, and sucrose were used to test carbohydrate fermentation. The major organic acid produced by carbohydrate fermentation is L-Lactate, with traces of formate, ethanol, and acetate also present. Mueller Hinton broth, "Soybean Casein Digest broth, King's broth, Mac Conkey broth, and Nutrient broth media" were used to assess the maximum growth and pigment production properties[4,5].

According to several studies, *Exiguobacterium profundum* can be used in bioremediation and toxic degradation by producing lipopeptide biosurfactants, which have stable chemical characteristics, are renewable, environmentally beneficial, and have low toxicity[2,6,7].

Currently used in industry, cosmetics, and pharmaceutical emulsions, foaming agents, wetting agents, emollients, and have antibacterial activity against gram-negative bacteria[2,8,9].

This research aimed to isolate and molecular identify *Exiguobacterium profundum* and study some physiological properties such as biosurfactant production, biofilm production, and salinity on growth and motility.

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Materials and methods

Sample Collection and Isolation

Fifteen samples were collected from washing machines, drained water in sterile containers and brought to the laboratory. Following, 0.1 mL from each sample was cultured on Trypto Soy Agar (TSA) by using sterile swab sticks (BIONOVO, Poland) . The plates were incubated at 37°C for 24-48 h. After the incubation, isolates were purified to obtain single colonies, then underwent to identification by using Gram-stain, colonies appearance and molecularly identified by using the 16S rRNA gene and the following assays[10].

Amplification of 16S rRNA gene and Phylogenetic Tree

DNA extraction

The isolate was grown at 37 °C for 24 h on a rotary shaker (250 rpm) in a falcon tube containing 20 mL of Tryptic Soy Broth (TSB). The method for extracting DNA was subsequently followed using the G- spin DNA extraction kit (intron biotechnology, Korea). The 16S rRNA genes were amplified by polymerase chain reaction (PCR) from genomic DNA of selected isolates according to the Maxime PCR (PreMix kit) methodology. For PCR reaction, 1.5µl of DNA template, 1µl of 10 picomols/µl of each conserved primers 1250R (5'- GGTTACCTTGTTACGACTT- 3') and 1250 F (5'- AGAGTTTGATCCTGGCTCAG- 3') from (Integrated DNA Technologies company, Canada), 5µl of Taq PCR PreMix and 16.5 µl ddH2O for a total volume of 25µl. PCR with the optimal cycle setting for the detecting 16S rRNA gene was carried out using a Bio-Rad MyCycler thermal cycler (Bio-Rad, USA) with an initial denaturation step at 95oC for 5 min, Denaturation step at 95oC for 45 sec, 35 cycles of 95oC for 45 sec, 58oC for 45 min, 72oC for 45 sec, followed by a final extension step of 72oC for 7 min. Amplification products were analyzed by electrophoresis in 1% (w/v) agarose gel.

Purification of PCR Products

According to the manufacturer's instructions, the PCR products were purified using the QIAquik PCR purification kit (QIAGEN). In brief, one volume of PCR product mixed with five volumes of Binding Buffer was mixed thoroughly and, after that, placed in a QIAquick spin column, followed by spinning for 60 seconds to bind the DNA, flow-through was removed, the column was then placed back into the collecting tube. DNA was centrifuged for 60 seconds after being cleaned with 0.75 ml of washing buffer. The flow was removed, and the tubes were centrifuged for another minute. After that, the column was put in a fresh 1.5 ml centrifuge tube. Fifty µl Buffer of Elution was added to the middle of the QIAquik membrane for DNA elution, and the tubes were centrifuged for 1 minute. Macro gene/Korea Sequencing Service sequenced the purified PCR products. (Following Kit instructions).

Phylogenetic tree

The DNA sequence was collected and matched to sequences in the gene bank databases. Homolog surveys were carried out using internet bioinformatics tools (www.ncbi.nlm.nih.gov.BLAST). Sequences had a lot of similarities (100%) with species *E. profundum*. In FASTA format, all isolates were retrieved and downloaded. A maximum-likelihood Multiple Sequence Comparative by Log Expectation (MUSCLE) software created phylogenetic trees with the 16S gene of Kh-Am2 strain using program MEGA version 7.0 and matched sequences. The confidence value of individual branches was calculated using 1000 bootstrap copies of the original sequences. The complete 16S rRNA gene sequences were deposited in the Gene Bank database.

Biosurfactant screening

Blood agar hemolysis assay

Sterile fresh blood in volume 1.5 mL was added to a 100 mL Erlenmeyer flask containing a sterile blood base medium. The selected isolate was streaked onto blood agar and cultured for 48-72 hours at 37°C[9].

Oil spreading assay

The method of (9) was used in the screening of biosurfactants production for Kh-Am2 isolate. Using a petri dish, 30 mL of distilled water was poured into it, 1 ml of coconut oil and crude oil were separately tubed and dropped into the center; in the center of the oil layer, 20 mL of the Kh-Am2 culture was placed.

Spread Plate assay

Following the method of Spread Plate assay with modified[10], the assay was done to conduct the ability of the selected isolate to consume crude oil and coconut oil. The plates containing Bushnell Hass medium (BHM) as a mineral salt medium comprised of (K2HPO4 1gm, MgSo4.7H2O 0.2 gm, FeCl3 0.05 gm, CaCl2 0.02 gm, NH4NO3 1gm, , Agar 15 gm) in 1L of distal water were prepared with added 1% of crude oil and coconut oil separately. Each plate was inoculated by 0.1 ml 1.5×10^{8} cells ml^{-1} of selected isolate suspension. Then plates were incubated for seven days at 37C˚. The growth of colonies after the incubation time is taken as a sign of their ability to use crude and coconut oil as a source of energy and carbon.

Biofilm formation detection using the tube method

The chosen isolate was inoculated in a tube containing ten trypticase soy broth and 1% glucose and cultured for 24 hours at 37°C. After incubation, tubes were poured and washed with phosphate buffer saline (pH 7.3) before drying. The tube was then colored with crystal violet (0.1 percent). Any residual discoloration was removed with deionized water and then dried inside out. The findings of the control were used to evaluate the tube method. It was considered a positive sign of biofilm development when a visible film lined the tube's wall and bottom[11,12].

Salinity tolerance

The Kh-Am 2 isolate was cultivated on YP culture media with NaCl concentrations of (0), (25) , (50) and (75) g/l. 75 hours at 25°C incubation with orbital agitation at 120 rpm. OD600 was measured in a UV–visible spectrophotometer UV 1800 to track growth.

Salinity's Influence on Motility

The selected isolate was cultivated on YP agar (0.3% agar / Difco) . A drop over the agar in the Petri plate's middle inoculated. The motility ratio were evaluated after 72 hours of incubation at 25 °C in (0) , (25) , (50)and (75) g /l NaCl[13].

Results and discussion

The streaking results on cultured medium plates revealed that the drain water samples contain various microbial communities with different gram-positive and gram-negative bacteria. A total of 42 bacterial isolates were isolated. The target Gram-positive rods bacteria of Exiguobacterium was the most numerous of other isolates, and this agrees with what was indicated by both[4,14] and was chosen for further studies.

16S rRNA gene sequence and phylogenetic analysis

The isolate designated as the Kh-Am2 and their 16SrRNA gene sequences was recorded in the NCBI Gene bank databases under the accession number MW447498.1. Once the 16S rRNA gene of the Kh-Am2 isolate was amplified and analyzed using a 1 percent agarose gel-electrophoretic method, the PCR result was 1500 bp, as shown in Gene data (http://www.ncbi.nlm.nih.gov) a search tool was used to perform BLAST analysis on partial 16S r RNA sequences. The Kh-Am2 isolate exhibited 99-to-100% matched with the NCBI Gen database's sequences. The isolate's Neighbour Joining tree as constructed in MEGA version 7 (Figure 1), identified that a neighbor-joining tree based on similar 16S rRNA gene sequences for the *E. profundum* Kh-Am2 is the most closely related to Exiguobacterium sp. 8A. They are both clustered with *E. profundum* strain fsznc-03 and *E. profundum* strain GN31a.

Blood agar hemolysis assay

The results of the hemolysis test on the blood agar medium showed a negative result indicating that no clear areas were formed around the inoculation line, which is consistent with what was indicated by both[9] as it does not produce hemolysin that breaks down the red blood cells.

Oil spreading assay

The results showed the ability of Kh-Am2 isolate to produce biosurfactant in terms of clear zones and oil displacement zones for both types of crude oil and coconut oil, as shown in (figure 2). The reason is due to the ability of Kh-Am2 to produce biosurfactant; in addition, *Exiguobacterium* strains produce alkali esterase enzyme

belonging to Lipolytic enzymes[22], and this is consistent with what was referred to by (9), who stated that The biosurfactant produced by *E. profundum* could be classified into the lipopeptide group, It also gave the results of the oil spreading assay presented by Ahmed's observation for *Bacillus cereus* ND 1, as it showed its ability to decompose crude oil[15], Biosurfactants work as emulsifiers by lowering surface tension whereas, biosurfactants are made up of polar and nonpolar parts[16].

Spread Plate assay

Kh-Am2 isolate showed its ability for growing on BHM containing crude oil and coconut oil separately, which indicates its ability to consume both crude oil and coconut oil, as shown in (figure 3), due to its ability to use crude oil and coconut oil as a source of energy and carbon. The researcher[17] pointed out the role of *Exiguobacterium sp*. AO-11 in removing crude oil pollutants from the microcosms of sediments, and The researcher Chen referred to the role of *Exiguobacterium sp* in the decomposition of crude oil[18].

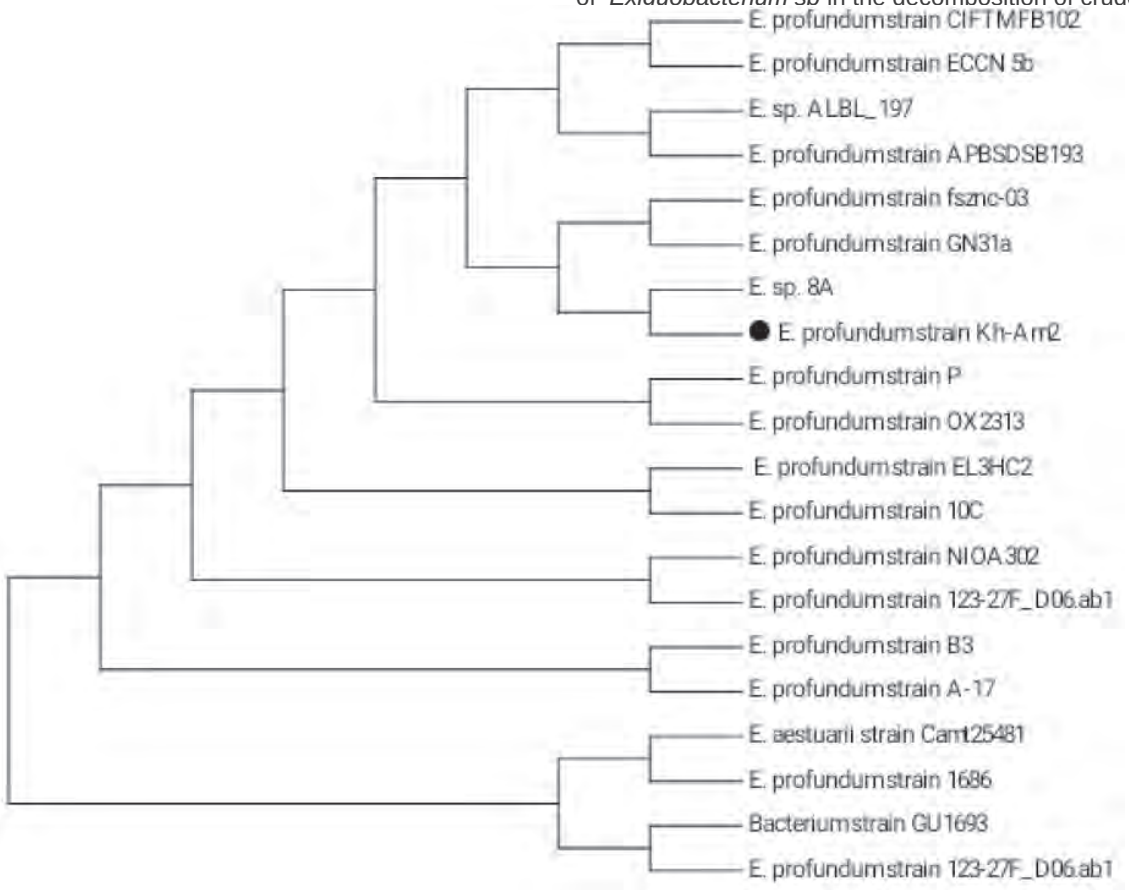


Figure 1. Phylogenetic tree (1000 bootstrap) constructed by MEGA7 tool from 19 merged nucleotide sequences of 16S rRNA gene homologous to that of *Exiguobacterium profundum* strain Kh-Am2 (indicated by black cycle).

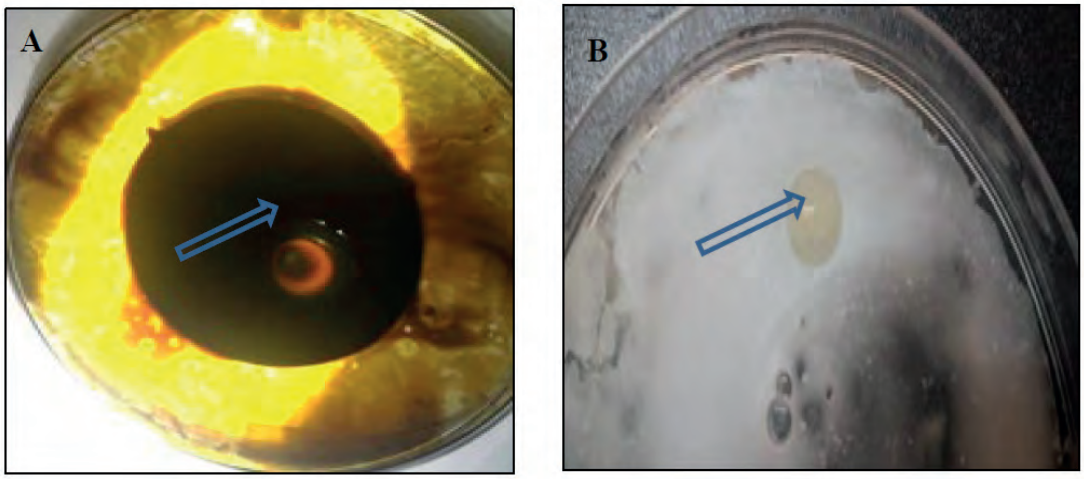


Figure 2. The clear zone begins to form in the oil spreading assay:- (A) crude oil (B) coconut oil.

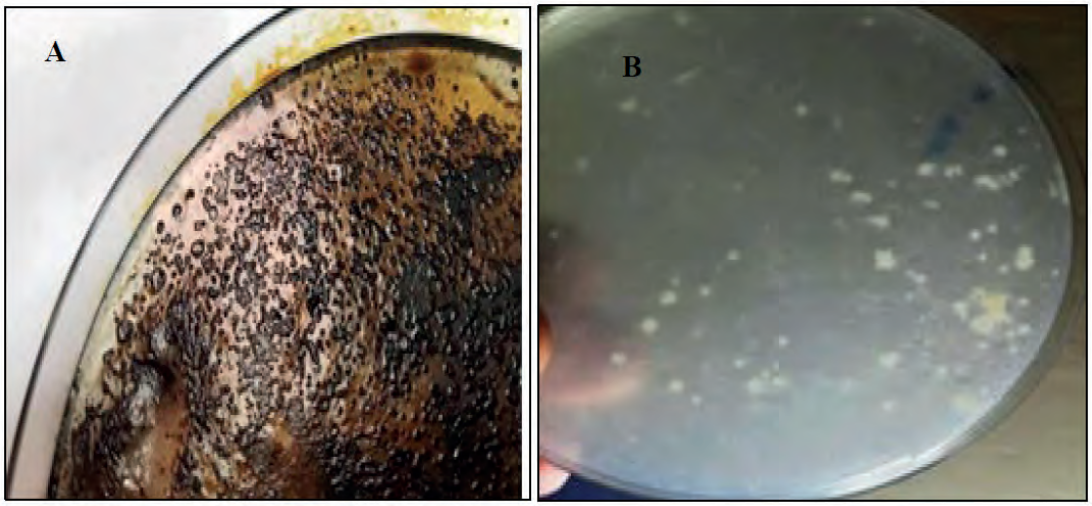


Figure 3. The ability of Kh-Am2 isolate to consume crude and coconut oil on BHM :- A(BHM with 1% crude oil) B (BHM with 1% coconut oil).

This could be a unique trait that helps to the survival of these populations and reflects the ability of these creatures to thrive in such harsh conditions in the washing machines, as it can depend on its growth on oils sticking to clothes when it comes out with drain water.

Biofilm formation detection using the tube method

The results showed the ability of Kh-Am2 to adhere and form a biofilm by using the tube method in terms of the deposition of the crystal violet on the inner side of the test tube, as shown in (figure 4). Biofilms are a community of bacteria that produce extracellular substances such as proteins, polysaccharides, DNA, RNA, and water. The essential component of a biofilm is the nutrient flow control matrix[19]. The ability of Kh-Am2 isolate to generate biofilms was demonstrated using the biofilm tube approach. Where the researcher[17] was referring to Exiguobacterium sp. ability to create biofilm. Biofilm production is one of the most important mechanisms that the Kh-Am2 isolate has for surviving in harsh settings.

Salinity tolerance

The results showed the ability of Kh-Am2 isolate to grow in high concentrations of salt. The best growth was found in (25) g/l of NaCl, where the absorbance reached 0.8 at OD_{600} nm. The concentrations (0), (50), (75) g/l of NaCl were 0.4, 0.3, 0.3 at OD600, respectively, as shown in (figure 5), where it gave a similar reading for both concentrations (50) and (75) g/l of NaCl, this result makes the Kh-Am2 isolate is classed as halotolerant bacteria, While the researcher[13] indicated that Exiguobacterium sp. SH31 were unable to grow at a concentration of (75) g/l NaCl percent, but they were able to grow at a salt concentration of(50) g /l NaCl percent[19] also noted that the growth of *E. profundum* PHM11 stopped at the concentration of 2500 mM. There is little published research on how *Exiguobacterium* tolerates salinity by fine-tuning its gene expression patterns and modifying its inherent physiology. Living in extreme environments causes their genes to adapt to harsh environmental conditions, and thus the enzymes and proteins encoded by the genes can withstand extreme conditions.

Salinity's Influence on Motility

The results showed that Kh-Am2 isolate moved (swimming movement) on YP agar at (0) , (25) and (50) g/l NaCl, where the highest percentage of movement was observed at a salt concentration of (25) g/l NaCl, which represented the best growth for the isolate as observed in (Figure 6). At the same time, the motility was wholly inhibited at (75) g/l NaCl as shown in (figure 6).

Some bacteria respond to osmotic pressure, as in *Pseudomonas putida*[21]. Although *Exiguobacterium* bacteria are described as motile[22], it suggests that specific salt concentrations make the cell less active or could be gover-

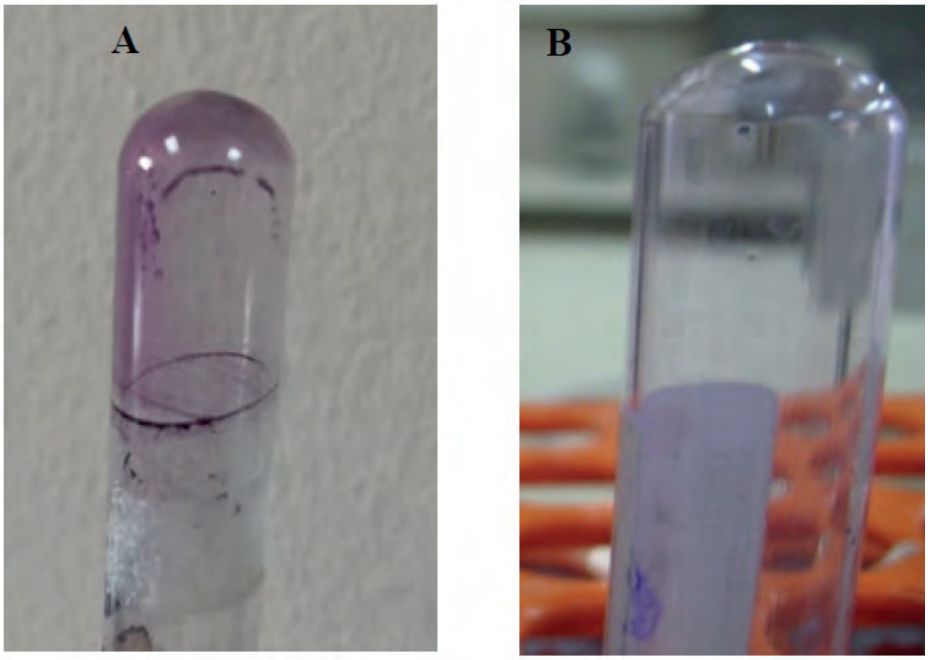


Figure 4. Biofilm formation by Kh-Am2 on the inner sides of the test tube. A:- Positive resul; B:-Control.

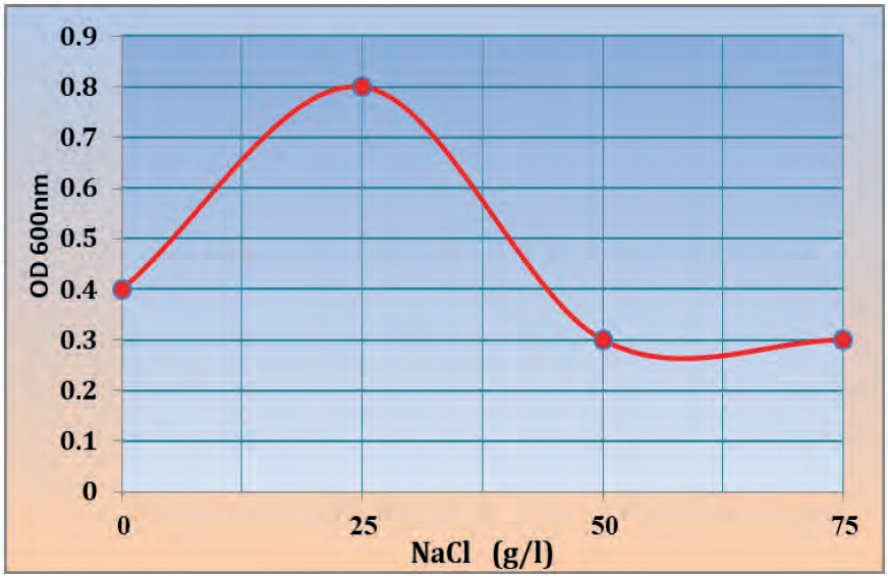


Figure 5. Growth of Kh-Am2 in 0, 25, 50 and 75 g/l NaCl.

ned by environmental factors somehow[23]. Flagella synthesis requires much energy, and bacteria can block specific processes in reaction to stress. Motility was reduced, and biofilm development was inhibited[13]. The results of salinity on motility showed that the highest motility (Swimming motility) was obtained at a concentration of (25) g/l NaCl. In contrast, motility was inhibited at a concentration of(75) g/l NaCl, and this agrees with the scientist (13) in obtaining the highest percentage of motility at a concentration of(25) g/l NaCl for *Exiguobacterium sp.* SH31 while the movement was inhibited at a (50) g/l NaCl concentration.

Conclusions

Under extreme conditions, salinity is a significant element in determining the spread of life. Our study suggests that *E. profundum* Kh-Am2 possesses a stressing regulation system that controls gene expression and supports the organism's survival and growth in salt environments. Our data revealed the potential of the *E. profundum* Kh-Am2 isolate to grow at a high salt concentration of 75 g/L and the ability to develop a biofilm. This was related to maintaining the integrity of bio-molecules in the physiological process and preserving cell architecture and function. *E. profundum* Kh-Am2 can degrade crude and coconut oil in an oil spreading assay, indicating that the bacteria can be used in bioremediation and toxic degradation.

More analyses are needed to comprehend further the gene regulation of salinity pathway of the E. profundum Kh-Am2 isolated from waste water of a washing machine.

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Conflict of interest

The authors declare that they have no conflict of interest.

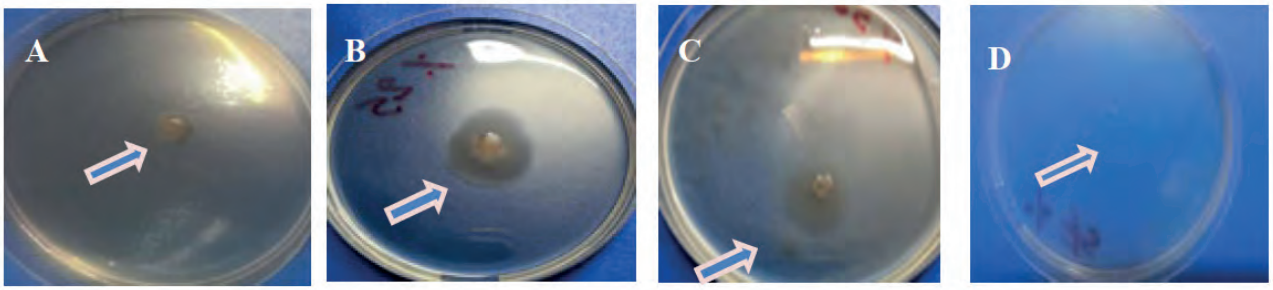


Figure 6. Effect of salinity in the motility of Kh-Am2 with different concentrations of NaCl after incubation 72 h at 25°C. A .Concentration 0 g/l of NaCl, B.Concentration 25 g/l of NaCl, C .Concentration 50 g/l of NaCl, D.Concentration 75 g/l of NaCl.

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ARTICLE / INVESTIGACIÓN

Synthesis and characterization of azo liquid crystal compounds based on 5H-Thiazolo [3,4-b][1,3,4] thiadiazole unit

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Abstract: A calamitic symmetric liquid crystalline consisting of an azo group containing 5H-Thiazolo[3,4-b][1,3,4]thiadiazole moiety compound[III] was synthesized via sequence reactions starting from reaction terephthaldehyde with mercaptoacetic acid and thiosemicarbazide in the presence of concentrated sulfuric acid to synthesized 5,5'-(1,4-phenylene)bis(5H-thiazolo[4,3-b][1,3,4]thiadiazol-2-amine)[I] then the azo compound [II] synthesized by coupling between diazonium salt of the compound [I] with phenol at (0-4)℃., after that the compound [III] was synthesized by the reaction of the compound [II] with methyl bromide in alkaline media. The compounds are characterized by melting points, FTIR and ^{1}HNMR spectroscopy. The mesomorphic behavior was studied by using polarized optical microscopy POM.

Key words: Azo compounds, liquid crystal, mesomorphic properties.

Introduction

Calamitic liquid crystals (LCs) are widely used in LC displays and optical systems because of the suitability of their anisotropic properties[1-3]. In the case of liquid crystals, the azo group is the most widely employed photochromic unit[4-9]. This is mainly due to its linearity, diversity in preparation[10], speed and stability of isomerization[11]. However, their thermal and optical properties can be adjusted by modifying the molecular geometries of the mesogenic compounds. Several calamitic azo LC derivatives have been investigated and evaluated based on their optical properties[12,13], a few groups have increasingly worked with calamitic azo LC derivatives with different core sizes to determine the location of azo linkages within the rigid portion, lateral groups and terminal flexible-chains length[14-21]. A rigid shape creates azobenzene molecules, essential for exhibiting mesomorphic phenomena[22,23]. Therefore, from these geometrical investigations and part of our study of liquid crystal derivatives containing heterocyclic units[24-29] the purpose of this research was to develop the synthesis of a new azo LC material containing 5H-thiazolo[4,3-b]-1,3,4-thiadiazole unite and methoxy terminal group and the correlation between the geometry of its mesogenic part and its mesomorphic properties.

Materials and methods

Experimental

The materials were taken from Aldrich, Fluka and Merck Co.

Techniques

FT-IR spectra were recorded by SHIMADZU (IR Affinity-1) FT-IR spectrometer in the wave number range 4000-600 cm^{-1}. ^{1}H-NMR spectra were measured by company: Bruker 400 MHz and were reported in ppm (δ); the compounds were dissolved in DMSO- d6 solution with the TMS as the internal standard. The melting point measurement by, Gallen Kamp apparatus. Liquid crystalline properties were investigated by using a (Meiji MT9000) Polarizing Optical Microscope attached to an INSTEC Hot stage.

Synthesis

The route of synthesized new compounds is represented in figure 1.

Synthesis of 5,5'-(1,4-phenylene)bis(5H-thiazolo[4,3-b][1,3,4]thiadiazol-2-amine)[I]

Terephthalaldehyde (1.34 gm, 0.01 mol) and mercaptoacetic acid (1.84 g, 0.02 mol) were mixed for 20-25 min. Then thiosemicarbazide (1.5 gm, 0.02 mol) was added, followed by the addition of concentrated sulfuric acid (15 mL) portion-wise upon cooling. The mixture was kept in the freezer for 24 h. Then treated with crushed ice 40 gm, adding aqueous sodium hydroxide 40% to obtain pH=7-8. The precipitate was filtered[30], dried and recrystallized from ethanol.

Molecular formula: $C_{14}H_{12}N_6S_4$, Yield 60 %, Color yellow, M.P= dec.270 °C. FTIR (v/cm^{-1}): 3456-3194 (asym.,sym NH_2), 3005 (Ar-H), 1643 (C=N), 1589 (C=C). ^{1}H NMR (400MHz, DMSO-d6) δ (ppm): 8.64-6.96 (4H, Ar-H), 5.31 (s,4H,2NH_2), 3.42(2H (2S-CH-N) in cyclic),3.27 (2H (2S-CH=C) in cyclic).

Synthesis of 4,4'- (5,5'- (1,4-phenylene) bis (5H-thiazolo [4,3-b][1,3,4] thiadiazole-5,2-diyl))bis (diazene-2,1-diyl) diphenol[II]

Compound [I] (0.32 g, 0.0017 mol) was dissolved by heating and stirring in 16 mL of 85% phosphoric acid, cooled to 0°C in an ice bath. 4 mL concentrated nitric acid, and

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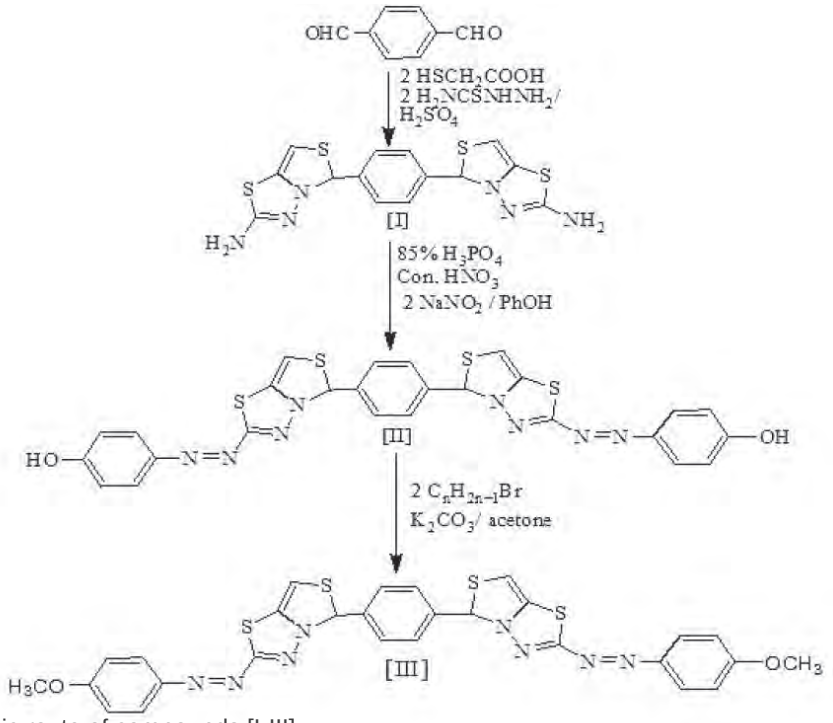


Figure 1. The synthetic route of compounds [I-III].

a solution of (0.23 g, 0.0034 mol) sodium nitrite in 4 mL water was added. React mixture was stirred at 5°C for 10 minutes, then a solution of (0.32 g, 0.0034 mol) phenol in 1 mL water was added to it; the brown product was formed, filtered, washed with water, then dissolved in 60 mL 10% NaOH, the solution was filtered, the crude product was precipitated during neutralization with 10% HCl, then filtered and washed with water several times, recrystallized from ethanol to give brown solid.

Molecular formula: $C_{26}H_{18}N_8O_2S_4$, Yield 80%, Color brown ,M.P>270°C.

FTIR (v/cm-1):3556(OH), 3050 (C-H arom), 1666 (C=N), 1600 (C=C), 1543(N=N)

1,4-bis(2-((4-methoxyphenyl)diazenyl)-5H-thiazolo [4,3-b][1,3,4]thiadiazol-5-yl)benzene [III]

A mixture of compound [II] (1.2 g, 0.0015 mol) and anhydrous potassium carbonate (0.80 g, 0.012 mol) dissolved in acetone 20 mL, then methyl bromide (0.004 mol) was added, the mixture was refluxed overnight. Afterward, the mixture was poured onto ice water; the precipitate was filtered and then washed with water.

Molecular formula: $C_{28}H_{22}N_8O_2S_4$, Yield 90%, Color deep yellow, M. P >300C°

FTIR (v/cm^{-1}):3005 (C-H arom), 2958-2835(C-H aliph), 1651(C=N), 1597(C=C arom), 1573(N=N), ^{1}H NMR (400MHz, DMSO-d6) δ (ppm): (7.75-6.44) (12H, Ar-H),(3.87-3.73) (4H (2S-CH-N) and (2S-CH=C) in cyclic), 2.30 (6H,2OCH$_3$).

Results

The new synthesized compounds characterization by FTIR and ^{1}HNMR spectroscopy gave analysis satisfactory for the proposed structures. Compound [I] was synthesized by treating terephthalaldehyde compound with mercaptoacetic acid and thiosemicarbazide in H_2SO_4 at reflux. This compound characterization by FTIR and ^{1}HNMR spectroscopy. FTIR spectroscopy for compound [I] showed absorption bands at (3456-3194) cm^{-1} which assigned to asymmetry and symmetry of NH_2 and NH groups (Tautomerism NH_2 with C=N in cyclic) also C=N groups were appeared at 1643 cm^{-1}. Also showed absorption bands at 3005 cm^{-1} and 1589 cm^{-1} for C-H and C=C aromatic groups, respectively.

The ^{1}HNMR spectrum (in DMSO-d6 as a solvent) of compound [I], showed: a singlet signal at δ11.49 ppm for protons of NH group (tautomerization NH_2 with C=N in cyclic); many signals in the region δ (8.64-6.96) ppm for four aromatic protons of benzene rings, a single signal at δ 5.31 ppm due to four protons of two (NH_2) groups, a single signal at δ3.42 ppm for two protons of S-CH-N groups also a singlet signal at δ3.27 ppm for two protons of S-CH=C groups.

The azo compound[II] synthesized by coupling between diazonium salt of the 5,5'-(1,4-phenylene)bis(5H-thiazolo[4,3-b][1,3,4]thiadiazole-2amine)[I] with phenol at (0-4)°C. The FTIR spectrum of compound [II] showed disappearance absorption stretching bands of NH_2 groups for starting material compound[I] and appearance stretching bands of hydroxyl 3556 cm^{-1}. Also showed bands at (3050), 1666 cm^{-1}, 1600cm^{-1} and 1543 cm^{-1} for C-H aromatic, C=N, C=C and N=N groups, respectively.

The compound [III] was formed from the reaction of the compound [II] with two moles from methyl bromide with K_2CO_3 in acetone. The FTIR spectrum for compounds [III] showed the disappearance of stretching bands for hydroxy groups for starting material and showed absorption stretching bands of C-H aliphatic of methoxy groups in the region 2958-2835cm^{-1}.

Discussion

Liquid crystalline properties

The phase transition temperatures and mesophase

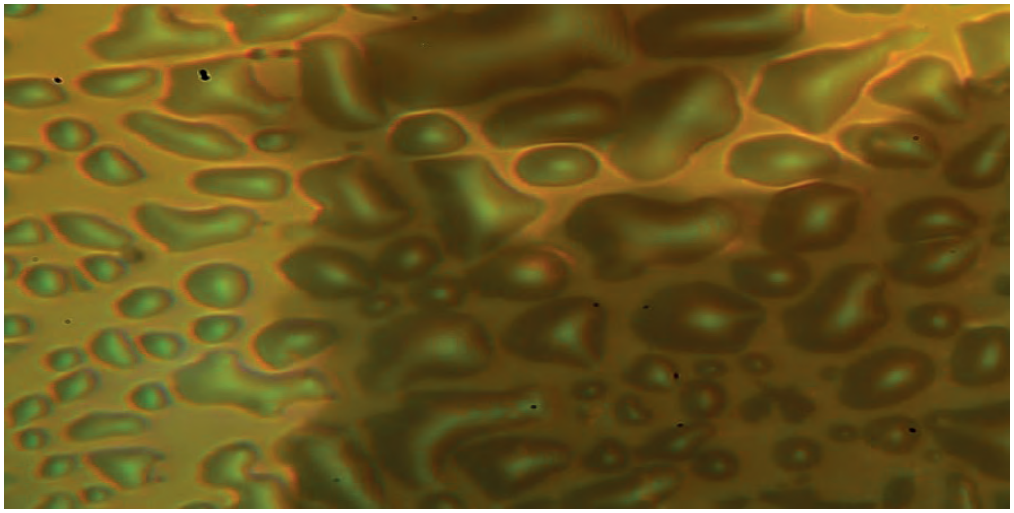


Figure 2. Cross-polarizing Optical textures of nematic phase obtained on heating at 190 C°(Magnification 200×) for compound [III].

type (texture identity) of the compound was investigated by using POM. The mesophases exhibited by compound of series [III] were identified according to their optical textures, which were observed by POM, using the classification systems reported by Sackmann and Demus and Richter[31, 32] and Gray and Goodby[33].The compound [III] showed droplets nematic phase as in figure 1

Conclusions

This work investigated the design and synthesis of a new azo liquid crystalline compound including 5H-thiazolo [3,4-b],[1,3,4] thiadiazole linked with a methoxy group as the terminal chain length, which was then characterized. The melting points, FTIR, and 1HNMR spectroscopy of the compounds are used to describe them. POM (polarized optical microscopy) was used to investigate the mesomorphic behavior of the material.

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ARTICLE / INVESTIGACIÓN

Comparison between immune-chromatography (ICT) and ELISA techniques for detection of anti-HAV antibodies among patients suspected to be infected with Hepatitis A virus (HAV)

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Abstract: Infection with Hepatitis A virus (HAV) is a significant cause of acute hepatitis, posing an important public health problem worldwide. This study aimed to compare the efficiency of the immune-chromatography test and the enzyme-linked immunosorbent assay (ELISA) in detecting infection with hepatitis A virus (HAV). For this purpose, 5 ml of blood was collected from 176 patients (92 males and 84 females) who attended the virology unit at Kirkuk General Hospital and the serological examination unit at the pediatric hospital, as well as private clinics in Kirkuk city for the presence of clinical signs or complaint directed towards infection doubt with hepatitis A virus (HAV), during the period between March 2020 and December 2021. The seropositive rate of anti –Hepatitis A immunoglobulin M antibody was 46(26.13%) of total patients of the study, while the ratio of the seronegative of HAV was 90(51.13%). The percentage of false-positive and false-negative results by immune-chromatography (ICT) was 34(19.38%) and 6(3.4%). These findings suggest that the fast test (ICT) is not a suitable screening test for detecting specific antibodies to the hepatitis A virus due to the high frequency of false-negative results caused by the test's low sensitivity and negative predictive value. This emphasizes employing ELISA or PCR methods to confirm hepatitis A virus diagnosis in public hospitals. Although ICTs are cheap, they alone are not useful in diagnosing and deciding treatment for patients infected with Hepatitis A virus, so it is recommended to use the ELISA test to confirm the diagnosis of infection and identify the Hepatitis A virus in public hospitals, healthcare units and blood transfusion centers.

Key words: Hepatitis A virus, Seroprevalence, immuno-chromatographic test, ELISA test, IgM.

Introduction

Hepatitis is a significant health problem in both developing and developed countries. An estimated 1.4 million clinical cases of hepatitis A occur worldwide every year[1]. Hepatitis A is an acute, necro-inflammatory liver infection caused by the hepatitis A virus (HAV)[2]. Hepatitis A virus (HAV), a small, non-enveloped with a single-stranded RNA genome, belongs to the Hepatovirus genus and is a member of the Picornaviridae family[3,4]. Hepatitis A virus is acquired during early childhood. It is transmitted by a fecal-oral method which happens either by direct contact with feces or indirectly by ingestion of food or water contaminated by fecal matter of the infected persons[5]. The clinical course of HAV infection is age-dependent and ranges from asymptomatic (commonly in children aged ≤ 5 years) to acute symptomatic hepatitis, with higher severity of disease in the older population[6]. The common symptoms of hepatitis infection include fever, nausea, vomiting, anorexia, general fatigue, and abdominal pain where the liver is located. Jaundice, leading to change the urine color to dark orange and stool to light yellow or similar to clay color[7,8].

Accurate detection of the virus is essential for controlling the transmission of the virus; for this reason, it is necessary to validate detection methods before allowing their use in diagnostic laboratories[9]. Different ways are used to diagnose hepatitis, including ICT, ELISA, and PCR. In several developing countries, rapid diagnostic tests such as ICT are widely used to detect hepatitis B and C virus for diagnosing and screening acute and chronic infections[9]. The current study aimed to determine the seroprevalence of HAV and compare ICT and ELISA in detecting cases of HAV, in addition to determining the percentage of a false positive and false negative in ICT tests through a statistical study of these results.

Materials and methods

A total of 176 patients of both sexes (92 males and 84 females) were suspected of being infected with hepatitis A virus from those attending Kirkuk General Hospital (virus unit), Pediatric Hospital (serology unit) and specialized outpatient clinics in Kirkuk-Iraq for the period from March 2020 to December 2021. 5 ml venous blood was collected from patients in dry plane tubes, then centrifuged at 3000 rpm for 3 minutes to obtain serum kept in Eppendorf tubes at -20°c. Patients whose ages ranged between (1-60) years were screened for HAV using Immuno-chromatographic (ICT) kit (CTK Biotech Inc: USA) and ELISA (WANTAI Bio-Pharm:

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China) technique according to manufacturer instructions.

Statistical analysis

Data was analyzed Statistically using SPPS version 20. The Chi-square test was used to find the difference between study variables (ages and sex). The p-value is less than 0.01were considered significant.

Results and discussion

Table (1) show the ratio of the prevalence of anti-HAV-IgM in study patients by using both tests (ICT and ELISA); out of the total 176 study cases, 46 (26.13%) were the seropositive case (ICT positive / ELISA positive), 90 (51.13%) were the seronegative case (ICT negative/ELISA negative), and 6 (3.4%) cases were false seronegative (ICT negative / ELISA positive). Finally, 34 (19.38%) subjects were false seropositive in the ICT test (ICT positive / ELISA negative).

Both ICT and ELISA tests were conducted on the study cases to show HAV prevalence in different age groups. The results in a table (2) showed that the infection rate was higher in the age group (1-10) years, reaching 30(17.04%) with a false seronegative rate of 6(3.4%) in the ICT test and false positivity at 18(10.23%), meaning that the total seropositive rate amounted to (20.44%) compared to the infection rate in the age group (11-20) years, which amounted to (9.09%) and the seropositive rate false 6(3.41%). The statistical analysis results showed significant differences between the age groups at a p-value <0.01, while no cases of infection were recorded in the older age groups.

The prevalence of hepatitis A virus (HAV) infection and its relationship with sex were also studied using both tests. The results in the table (3) showed that the prevalence of HAV infection was higher in female patients, 24(13.6%) of the total study patients (with a rate of false seronegative 2(1.14%) in the ICT test), compared to 22(12.5%) in male patients (with a rate of false seronegative 4(2.3%).

Hepatitis A viruses of different types spread very quickly in the community, so the availability of appropriate screening techniques to detect possible infection with these viruses in public hospitals can contribute significantly to the promotion of these viruses, as the confirmation of the diagnosis of hepatitis virus infection based on several immunological, molecular and histological tests. Immunological techniques to detect specific antibodies in serum include enzyme-linked immunosorbent assay (ELISA) test and rapid methods. These methods are inexpensive and available compared to molecular and histological methods that are very expensive and unavailable, especially in economically poor countries[10].

The current study showed that the prevalence rate of HAV in the study patients was (26.13%) in the ICT test, compared to (29.53%), in the ELISA test, which indicates that (3.4%) of the study patients were negative in the ICT test appeared optimistic in the ELISA test. These results show that rapid tests such as ICT that are used to detect antibodies against hepatitis A virus in serum are not considered ideal screening tests because they do not predict the accurate picture of hepatitis A virus prevalence due to the high rates of false seronegative results due to the low sensitivity and specificity of these tests compared to ELISA test, which has high sensitivity and specificity[11,12].

Hepatitis A virus infection is detected in some hospitals by using rapid tests. However, those tests are sensitive and

Total number of Study group	ELISA/ICT -/-		ELISA/ICT +/+		ELISA/ICT +/-		ELISA/ICT -/+		Total	
	N	%	N	%	N	%	N	%	N	%
176	90	51.13	46	26.13	6	3.4	34	19.38	176	100

Table 1. The prevalence rate of Anti-HAV-IgM among stupatients using ICT and ELISA.

Age group	Total number		ELISA/ICT -/-		ELISA/ICT +/+		ELISA/ICT +/-		ELISA/ICT -/+	
	N	%	N	%	N	%	N	%	N	%
(1-10)year	102	58	48	27.27	30	17.04	6	3.4	18	10.23
(11-20)year	36	20	14	7.95	16	9.09	0	0	6	3.41
(21-30)year	14	8	10	5.68	0	0	0	0	4	2.3
(31-40)year	10	6	10	5.68	0	0	0	0	0	0
(41-50)year	4	2	2	1.14	0	0	0	0	2	1.14
(51-60)year	10	6	6	3.41	0	0	0	0	4	2.3
Total	176	100	90	51.13	46	26.1	6	3.4	34	19.38
			** X^2=15.019 P = 0.010		** X^2=20.263 P = 0.001		ns X^2=4.266 P = 0.312		* X^2=8.669 P = 0.036	
			** Chi-Square = 33.653 P-Value = 0.004							

Table 2. The prevalence rate of anti-HAV-IgM among patients according to age group using ICT and ELISA.

Sex	Total number		ELISA/ICT -/-		ELISA/ICT +/+		ELISA/ICT +/-		ELISA/ICT -/+	
	N	%	N	%	N	%	N	%	N	%
Male	94	52	50	28.1	22	12.5	4	2.3	18	10.23
Female	82	48	40	22.73	24	13.6	2	1.14	16	9.1
Total	176	100	90	50.83	46	26.1	6	3.44	34	19.33
			ns x^2=0.796 P = 0.372		Ns x^2= 1.932 P = 0.165		ns x^2= 0.516 P = 0.473		Ns x^2 =0.008 P = 0.931	
			Ns Chi-Square = 2.319 P-Value = 0.509							

Table 3. Seroprevalence rate of anti-HAV-IgM among patients according to sex group using ICT and ELISA.

specific. The possibility of some false negative or positive results cannot be ignored, as was proven in the results of the current study, which reinforces the necessity of using the ELISA test for its superiority in terms of sensitivity and specificity over the ICT test in investigating the spread of HAV cases in public hospitals.

False-negative and positive results were evaluated in the ICT test, as the current study indicated that the percentage of false-negative results in the ICT test was large (3.4%). This could be attributed to a decrease in sensitivity caused by the difficulty of detecting antibodies with low concentrations and poor affinity; compared to ELISA, the current study results agreed with other studies in the world[10,13-15].

Conclusions

Although ICTs are cheap, they alone do not help diagnose and decide treatment for patients infected with Hepatitis A virus, so it is recommended to use the ELISA test to confirm the diagnosis of infection and identify the HAV in public hospitals healthcare units and blood transfusion centers.

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ARTICLE / INVESTIGACIÓN

Biological activities of Ethanolic Extract Produced by *Cucurbita pepo* plant

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Abstract: Antibacterial and antioxidant effects of alcoholic extract for the *Cucurbita pepo* plant were studied. Properties optical (UV-visible spectrophotometer) and morphological surface(scanning electron microscopy) detection of the alcoholic extract was done, and the antibacterial activity was investigated against pathogenic microbes (*E.coli*, *Staphylococcus aureus*). Results showed the highest effect for crud extract in the growth of *E.coli* by the zone of inhibition diameter reached (16.33±0.58), followed by Staph. Aureus by the diameter of the inhibition zone reached (12.33±2.30mm). The results indicate that the plant C. pepo can be considered a valuable source of effective antioxidant agents at a 0.8 µg/ml concentration.

Key words: Cucurbita pepo plant, alcoholic extract, antioxidant, antibacterial activity.

Introduction

Herbal medicine has long been utilized in India and China as a less expensive means of treating various health problems[1], and it is still the principal therapy for treating infections in several underdeveloped nations[2]. When compared to synthetic drugs, herbal remedies are frequently less toxic and have fewer adverse effects. The World Health Organization has also advised that research be launched to identify and describe novel herbal remedies derived from historically recognized plants and create new effective therapeutic agents, particularly in places where modern, safe pharmaceuticals are lacking. For the treatment of long-term illnesses[3].

Aromatic herbs are commonly used as nutritional supplements[4], and they are treasured as a source of natural antioxidants[5]. Because of the rising safety issues linked with the intake of synthetic antioxidants, it's more crucial than ever to seek cheaper and safer antioxidants from natural sources, mainly plants[6]. *C. pepo*, a native squash plant, is one of the 15 species of Cucurbita genus in the Cucurbitaceae family[7]. Gourds are utilized for various aesthetic and traditional purposes, while pepo vines and fruit are used as cattle feed[8,9]. *C. pepo* is a superb protein, carbohydrate, mineral, and fat supplement[10]. The cucurbits family, often known as cucurbits, is a vast collection of plants that may be cultivated in warmer climates worldwide and yield popular food crop plants. Some of these kinds are squash, gourd, watermelon, and gourd are some of these varieties. One of the first known cultivated species is the peppo squash. Fruits that are not fully ripe are eaten as vegetables. Sweet, ripe fruit is utilized in sweets and roasted or boiled beverages[11]. This study aims to determine some biological activities of the *C. pepo* plant.

Materials and methods

Extract Preparation

The extraction was done by Soxhlet through 75% ethanol; then the solvent was removed using a rotary evaporator, then transferred to the oven to dry and stored at 4 ℃ until used[12].

Phytochemical Screening of C. pepo extract

The crude extract screening for the determination of phytoconstituents[13].

Characterization *C. pepo* extract.

UV-Visible and SEM

UV-Vis absorption spectra were employed for the optical test of C. pepo extract at various preparation conditions within range (365-540 nm); the solution was measured by UV-Vis double beam astrophotometers SP-3000 plus (CE OPTMA TOKYO). All spectra were measured at room temperature. The size and shape of *C. pepo* extract were passed out by (SEM), and were applied in the University of technology-Iraq.

Antioxidant activity

Antioxidant activity of C. pepo was studied by DPPH assay with modified according to (13), ten µl of the sample was mixed up with 490 µl of DPPH and finally added 500 ethanol. The equation formula examined the optical density (O.D.) was examined by 517 nm by the equation formula[13,16].

$$\text{Antioxidant activity \%} = \frac{^{OD}\text{control} - ^{OD}\text{sample}}{^{OD}\text{control}} \times 100$$

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OD= Optical density

Preparation of bacterial isolates

Microbial isolates were taken from the Biotechnology department at the University of technology. The microbes were grown overnight at 37 ℃ on nutrient broth to prepare the suspensions cell to 0.5 McFarland standards (1 × 105 CFU/mL)[16].

Antimicrobial activity

Isolates were obtained from the Biotechnology Branch/ Applied Science Department at the University of Technology. The diffusion agar method was used to detect antimicrobial activity. The inhibition zone was measured around each well, compared with the control, and conducted in triplicate[12,13,16].

Results

The UV-visible spectroscopy was used to check the formation alcohol extract of C. pepo. The surface of the alcohol extract band at 390 nm (Fig. 1).

SEM assay

SEM technique was employed to visualize the shape and size of the ethanolic extract by *C. pepo* plant shown in figure 2.

Phytochemical Screening of extract

The result of the Phytochemical Screening of plant extract ware dark green color suggests the presence of phenolics and tannin, respectively. Yellow to colorless (flavonoids), volatile oil by yellow precipitate, reddish-brown alkaloids, flavonoids by yellow precipitate is indicated by the formation of a precipitate, such as yellow precipitate, the presence of saponins is indicated by the persistence of frothing. While Glucosides, Steroids, and Terpenoids were absent in Ethanolic extract (Table 1).

Antioxidant activity of C. pepo plant

The DPPH was found to be proportional to the increase in concentration. The free radicals were given 56.50%, 62.42%.,66.12%,74.33% by the concentration of 20,40,60 and 80 µg/ml respectively in figure.3.

Antibacterial activity

C. pepo extract effect was studied against two types of pathogenic microbes. The effects of the ethanolic extract varied on pathogenic microbes' growth. The highest effect observed on the growth of *E. coli* by a zone of inhibition reached (16±0.13mm), followed by *S. aureus* (15±0.58 mm) (Figure 4, and Table 2).

Discussion

C. pepo extract exhibited antibacterial action related to Gram-negative and Gram-positive bacteria[13]. The activity of *C. pepo* extract against bacteria was credited to phytochemical materials like Flavonoids, phenolics, alkaloids, and tannins which affect the cell wall bacteria[14]. Antibacterial activity differed depending on the cell wall structure of G +ve and G-ve bacteria[15,18], which leads to the destruction of bacteria[16,18]. In-creased phenolic elements, such as flavonoids, phenolic diterpenes, and phenolic acids, increased DPPH radical scavenging activity. Phenols, Flavonoids, and Alkaloids isolated from leaves of plants decreased the oxidant action[17].

Conclusions

C. pepo plants have wide phytochemical compounded and clinical investigations. Scientific studies have shown most of the claims of traditional medicines through has antibacterial activity against G+ve and G-ve bacteria, the ability of the extract to inhibitor 75%, and antioxidants.

Funding

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Informed Consent Statement

Written informed consent has been obtained from the biotechnology department and biomedical engineering at the University of technology.

Data Availability Statement

The results might be achieved using an evaluation software or assessment table, available at www.diagnostics.sk/idmicro.

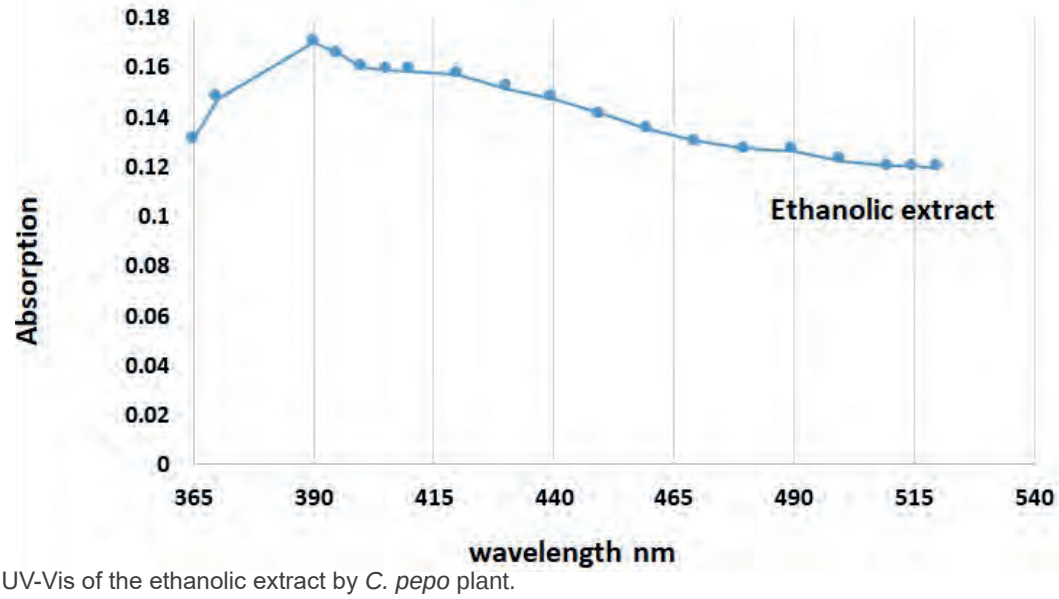


Figure 1. UV-Vis of the ethanolic extract by *C. pepo* plant.

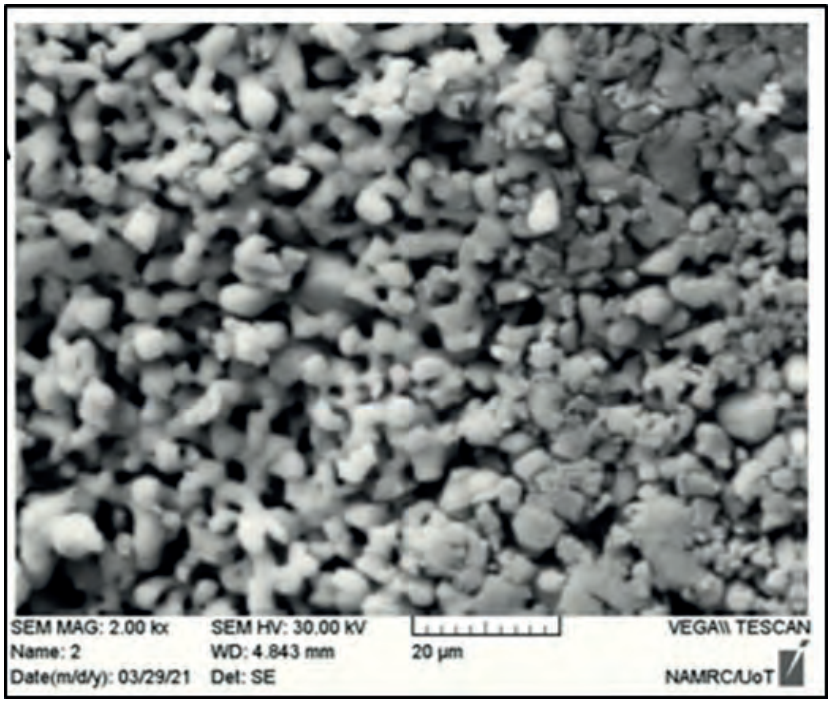


Figure 2. SEM of the ethanolic extract by *C. pepo* plant.

Constituent	Test
Tannins	**Presence**
Saponins	Presence
Phenol	Presence
Alkaloids Wagner's test	Presence
Flavonoids Ferric chloride test	Presence
Volatile oil	Presence
Glucosides	Absent
Steroids	Absent
Terpenoids	Absent
Comarines	Presence

Table 1. Phytochemical Screening of *C. pepo* plant.

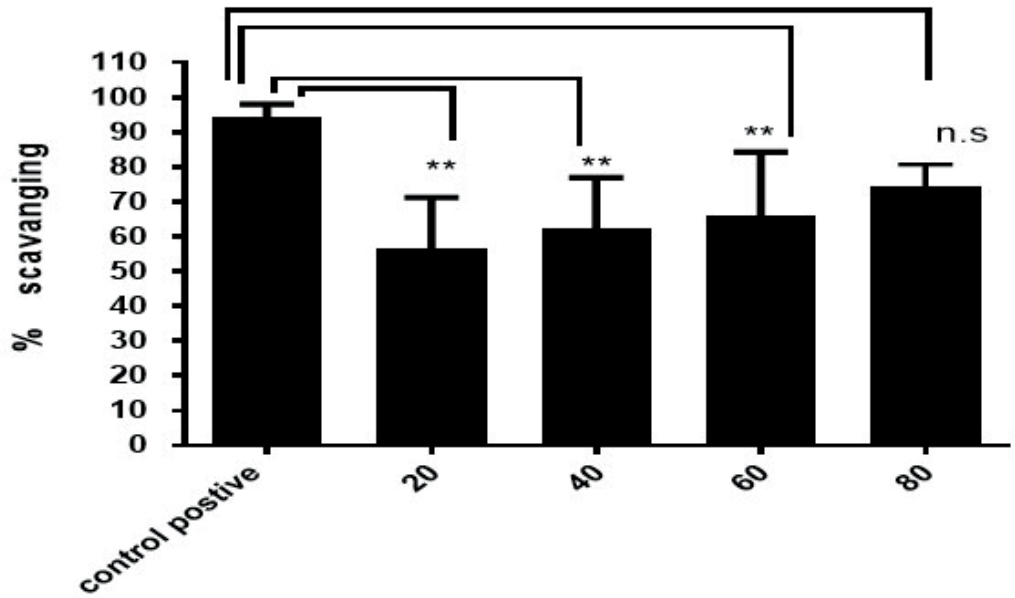


Figure 3. DPPH radical scavenging of *C. pepo* plant.

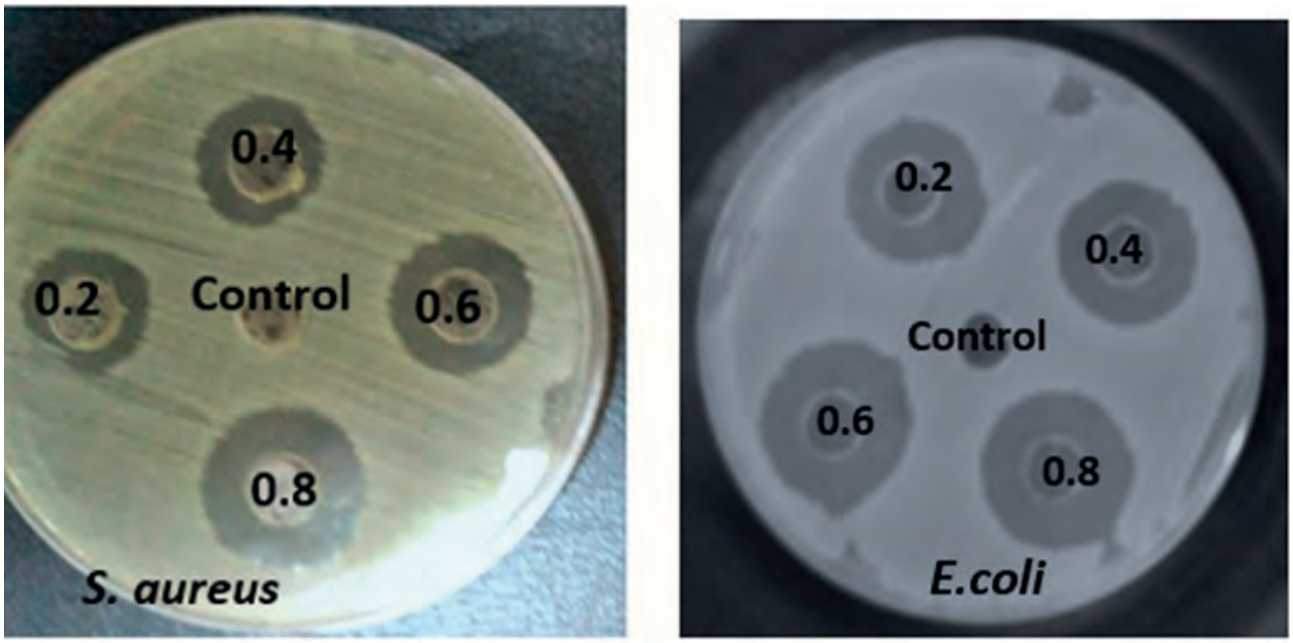


Figure 4. Antibacterial activity of ethanolic extract toward. *E.coli*, *S. aureus*.

Microorganism pathogenic	Concentrations µg/ml				
	0.2	0.4	0.6	0.8	Control
S. aureus	7±0.58	9±2.30	12±1.15	15±0.58	-
E.coli	10±0.58	12±0.02	14±0.23	16±0.13	-

Table 2. The effect of ethanolic extract against some pathogenic.

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Conflicts of Interest

None.

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ARTICLE / INVESTIGACIÓN

Efficacy of Bio-fertilizer and Chemical Fertilization on Flavonoids Distribution in Different Plant Parts of *Stevia rebaudiana* (Bertoni.)

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Abstract: This study aims to investigate the effect of the biological and chemical fertilizers on the content of the flavonoid compounds distributed within the different plant parts (leaves, stems, branches, and roots) of *Stevia rebaudiana* (Bertoni.) grown in Iraq. The results showed that the treatments of the biological fertilizers, including Mycorrhiza (C2) achieved the highest content of the most flavonoids in different parts of the plant. The treatment C2 recorded a rise of the flavonoid compounds Naringin, Naringenin and Luteolin 7-glucose in the leaves, Naringin, Rutin, and Acacetin7-neorutinoside in the stems and branches, and Apiening6-rhamnose8- glucose, Apigenin7-o neohespiroside, Kampferol3-7dirmmoside, Quercetrin, Narengenin, Acacetin7-neorutinoside, Kampferol, and Luteolin 7-glucose in the roots. On the other hand, treatment C1 recorded the highest content of Quercetin in the leaves, Quercetrin3-O glucose in the stems and branches, and Quercetrin3-O glucose, Naringenin, and Acacetin7-neorutinoside in the leaves .

Key words: Flavonoid, Stevia, Mycorrhiza and chemical Fertilization.

Introduction

Flavonoid compounds are among the most significant secondary metabolic compounds naturally produced by plants. They consist of more than 1000 structurally varied compounds[1,2]. Half of the flavonoid compounds are accumulated in the plant vacuoles as a form of aglycone, glycosides, and methylated derivatives. Some flavonoids are released from roots to the aria surrounding the roots, known as rhizospheres. Some flavonoids can release from roots and make biological contact with the microorganism in the rhizospheres and establish a symbiotic relationship with them, such as rhizobia, arbuscular mycorrhizal fungi, and plant growth-promoting rhizobacteria, Pathogens, and nematodes in addition to some other plants. This has been confirmed by finding aglycones and glycosides in the flavonoid compounds, the root extract, and the soil containing these organisms[3-5]. *Stevia rebaudiana* is classified among the perennial herbaceous plants of the Asteraceae family. It is a medicinal and nutritional plant that has gained particular nutrient and healthy importance in terms of nutrition and health for its leaves containing Steviol Glycosides that led to using the plant leaves as calorie-free food additives in Japan, Brazil and Europe[6,7].Containing a group of flavonoid compounds essential for the human body as antioxidants that have a role in protecting against cancer, diabetes and diseases of cardiovascular and kidney, increased the importance of Stevia as a medicinal plant; furthermore, it is considered a fungal and bacterial antibiotic and it is used as a diuretic as well as it has many other benefits[8,9]. Creating biologically activated compounds in plants is affected by various environmental and agricultural factors, including fertilizers such as NPK[10]. Flavonoid contents in plant tissues of plant parts were increased when the chemical fertilizers were used while other research proved the revers[11-15]. 80% of plants can form a symbiotic relationship with the mycorrhiza fungus[16]. Mycorrhiza is one of the bio-fertilizers characterized by the capability to increase the secondary metabolism compounds in plants, and 80% of plants have the ability to form a symbiotic relationship with the mycorrhizal fungus[16,17]. Adding Mycorrhiza to medicinal plant roots changed primary metabolism processes, including photosynthesis, water absorption, and plant tolerance to drought[18]. In addition, the changes in secondary metabolism compounds such as the change in the dynamics of plant hormones and the structural manipulation and activating the defensive mechanism[19].The reason was attributed to the excellent compatibility between the Mycorrhiza and the host plant, As well as the nutritional status of the plant. This increased the plant content of secondary metabolites such as phenols and total flavonoids[20].

Due to the nutritional and medicinal importance of the Stevia plant and the absence of studies about it in Iraq, this research aims to probe the effect of the chemical and biological fertilizers on the content of flavonoid compounds in different parts of the Stevia grown in Iraq.

Materials and methods

The experiment was conducted in the greenhouse at research station B affiliated with the Department of Horti-

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culture and Garden Engineering, College of Agricultural Engineering Sciences, the University of Baghdad during the period from 1/3/2017 to 15/7/2017. The design Randomized Complete Block Design (RCBD) was used to lay out this experiment with three replicates. Each replicate contained 10 plants 6 weeks old, knowing that the seedlings had been produced through the tissue culture and been adapted previously. The experiment treatment included: Control treatment: without applying any fertilizer. C0 symbolizes it. Chemical fertilizer treatment: according to the NPK fertilization recommended[21] where the fertilizer contains the combination of 30 N: 10P: 10K. The treatment was symbolized by C1. Biological fertilizer treatment: the *Glomus mosssaea* was added at 15 g .plant^{-1} in the pits in contact to the seedling roots[22] and 250 g.plant^{-1} of sterilized Peat moss[23]. The Mycorrhizae were obtained from the Ministry of Sciences and Technology, Department of Agricultural Research. It was loaded on the soil at 51 spores. g^{-1} soil. C2 symbolized the treatment. The seedlings were planted on terraces of 50 cm in width in a non-warmed greenhouse covered by polyethylene. The distance between plants from each other was 20 cm[24]. Inside the greenhouse was equipped with a thermometer and a hygrometer, also was equipped with non-woven polypropylene spun bounded (characterized by thickness GSM 17 and UV3%) was placed 2.5 m above the plants to decrease the temperature[25] (Table 1)

Estimate the concentration of the flavonoid compounds in different plant parts(roots, stems and leaves)

The flavonoids concentration was estimated with High-Performance Liquid Chromatography (HPLC) according to (26) included the following steps:

Extraction and separation of flavonoids

When leaves reached the full enlargement stage preceding the flowering and after the last batch of the soil NPK fertilization, samples including the leaves, stems, branches, and roots were collected separately after the first harvest in the evening in order to analyze the flavonoid compounds. The plant parts were dried at the room temperature of 25-30 C° until the weight stabile in a room equipped with an evacuator. The samples were ground using a special industrial grinder and kept in airtight paper bags. The bags were also placed inside sealed zipper freezer bags (type Falcon) for food preservation. Then, they were kept in a refrigerator under 4-5 C°. The sample extraction was prepared according to (26). The standard solutions of the compounds studied were imported from the Sigma- Aldrich Co.

The device conditions

All concentrations of flavonoid compounds in the Stevia plant were assessed at the National Agricultural Research Center laboratories - Food Technology Research Institute / Arab Republic of Egypt. The Chromatography approach

Inside the greenhouse					
Month	dayss	Maximum Temperature C°	Minimum Temperature C°	relative humidity %	Soil Temperature
March	1*	21.5	12.8	70.3	25
	2*	24.5	14.2	68.2	25
	3*	27.5	15.1	62.2	25
April	1*	32.6	16.5	58.9	26
	2	30.9	18.4	59.4	26
	3	31.6	19.9	39.9	26
May	1	33.8	20.9	38.1	26.5
	2	36.1	23.7	35.4	28
	3	35.2	26.1	36.7	27.5
June	1	38.1	27.3	35.1	28
	2	40.1	28.1	33.2	28.5

Table 1. Maximum and minimum temperature (C°) and relative humidity (%) as well as the soil temperature inside the greenhouse. * (1) The average of the first ten days of the month .* (2) The average of the second ten days of the month.* (3) The average of the last ten to eleven days of the month.

was used to estimate the compounds of flavonoids studied . HPLC device (type Agilent, model 1200) was used for determining the retention time and area of the standard and sample solutions. The separation column type C18 (4.5 x 250 mm) was used, and the movable phase acetonitrile: Phosphoric acid was pushed (at a Flow rate 14: 80 ml. min^{-1}). The results were measured at the wavelength of 330 nm and a temperature of 35C°. Next, the measurements of the compounds of the package space of the samples were compared to those the standards to recognize the compounds Apiening6-rhamnose-glucose, Naringin, Rutin, Quercetrin 3-O glucose, Apigenin7-o-neohespiroside, Kampferol3-7dirmmoside, Quercetrin, Quercetin, Naringenin, Acacetin7-neorutinoside, Hespirtine, Kampferol, Apeginen, and Luteolin 7-glucose according to the approach followed by (26).

Statistical analysis

The experiment was designed using the Randomized Complete Block Design (RCBD) with three replicates for each treatment[27]. Genastat software was used to compare the means relying on the L.S.D 5 %.

Results

Effect of the chemical and biological fertilizers on the flavonoid content in Stevia plant leaves (ppm)

Results in Table 2 show non -significant effect of the chemical fertilizer treatment C_1 and the biological fertilizer treatment C_2 compared to the control treatment on the compounds Apiening6-rhamnose8-glucose,Rutin,Quercetrin 3-O glucose, Apigenin 7 glucose, Apigenin7-o- neohespiroside, Kampferol3-7dirmmoside, Acacetin7-neorutinoside, Hespirtine, Kampferol, and Apeginen. The treatment of the biological fertilizer C_2 showed superiority in the leaf content of the flavonoid compound Naringin reached 1669.7 ppm compared to the C_1 and C_0 (1430.5 and 978.6 ppm), respectively. The treatment C_2 was superior in the content of Narengen in the Stevia plant leaves (32.6 ppm) compared to C_0. (13.4 ppm) . The treatment C_2 also showed superiority in the plant leaf content of Luteolin 7-glucose (5670.5 ppm) compared to C_1 and C_0 (1562.3 and 1665.0 ppm, respectively). The treatment C_1 recorded the highest value of Quercetin in the leaves (58.6 ppm) compared to the treatments of C_2 and C_0 (39.77 and 34.84 ppm, respectively).

Effect of bio and chemical fertilizers on the flavonoid compounds content of *S. rebaudiana* (Bertoni.) leaves (ppm) Table 2

Effect of bio and chemical fertilizers on the flavonoid compounds content in the stems and branches of Stevia plants

Results in Table 3 illustrate that the treatments C1 and C2 were non-significant on the content of the compounds Apiening6-rhamnose8-glucose, Apigenin7-o-neohespiroside, Kampferol3-7dirmmoside, Quercetrin, Narengenin, Hespirtine, Kampferol, Apeginen, and Luteolin 7-glucose in the stems and branches of Stevia plant. The treatment of C2 recorded an increase in the Naringin content in the stems

Treatment / Flavonoids compounds content	C0	C1	C2	LSD 5%
Apiening6-rhamnose8- glucose	519.5	561.2	598.5	N.S
Naringin	978.6	1430.5	1669.7	39.91
Rutin	4763.9	5764.1	10153.3	N.S up normal
Quercetrin3-O glucose	-	-	-	-
Apigenin 7 glucose	291.0	439.0	346.3	N.S up normal
Apigenin7-o neohespiroside	386.1	420.0	415.1	N.S up normal
Kampferol3-7dirmmoside	173.2	205.0	145.1	N.S up normal
Quercetrin	743.0	1114.1	1116.2	N.S up normal
Quercetin	34.84	58.63	39.77	6.63
Narengenin	13.4	26.4	32.6	16.49
Acacetin7-neorutinoside	165.1	242.4	268.1	N.S up normal
Hespirtine	66.0	68.1	83.0	N.S up normal
Kampferol	74.0	96.4	108.2	N.S up normal
Apeginen	19.1	32.4	23.9	N.S up normal
Luteolin 7-glucose	1665.0	1562.3	5670.5	483.1

C0:control treatment,C1: chemical fertilizer treatment and C2: Biological fertilizer treatment

Table 2. Effect of bio and chemical fertilizers on the flavonoid compounds content of *S. rebaudiana* (Bertoni.) leaves (ppm)

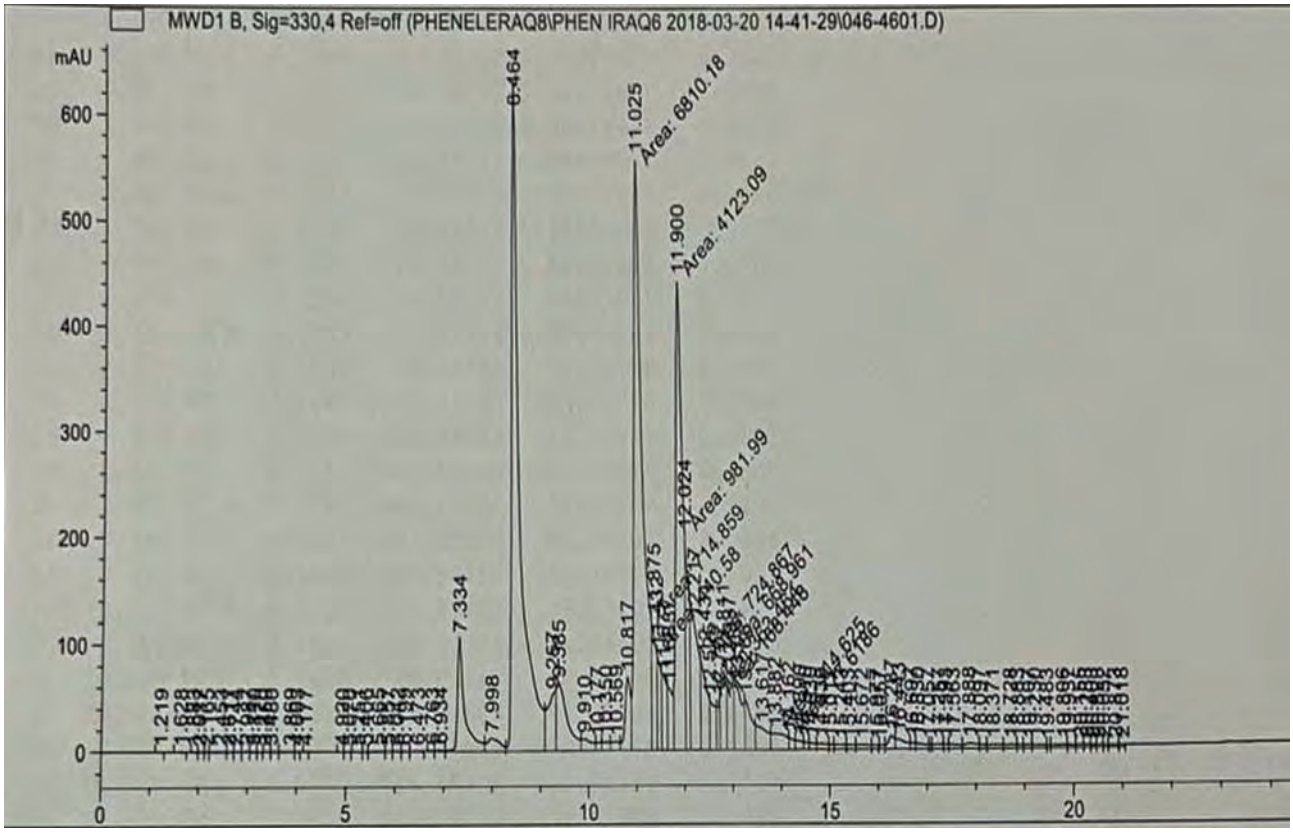


Figure 1. The flavonoid compounds content of *S. rebaudiana* (Bertoni.) leaves in cntrol treatmeant (ppm).

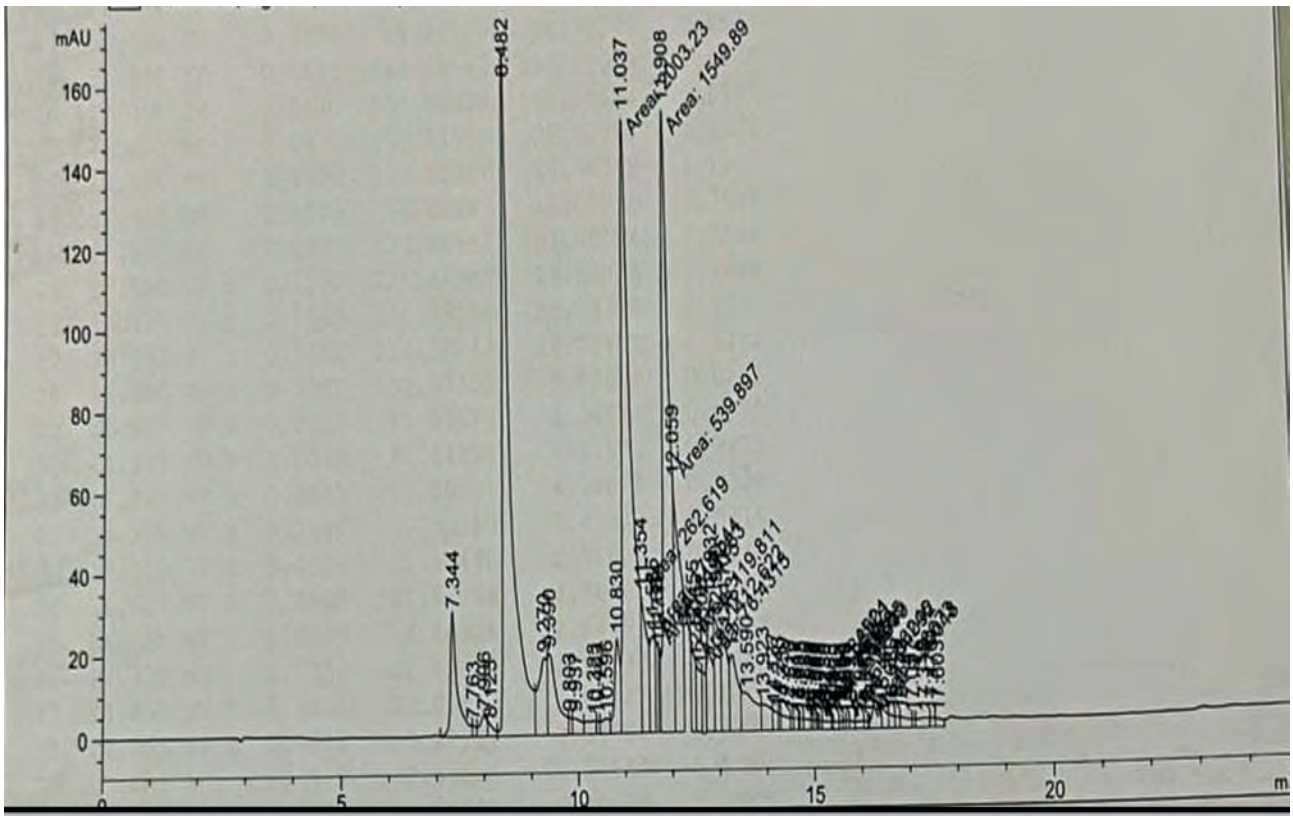


Figure 2. The flavonoid compounds content of *S. rebaudiana* (Bertoni.) leaves in chemical fertilizer treatmeant (ppm).

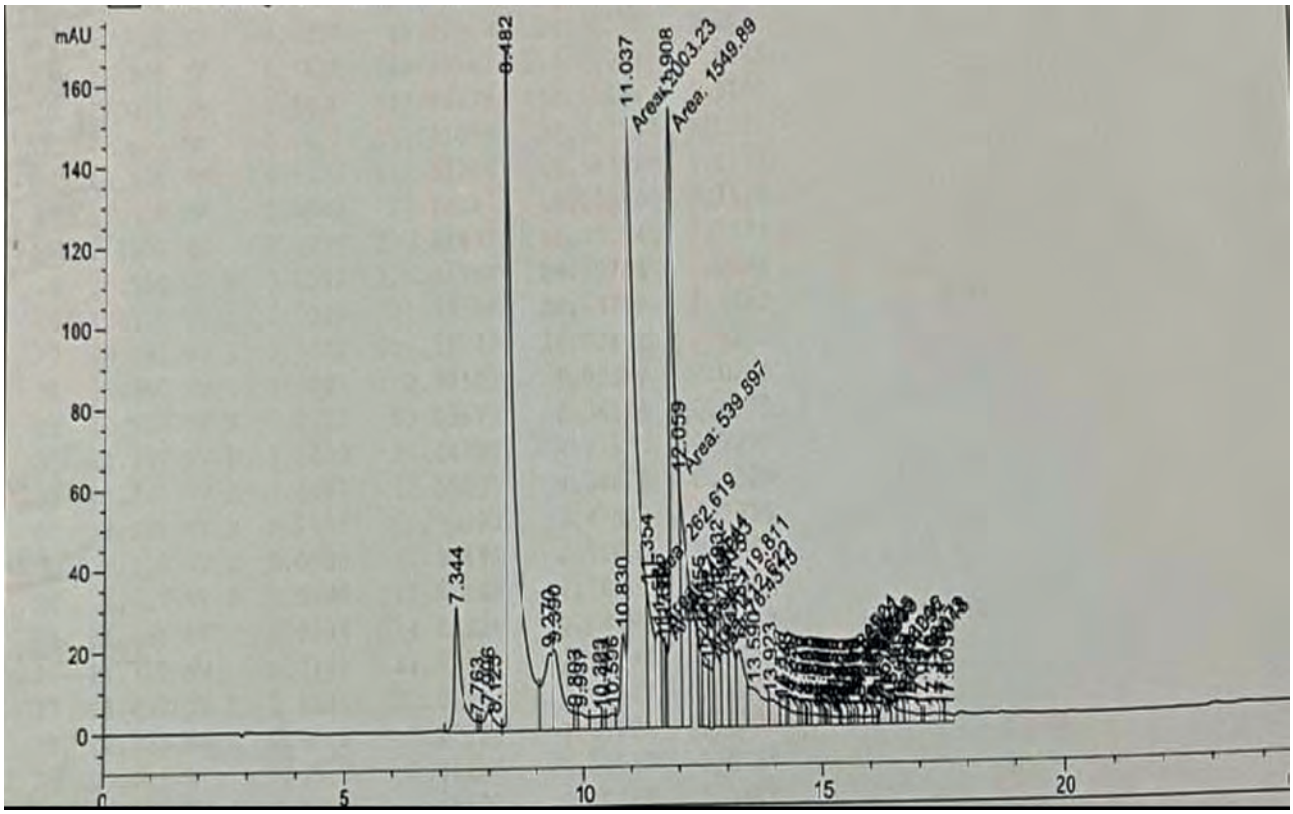


Figure 3. The flavonoid compounds content of *S. rebaudiana* (Bertoni.)leaves in Biological fertilizer treatment fertilizer (ppm).

Treatment / Flavonoids compounds content	C0	C1	C2	LSD 5%
Apiening6-rhamnose8- glucose	184.0	137.4	117.0	N.S up normal
Naringin	320.0	222.2	2175.4	1043.4
Rutin	272.4	611.4	1222.3	629.7
Quercetrin3-O glucose	11.0	18.1	0.0	14.51
Apigenin7-o neohespiroside	43.0	62.5	89.1	N.S up normal
Kampferol3-7dirmmoside	59.0	61.0	99.1	N.S up normal
Quercetrin	40.0	40.1	95.1	N.S up normal
Quercetin	4.6	10.3	8.6	N.S up normal
Narengenin	2.12	2.68	2.82	N.S up normal
Acacetin7-neorutinoside	28.9	33.8	80.3	13.00
Hespirtine	6.5	10.4	10.5	N.S up normal
Kampferol	5.2	5.3	8.2	N.S up normal
Apeginen	1.87	2.83	2.10	N.S up normal
Luteolin 7-glucose	337.1	193.0	239.0	N.S up normal

C0: control treatment, C1 : chemical fertilizer treatment and C2: Biological fertilizer treatment.

Table 3. Effect of bio and chemical fertilizers on the flavonoid content in the stems and branches of *S. rebaudiana* (Bertoni.) (ppm).

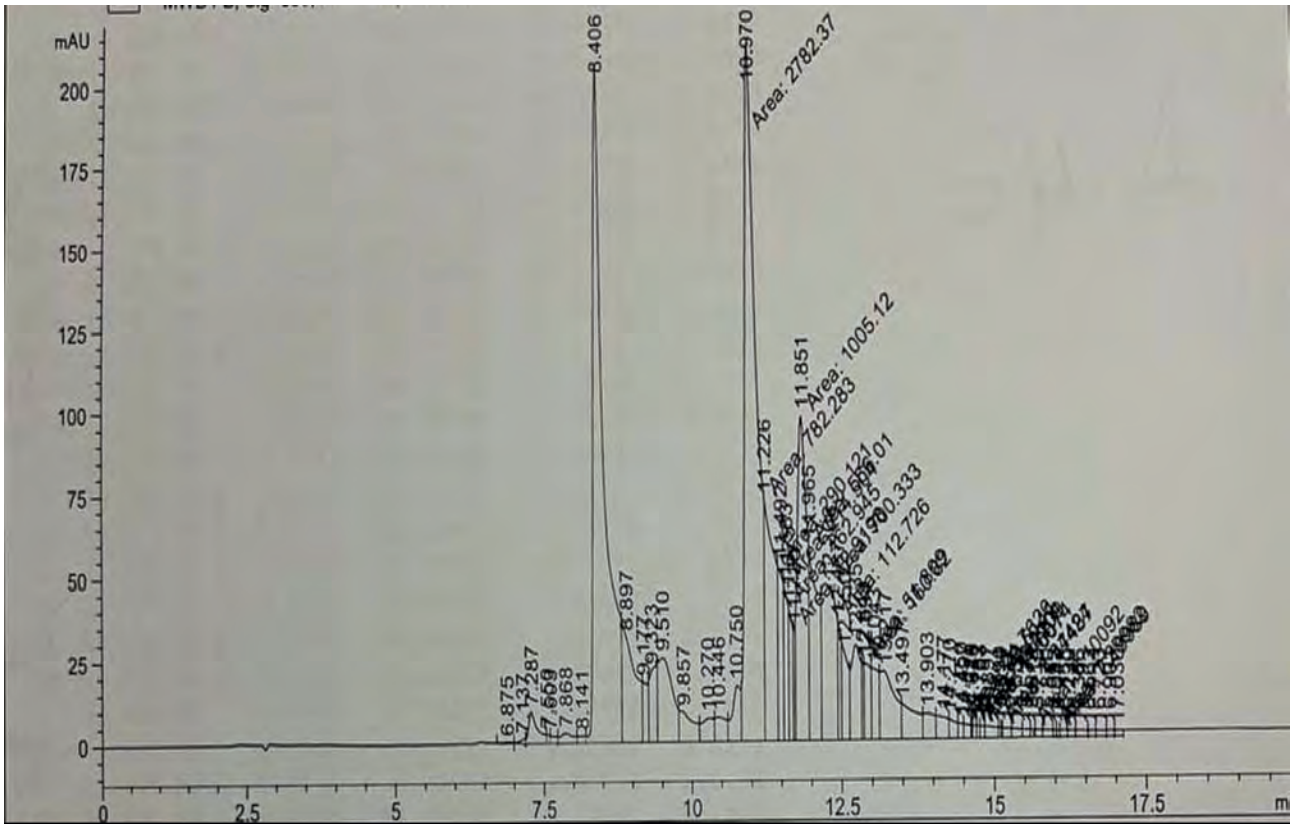


Figure 4. The flavonoid compounds content of *S. rebaudiana* (Bertoni.) stem and branches in cntrol treatmeant (ppm).

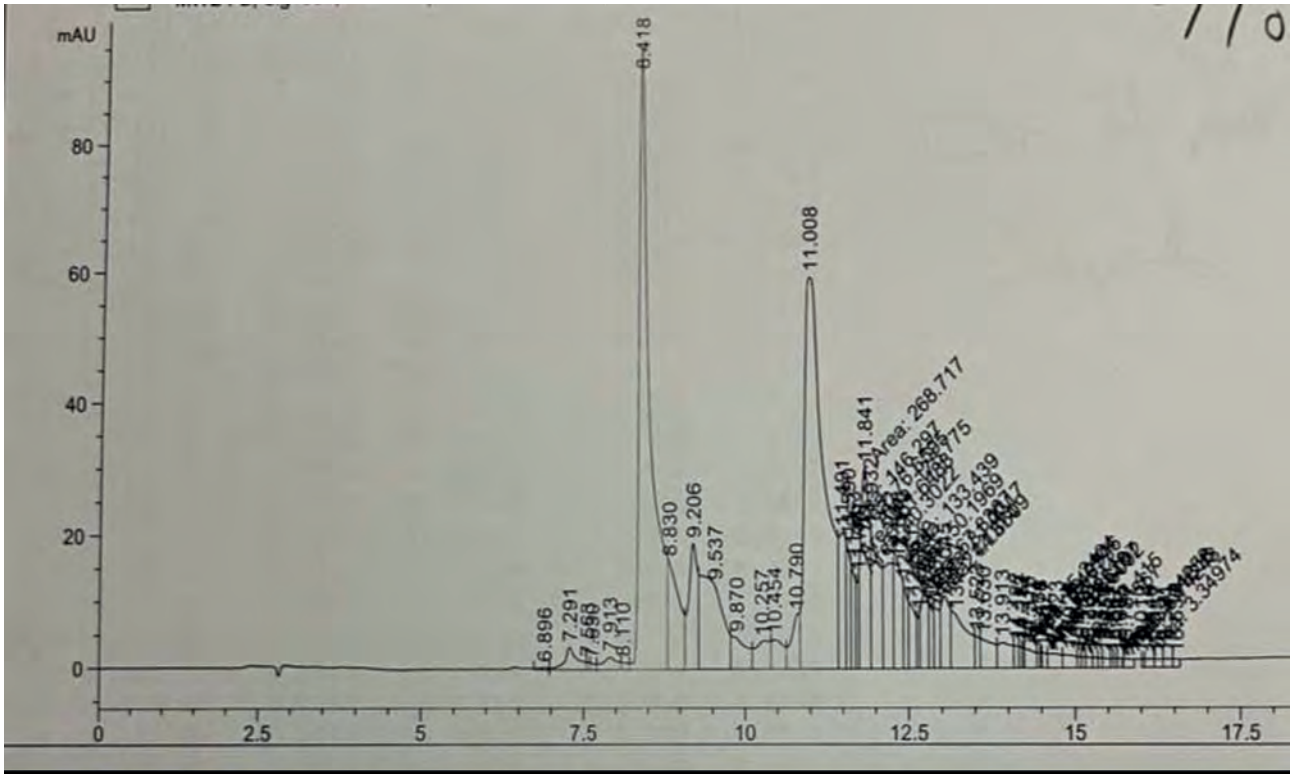


Figure 5. The flavonoid compounds content of *S.rebaudiana*(Bertoni.)stem and branches in chemical fertilizer treatmeant (ppm).

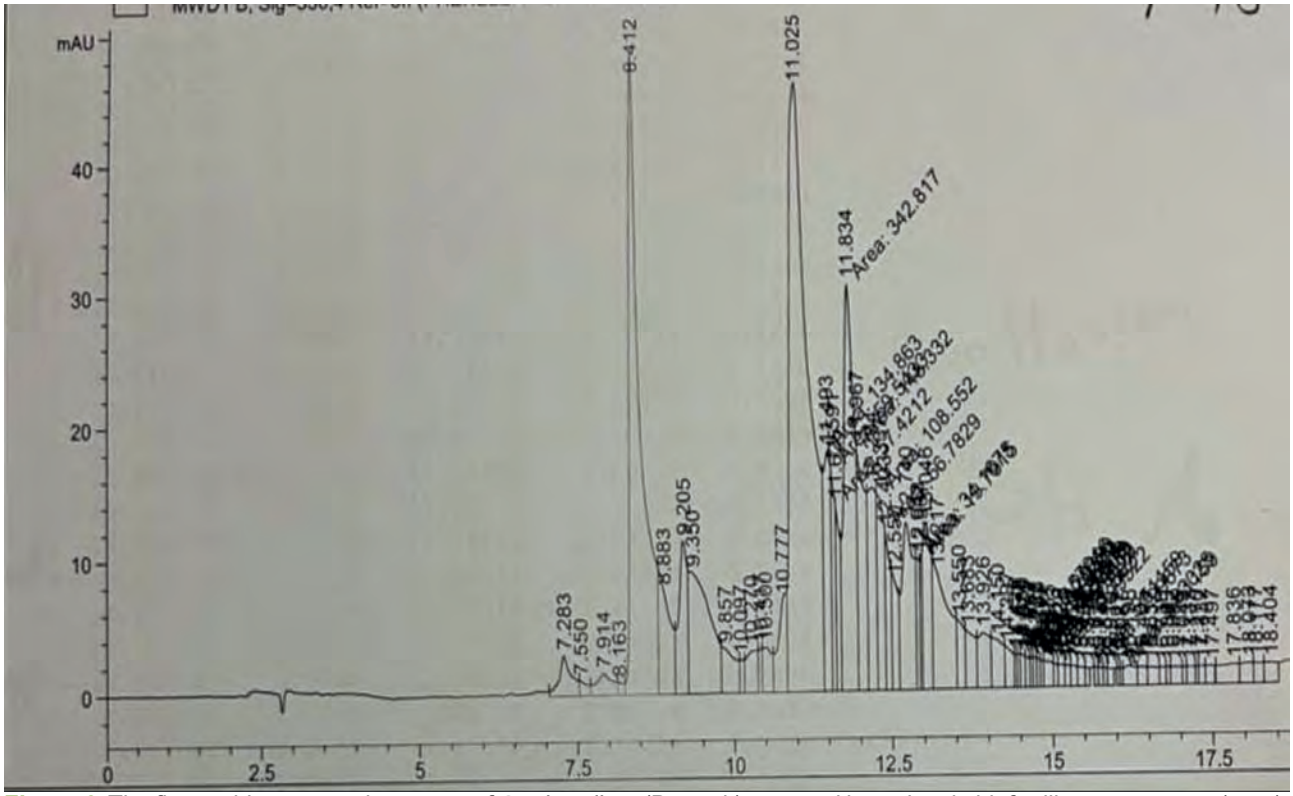


Figure 6. The flavonoid compounds content of *S.rebaudiana*(Bertoni.)stem and branches in biofertilizer treatmeant (ppm).

and branches (2175.4 ppm) compared to the treatments C0 and C1 (320.0 and 222.2 ppm respectively). The treatment C2 also recorded an increase in the Rutin content in the stems and branches (1222.3 ppm) compared to C0 (272.4 ppm). On the other hand, the two treatments, C1 and C0 recorded a noticeable increase of the compound Quercetrin3-O glucose content in the stems and branches to 18.1 and 11.0 ppm respectively. The C2 treatment was superior recording the highest content of Acacetin7-neorutinoside of 80.3 ppm compared to C1 and C0 which recorded 33.8 and 28.9 ppm respectively.

Effect of bio and chemical fertilizers on the flavonoid content in the roots of Stevia plants (ppm)

Results in Table 4 showed a non-insignificant difference among the study treatments in the content of the compounds Naringin, Rutin, Apigenin 7 glucose, Hespirtine, and Apeginen. However, treatment C2 recorded an increase in the content of Apiening6-rhamnose8- glucose compared to C1 and C0 which recorded 251.2 and 146.0 ppm, respectively. The treatment was also superior in the content of Apigenin7-o neohespiroside compared to C0 that recorded 40.9 ppm and recorded an increase in Quercetrin to 88.6 ppm compared to C1 and C0 (44.8 and 43.3 ppm, respectively). The treatment C2 recording 88.6 ppm also increased the Quercetrin content compared to C1 and C0 recording 44.8 and 43.3 ppm, respectively. Moreover, the treatment C2 had the highest in the content of Kampferol recording 15.8 ppm compared to C1 which recorded 6.2 ppm. The treatment C1 was superior in the content of Quercetrin 3-O glucose recording 75.8 ppm compared to C2 and C0 which recorded 30.4 and 3.80 ppm respectively. The superiority of the treatment C2 in the content of Luteolin 7-glucose was observed in comparison to C0 and C1 which recorded 948.2, 497.3, and 478.1 ppm respectively. The two treatments C2 and C1 were superior in the content of Acacetin7-neorutinoside recording 97.3 and 60.3 ppm respectively compared to C0 which recorded (35.0 ppm). Concerning the content of Narengenin, the two treatments C2 and C1 were superior recording 1.67 and 1.66 ppm respectively compared to C0 which recorded 0.71 ppm.

Discussion

The results illustrated in Tables 2, 3, and 4 showed a significant non-effect of the studied treatments on Hespirtine and Apeginen in all plant parts (leaves, stems, branches, and roots). Also the treatments did not significantly affect the content of Apiening6-rhamnose8- glucose, Apigenin 7 glucose, Kampferol3-7dirmmoside, Quercetrin, Hespirtine, Hespirtine, Kampferol, and Apeginen found in the leaves, stems, and branches. Moreover, they did not significantly affect the content of Apigenin 7 glucose and Rutin in the leaves and roots. Further, the content of Quercetrin and Acacetin7- neorutinoside in the leaves, and Quercetin, Naringenin, and Luteolin 7-glucose in the stems and branches were also not significantly affected.

The former results illustrated in Tables 2, 3, and 4 show an insignificant effect of the studied treatments on the content of the compounds this is due to the plant tissue content of the flavonoid compounds in any plant part was not affected by the use of any type of fertilizer[14,15]. Whether they are treated or not by the NPK fertilizers, the medicinal plants' flavonoids in the plant tissues are not affected significantly; as a result, each plant needs a certain critical NPK level for the synthesis and metabolism the flavonoid compounds[10]. Among the flavonoid compounds contained in all Stevia plant parts are Apiening6-rhamnose8- glucose, Apigenin 7 glucose, Kampferol3-7dirmmoside, Quercetrin, Hespirtine, Hespirtine, Kampferol, and Apeginenwhich are not affected significantly by the studied treatments. The treatment of

Treatment / Flavonoids compounds content	C0	C1	C2	LSD 5%
Apiening6- rhamnose8- glucose	146.0	251.2	506.4	N.S **up normal**
Naringin	561.0	563.1	983.1	N.S **up normal**
Rutin	92.0	219.3	247.1	33.26
Quercetrin3- O glucose	3.80	75.8	30.4	N.S **up normal**
Apigenin 7 glucose	15.5	24.1	25.6	18.52
Apigenin7- o neohespiroside	40.9	91.2	113.4	26.50
Kampferol3- 7dirmmoside	30.0	41.4	50.5	26.83
Quercetrin	43.3	44.8	88.6	0.75
Quercetin	3.59	5.50	4.50	0.20
Narengenin	0.71	1.66	1.67	42.14
Acacetin7- neorutinoside	35.0	60.3	97.3	N.S **up normal**
Hespirtine	13.3	13.8	14.1	9.08
Kampferol	12.3	6.2	15.8	N.S **up normal**
Apeginen	2.72	6.34	5.82	167.2
Luteolin 7- glucose	497.3	478.1	948.2	N.S **up normal**

C0:control treatment , C1 :chemical fertilizer treatment and C2: Biological fertilizer treatment

Table 4. Effect of bio and chemical fertilizers on the flavonoid content in the roots of S.rebaudiana(Bertoni.)(ppm).

biological fertilizer also did not show any significant effect on the flavonoids mentioned above which may be due to the type of the biological fertilizer or to the plant part ability extent to synthesize these flavonoid compounds[28]. Results listed in Tables 2-4 show that some flavonoids are found in specific plant parts but not in others of Stevia for instance the compound Quercetrin3-O glucose was not found in the leaves of a plant treated with both fertilizer types, while it was found in the stems and roots although the plants in study were treated with different fertilizer treatment the biological fertilizer (Table 3). Apigenin 7 glucose was not observed in the studied plant stems of all treatments. The results, on the one hand, maybe due to that the distribution of the flavonoid contents formed within plants are various according to the plant part so they may appear in one part but disappear in another; on the other hand, may be due to the effect of the agricultural operations including the fertilization. It indicates a relation between the studied treatments and flavonoids contained in the specific plant part. The reason also may be due to other factors such as the sampling time and rising the temperature during the second and third ten days of June (Table 1). The treatment with Mycorrhiza increased the plant tolerance to the environmental conditions that led to raising the contents of the most flavonoid compositions[29] in addition to the variation in distribution the compounds may be characterized according to the plant species[11-13] concluded that using chemical fertilizers increases the content of the flavonoids in plant parts and some other research papers proved the opposite[14,15].

Conclusions

Using the biofertilizers, Mycorrhiza achieved the highest content of the flavonoid compounds in different plant parts (leaves, stems, branches, and roots) of Stevia followed by using the chemical fertilizers NPK.

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ARTICLE / INVESTIGACIÓN

The effect of subclinical thyroid dysfunction on B- type natriuretic peptide level

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Abstract: Thyroid hormones (THs) have a significant effect on the cardiovascular system. THs increase myocardium stretch, leading to the release of B-type Natriuretic Peptide (BNP), which is considered a diagnostic biomarker of heart failure (HF). Thyroid dysfunctions (subclinical hypothyroidism; SCH and subclinical hyperthyroidism; SCHyper) stimulate several changes in the heart by causing either diastolic or systolic left ventricular dysfunctions leading to HF. This study aims to measure the changes of B- type NP levels in cases of subclinical hypo and hyperthyroidism. The present study aims to measure the changes in B-type Natriuretic Peptide (BNP) levels in subclinical hypo and hyperthyroidism (SCH and SCHyper). A theoretical study was also conducted using a docking program to find the effectiveness of some drugs in inhibiting or promoting B-type Natriuretic Peptide (BNP). A case study was conducted in a private clinic, Mosul- Iraq, from (April 1st – Sep 1) 2021, with 25 healthy participants with normal functioning thyroids as a control group (EU). A newly diagnosed 25 SCH and 17 SCHyper patients participated in this study, considering that none of them have thyroid dysfunctions taking medicine, hypertension, heart diseases, renal failure, and pregnant women. They all were checked for Thyroid Function Tests (TFTs), Free Triiodothyronine (FT3), Free Thyroxin (FT4) and Thyroid Stimulating Hormone (TSH). The plasma level of BNP was measured in all participants of the three groups. The results showed that the plasma level of BNP was higher in SCHyper patients (10.97 pg/ml) as compared to that of SCH patients (8.09 pg/ml) and EU subjects (8.27 pg/ml). Hereby, we could state that subclinical hyperthyroidism, SCHyper, triggers BNP release. Therefore, it should be kept in mind that any high BNP levels due to SCHyper should be considered a reliable diagnostic biomarker of heart failure (HF).

Key words: Thyroid hormone(TH), Subclinical hypothyroidism(SCH), Subclinical hyperthyroidism(SCHyper), Chronic heart disease(CHD), Heart failure(HF), B-type natriuretic peptide(BNP), Docking Study.

Introduction

Thyroid hormones (THs) have essential effects on the cardiovascular system, including hemodynamic alterations; these effects are mediated on the cardiac myocytes through gene expression[1,2].

Subclinical hypothyroidism (SCH) is defined by high values of Thyroid Stimulating Hormones (TSH) along with normal serum levels of THs (T3 and T4)[3-6]. SCH is present in 15% of women over 60 years old, and etiologically most cases of SCH cases might be regarded as a temporary transition into overt thyroid disease usually caused by autoimmune thyroiditis, but this transition time might vary considerably. It is thought that most of these cases progress to clinical hypothyroidism[1,7,8].

SCH is associated with an increased risk of Chronic Heart Disease (CHD) related events, including mortality and heart failure (HF), especially when TSH levels ≥ 10.0 mIU/L[9-12]. In patients with or without underlying heart disease, persistent SCH can be associated with HF development[13]. Through various mechanisms, the abnormalities of THs in SCH may lead to the development of HF complications such as systolic and diastolic dysfunction, blood pressure alterations, and endothelial and vascular dysfunction[14-17] since SCH increases systemic vascular resistance(SVR) and arterial stiffness by impairing vascular smooth muscle cells relaxation[18] a reduction of Nitric Oxide(NO) availability[19] leading to decrease in stroke volume and cardiac index[20,21]. SCH is usually correlated with left ventricular diastolic dysfunction due to impaired ventricular filling and relaxation[22-26]; thus, poor exercise tolerance is attributed to both diastolic and systolic dysfunction in SCH[27].

The symptoms and signs of SCH are not pathognomonic; therefore, the diagnosis and treatment monitoring depends fundamentally on the measurement of plasma THs and TSH[28].

Subclinical hyperthyroidism (SCHyper) is defined as a subnormal serum level TSH level and serum-free T4 and T3

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concentrations within the normal reference ranges[29-31]. As overt hyperthyroidism (OHyper), SCHyper can be caused by exogenous (secondary to excessive THs replacement therapy)[32] or endogenous (thyroid disease causing thyroid over-activity) factors[32-33].

SCHyper showed a higher heart rate and greater left ventricular mass than EU individuals[34-35] and impaired diastolic function compared to OHyper[20,36]. SCHyper has a potentially arrhythmogenic effect with an increased risk of developing atrial fibrillation, especially in people over 65 years old[37]. This may explain why cardiac dysfunction has been found in patients with SCHyper rather than OHyper. Thus, SCHyper is associated with increased cardiovascular mortality[38-39].

Progression and development of HF[40-41] and cardiovascular mortality have been associated with persistently untreated SCHyper[38].

The risk and severity of HF were associated with both higher and lower TSH levels significantly when TSH >10 µIu / ml and for TSH<0.01 µIu / ml[42], BNP level rises proportionately to systolic, diastolic dysfunctions and the severity of HF[43].

In 1988, a peptide was purified from the porcine brain; it was named "Brain Natriuretic Peptide" (BNP)[44]. Later, this peptide was known as a" B-type natriuretic peptide" and synthesized primarily in the myocardium[45,46]. BNP is synthesized as a 134 amino-acid 'pre-proBNP' which is cleaved to 'proBNP'. Further processing gives rise to the inactive N-terminal pro-BNP (NT-proBNP) (76-residues) and the biologically active C-terminal BNP (32-residues)[47].

BNP belongs to the natriuretic peptide family with different physiological effects, including a diuretic, natriuretic, and vasorelaxant actions[48,49]. Excessive stretching is the primary stimulus triggering BNP secretion by the ventricular myocytes of the heart[50] rather than the trans-mural pressure load[51-56]. It was noted that FT3 has a direct stimulus for the BNP secretion from myocardial cells by increasing the gene expression. Thus, THs in a direct action increase myocardial BNP gene expression[57,58].

It acts as a blood pressure regulatory hormone that is physiologically opposed and suppresses the renin-angiotensin-aldosterone system, endothelin-1, and the sympathetic nervous system[59]. Thus, it is considered a cardioprotective peptide[60]. An increase in heart rate, total blood volume, left ventricular end-diastolic volume and cardiac output in hyperthyroidism exerts a "stress" effect on the cardiac wall, BNP secreted from the myocardial ventricle is a result of a stretch of the myocardial wall. A possible stimulus for BNP secretion could subsequently increase plasma BNP levels[61]. Plasma BNP level has been recommended as a diagnostic and prognostic marker for patients with HF[62-65].

Results

The participant's clinical and biochemical characteristics with EU, SCHyper and SCH are summarized in table (1), which shows no significant difference between the mean values of age, body mass index (BMI), and systolic BP diastolic BP among the three groups.

The mean FT3, FT4, TSH and BNP distribution values showed significant differences among the three groups. Table (2).

Figure 1 shows the distribution of BNP among the three groups. A high value of BNP (10.97 pg/ml) for SCHyper group.

Parameters	EU [n = 25] Mean ± SD	SCHyper [n = 17] Mean ± SD	SCH [n = 25] Mean ± SD	*P-value
Age (years)	37.3 ± 12.69	37.7 ± 8.80	39.4 ± 10.79	0.776
BMI (kg/m^2)	32.9±6.66	29.8±6.65	30.3 ± 7.17	0.253
SBP (mmHg)	120.4 ± 8.41	119.4 ± 9.66	123.2 ± 9.88	0.379
DBP (mmHg)	78.0±5.77	78.5±6.06	76.6±8.00	0.622

* One-way ANOVA-test with Tukey's Pair wise comparisons was used.

Table 1. Personal characteristics of the study sampled groups.

Parameters	EU [n = 25] Mean ± SD	SCHyper [n = 17] Mean ± SD	SCH [n = 25] Mean ± SD	*P-value
F T_3(Pmol/l)	4.13 ± 0.71	5.02 ± 0.87	4.79 ± 0.92	0.002
FT_4(Pmol/l)	12.57±2.75	11.53±2.37	10.76 ± 2.69	0.049
TSH (µIu/ml)	2.12 ± 1.58	0.11 ± 0.21	9.58 ± 4.67	0.001
BNP(pg/ml)	8.27±4.44	10.97±2.68	8.09±3.66	0.036

* One-way ANOVA-test with Tukey's Pair wise comparisons was used.

Note: p-value ≤ 0.05 consider statically significant.

Table 2. Comparison of TFTs among the three study sampled groups.

Results of docking studies

Comparing the binding concerning to docking score from the Patchdock server is the main idea of the theoretical part in this study. Figure 3 and Figure 4 illustrate the 2D binding of T3 with NP receptor and T3 with its specific receptor, respectively.

It is evident from figures and Tables 3 and 4 that the binding T3 is stronger to the NP receptor than its specific receptor, which may lead to an increase in the production of NPB, because the values of Global energy have a slight difference. When data of docking scores in Tables 3 and 4 are compared, it can be noticed that the binding of T3 to the receptor of NP is possible because the active site of binding is similar to the thyroid hormone receptors. The highest docking score was obtained from binding with of T3 with NP receptor (5430), while the highest docking score was obtained from T3 with thyroid hormone receptor (4710). The interface area of T3 binding to NP receptor is (649.90) compared to the interface area of T3 with its receptor (613.80). The results indicate that T3 may bind strongly and affect the response of increasing the concentration of NP. The docking scores and interface area can theoretically explain the relationship between THs and NP. Three-dimensional pictures showing the interaction of T3 to NP receptor and to thyroid hormone receptor have been implemented in figure 5 and figure 6, respectively.

Patients and methods

A case-control study has been done in a private surgical clinic in Mosul, Iraq, from Apr 1 to Sep 1. 2021. Permission has been asked from all involved subjects after explaining the outlines of the study to them.

A questionnaire has been designed and fulfilled by all patients, including the information of name, age, height, weight, BMI, blood pressure (systolic and diastolic), presence of any diseases (diabetes mellitus, heart disease, hypertension, thyroid disease). The participants were selected according to the exclusion criteria (heart disease, hypertension, renal failure and pregnancy). Twenty-five subjects with SCH, seventeen subjects with SCHyper, and twenty-five healthy subjects were regarded as a control group, all the subjects in the three groups were females with ages between 20 - 67 years.

The patients were segregated based on thyroid function tests (TFTs), THs (free Tri-iodothyronine ;FT3 and Free thyroxin ;FT4) and TSH. The FT3 reference range(RR) (4.14-6.09 pmol/L), FT4 RR (8.4-14.42pmol/L) and TSH RR(0.38 -5.33μIU/L).[66] SCH was defined by elevated TSH levels in the serum above 5.33 μIU/L in the presence of THs levels within normal range, while SCHyper defined by level of TSH lower than 0.38 μIU/L with normal levels of THs. A plasma level of BNP hormone was measured in all patients with RR (≤ 26.5 pg /ml).[67]

A blood sample (5 ml) was taken from all patients, 2 ml were put in EDTA tube, then shaking for half minute and centrifugation. Plasma samples were needed to measure BNP by Tosoh AIA- 360, Japan through immune Enzymatic Assay system[67]. Another 3ml of blood was put in gel tube, serum was obtained by centrifugation, and TFTs estimation was done through Access 2 (Beckman coulter), NHANES, USA[68-70]. An electrochemiluminescence assay was performed for TFTs estimation.

Approval has been obtained from the Committee of Ethics at Nursing college, Mosul University, Iraq.

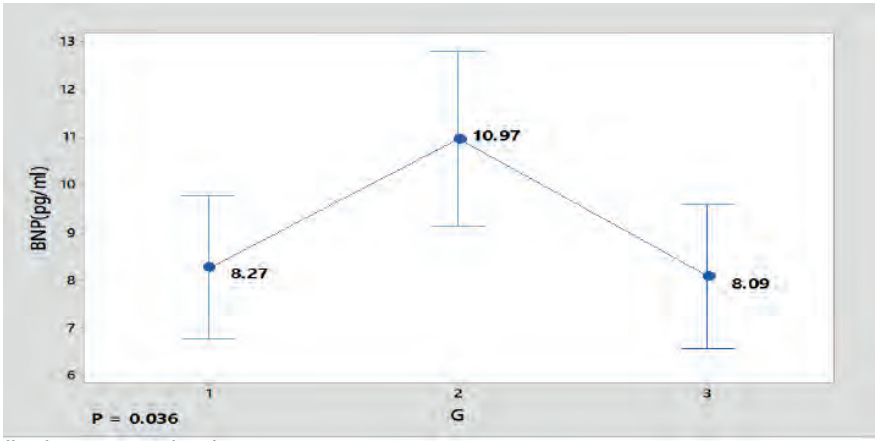


Figure 1. BNP distribution among the three groups.

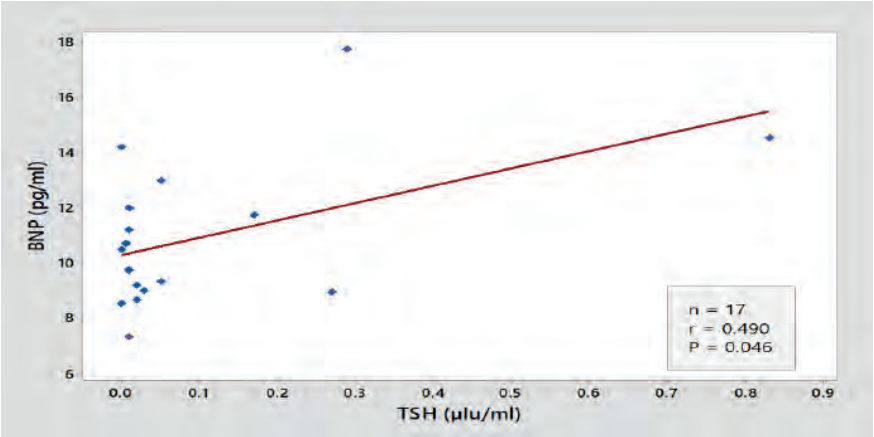
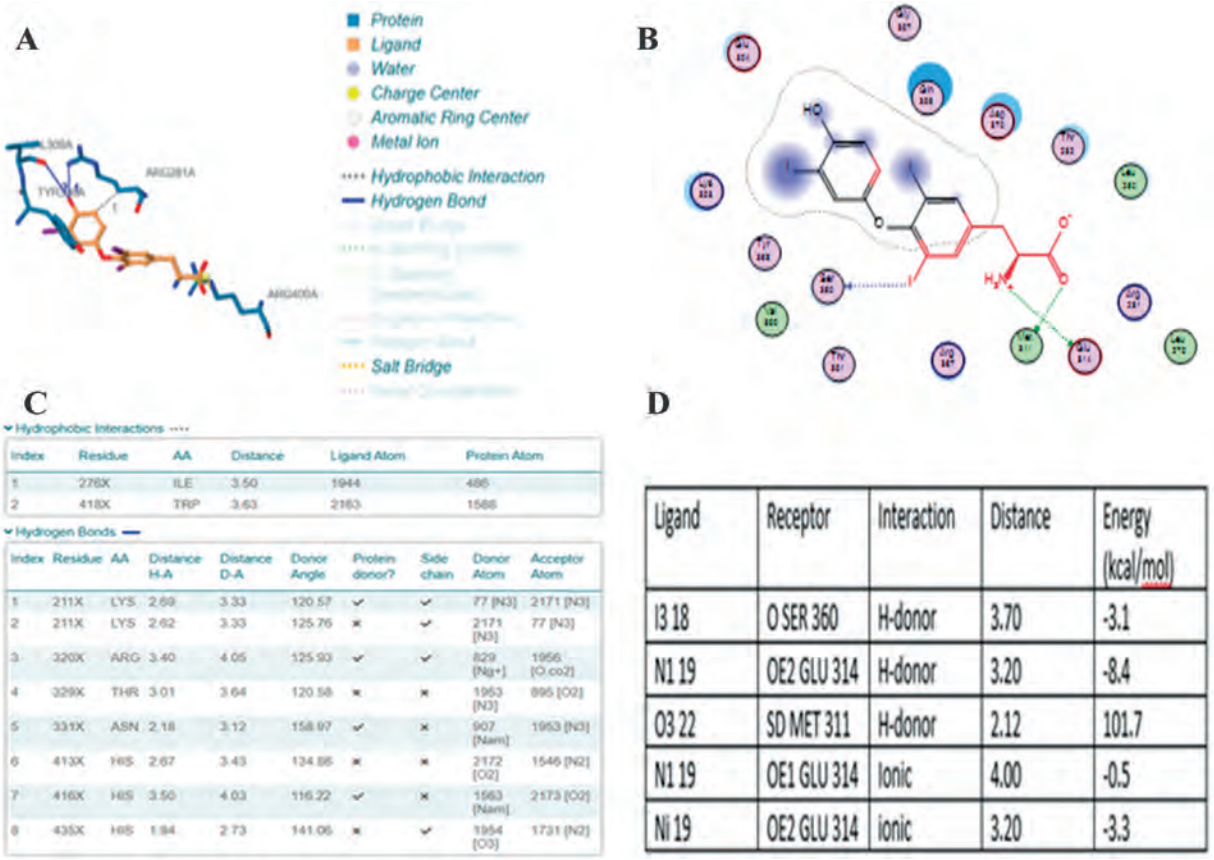


Figure 2. Correlation between TSH and BNP Level.



Ligand	Receptor	Interaction	Distance	Energy (kcal/mol)
I3 18	O SER 360	H-donor	3.70	-3.1
N1 19	OE2 GLU 314	H-donor	3.20	-8.4
O3 22	SD MET 311	H-donor	2.12	101.7
N1 19	OE1 GLU 314	Ionic	4.00	-0.5
Ni 19	OE2 GLU 314	ionic	3.20	-3.3

Figure 3. Two-dimension docking visualization of T3 with NP receptor using PLIP (a), 2D visualizing docking using MOE (b), types of interaction and distance according to PLIP (c), and interaction distance and energy according to MOE74.

Docking studies

Two docking studies have been carried out using patchdock server trying to prove the practical data. tri-iodothyrionine (T3) was selected as the ligand to investigate its binding to the natriuretic peptide receptor and compared the results with T3 binding to the specific receptor (thyroid hormone receptor). Docking was carried out using PatchDock[71,72] server (http://bioinfo3d.cs.tau.ac.il/ PatchDock/). The proteins in this study were receptors of T3 hormone with PDB ID (3gws) and NP receptor with PDB ID (1ky1), while the structure of T3 was designed and energy minimized using MOE. The protein-small ligand was chosen as the complex type, with a clustering RMSD of 1.5 Å. Protein ligand interaction profiler (PLIP)[73] was used to visualize the 2D and 3D structures as well as MOE v14.

Statistical analysis

Descriptive statistical methods were used to tabulate and summarize data. One-way ANOVA-test with Tukey's Pairwise comparisons was used. The Data was expressed as a mean with standard deviation (SD), P-values ≤0.05 were considered statistically significant. Pearson correlation test was used to assess the strength and direction of the relation between BNP and FT3, FT4 and TSH levels in each sample group. All statistical procedures were performed using Minitab version 18 software statistical program.

Discussion

THs have the leading role in the homeostasis of the cardiovascular system. Several processes, including the maintenance of heart structure, function, and cardiac contractility, are all regulated by THs[75]. These lead to many changes, such as hemodynamic changes, including myocardial contractility, cardiac output, and SVR changes[76]. Arises in THs cause stretching of the myocardium, through genomic and non-genomic pathways[77,78]. Therefore, thyroid dysfunctions lead to the pathogenesis of HF[79].

Our study shows that the value of BNP in patients with SCHyper is higher than that of EU subjects and SCH patients and is statistically significant. Ohba *et al.* (2020)[80] measured the BNP levels in patients with SCHyper, SCH and EU subjects; they reported that BNP levels were higher in SCHyper patients than that of SCH and EU subjects and statically different. Pakula *et al.* (2011)[81] showed a significantly higher mean of NT-proBNP in SCHyper patients than in SCH patients and EU subjects; these results are similar to the results of this study. The results reported in this study agreed with that of Ertrugul *et al.*(2008)[54]. They found an increase in BNP levels for SCHyper patients compared to that of SCH patients and EU subjects and statically differences among the three groups.

The preferred binding orientation of a ligand into a receptor is predicted through docking[82] and docking scores can be used just for classifying active ligands from in-actives; but furthermore, we should consider binding assays. Docking gives us the protein-ligand complex, where ligands get bound in the same active site as predicted experimentally or any predicted active site in case of another protein or homology modeled protein. It is the first time studying the association of T3 with NP receptors, which gives good indications and may explain the results of T3 and T4 with NP in our case study.

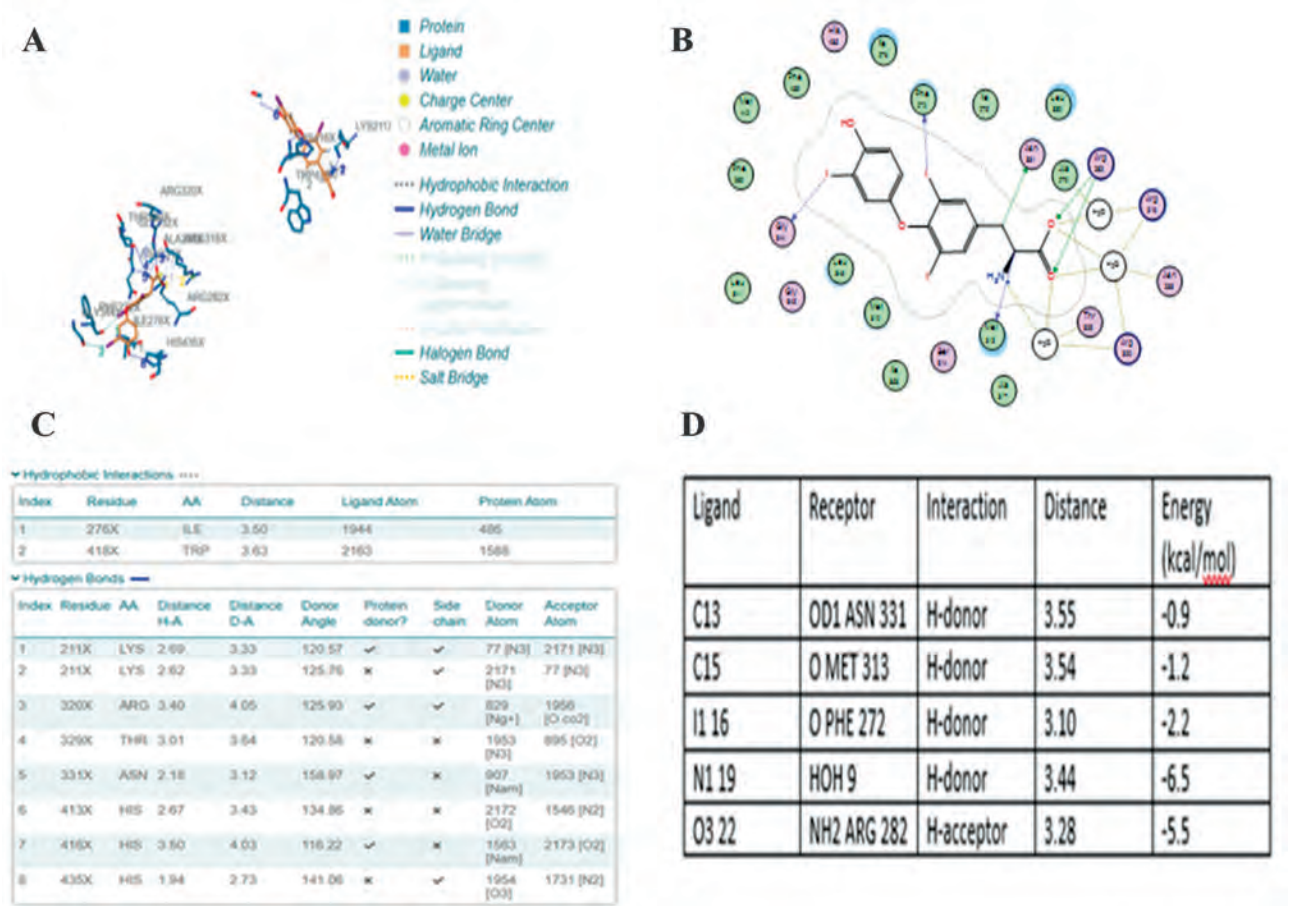


Figure 4. Two-dimension docking visualization of T3 with thyroid hormone receptor using PLIP (a), 2D visualizing docking using MOE (b), types of interaction and distance according to PLIP (c), and interaction distance and energy according to MOE74.

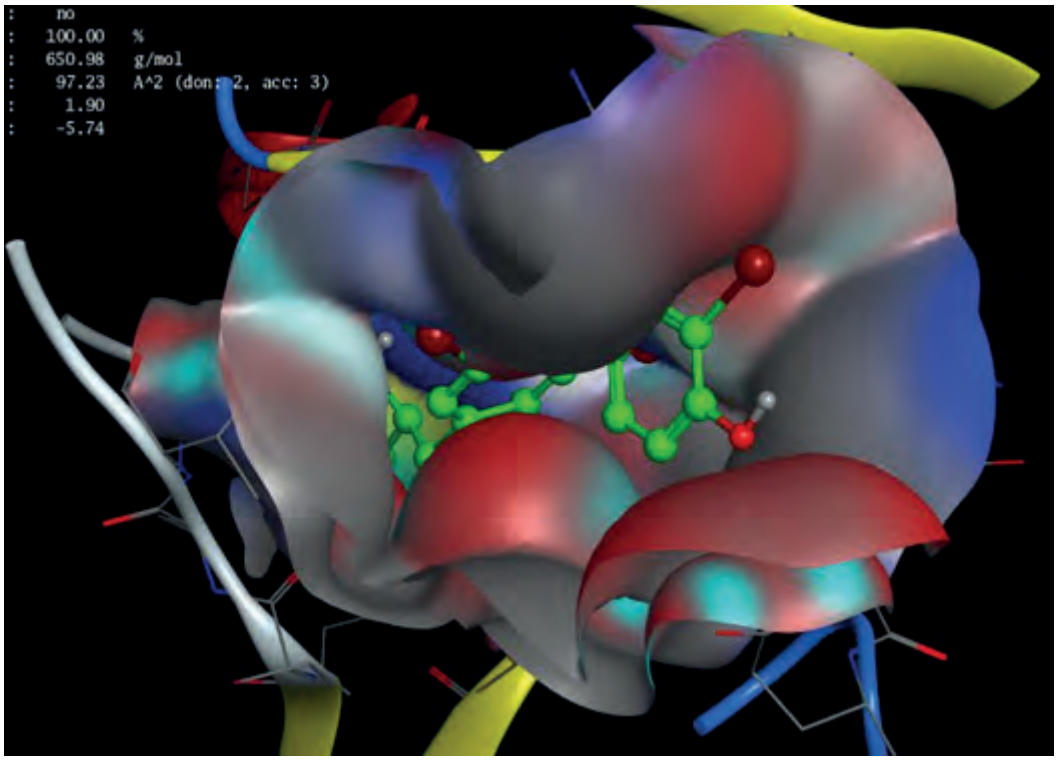


Figure 5. 3D of T3 interaction to NP receptor using MOE.

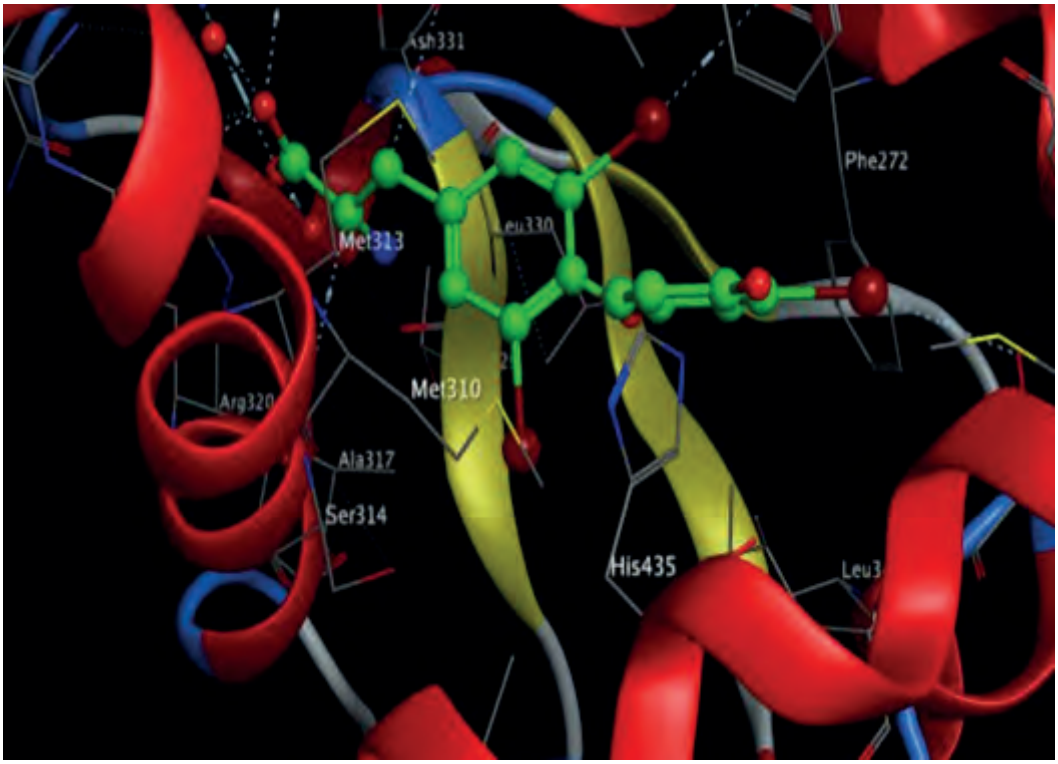


Figure 6. 3D of T3 interaction to thyroid hormone receptor using MOE.

Solution No	Score	Area	ACE	Transformation
1	5430	649.90	-75.47	-2.45 -0.18 -2.35 -1.62 21.54 86.40
2	5418	626.10	-64.16	-2.23 0.16 -1.99 -12.63 38.52 72.44
3	5332	602.70	-162.52	-0.16 1.25 0.08 102.48 -26.23 -26.18
4	5224	598.40	-67.67	0.60 0.51 2.52 -15.71 -10.25 -77.91
5	5176	612.20	-144.96	0.37 -0.55 -1.04 12.15 50.95 -82.94
6	5144	631.40	-147.56	0.65 -0.90 -1.23 40.22 77.23 -54.17
7	5108	612.10	-112.80	-1.23 -0.14 0.53 66.94 -90.58 -16.46
8	5104	619.20	-198.98	2.91 0.52 -0.15 -21.51 52.77 79.00
9	5094	628.50	-102.53	2.28 -0.73 3.00 -1.96 -81.84 39.48
10	5086	590.20	-120.26	-2.79 -0.51 -2.14 -17.70 -25.69 93.85

A

Rank	Solution Number	Global Energy ↓	Attractive VdW	Repulsive VdW	ACE
1	8	-20.97	-11.43	5.33	-7.21
2	9	-19.41	-11.61	5.16	-5.20
3	10	-15.86	-9.22	2.88	-4.03
4	2	-11.50	-10.34	5.04	-1.65
5	4	-8.05	-5.57	2.87	-4.20
6	3	-5.75	-8.64	4.99	-0.13
7	7	-4.36	-4.61	0.64	-0.53
8	6	-0.96	-11.93	22.60	-2.74
9	1	1.63	-4.52	1.21	-1.54
10	5	30.88	-11.79	65.86	-3.71

b

Table 3. Docking score from patchdock server of T3 with NP receptor (a) and Fast Interaction Refinement in Molecular Docking "FIRE DOCK" (b).

Solution No	Score	Area	ACE	Transformation
1	4710	613.80	-111.99	2.52 -0.65 2.55 -68.02 -12.63 53.07
2	4648	544.20	-18.75	0.49 0.45 -1.64 20.41 -7.20 -86.67
3	4612	590.90	-114.88	0.44 -0.06 -0.56 7.69 51.07 -96.13
4	4594	589.80	-100.87	-2.41 -0.98 -1.34 -16.91 -54.39 65.48
5	4514	583.50	-124.19	1.02 0.92 -0.01 62.07 86.92 -30.97
6	4496	556.70	-114.83	-1.43 0.85 1.51 109.37 8.82 15.27
7	4492	524.80	-79.50	1.36 1.46 -2.31 29.91 -59.68 15.30
8	4470	526.70	-140.39	-2.60 0.31 1.44 40.34 -14.95 99.41
9	4460	549.30	-139.79	-1.00 0.36 -2.13 -66.45 63.21 -18.20
10	4372	610.50	-77.32	0.58 0.50 -1.49 38.88 -6.93 -82.76

A

Rank	Solution Number	Global Energy ↓	Attractive VdW	Repulsive VdW	ACE
1	6	-17.65	-13.48	6.59	-4.74
2	10	-12.48	-8.75	1.49	-2.77
3	7	-7.37	-5.63	1.35	-1.20
4	2	-6.27	-16.05	16.95	-4.55
5	3	-4.62	-11.69	23.78	-6.87
6	1	0.90	-2.93	0.10	0.00
7	4	32.43	-23.01	95.52	-6.63
8	8	65.68	-18.22	134.45	-10.20
9	5	80.49	-15.55	140.71	-9.07
10	9	138.54	-24.01	224.05	-6.25

b

Table 4. Docking score from patchdock server of T3 with thyroid hormone receptor (a) and Fast Interaction Refinement in Molecular Docking "FIRE DOCK" (b).

Conclusions

This study, from the results, shows that SCHyper has a significant increase in plasma levels of BNP more than SCH influence. Since SCHyper induces significant hemodynamic changes in the cardiovascular system, thyroid function affects the BNP level, and THs stimulate BNP release. Therefore, SCHyper should be considered in any patient presented with mildly elevated BNP levels. Also, we should consider any elevation of BNP levels in the presence of thyroid dysfunction that could lead to HF.

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Conflict of interests

The author declares no conflict of interest.

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ARTICLE / INVESTIGACIÓN

Study of genetic dimension for local varieties of bread wheat

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Abstract: In this study, nine types of bread wheat (*Triticum aestivem* L.) were used. To analyze it at the molecular level, Random Amplified Polymorphic D.N.A. (R.A.P.D.) markers based on PCR technology were used to study the variations of the cultivars. It is absent in other varieties and can be used as a distinctive genetic fingerprint to preserve plant breeders' rights. Genetic banks can use it to distinguish the mentioned varieties and models, confirming the importance of this technique in molecular characterization and genetic fingerprinting studies. From the results of the molecular aspect, the similarity matrix was implemented based on the coefficients of (Nei & Lie) for the nine studied varieties.
The highest similarity value was between the variety (Taymoz/2 and Baghdad/3), and its value was 0.9167. Most of the traits showed a similarity in the mean between them for all the genetic traits, but the lowest similar value was between the two varieties (Rashid and Baghdad/1). Genetic level, the cultivar seeds were sown under arid conditions in the fields. The experimental unit contained two lines for each category, the length of the line was 2 m, and the distance between the lines was 30 Cm. The characteristics of the number of days of 50% flowering, plant height (Cm), biological yield (gm/plant), grain yield (gm/plant) and weight of 100 grains (gm) were studied. The inputs differed significantly at the 1% probability level for all studied traits. There was high genetic variance for the characteristics of the biological yield (kg/ha) and seed yield (kg/ha), and the weight of one hundred seeds (g). The highest values of genetic variation coefficients for seed yield were superior. The percentage of heritability in its broad sense was high for all the studied traits. The values of the expected genetic improvement were a high percentage of seed yield, vital yield, harvest index, number of total branches per plant, the average for plant height, first pod height (Cm), number of seeds/pod, the weight of 100 seeds, and few numbers of days to flowering at 50%.

Key words: Triticum aestivem, R.A.P.D., Phylogenetic, Molecular markers.

Introduction

Bread wheat (*Triticum aestivum* L.) is one of the most important cereal crops globally and the most cultivated and productive. The area planted with wheat in 2018 reached 214 million hectares, and its productivity was about 34.254 tons/hectare[1]. In Iraq, the area planted with wheat reached about 6331 thousand dunams for the winter season of 2019, and its production capacity for the same year is about 4,343,000 tons[2].

The study of genetic parameters has a preliminary indicator for evaluating the varieties and the extent of variation between them. Several researchers estimated these genetic parameters in the wheat crop were estimated by several researchers where (3) indicated that the values of genetic, phenotypic, and environmental variations were significantly high for plant height and leaf area. The environmental variance values were higher than the genetic variance values. (4) reported that the values of genetic variance were significant for most of the traits except for the traits of several days to flowering, the number of spikes/m^2, biological yield g/m^2, and grain yield g/m^2. Grains/spike and the weight of 1000 grains in bread wheat.

(4) showed that the phenotypic variation coefficient values were high for grain yield, g/m^2, medium for the number of spikes/m^2, biological yield, g/m^2, and the number of grains/spike, and low for the rest of the traits in bread wheat. As for the genetic variation coefficient values were high for the number of grains/spike and grain yield g/m^2 and the average for the rest of the traits, and the values of the phenotypic variation coefficient were more significant than the genetics for all traits.

(3) showed that the values of heritability in the broad sense were high for plant height, the number of spikes per plant, The numerical quantity of wheat grains in the spike grain yield and weight of 100 grains, and (5) indicated that the expected genetic improvement values were high for the characteristics of grain weight/spike, plant height, 1000-grain weight, and spike length in coarse wheat.

Since the R.A.P.D. Technique high efficiency has been used Widely in the genetic variation analysis (6) have estimated the genetic variation using 23 random primers with various bread wheat varieties, and they found that gene diversities were 0.8028 and 0.927. Tease high values indicate that the random primers used in the R.A.P.D. can identify the variation between the wheat varieties depending on the

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presence of the prominent bands and their numbers. (7) has reported that the primers he used showed an apparent genetic variation among the refined wheat under study. 8reported the genetic distance for (8) tetraploid wheat varieties using 24 random primers; two of them showed standard bands while 22 primers showed 141 bands, 101 of them were variable. The genetic distance percentage was 72.6% using R.A.P.D. markers.

The study of the genetic dimension is important because it is one of the goals that plant breeders aspire to evaluate varieties for each breeding program, whether (selection, crossbreeding, introduction), and to locate quantitative traits and preserve genetic assets, in addition to that it does not require hypotheses about the nature of the data distribution, and The reflection of the field assessment of varieties with the laboratory through the use of polymorphic random multiplication indicators[9].

This study aims to evaluate the performance of cultivars and their adaptation to environmental conditions by estimating genetic parameters and estimating the degree of genetic divergence between the studied cultivars.

Materials and methods

The study included two phases:

The field study

The study was carried out in the fields of the College of Agriculture and Forestry, University of Mosul, and included the cultivation of seeds of fifteen varieties of bread wheat (*Triticum aestivum* L. sourced from the Plant Breeding Center / Ministry of Science and Technology - Baghdad and Table (1) show the genealogy of the varieties. They were planted on 16/12 /2018, according to the design of complete randomized sectors. RCBD[10], with three replications, where the items were randomly distributed in each repetition and by two lines for each item. The line length was 2 meters, the distance between the lines was 30 cm, and urea fertilizer was added at a rate of 20 kg (nitrogen). per dunum[11], which is equivalent to 43.47 kg of urea, in two batches, the first - when planting and the second before the spikes were expelled. The study was conducted on 10 plants taken randomly, excluding the terminal plants from each line.

The amount of rain falling during the growing season was (812.5 mm). The study was conducted on 10 plants randomly taken from every two lines, and the following characteristics were studied:

1- The number of days from planting until 50% of the spikes left the flag leaf sheath.

2- Plant height (Cm): The height of the plants was measured from the surface of the soil to the top of the ears, without the ridge.

3- The length of the spike (Cm): It was measured from the base of the spike to the top of the spike without the spur estimated.

4- The number of spikes/m^2.

5- The number of grains of a spike: the average number of grains in each spike were calculated.

6- The vital yield: It represents the weight of the product of two lines (spikes + straw) and converted to kg/ha.

7- Grain yield: This is from the yield of two lines and then converted to kg/hectare.

8- Harvest index (%): It was calculated using the formula: Harvest index % = (grain yield/biological yield) x 100 mentioned by (12).

9- The weight of 1000 grains is estimated in grams. The data were statistically analyzed according to the randomized complete block design (R.C.B.D) using the S.A.S. program (2004); genetic, phenotypic and environmental variances were estimated according to the method explained by (13) according to the randomized complete block design (R.C.B.D) as reported by (10).

$$\sigma^2_g = (M.S1 - M.s.e)/r \qquad \sigma^2_e = M.S.e \qquad \sigma^2_p = \sigma^2_g + \sigma^2_e$$

As for the standard error of the phenotypic variance, it was estimated according to what was mentioned by (14) according to the equation:

$$SE(\sigma^2 G) = \sqrt{\frac{1}{r^2}\left[\frac{2(msg)^2}{k+2} + \frac{2(mse)^2}{k+2}\right]} \quad SE(\sigma^2 E) = \sqrt{\frac{2(mse)^2}{k+2}} \quad SE(\sigma^2 p) = \sqrt{\frac{2(\sigma^2 p)^2}{N}}$$

Note that k = degrees of freedom for each source (genotypes or experimental error), r = the number of replicates, where N = the sum of the degrees of freedom for genotypes and experimental error, and the values of the coefficients of phenotypic variation (PCV) and genetic variation (G.C.V.) were calculated according to the method explained by (15), and based on the ranges used by (16) it is less than 10% low, 10-30% medium, and more than 30% high.

$$Phenotypic Coefficient of Variation (P.C.V.) = \frac{\sqrt{\sigma^2_P}}{\bar{X}} \times 100$$

	Genotype	Source
1	Sally	Plant Breeding Center, Ministry of Science and Technology, Baghdad
2	Taymoz/2	Plant Breeding Center, Ministry of Science and Technology, Baghdad
3	Rashid	Plant Breeding Center, Ministry of Science and Technology, Baghdad
4	Abu Ghraib	Plant Breeding Center, Ministry of Science and Technology, Baghdad
5	Sham	Plant Breeding Center, Ministry of Science and Technology, Baghdad
6	Baghdad/1	Plant Breeding Center, Ministry of Science and Technology, Baghdad
7	Babylon/113	Plant Breeding Center, Ministry of Science and Technology, Baghdad
8	Baghdad/3	Plant Breeding Center, Ministry of Science and Technology, Baghdad
9	Adana	Plant Breeding Center, Ministry of Science and Technology, Baghdad

Table 1. Genealogy of genotypes and their numbers used in the study

And finding the values of inheritance in the broad sense in the way they explained[17]. The ranges explained by (18) were adopted. The values of heritability in the broad sense are less than 40%, low, 40-60% is medium, and 60% or more are high.

$$Genotypic Coefficient of Variation (G.C.V.) = \frac{\sqrt{\sigma^2 g}}{\bar{X}} \times 100$$

$$H^2 = \frac{\sigma_G^2}{\sigma_P^2}$$

The expected genetic improvement when 5% of the plants were selected was estimated[19]. The ranges suggested by (16) for the expected genetic improvement were adopted as a percentage of the average: less than 10% is low, 10-30% is medium, and more than 30% is high.

The Laboratory Study

Although the R.A.P.D. The technique is a relatively old and simple molecular method, but it is still widely used due to its ease of application and economics compared with other high accuracy techniques.

DNA Extraction

Genomic D.N.A. was extracted from plant tissue according to the protocol of Wizard Genomic D.N.A. Purification Kit (Promega, U.S.A.).

Quantitation of D.N.A.

To detect the extracted D.N.A., a fluorescent indicator was used to detect the goodness of samples for downstream applications. For 1 µl of D.N.A., 199 µl of diluted Quanty Flour Dye was mixed. After 5min incubation at room temperature, D.N.A. concentration values were measured.

PCR Amplification

Thermal Cycling Protocol

Gene: ITS

No. of Reaction	48	rxn	Annealing temperature of primers	55
Reaction Volume /run	10	µl	Length of PCR product (bp)	729bp
Safety Margin	5	%	No. of PCR Cycles	30

Table 2. Thermal Cycling Protocol.

Primers

Primer Name	Seq.	Tm	Product Size
ITS1	TCCGTAGGTGAACCTGCGG	55	729bp
ITS4	TCCTCCGCTTATTGATATGC		

Table 3. Primers.

RAPD Technique

The R.A.P.D. The technique was applied using nine random primers in the sequences shown in table (4) to study the discrepancy between 9 varieties of wheat entering Iraq for the first time (Table 1).

T	Primer	Sequence
1	R1	GGTTCGGGAA
2	R2	GAAACGGGTG
3	R3	CACACTCCAG
4	R4	CAAACGTCGG
5	R5	GCATATCCGG
6	R6	CTGACCAGCC
7	R7	ACCAGGTTGG
8	R8	GGACCCCGCC
9	R9	CTTTCGTGCT

Table 4. Names and sequences of the random primers used in the experiment.

Agarose Gel Electrophoresis

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification at 80 volts for an hour and 40 minutes. The results of the R.A.P.D. Random multiplication was examined by imaging the electrophoresis patterns for each primer and identifying the bands, where the presence of the bands is represented by the number (1) and the absence of the band by the number (0). Cluster analysis was also conducted using the Euclidean program, and the scale through the genetic distance was estimated according to the R.A.P.D. Indicators and based on the similarity scale (Jaccard Index), after calculating the similarity ratios according to the previous laws, the other stage begins to calculate the genetic kinship and the Phylogenic evolution tree was drawn for a class of similar individuals next to each other in one cluster. If the genetic material is identical between two classes, this indicates that the genetic dimension must be equal to zero, and the similarity ratio is equal to 1 (100%)[20].

Results and discussion

It is noted from Table (6) that the averages of the cultivars' squares differed significantly at the 1% probability level for all the studied traits except for the trait of the number of days to flowering at 50%, which did not reach the level of significance. Such differences between varieties in the characteristics give the appropriate opportunity for plant breeders to benefit from them in future breeding programs.

Table (7) indicates the averages of the cultivars and for nine studied traits, during which it becomes clear to us that there are differences in these cultivars and for all traits, where the cultivar Adana and in the desired direction outperformed the rest of the cultivars in the short period of days for flowering at 50% as it reached (94 days). As for the plant height characteristic, the cultivar Sally showed its superiority in the desired direction over the rest of the cultivars in length and reached (101.733 cm). The characteristic of the area of the flag leaf showed the highest value in the variety Babylon/113 in the desired direction, as it reached (42.079 cm^2). The cultivar Sally showed its superiority over the rest of the cultivars and in the desired direction, as it reached a value of (5.800) for the characteristic of the number of spikes/plant thus it is possible to benefit from the cultivar Sally by including it in the crossbreeding programs. It is noted that the highest value of the average spike length was in the cultivar Babylon/113 in the desired direction, and it reached (12.367 cm). As for the characteristic of the number of grains/spike, the Rashid variety excelled in the desired direction and reached (68,333) over all other varieties, and from this, it is possible to benefit from the variety Rashid in improving the grain yield of gm/plant by increasing the number of grains/spike.

The genetic structure of Sally gave the highest value in the trait of biological yield, g/plant, and in the desired direction, which amounted to (23.415 g). The results of the average values of the grain yield, g/plant, indicate the superiority of the cultivar Sally over the rest of the cultivars and in the desired direction, as it reached (7.805 g). Therefore, it is possible to select the variety Sally and replanted in subsequent studies and in multiple locations to ensure its superiority and stability of its characteristics, to benefit from it either directly as a new variety or by entering it into crossbreeding programs with other varieties. As for the 1000-grain weight

Steps	Temperature °C	Time	Cycle no.
Initial Denaturation	95	5 min.	1
Denaturation	95	45 sec.	35
Annealing	35	1 min.	
Extension	72	1 min.	
Final extension	72	7 min.	1
Hold	4	5 min	1

Table 5. PCR Program.

traits Sources	Degrees of freedom	Number of flowering days at 50%	Plant height (cm)	Flag leaf area (cm^2)	Number of spikes/plant	spike length (cm)	Number of grains/spike	biological yield (gm)/plant	grain yield (gm)/plant	weight of 1000 grains (gram)
Repeats	2	0.111	506.139	116.432	14.801	9.731	53.444	66.830	7.425	42.884
varieties	8	1.083 n.s.	424.495 **	201.084 **	4.469 **	15.758 **	57.666 **	47.664 **	5.296 **	58.512 **
error	16	0.736	63.866	46.606	0.611	1.398	11.361	8.089	0.899	11.048

Table 6. Analysis of variance for the studied traits in bread wheat. **** Significance at 0.01

trait, Rashid showed a high superiority over the rest of the cultivars in the desired direction and reached (60.564 g); and thus, Rashid could be entered into the crossbreeding program with other cultivars to transfer the genes of the high yield, as the 1000-grain weight trait is one of the main yield components.

Table (8) shows that the values of phenotypic, genetic and environmental variances were significant from zero for all studied traits except for the number of days to flowering at 50%. Environmental variations were significant for most traits except for the traits of a number of days to flowering at 50%, several spikes/plant, spike length, and the grain yield, and concerning the phenotypic variations, it was significant for all traits except for the number of days to flowering at 50%, the number of spikes/plant and the grain yield and this is in line with what was mentioned by (21),(22) and (4) and (23) As for the coefficients of phenotypic and genetic variation, they were medium and low for most of the traits, and these results are in line with what was reached by4. As for the values of heritability in the broad sense, they were high for the characteristics of plant height, the number of spikes/plant, spike length, biological yield and grain yield, medium and low for the rest of the traits is consistent with (3). The expected genetic improvement values were high for the characteristics of the leaf area/cm^2, the number of spikes/plant, spike length; biological yield, grain yield, medium and low for the rest of the traits, and these results are in line with what[4,5].

PCR expansion

Figure. 1 ITS region amplification fractionated on 1% agar gel electrophoresis stained with Eth.Br. M: 100bp D.N.A. marker.

Polymorphism resulting from the use of R.A.P.D. technique

Eight primers were highly efficient in detecting the divergences between the varieties, according to Table (9).

Depending on the primer used, the results showed a clear difference in the number of alleles, in addition to the presence of primers that did not show any bands, and the reason may be due to the absence of a complementary linkage region for the sequence of bases for this primer on a complementary site within the D.N.A. sequences of the genome of the studied species. Several studies showed the presence of differences in results for primers[24,25]. The rest of the primers had varying abilities to detect the magnitude of genetic variation among the studied genotypes, while others gave a less number of bands. We also note that the nine primers gave 30 allelic loci, including 9 major alleles for their appearance in all genotypes, while the different alleles are of the characteristic alleles of the genotypes, and 21 alleles are relied upon to determine the genetic link between the genotypes.

Primer R1

This primer was used in genotypic D.N.A. replication, and 4 different allelic binding sites were obtained, and the percentage of them was 100%. This primer showed an apparent variation in bands position and a molecular weight ranging between 1000-1100bp.

From Figureure (1), it is clear that cultivars 6 and 4 do not have any linkage site with this primer, and the reason is due to the absence of a complementary linkage region for the base sequence of this primer on a complementary site within the genome D.N.A. sequences of the studied species.

Primer R2

The primer was able to identify 3 sites of its complementary sequences in the D.N.A. of the genome; all of the first structures with a size of 35 bp and the second 1100 bp are present in all varieties. The third link site has complementary sites in the first and second variants, with a size of 1000 Pb. This primer showed a 33.33% variation.

Primer R3

The number was 3, whose sizes ranged from 390bp to 800bp. Cultivar 6 was distinguished by the absence of bands and was considered a distinctive feature of this cultivar, and the variance ratio was 100%.

Primer R4

This primer has many 32 packets that resulted from 4 alleles, all bands are different, so the percentage of divergent bundles becomes 100%. The primer was able to

Genotypes	Number of flowering days at 50%	Plant height (cm)	Flag leaf area (cm^2)	Number of spikes/plant	spike length (cm)	Number of grains per spike	grain yield (gm)/plant	biological yield (gm)/plant	weight of 1000 grains (gram)
Sally	95.000	101.733	24.497	5.800	8.567	56.667	23.415	7.805	48.089
Taymoz/2	95.333	61.200	23.034	2.533	9.267	62.667	10.662	3.554	54.652
Rashid	95.000	80.933	41.097	3.200	15.900	68.333	15.836	5.279	60.564
Abu Ghraib	94.333	76.400	31.163	5.133	9.100	63.333	18.743	6.248	54.623
Sham	94.333	77.467	27.845	4.467	10.167	57.333	17.471	5.824	48.786
Baghdad/1	95.000	72.200	37.180	3.267	11.267	59.000	21.789	7.263	51.652
Babylon/113	96.000	83.067	42.079	4.933	12.367	67.333	17.789	5.930	58.775
Baghdad/3	95.000	71.733	21.236	2.467	9.733	60.333	12.811	4.270	52.280
Adana	94.000	64.000	22.645	3.133	9.467	57.000	18.295	6.098	48.986
Average	94.889	76.526	30.086	3.881	10.648	61.333	17.423	5.808	53.156
L.S.D (0.05)	1.485	13.833	11.817	1.353	2.047	5.834	4.923	1.641	5.753
L.S.D (0.01)	2.046	19.060	16.282	1.864	2.820	8.039	6.783	2.261	7.927

Table 7. Average varieties of bread wheat for the studied traits.

Traits genetic parameters	Number of flowering days at 50%	Plant height (cm)	Flag leaf area (cm^2)	Number of spikes/plant	spike length (cm)	Number of grains per spike	biological yield (gm)/plant	grain yield (gm)/plant	weight of 1000 grains (gram)
genetic variance	0.116 ± **0.696**	120.210 ± **12.825**	51.493 ± **5.231**	1.286 ± **0.125**	4.787 ± **0.388**	15.435 ± **1.828**	13.192 ± **2.163**	1.466 ± **0.240**	15.821 ± **1.880**
Environmental variance	0.736 ± **1.461**	63.866 ± **31.964**	46.606 ± **23.346**	0.611 ± **1.447**	1.398 ± **1.578**	11.361 ± **5.854**	8.089 ± **4.285**	0.899 ± **1.484**	11.048 ± **5.702**
Phenotypic variance	0.852 ± **4.023**	184.076 ± **92.125**	98.099 ± **49.212**	1.897 ± **4.111**	6.185 ± **5.056**	26.796 ± **13.982**	21.281 ± **11.367**	2.365 ± **4.171**	26.869 ± **14.017**
genetic variation coefficient	0.349	12.533	13.082	5.756	6.705	5.017	8.701	5.024	5.456
phenotypic variation coefficient	0.947	15.509	18.057	6.991	7.621	6.610	11.052	6.381	7.110

Table 8. Genetic parameters of the studied traits in bread wheat.

identify the complementary sequences in the D.N.A. of the genome for the genotypes, and it showed an apparent variation in the molecular weight ranging between 850-1700 bp; from Figureure (4) we note that the primer did not find its complementary series at genotype 3, so no bundle appeared; thus it can be considered as an imprint of this genotype in this prim.

R5

This primer distinguished variant 1 with a band size of 1700 bp. It also able to identify the complementary sequences in the D.N.A. of the genome, as it showed a clear variation in the molecular weight, ranging between 600-300 bp.

R6

This primer showed two complementary genomic loci, the first was associated with a size of 800 bp, and the second site distinguished a locus with a size of 1500 pb. As for variants 2 and 6 respectively, it was distinguished by the absence of any complementary linkage to the primer, and the divergence ratio was 100%.

R7

This primer was distinguished by the presence of 4 identical bans for all types, the similarity rate was 0%, and their sizes ranged from 900 to 1700bp.

R8

This primer produced 5 different bands, ranging from 400 to 1700bp, with a variance of 100%.

R9

This primer produced 3 bands ranging from 400 to 1200pb. Variant 8 was distinguished by the absence of a

Primer	Binding site	Polymorphic	Polymorphism (%)	monomorphic	Bands size
R1	4	4	100	0	900-1000
R2	3	1	33.33	2	35-1000
R3	3	3	100	0	800-390
R4	4	4	100	0	850-1300
R5	1	1	100	0	1300-600
R6	2	2	100	0	800-1150
R7	4	0	0	4	900-1300
R8	5	5	100	0	300-1300
R9	4	1	66.6	3	400-1200
	30	21			
	3.33	2.33			

Table 9. Genetic variances for the primers used.

band with a size of 400pb in all varieties, and the variance ratio was 66%. This is considered a distinctive feature of variant 8 because the initiator did not find a complementary site for it.

The relative relationship between the wheat cultivars under study based on R.A.P.D. data

According to the previous results, the R.A.P.D. The technique was able to find unique and distinct bands capable of distinguishing between the studied wheat varieties; that is, these bundles were found in a particular variety and absent in other varieties. Genetic banks can use it to distinguish the mentioned cultivars. This confirms the importance of this technique in studies of molecular characterization and genetic fingerprinting.

A genetic relation tree was made for the nine taxa, where U.P.G.M.A. similarity values were estimated using the studied R.A.P.D. method based on data analysis.

The similarity between the studied wheat cultivars was Estimated based on the results shown by the primers, where the results that appeared in the gel electrophoresis were transferred to the characterization tables by placing one in the presence of a band and zero in its absence after that the symmetry matrices are formed based on the coefficients Table (3) results of the similarity matrix between the studied varieties. The highest similarity was between the variety (2 and 8), and its value was 0.9167; most of the traits showed closeness in the arithmetic means between them for all genetic traits, while the lowest similarity value was between the two variants (3 and 6), where the result of reading the arithmetic mean was significantly different in most of the phenotypic traits between these two variants, these results between varieties using R.A.P.D. Indices were associated with phenotypic traits and were consistent with what was found before[8], where the results showed that the lowest genetic distance was between two cultivars that have specific significant characteristics in common.

Cluster analysis of genotypes of bread wheat based on R.A.P.D. Markers

Cluster analysis shows the evolutionary relationship of a group of living organisms that arose from a common lineage containing the largest possible number of genotypes in plant breeding and development programs,. The accessions may be collected in one group according to their geographical location or origin and lineage Figureure (11).

The nine bread wheat varieties cluster tree showed two main groups; the first leading group was divided into two secondary groups, and the first secondary group was divided into two branches. The genetic material of these genotypes may be similar according to the number of D.N.A. segments to the gene of these complementary structures of the primer sequences used, while the second secondary group included variants 4 and 6, and the reason for this relationship is due to sharing most of the genetic traits and their possession of a number of similar D.N.A. pieces to show the extent related to each other, while the second main group included only variants 3 and 9, due to their uniqueness of their behavior with most of the primers used in the study.

Conclusions

Among the results obtained from both aspects, the molecular study proved its effectiveness of and usefulness for plant breeders and the as the building block for the success of any plant breeding program, and the shortening of time in determining the appropriate varieties to conduct crosses and improving the characteristics of the yield.

Funding

Self-funding

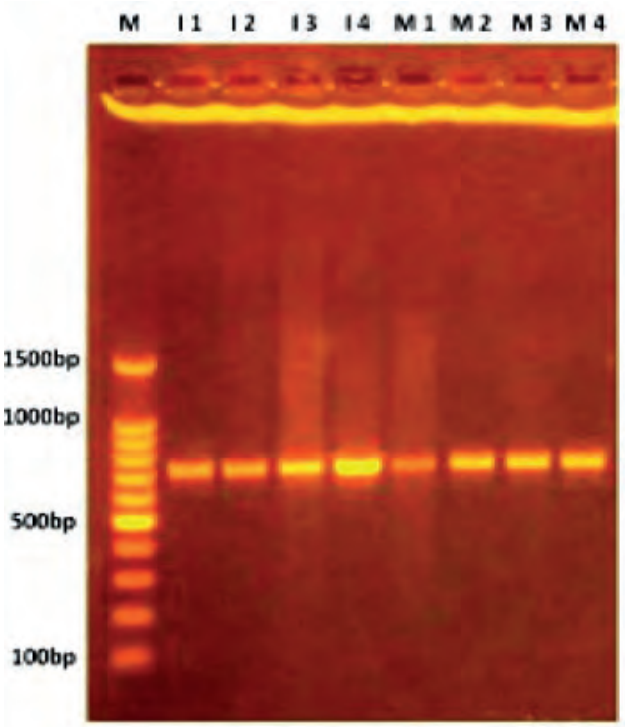


Figure 1. ITS region amplification fractionated on 1% agar gel electrophoresis stained with Eth.Br. M: 100bp D.N.A. marker.

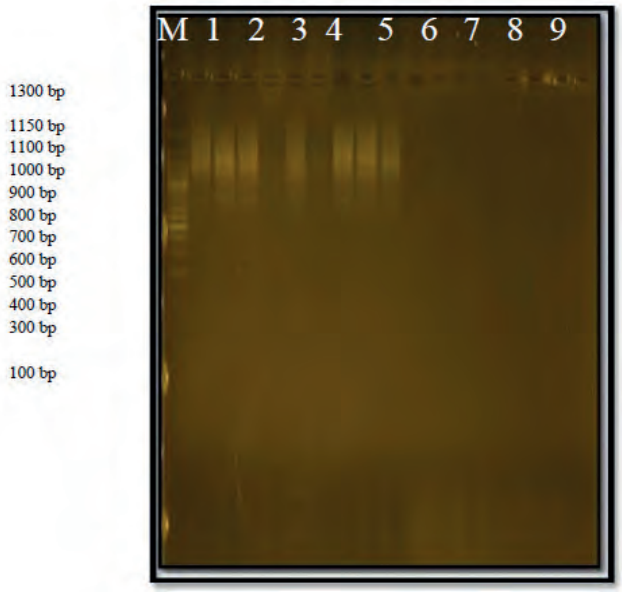


Figure 2. The product of the random amplification reaction of D.N.A. using the R1 primer.

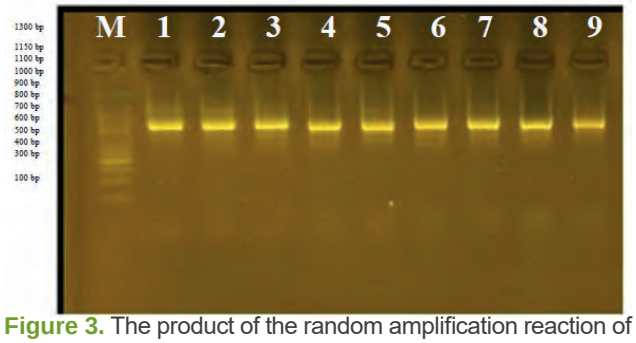


Figure 3. The product of the random amplification reaction of D.N.A. using the R2 primer.

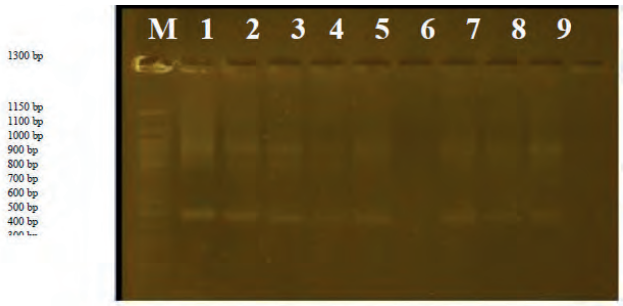


Figure 4. The product of the random amplification reaction of D.N.A. using the R3 primer.

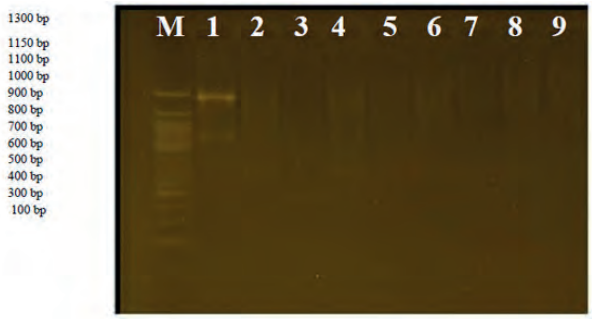


Figure 5. The product of the D.N.A. random amplification reaction using the primer R5.



Figure 6. The product of the DNA random amplification reaction using the primer R6.

Acknowledgments

In this section, we acknowledge any person who supports us in completing this project.

Conflicts of Interest

There is no conflict.

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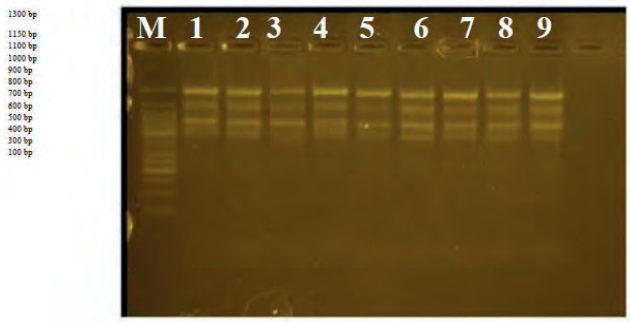


Figure 7. The product of the D.N.A. random amplification reaction using the initiator R7.

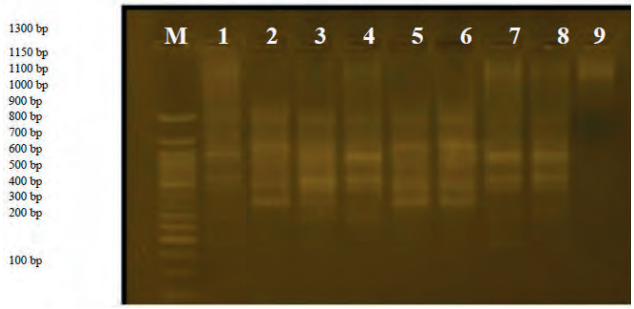


Figure 8. The product of the D.N.A. random amplification reaction using the initiator R8.

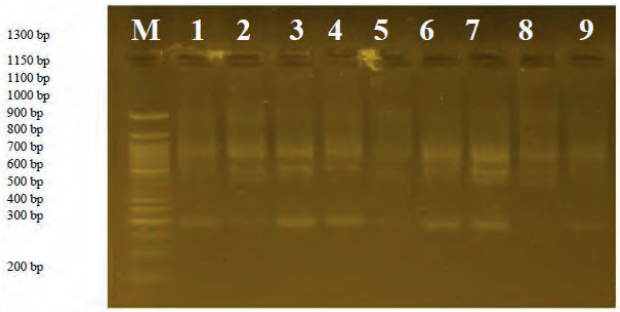


Figure 9. The product of the D.N.A. random amplification reaction using the initiator R9.

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 +=    1.00000, -=    0.00000
Matrix type = 3, size = 9 by 9, missing value code = "none"  (similarity)

      A1      A2      A3      A4      A5      A6      A7      A8      A9
 ------------------------------------------------------------------------
A1 | 1.0000
A2 | 0.7273 1.0000
A3 | 0.5000 0.6000 1.0000
A4 | 0.7368 0.7368 0.3529 1.0000
A5 | 0.7273 0.9091 0.5000 0.8421 1.0000
A6 | 0.5000 0.6250 0.1429 0.7692 0.6250 1.0000
A7 | 0.6957 0.8696 0.7619 0.6000 0.7826 0.4706 1.0000
A8 | 0.8333 0.9167 0.5455 0.7619 0.9167 0.5556 0.8000 1.0000
A9 | 0.6667 0.7619 0.7368 0.5556 0.6667 0.4000 0.7273 0.7826 1.0000

---- next dataset ----

Comments:
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 +=    1.00000, -=    0.00000
Matrix type = 3, size = 9 by 9, missing value code = "none"  (similarity)

      A1      A2      A3      A4      A5      A6      A7      A8      A9
 ------------------------------------------------------------------------
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Figure 10. The kinship relationship between the studied wheat cultivars based on R.A.P.D. data.

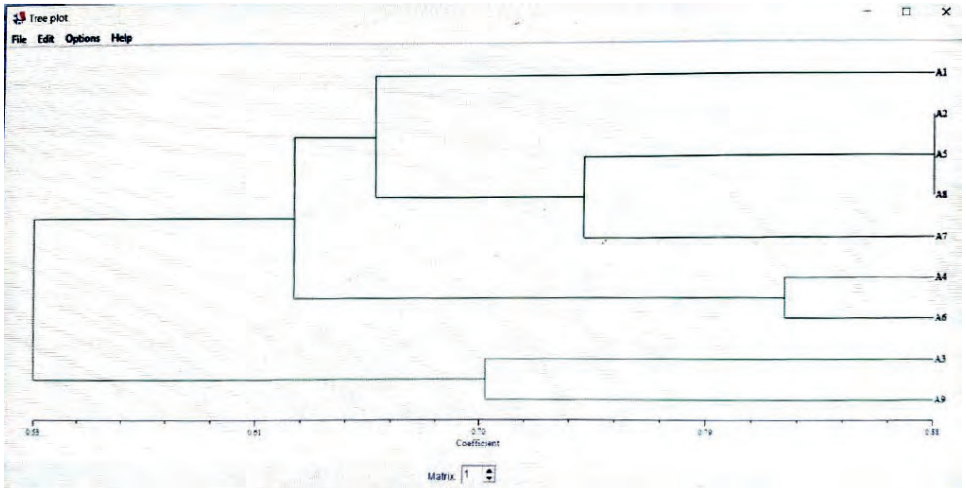


Figure 11. Neighbor-joining tree for the studied varieties using unequal pairs groups (U.P.G.M.A.).

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ARTICLE / INVESTIGACIÓN

Molecular identification of some allergenic fungi found in household dust in Mosul city

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Abstract: The study included isolating and diagnosing the fungi found in dust samples from homes and buildings such as basements and walls containing fungal growth in separate areas from the left side of the city of Mosul in northern Iraq, such as Al-Ghufran neighborhood, Al-Mazare' neighborhood, and Al-Mohandesin neighborhood during October and November, and the relationship of these fungi to human diseases, including allergies and asthma. The isolation results showed many fungal genera, including Cladosporium, Penicillium, Aspergillus, Alternaria and Trichoderma. The research aims to study the fungi Alternaria and Trichoderma, where the percentage of the presence of Alternaria in the wall sample containing the previous fungal growth was 28.57%. Whereas the percentage of the presence of Trichoderma fungus in dust and gypsum falling on the surfaces of poorly ventilated rooms in the cellars was 42.86%, and the molecular diagnosis of fungal isolates was carried out, as it was confirmed that there is a match with the standard strains found in the gene bank. The Alt a1 and Exp genes responsible for asthma were also examined and detected in fungal isolates using PCR technology and polymerase chain reaction; the new genes in both isolates were recorded. On behalf of both the supervisor and the researcher with international numbers in the global gene bank.

Key words: Household dust, allergies and asthma, indoor environments, Alternaria and Trichoderma, Mosul local fungi.

Introduction

Fungi are ubiquitous in the air, and their composition is essential to human health[1] and poses a health threat, especially to immunocompromised patients[2]. House dust is an environmental measure commonly used as an indicator of exposure to human for fungi[3,4]. Alternaria is among the airborne genera responsible for allergic rhinitis or asthma[5]. In addition, some species of Trichoderma, which are filamentous fungi responsible for infection-causing deaths in up to 53% of patients with immunodeficiency[6]. Fungi contain many known allergens, including Alt a1, one of the main fungal allergens present in the ubiquitous species. Alt a1 is a highly present allergen in Alternaria sp and other fungi that can produce Alt a1 allergen present in 95-99% of homes[7]. Therefore, the response to allergies, whether nasal allergy or asthma, can be considered natural problems Associated with the inhalation of fungi in the air[8]. Exposure to fungi occurs through inhalation of dust[9]; exposure to airborne germs has been linked to upper and lower respiratory diseases[10]. Indoor fungal exposure has been associated with increased risk and severity of asthma and allergies. Asthma and respiratory allergies are chronic inflammatory diseases that affect the airways and are characterized by bronchial hyperactivity and altered airway obstruction, which leads to recurrent episodes of coughing, shortness of breath, chest tightness, and wheezing, the severity of which can vary over time[11,12]. Early exposure to microbes or air pollutants and asthma is complex, and this depends on several factors, such as the nature of exposure to the pathogen, how it occurs and when the genetic susceptibility of the host[13]. These factors indicate the importance of the surrounding environment in developing and exacerbating asthma[14]. Some health studies have revealed the presence of some fungi in the respiratory tracts, including those of the genera Cladosporium, Eurotium, Penicillium and Aspergillus. Other genera such as Candida have been detected but in low proportions[13].

Materials and methods

Dust samples were collected indoors, where dust from the air-conditioning filter, dust accumulated on furniture, and dust samples were collected in ground rooms with poor ventilation and lighting. Finally, dust samples were collected from the damp walls containing former fungal growth. Fungi were isolated in dust samples using the dilution and dishes method to obtain dilutions of 10-1, 10-2,10-3, then 1 ml of each dilution was transferred to sterile Petri dishes, and 10 ml of PDA medium was added to each dish in three replicates. These dishes are then placed in nylon bags for each dilution and incubated at 28 °C for 7 days. The numbers of developing fungal colonies were counted, then the most common fungi were purified by transferring a small portion of the outer edges of the fungal colony to plates prepared with a PDA medium and preserving them for diagnostic purposes.

Preparation of Potato Dextrose Agar (PDA)

It was prepared by dissolving (39) grams of prepared nutritional medium (Lab.M.Limited, UK) in a liter of distilled

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water, then placing the medium in a Glass Flask and sterilizing it in an autoclave and adding the antibiotic gentamycin, then pouring the medium into Petri dishes and leaving the medium until it solidifies for use it later[15].

Preparation of Potata Sucrose Broth (PSB)

Potato tubers were taken in the amount of 200 g, peeled and cut into small cubes, boiled in 500 ml of distilled water for 20 minutes, then filtered with a gauze cloth and 20 g of sucrose dissolved in it, then completed the volume to 1 liter with distilled water Then pour the medium into Petri dishes and leave the medium to solidify for later use[15].

DNA isolation

Several steps were followed based on the instructions of the company's "Genomic DNA mini Kit of fungi" From Geneaid Company for DNA extraction.

DNA molecular size estimation

DNA molecular sizes were estimated by performing electrophoresis of samples on an agarose gel using a molecular ladder; 100 base pairs, then mixing the DNA with the loading dye and running it in a 2% agarose gel for min.

Amplification of a highly conserved ITS region in fungi using PCR technology

The polymerase chain reaction was performed using universal primers that amplify the ITS regions in genomic DNA, Table (1).

Then the reaction tubes were inserted into the German-origin thermal cycler to conduct the reaction using the particular program for the reaction, as shown in the following table (2).

Primer	Sequence
Forward	5'TGAATCATCGACTCTTTGAACGC 3'
Reveres	3'TTTCTTTTCCTCCGCTTATTGATAT 5'

Table 1. Polymerase chain reaction (PCR) program for ITS region amplification.

DNA extraction from agarose gel

The packets resulting from the PCR reaction were extracted from the gel to be purified and sent for nucleotide sequencing testing, depending on the analysis kit supplied by (Geneaid) company.

Detection of the presence of ATL1 a and EXP genes in the Fungal isolates using PCR technique

Both ATL 1 a and EXP genes were detected in the fungal samples. The DNA template was 4 µl (100 nanograms), and each gene-specific primer 1 µl (10 picomols) were added to the contents of the Master mix, as shown in table (3).

Then the reaction tubes were inserted into the thermocycler to conduct the amplification reaction, using the particular program for the reaction, as shown in the following table (4).

Determination Of Nucleotide Sequences For Amplified Pieces Using DNA Sequencing

The sequence of the nitrogenous bases of the fungi samples under study was determined. The PCR reaction products extracted from the gel were sent for the above-mentioned samples. The sequence was read for the genes based on the 3130 Genetic Analyzer device supplied by the Japanese company Hitachi, and the sequences of the genes were matched with the sequences Genes documented in the National Center for Biotechnology Information NCBI the results were analyzed using BLAST software.

Results

Isolation and diagnosis of fungi from dust samples

The results of isolating fungi from household dust samples showed the presence of different types of fungi (Cladosporium, Penicillium, Aspergillus, Alternaria and Trichoderma) and after purifying the most prevalent isolates and growing them on PD. The following fungal isolates were obtained in a medium (Fig 1 and adding table 5.

Air pollution with fungal spores was of varying degrees in indoor environments, buildings and closed homes, as the largest number of fungi found in homes was recorded in dust samples collected from the surfaces of poorly ventilated ground rooms on the ground floor of some homes, and in gypsum samples falling from the walls of these rooms,

No.	Stage	Temperature(°C)	Time	Cycle number
1.	**Initial denaturation**	**95**	**5 min.**	1
2.	Denaturation	95	45 sec.	35
3.	**Annealing**	**55**	**1 min.**	
4.	Extension	72	1 min.	
5.	**Final extension**	**72**	**7 min.**	1

Table 2. Formulas and variables for calculating the components of the productive process' cost.

Primer	Sequence
ATL- F	5'ATGCAGTTCAC CACCATCGC 3'
ATL-R	3'ACGAGGGTGAYGTAGGCGTC 5'
EXP-F	5'CAAATACGGCTGCCCTCTTC 3'
EXP-R	3'AAATAGTACGGCCCTCCACC 5'

Table 3. Alta1 a, EXP genes primer.

No.	Stage	Temperature(°C)	Time	Cycle number
1.	Initial denaturation	95	5 min.	**1**
2.	Denaturation	95	45 sec.	**35**
3.	Annealing	55	1 min.	
4.	Extension	72	1 min.	
5.	Final extension	72	7 min.	**1**

Table 4. Polymerase chain reaction (PCR) program for Alta1 a, EXP genes amplification.

NO.	Sample Region and Kind	Fungal Genus	% presences
1	Wall Sample (Al-Mohandesin)	*Alternaria alternata*	28.57%
2	Filters Conditioner (Al-Mazare')	*Aspergillus niger*	85.71%
3	Basement Rooms(Al-Ghufran)	*Cladosporium*	14.31%
4	Basement Rooms (Al-Mohandesin)	*Trichoderma atroviride*	42.86%
5	Wall Sample (Al-Mohandesin)	*Penicillium consobrinum*	71.43%

Table 5. Shows the percentage of races obtained and the regions from which they were isolated..

followed by dust samples from walls containing previous fungal growth while, dust samples from air conditioner filters and dust accumulating on furniture recorded lower fungal growth, respectively. Fungal contamination of samples and surfaces differs from one house to another, and this mainly depends on the available conditions and factors, which vary according to the environment.

The results of isolation showed many fungal species present in the air of rooms and homes, Penicillium was the most common and frequent in a dust sample that was taken from a wall containing a previous fungal growth, where the percentage of its recurrence in the dishes was the highest and the percentage of its appearance in the dish was 71.43% Then followed by Alternaria, whose frequency was lower than Penicillium, and its appearance in dishes was 28.57%, and this was partially identical to what was isolated before[16,17].

As for the dust samples of air conditioner filters, the frequency of Aspergillus fungus was the most prevalent, and the percentage of its appearance in dishes was 85.71%, while the percentage of other species that appeared in the dish was 14.29%, and this indicates that the air conditioner can cause the spread of fungal spores in house rooms. According to (18), fungal contamination of the air-conditioning filter, through which the air current passes, distributes the fungal spores throughout the room's atmosphere. Sometimes a foul smell or a cough occurs when the air conditioner is running, which is related to the fungal contamination inside the air conditioner as the air conditioners provide enough moisture to make the fungi grow and multiply on the filter.

As for the dust and gypsum samples falling on the surfaces of the ground rooms in the basements of some houses, several types were isolated, including Trichoderma, whose frequency was more common, and the percentage of its appearance in the dishes was 42.86%. In comparison, the appearance percentage of Aspergillus was 28.6%, the

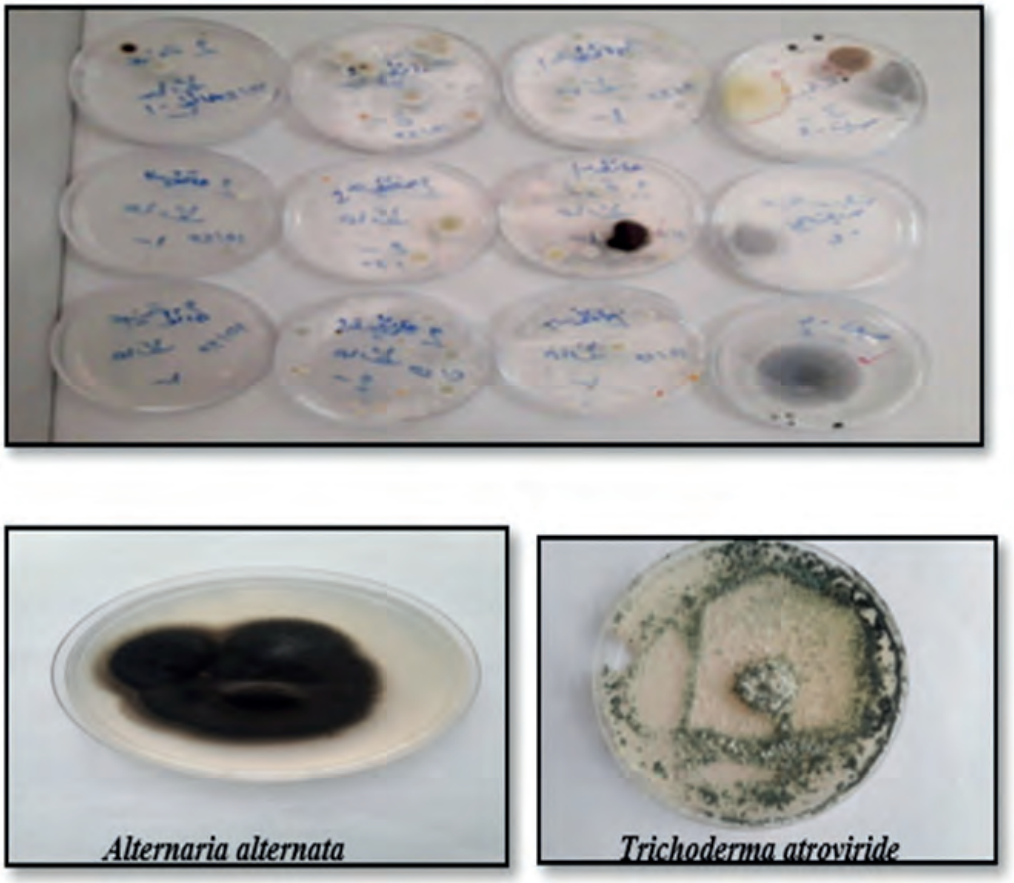


Figure 1. Fungi isolated from dust samples of some house's dust.

appearance of Penicillium fungi was 14.29%, and Cladosporium 14.31%. This indicates that the conditions of these rooms were a source of fungal contamination that made them rich in several similar fungal species. For those isolated by Lugauskas and Jaskeleviius[19] from the basement rooms of some buildings that were used as gymnasiums and some were used as book stores.

DNA Extraction and PCR with Sequencing detection

Using a DNA extraction kit has several advantages, including the speed of extraction and obtaining high purity DNA. It also has a system capable of removing degrading enzymes as well as removing PCR inhibitors, thus resulting in high-quality DNA within a period not exceeding 60 minutes without resorting to the use of phenol or chloroform, and after the process of DNA isolation and extraction, electrophoresis was performed by adding of fungal DNA 1 µl in the presence of a DNA ladder in an agarose gel. The extracted fungal DNA samples were diagnosed as shown in the following figure (2). The results showed the amplification of ITS region for all samples and with high purity as shown in the following figure (2).

After the first fungus was isolated in pure form, PCR technology was performed to confirm its molecular diagnosis by investigating the duplication results representing the gene's DNA segment. The nitrogenous base sequences of the gene within the National Center for Biotechnology Information (NCBI) using the BLAST program, the name of the fungus for the isolate was obtained, and the sequences of the nitrogenous bases for the isolate gave the genus *Alternaria alternata*, which showed the percentage of match between the local isolate and the isolate recorded in the genebank by 100% as shown in the figure(4).

The result of the sequence of nitrogenous bases, the second isolate Trichoderma, was the Identities % between the local isolate and the isolate recorded in the gene bank was 99%, as shown in the following figure (5).

The results confirmed that techniques based on nucleic acid amplification could detect DNA quantities as they are usually fast and sensitive assays, thus overcoming the limitations of traditional diagnostics that are slow or have insufficient sensitivity[20].

Detection of the presence of ATL 1 a and EXP genes in the fungi isolates

Specialized tools were used to detect and investigate the presence of ATL and EXP genes responsible for allergy and asthma in the selected local fungal isolates (Alternaria and Trichoderma) by conducting multiplex polymerase chain reactions, The appearance of the bundles for each gene, where the length of the ATL 1 gene bundles is 510 base pairs, while the EXP gene bundles is 180 base pairs long. This confirms that the selected isolates contain their own gene that causes allergy and asthma, as shown in Fig (6).

During the investigation and detection of ATL and EXP genes, new genes were globally registered in the International Information Bank (NCBI), where both isolates were registered with the name of both the supervisor and the researcher, the international number of the first isolate Alternaria LC632931 and the second isolate Trichoderma has the international number LC632932, and they were named MIK-AYJ1 and MIK-AYJ2 which is an abbreviation of the name of the supervisor and the researcher as shown in the fig (7) and fig (8).



Figure 2. Extraction of DNA from Genomic DNA, 1: *Alternaria*, 2: *Trichoderma*.

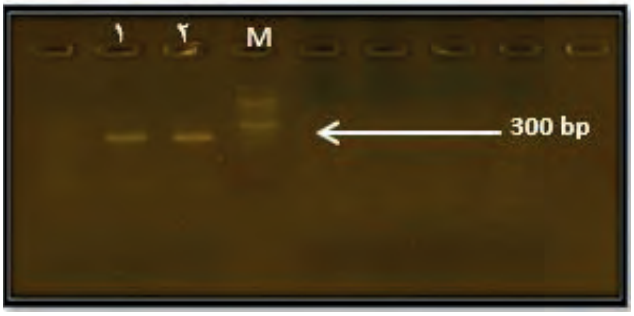


Figure 3. Amplification of ITS region, 1: Alternaria, 2: *Trichoderma*.

Alternaria alternata strain GRSH10 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

Sequence ID: KY788027.1 Length: 527 Number of Matches: 1

Range 1: 293 to 527 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
435 bits(235)	1e-117	235/235(100%)	0/235(0%)	Plus/Plus

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Query  1    TGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGTCTCC  60
Sbjct  293  TGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGTCTCC  352
Query  61   AGTTCGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAG  120
Sbjct  353  AGTTCGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAG  412
Query  121  CACAAGTCGCGCTCTCTTCCAGCCAAGGTCAGCATCCACAAAGCCTTTTTTCAACTTTTG  180
Sbjct  413  CACAAGTCGCGCTCTCTTCCAGCCAAGGTCAGCATCCACAAAGCCTTTTTTCAACTTTTG  472
Query  181  ACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA  235
Sbjct  473  ACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA  527
```

Figure 4. The sequences of nucleotides of *Alternaria alternata*.

Trichoderma atroviride internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: KM099499.1 Length: 1343 Number of Matches: 1

Range 1: 295 to 567 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
488 bits(264)	9e-134	270/273(99%)	0/273(0%)	Plus/Plus

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Sbjct  415  GCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGCGTCCAC  474
Query  181  GTCCGTAAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGA  240
Sbjct  475  GTCCGTAAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGA  534
Query  241  ACTTAAGCATATCAATAAGCGGAGGAAAAGAAA  273
Sbjct  535  ACTTAAGCATATCAATAAGCGGAGGAAAAGAAA  567
```

Figure 5. The sequences of nucleotides of *Trichoderma atroviride*.

Alternaria alternata has been significantly linked to asthma, and (21) have reported that *Alternaria alternata* is the most common allergen among those studied to date. It is considered one of the common biological pollutants in many countries and environments, it colonizes indoor environments, and the presence of these fungi in indoor environments makes people, especially those with immunodeficiency in these environments more susceptible to respiratory diseases, allergies and asthma, This is due to the ability of this fungus to produce Alt a1, which when inhaled provokes allergic reactions in patients allergic to mold. In this study, the Alt a1 gene was actually detected in *Alternaria alternata*, which was identical to researcher (3). As for the inhalation of the spores of this fungus by people who suffer from asthma or allergies, this will lead to severe attacks of shortness of breath. In contrast, the Trichoderma plays an essential role in human health, as it is responsible for many diseases and causes death to reach to the highest levels of 53% in immunocompromised patients. Several potential human pathogens have been reported, such as *Trichoderma atroviride, Trichoderma longibrachiatum, Trichoderma pseudokoningii* and *Trichoderma reese*, which cause sinusitis, skin infections, pneumonia and stomatitis, as referring from (5) that reported the first confirmed case of lung infection. Due to the genus Trichoderma, which appears as invasive pulmonary aspergillosis in the patient, also, some



Figure 6. Alt and EXP gene , 1,*Alternaria alternata*, 2: *Trichoderma atroviride*.

NCBI Resources How To

Nucleotide Nucleotide Advanced

GenBank Send to:

Alternaria sp. MIK-AYJ1 genes for 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence

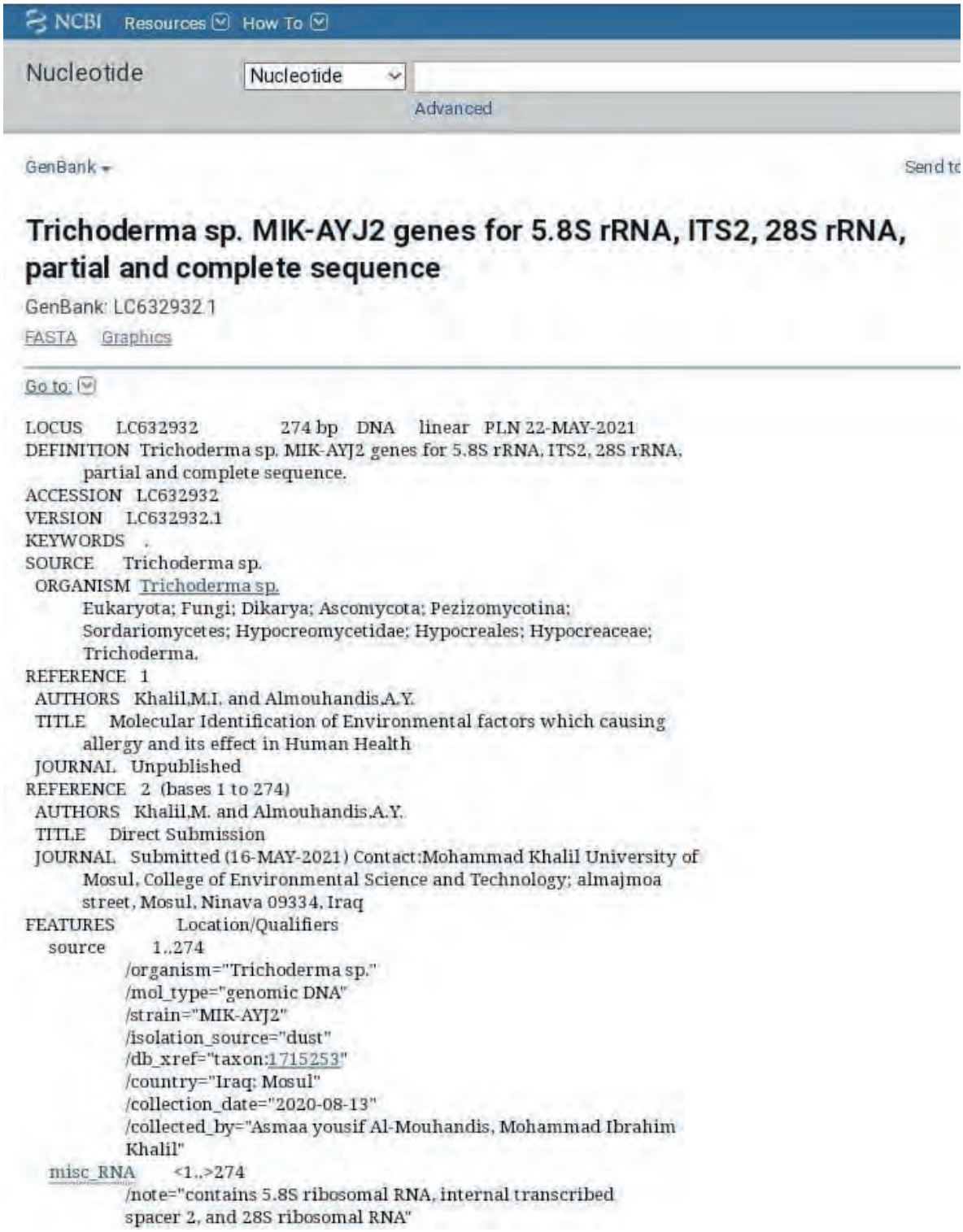
GenBank: LC632931.1

FASTA Graphics

Go to:

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ACCESSION   LC632931
VERSION     LC632931.1
KEYWORDS    .
SOURCE      Alternaria sp.
  ORGANISM  Alternaria sp.
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REFERENCE   1
  AUTHORS   Khalil,M.I. and Almouhandis,A.Y.
  TITLE     Molecular Identification of Environmental factors which causing
            allergy and its effect in Human Health
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 239)
  AUTHORS   Khalil,M. and Almouhandis,A.Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-MAY-2021) Contact:Mohammad Khalil University of
            Mosul, College of Environmental Science and Technology; almajmoa
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                     /collected_by="Asmaa Yousif Al-mouhandis, Mohammad Ibrahim
                     Khalil"
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                     /note="contains 5.8S ribosomal RNA, internal transcribed
                     spacer 2, and 28S ribosomal RNA"
```

Figure 7. *Alternaria aletrnata* NCBI BLAst.


NCBI Resources How To

Nucleotide Nucleotide Advanced

GenBank Send to

Trichoderma sp. MIK-AYJ2 genes for 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence

GenBank: LC632932.1

FASTA Graphics

Go to:

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VERSION     LC632932.1
KEYWORDS    .
SOURCE      Trichoderma sp.
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            Sordariomycetes; Hypocreomycetidae; Hypocreales; Hypocreaceae;
            Trichoderma.
REFERENCE   1
  AUTHORS   Khalil,M.I. and Almouhandis,A.Y.
  TITLE     Molecular Identification of Environmental factors which causing
            allergy and its effect in Human Health
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 274)
  AUTHORS   Khalil,M. and Almouhandis,A.Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-MAY-2021) Contact:Mohammad Khalil University of
            Mosul, College of Environmental Science and Technology; almajmoa
            street, Mosul, Ninava 09334, Iraq
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                     spacer 2, and 28S ribosomal RNA"
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Figure 8. *Trichoderma atroviride* NCBI BLAst.

people have been identified as having a respiratory allergy caused by Trichoderma fungus through skin prick tests of patients, where it was reported that the most critical respiratory allergen is *Trichoderma harzianum*[21].

Conclusions

There is a strong relationship between exposure to house dust fungi and asthma and allergies.

Household air conditioner filters contain a high percentage of respiratory fungi, including Cladosporium, Penicillium and Aspergillus

The extent of the importance of the polymerase chain reaction technology in detecting and investigating new genes and identifying mutations and strains for many organisms.

Fungi isolated from home contained Alta1 and Exp allergens.

The extent of the importance of air quality in the surrounding environment of humans.

Acknowledgments

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ARTICLE / INVESTIGACIÓN

Molecular diagnosis of *Streptomyces* genus and bioactive potential against pathogenic microbes

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DOI. 10.21931/RB/2022.07.02.24

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Abstract: This study (40) locally isolated the genus *Streptomyces* from soil samples collected from different regions of Iraq (Nineveh , Erbil , Duhok) and evaluated their antagonistic. The isolates were found to have bioactivity against gram-positive and negative bacteria and fungus. *Streptomyces* were isolated on (S.G. medium), and morphological similarities and the 16 srRNA sequencing were used to characterize them. The results of a polymerase chain reaction (PCR) with eight strands of DNA gene picked from local bacteria isolates in a volume range of (900–1000) base pairs. The nitrogenic base sequence determined the polymerase chain reaction products of DNA samples selected from 6 local isolates. These strands preserved the employed DNA ladder volume. According to DNA Blast NCBI data, the species are Streptomyces atrovirens, Streptomyces SP.S. coeuleroubidus, and Streptomyces bellus.

Key words: *Streptomyces*, Molecular, Pathogenic Microbes.

Introduction

Among the microbe, actinomycetes are one of the critical sources for the production of antibiotics. About two–Streptomyces alone[1] produce thirds of the antibiotics. *Streptomyces* are filamentous bacteria gram a positive with a high (G+C) content (70%) and found in all environmental[2].

The genome code includes more than 20 genetic groups for secondary metabolites with high medicinal potential, such as antibiotics, which help fight against microbes[3].

Many studies have found that Streptomyces has a considerable potential for producing secondary products such as antibiotics, growth factors, and pesticides[4]. Streptomyces can be distinguished from other actinobacteria by their 16 srDNA analysis and DNA - DNA hybridization[5].

Materials and methods

Collecting of samples

(20) Soil samples were gathered from various farms in Nineveh at depths ranging from 5 to 10 cm below the earth's surface. Calcium carbonate (CaCO3) treated the samples at a 1:10 ratio. They were then dried for 4 days at (40–45) degrees centigrade. Samples are collected in plastic bags, closed tightly, and then transferred to a Freezing container until needed[6].

Isolation of Streptomyces

(1) gram of dirt was well mixed in tubes with 10 ml of distilled water; then, a series of dilutions were performed. produced until the desired concentration was reached.10^{-6}, 1 ml was taken from the last dilutions (10^{-5} - 10^{-6}) and placed on culture medium Starch – casein medium (S.G.)

This was done three times for each sample (chilled to 45 degrees centigrade). The inoculum was then disseminated evenly using a sterile glass spreader and incubated for 7 days at 28 C0. Plates with 10–35 colonies were chosen, and the number of solitary colonies was re-cultured in the same medium to generate pure culture[7,8].

The media

Starch – casein medium

It was made by using the following ingredients: (18) gm Agar (10) gm starch, (0.3) gm casein, (2) gm KNO3, (2) gm NaCl, (0.02) gm CaCO3, (2) gm KH2 PO4, (0.05) gm MgSO4 7H2 O (0.01) gram FeSO4. 7H2O, in (1) liter distilled water with a pH of (7.2). After that, the Autoclave apparatus was used to sterilize them[9].

Nutrient agar medium

By melting 23 grams of the medium in (1) liter of distilled water with a pH of (7.2) and sterilizing everything in the autoclave, this medium was created according to (oxoid) the supply firm's recommendations.

Starch mineral salts media

The following ingredients were used to make it: (10) gm Starch, (2) gm (NH4)2 SO4, and (2) gm CaCO3. (1) gram of K2 HPO4, (1) gram NaCl, (20) gm Agar in distilled water with a pH of (7.0), and they were all autoclave sterilized[10].

2.3.4 Glycerol asparagine agar medium

Prepare this medium by dissolving: (1) gm asparagine, (10) gm glycerol, (1) gm K2HPO4, (20) gm agar, (1) ml of trace salt solution, (0.64) gm CuSO4. 5H2O. (0.11) gm FeSO4 . 7H2O, (0.79) gm MnCl2. 4H2O, (0.15) gm

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ZnSO4. 7H2O, The materials were dissolved in (1) liter of distilled water at pH (7.4) and sterilized in an autoclave[11].

Antibiotics Production Medium

The medium for enhanced antibiotic synthesis was created by mixing (0.8) gm NaCl, (1) gm NH4Cl, (0.1) gm K2HPO4, (0.2) gm MgSO4 7H2O, (0.1) gm CaCl2, (10) gm glucose, and (3) gm yeast extract. The materials were dissolved in (1) liter of distilled water at pH (7.3) and sterilized in an autoclave[12].

Isolation and characterization of Streptomyces

The Gram staining, colony morphology, and color of colonies, the starch mineral agar media, and using the slide culture technique been noticed the shape of the aerial and substrate mycelium, as well as the spore arrangement were used to characterize *Streptomyces*[12].

Assay the antagonistic activity of isolates in this experiment

Staphloccocus aureus Proteus vulgris, E. coli, Klebsilla, Pseudomonas aeruginosa (and pathogenic fungi (Candida albicans, Rhizoctonia solani, Fusarium solani, Alternaria alternata .

Antifungal activity against phytopathogenic fungi

To evaluate the effect of *Streptomyces* isolates on the growth mycelium of plant pathogenic fungi, the *Streptomyces* isolates were transferred to the middle of a petri dish, distributed vertically in the petri dish by a sterile loop and incubated at 28-+1 ^{0}C for (5) days, after the incubation period two discs from the eight-day-old fungal culture were transferred to half dishes (1.5) cm away from the growth line of the *Streptomyces* isolates. The control treatment contains fungi discs only, which are incubated 28-+ 1 for 5 days (the incubation period depends on the arrival of the mycelium fungi to the edges of the dish[13].

Antibacterial activity against bacteria pathogen

The bioactivity of the *Streptomyces* was studied against the microbial Agar diffusion method[14]. Asparagine glycerol agar plates were inoculated with Streptomyces isolates and incubated at 28-+ 1 ^{0}C FOR (7) days. After the isolates were grown, 8 (mm) discs were taken by a sterile cork borer from the developing colonies of *Streptomyces* and transferred to the nutrient agar medium inoculation with pathogen bacteria.

Diagnosis of selected samples of bacteria *Streptomyces*

The selected Streptomyces was diagnosed to the species level using the diagnostic tests[12,15-17].

Preparation of inoculation

The elements in the inoculation medium are the same as those in the production media. They were cultivated in autoclave-sterilized 250 mL flasks with 50 mL each. They were inoculated by transferring a tip of Streptomyces cultured on Glycerol asparagine agar to the inoculum. The flask was then shaken in a shaker incubator for 3 days at 140 rounds per second at (28-+1) Celsius.

Cultural Condition

In a 250 mL flask, 50 mL of antibiotic manufacturing media was created. The flasks were tightly sealed, and sterilization in the autoclave was conducted. They were all allowed to cool before injecting inoculum prepared from the 3-day-old chosen isolation at a 2:1 (v/v) ratio. The flaks were kept in an incubator vibrator for 7 days at (28+- 1) degrees Celsius and 240 round minutes.

Purification and acquisition of DNA from *Streptomyces*

The DNA from the *Streptomyces* samples was extracted using Geneaid's analysis kit.

PCR reactions: Genomic DNA Extraction

The T.E. (the solution was used to optimize the DNA concentration in all of the samples used in this experiment, and it was successful in obtaining the desired concentration for the PCR reaction to continue (50 nanograms per microliter).

In a (0.2 ml) Eppendorf tube provided by the British business, the DNA sample was mixed with the specific primer for each gene to make the master reaction for each PCR reaction (bio labs). Using distilled water, the reaction volume was set to 20 microliters, and the reaction was placed in a microfuge for 3–5 seconds. The tubes were then placed within a heat cycler, which used a customized program for each reaction to achieve the polymer reactions. The samples were then electrophoresed using a bio labs ladder in a well of a 2 percent agarose gel for 60–70 minutes, and the gel was filmed using Gel documentation.

Streptomyces molecular diagnostics based on 16srRNA amplification

To identify the amplification area, Add 4 microliters (100 nanograms) of DNA template and 1 microliter (10 picomols) of each gene primer were added to the final mix

Primer Forward: AAGCCC TGG AAACGGGGT

Primer Reverse : CGTGTGCAGCCC AAGACA

After that, the reaction tubes were placed in the thermal cycler, and the polymers reaction was carried out according to the program listed in the table. (1).

DNA Sequencing analysis

DNA sequencing is the most frequently and commonly utilized technique for finding mutations and SNP variants in DNA samples. The sequence of amplified portions of DNA is used to determine, and PCR reactions usually determine research mutations. DNA sequencing results have gotten more precise in determining mutations in recent years[18,19]. If the PCR reaction produces many strands, they are purified, and the desired section of the DNA is recovered from the gel; however, if the reaction produces just one strand, it will be the predominant strand and will be used to identify the sequence[20].

Using DNA sequencing to determine the nucleotides of the amplifier section

The genetic sequences of the samples were determined using the PCR technique outlined previously. Used in the study, and the primers were analyzed with the Hitachi 3130 Genetic Analyzer device. The results were analyzed using the BLAST tool, which compared the genetic sequences to those in the National Center for Biotechnology Information) NCBI(database.

Diagnosis

The Streptomyces samples were identified using the slide culturing technique, one of the best techniques (on a genus level) to display substrate and hyphae, which are the differentiating traits that identify thread-like bacteria[16].

NO.	Stage	Temperature	Time	Cycle number
1	Initial denaturation	95	6 min	1
2	Denaturation	95	1-30 min	35
3	Annealing	58	1-30 min	
4	Extension	72	2 min	
5	Final extension	72	5 min	1

Table 1. Shows the PCR reaction inside the thermocycler device.

The substrate hyphae are spore-free, well-branched, and unsegmented. While the aerial hyphae appeared to be a darker, thicker, and less branched thread than the substrate mycelium, the aerial hyphae have a sporophore containing chain of spores, which can be erect (rectus), spiral (spiral), or Crict with waves (rectus – flexible) depending on how these spores are arranged21. figure (1) .

When the isolates were cultivated on different media types, they revealed a variety of colors. They could not produce melanin and other colors, with gray colonies being the most common[21].

Genomic DNA polymers reaction

Using the forward primer, the following DNA,s reaction was performed on pure DNA acquired from organisms gathered from samples depending to the Geneaid protocol:

(AAGCCCTGGAAACGGGGT) And the revers:(CGTGTG CAGCCCAAGACA). (Maleki et al.,2013) In Figure 3, it can be seen that strands of pure DNA from the samples are of the same length (1000–9000) base pairs generated from the Streptomyces DNA specified polymer process. It has been discovered that they have the same length, indicating that there are nitrogenous base sequences in DNA that are mutual.

These isolates' genomic DNA can link with the primer and continue the reaction, resulting in additional DNA strands of the same length. These results are similar to what was found[22] When utilizing the polymers reaction approach to identify local bacterial samples and acquire a positive result, the length of the DNA created was (1000) nitrogenous base pairs, and so on. When using the polymers reaction method to analyze a bacterial local sample identification, the length of the DNA obtained was 1000 nitrogenous base pairs and so on (μ) marker evaluating and assessing the sequence of nitrogenous bases obtained from particular PCR., from local samples.

The findings of the specific polymer process on isolated DNA from local samples were forwarded to the NCBI, where they were compared to sequences in the gene bank figure (4).

Antifungal activity against phytopathogenic fungi

In this study of the ability of *Streptomyces* isolates to inhibit plant pathogenic fungi Table (2). The isolates *Streptomyces satrovirnes* showed inhibit against fungi (F*usarium solani , Rhizoctonia solani , Alternaria ignifica , Candida albicans*), while the isolates *S. coeuleorubidus*, S. sp, *S. bellus, inhibited fungi C. Albicans, F. solani, and A. alternate* respectively Figure(5).

The *Streptomyces* can be biologically controlled by resistance / or killing fungi and bacteria pathogens; they are often Bioactive before the pathogen thoroughly infects its host. Recently a focus has been placed on different methods of developing biocontrol strategies against soil-borne fungi[23]. For example, the seed of the *Arabidopsis thaliana*.

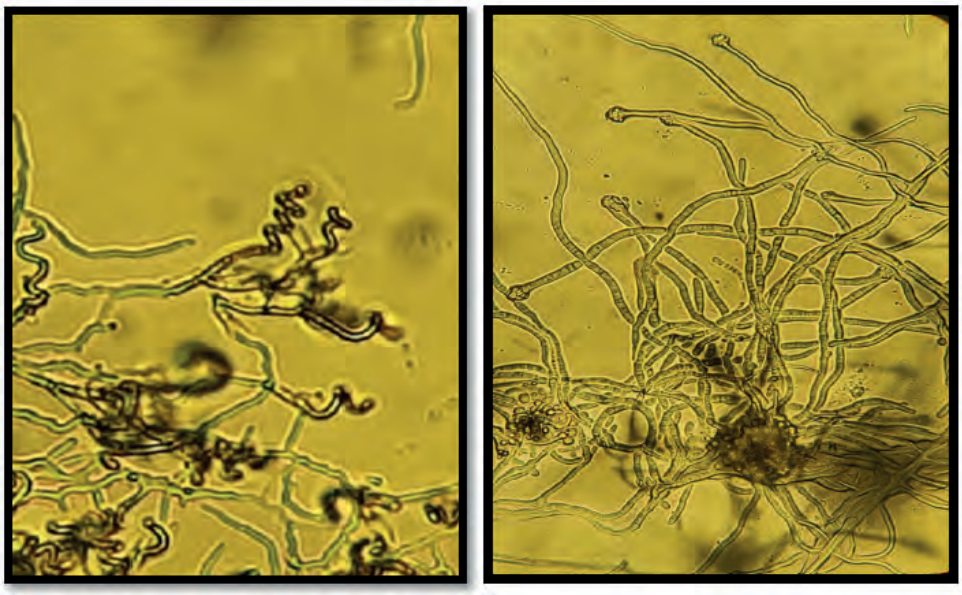


Figure 1. The shape of the substrate and aerial mycelium of *Streptomyces* SPP.



Figure 2. The shape of colonies and pigment production of *Streptomyces*.

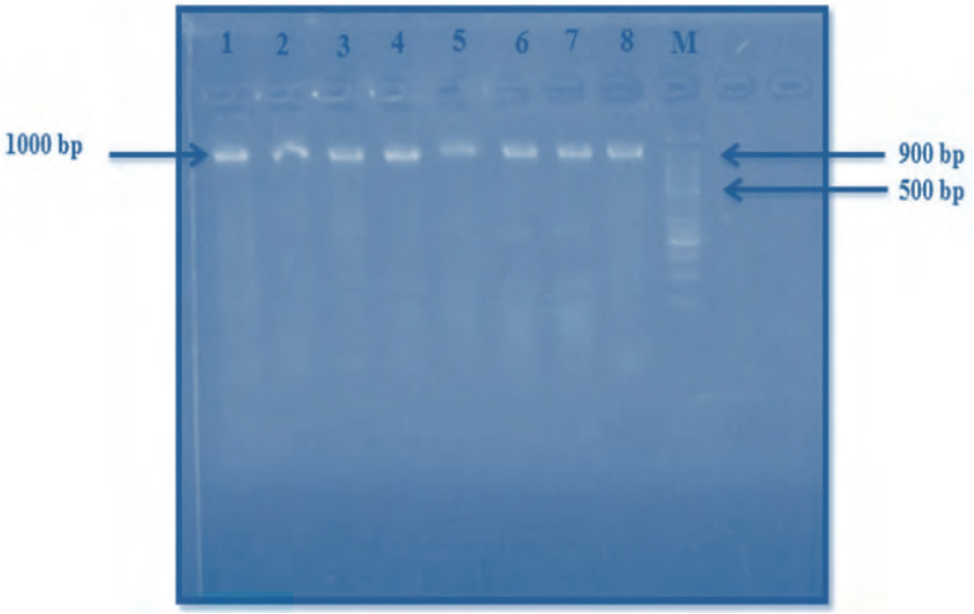


Figure 3. The specific polymerase reaction of *Streptomyces spp.* Primer 16s r RNA.

Before sowing, treatment with Streptomyces and Micomonospora SP was protected from infection by *Erwinina carotovra* and *F. oxysporum*.

It has been observed that *Streptomyces* have an antagonistic ability to stimulate the defense mechanisms in the plant[24].

In this study[25]. Indicated a signification inhibition of mycelium growth in dual culture, using three strains of *Streptomyces*. S.P.ALP 07R, Streptomyces S.P. MRA 1W, against six types of plant pathogenic fungi *Sclerotinia sclerotiorum, Fusarium oxysporm, f. S.P. Lactuca, Rhizoctonio solani Thielvopsis basicola, Phytophthora S.P. Pythium ultimum*. According to (26), one strain of Streptomyces was found among Streptomyces' one hundred seven isolates. *In vitro* and *in vivo*, S.P. SI RO3 reduced the growth of mycelium *Pestalotipsis theae* by a percentage percent 86.15 percent 93.85, respectively.

Antibacterial activity against bacteria pathogen

In this study, the biological activity of *Streptomyces* against some pathogenic bacteria was also evaluated. The *Streptomyces Bellus* give inhibition against all pathogenic bacteria used in this study, while the *Streptomyces* SP. and *S.atrovirence* were effective against all bacteria except Klebisla Penumonia Table (3). The researchers[27]. Showed that the inhibition extracted by acetate was effective against E SBL – producing *E- coli*. Numerous studies have indicated that the metabolites produced by actinomycetes showed high activity against Gram-negative, including *E. coli*[28]. The *Streptomyces* SMO1 gives a high inhibition against bacteria *Staphylococcus aureus* at a concentration (5 ml) when grown on medium AIA at P.H. (7) for 7 days compared with Streptomycin (5 mg) ampicillin (5 mg) also indicated that among 8 isolates, one isolates S MO1 give inhibition against six bacteria strains except for *K. pneumonia* and *S. typhimurium*[29]. However, due to a double membrane barrier and brown transmembrane efflux, Gram-negative bacteria are more resistant to antimicrobial agents than Gram-positive bacteria[28].

Conclusions

Among the results obtained from both aspects, the molecular study proved its effectiveness and usefulness for plant breeders and the building block for the success of any plant breeding program and the shortening of time in determining the appropriate varieties to conduct crosses, improving the characteristics of the yield.

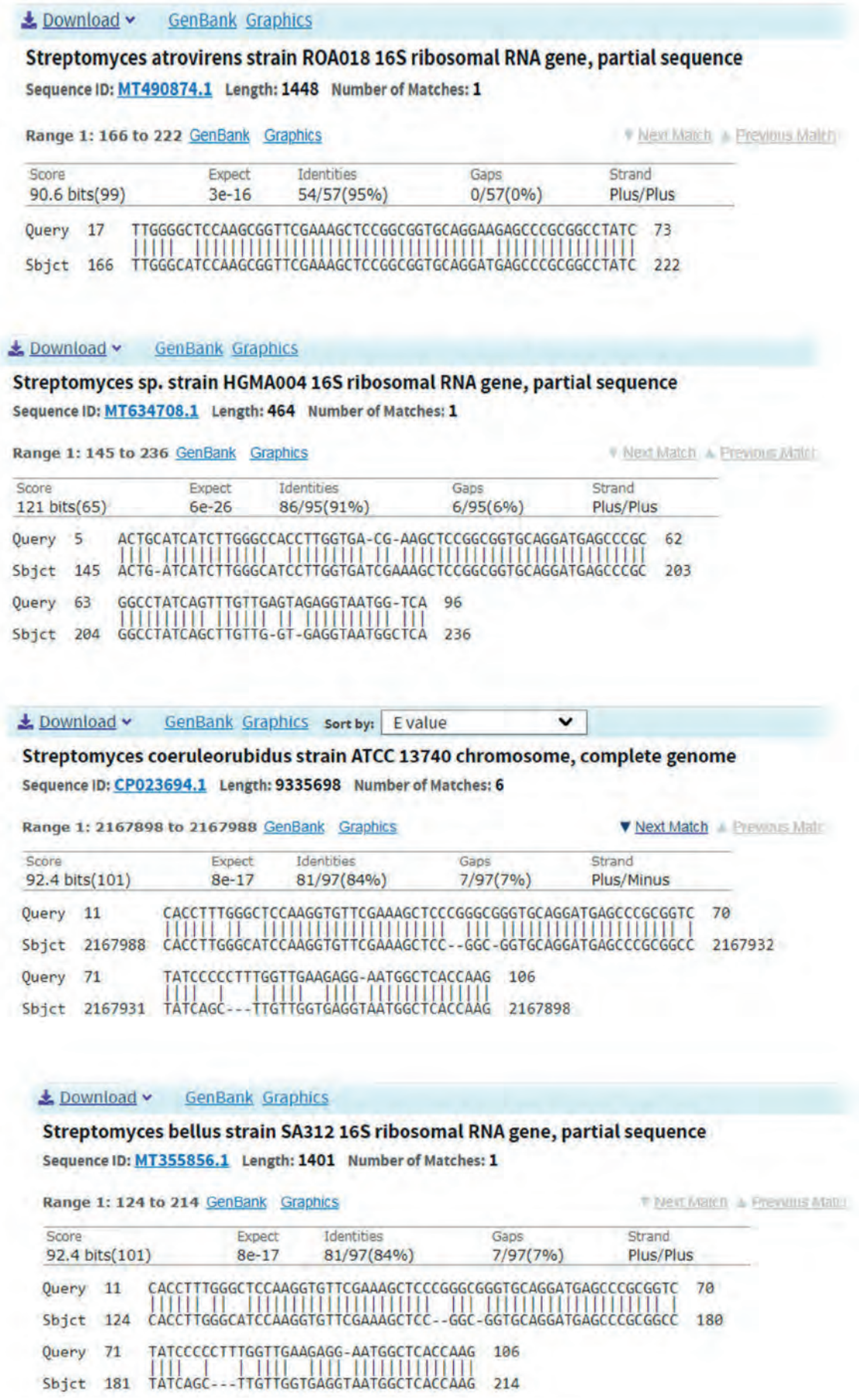


Figure 4. Shows an analysis of the nitrogen base sequence of the gene bank.

Fungi	*Streptomyces SP.*	*S. atrovirens*	*S.Ceouleroubidus*	*S. bellus*
Fusarium Solani	–	14	10	–
Rhizoctonia Solani	–	15	–	–
Alternaria Alternate	–	13	11	8
Candida Albicans	17	14	–	15

Table 2. The effect of *Streptomyces* isolates of phytopathogenic fungi (mm).



Figure 5. The effect of *Streptomyces* on phytopathogenic fungi.

Bacteria Pathogen	*Streptomyces_SP.*	*S. atrovirens*	*S. Ceauleroubidus*	*S. Bellus*
Staphylococcus Aureus	12	16	–	13
Escherichia Coli	14	11	–	13
Klebsiella Pneumonia	–	–	17	11
Pseudomonas aeuraginosa	14	15	11	14
Proteus Vulgaris	11	12	12	11

Table 3. The effectiveness of *Streptomyces* on bacteria pathogen inhibition zone (mm).

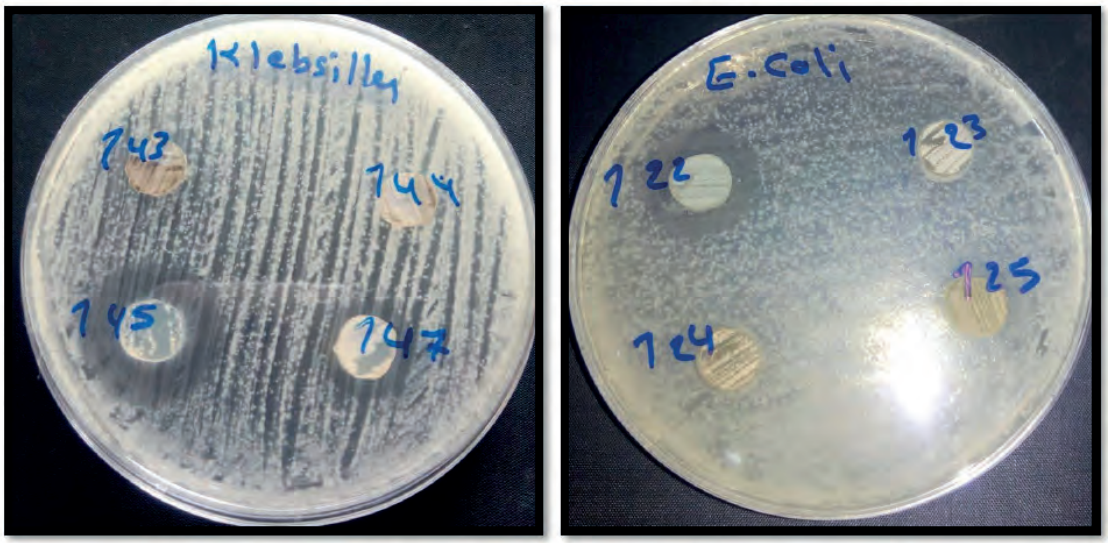


Figure 6. The effect of *Streptomyces* isolates on pathogenic bacteria.

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Conflicts of Interest

There is no conflict.

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REVIEW / ARTÍCULO DE REVISIÓN

Mecanismos de defensa en plantas de *Capsicum* contra *Phytophthora capsici*

Defense mechanisms in *Capsicum* plants against *Phytophthora capsici*

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Resumen:P*hytophthora capsici* es un oomiceto causante de la pudrición de raíz, tallo, frutos y tizón foliar en varias especies vegetales de importancia agrícola, principalmente en Solanáceas del género *Capsicum* como ají y pimiento. Este fitopatógeno cosmopolita posee mecanismos de ataque que favorecen la rápida infección, colonización y reproducción en huéspedes susceptibles. Contrariamente, estos procesos son retrasados o evitados fuertemente por genotipos resistentes, debido principalmente a sus mecanismos de defensa. En esas interacciones incompatibles, las plantas resistentes de *Capsicum* reconocen el oomiceto y rápidamente expresan múltiples genes que posteriormente señalizan moléculas, que permiten la acumulación de compuestos fenólicos, fitoalexinas y especies reactivas de oxígeno, la actividad de diferentes enzimas, que pueden permitir incluso la formación de barreras físicas. Esta revisión aborda, expone y discute los avances y el progreso de las investigaciones a lo largo de los ultimos veinte años, referente a los mecanismos de defensa estructurales, bioquimicos y moleculares que utilizan las plantas resistentes de *Capsicum* para defenderse de *P. capsici*.

Palabras clave: Ají, pimiento, pudrición de raíz y corona, tizón foliar, resistencia vegetal.

Abstract:P*hytophthora capsici* is an oomycete that causes root, stem and fruit rot, and leaf blight in several plant species of agricultural importance, mainly in Solanaceae of the genus *Capsicum* as chili pepper and pepper. This cosmopolitan plant pathogen has attack mechanisms that favor a fast infection, colonization, and reproduction in susceptible hosts. In contrast, these processes are strongly delayed or avoided in resistant genotypes, mainly due to their effective defense mechanisms. In these incompatible interactions, the resistant plants of *Capsicum* recognize the oomycete and rapidly express multiple genes that subsequently signal molecules, which allow the accumulation of phenolic compounds, phytoalexins, and reactive oxygen species, activity of different enzymes, which can even allow the formation of physical barriers. This review address exposes and discusses the advances and progress of research over the last twenty years regarding the structural, biochemical, and molecular defense mechanisms *Capsicum* resistant plants use to defend against *P. capsici*.

Key words: Chili pepper, pepper, root and crown rot, leaf blight, plant resistance.

Introducción

Phytophthora capsici es un oomiceto descrito en 1992 por Leonian[1], causando pudrición de raíz y tallo en plantas de ají (*Capsicum chinense* Jacq.). Desde su reporte, ha expandido su rango de hospederos hasta superar las 70 especies vegetales que incluyen Solanaceas del género *Capsicum* (*C. baccatum, C. chinense Jacq. y C. frutescens L y C. annuum L)*[2,3]. En condiciones ambientales favorables, las enfermedades causadas por *P. capsici* pueden reducir totalmente la producción de frutos[4]. Además, el patógeno puede sobrevivir en ausencia del huésped, mediante la producción de oosporas, estructuras de hibernación que forman el inóculo primario y así iniciar la infección en plantas susceptibles[5]. Las plantas infectadas presentan amarillamiento y marchitamiento repentino en hojas, al limitarse la absorción y transporte de agua, producto de la destrucción del sistema radicular (pudrición)[6]. De igual manera, tallos, y frutos también pueden verse afectados[7], por lo que el complejo manejo del oomiceto deriva en el uso de fungicidas (oomiceticidas) de forma indiscriminada, afectando esta práctica negativamente el agroecosistema a largo plazo. Así, el uso de genotipos resistentes se vislumbra como la estrategia más conveniente y eficaz para el control de la pudrición de raíz y cuello (PRC)[8]. Hasta hace algunos años atrás, se conocía la presencia de fuentes de resistencia a *P. capsici* únicamente en variedades criollas de pimiento CM334 y de ají habanero criollo naranjal[9,10].

En la actualidad, existe evidencia de resistencia al oomiceto en diferentes genotipos criollos y comerciales de *Capsicum* en algunos países del mundo como México[11] y Ecuador[3], que son poco o nada afectados por el patógeno, mostrando incluso una tenue colonización de tejidos de la raíz y del hipocótilo. La interacción entre plantas y fitopatógenos son asociaciones complejas que se caracterizan por el reconocimiento de patrones moleculares asociados a patógenos por parte del sistema inmunológico de una planta y las respuestas posteriores por efectores secretados codificados por patógenos[12,13]. En *Capsicum*, el exiguo avance del patógeno en sus tejidos, podría resultar de la activación de mecanismos de defensa como la acumulación de compuestos fenólicos[14], actividad de peroxidasa y fenilalanina

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amonio-liasa[15,16], contenido de lignina y biosíntesis de fitoalexinas[15], acumulación de transcritos[9], formación de barreras físicas[17,18], entre otros.

Las fuentes de resistencia y el conocimiento de la interacción entre *Capsicum spp*. y *P. capsici*, mejorarían notablemente la durabilidad de esta en los genotipos resistentes, principalmente en condiciones de campo[3]. Aunque existe información científica sobre mejoramiento genético de *Capsicum spp.*, los mecanismos de defensa relacionados a la resistencia a *P. capsici* han sido abordados con poca frecuencia, exeptuando algunos trabajos que han sido cubiertos en esta revisión como los de Castro, Fernández, y Osuna[19] y Chávez-Díaz y Zavaleta-Mejía[20], que exploran los mecanismos de defensa y la comunicación molecular en el patosistema *Capsicum spp. - P. capsici*.

Esta revisión aborda, expone y discute los avances y el progreso de las investigaciones a lo largo de los ultimos veinte años, relacionado especificamente a los mecanismos de defensa bioquimicos, fisicos y moleculares, usados por plantas de *Capsicum spp.* para defenderse de*Phytophthora capsici*.

Etiología

Phytophthora capsici, taxonómicamente pertenece al Reino Chromista, Phylum Oomycota, Clase Oomycetes, Orden: Peronosporales, y Familia Peronosporaceae[10], ubicándose en el clado número 2, dentro de los diez propuestos para *Phytophthora* en el 2008 por Blair[21], guardando estrecha relación filogenética con otras especies como *P. mexicana*, *P. tropicalis* y la nueva especie P. sp. “glovera”. El patógeno posee micelio cenocítico y produce zoosporangios ovoides, elipsoides y papilados que contienen zoosporas reniformes y biflageladas[10,22], que pueden ser liberados rápidamente, aislados o agrupados, cuando están bajo estrés térmico[23]. Este oomiceto también produce un gametangio masculino (anteridio) y otro femenino (oogonio), con los que mediante meiosis forman oosporas (esporas sexuales), que pueden servir como estructura de hibernación[24]. Aproximadamente la mitad de las especies del género *Phytophthora* son homotálicas o auto fértiles, mientras que las restantes como *P. capsici* son heterotálicas, lo que contribuye al proceso de recombinación genética entre especies de *Phytophthora*, como, se describe entre *P. capsici* y *P. drechsleri*, en *Petunia* x *hybrida* como hospedero[25,26].

Sintomatología

Los síntomas observados en varios órganos y en diferentes etapas fenológicas vegetativas o reproductivas (Figura 1), pueden estar asociados a la agresividad de P. capsici, a los niveles de resistencia del genotipo, y a las condiciones climáticas i.e. clima cálido y húmedo, temperaturas entre 25 y 30 °C y humedad relativa entre 60 a 80%[2,27]. El oomiceto puede afectar prácticamente todos los órganos de ají o pimiento[6,25,28,29].

Entre los síntomas observados están las lesiónes distintivas de color negro-marrón tanto en raices (Figura 1A) como en la corana (cuello, Figura 1B), pudiendo incluso visualizarce lesiones empapadas de agua en esta última región. Una vez que los tallos de las plantas son infectados, se desarrolla un marchitamiento irreversible que comienza en el follaje, propagándose luego a la parte aérea de la planta (Figura 1C). Todas estas afectaciones derivan en la muerte anticipada de las plantas (Figura 1D). En las hojas pueden observarse pequeñas manchas de color verde oscuro, que a medida que avanza la infección se agrandan y se decoloran, denominándose este síntoma como tizón foliar (Figura 1E). Mientras tanto, los frutos infectados al inicio muestran una coloracion verda oscura, que posteriormente se tornan marrón con manchas oscuras acuosas cubiertas de micelio y esporas de color blanco del patógeno (Figura 1E).

Mecanismos de defensa en *Capsicum spp*.

Durante el proceso evolutivo los fitopatógenos han desarrollado mecanismos para obtener nutrientes de su hospedante. En respuesta, las plantas presentan barreras físicas (figura 3), químicas y moleculares (figura 4) para dificultar el progreso de esos microorganimos[20], desarrollando

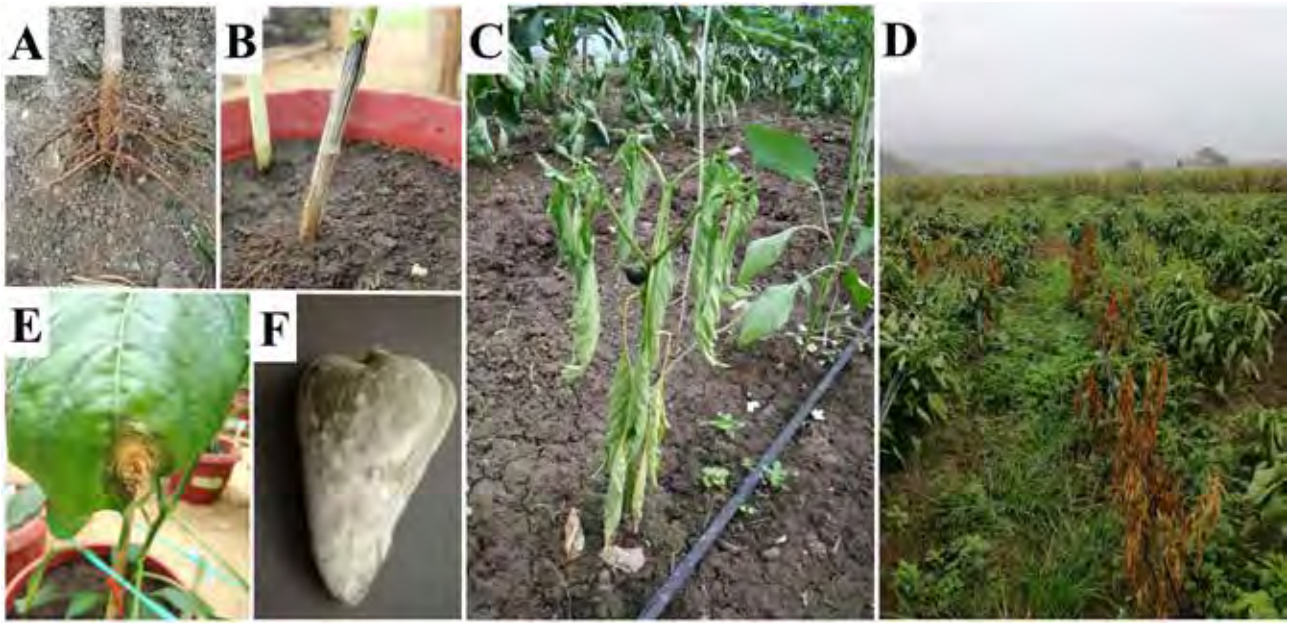


Figura 1. Síntomas de pudrición de raíz (A) y tallo (B), marchitez (C) y muerte (D) en plantas, tizón foliar (E), y pudrición de frutos (F), en *Capsicum spp.* causados por*Phytophthora capsici*, en condiciones controladas (A, B, E y F) y de campo (C y D).

la capacidad de reconocer Patrones Moleculares Asociados al Daño (DAMP), así como a Microbios o Patógenos (MAMP o PAMP, respectivamente), a través de Receptores de Reconocimiento de Patrones (PRRs) localizados en la membrana celular[12,13]. *P. capsici* secreta efectores (proteinas o moléculas) citoplasmáticos durante la infección del huesped, para manipular su fisiología y defensa, como las proteinas crinkler (CRN) y la quitina (polisacárido), que se trasladan a las células vegetales, para alcansar exclusivamente el núcleo del huesped[12,30]. La familia de proteinas CRN pueden modificar la señalización de la célula huésped en sus distintos compartimentos subnuclares, poseyendo una importancia en la patogénesis de los oomicetos en las plantas, participando en la muerte celular inducida por PAMP y aumentando la virulencia de *P. capsici*[13]. Por otro lado, la quitina, componente habitual de la pared celular de hongos verdaderos, puede estar presente en zoosporas y esporangios liberados de *P. capsici*, empleandose como un PAMP sobre *Arabidiopsis thaliana*[30].

Otros efectores como el PcAvh1 induce la muerte celular no solo en pimiento morrón, sino tambien en *Nicotiana benthamiana* y tomate[31]. Así mismo, el RxLR48 facilita la infección suprimiendo incluso la señalización de ácido salicílico (SA) y de la inmunidad activada por patrones (PTI) en *A. thaliana*[32]. Otro efector como PcAvh103, puede facilitar la infección mediante la supresión de la inmunidad de la planta, siendo necesario para la virulencia de *P. capsici*[33].

En una interacción incompatible, genotipos resistentes de *Capsicum* tienen la capacidad de retrasar o evitar el crecimiento de *P. capsici* en sus tejidos[8]. Como resultado, las plantas presentan síntomas imperceptibles, o casi nulos tanto interna como externamente, en tejidos subterraneos o aereos, debiendose, posiblemente a que el oomiceto llega con un retraso considerable a los tejidos superiores como hipocótilos[3]. Así la colonización y los síntomas producidos por *P. capsici* es mayor e intensa en tejidos susceptibles comparado con los resistentes (Figura 2).

Los posibles mecanismos bioquímicos, físicos y moleculares usados por plantas resistentes de ají o pimiento para defenderse de *P. capsici* son descritos a seguir.

Mecanismos estructurales (físicos)

Estos mecanismos son la primera barrera en la defensa vegetal, apoyados por el reconocimiento genético y la activación de compuestos bioquímicos, que producen cambios estructurales para fortalecer los tejidos en órganos afectados por *P. capsici*, derivando en una serie de eventos con la finalidad de detener o reducir la infección del oomiceto (Figura 3).

Refuerzo de la pared celular

La cutícula es la primera barrera física que presentan las plantas para contrarrestar los fitopatógenos. Esta capa protectora situada en la superficie más externa en las partes aéreas de plantas superiores está constituida principalmente de los polímeros lipofílicos cutina y ceras cuticulares, los cuales son alterados tanto en su composición como ultraestructura por factores genéticos, fisiológicos y ambientales[4]. Aunque el refuerzo de la cutícula es un mecanismo físico presentado por las plantas para frenar el avance de la infección de fitopatógenos, organismos hemibiotrofos como *P. capsici* podrían fácilmente superar esta barrera y continuar con la infección[5,18].

Ante esto es posible el reforzamiento o endurecimiento de la pared celular especialmente de celulas epidérmicas, mediante la acción de multiples enzimas como las peroxidasas (POX), que sintetizan compuestos como lignina y suberina, generando rigidez en las paredes celulares y téjidos, frenando el progreso de la infección de *P. capsici* en getipos resistentes (Figura 3)[19]. Además, el engrosamiento o formación de paredes de la periderme (producida ante heridas), formados entre la parte inferior de las células epidérmicas y superior de las células corticales de *Capsicum*, resulta en un aumento del grosor de la laminilla media, evitando la colonización cortical por parte del oomiceto (Figura 3)[17]. Finalmente, aunque el polisacárido calosa o callosa sea un mecanismo bioquímico, su acumulación en células vasculares y medulares puede blindarlas, frenando el avanace de *P. capsici* (Figura 3)[18].

Formación de aposiciones

Una de las características estructurales más prominentes en plantas resistentes son la formación de aposiciones (papilas) en la pared celular, localizadas en el sitio de infección de *P. capsici*. Estas papilas gruesas y densas encierran al haustorio del oomiceto restringiendo su desarrollo y penetración en los tejidos (Figura 3)[36]. En algunas ocasiones se ha podido observar daño celular del oomiceto dentro aposiciones, sugiriendo que este mecanismo además de blindar a la célula, pueden estar compuestas de enzimas como β-glucanasas (GLU) y quitinasas (CHI), conocidas por degradar la pared celular de fitopatógenos[20].

Producción de exudados

Las paredes celulares de raíces de genotipos susceptibles son parcialmente degradadas estructuralmente por la acción enzimática de *P. capsici*. En contraste, en tejidos resistentes el fitopatógeno es atrapado por exudados radicales en la rizodermis de raíces, compuestos de proteínas y polisacáridos, que se acumularían cerca de la laminilla media de células rizodérmicas, permitiendo no solo el control de microrganismos nocivos sino también la comunicación con plantas vecinas (Figura 3)[17]. De cualquier manera, la cantidad del patógeno en tejidos radiculares no siempre se correlaciona con la resistencia o susceptibilidad en *Capsicum*. De hecho, en un trabajo reciente donde se estudió la colonización de tejidos por *P. capsici*, empleando herramientas convencionales y moleculares se encontró que el patógeno coloniza de forma más intensa los tejidos subterraneos resistentes que los susceptibles, mientras que en tejidos del hipocótilo fue lo opuesto[3].

Resistencia relacionada con la edad

Este mecanismo puede ser observado tanto en plantas[28] como en frutos[37]. En plantas, la susceptibilidad a la enfermedad puede estar relacionada con la edad de las plantas, es decir, plantas adultas son más resistentes que las jóvenes, debido principalmente a la dureza de los tejidos por el aumento de la lignificación. En el caso de frutos, la susceptibilidad disminuye al aumentar la etapa de maduración (senescencia), debido a que la cutícula de frutos rojos es mucho más gruesa que la de los verdes, constituyendo un factor de resistencia estructural contra *P. capsici*. Este engrosamiento es correlacionado por el aumento de POX, contribuyendo con la polimerización de compuestos similares a la lignina, y formando una barrera ante la infección (Figura 3). En frutos de otros cultivos como calabaza (*Cucurbita duchesne*), generalmente muy susceptible a *P. capsici*, se ha observado una alta correlación entre la formación de una cutícula y epidermis gruesas en frutos maduros[38].

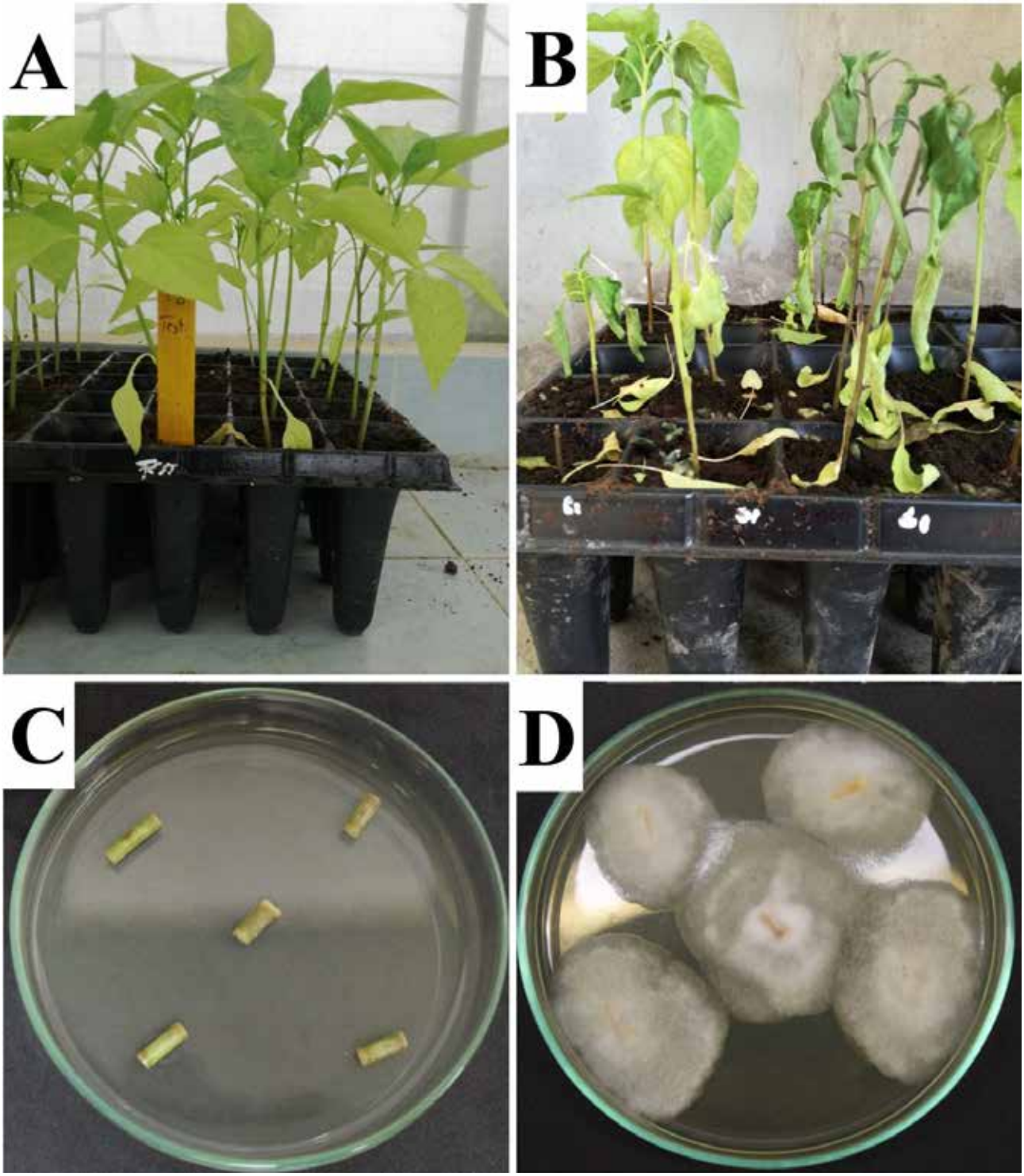


Figura 2. Síntomas de amarillamiento tenue en hojas bajeras (A), marchitamiento generalizado (B), y colonización de hipocótilos (C y D) en plantas resistentes (A y C) y susceptibles (B y D) de pimiento a los 17 días despúes de la inoculación conP*hytophthora capsici*.

Mecanismos bioquímicos

Las plantas pueden responder al ataque de fitopatógenos, mediante la producción de sustancias enzimáticas o precursores y sus productos, especies reactivas de oxígeno, fitoalexinas y hormonas. Estas sustancias logran crear un ambiente altamente tóxico, o a su vez intervienen en la deposición del refuerzo de la pared celular, formando parte de la barrera mecánica frente a fitopatógenos[39].

Capsidiol

El capsidiol (sesquiterpno antifúngico, $C_{15}H_{24}O_2$) es una fitoalexina aislada por primera vez a partir de frutos de pimiento infectados por diversos fitopatógenos[40], siendo producida en diferentes especies de *Capsicum*, y formada tanto en la vía Isoprenoide a partir de genes biosintéticos EAS (5-epi-aristolochene sintasa) y de enzimas como hidroximetilglutaril-CoA reductasa (HMGR, EC:1.1.1.34), así como en la vía del mevalonato (ácido mevalónico) donde

participan los genes *HMGR2*[41,42] (Figura 4). En frutos de pimiento, el capsidiol también puede ser inducido por el tratamiento exógeno de peróxido de hidrógeno (H_2O_2), junto con una respuesta que incluye la producción de especies reactivas de oxígeno (ROS)[43].

La fitoalexina puede ser fungitástica o fungitóxica a niveles de 3,8 o <5 mM, siendo esta concentración equivalente a 1180 µg de capsidiol mL^{-1}, correspondiendo aproximadamente a la concentración observada a los 6 dias despues de la inoculación (DPI) con *P. capsici* (204 µg mL^{-1}), en el área necrosada del genotipo resistente de pimiento Smith-5 (Figura 5). De igual manera, el genotipo CM334 presenta concentraciones elevadas de capsidiol a las 12 y 24 horas posterior a la inoculación (HPI) con el oomiceto[15].

Calosa

La calosa es un polisacárido producido principalmente en los plasmodesmos de células vegetales a partir de la enzima Calosa sintasa (CalS, EC 2.4.1.34), para blindar la pared y frenar el avanace de *P. capsici* en las células vasculares y medulares, reflejandose daños menores en plantas resistentes debido a su deposición, sirviendo además en la comunicación celular por su acumulación cercana a los sitios de interconección celular (Figura 5)[18,44]. En trabajos como el de Piccini *et al.* (2019)[18], se observó una deposición de este polisacárido únicamente en células xilemáticas de plantas resistentes, infectadas y no infectadas por *P. capsici*. sugiriendo ser un importante mecanismo de defensa/resistencia contra el oomiceto.

Enzimas involucradas en la biosintesis de compuestos fenólicos

La pared celular vegetal es una estructura compleja que actúa como una barrera física, restringiendo el acceso a la mayoría de microbios hacia el interior de la célula, debido principalmente a la acumulacion de compuestos fenólicos como la lignina, polímero hidrófobo de monolignoles sintetizados en el citoplasma y transportados al apoplasto, cuya funcion es dar duresa y rigidez a la pared celular, sintetizada por la activación de enzimas[45]. Por lo general, el nivel de enzimas aumenta en tejidos vegetales para contrarestar la infección de fitopatógenos vehiculados por el suelo[46].

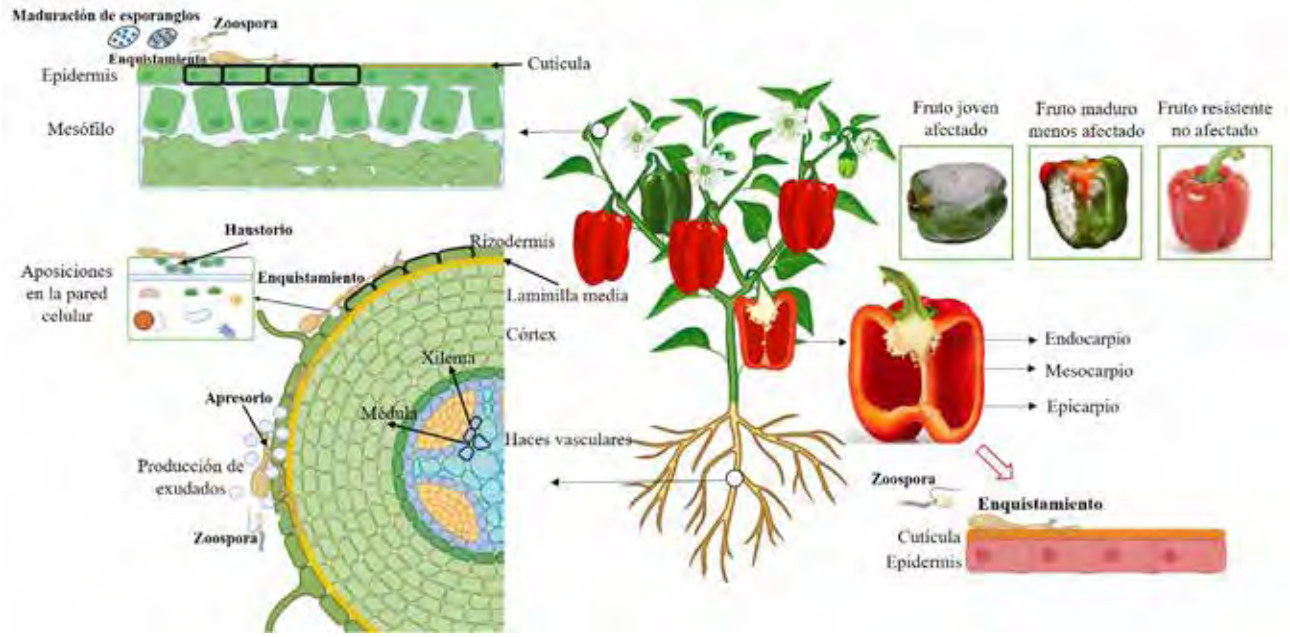


Figura 3. Mecanismos de defensa estructurales (físicos) en plantas de *Capsicum spp.* contra P*hytophthora capsici.* Defensa física de la celula por el engrosamiento de la pared celular en la epidermis y rizodermis[20]. Aumento de la laminilla media entre las células epidérmicas y corticales para limitar el avance de las hifas del fitopatógeno[17]. Formación de aposiciones (papilas) en células rizodermicas que encierran el haustorio de *P. casici*, limitando su movilidad e incluso degradando sus estructuras[36]. Producción de exudados radiculares compuestos de proteínas y polisacáridos que reprimen el desarrollo de la infección por el oomiceto[17]. Resistencia asociada a la edad en frutos maduros debido al aumento del grosor de la cutícula[37].

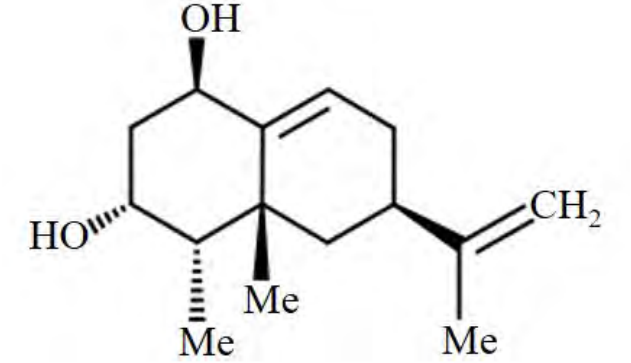


Figura 4. Estructura de la fitoalexina capsidiol, un sesquiterpeno encontrado en tejidos de *Capsicum spp.*

Peroxidasas

El grupo de las peroxidasas (POX, EC 1.11.1.14) como guayacol peroxidasa (GPX, EC 1.11.1.7), ascorbato peroxidasa (APX, EC 1.11.1.11)[46], entre otras, son las principales enzimas presentes en el sistema antioxidante de plantas, transformando varios compuestos en productos oxidados o polimerizados, como la lignina (Figura 5), a partir de la sintesis de mognolignoles mediante actividad redox (H_2O_2 + $2AH_2$ + H_2O + 2. AH)[47]. En tallos y hojas de plantas resistentes de ají a *P. capsici*, se ha observado el constante incremento de POX a los 2 DPI, elevándose su actividad 36 % más a los 6 DPI. El aumento de la actividad de POX es tanto a nivel extracelular como en la pared celular, indicando un papel clave en la resistencia local y sistémica en *Capsicum* contra *P. capsici*[48,49].

Polifenol Oxidasa

Polifenol Oxidasa (PPO, EC 1.14.18.1) está involucrada en la lignificación, la oxidación de compuestos fenólicos existentes y su posible esterificación en la pared celular durante la invasión de fitopatógenos hemibiotrofos (Figura 5), participando además en la respuesta de defensa hipersensible[50,51]. En raíces de *Capsicum*, puede observarse un incremento de la actividad de PPO a los 3 DPI con *P. capsici* como una respuesta temprana de la infección. Además, como resultado del ataque del patógeno existe una ruptura de la membrana que puede también inducir la formación de quininas (alcaloide antimicrobiano)[48].

Fenilalanina amonio liasa

La fenilalanina amonio liasa (PAL, EC 4.3.1.5) es una enzima clave involucrada tanto en el primer paso de la vía de fenilpropanoides que convierte la L-fenilalanina en ácido transcinámico[52], como en la biosíntesis de fenoles, conduciendo principalmente a la producción de lignina en las células vegetales. La actividad de PAL puede aumentar en raíces, hojas y tallos de plantas resistentes de *Capsicum* a los 2 o 3 DPI con P. capsici[16,53]. Inclusive se ha comprobado un aumento de los niveles de compuestos fenólicos y de la actividad enzimática de su precursor PAL (figura 5) al sexto DPI con el oomiceto[49,54].

Enzimas antimicrobianas (β-1,3-glucanasa y quitinasa)

La enzima β-1,3-glucanasa (GLU, EC 3.2.1.39) hidroliza las paredes celulares de patógenos fúngicos comúnmente en combinación con otra enzima, la quitinasa (CHI; EC 3.2.1.14)[55], y pertenecen al grupo de proteinas PR (PR2 and PR3, respectivamente). La enzima GLU inhibe el crecimiento micelial (Figura 5) y la formación de esporangios de *P. capsici* en plantas resistentes como CM334, detectándose concentraciones máximas de 77.2 µg a las 24 HPI, mediante la degradación de glucanos (β-1,3-glucanos) presentes en la pared celular del oomiceto, y que actúan como una MAMP que desencadenan la defensa basal de la planta[56,57] (Figura 5). De hecho, una acumulación temprana y rápida de GLU está asociada con la reacción de defensa que se desarrolla en tallos de pimiento infectados con *P. capsici*[58]. Incluso esta enzima puede actuar sinergicamente con otras para defenderse de oomicetos, por ejemplo, con PAL para inhibir el crecimiento micelial y la formación de esporangios del oomiceto[56].

Por otro lado, la quitinasa (EC 3.2.11.14) CaChiIV3 encontrada en tejidos de pimiento cv. Yanshan01, además de estar relacionada con β-1,3-glucanasa, esta enzima es producida por la planta para degradar la quitina de la pared celular de *P. capsici*, o funcionar como un receptor, desencadenando respuestas de defensa en la planta[59].

Otras enzimas vegetales

Transglucaminasas

Las transglucanasas (TGasas, EC 2.3.2.13) son una clase de enzimas que catalizan el entrecruzamiento de cadenas peptídicas mediante la formación de enlaces isopéptidos entre los donantes de amina y el grupo γ-carboxiamida de las cadenas laterales de glutamina[60] (Figura 5). Aunque la actividad de TGases se observe tanto en el xilema (pared celular cercana a la laminilla media) de plantas resistentes como susceptibles de pimiento a *P. capsici*, esta es mayor en las resistentes, fortaleciendo la pared celular y evitando que las hifas penetren dichas células (Figura 5)[18].

Nitrato reductasa

La enzima nitrato reductasa (NR, EC 1.6.6.1) puede generar óxido nítrico (NO) principalmente a partir de NO^-_2, jugando un papel en las respuestas de defensa inducidas tanto por PAMPs y mecanismos de gen por gen[61]. En *C. chinense* la actividad de NR aumenta constante y progresivamente entre 6 y 72 HPI con *P. capsici*, incluso en plántulas tratadas con SA y etefón (ET), pudiendo esta respuesta ser relacionada como un mecanismo de defensa específica del huésped (Figura 5)[9]. Sin embargo, el hallazgo de que *P. capsici* induce la actividad de NR dependiente de NADH en plantas susceptibles, podría significar que la vía de defensa mediada por el ON podría ser manipulada por el patógeno[62]. Así, no estaría aun totalmente clara la actividad de esta enzima en plantas de *Capsicum*.

Regulación de la muerte celular y especies reactivas de oxígeno

La muerte celular o respuesta hipersensible (HR) es una de los principales mecanismos en la defensa vegetal contra patógenos, pero su inducción no siempre es necesaria, de tal manera, varias enzimas se involucran en inducir y mediar la producción de especies reactivas de oxígeno (ROS) *i.e.* H_2O_2, O^-_2 y OH^-, principales moléculas inductoras de HR[49,63]. Este metabolismo oxidativo es regulado por un mecanismo enzimático complejo, que incluye NADPH-oxidasa (NOX, EC 1.6.3.1), GPX y lipoxigenasa (LOX, EC 1.13.11.12) como productoras de ROS, y superóxido dismutasa (SOD), APX y catalasa (CAT, EC 1.11.1.6) como captadores[46].

Un aumento de la actividad tanto de la enzima LOX como de SOD (dependiente de Calcio – Ca^+), puede ser observado en genotipos resistentes de *Capsicum* como 23CherryP-Orsh a los 7 DPI con *P. capsici*[20,54], siendo de suma importancia dentro de la producción de ROS. Así mismo, NOX puede mediar la muerte celular por peroxidación lipídica, sirviendo además de moléculas de señal. Además, O^-_2 producido por LOX puede fusionarse con el NO formando peroxinitrito (ONOO-), que puede ser tóxico para varios patógenos[20,46].

Otra enzima como CAT puede aumentar su actividad en plantas resistentes en comparación a las susceptibles, tanto al inicio de la infección, como hasta los siete DPI con altas concentraciones de inoculo de *P. capsici*, limitando la excesiva producción de H_2O_2 y la peroxidación lipídica[16]. Esto hace que se evite el estrés oxidativo en la planta y la muerte celular innecesaria por la degradación de la mem-

brana plasmática, gracias a la actividad de enzimas como CAT y POX (Figura 5)[20,54]. La infección causada por patógenos vehiculados por el suelo, puede modificar el metabolismo oxidativo de manera diferente en tejidos de susceptibles y resistentes[46].

Fitohormonas

Las fitohormonas son sustancias producidas en las células vegetales que se acumulan en cantidades variables tras el reconocimiento de microbios, dando lugar a la activación de diversos mecanismos de defensa. La resistencia inducida por fitohormonas como SA es más eficaz contra patógenos biótrofos ya que su acción va dirigida a la muerte celular, mientras que la resistencia inducida por ácido jasmónico (JA) y etileno (ET) es generalmente operativa contra el ataque de patógenos necrótrofos, pudiendo incluso servir como moléculas de señalización en la defensa vegetal (Figura 5)[64]. De hecho, en cultivares resistentes como CM334, la expresión de JA aumenta en hojas hasta alcanzar los 80 mg g^{-1} a los 30 minutos posterior a la inoculación, alcanzando su nivel máximo a las 24 HPI. Sin embargo, a medida que los niveles de JA disminuyen los niveles de SA aumentan drásticamente, dando paso a una muerte celular mediada también por la producción de H2O2 (Figura 5)[65].

De igual forma, ET es otra hormona que estaría correlacionada con la resistencia de *Capsicum* a *P. capsici*. Por ejemplo, la aplicación exógena de ET induce resistencia a *P. capsici*, 24 horas antes de la infección con el oomiceto, reduciendo los sintomas en hasta un 30%, y aumentando la actividad de PR1, un marcador genético relacionada a la resistencia sistémica adquirida (SAR). Con este se deduce que la planta requiere de ET para activar su defensa vegetal, ya que al bloquear el receptor de la hormona no se manifiesta la resistencia. Así mismo, la acción ET es justificada también por los aumentos o cambios transcripcionales presentados en plantas resistentes, como *CaPTI1*, conocido como un factor de transcripción de respuesta al ET, involucrado en la expresión de resistencia a *P. capsici*[66,67].

Mecanismos moleculares

Patógenos como *P. capsici* necesitan inhabilitar los procesos nucleares de defensa del hospedero, provocando alteraciones en el proteoma nuclear. Sin embargo, en plantas resistentes se producen cambios a nivel molecular que inhiben el proceso de infección del patógeno, mediante la activicación de mecanismos como los genes R, que deriva en la síntesis y producción de diferentes compuestos antimicrobianos[68,69]. Por ejemplo, en pimiento se han descubierto nuevos genes candidatos expresados diferencialmente (DEGs) denominados *XLOC*, relacionados al transporte de azucares y activación de POX (Figura 5)[70].

Líneas resistentes a *P. capsici* como PI201234 presentan 211 genes candidatos involucrados en respuestas de defensa, que participan en la modificación de la pared celular, la biosíntesis de fitoalexinas, el desarrollo de síntomas y las vías de señalización de fitohormonas[71]. Además, la resistencia presentada por la línea PI201234 contra la raza 2 de P. capsici podría estar controlada por *CaPhyto*[72]. Este y otros hallazgos en PI201234 hace pensar que la resistencia al oomiceto podría ser de tipo horizontal, por conferir inmunidad tanto a la raza 1 como a la 2 de *P. capsici*[73].

Receptores de reconocimiento de patrones

En una interacción compatible de *Capsicum – P. capsici*, las plantas no activan una eficiente respuesta de defensa, permitiendo al patógeno infectar, colonizar y reproducirse en sus tejidos, y finalmente producir los síntomas característicos. El patógeno hemibiotrofo produce diversos péptidos y moléculas pequeñas conocidas como efectores, que usa para manipular o inhibir las respuestas de defensa del huésped. Uno de esos es el efector SCR82 (proteína rica en cisteína), que en interacciones incompatibles genera un aumento de la agresividad del oomiceto mediante una regulación positiva de ROS[74].

Contrariamente, las plantas resistentes (interacciones incompatibles) desarrollan una inmunidad activada por patrones, como los receptores de reconocimiento de patrones (PRR), localizados en la membrana plasmática de las células del huésped i.e. repeticiones ricas en leucina (LRR) y MAPK quinasas, y pueden percibir moléculas extrañas y emitir alertas al interior de la célula, activando diferentes mecanismos de defensa (Figura 5)[20]. Además, en semillas, raíces, tallos y hojas de *Capsicum spp.* se han encontrado expresándose genes putativos de quitina inducidos después de la infección de *P. capsici*, destacándose *CaChiV3* y *CaChiIV1*, que señaliza positivamente la muerte celular y regula la expresión de genes PR (relacionados con la patogénesis), respectivamente (Figura 5)[75,76]. Estos genes son codificados por proteínas quitinasas que interactúan con la proteína quinasa citoplasmática, similar al receptor CaPIK1 ubicado el retículo endoplásmico (Figura 5). Esta proteína mejora las respuestas a *P. capsici* i.e. muerte celular, y producción de ROS y NO[75]. Por otro lado, en *Arabidopsis thaliana* el efector PcAvr3a12 de *P. capsici*, es dirigido al retículo endoplásmico para suprimir la expresión de la proteína FKBP15-2 relacionada a la inmunidad vegetal[78]. Así, se podría inferir que en Capsicum dicho efector podría comprometer la expresión de CaPIK1, afectando la defensa al oomiceto.

Proteínas similares a la calcineurina B (CBL) pueden también interactuar con proteínas quinasas (CIPK). En el genoma de pimiento se han encontrado nueve genes *CaCBL* y 26 *CaCIPK*, y su expresión de la mayoría de estos se alteró cuando las plantas estuvieron expuestas a *P. capsici*. De hecho, la sobreexpresión de *CaCIPK1* potencia la acumulación de H_2O_2 y la muerte celular programada, así como la expresión de genes relacionados con la defensa vegetal (Figura 5)[79].

Factores de transcripción

Los factores de transcripción (TFs) son proteínas de unión a ADN específicas y de secuencia, que reconocen elementos específicos en los promotores de genes diana para controlar su expresión ante estímulos exógenos como patógenos, y activar procesos bioquímicos y fisiológicos (Figura 5) en plantas[80]. Recientemente, se encontró que la inoculación de P. capsici en raíces de pimiento induce la expresión del TFs *CanTF* (factor de transcripción de *C. annuum*), regulando a su vez la expresión de genes relacionados con la defensa como *CaPR1*, *CaDEF1* y *CaSAR82*, y el aumento de la actividad de POX (Figura 5)[81].

Familias de multigenes también se desempeñan como TFs, por ejemplo, WRKY. Estos TFs actúan directamente en la resistencia de pimiento contra *P. capsici*. La expresión de estos genes en las plantas, podrían dividirse en tres subgrupos: respuesta temprana (3 HPI), respuesta media (24 HPI) y respuesta tardia (>24 HPI). El silenciamiento de genes como *CaWRKY03*-7, *CaWRKY06-5* y *CaWRKY10-4*, aumenta significativamente la susceptibilidad de pimiento a *P. capsici*, incluso en genotipos resistentes[82]. Otros TFs

como los relacionados a la respuesta al etileno (ERF), así como los genes miembros de la familia APETALA2/ERF, ambos pueden ser inducidos por moléculas de señalización como el SA, JA y H_2O_2, destacando genes como *CaPTI1* y *CaAP2/ERF064*[66,83].

Loci de resistencia

Se conoce que la resistencia en *Capsicum spp.* contra *P. capsici* es controlada por numerosos *loci* en el cromosoma 5, en especial los de rasgos cuantitativos (QTLs)[84]. Mediante un mapeo de QTLs se determinaron 3 *loci* (5.1, 5.2, 5.3) que producen los efectos principales de resistencia, logrando identificar 2 genes de dominio Quinasa RLK (PHT81227.1 y PHT81229.1), 2 de unión de nucleótidos y de dominio de repetición ricos en leucina NBS-LRR (PHT81215.1 y PHT81216.1), y uno de la proteína precursora SAR8.2 (S05_28825990), convirtiéndolos en fuertes genes candidatos en la resistencia de *Capsicum* a *P. capsici* (Figura 5)[85].

En genotipos resistentes de *Capsicum* a *P. capsici* como CM334, aparte de los genes tipo NBS (sitio de unión de nucleótidos)-ARC (apoptosis, proteínas R, CED-4), 32 de ellos agrupados en los cromosomas 3, 5 y 7 y exhibiendo respuesta a la infección del oomiceto, también se encontró la presencia del gen *Snakin-1* (Figura 5)[86]. Parece ser que los genes de la proteína NBS-ARC-LRR constituyen la mayoría de genes predominantes de resistencia a enfermedades (genes *R*).

Conclusiones

Phytophthora capsici es uno de los principales agentes causales de enfermedades en cultivos pertenecientes al género *Capsicum* alrededor del mundo, especialmente ají y pimiento, debido principalmente a su agresividad (ataca prácticamente todos los tejidos), y poder de diseminación, infección, colonización y reproducción. De tal manera, se ha venido usando genotipos resistentes que poseen diferentes mecanismos de defensa que actúan de manera aislada o conjunta para contrarrestar al oomiceto. Esta revisión explora los principales mecanismos de defensa físicos, bioquímicos (enzimáticos o no), y moleculares que

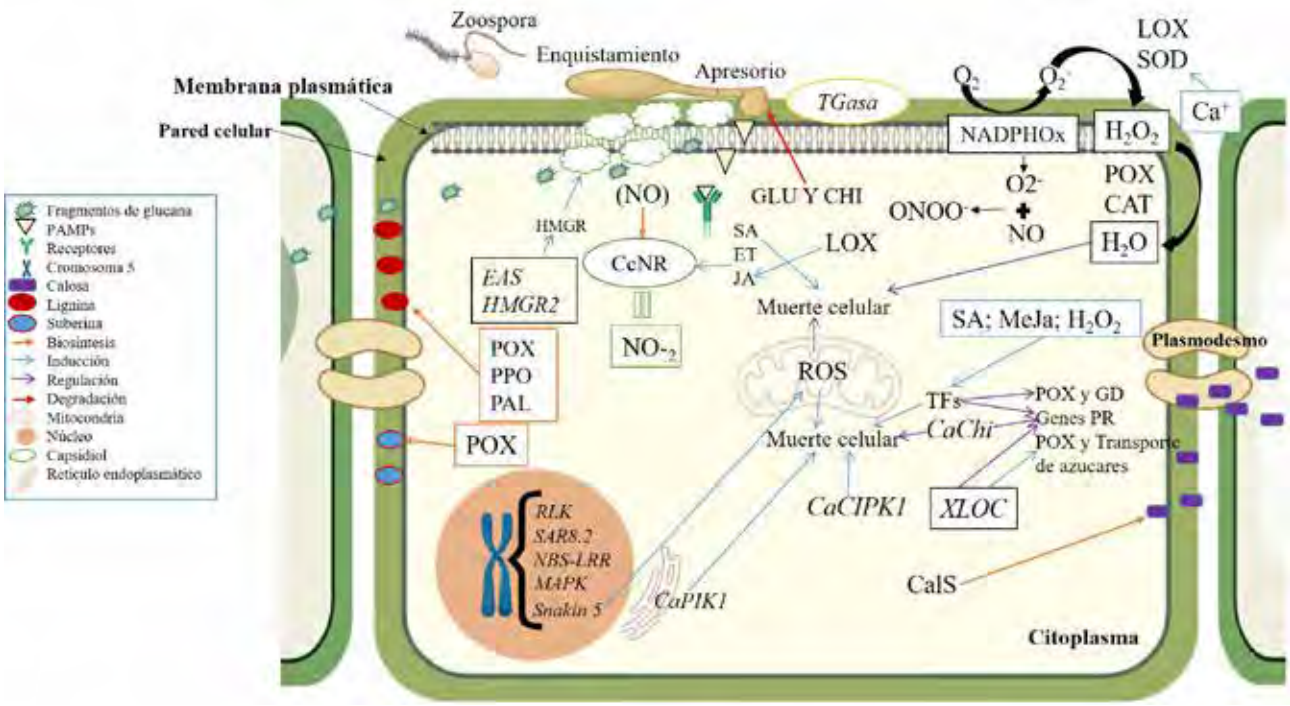


Figura 5. Mecanismos bioquímicos y moleculares de resistencia en *Capsicum spp.* contra*Phytophthora capsici.* El reconocimiento de DAMPS por PRRs genera señales mediante DF (ET-JA)[64] y genes como *CaChiV3*[75], activación de fitoalexinas como Capsidiol[40,43] a partir de la enzima HMGR (inducida por genes *EAS y HMGR2*)[41,42], además de la inducción de las enzimas POX[48,49], PPO[48] y PAL[16,53,49,54], involucradas en la biosintesis de compuestos fenólicos que podrían blindar la pared celular, al igual que CalS en la producción de calosa, cuya deposición cercana a los plasmodesmos posibilita la comunicación intercelular[18,44]. De igual manera TGasa, otra enzima presente en la pared celular que cataliza el entrecruzamiento de cadenas peptídicas mediante la formación de enlaces isopéptidos entre donantes de amina y el grupo γ-carboxiamida de las cadenas laterales de glutamina[18,60]. GLU y CHI actúan directamente degradando las estructuras del oomiceto como β-1,3-glucanos y quitina, respectivamente, presentes en su pared celular, pudiendo incluso actuar como MAMPs, desencadenando la defensa basal de la planta[56-59]. CcNR inducida por ET y JA, interviene en la biosintesis de nitrógeno, elemento esencial en la defensa vegetal[9,62]. Producción y regulación de HR por el complejo enzimático NADPHOx, GPX y LOX como productoras de ROS, y SOD, APX y CAT como captadoras o reguladoras[46], proceso en el que además se genera ONOO- que es tóxico para varios patógenos[20,46]. También puede evidenciarce la muerte celular inducida por SA[64]. TFs pueden regular la actividad POX[81], genes PR[83], genes de defensa[81] y de respuesta al ET[66]; genes como *Snakin-1*[86], genes R de tipo RLK, NBS-LRR y MAPK[87,88]. La sobre expresión de otros genes como *CaPIK1*[75,77] (ubicado en el ER) y *CaCIPK1* pueden potenciar la acumulación de H_2O_2[78]. Así mismo, el gen *CaChiV1* regula la muerte celular y la expresión de genes PR[74]. Finalmente, genes como *XLOC* actúan tanto en la regulación de la actividad POX, genes PR y el transporte de azucares[70].

hacen que el patógeno infecte, colonice o se reproduzca lentamente en los tejidos vegetales. A pesar de los avances actuales y recopilados en la presente revisión, aun es amplio el panorama por explorar concerniente al estudio de posibles mecanismos de defensa en genotipos resistentes a *P. capsici*.

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ARTICLE / INVESTIGACIÓN

Study the quality of drinking water in the holy city of Karbala

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Abstract: This study aims to assess the quality of drinking water in Karbala. Furthermore, the quality of tap and bottled water are compared in this study. Materials and method: Physical, chemical, and bacteriological parameters were all measured in the study. The bacteriological test includes a total count of coliform and fecal coliform, which may be detected using standard microbiology procedures. Results: In bottled water, total dissolved salts (TDS), Ca, Cl, and Mg were within acceptable ranges, like that in tap water, the mentioned parameters were within normal ranges except for regions Aborwayah, Al-wend, Al-khayrat and Al-husainia which were beyond the expected value. In terms of bacteriological tests, coliform bacteria were found in bottled water (Al-katara water and Al kafeel) samples with no *E. coli* bacteria. In the case of tap water samples, the findings revealed that most of the regions (9 out of 12 = 75%) had coliform bacteria growth, so (4 out of 9 = 44. 4%) had E. coli bacterium growth does not comply with the approved standards for drinking water, which must be free of the microbial content of *E. .coli* and coliform. Conclusion: According to the study, drinking water treatment operations are not up to par, particularly in locations outside of the city center, and bottled water is preferable to tap water for drinking.

Key words: Drink water, coliform, TDS, Chloride, turbidity.

Introduction

Water is a significant natural resource globally, and sustaining human life is the essential thing on earth[1]. The physicochemical characteristics of water are often a crucial factor in determining and assessing the quality of drinking water. Various chemical and physical criteria may be used in the evaluation of water quality. Several guidelines were formulated to individually test the chemical and physical parameters of the quality of drinking water (World Health Organization, 2004[2]. Transmitted by disease-causing microbes via drinking water is primarily of fecal origin, and they are recognized as enteric pathogens[3,4]. The World Health Organization (WHO) criteria indicate that no drinking water should contain any microorganism considered to be pathogenic or some other microorganism fecal pollution-indicative bacteria[5].

Approximately 250 thousand people die every day worldwide due to diseases (typhoid, cholera, etc.) caused by unsafe water use, according to WHO (2006) evidence. Problems with water supplies have been one of the most critical concerns throughout human life[6]. As a result of poor treatment and disposal of waste, industrial discharge and overuse of limited water resources, pollutants such as bacteria, viruses, heavy metals, nitrates and salt have made their way into water supplies[6]. Working in close cooperation with the Government of Iraq for many years, WHO and UNICEF have been profoundly concerned about this situation. They are keen to provide assistance to improve the health status of the general population and minimize morbidity and mortality through activities aimed at protecting and promoting the quality and protection of the supply of water[2]. To ensure that water supplied to customers is well regulated and follows national standards and WHO guidelines on drinking water quality, an expanded network of water quality control laboratories has been established. The study aims to analyze and compare the quality of drinking water in the areas studied in Karbala city.

Materials and methods

Sample Collection

Eighteen water samples were collected from different places in Karbala governorate from November 2020 up to September 2021, where sex samples of bottled water distributed the samples and twelve samples of tap water, and regions numbered from 1 to 18 as in table (1).

The samples were collected in germ-free plastic bottles, taken to the laboratory and analyzed directly.

Chemical analysis

A turbid meter device HACH 2001N was used to measure the turbidity of the water. In addition, total dissolved salts (TDS) were estimated using the inolab-cond 7110 devices and the pH of the water using a pH meter. The chloride, magnesium, and calcium concentrations were determined using the titration method.

Bacteriological analysis

Many culture media were used for isolation of bacteria such as nutrient agar (Oxoid) for total aerobic bacterial count, MacConkey agar (Himedia) for coliform count and

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No.	Region name	Type of water
1	Al-kawther	Bottled water
2	Al- harameen	Bottled water
3	Salsabeel	Bottled water
4	Al-Katara	Bottled water
5	Al-kafeel	Bottled water
6	Al-wareth	Bottled water
7	Al-Khadeer	Tap water
8	Al-mowathafeen	Tap water
9	West of Al-abaseia	Tap water
10	Al-seglah	Tap water
11	Aborwayah	Tap water
12	Eshan	Tap water
13	Al-wafaa	Tap water
14	Al-wend	Tap water
15	Karbala	Tap water
16	Al-khayrat	Tap water
17	Al-husainia	Tap water
18	Al-Imel quqrter	Tap water

Table 1. Numbering of regions involved in the study.

brain-heart infusion agar (Himedia) for fecal streptococci count. These media were sterilized in an autoclave at 121 C^0 for fifteen minutes. Samples were appear growth at tubes of multiple fermentation tube technique cultured on the prepared medium in duplicate and incubated aerobically at 37 C^0 for TBC and TC. The colonies were counted and expressed as colony-forming unit's per100 milliliter (CFU/100ml) of the samples as described by APHA (1998). The presence of these bacteria was expressed in the results table with the presence or absence (+/-). Various biochemical tests were carried out for bacterial identification; in addition, the diagnosis of *E. coli* was confirmed by the VITEK-2 technique.

Results and discussion

The chemical analysis results of some biochemical parameters of drinking water (Table 2) show normal ranges for all parameters with the mean values. A high turbidity value appeared in Al-kawther region (4.9) and a lower reading at Al-kafeel region (2); these results reach the acceptance of Iraqi limits specification (417)[7] and the WHO guidelines for drinking-water quality[2]. also, the results have come in contact with other Iraqi researches[1,6,8] in Baghdad, Erbil and Hilla respectively. TDS shows a common mean value and recordings in all regions with a high record at (247) and low at Al-Katara (38), also, the result was acceptable in Iraqi limits specification and WHO[2,7] and (1,6,8). The normal pH range for drinking water is between 6.5 – 8.5; the measurement of pH was normal in all regions except for the region that appeared highly alkaline with a pH 8.5 in salsabeel sample, and the lower pH was in Al-Katara (6.6) which appeared near to results of researches in Baghdad, Erbil, Diwanyia and Al-Kut[1,3,4,8]. Chloride (Cl) is added as a disinfectant to water which leads to the formation of acids[8]; the purpose is to kill pathogens causing disease, such as bacteria, viruses, and protozoans, that commonly grow in water supply reservoirs[10], with a high level in salsabeel (71 mg/l) and the lower level was in Al-Katara (14 mg/l), all of these are within normal level according to Iraqi limits specification (417)[7] and the WHO guidelines for drinking-water quality[2], and agree with many studies[1,6,8] in Iraq. Ca++ and Mg ions are also within acceptable and safe levels; the results were agreed with other studies in Iraq, Baghdad[8,11,12], Erbil[1,13].

Table (3) shows the presence of bacterial contamination in drinking water. Only two regions (Al-Katara and Al-kafeel) found bacterial coliform contamination over the zero average counts, and all samples were negative for *E. coli.* These results found agreement with other Iraqi studies[8] in Baghdad, Mosul[14] and Hilla[6].

The study also investigated the tap water for chemical constituent and bacterial contamination, as shown in tables (4 and 5) in which there was a high variation in chemicals level and coliform bacteria contamination with or without the presence of *E. coli*. The turbidity index shows a standard value except in region 1 (5), and the results disagreed with a study in Baghdad that found that some regions were above (60)[15]. All regions were at the threshold of TDS, and three of them were above (1000), which appear near the same study results in Baghdad. The primary sources of high TDS in river water may result from agricultural runoff, soil erosion, house waste, and other human activities[18]. The other parameters

Regions No.	Turbidity	TDS mg/L	pH	Cl mg/L	Mg mg/L	Ca^{++} mg/L
1	3.6	216	7.4	63	20	2
2	3.1	247	8.5	71	16	5
3	3.9	38	6.6	14	4	2
4	2	214	7.8	49	12	5
5	3	175	8	28	6.4	8
6	4.9	102	7.1	35	8	5
Norma value	Up to 5	33-524 mg/L	6.5-8.5	Up to 250 mg/L	Up to 75 mg/L	Up to 100 mg/L

** TDS: Total Dissolved Solids*

Table 2. Results of chemical analysis of bottled drinking water (RO) samples in different Karbala regions

Regions No.	Coliform cfu/100ml	*E. coli* cfu/100ml
1	-	-
2	-	-
3	-	-
4	+	-
5	+	-
6	-	-

* (+) contain coliform bacteria over normal count.

Table 3. Results of bacteriological investigation of drinking water samples in different Karbala regions.

Regions No.	Turbidity	TDS mg/L	pH	Cl mg/L	Mg mg/L	Ca^{++} mg/L
7	5	914	8.5	95	100	24
8	4.5	954	8	113	80	46
9	4.8	937	8.5	96	80	29
10	3.2	940	8.4	120	64	40
11	3	1051	8.3	106	120	29
12	4.7	958	8.3	106	72	28
13	4	865	8	127	88	34
14	4.5	1048	8	120	80	34
15	2.5	925	8.1	127	72	33
16	5	1088	8.4	149	96	38
17	4	1017	8.1	114	132	21
18	3.4	909	8.5	127	96	34
Normal value	Up to 5	Up to 1000 mg/L	6.5-8.5	Up to 350 mg/L	Up to 100 mg/L	Up to 150 mg/L

Table 4. Results of chemical analysis of tap water samples in different Karbala regions.

show an undulation between normality and abnormality; the pH was within normal standardized but tended to alkalinity with a mean value (8.3). The calcium also appeared normal in all regions, Mg was above stander measurement in three regions (7, 11 and 17), and the Ca^{++} showed normality. These parameters converge with that study in Baghdad[15].

The bacterial analysis of tap water shows a diversity of contamination with coliform bacteria in several regions, and only three regions (7, 15 and 18) give negative culturing of coliform bacteria. This indicates poor water sanitation in Karbala in general; these results also agree with other Iraq studies (6, 8, 14, 16 and (17). At the same time, *E. coli* culturing on EMB media shows a negative in all regions except (11, 14, 16 and 17), a positive culture for *E. coli* isolation from these regions as in table (5). Coliform bacteria are sources of human and animal excreta pollution in water. Fecally contaminated water contains harmful pathogens, and so it is not safe in use by human daily works[19]. Also, the high bacterial count was due to old piped city projects or inefficient filtration and precipitation process[20], the chlorination time during sterilization was not enough[21].

The existence of these organisms in some of the drin-

Regions No.	Coliform	*E. coli*
7	-	-
8	+	-
9	+	-
10	+	-
11	+	+
12	+	-
13	+	-
14	+	+
15	-	-
16	+	+
17	+	+
18	-	-

* (+)contain coliform bacteria up to average count.

Table 5. Results of bacteriological investigation of tap water samples in different Karbala regions.

king water samples under investigation is significant in terms of public health. It's possible that this is due to poor sanitation in the neighborhood. Drainage and floods of contaminated surface water into water sources could also be causes. These organisms could also be due to the openness of water sources, which allows particles from the environment to enter quickly, or contaminated equipment to draw water from the sources. Also, the factor of distance from the city affects the high rate of contamination due to the lack of residual chloride or its absence, which leads to the presence of these harmful bacteria in drinking water in remote areas humble point of view.

Not to mention that most rural residents dump their homes' sewage into the mouths of rivers, which are the source of water pumping stations for the filtration and treatment units for drinking water? This investigation revealed that bacterial contamination in drinking water samples from most regions of Karbala, especially those furthest from the city center, exceeds the legal limit, implying that the processes employed to filter and sterilize drinking water are not up to par. Not to mention that most individuals in rural areas dump their sewage into rivers, which serve as a supply of water for water pumping stations and filtration and treatment units for drinking water.

Conclusions

The study suggests working to develop drinking water distribution networks following international standards and using alternatives to chlorine sterilization and strict government oversight to provide safe, healthy water to residents of the governorate in all of its regions without discrimination. The current study showed that there is bacterial contamination that exceeds the permissible limit in drinking water samples for most areas of Karbala, especially areas far from the city center, as well as bottled water, and this gives the impression that the procedures used to filter and sterilize drinking water are not at the required level.

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Authors' Contributions

All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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None.

Data Availability

All datasets obtained or studied during this study are incorporated in the manuscript.

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ARTICLE / INVESTIGACIÓN

The Incidence of Extended Spectrum β-Lactamase Enzymes and Their Connection to Virulence Genes in Community-Acquired Urinary Tract Infection

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Abstract: In community-acquired urinary tract infections, *Klebsiella pneumoniae* is considered one of the most common etiological agents. Multidrug resistance and virulence are common in *Klebsiella pneumoniae* populations. In this study, fifty *Klebsiella pneumoniae* isolates were isolated from urine samples and identified using a vitek 2 compact device. The Kirby–Bauer disk diffusion technique used the antibiotic susceptibility test. According to the findings, approximately [n = 46 (92%)] of *Klebsiella pneumoniae* isolates are multidrug-resistant (MDR). To detect the production of Extended Spectrum β-lactamase (ESBL) enzymes, the Modified Double Disc Synergy Test (MDDST) was used. The results show that approximately [n=45 (90%)] of the isolates produce ESBLs. The most common ESBL genes (TEM, SHV, and CTX-M) were investigated in isolates. The results show that the SHV gene had the highest prevalence among ESBL genes [n = 34 (68%)], followed by the CTX-M gene [n = 33 (66%)]. while none of the isolates possessed the TEM gene. The virulence factor type 3 fimbriae (MrKD) gene and biofilm (BssS) gene were revealed. The results found that the isolates contain the MrKD gene at [n = 41 (82%)]. At the same time, the results found that the isolates contained the BssS gene at [n =36 (72%)]. The prevalence of Virulence genes within ESBL-producing *Klebsiella pneumoniae* isolates shows that only [n = 3 (6%)] of isolates that are non-ESBL producers carry one or both virulence genes, while [n=41 (82%)] of ESBL-producing isolates contain one or both virulence genes. The prevalence of ESBL-producing *Klebsiella pneumoniae* in community patients was high in this research. There may also be a correlation between ESBL production and some virulence factors.

Key words: *Klebsiella pneumoniae*, Antibiotic Resistance, Virulence Gene, ESBL, Urinary Tract Infection, CTX-M.

Introduction

Urinary tract infections (UTIs) have severe health and financial consequences for society. These infections are considered the most prevalent type of bacterial infection, and they can strike at any moment during a person's life. People in hospitals and the general public are susceptible to urinary tract infections. CAUTIs (community-associated urinary tract infections) are frequently identified in individuals with risk factors such as age, prior UTI history, sexual activity, and diabetes mellitus. Antibiotics are the most common therapy for bacterial UTIs[1]. After *E.coli*, *K. pneumoniae* is the most medically significant species in the Enterobacteriaceae family[2]. One of the frequent pathogens linked to both community and hospital-acquired urinary tract infections is *K. pneumoniae*[3]. Because bacterial etiology is common in UTIs, broad-spectrum antibiotics are frequently used, resulting in a rise of resistant uropathogens[1]. Antibiotic resistance among *K. pneumoniae* strains is a major public health concern and a significant financial burden for UTI sufferers. Antibiotic resistance of these isolates is acquired by various processes, including the creation of extended spectrum-lactamases (ESBLs). The formation of extended-spectrum beta-lactamase, an enzyme that attacks the beta-lactam ring in medicines and renders them useless, causes ESBL resistance[4]. Several ESBL families have been identified; however, the prominent families such as SHV, TEM, and CTX-M account for the bulk of ESBLs[5]. The rapidly increasing resistance of ESBL producers to multiple antibiotic families is a significant issue that limits the treatment options available to ESBL producers[6]. In addition, lipopolysaccharide (LPS), capsular polysaccharide, adhesions, and siderophores are among the virulence components found in *K. pneumoniae* that contribute to its pathogenicity[7]. Adhesion is an essential phase in the infection that must be strong enough to overcome the host's defensive mechanisms. Fimbriae, also known as pili, are proteinaceous structures that extend from the bacterial cell surface to a distance of 100 nm to several microns and are composed of adhesions that are thought to aid bacterial adhesion[8]. There are two kinds of fimbriae or pili in *K. pneumoniae*, types 1 and 3[9]. Type 3 fimbriae have a helix-like structure that gives them spring-like flexibility and stretch-ability. This fimbrial type is encoded by a chromosomally borne gene cluster that has previously been demonstrated to consist of five genes in various strains of *K. pneumoniae*. The mrkABCDF gene cluster encodes the primary structural component (mrkA) and the fimbrial adhesin (mrkD), whereas the genes mrkB and mrkC encode the chaperone and usher proteins, respectively[10]. Type 3 fimbriae have been shown to mediate adhesion to

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various structures in human kidney and lung tissue and epithelial cells from human urine sediments and endothelial and bladder epithelial cell lines in vitro investigations[11]. *K. pneumoniae* biofilm development on biotic and abiotic inert surfaces is commonly linked to Type 3 fimbriae[12]. Infections caused by *K. pneumoniae* producing biofilms are more resistant to therapy than other infections. Biofilms serve as shields for bacterial populations, allowing them to evade host defenses. The bacteria are also protected from severe circumstances such as pH changes, forces of shear, and nutritional deficiencies[13]. As a result, the current study was designed to look into the distribution of extended-spectrum β-lactamase enzyme genes (TEM, SHV, CTX-M), virulence genes (type 3 fimbriae MrKD gene, biofilm BssS gene), and the relationship between them in *K. pneumoniae* isolates from urine samples.

Materials and methods

Bacterial Isolation and Identification

From different laboratories in Baghdad, (50) isolates of *K. pneumoniae* were collected from (133) bacterial isolates from urine samples from the period (2020/07/1) to (2020/09/25). For their identification, *K. pneumoniae* isolates were diagnosed by the vitek 2compact (bioMérieux/France) device.

Antibiotic Susceptibility Test

The Kirby–Bauer disk diffusion technique was used to perform Antibiotic susceptibility testing for all isolates[14]. Antibiotics listed below were utilized: Ampicillin (10 µg), Piperacillin (100 µg), Amoxicillin-Clavulanate (20/10 µg), Ceftazidime/Avibactam (30/20 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Ceftazidime (30 µg), Cefuroxime (30 µg), Cefazolin (30 µg), Loracarbef (30 µg), Cepodoxime (10 µg), Cefixime (5 µg), Aztreonam (30 µg), Imipenem (10 µg), Meropenem (10 µg), Gentamicin (10 µg), Tobramycin (10 µg), Amikacin (30 µg), Doxycycline (30 µg), Tetracycline (10 µg), Ciprofloxacin (5 µg), Levofloxacin (5 µg), Nalidixic acid (30 µg), Trimethoprim-Sulfamethoxazole (1.25/23.75 µg), Chloramphenicol (30 µg), Nitrofurantoin (300 µg).

Modified Double Disc Synergy Test (MDDST)

Detect ESBL enzymes production by using the Modified Double Disc Synergy Test through the following steps[15]: After uniformly spreading the inoculum onto sterile Mueller–Hinton agar (biolab/Hungary), the disc which contained amoxicillin-Clavulanate (20/10µg) was placed in the center of the plate and the discs of third-generation cephalosporin [cefotaxime (30µg), ceftriaxone (30µg), and cefpodoxime (10µg)], and fourth-generation cephalosporin [cefepime (30µg)], were placed 15mm and 20 mm apart respectively, center to center to that the amoxicillin-Clavulanate disc. After being Incubated for 18–24 h at 37 °C, any increase in the inhibition zone toward the amoxicillin-Clavulanate disc was considered positive for ESBL production.

Detection of Antibiotic Resistance and Virulence Factor Genes

DNA extraction

For the isolation and purification of DNA from K. pneumoniae isolates, DNA was extracted using G-spin™ Total DNA Extraction Kit (Intron biotechnology/Korea) according to the manufacturer's instructions. Using Nanovue plus™ spectrophotometer (GE Healthcare/UK), estimated concentration and purity.

Polymerase Chain reaction of Antibiotic Resistance and Virulence Genes

The PCR method detects genes encoding virulence factors (type 3 fimbriae MrKD, biofilm BssS) and extended-spectrum -lactamase genes (TEM, SHV, CTX-M). Primers used in this study were purchased from (Alpha DNA/Canada) in lyophilized form, as shown in Table 1. The PCR amplification reaction mixture components (20µl) used to detect each gene contain the following: DNA sample, primers, nuclease-free water, and 5x FIREPol® Master Mix (Solis Bio Dyne/Europe) 16. After preparing the reaction volume in the PCR tube, the mixture was spun down via (Centrifuge/Vortex for PCR plates) and then PCR tubes were placed in the PCR thermal cycler (Bio-Rad/USA) to amplify the target DNA for (TEM, SHV, CTX-M, BssS, MrKD primers) using the following program17 as shown in Table 2.

Gene	Primers	PCR Product (bp)[c]	Ref
TEM-F[a] TEM-R[b]	AT GAG TAT TCA ACA TTT CCG TT AAT CAG TGA GGC ACC TAT	717	[11]
SHV-F SHV-R	AGCCGCTTGAGCAAATTAAAC ATCCCGCAGATAAATCACCAC	525	[12]
CTX-M-F CTX-M-R	GCT GTT GTT AGG AAG TGT GC CCA TTG CCC GAG GTG AAG	515	[13]
BssS-F BssS-R	GATTCAATTTTGGCGATTCCTGC TAATGAAGTCATTCAGACTCATCC	225	[14]
MrKD-F MrKD-R	AT GGA ACC CAC ATC GAC ATT AT GAG TAT TCA ACA TTT CCG	390	[15]

Table 1. Primers Used for Detection Genes.

Gene	Thermocycler conditions				
	Denaturation	Annealing	Extension	Final Extension	cycles
TEM	95°C 3 min.	55 °C 45 sec	72°C 50sec	72°C 7 min	32
SHV	95°C 3 min.	61 °C 45 sec	72°C 50sec	72°C 7 min	32
CTX-M	95°C 3 min.	59 °C 45 sec	72°C 50sec	72°C 7 min	32
BssS	95°C 3 min.	66 °C 45 sec	72°C 50sec	72°C 7 min	32
MrKD	95°C 3 min.	59 °C 45 sec	72°C 50sec	72°C 7 min	32

Table 2. PCR Thermocycler Conditions for Each Gene.

Results

In the current study, 50 *K. pneumoniae* isolates were collected from urine samples of community-acquired urinary tract infection patients. All samples were identified by vitek 2compact device (bioMérieux/France).

Antibiotic Susceptibility Test

The antibiotic susceptibility test was done by the Kirby–Bauer disk diffusion method, then interpreted the results were using criteria published by the Clinical and Laboratory Standard Institute[18]. As seen in Table 3. The results showed that about [n=46(92%)] of K. pneumoniae isolates were multidrug resistance (MDR).

The Modified Double Disc Synergy Test (MDDST) was used to detect ESBL production. As a result, any increase in the inhibition zone of cefeime, cefpodoxime, ceftriaxone, and cefotaxime toward the amoxicillin clavulanate disc was interpreted as a sign of ESBL generation. The results showed that approximately [n=45 (90%)] of the isolates produced ESBLs, as shown in Figure 1.

Detection of Antibiotic Resistance and Virulence Factors Genes

The ability of *K. pneumoniae* isolates to produce ESBLs (TEM, SHV, and CTX-M) genes and the capacity of isolates to generate Virulence factor type 3 fimbriae (MrKD) genes and biofilm (BssS) genes are determined using PCR, which uses sets of primers that amplify the genes. The results showed that the SHV gene was the most prevalent among ESBL genes [n=34(68%)], then the CTX-M gene [n=33(66%)]. While none of the isolates possessed the TEM gene, as showed in Figure 2.

The results found that the isolates contain MrKD gene at [n=41 (82%)] as shown in the figure A (3). It also, found that [n=36 (72%)] of isolates contain the BssS gene as shown in the figure B (3).

The prevalence Virulence genes within ESBL-producing *K. pneumoniae* isolates show that only [n=3 (6%)] of isolates which are non-ESBL producing carry one or both virulence genes, while [n=41 (82%)] of ESBL-producing isolates contain one or both virulence genes as seen in Table 4.

Discussion

In both community and hospital settings, *K. pneumoniae* is a leading cause of serious infections such as urinary tract infection, pneumonia, skin and soft tissue infection, intra-abdominal infection, bloodstream infection, meningitis, and pyogenic liver abscess. In humans, antimicrobials have long been used to treat *K. pneumoniae* infections[19]. The emergence of multidrug-resistant (MDR) *K. pneumoniae* strains worldwide is a major source of worry[20]. In this study, the results showed that about [n=46(92%)] of *K. pneumoniae* isolates were multidrug resistance (MDR). MDR is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories[21]. Different rates of MDR were reported in many studies, such as the study done by Mohamed *et al.*, which reported that *K. pneumoniae* had the highest level of resistance to conventional antibiotics, with 86.66% of isolates being MDR22. Also, a study done by Pishtiwan and Khadija concluded that most of the *K. pneumoniae* isolates were multidrug-resistant (MDR). Where 75% of *K. pneumoniae* isolates showed the MDR phenotype23. MDR obstructs disease control by increasing the risk of resistant microorganisms spreading, lowering treatment efficacy and, as a result, causing patients to be infected for more extended periods of time[24]. A variety of virulence factors contribute to *K. pneumoniae's* pathogenicity[25]. As well as the ability to rapidly develop antibiotic resistance[26]. *K. pneumoniae* is, in fact, a significant ESBL host. The development of ESBLs has significantly increased bacterial resistance to beta-lactams in human infections, causing significant morbidity and death[27]. Extended-spectrum beta-lactamases (ESBLs) are enzymes that can give resistance towards β-lactam antibiotics such penicilins, scephalosporins, and aztreonam. This is achieved by hydrolyzing the antibiotics, and beta-lactamase inhibitors like clavulanic acid block these enzymes[28].

This study used Modified Double Disc Synergy Test (MDDST) to detect ESBL production. The results showed that approximately [n=45 (90%)] of the isolates produced ESBLs. These results were close to those obtained by Shakib *et al.*, who showed that [n=62 (88.6%)] of *K. pneumoniae*

Antimicrobial Agent	Susceptible No (%)	Intermediate No (%)	Resistant No (%)
Ampicillin	0 (0.0)	0 (0.0)	50(100)
Piperacillin	0 (0.0)	0 (0.0)	50(100)
Amoxicillin/Clavulanate	0 (0.0)	0 (0.0)	50(100)
Ceftazidime/avibactam	20 (40)	6 (12)	24(48)
Cefotaxime	2 (4)	0 (0.0)	48 (96)
Ceftriaxone	3 (6)	1 (2)	46 (92)
Ceftazidime	3 (6)	0 (0.0)	47 (94)
Cefuroxime	2 (4)	5 (10)	43 (86)
Cefazolin	4 (8)	0 (0.0)	46 (92)
Cefepime	4 (8)	0 (0.0)	46 (92)
Loracarbef	4 (8)	0 (0.0)	46 (92)
Cefixime	3 (6)	2 (4)	45 (90)
Cefpodoxime	3 (6)	4 (8)	43 (86)
Aztreonam	2 (4)	0 (0.0)	48 (96)
Imipenem	19 (38)	18 (36)	13 (26)
Meropenem	26 (52)	14 (28)	10 (20)
Gentamicin	34 (68)	2 (4)	14 (28)
Amikacin	40 (80)	7 (14)	3 (6)
Tetracycline	2 (4)	0 (0.0)	48 (96)
Doxycycline	3 (6)	0 (0.0)	47 (94)
Tetracycline	2 (4)	0 (0.0)	48 (96)
Doxycycline	3 (6)	0 (0.0)	47 (94)
Ciprofloxacin	36 (72)	1 (2)	13 (26)
Levofloxacin	37 (74)	0 (0.0)	13 (26)
Nalidixic acid	11 (22)	7 (14)	32 (64)
Trimethoprim-sulfamethoxazole	19 (38)	0 (0.0)	31(62)
Chloramphenicol	27 (54)	8 (16)	15 (30)
Nitrofurantoin	1 (2)	1 (2)	48 (96)

Table 3. Antibiotic Susceptibility of 50 *K. pneumoniae* Isolates.

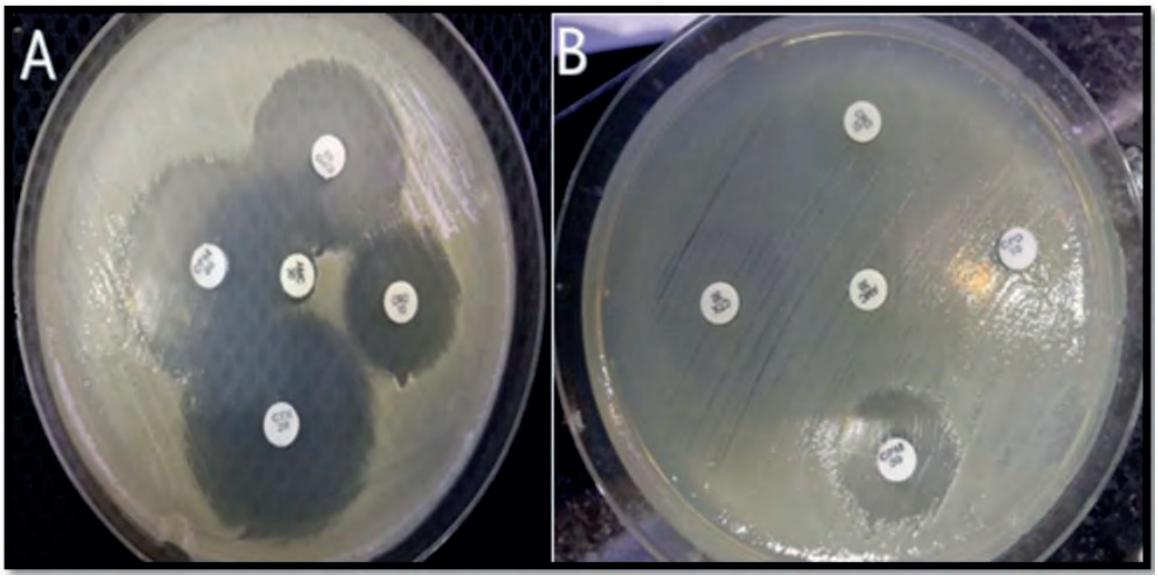


Figure 1. Modified Double Disc Synergy Test (MDDST) (A) shows synergism of cefeime, cefpodoxime, ceftriaxone and cefotaxime with amoxicillin-Clavulanate, which indicates ESBL production, while in (B) showed no synergism with amoxicillin-Clavulanate, which indicates none of ESBL production.

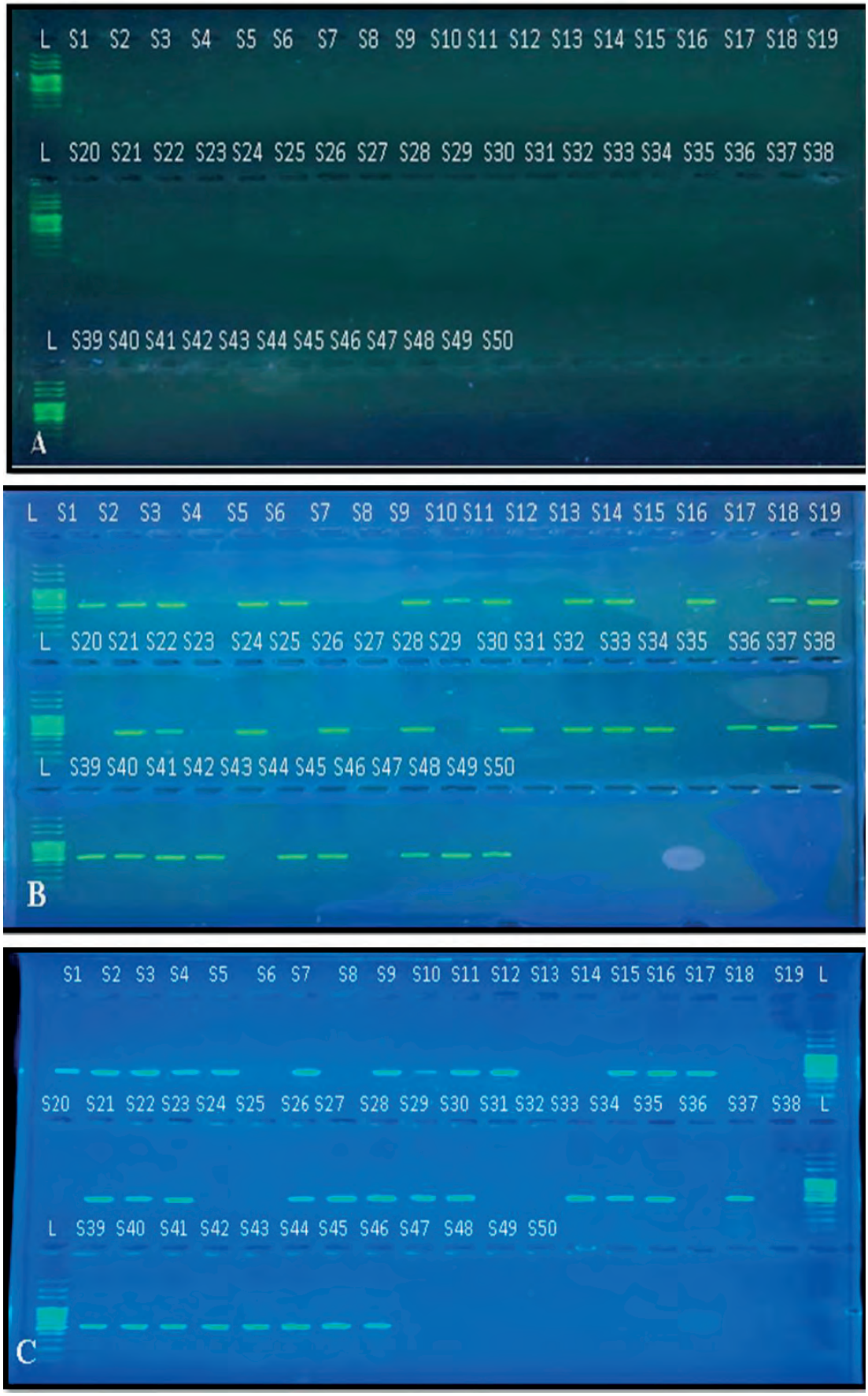


Figure 2. Agarose gel electrophoresis of PCR products of (A) TEM gene, (B) SHV gene and (C) CTX-M gene in *K. pneumoniae* isolates visualized under UV after staining by RedSafe™ nucleic acid staining solution for 2% agarose gel at 100 volts, 70 amps for 60 min. L: ladder DNA (100pb), S: Sample.

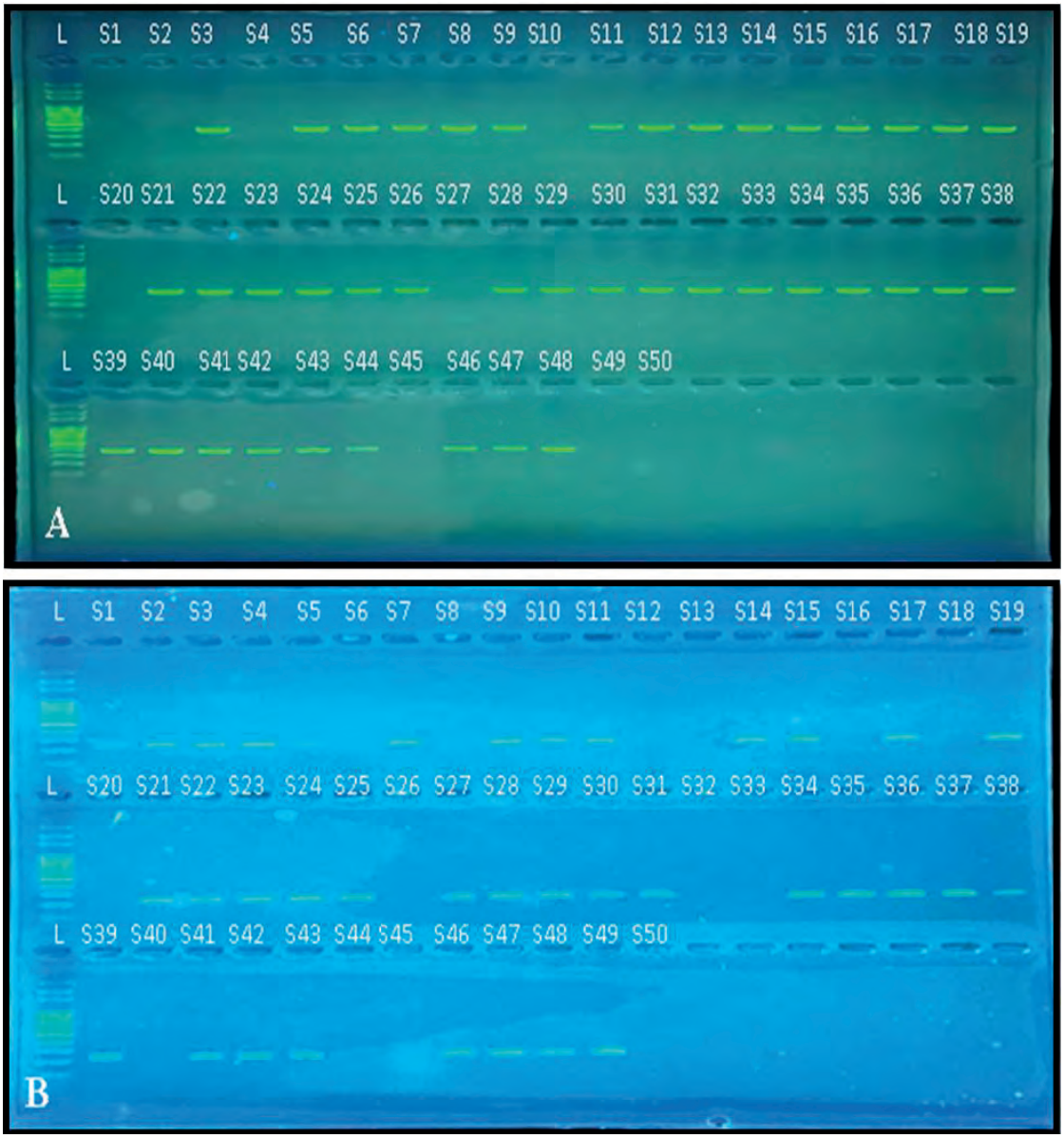


Figure 3. Agarose gel electrophoresis of PCR products of (A) MrKD gene and (B) BssS gene in *K. pneumoniae* isolates visualized under UV after staining by RedSafe™ nucleic acid staining solution for 2% agarose gel at 100 volts, 70 amps for 60 min. L: ladder DNA (100pb), S: Sample.

isolates were ESBL-producing[29]. Pishtiwan and Khadija *et al.*'s research revealed that [n=17(85%)] of *K. pneumoniae* isolates were positive for ESBL production[23]. The prevalence of ESBL-producing clinical isolates is related to risk factors such as current antibiotic use and hospitalization[29].

The ability of *K. pneumoniae* isolates to produce ESBLs (TEM, SHV, and CTX-M) genes showed that the SHV gene was the most prevalent among ESBL genes [n=34(68%)], then the CTX-M gene [n=33(66%)]. At the same time, none of the isolates possessed the TEM gene. The study done by Malekjamshidi *et al.* showed that the most prevalent β-lactamase gene was SHV, followed by TEM and CTX-M[30]. Also, Pishtiwan and Khadija *et al.* reveal that the TEM gene has the highest prevalence in *K. pneumoniae* isolates at 64.7%, followed by the CTX-M gene at 41.1% and the SHV gene at 35.2% 23. In addition, a study by Mbelle et al. found that all isolates contained the CTX-M gene, while (97%) contained TEM, and (83%) harbored SHV genes[31]. There are significant geographic differences in the prevalence of resistant bacteria and their genes. Low-and middle-income countries generally have higher endemic antimicrobial resistance than high-income countries, mainly driven by antibiotic overuse in humans and animals[32]. International travel can facilitate the transfer of resistant bacteria and their genes from their endemic regions to other locations[33].

Conclusions

The capacity of *K. pneumoniae* isolates to generate Virulence factor type 3 fimbriae (MrKD) genes and biofilm (BssS), found that the isolates contain MrKD gene at [n=41 (82%)]. While the results found that [n=36 (72%)] of isolates contain the BssS gene. The prevalence Virulence genes within ESBL-producing *K. pneumoniae* isolates show that only [n=3 (6%)] of isolates that are non-ESBL producing carry one or both virulence genes, while [n=41 (82%)] of ESBL-producing isolates contain one or both virulence genes. This is consistent with the study done by Gharrah *et al.* that suggests a correlation between ESBL production and

No.	Isolate symbol	Virulence genes	ESBLs producing isolates
1	T969	MrKD	+
2	A1762	MrKD, BssS	+
3	T971	BssS	+
4	T973	MrKD, BssS	+
5	T975	----------	+
6	T982	MrKD, BssS	+
7	A1730	----------	+
8	A1553	MrKD, BssS	+
9	T985	MrKD, BssS	+
10	A1394	MrKD, BssS	+
11	A1095	----------	_
12	T976	MrKD, BssS	+
13	A1789	MrKD, BssS	+
14	A1100	MrKD, BssS	+
15	T967	MrKD, BssS	+
16	A1788	MrKD, BssS	+
17	76-8	MrKD	+
18	A1743	----------	+
19	A1279	MrKD, BssS	+
20	A1794	MrKD, BssS	+
21	A1144	MrKD	+
22	A1516	MrKD, BssS	+
23	A1054	MrKD, BssS	+
24	T970	MrKD, BssS	+
25	A1557	MrKD, BssS	+
26	T979	MrKD, BssS	+
27	A1274	MrKD, BssS	+
28	A1424	MrKD, BssS	+
29	L44	MrKD, BssS	_
30	T980	MrKD, BssS	+
31	T1191	MrKD, BssS	+
32	A1984	BssS	+
33	A1993	BssS	_
34	A1994	MrKD, BssS	+
35	A1971	MrKD, BssS	+
36	A1965	MrKD	+
37	A1957	MrKD, BssS	+
38	A1946	MrKD	+
39	A1787	MrKD, BssS	+
40	T1188	MrKD, BssS	+
41	A1612	MrKD	_
42	T966	----------	_
43	A1492	MrKD	+
44	A1767	MrKD	+
45	A1859	MrKD, BssS	+
46	A1875	----------	+
47	A1845	MrKD, BssS	+
48	T974	MrKD, BssS	+
49	T981	MrKD, BssS	+
50	A1577	MrKD, BssS	+

Table 4. Distribution Virulence genes within ESBLs and non-ESBLs producing *K. pneumoniae* isolates.

some virulence factors[34]. In conclusion, the prevalence of ESBL-producing *K. pneumoniae* in community patients was high in this research. It's also possible that ESBL production and virulence factors are linked. Antibacterial resistance is a developing problem in clinical practice. As a result, improved clinical awareness and laboratory testing are required to decrease treatment failure and prevent the spread of ESBL-producing *K. pneumoniae*.

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ARTICLE / INVESTIGACIÓN

Isolation of different parasites from the freshwater of Euphrates River in Anbar Province, Iraq

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Abstract: Intestinal parasites present in freshwater from the Al- Fallujah, Al- Habbaniyah and Al-Alwarar, of the Euphrates river in Iraq are *Cryptosporidium spp* (25.3%), *Giardia sp* (3.3%), *Eimeria sp* (3.3%), *Pinworm* eggs (3.3%), *Naegleria sp* (15.3%), *Lecane niwati* (1.3%), *Trichomonas hominis* (19.3%), Acanthamoeba spp (24.6%), *Entamoeba coli* (20.6%), *Balantidium coli* (12%), *Ascaris sp* (3.3%), *Volvox sp* (26%), *Chilomastix mesnili* (4%), *Pelomyxa palustris* (2.6%), *Trinema enchelys* (2.6%), *Actinophrys Sol* (7.3%), *Amobea Vespertilio* (9.3%), *Rhabditea* (5.3%), *paramecium bursaria* (9.3%), cyst of cestode (6%), *Oocyst protozoa* (16%), *Euglena gracilis* (10.6%).were isolated. The study's goal was to isolate some of the parasites that pollute the Euphrates water in Anbar Province and their impact on the health of living species and swimmers.

Key words: *Acanthamoeba spp*, *Cryptosporidium*, Fresh water, *Giardia*, *Rotifer*.

Introduction

Water pollution is a major global issue requiring ongoing analysis and reform of water resource programs in all situations, from the international level to individual aquifers. As a result, water contamination is a leading source of mortality and disease worldwide[1,2]. Contamination of community water systems can cause disease in many people[3].

Waterborne parasites are parasitic protozoans that infect humans, domestic animals, and wildlife all over the world, at least 325 parasitic protozoan infection outbreaks linked to water have been proved around the world[4]. There's a direct link between the prevalence of some sponger infections and the presence of the causative agent in water[5]. In certain countries, parasitic illnesses are relatively widespread.

In Iraq, the parasite infection is widespread and has a diverse distribution. Helminth and Protozoa parasites have been found in various water sources, with a higher prevalence of contamination in rural villages of marshland. This higher prevalence of water supply contamination may be explained by poor sanitation and hygiene, low socioeconomic status, a lack of acceptable health and biological process education, and contamination of water with animal and human waste material[6]. This study aims to show the prevalence of protozoa and parasites in the Euphrates river, which is a public health threat when consumed or gone swimming in.

Materials and methods

Study area of Anbar Province

Area 1

Al- Fallujah

Area 2

Al- Habbaniyah

Area 3

Al-Alwarar

Samples collection

150 samples were collected from three locations in Anbar Governorate (Al- Fallujah, Al- Habbaniyah, and Al-Alwarar), with 50 samples distributed from each area. All of the samples were collected in a 300 ml bottle labeled with the location from different parts of the river (from the cliff) and collection date. The water samples were sedimented by clinical centrifuge (approximately 2300rpm) for 1 minute[7]. Transfer several loop-fulls of material from the surface film to a clean slide with a bacteriologic loop after 1 to 2 minutes, and cover with a coverslip. The samples were also diagnosed using direct and an iodine stain. Examine under a low magnification of 10x, then under a high magnification of 40x and 100x[8].

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Results

The recent research found that In freshwater from opposing banks of the Euphrates river, A total of Twenty two genera and species of harmful and nonpathogenic parasites were found In freshwater of the Euphrates river, as indicated in Table 1, Figures (1 to 21). *Cryptosporidium spp* (25.3%), *Giardia sp* (3.3%), *Eimeria sp* (3.3%) *Pinworm eggs* (3.3%), *Naegleria* sp (15.3%), *Lecane niwati* (1.3%), *Trichomonas hominis* (19.3%), *Acanthamoeba spp* (24.6%), *Entamoeba coli* (20.6%), *Balantidium coli* (12%), *Ascaris sp* (3.3%), *Volvox sp* (26%), *Chilomastix mesnili* (4%), *Pelomyxa palustris* (2.6%), *Trinema enchelys* (2.6%), *Actinophrys Sol* (7.3%), *Amobea Vespertilio* (9.3%), *Rhabditea* (5.3%), *paramecium bursaria* (9.3%), cyst of cestode (6%), *Oocyst protozoa* (16%), *Euglena gracilis* (10.6%).

Discussion

The current study revealed that freshwater was contaminated with Twenty two genera and species of parasites and protozoa, with varying frequencies among the study areas. This discrepancy arises from mixing sewage with river openings in some regions. This study is similar to others that have documented parasite contamination of river water, such as (9), which found pathogenic intestinal parasites in the freshwater of the Tigris River in Baghdad, Iraq. Furthermore, the current study found the most significant percentage of *Cryptosporidium sp* infection (33.3%), similar to (9), which found 31.6 % in Tigris River freshwater. In freshwater of the Tigris River, Diyala province, (10) found the highest infection rate with *Cryptosporidium sp* (60 %), followed by *Acanthamoeba sp* (50 %), Rabditae larve (40 %), and *Enta-*

Parasite (Species)	phylum	Stage	Area 1	Area 2	Area 3	Percentage%
Cryptosporidium spp	Apicomplexa	Oocyst	10	15	13	25.3
Giardia sp	Metamonads	cyst	-	5	-	3.3
Eimeria sp	Apicomplexa	Oocyst	5	-	-	3.3
Pinworm eggs	Nematoda	Egg	5	-	-	3.3
Naegleria sp	Percolozoa	Trophozoite	8	10	5	15.3
Lecane niwati	Rotifera	-	-	2	-	1.3
Trichomonas hominis	Metamonads	Trophozoite	9	15	5	19.3
Acanthamoeba spp	Amoebozoa	Trophozoite	12	18	7	24.6
Entamoeba coli	Amoebozoa	cyst	2	19	10	20.6
Balantidium coli	Ciliophora	Trophozoite	8	6	4	12
Ascaris sp	Nematoda	Egg	2	3	-	3.3
Volvox sp	Chlorophyta	-	15	20	4	26
Chilomastix mesnili	Metamonads	cyst	-	4	2	4
Pelomyxa palustris	Amoebozoa	flagella	2	2	-	2.6
Trinema enchelys	Cercozoa	-	-	4	-	2.6
Actinophrys Sol	Ochrophyta	-	3	6	2	7.3
Amobea Vespertilio	Amoebozoa		8	6	-	9.3
Rhabditea	Platyhelminthes	larvae	3	5	-	5.3
paramecium bursaria	Ciliophora	-	7	5	2	9.3
cyst of cestode	Platyhelminthes	cyst	4	3	2	6
Oocyst protozoa	Protozoa	Oocyst	9	15	-	16
Euglena gracilis	Euglenozoa	-	-	-	16	10.6

Table 1. Prevalence of parasites and protozoa in the freshwater of studied areas



Figure 1. *Cryptosporidium spp oocyst*.



Figure 2. *Giardia sp cyst*.



Figure 3. *Pinworm eggs*.



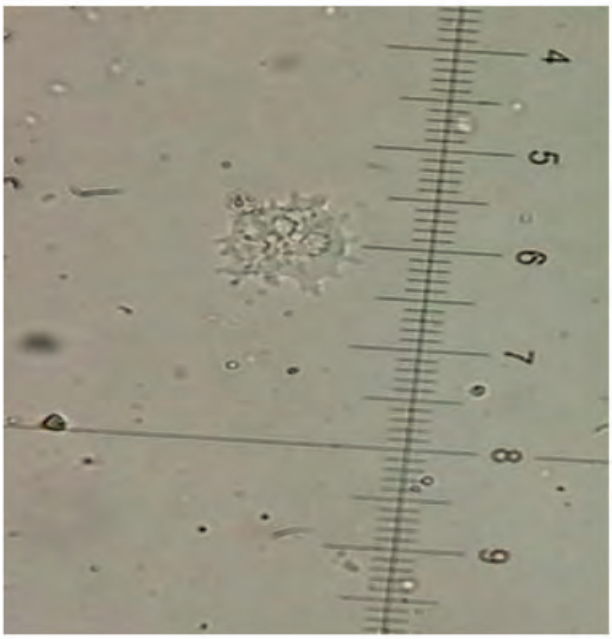
Figure 4. *Naegleria sp* trophozite.



Figure 5. *Lecane niwati* (Rotifera).



Figure 6. *Trichomonas hominis* (Trophozoite).



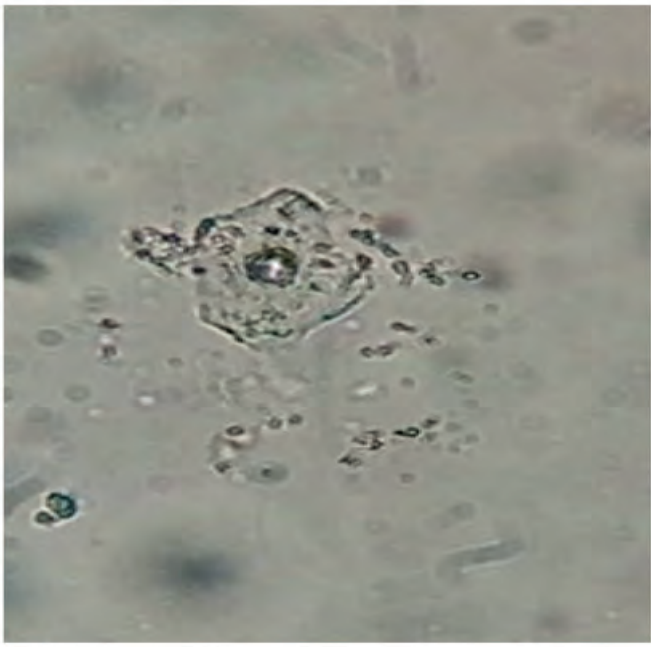


Figure 7. *Acanthamoeba spp* (left and right).

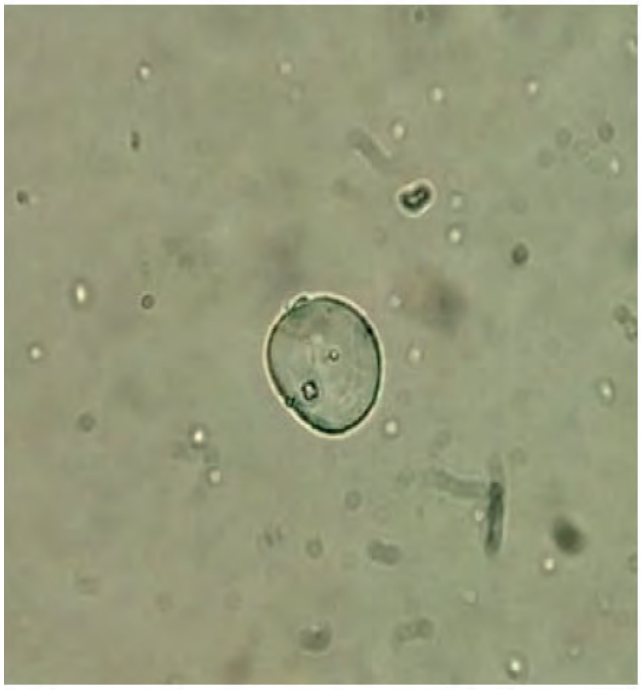


Figure 8. *Eimeria Oocyst*.

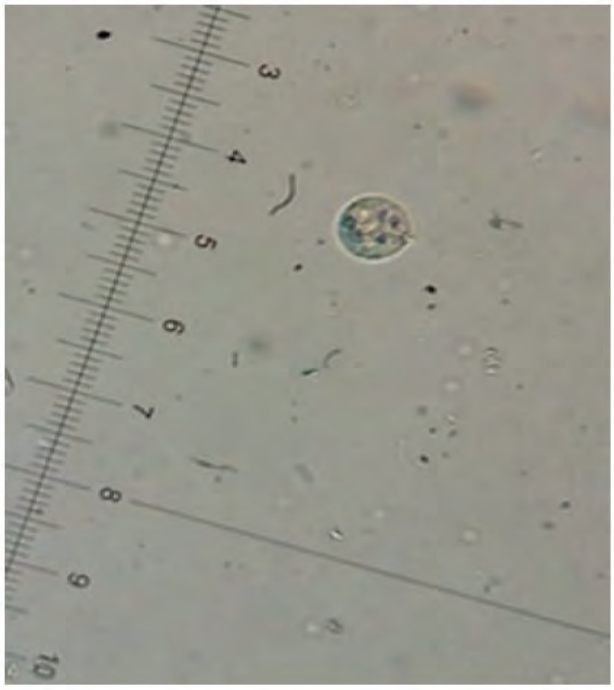
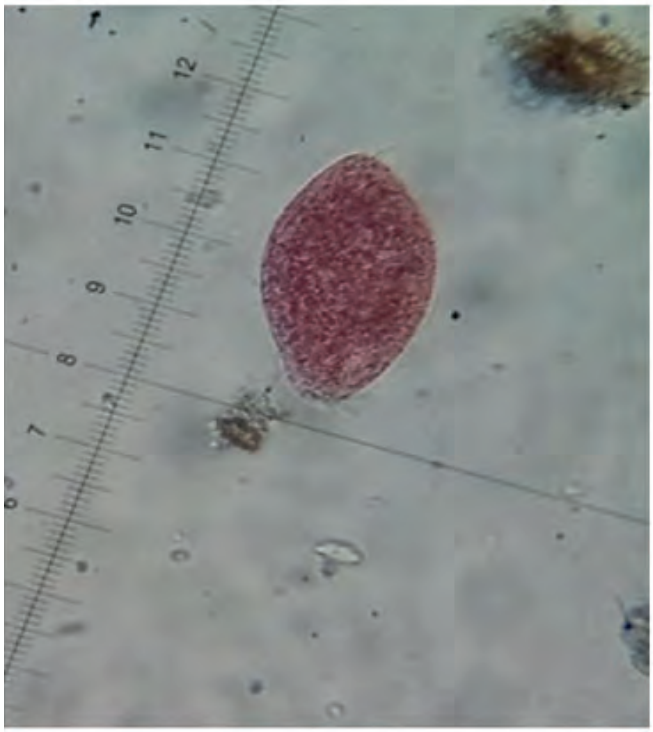


Figure 9. *Entamoeba coli* (cyst).



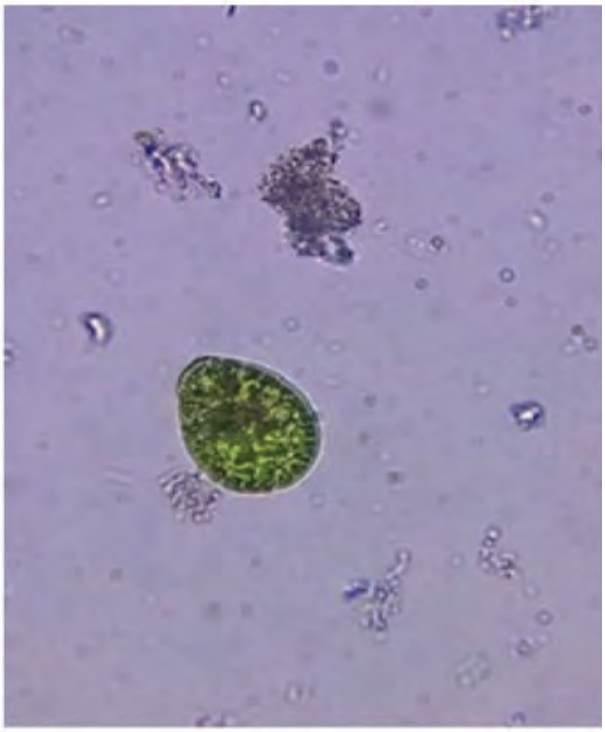


Figure 10. *Balantidium coli* (left and right) Trophozoite.



Figure 11. *Ascaris egg.*

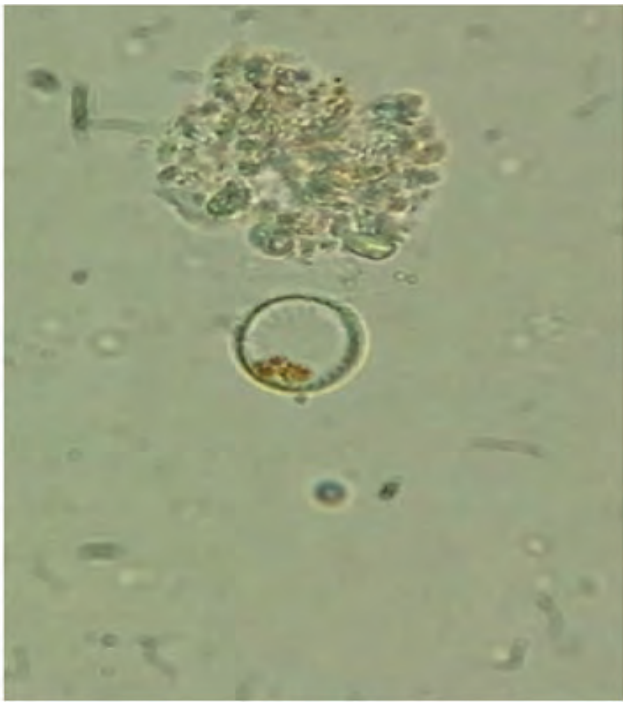


Figure 12. *Volvox sp*.



Figure 13. *Chilomastix mesnili (cyst)*.

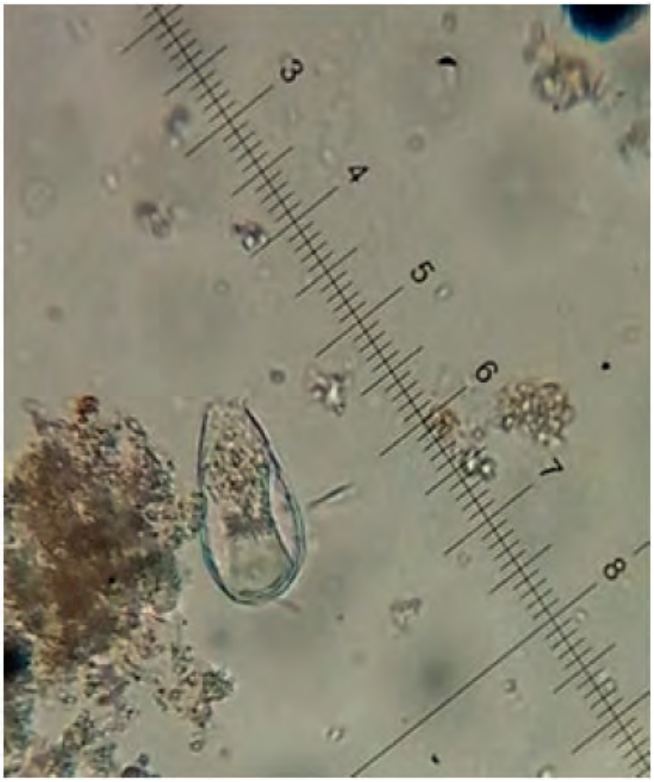


Figure 14. *Pelomyxa palustris*.

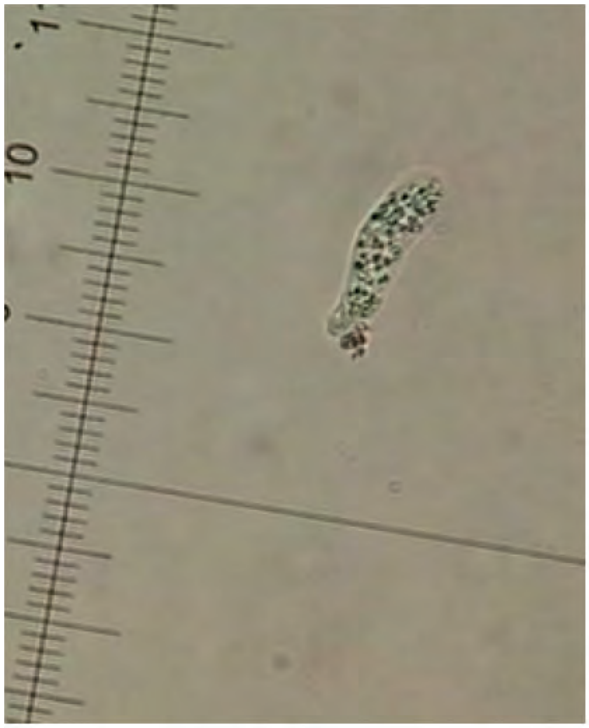


Figure 15. *Trinema enchelys*.



Figure 16. *Actinophrys Sol*.



Figure 17. *Amobea Vespertilio*.



Figure 18. *Rhabditea larvae*.



Figure 19. *Paramecium bursaria*.



Figure 20. *Cyst of cestode*.

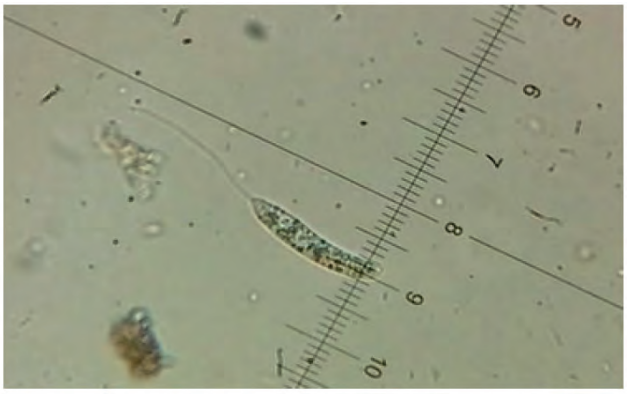


Figure 21. *Euglena gracilis*.

moeba coli (40%). This investigation confirmed (11,12) that the Tigris river water was contaminated with pathogenic parasites.

Al-Dulaimi *et al.* revealed the following parasites in the drinking water of Babylon province: *Giardia lamblia*, *Cryptosporidium sp. Entamoeba coli* and *Balantidium coli*[13] revealed a concern to public health. In the current study, *Entamoeba coli* was found in 20.6 %, similar to the results of (13), who found *Entamoeba coli* in 19.5 % of tank water, rivers, and wells water in three districts (Abi-Gharaq, Al-Kefel, and Al-Neil).

A recent study found that the fresh water in these three places contains several pathogens and nonpathogenic parasites, making the water unsafe to consume without treatment and unsafe for swimming for people of all ages, particularly youngsters[14,15]. Water contamination with various pollutants for the spread of parasitic illnesses. In addition to sanitation and a lack of personality for cleaning[16]. Many people living in these areas, particularly farmers, were more infected with these parasites[17], which cause acute diarrhea, which could be a severe public health problem in Iraq, especially during hot seasons when drinking raw water.

Nonparasitic protozoans that live in freshwater are known as free-living amoebae. These amoebae have been proven to be infective in humans, causing a deadly disease that affects the central nervous system[18]. The *Acanthamoeba* genus is primarily recognized for infecting and inflaming the eyes[19-21]. When the environment's temperature is high enough, these amoebae multiply in sufficient numbers to cause illness[22]. In the freshwater of the Tigris River, completely distinct morphologies of free amoebae have been recorded.

Conclusions

The current study concludes that the freshwater of the Euphrates River in Anbar Governorate is contaminated with pathogenic and nonpathogenic parasites by isolating several types of parasites that have a pathological effect on living organisms (human and animal).

Author Contributions

The current study was based on references borrowed from the Natural History Research Center and Museum / University of Baghdad / Iraq. The diagnosed samples were photographed, and the article was edited and written with the help of scientific sources.

Funding

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Institutional Review Board Statement

The article's title is approved by the scientific institution at the university, as it shows the significant impact of water on the health of animals and living organisms in general.

Acknowledgments

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Conflicts of Interest

The authors declare no conflict of interest.

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ARTICLE / INVESTIGACIÓN

Evaluación de diferentes combinaciones de polímeros en la microencapsulación de licopenos procedentes de residuos de tomate de árbol (*Solanum betaceum*)

Evaluation of different polymer combinations in the microencapsulation of lycopenes from tree tomato (*Solanum betaceum*) residues

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Resumen: El consumo de tomate de árbol en el Ecuador es muy amplio a lo largo de las diferentes zonas, es decir, tiene una gran demanda, a su vez no es aprovechado en su totalidad generando gran cantidad de residuos orgánicos. Mediante la microencapsulación se pueden elaborar productos derivados de los principios activos que se generan de esta fruta. En este trabajo se extrajeron y concentraron carotenoides de la piel de *Solanum betaceum*. Además, se microencapsuló los principios activos contenidos en el extracto con polímeros (goma arábiga y maltodextrina) en una concentración del 35 y 40 %. Es necesario controlar la temperatura de microencapsulación para evitar la degradación del material de interés. Es importante determinar la actividad que presentan dichos metabolitos al ser microencapsulados, se evaluó la actividad antioxidante utilizando un método colorimétrico cuantitativo llamado DPPH. Por otro lado, se usó un modelo *in vivo* con *Saccharomyces cerevisiae* en el cual se midió la actividad antioxidante considerandondo de la curva de crecimiento del microorganismo en cuestión sometiéndolo a diferentes factores, uno de ellos el crecimiento normal sin agentes externos, en los dos siguientes se emplearon concentraciones de agentes oxidantes, para simular estrés alto y bajo con hipoclorito de sodio y peróxido de hidrógeno.

Palabras clave: Carotenoides, extracción, secado por aspersión, actividad antioxidante, *Saccharomyces cerevisiae*, razón de crecimiento.

Abstract: Tree tomato consumption in Ecuador is pervasive across the different areas; it has a great demand, but it is not used in its entirety, generating a large amount of organic waste. Through microencapsulation, products derived from active ingredients generated from this fruit can be developed. Carotenoids were extracted and concentrated from Solanum betaceum's rind in this work. In addition, active ingredients contained in the extract were microencapsulated using polymers (gum arabic and maltodextrin) at 35 and 40% of concentrations. It is necessary to control the microencapsulation temperature to avoid degradation of the material of interest. It is essential to define metabolites' activity when microencapsulated; the antioxidant activity was evaluated using a quantitative colorimetric method called DPPH. On the other hand, an *in vivo* model with *Saccharomyces cerevisiae* was used considering the growth curve of the microorganism subjecting to different factors; one of the expected growth without external agents, in the following two concentrations of oxidizing agents, were used to simulate high and low stress with sodium hypochlorite and hydrogen peroxide.

Key words: Carotenoids, extraction, spray drying, antioxidant activity, *Saccharomyces cerevisiae*, growth rate.

Introducción

Una de las causas más comunes que provoca un aumento en la tasa de mortalidad humana son las enfermedades no transmisibles (ENTs), un 71 % de la población representada aproximadamente por 41 millones de personas son afectadas cada año según la Organización Mundial de la Salud (OMS)[1]. El Instituto Nacional de Estadística y Censos[2], reportó que el 16,33 % de la población ecuatoriana muere a causa de patologías como: diabetes mellitus, enfermedades cerebrovasculares e hipertensivas.

El estrés oxidativo es uno de los mediadores claves de los efectos producidos por las ENTs como la hiperglucemia[3]; este es el principal factor de riesgo para el desarrollo de complicaciones crónicas diabéticas ya que contribuye a la respuesta inflamatoria que caracteriza a las enfermedades cardiovasculares (ECV)[4]. Por otro lado, el estrés oxidativo se ve involucrado en el proceso de degeneración celular debido al alto índice de toxicidad al que se expone la célula al no ser capaz de mediar los productos resultantes del metabolismo, provocando así su necrosis[5].

En el Ecuador se generan alrededor de 2 074 tone-

Citation: Pazmiño D, Fernández D, López OD, Iraizoz A. Evaluación de diferentes combinaciones de polímeros en la microencapsulación de licopenos procedentes de residuos de tomate de árbol (Solanum betaceum). Revis Bionatura 2022;7(2) 29. http://dx.doi.org/10.21931/RB/2022.07.02.29
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ladas de residuos al día, de los cuales el 53,81 % son de origen orgánico[6], el licopeno está presente en estos, en una proporción aproximada de 600-800 µg de carotenos/100 g de cáscara de diferentes tipos de tomate[6]. Se ha demostrado que el licopeno es un compuesto bioactivo, químicamente inestable y de rápida degradación al estar expuesto a condiciones ambientales externas diferentes a las de su fuente natural de obtención, como el oxígeno, la temperatura, la luz, el pH y algunas otras sustancias reactivas[7]. Tales causas han despertado interés en el desarrollo de una tecnología que brinde estabilidad a este principio activo, razón por la que se ha escogido a la microencapsulación con polímeros inocuos para el consumidor como objeto de estudio del presente trabajo de investigación.

Materiales y métodos

Obtención del material vegetal

Se recolectaron cáscaras de tomate de árbol Solanum betaceum, se secaron a 60 ºC en un horno de circulación forzada de aire (Gander, Canadá), se redujo a polvo en un molino de cuchillas (INOX-EQUIP, Reino Unido). El material en polvo se almacenó en doble funda de polietileno de baja densidad transparente.

Obtención de extractos vegetales

La relación que se ocupó es de 1:70, es decir una parte de material vegetal y setenta partes de etanol al 96 %, se calentó el disolvente a 50 ºC y posteriormente se añadió el material vegetal. La mezcla se agitó a una velocidad de 6000 rpm por 30 minutos. Se filtró y el sobrenadante se almacenó en botellas color ámbar a 4 ºC[8].

$$C = \frac{A*10^4}{E}$$

Concentración del extracto vegetal

Se colocó el extracto en el balón del rotoevaporador (IKA, Alemania) y se calentó a baño María a 60 ºC con constante rotación a 2000 rpm hasta evidenciar ausencia del etanol.

Cuantificación de licopeno presente en el concentrado obtenido

Para la determinación de la concentración de carotenos (C) se empleó el espectrofotómetro UV-Vis (Fisher Scientific, Estados Unidos) midiendo la absorbancia (A) a 472 nm[8] y empleando la Ecuación 1 utilizando como blanco el etanol. Se empleó un valor de 3950 para la absortividad (E) del etanol[9].

Obtención de microencapsulados con diferentes combinaciones de polímeros

Basándose en las técnicas de microencapsulación empleadas[10].Haz clic o pulse aquí para escribir texto.se realizó la microencapsulación utilizando el equipo mini spray dryer (BÜCHI) B-290 utilizando los polímeros goma arábiga y maltodextrina. Las variables evaluadas fueron las proporciones polímero-licopeno y las relaciones temperatura de entrada y salida del equipo (Tabla I).

Determinación el porcentaje de eficiencia de la microencapsulación (EME)

Se determinó el porcentaje de eficiencia de la microencapsulación utilizando la Ecuación 2, para lo cual se disolvieron 0,01 g de los microencapsulados en 1 ml de hexano y agua de forma independiente. Se cuantificó la concentración de licopenos en ambas soluciones obteniendo la concentración total de licopenos presentes en las microcápsulas en la disolución con agua (DT) y la concentración parcial de licopenos en la disolución con hexano (DP)[11] que corresponde a los que se encuentran en la superficie de las microcápsulas.

$$\%Eficiencia\ de\ encapsulación = \frac{DT-DP}{DT}*100$$

Determinación *in vitro* de la actividad antioxidante por el método del radical 2,2-diphenil-1-1-picrylhydrazil (DPPH)

En una placa de 96 pocillos se colocaron 20 µL de extracto diluido o disolución estándar, con 180 µl del reactivo radical 2,2-diphenil-1-1-picrylhydrazil (DPPH) (Sigma-Aldrich, USA) disuelto en metanol-agua en una proporción (80:20) a una concentración de 150 µmol/L y se agitó por 60 segundos. Se incubó la placa por 40 minutos en ausencia de la luz a temperatura ambiente, posteriormente se leyó la absorbancia a una longitud de onda de 515 nm a 25 ºC en el espectrofotómetro (Fisher Scientific, Finlandia).

Se determinó el porcentaje de inhibición de DPPH (Ecuación 3), a partir de la curva de calibración realizada con trolox, utilizando la ecuación de la recta se determinó la concentración equivalente de trolox al porcentaje de inhibición obtenido en los extractos. En la Ecuación 3, la absorbancia de la muestra a analizar está representada por A, muestra y la absorbancia obtenida por la muestra control, es decir la conformada por metanol y agua en la proporción 80:20 por A control. Los resultados se expresaron como µmol de trolox/g de muestra.

$$\%\ Inhibicion\ de\ DPPH = \left[1 - \left(\frac{A\ muestra - A\ control}{A\ control}\right)\right] * 100$$

Determinación *in vivo* de la actividad antioxidante usando Saccharomyces cerevisiae

Se analizó la actividad antioxidante de los microencapsulados de licopenos utilizando como modelo de estudio la Saccharomyces cerevisiae. En este experimento se comparó la razón de crecimiento entre la curva de crecimiento de la levadura sin agentes oxidantes con respecto a la curva de crecimiento de la levadura sometida a diferentes concentraciones de antioxidantes y oxidantes tales como peróxido de hidrógeno e hipoclorito de sodio, obteniendo las curvas resultantes “Curvas Efecto”[12]. El estrés oxidativo al que se sometió la levadura consistió en colocar agentes oxidantes a concentraciones de 0,5 mmol/L (estrés bajo) y 50 mmol/L (estrés alto). Los reactivos utilizados fueron peróxido de hidrógeno (H_2O_2) e hipoclorito de sodio (NaOCl)[13,14].

Se aislaron levaduras a partir de un sobre de levadura comercial (Levapan, Ecuador) en medio PDA (Merck, Alemania), con incubación a 28 ºC por 72 horas. Se tomó una colonia de *S. cerevisiae* y se inoculó en 5 ml de medio YPD [2 % (m/v) glucosa, 1 % (m/v) extracto de levadura (Conda, España) y 2 % (m/v) peptona (BD, España)] manteniéndose a una temperatura de incubación de 28 ºC por 6 horas y una velocidad de agitación de 100 rpm. Se tomaron 5 µL de una dilución 1/10 del cultivo incubado y se inocularon en un tubo con medio YPD manteniéndose en incubación por 18 horas a 28 ºC y 100 rpm. Posteriormente se centrifugaron los tubos a 2 700 rpm por 20 minutos a temperatura

Polímeros	polímero – Extracto de carotenoides	Temperatura de entrada (°C)	Temperatura de salida (°C)
goma arábiga + maltodextrina (GAMD) C15T16090 (E1)	35:35:30	160	90
goma arábiga + maltodextrina (GAMD) C15T12080 (E2)	35:35:30	120	80
goma arábiga + maltodextrina (GAMD) C10T16090 (E3)	40:40:20	160	90
goma arábiga + maltodextrina (GAMD) C10T12080 (E4)	40:40:20	120	80

Tabla 1. Proporciones polímero – licopeno y temperatura de entrada/salida.

ambiente, se lavó el pellet con buffer fosfato salino (PBS) pH 7,4. Se disolvió el pellet con PBS hasta llegar a una absorbancia a λ de 600 nm (A600) de 0,1 dejando en reposo por 30 minutos a 28 ºC.

Se colocó el peróxido de hidrógeno e hipoclorito de sodio (Ecuaquímica, Ecuador) a concentraciones de 0,5 mol/L y 50 mol/L, se incubó a 28 ºC durante 1 hora sin luz. A continuación, se centrifugó a 2700 rpm por 20 minutos a temperatura ambiente, se lavó el pellet con PBS y se disolvió en 3 mL de medio YPD. Se sembró 180 µL de cada cultivo por triplicado en una placa de 96 pocillos y se llevó a incubación a 28 ºC en el lector multiplaca (Fisher Scientific, Finlandia) con control de crecimiento mediante la A_{600} por 24 horas a intervalos de 20 minutos con agitación antes de cada lectura.

La razón de crecimiento se determinó con la Ecuación 4 donde relaciona la absorbancia (A) de la curva estándar o del crecimiento de la levadura sometida a varias concentraciones de extractos y agentes oxidantes con respecto a la del crecimiento de la levadura sin tratamiento[15].

$$Razón\ de\ crecimiento = \frac{A_{600}\ de\ la\ curva\ estándar}{A_{600}\ de\ la\ curva\ control}$$

Resultados y discusión

Cuantificación de licopeno en el concentrado obtenido

Una vez secadas y molidas las cáscaras de tomate de árbol *Solanum betaceum*, el material particulado obtenido se empleó para la extracción de sus compuestos bioactivos. Posteriormente, se concentró y se le realizó la cuantificación de licopenos, tomando así un valor de 0,55 ± 0,02 mg de carotenos/L. Estudios realizados por Pérez[8], en los que obtuvo 0,51 mg/L muestran relación con los resultados obtenidos. Por otra parte, Arándiga & Díaz[3] tras realizar experimentos en el laboratorio consiguieron una cantidad de 0,44 mg/l ya que utilizaron el fruto maduro, lo que indica que el tiempo de cosecha influye en el proceso de obtención de carotenoides[16].

Determinación del porcentaje de eficiencia de la microencapsulación (EME)

Mediante un diseño experimental 2^2, con un nivel de confianza del 95 %, se analizó la influencia de las variables proporción polímero-licopeno y las relaciones temperatura de entrada y salida del equipo.

La ecuación del diseño obtenida es la siguiente:

-217,22 + 13,04*A + 1,59*B - 0,08*A*B con un R^2= 98,52 (Figura 1).

El gráfico de superficie de respuesta muestra el valor óptimo del porcentaje de eficiencia de la microencapsulación (EME) siendo este del 64,42 % que representa a una carga de licopeno del 30 % con una temperatura de entrada de 120 ºC y una temperatura de 80 ºC para la salida.

Comparando los resultados obtenidos por Soto[17], quien realizó microencapsulación con goma xantana se observó un 77 % de esta variable. Así mismo Rocha[18] determinó intervalos del 25 al 80 % empleando goma arábiga y maltodextrina, comparando con los valores obtenidos en el presente trabajo se encuentra dentro de este intervalo. Es importante recalcar que la variación de temperatura afecta tanto a los polímeros que son cadenas alifáticas como es el caso de la maltodextrina y mezclas de ciclo alquenos que brindan mayor estabilidad al compuesto de interés microencapsulado, en este caso licopeno y carotenoides que en su estructura presentan largas cadenas con dobles enlaces y grupos alquilo. Por tal razón es vital controlar esta variable evitando degradación de los componentes. Por otro lado, se ha reportado valores entre el 12 – 88 % de la misma variable utilizando maltodextrina como polímero y temperatura de entrada del equipo de 120 ºC[19].

Determinación *in vitro* de la actividad antioxidante por el método del radical 2,2-diphenil-1-1-picrylhydrazil (DPPH)

La ecuación obtenida del diseño para la actividad antioxidante es la siguiente:

-44,52 + 15,09*A + 0,76*B - 0,05*A*B, con un valor de R^2= 97,43 (Figura 2)

El microencapsulado que presentó mayor actividad antioxidante fue la combinación que presentó 30 % de carga de licopeno, 35 % de maltodextrina y 35 % de goma arábiga

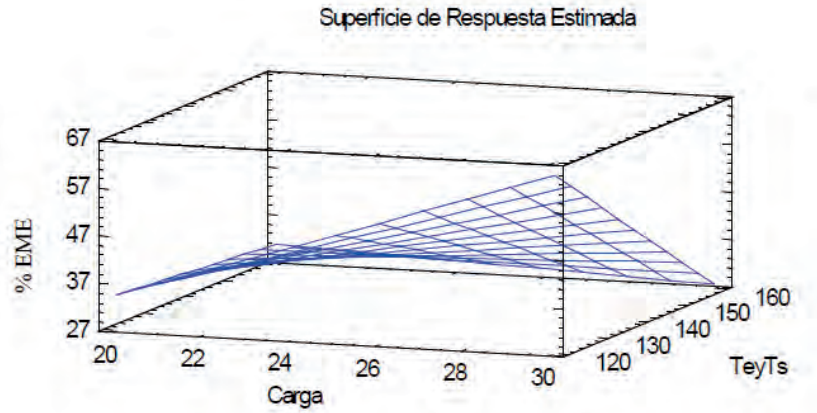


Figura 1. Diagrama de superficie de respuesta para el análisis del porcentaje de eficiencia de microencapsulación (EME).

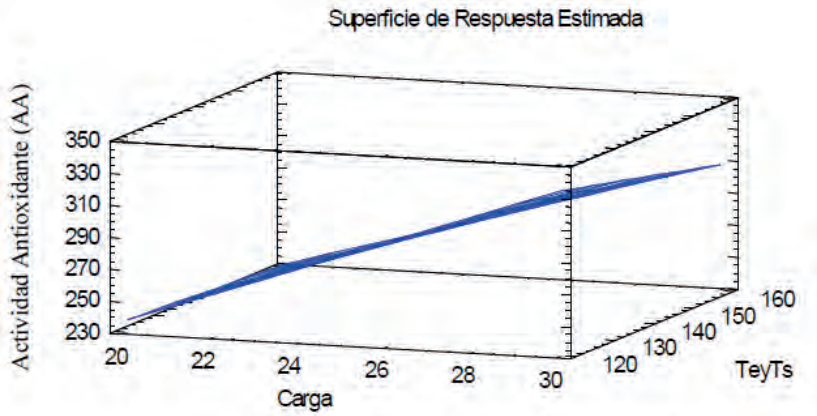


Figura 2. Diagrama de superficie de respuesta estimada de la actividad antioxidante determinada por el radical libre DPPH.

con una temperatura de entrada (Te) de 120 ºC y de salida (Ts) de 80 ºC, obteniendo un porcentaje de inhibición del 81,84 % lo que equivale a 333,78 ± 4,53 µmol de Trolox/g muestra. Este valor es menor al obtenido por Mera[20], en el cual el extracto de *G. mulfordii* obtuvo una concentración de 776,42 µmol trolox/g de extracto, al igual que los valores reportados por Hurtado[21] para el extracto hidroalcohólico de nogal (*Juglans Neotropica*) con un valor de 1019,10 µmol trolox/g extracto seco. Por otro lado, tiene una actividad antioxidante mayor a los datos de Moreno[22], quien realizó estudios en aguacate y encontró que la variedad Hass, tiene un valor de 189,80 ± 10,80 µmol trolox/g muestra, uvilla 70,30 ± 9,60 µmol trolox/g extracto[23], naranjilla 62,45 ± 13,22 µmol trolox/g muestra y taxo 55,47 ± 7,42 µmol trolox/g muestra[24].

Determinación *in vivo* de la actividad antioxidante usando *Saccharomyces cerevisiae*

Para poder determinar de manera preferible la mejor combinación de los microencapsulados se tomó en cuenta que el valor máximo representa a la actividad antioxidante, mientras que el valor mínimo se refiere a la actividad pro-oxidante y la capacidad que muestra el licopeno para ayudar a la levadura para activar su respuesta frente al estrés[25] (Figuras 3, 4, 5, 6).

El pico más alto que muestra la Figura 3 es 1,37 para el microencapsulado 2 seguido del 4 con 1,35 sometidos a un estrés bajo con 0,5 mmol/L de NaOCl, mientras que con peróxido de hidrógeno a 0,5 mmol/L el microencapsulado 2 tomó un valor de 1,45 (Figura 4). En cuanto al estrés alto el punto que sobresale es de 1,46 para el tratamiento con hipoclorito de sodio (Figura 5) y 1,29 con peróxido de hidrógeno (Figura 6).

Comparando cada uno de los valores antes mencionados es importante conocer la respuesta que presenta la levadura frente al estrés, además del mecanismo que presenta el licopeno frente a los radicales con los que interactúa. Por lo que, en el caso del hipoclorito de sodio el licopeno se fragmenta generándose metabolitos que interactúan con los iones (OCl^-) donándoles un hidrógeno[26]. Con respecto al peróxido de hidrógeno Plasencia[27], menciona que el H_2O_2 actúa a nivel del citosol activándose la respuesta transcripcional de la levadura. Según Martínez & Estruch[28], la levadura presenta dos tipos de catalasa que transforman los radicales superóxido en agua y oxígeno.

Ensayos realizados por Peláez[12], muestran que extractos etanólicos de cacao presentan una razón de crecimiento superior a 1,5 a 0,5 mmol/L de peróxido de hidrógeno lo que indica una actividad antioxidante similar a la del tomate de árbol. Por otra parte, Mera[20], reporta resultados de 2,5 para liposomas de *Greigia mulfordii* en condiciones de estrés bajo. En el caso del microencapsulado de licopeno se nota una actividad antioxidante con una razón de crecimiento 1,46 lo que indica que el microencapsulado de carotenos presenta características similares a los extractos de cacao y que los grupos fenólicos que contiene Greigia mulfordii tienen mayor actividad antioxidante.

Los microencapsulados tienen la ventaja de la liberación controlada de compuestos, se ha reportado que la capacidad antioxidante depende del polímero utilizado para su protección e interacción con la membrana plasmática de la célula para asegurar su biodisponibilidad[29]. La liberación lenta puede estar asociada a la interacción entre las bicapas lipídicas y el licopeno al ser un compuesto hidrofóbico se acopla fácilmente a las lipoproteínas de la membrana celular[30].

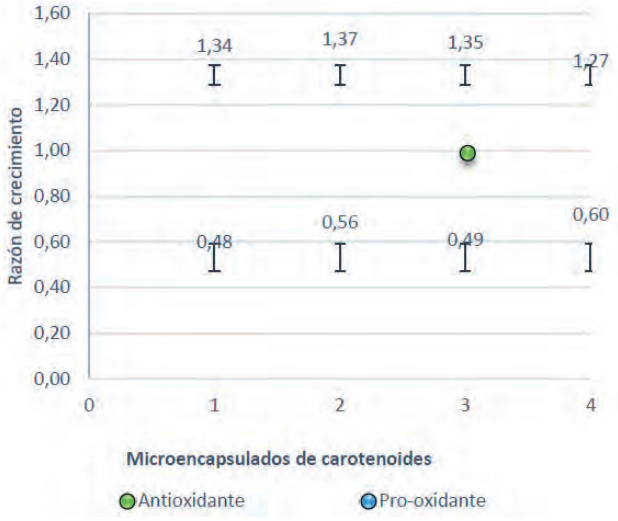


Figura 3. Razón de crecimiento de *S. cerevisiae* con actividad pro-oxidante y antioxidante a 0,5 mmol/L NaOCl.

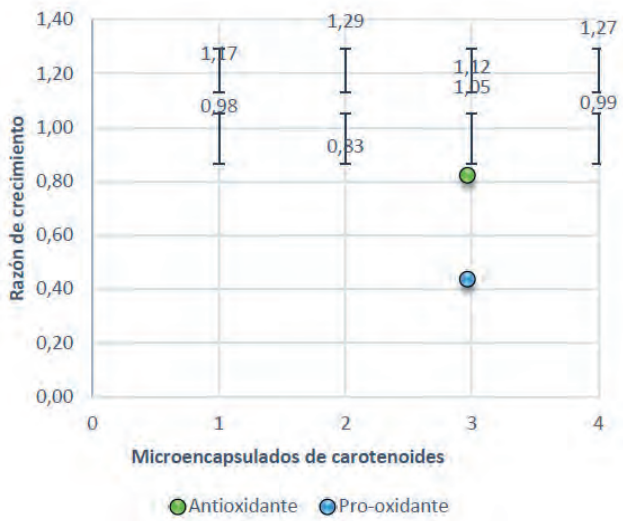


Figura 4. Razón de crecimiento de *S. cerevisiae* con actividad pro-oxidante y antioxidante a 50 mmol/L NaOCl.

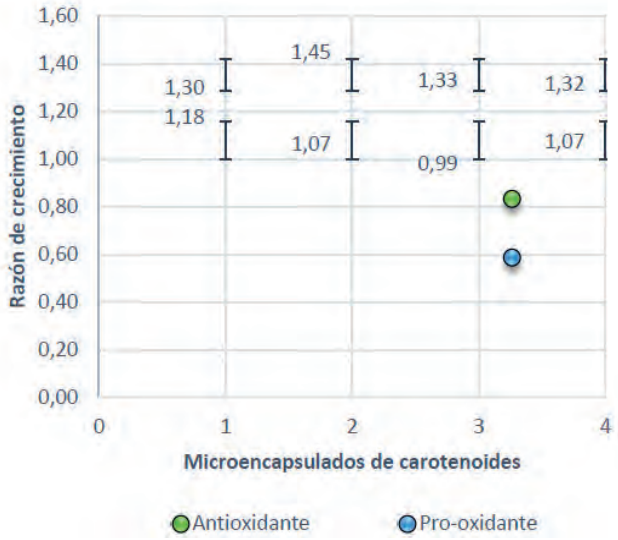


Figura 5. Razón de crecimiento de *S. cerevisiae* con actividad pro-oxidante y antioxidante a 0,5 mmol/L H_2O_2.

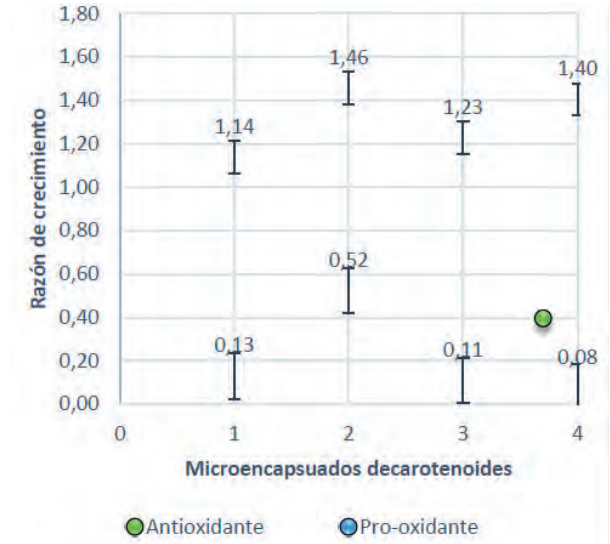


Figura 6. Razón de crecimiento de S. cerevisiae con actividad pro-oxidante y antioxidante a 50 mmol/L H_2O_2.

Es importante recalcar que en este trabajo la razón de crecimiento mínima alcanzada por el microencapsulado 2 (30 % licopeno Te/Ts 120 ºC/80 ºC) en la fase pro-oxidativa fue 1,07 y 0,56 mientras que en la actividad antioxidante tomaron valores de 1,45 y 1,37 frente a un estrés de 0,5 mmol/l de H_2O_2 y NaOCl respectivamente una concentración de 700 mg/mL de microencapsulado de carotenos.

Conclusiones

Se evaluaron las diferentes combinaciones de polímeros en la microencapsulación de licopeno de tomate de árbol (*Solanum betaceum*) encontrando que la mejor combinación fue la del microencapsulado que contiene 30 % de carga, 35 % goma arábiga y 35 % de maltodextrina a una temperatura de entrada (Te) de 120 ºC y de salida (Ts) de 80 °C que presentó el mayor porcentaje de eficiencia de microencapsulación con un valor de 64,42 %.

Se comparó la actividad antioxidante *in vitro* de los diferentes microencapsulados y del concentrado de licopeno mediante el método DPPH en el cual se observó que el valor máximo coincidió con las microcápsulas obtenidas con las mismas condiciones (30 % de carga, 35 % goma arábiga y 35 % de maltodextrina a una temperatura de entrada (Te) de 120 ºC y de salida (Ts) de 80 °C) con un porcentaje de inhibición del 81,84 % que representa una concentración equivalente de Trolox de 333,78 ± 4,53 µmol de Trolox/g muestra.

Se evaluó la actividad antioxidante in vivo de los diferentes microencapsulados de licopenos empleando *Saccharomyces cerevisiae* encontrando que este microencapsulado a una concentración de 700 mg/ml presenta mayor actividad antioxidante tomando valores de 1,37 y 1,45 en la razón de crecimiento sometida a una concentración de 0,5 mmol/L de hipoclorito de sodio y peróxido de hidrógeno respectivamente.

Al someter a la levadura a un estrés alto, es decir, a 50 mmol/L de agentes oxidantes se evidenció que este microncapsulado presentó mayor efecto antioxidante sobre la célula tomando un valor máximo de razón de crecimiento

de 1,26 con hipoclorito de sodio, mientras que al utilizar peróxido de hidrógeno esta variable aumentó a 1,46.

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ARTICLE / INVESTIGACIÓN

Detect the Antibacterial and Antitumor of synthesized Silver Nanoparticles Using *Microbacterium sp*

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Abstract: Metal nanoparticles are widely utilized in biotechnology and biomedicine for various applications, including medication delivery, imaging, and bacterial growth control. Silver nanoparticles (AgNPs) were synthesized by bacteria, fungi, algae, and plants. The Study aimed to synthesize nanomaterial with a cost-effective, environmentally friendly, and the uses of AgNPs as antibacterial (against pathogenic bacteria) and anticancer (on MCF7 cell line). In this Study, bacteria were collected from different soil samples. Isolated, purified by selective media, identification genotypically by 16rRNA sequencing analysis, then compared with NCBI, GenBank as *Microbacterium sp.* Biosynthesis of silver nanoparticles using *Microbacterium* for extracellular synthesis by reducing silver ions to silver nanoparticles. The color change to brown and reddish-brown was the first indication of the AgNPs formation; physical characterization using UV-Visible spectroscopy showed a wavelength in 489 nm, while X-ray diffraction (XRD) revealed that the silver nanoparticles were crystalline; transmission electron microscope (TEM) image showed that AgNPs spherical in shape with an average diameter of around 50 nm, in SEM (Scanning electron microscope) AgNPs formed with a diameter of 41-44 nm, spherical and uniform, while Energy-dispersive X-ray show very high silver peaks. Bioactivity of AgNPs by antimicrobial on pathogenic bacteria, which collected from Al- Sadr hospital in Misan (identified by using VITEK). This experiment showed that the inhibition zone was rung from (6- 38mm) on pathogenic bacteria; it was tremendous compared with antibiotics (Gentamycin in this project ranged from(7-28.5mm). Antitumor activity of extracellular biosynthesized AgNPs was determined using the MTT test against breast cancer cells (MCF7 cell line), which showed very high results. AgNPs inhibition breast cancer cell line by about 81% at 100ug/ml, indicating that the rate is outstanding. Finally, different biomedical approaches can benefit from AgNPs as antibacterial agents and anticancer agents with many results.

Key words: Silver Nanoparticles, biosynthesis, antibacterial, and antitumor.

Introduction

Recently, the immunological compatibility of humans has greatly enhanced the emergence of microbial diseases. As a result, many novel antibiotics and therapeutic pharmaceutical substances with a wide range of applications have been made available on the market to protect humans from various diseases[1], but they can potentially affect the environment, especially in developing nations[25]. Metals containing nanoparticles have the potential to be used in the control of several types of infection, but little is known about their antibacterial capabilities18. Due to a growing desire to generate environmentally friendly products, the synthesis of nanoparticles has become a hot topic at the junction of nanotechnology and biotechnology[2,26,27]. Using bacteria, fungus, algae, actinomycetes, plants, and other organisms, biogenic synthesis of metal nanoparticles has been demonstrated[3], Actinobacteria such as *Streptomyces sp.*[4], *Nocardia sp.*[5], and *Rhodococcus sp.*[6], have been reported to synthesize and characterize silver and gold nanoparticles. Metals such as zinc, silver, titanium, and copper have antibacterial properties that have been recognized for decades, allowing them to be used in various current medical applications to manage microbial infection disorders[33]. According to one idea, free metal ion toxicity arising from nanoparticle surfaces may play an essential role in infection prevention[34]. The catalytic, electrical, and optical characteristics of AgNPs are well-known[7,8]. A new generation of dressings comprising antimicrobial compounds like silver was created to prevent or minimize infection[9]. Hybrids of silver nanoparticles with amphiphilic hyperbranched macromolecules have recently been proven to have efficient antibacterial surface coating[10]. Because of their widespread use, silver nanoparticles are in high demand. Silver nanoparticles have attracted much attention among noble metal nanomaterials because of their appealing physicochemical feature[11]. Individual silver nanoparticles are great candidates for molecular labeling because of their surface Plasmon resonance and large effective scattering cross section[12,13]. Even Antifungal candidates could be AgNPs[19]. Silver ions have a well-known bactericidal action on microorganisms; however, the bactericidal process is only partially understood. It's been proposed that ionic silver interacts significantly with the thiol groups of essential enzymes, rendering them inactive[29].

Experiments show that when bacteria are exposed to silver ions, their DNA loses its replication capacity. Other research has found indications of structural alterations in cell membranes and the creation of tiny electron-dense granules from silver and sulfur[30,31]. Chemotherapy is one of the

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most common treatments for cancer, and a large number of antitumor compounds are found in nature as a whole or as derivatives, mostly formed and produced by microorganisms, particularly Actinomycetes, which produce a large number of natural products with various biological and bioactive properties, as well as antitumor properties, they work by inducing apoptosis through one of the suitable mechanisms. Topoisomerase, I or II inhibition, causes such DNA cleavage. Inhibition of essential enzymes affects signal transduction, such as proteases, mitochondrial permeabilization, cellular metabolism, and tumor-induced angiogenesis in some situations[32]. The Study's goal was to isolate and identify *Microbacterium* from the soil. Biosynthesis of silver nanoparticles, which were characterized using physicochemical methods such as UV-spectroscopy, XRD, SEM, TEM, EDX, and Study of the antibacterial activity of the AgNPs synthesized biologically against pathogenic bacteria; the last goal was evaluation the activity of AgNPs that synthesized in the lab as antitumor on MCF7 cell line (breast cancer cells).

Materials and methods

Soil Samples Collecting

Tn sterile polythene bags, soil samples were collected from sugar cane fields and gardens in Misan at 11cm below the surface. The samples were named with numbers. Closely tightened, and were taken to the laboratory[14].

Isolation of Actinomycetes

The soil samples were dried in the oven at 60oC for three hours to reduce the number of bacteria other than Actinomycetes in the soil. Actinomycetes form spores and then grow in the media. Serial dilution was done for each sample. The isolation media SCN Agar (Starch-casein-nitrite agar)[28] contained 1 ml of Cycloheximide (100ùg/ml) as antifungal agents; the samples were incubated for 5 days 30oC. The isolation bacteria were Isolated in pure culture on transfer medium YEG agar (Yeast Extract Glucose agar)[28], with one colony on each plate.

16S rRNA Gene Sequencing of Isolated Bacteria

Several methods for determining DNA sequence have been used using a universal primer (Macrogen/Korea), as shown in table1.

Genomic DNA was extracted from isolates using DNA Kit (Presto' Mini g DNA Bacteria Kit, Geneaid, Taiwan), PCR reaction mixture with a final volume of 20µl consisting of 2µl for each 27F and1492R primers (10 picomoles), 9µl De-ionized water, and 7µl of the DNA of the isolate, were added into the Maxime TM PCR Premix i-Taq (Intronbio/Korea), then amplified by polymerase chain reaction (PCR) technique under the following conditions: An initial denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 1 min. Annealing at 58°C for 30 sec. and 72°C for 1 min, with a final extension at 72C° for 7 min. The PCR product that amplified was then Electrophoresis on Agarose Gels[34]. The isolation strain was identified genotypically by 16S rRNA sequencing analysis and then compared with (NCBI) GenBank[14].

Biosynthesis of AgNPs Laboratory

Colonies transfer into a conical flask containing 200 ml of MGYP (Malt extract glucose yeast extract peptone broth)[7] at PH 7.0 and put in a shaker incubator (rpm 150) at room temperature for 7 days. After that colony developed on the medium, filtrated through Whatman filter paper No.1 (Sigma/USA). The supernatant was added to 2mM of AgNO3(V/V) and incubated in an orbital shaker (rpm 150) for 7days at room temperature; after that, the color changed into dark color (reddish-brown dark) indicating the formation of AgNPs in the culture solution[7].

Characterization of Biosynthesis AgNPs

The AgNPs were Characterized physically by using:

UV-Visible spectroscopy

UV-Vis analyzed silver nanoparticles that were biosynthesized laboratory. Spectroscopy (Elettrofor/Italy) to determine the absorption spectrum, The sample of bio- AgNPs collected in a quartz cuvette (1cm path length) contains 2ml of the solution to fill past the instrument light path. At room temperature, the untreated supernatant was set as reference control while treated supernatants were used to monitor their UV-Visible absorbance Spectra between 300-800 nm wave length[36].

X-ray diffraction (XRD) analysis

X-ray diffraction (Broker/Germany) is one of the most widely used techniques for characterizing NPs. XRD usually provides information regarding crystal structure, phase nature, lattice dimensions, and crystal sizes[20].

Transmission Electron Microscopy (TEM) examination

The formation type (shape) and size of the generated silver nanoparticles were determined by Transmission Electron Microscopy examination (Broker/Germany), according to magnification TEM micrographs[37].

Scanning Electron Microscopy (SEM)

SEM (Buker/Germany) was used to examine the AgNPs in the sample. Thin films of the sample were made on carbon-coated copper grids by dropping an amount of the filtrate on the grid and blotting away the excess solution using blotting paper, then allowing the films to dry overnight at room temperature under sterilized conditions. The silver nanoparticles were imaged using a scanning electron microscope equipped with EDX attachment[38,39].

Primer	Sequence (5´- 3´)	Length	Amplicon size	Reference
27F	AGAGTTTGATCCTGGCTCAG	20bp	1500bp	36
1492R	GGTTACCTTGTTACGACTT	19bp		

Table 1. This is a table of the sequence of Universal primer kite[36] used in this experiment.

Antibacterial Activity

The antibacterial activity of synthesized AgNPs was tested using the disc diffusion method[15] against some human pathogens from both gram-negative and gram-positive bacteria collected from Al-Sadr hospital in Misan/Iraq, as shown in table 2.

NO.	Pathogenic bacteria
1	*Staphylococcus* aureus
2	*Staphylococcus haemolytic*
3	*Staphylococcus hominis*
4	*Escherichia coli*
5	*Pseudomonas aeruginosa*
6	*Klebsiella pneumonia*
7	*Salmonella typhi*
8	*Enterobacter cloacae*
9	*Staphylococcus lentus*
10	*Proteus mirabilis*

Table 2. This is a table of pathogenic bacteria that were collected from Al-Sadr hospital in Misan and used in our experiment.

Using sterile cotton swabs, each strain was swabbed uniformly into the individual Muller Hinton agar plates. 30ul of synthesized AgNPs were placed onto a plate using a sterile micropipette. It was then applied to a sterile paper disc (0.6 mm) and left to dry. After putting the AgNPs disc on the plates then incubation for 24 hours at 37°C, inhibitory zones appeared around the filter paper disc, showing the bioactivity of produced AgNPs[40]. The clear zone diameters were measured and compared to Gentamycin (30ul).

Antitumor activity

Mcf7 (breast cancer cells) were obtained from the IRAQ Biotech Cell Bank Unit in Basrah and cultured in RPMI 1640 (Gibco/USA) with 10% Fetal bovine serum (Sigma/USA), 100 units/mL penicillin, and 100 g/mL streptomycin. Cells were passaged twice a week with Trypsin-EDTA, reseeded at 50% confluence, and incubated at 37°C with 5% CO2[41]. The cytotoxicity test (measured by MTT assay) performed the MTT cell viability assay on 96 well plates to detect the cytotoxic effect. The mcf7 cell line was planted at 1 $\times 10^4$ cells per well. Cells were treated with the tested substance at a final concentration of 1000ug/ml after 24 hours or when a confluent monolayer was attained. After 72 hours of treatment, cell viability was determined by removing the medium, adding 28 liters of a 2mg/ml MTT solution (Gibco/USA) and incubating the cells for 2 hours at 37°C. Following removal of the MTT solution, the crystals in the wells were solubilized by adding 100ul of DMSO (Dimethyl Sulfoxide) and incubating at 37°C for15minutes with shaking[42]. The absorbency was measured using a microplate reader at 620 nm (test wavelength), and the assay was done three time[16,17].

Results

After incubation period the bacteria was grown on culture media, then purified on transfer media. The isolates were identified genotipically[14].

Identification of the Isolated Strains genotypically

After DNA extraction from isolated bacteria, the DNA must be amplified by polymerase chain reaction (PCR) technique, then the nucleotide sequences of the 16S rRNA gene were compared to the nucleotide sequences of reference strains retrieved from the GenBank database. One of the isolated was a new strain, so it was registered in my name on GenBank the other one was already registered on GenBank; the first isolation (the new one) was:

1. *Microbacterium paraoxydans* strain shahooda, 16S rRNA gene, partial sequence100% identical, Sequence ID: MZ701742.1, Length: 1388bp. The second isolation:
2. *Microbacterum lacticum*, strainSTM54,16S rRNA sequence gave 949 base pair, an NCBI, BLAST search revealed that the sequence was 100% identical to the sequence of *Microbacterium lacticum*. strain STM54 16S rRNA gene, partial sequence, Sequence ID: KY393059.1, Length: 949bp.

Producing Nanoparticles

This Study was focused on the extracellular synthesis by supernatant to form AgNPs using *Microbacterium sp*, after incubation period the color change from white to reddish brown was the first indicating of silver nanoparticles formation[7], as shown in figure 1.

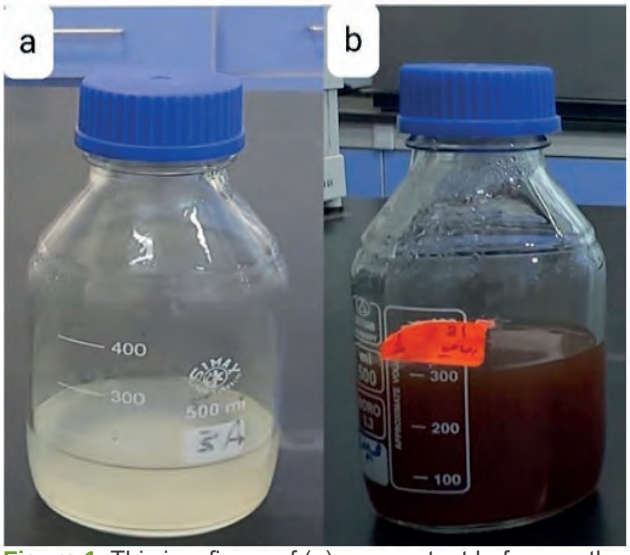


Figure 1. This is a figure of (a) supernatant before synthesis. (b) supernatant after the formation of AgNPs.

Characterization of Ag Nanoparticles

UV-Vis spectroscopy

Metallic bio-nano particles have a distinct optical absorption spectrum in the UV visible region (300-800)[36], and the optical absorption spectrum in this Study was at 489nm wavelength, as shown in figure 2.

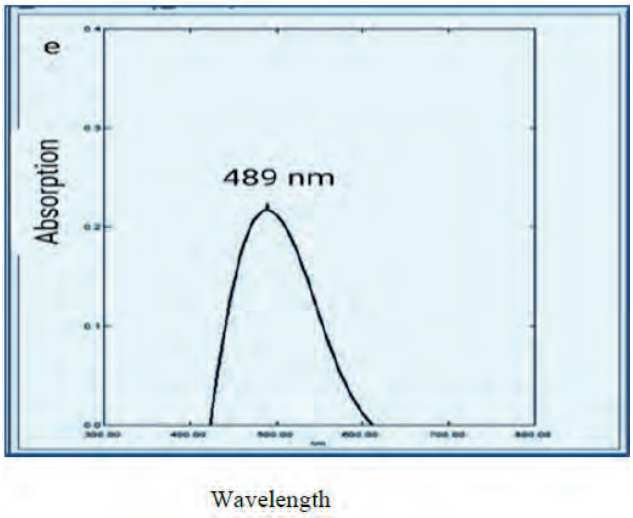


Figure 2. This figure of UV-Vis spectroscopy of AgNPs showed a peak at 489nm wavelength.

As the process progressed, the spectra indicated a rise in silver solution intensity with time, indicating the creation of more silver nanoparticles in the solution. Shows that after 72 hours, there is no discernible difference in the UV-Vis spectra of the reaction product, indicating that the process has reached equilibrium.

XRD analysis

Further studies were carried out on silver nanoparticles using X-ray Diffraction. Depicts the evaluation of the XRD phase and crystal structure analysis of green produced AgNPs. In the XRD investigation, 2□degrees = 32.5, 28.3, and 48.1 values were used to determine the reflections (122), (111) and (200), respectively, diffraction Standards (JCPDS) silver file No. (04–0783)[23], as shown in figure 3.

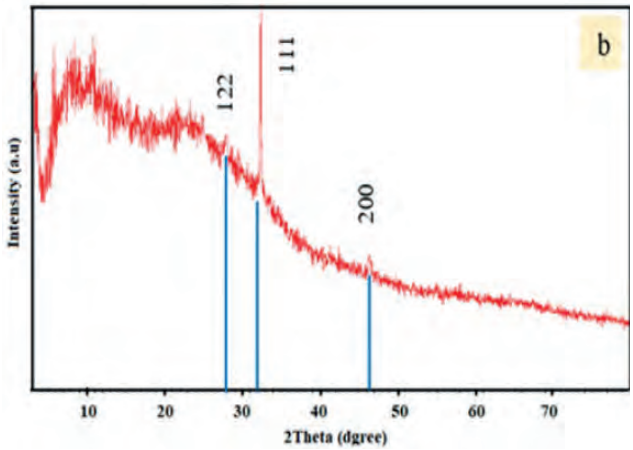


Figure 3. This figure of X-ray diffraction of synthesized AgNPs analysis, diffraction standards (JCPDS), silver file No. (04–0783).

AgNPs have an elemental (Ag^0) and spherical and generated crystalline, indicating that they are face-centered and cubic. Similar results have been presented in another research[46-48].

TEM analysis

TEM was used to determine the size and shape of particles. Silver nanoparticles which examine with TEM were of an average diameter of around 50 nm as shown in figure 4.

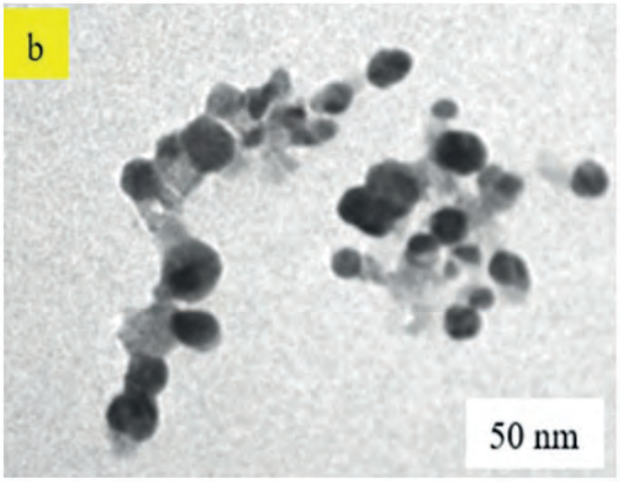


Figure 4. This figure of Transmission Electron Microscopy image showed the size of silver nanoparticle with an average diameter of around 50 nm.

SEM analysis

As showed in the previous studies on biosynthesized silver nanoparticles on SEM image found to be generally spherical and uniform[49,50], and the size was between 41-44 nm, as shown in figure 5.

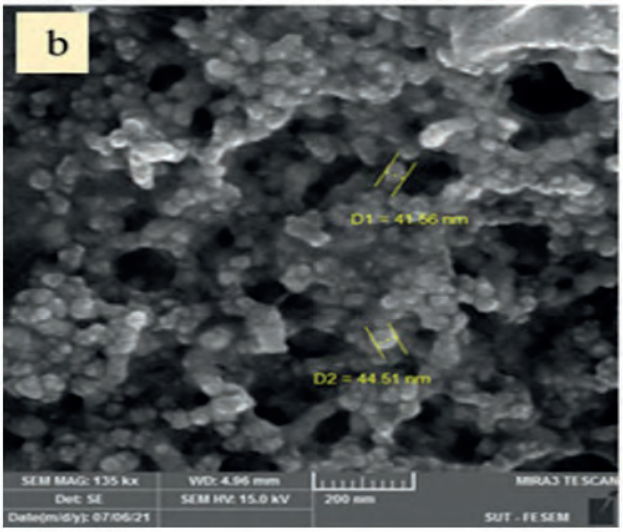


Figure 5. This is a figure of a Scanning Electron Microscopy image of AgNPs fabricated by *Microbacterium sp.* with a 41-44 nm diameter.

Biological Characterization of Synthesized Silver Nanoparticles

Antibacterial Activity

The data from energy dispersive spectroscopy X-ray (EDX) show very high silver peaks, indicating that the reduction of silver ions to elemental silver may have come

from molecules connected to the AgNPs' surface. The silver's dense peak was a clear indicator of the formation of silver nanoparticles from silver ions[20]. As shown in figure 6.

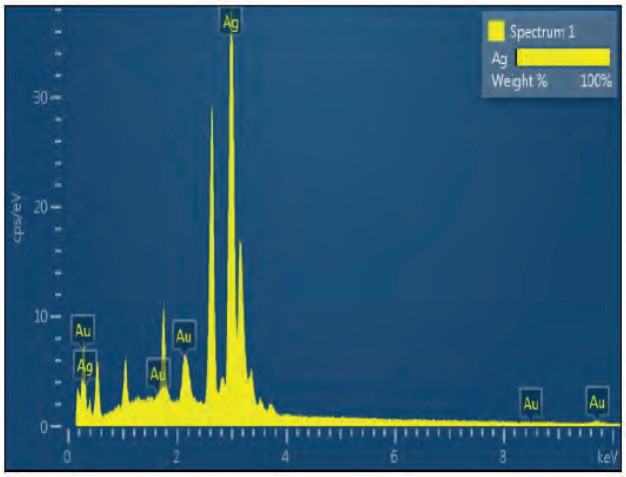


Figure 6. This figure of EDX analysis showed a strong peak of silver at 3kev.

Antibacterial testing revealed that the Nanoparticle is effective against bacterial pathogens[21,22]. Both Gram-negative and Gram-positive bacteria alike (identified by using-VITEK-2)[23] and compared with Gentamycin antibiotic, as shown in figure 7 and figure 8.

Microbacterium's silver nanoparticles displayed the most significant inhibition zones (38mm) against *S.aureas* bacteria; it was enormous compared with antibiotics (Gentamycin) in this project; the lowest inhibition zone was 7 mm on pseudomonas aeruginosa bacteria, as shown in table 3.

Antitumor activity of AgNPs biosynthesized laboratory

Silver nanoparticles play important role as antitumor[24]. At low concentrations demonstrated very great activity on MCF-7 cell line, AgNPs inhibition breast cancer cells by about 81.732 % at 100ug/ml, and only18.268 % of MCF7 were able to form formazan product and remained as alive cells (viability, after 72hr of incubation period. There was significant change in cancer cell that inhibition with synthesized silver nanoparticles at a different concentration (10,30,60,80,100ug/ml) of AgNPs, by about (9.263%,65.252%,81.565%, 80.447%,81.732%), as shown in) figure 9.

The median inhibitory dose (IC50) value of 24.93ug/ml. This means that biosynthesis AgNPs have excellent antitumor activity on breast cancer cells. These results agreed with those described by (24).

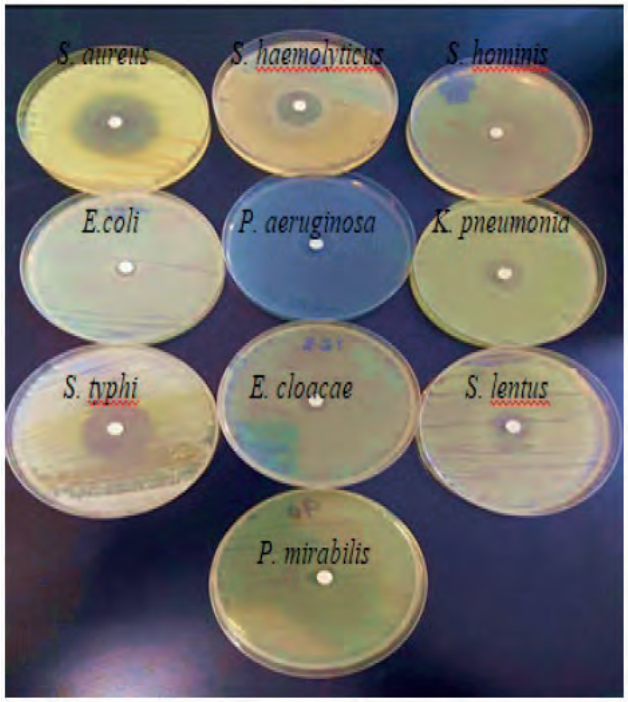


Figure 7. This figure of the Antibacterial activity of synthesized AgNPs against ten pathogenic bacteria.

Discussion

Compared to other bio-reductants, the manufacture of metallic nanoparticles using microorganisms is more fruitful in terms of ease[51]. Phytochemicals are well known for converting Ag^{1+} to Ag^{0} and capping these nanoparticles, making them highly stable[52]. In this Study, *Microbacterium* was used after being isolated from soil to green synthesis of AgNPs, and This indicates that this isolated bacteria had the enzyme that reduced Ag^{+} to Ag^{0} by Nitrate reductase, then the aggregation of silver atoms was formed AgNPs. Then was tested of the synthesized AgNPs were for bactericidal and cytotoxic activity. Spectroscopy in the ultraviolet and visible ranges was used to determine metallic nanoparticles' formation and exploit their optical properties. Plasmon bands were recorded at various points during the bioreduction process. The AgNPs were prepared principally by the emergence of a reddish-brown color after 72h. The reaction was finished and validated by the Plasmon absorption peak, which reached a constant value. The SPR (surface Plasmon resonance) band of the spherical AgNPs at 489 nm is visible

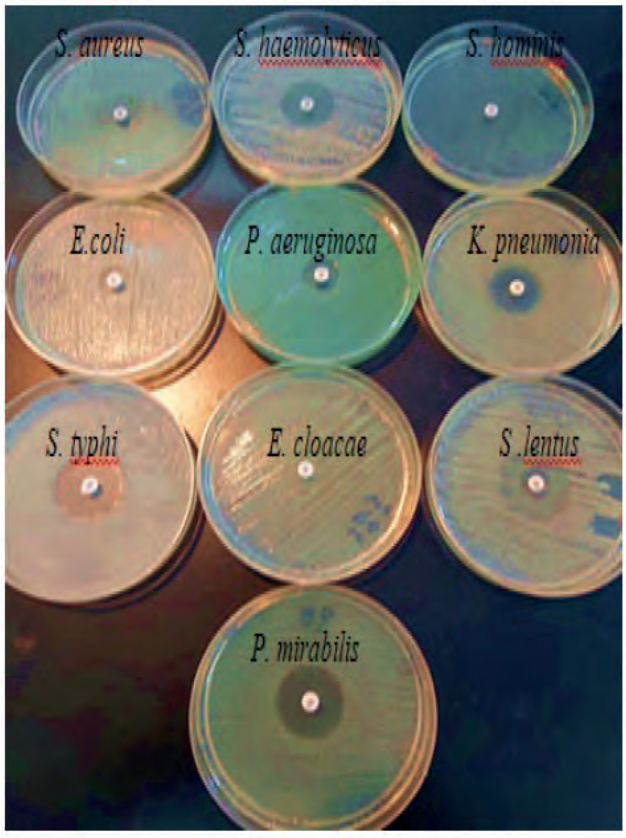
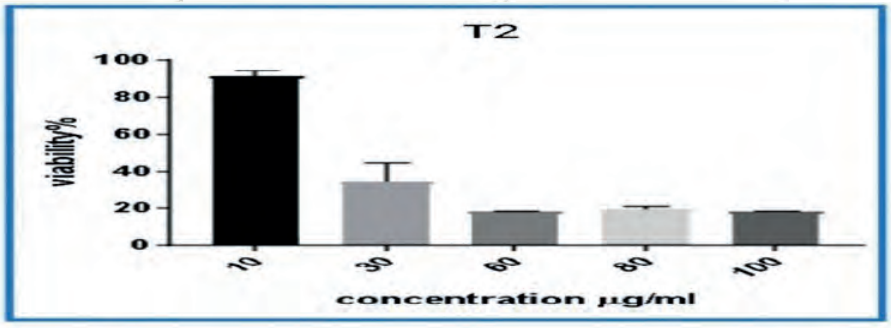


Figure 8. This figure of the Antibacterial activity of antibiotic (Gentamycin) against the same pathogenic bacteria.

Pathogenic bacteria	Inhibition zone of Gentamycin (antibacterial agent) (mm)	Inhibition zone (mm) of synthesized AgNPs
Staphylococcus aureus	9 mm	38 mm
Staphylococcus haemolytic	24.5 mm	22.5 mm
Staphylococcus hominis	7 mm	13 mm
Escherichia coli	14 mm	9.5 mm
Pseudomonas aeruginosa	13.5 mm	7 mm
Klebsiella pneumoniae	16.5 mm	18 mm
Salmonella typhi	24.5 mm	33.5 mm
Enterobacter cloacae	8 mm	12 mm
Staphylococcus lentus	28.5 mm	21 mm
Proteus merabilis	24.5 mm	10 mm

Table 3. This table of Inhibition zone on ten pathogenic bacteria by synthesized AgNPs and Gentamicin.



Compound µg/ml	10	30	60	80	100
Viability%	90.837	34.748	18.435	19.553	18.268

Figure 9. This figure of viability percent on the mcf7 cell line in different concentrations of synthesized AgNPs (10, 30, 60, 80, 100) ug/ml.

in the spectra, showing that the synthesis of AgNPs in the reaction mixture is consistent with the AgNPs synthesized before[53]. After one month under ambient circumstances, the stability of the nanoparticles was assessed throughout time (30 days), and no change in absorption peak value was detected, indicating that the nanoparticles are highly stable.

The particle size and shape of the produced AgNPs were studied using TEM and SEM. The synthesized AgNPs were monodispersed, spherical in shape, and ranged in size from 41 to 44 nanometers. The interaction of hydrogen bonds and electrostatic interactions between the bioorganic capping molecules linked to the AgNPs resulted in silver nanoparticles. Even within the aggregates, the nanoparticles were not in direct contact, indicating that the nanoparticles had been stabilized by a capping agent and crystalline in nature[45], and well dispersed, primarily spherical, which is agreed with other reports[44,46].

Correlated to the (111), (200), and (220), and indicating that AgNPs have been prepared. Several Bragg reflections with 20 values of 32.5°, 28.3°, and 48.1° are achieved

and related with the diverse set of (111), (200), and (220), which can be recorded as the band for face-centered cubic (JCPDS file no. 89-3722). As a result, XRD indicates that the samples are pure AgNPs that are highly crystalline. The silver particle size histograms revealed that the particles varied in size. Planes appear in the selected area Electron Diffraction Pattern (EDX), indicating that the produced AgNPs are crystalline. The dots are aligned with a face-centered cubic structure. The AgNPs are crystalline, according to the EDX pattern. The elemental detection of AgNPs was also done with the EDX. The presence of a prominent peak of silver at 3 KeV can be seen in the EDX pattern; the produced AgNPs have outstanding antibacterial effectiveness. When clinical bacterial pathogens are exposed to AgNPs, the membrane permeability is altered, resulting in cellular leakage, restricting cell growth and replication. Some bacterial macromolecules can be affected by AgNPs, resulting in disintegration and cell death[54]. Compared to chemically synthesized AgNPs, green produced AgNPs are more biocompatible and have a stronger antibacterial impact[55]. The AgNPs first bind the cell membrane at numerous points before quickly penetrating it, causing structural changes and, as a result, perforations that allow compounds from intracellular storage to flow out[56]. When AgNPs reach the interior, silver ions are released, resulting in the production of reactive oxygen species (ROS), which can affect membrane proteins, causing the electron transport chain to be disrupted[57].

The MTT assay findings show that AgNPs have high cytotoxicity against MCF7. The MTT assay was carried out in triplicate. AgNPs have higher toxicity at lower concentrations as they were incubated for 24 hours. At doses of 10,30,60,80, and 100ug/ml of AgNPs at a different concentration, respectively, toxicity if there were (9.163%,65.252% ,81.565%,80.447%,81.732%) found after 72 hours of incubation respectively this indicates that as the concentration of AgNPs rises so does its toxicity. The results showed that AgNPs strongly suppressed MCF-7 cell proliferation. For AgNPs-treated MCF7, IC50 values were calculated over a wide concentration range and incubation duration. Several studies have previously concentrated on using cell culture methods to perform AgNPs cytotoxicity tests[58,59]. The cytotoxicity of any natural or synthetic substance on an established cell line must be determined before moving to in vivo experiments[60]. When tested against breast cancer cells, our biogenic AgNPs have high cytotoxicity.

Conclusions

The soil was shown to be a rich supply of various bacteria in the current study, and even a novel strain was discovered (*M. paraoxydans* strain shahooda). As a result of this research, it can be concluded that it has been employed effectively for AgNPs extracellular synthesis using soil microbes. The presence of elemental silver and its crystalline structure and size were confirmed by using UV-Vis. Spectroscopy, EDX, SEM, TEM, XRD. Both Gram-negative and Gram-positive pathogenic bacteria are susceptible to AgNPs. The anticancer effects of extracellular AgNPs on MCF7 (breast cancer cells) yielded the best results, with AgNPs significantly suppressing MCF7 cell multiplication. Finally, compared to commercially available antimicrobial drugs, the current Study provides an environmentally friendly and cost-effective approach for synthesizing powerful antibacterial silver nanoparticles (biologically) against pathogenic bacteria and the capacity of silver nanoparticles as antitumors on breast cancer cells line.

Authors contributions

Conceptualization, TMD and RRH; methodology, TMD; software, TMD; validation, TMD and RRH; formal analysis, TMD; investigation, TMD; resources, TMD; data curation, TMD; writing—original draft preparation, TMD; writing review and editing, TMD; visualization, TMD; supervision, RRH; project administration, RRH. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board

Al- Sadr hospital in Misan had been informed about the aims of the Study before collecting samples and declared their agreement to give samples (175; 1/6/2021). The Study follows the rules of scientific research at Misan University, Iraq.

Informed Consent

Informed consent was obtained from all subjects involved in the Study. The patient's consent was oral.

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Conflicts of interest

The authors declare no conflict of interest.

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ARTICLE / INVESTIGACIÓN

Serological estimation of serum level of Vitamin D3 among Psoriasis patients

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Abstract: Background: psoriasis is a prevalent, persistent, non - contagious skin disorder of unknown origin or cure. It has a significant detrimental influence on people's life. It was hypothesized that there is a relation between reducing vitamin D levels and psoriasis. Psoriasis is a severe global condition that affects at least 100 million people; the incidence of psoriasis ranges from 0.09 to 11.43 %. The incidence of psoriasis is 0.09 % to 11.43 %. Objectives & Methodology: Study the frequency of and incidence of psoriasis and its correlation with serum vitamin D3 level; 150 people aged between 1-45 years old and from both sexes were selected for the current study. All of them were recruited from Hilla Teaching Hospital and TelAfer from November 2018 to February 2019 in Babylon Governorate. Of them, 50 were suffering from Psoriasis symptoms, 50 were presented with a positive family history of psoriasis, and the last 50 people were healthy and considered a control group. They were all subjected to estimating the serum level of vitamin D3 by using the ELISA technique. Results: a significant decrease in the serum level of vitamin D3 was found among patients with psoriasis compared to the control and family history groups. The study also indicates that the onset frequency of psoriasis has a hereditary component. In comparison to the age groups (1-15) and (16-30), the results showed a considerable increase in the age groups (31-45). The incidence was more in males than in females. Conclusions: A significant decrease in vitamin D3 in patients with psoriasis.

Key words: Family history, gender types, Vitamin D3, psoriasis.

Introduction

Psoriasis is a common, persistent, non - contagious skin disease with an unknown cause or cure. It has a significant detrimental influence on people's life. Psoriasis influences people of different ages in every country. Psoriasis is a severe global condition that affects about 100 million persons. The estimated incidence of psoriasis is from 0.09 % to 11.43 %[1].

Psoriasis symptoms are unexpected, and there are a lot of extrinsic triggers and significant comorbidities, like arthritis, heart disease, metabolic syndrome, depression, and inflammatory bowel disease[2].

The etiology of this illness is undefined, with emerging evidence of a genetic predisposition[3]. The role of immunity in the development of psoriasis is also a hot topic in research. Although it has been suggested that psoriasis is an autoimmune illness, no autoantigen has yet been identified as the cause. Extrinsic and intrinsic factors involving moderate trauma, sunlight, infections, systemic medicines, and stress can cause psoriasis[4].

Psoriasis affects the nails and skin, and it's linked to a slew of other conditions. Localized or widespread skin lesions are usually symmetrical, with crimson papules and plaques with highly defined borders that are typically covered in white or silver scales. Itching, stinging, and pain are all symptoms of lesions[5]. Psoriasis was reported to have increased from 4.8 percent in 1979-1980 to 11.4 percent in 2007-2008. Recent research has revealed a rise in the prevalence of psoriasis; between the 1970s and 2000, the number of instances roughly doubled, with the cause still unknown[2]. the study aims to explain the correlation between vitamin D3 levels.

Materials and methods

Study population

The study included three groups of people (50 persons in each group) who attended Hilla Teaching Hospital in Babil Governorate and TelAfer during the period (Nov. 2018 – Feb. 2019), their ages were between (1-45) years old and both genders. Of the fifteen patients suffering from a skin lesion and dermatitis, fifteen persons have a positive family history of psoriasis. The last fifteen were healthy as a control group. They were divided into three age groups (1-15, 16-30, and 31-45). By excluding any healthy control with the abnormal value of vitamin D.

Study samples

Three ml of venous blood samples were collected from the study participants in sterile coagulation tubes and then left for (60 min) at room temperature (25C˚). Blood samples were centrifuged at 3000 rpm for 15 minutes to obtain serum. The serum samples were used for the estimation of vitamin D3 levels.

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Estimation of serum vitamin D3 level

Serum samples obtained from the study populations were subjected to determining vitamin D3 levels. Enzyme-Linked Immune Fluorescent Assay technique was depended for this purpose by using VIDAS 25 OH Vitamin D total kit performed by (bioMérieux SA, 376 Chemin de l'Orme, 69280 Marcy-l'Etoile – France).

Statistical Analysis

Study results were analyzed by using SPSS software package version 23 (performed by IBM Com. the USA). Chi-square and t-test were used in the analysis considering a P-value of ≤ 0.05 as a significance level.

Results

Serum vitamin D3 Level

The results of the current study found a significant decrease (P-value < 0.01) in the level of serum vitamin D3 in psoriasis patients and family history groups compared to the healthy persons (control group) as presented in figure (1). According to the age group, figure (2) shows that serum vitamin D3 levels also decreased significantly in all age groups of psoriasis patients and family history groups compared to the control group.

The Relationship between vitamin D3 and the gender

A considerable drop in vitamin D3 was found in this study among psoriasis patients and family history groups compared to the control group and for all age groups and both genders.

Both genders presented a statistically considerable decrease in the level of vitamin D3 among psoriasis patients (16.15±13.9 IU/ml for males and 9.07±1.50 IU/ml for females) in the first age group 1-15years old (p<0.01) compared to the control group as presented in table (1). Similar decreasing the level of serum vitamin D3 levels were also found in the age groups (16-30 and 31-45 years old). These results were also statistically significant (p<0.01and p<0.05). However, we noticed that the serum vitamin D3 level was higher in psoriasis males from the age group (1-15years old), which was the opposite of the vitamin level of healthy people belonging to the same age group. For the other age groups, psoriasis females had higher levels of vitamin D3 level compared to psoriasis males; these results were utterly inverted in the healthy subjects from the same age groups, table (1).

Similarly, table (2) showed that the family history group also had lower serum vitamin D3 levels compared to healthy groups for both genders and all age groups involved in the present study. Differences exist between the vitamin D3 levels according to the age and sexes among family history persons and a control group.

Types of psoriasis

Out of (50) psoriasis patients that were included in this study, it was found (73.3%) of the cases were positive for plaque psoriasis type, followed by (20%) with inverse psoriasis and (6.7%) nail psoriasis.

There was a highly significant association between plaque psoriasis type compared to other types of psoriasis among psoriasis patients (P < 0.01), as seen in the table (3).

Discussion

Vitamin D3

Our study proved that vitamin D3 is low when psoriasis patients are severely reduced; this proves that psoriasis associated with vitamin D3 compared with control suggests that the control result was expected. The usual range of vitamin D3 in the human body, since possessing a level of less than 10 ng/ml, indicates a severe deficiency, while reading ranging from 10 - 25 ng/ml suggests an average lack. If reading between 25 - 85 ng/ml is considered adequate and normal for healthy people[6]. In both groups (controls and patients), blood 25-hydroxycholecalciferol levels remained below the standard limit suggested by the American Society for Bone and Mineral Research. Patients with psoriasis had significantly lower levels of 25-hydroxycholecalciferol than healthy people, with the lowest level coming from patients

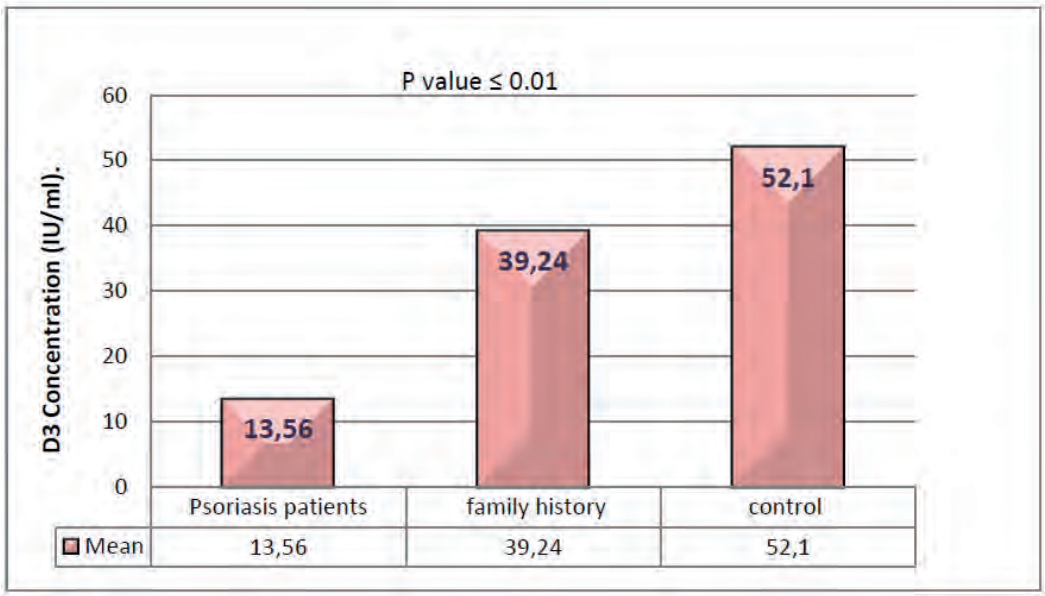


Figure 1. Mean vitamin D3 concentration in psoriasis patients, family history groups and control group.

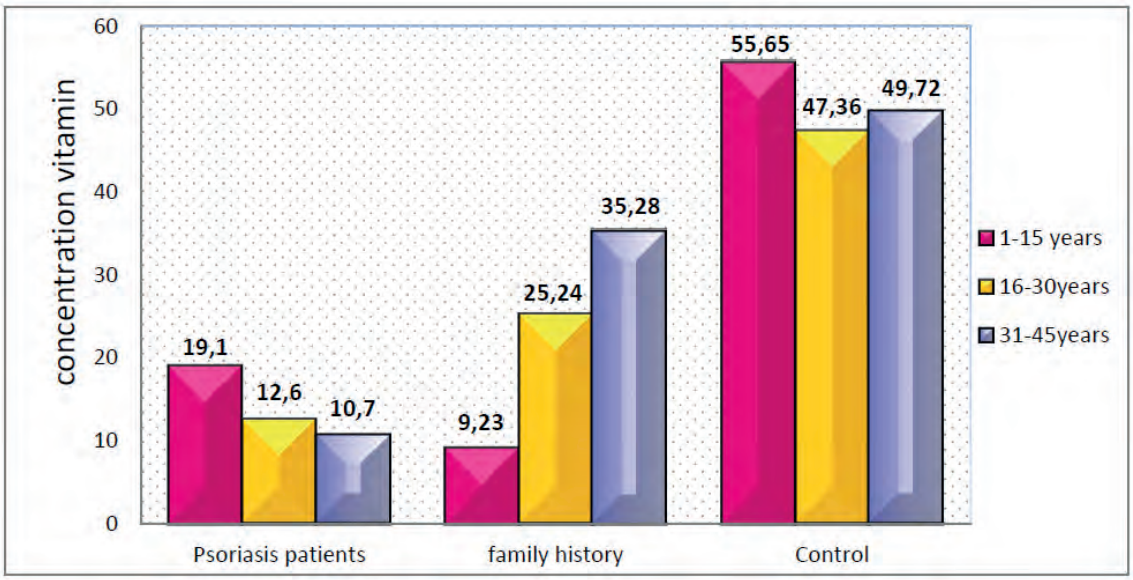


Figure 2. Mean vitamin D3 concentration concerning age among the study groups.

Age groups (years)	Psoriasis patients Mean ± SD (IU/ml)		Control Mean ± SD (IU/ml)	
	Male	Female	male	Female
1-15	**16.15±13.9	**9.07±1.50	51.33 ±20.68	53.16±17.24
16-30	**19.94± 130	*25.1±15.3	55.3±40.2	40.65±16.3
31-45	**21.34± 10.32	*23.56± 4.2	45.1±12.3	42.38±12.9

* The results are significant at potential level ($p<0.05$).
-Normal range between 30-100 ng/ml in serum

Table 1. The relationship between gender and age with vitamin D3 concentration in psoriasis patients and control group.

Age groups (years)	Family history Mean ± S.D(ng/ml)		Control Mean ± S.D(ng/ml)	
	Male	female	male	Female
1-15	*29.45±15.23	*33.70±12.2	51.33 ±20.68	53.16±17.24
16-30	*37,94± 13.09	*22.1±15.32	55.3±40.2	40.65±16.3
31-45	*26.34± 10.32	*38.56±11.29	45.1±12.3	42.38±12.9

** The results are significant at the potential level ($p<0.01$).
* The results are significant at the potential level ($p<0.05$).
-Normal range between 30-100 ng/ml in serum

Table 2. The relationship between gender and age with vitamin D3 concentration in family history groups and control group.

Psoriasis Types	No.%
Plaque Psoriasis (Psoriasis Vulgaris)	**22 (73.3%)***
Inverse Psoriasis	**6 (20%)**
Nail Psoriasis	2 (**6.7%**)
Total	**30** (100%)
***Significance P < 0.01**	

Table 3. Frequencies of psoriasis types in studied patients.

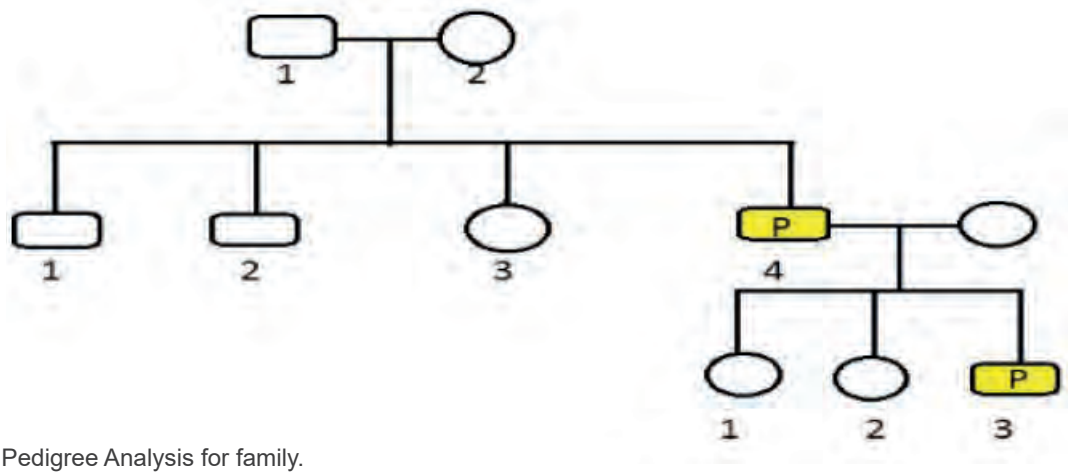


Figure 3. Pedigree Analysis for family.

with severe psoriasis[7]. The blood levels of 25 (OH) D in psoriatic patients were substantially lower (21.8ng/mL) than in healthy controls (34.3ng/mL)[8].

Frequency of psoriasis in different sexes and age groups

Psoriasis and its relationship with sex did not notice a significant variation in the incidence of psoriasis in both sexes. Psoriasis does not affect the specific sex; both sexes are vulnerable to psoriasis. The males who enrolled in the present study were 19, while the female was 11; the severity of psoriasis was similar according to age and gender. Compared to patients aged between 46 to over -65 years old, patients aged 18 to 29 and 30-to-45 showed higher frequently (P-value more than 0.05) concerns with appearance/socialization and occupation/finances. Men reported more work-related stress than women did, although there were no gender differences in the frequency of items related to appearances and socialization (P-value more than 0.05)[9]. This disease is common among Caucasians, affecting an estimated 125 million people worldwide[10]. Psoriasis influences about 3% of the population and affects men and women equally, with a 1:1 female to male ratio of (11,12). Psoriasis Vulgaris, which accounts for about 90% of all cases, is distinguished by well-defined, dark red, scaly lesions and plaques, usually appearing on the elbows, knees, scalp and other skin areas[13]. Chronic plaque is the most common type of psoriasis, accounting for approximately 70% to 80% of all patients[14]. Griffiths *et al.*[15] also stated that persistent plaque psoriasis was the most common type of psoriasis. The most prevalent clinical phenotype was chronic plaque-type psoriasis, with guttate and erythrodermic variations accounting for fewer than 2% of all cases[16].

According to Kumar *et al.*[17], the most common pattern of involvement was plaque psoriasis type lesions located over pressure bearing areas on the palms, and isolated plaques present randomly over the soles. Over five years, Kaur *et al.*[18] investigated nail alterations in 167 psoriasis patients. Of the overall cases, 3% showed isolated nail involvement. Inverse psoriasis is an uncommon type that affects about 3% to 7% of people living with psoriasis[19]. Psoriasis Vulgaris was shown to be more common on the Iberian Peninsula, according to a study (79.8 %)[20]. Another Japanese study of nearly 28000 psoriasis patients found that 86.0 % had plaque psoriasis, and 2.8 percent had guttate psoriasis[21]. Psoriasis affects more than 2% worldwide, with guttate psoriasis accounting for 2% of these instances[22].

Frequency psoriasis in families of patients and analysis of the genealogy

Family history and effect on psoriasis of family history has an apparent impact on psoriasis, which is also a genetic disease. We have noticed this through the collected samples, and they were infected with psoriasis, of which about 70% have a family history. This result indicates that psoriasis has a strong relationship with inheritance. We have demonstrated this relationship through the work of the family tree of the disease. For a long time, psoriasis has been known to run in families. In his famous epidemiological research of psoriasis among 10,000 residents of the Faroe Islands, Lomholt discovered that the prevalence of psoriasis was much greater among first- and second-degree relatives of sufferers than among unaffected control persons[23]. Another way to look into the heredity and penetrance of a disease is to compare illness concordance in monozygotic (MZ) twins (who have identical genomes) and dizygotic (DZ) twins (who only share half of their genomes). The heritability of psoriasis, h2 (the amount of phenotypic variation of a variable attributed to genetic differences), ranges from 60% to 90% based on twin and family studies, making it one of the highest for all multifactorial genetic illnesses. Psoriasis concordance in MZ twins is increased substantially compared with DZ twins, suggesting a genetic predisposition to the disease[24,25].

Conclusions

Many theories have been proposed to explain the inheritance of psoriasis. Earlier, It hypothesized a dominant inheritance model with imperfect penetration. Psoriasis is thought to be inherited in a complex manner nowadays. Psoriasis' genetic contribution has been widely characterized, with at least 20 distinct psoriasis susceptibility loci identified[26].

Author contribution and funding

AM designed the tables and supervised and reviewed the manuscript, RM drafted the manuscript and compiled information from the literature. There is no source of funding, and the authors bear the expenses.

Ethics statement

This study was done in conformity with the testament of the committee of ethical standards in the College of Science, University of Al-Mustaqbal University College. Babylon Governorate.

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ARTICLE / INVESTIGACIÓN

The role of TNFα in type2 diabetes mellitus

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Abstract: Type2 Diabetes mellitus is a chronic and most common form of diabetes characterized by hyperglycemia arising from problems with the utilization or production of insulin. Various agents related to an increased risk of developing T2DM include pro-inflammatory cytokines that are critically associated with the progressive resistance of insulin and the pathogenesis of newly diagnosed T2DM. The study aimed to study a possible association of TNFα serum level in diabetes type2 initiation using enzyme-linked immunosorbent assay (ELISA). In this study, 50 newly diagnosed T2DM patients with an age range between 25 and 70 years were included; in addition to fifty healthy volunteers whose sex and age were matched with the T2DM patient's group to act as a control, serum samples were collected to estimate serum levels of TNFα by Enzyme-Linked Immunosorbent Assay (ELISA). The TNFα revealed a statistically significantly higher serum level in the newly diagnosed T2DM patients compared with the healthy control group. On the other hand, there is no statistically significant difference between obese and non-obese T2DM. The higher level of TNFα in T2DM patients compared to the control group may have a role in the development and pathogenesis of T2DM. The current study showed an association between the serum TNFα level and newly diagnosed type 2 DM, and this studied marker might help in the prediction of T2DM

Key words: T2DM, TNFα, ELISA.

Introduction

Type 2 diabetes is related to insulin resistance associated with metabolic stress and inflammation and other factors, such as genetic[1], that can lead to cardiovascular disease and atrophy[2]. Although type 2 diabetes (T2D) is characterized by insulin resistance which means that insulin responding to its target tissues (especially the liver, muscle, and fat) will be the decline, in this case, insulin inoperative with rising its generation to sustain glucose level as a consequence, blood sugar is unable to enter these cells and be used for energy storage. When sugar is unable to enter cells, the blood sugar level rises. This is referred to as hyperglycemia. The body is incapable of using glucose as an energy source. This results in type 2 diabetes symptoms[3-5]. Type 2 diabetes commonly advances misdiagnosed for several years due to slow of hyperglycemia and, through earlier stages, is mostly insufficient for the patient to observe the usual manifestations of diabetes. However, patients who are undiagnosed have rising danger progressing of microvascular and macrovascular problems[6]. Hence, due to the immediately developing frequency and ubiquity of T2DM rises necessary urgency to advance competent or powerful means being used for initial and earlier detection of the disease in order to avoid the development of T2DM and ameliorate clinical outcomes of patient[7]. Tumor necrosis factor-alpha (TNF-α) is one the most important pro-inflammatory mediator. It is yielded chiefly from adipocytes and/or peripheral tissues and activates inflammation of particular tissue via the production of reactive oxygen species and induction of diverse intermediate transcriptional pathways[8]. When TNF-α consistent elevated it will activate resistance of insulin in both peripheral tissues and adipocytes via hindering the insulin signaling through serine phosphorylation which promote arising of T2DM[9]. It is a cytokine that is secreted by chronically inflamed cells. The immune system produces it. Type 2 diabetes mellitus is believed to be connected with low-grade chronic pancreatic inflammation[10].

Materials and methods

This case-control study was conducted on 100 Iraqi adults who were divided into two groups

Group (1): includes 50 newly diagnosed patients with type2 diabetes mellitus as a case group.

Group (2): includes 50 healthy subjects as a control group.

Diabetic pregnancy, patients on insulin or any other drugs that would affect glucose homeostasis, smokers' and alcoholism patients were excluded from the study. This study was established in AL-Emamain AL-Kadhemain Medical City and Diabetes Disease center in Baghdad during the period between October 2020 till September 2021.

Blood sample collection

Five ml of venous blood from each subject were distributed into two tubes. The first 3ml was dispensed into a gel tube containing a clot activator, left at room temperature

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to clot for about (10) minutes, and then centrifuged for (10 min at 4000 rpm). In this study, the serum was isolated and utilized to assess glucose, cholesterol, and triglyceride. The residual serum was frozen at -20 °C till the quantified of TNFα in serum using a sandwich ELISA kit. The other 2ml was transmitted to the ethylenediaminetetraacetic Acid tube (EDTA) for routine work glycated Hemoglobin assessment.

HbA1C, cholesterol, TG, CRP

The healthy subjects and patients were investigated for the following parameters: hemoglobin A1c, fasting blood sugar FBS, TG, cholesterol, CRP was measured by Cobas c111 system. A ready commercial Kit Mybio-source Catalog No: MBS455676 / USA was used for the in vitro quantitative measurement of TNFa in human serum.

Statistical analysis

To do statistical analysis, SPSS 20.0 and Microsoft Excel 2013 were used. The Shapiro-Wilk test was used to examine the normality of numerical data. The Mann–Whitney U test was employed to compare the median and 25-75 confidence intervals of the data for comparing two groups. Percentage of the total number of categories in the data set. The use of the chi-square test estimated the connection between variables. Below or equal to 0.05 is the minimum statistical significance level that may be acceptable.

Results

The mean age of patients was 46.00± 10.42 years, and there was female predominance among patients; there were 52.0% females, while 48.0 % were males. There was no statistically significant difference between patients and the control group (p> 0.05) in age or sex present. According to the (BMI) The results in this study demonstrated a statistically significant difference in (BMI) between the studied groups (p ≤ 0.001).

Fasting Blood sugar and HbA1c

The results in table 3 illustrated a highly significant difference in median FBS and HbA1c among study groups (P ≤ 0.001).

		Study groups	
		T2DM	Control
Age/years	Mean	46.00	45.64
	SD	10.42	9.73
P value	0.909 NS		

NS= nonstatistical significant difference (p>0.05)

Table 1. The age distribution of study groups.

Sex	Study groups	
	T2DM	Control
Female	26	26
%	52.0%	52.0%
Male	24	24
%	48.0%	48.0%
Total	50	50
P value	0.841 NS	

NS= nonstatistical significant difference (p>0.05)

Table 2. Sex type distribution in T2DM patients and control.

Regarding TG and cholesterol, LDL, AI with median value (177.50 Mg/dl), (200.00 Mg/dl), (137.00 Mg/dl) (0.64) respectively, was higher in T2DM than in control group (102.94 mg/dl), (164.69 Mg/dl), (94.99 Mg/dl), (0.30). with p-value =< 0.001, While the HDL results were shown that the median of patients' T2DM was lower than the control group (38.84, 51.18 Mg/dl) respectively, with p-value = < 0.001.(table 4). On the other hand, concerning CRP, the median value for T2DM was (4.47 Mg/L) higher than the median of the control group (1.36 Mg/L). So, there was a significant difference between the T2DM patients and control groups shown in table 5. Also, The current result revealed a higher concentration in the serum of patients TNFα compared with controls, as shown in Table 6.

The results of the current study concerning TNFα concentration between obese and non-obese patients showed a non-significant difference, as revealed in Table (7). Recent study results revealed a non-significant association between TNF alpha and HbA1C in patients with T2DM, as shown in Table 8.

Level of HbA1c, CRP, TNFα in obese and non-obese T2DM

According to the results demonstrated in Table (9), there was no statistically significant difference between obese and non-obese regarding HbA1c, and TNFα in patients with T2DM, except CRP. There was a statistically significant difference between obese and non-obese T2DM with a p-value (0.020). Also, this study revealed no statistically significant correlation between TNFα and group of demographic and laboratory findings (Age, BMI, FBS, HbA1c, Lipid profile, AIP and CRP) as in Table 10.

Discussion

This study revealed a significant difference in TG levels between T2DM patients and control groups, similar to research 11that established a link between high TG and type 2 diabetes. There is a significant difference in cholesterol levels between the patients and the control group. This is consistent with the findings[12], which showed that diabetes individuals had considerably greater CHO levels than non-diabetic individuals. The present results on TG and Cholesterol levels disprove those of other studies[13], which found no significant difference between T2DM and healthy controls. The rise in cholesterol levels appears to be due to increased cholesterol synthesis due to a lack of control or poor management of hyperglycemia, which returns

		Study groups		P-value
		T2DM	Control	
FBS (Mg/dl)	Median	182.50	86.56	<0.001**
	Percentile 25	131.00	78.22	
	Percentile 75	265.00	94.34	
HbA1C%	Median	9.05	4.90	<0.001**
	Percentile 25	7.00	4.69	
	Percentile 75	11.80	5.20	

**= highly statistical significant difference (p<0.001)

Table 3. Serum laboratory findings distribution in both T2DM patients and control.

		Study group		P-value
		T2DM	Control	
TG (Mg/dl)	Median	177.50	102.94	<0.001**
	Percentile 25	129.50	75.40	
	Percentile 75	255.00	118.09	
Cholesterol (Mg/dl)	Median	200.00	164.69	<0.001**
	Percentile 25	182.00	147.19	
	Percentile 75	231.00	180.00	
HDL (Mg/dl)	Median	38.84	51.18	<0.001**
	Percentile 25	34.00	46.64	
	Percentile 75	44.00	58.23	
LDL (Mg/dl)	Median	137.00	94.99	<0.001**
	Percentile 25	115.20	78.49	
	Percentile 75	167.00	110.24	
AI	Median	0.64	0.30	<0.001**
	Percentile 25	0.53	0.17	
	Percentile 75	0.79	0.36	

**= highly statistical significant difference (p<0.001)

Table 4. Lipid profile and Atherogenic index distribution in T2DM and control groups.

		Study groups	
		T2DM	Control
CRP (Mg/L)	Median	4.47	1.36
	Percentile 25	2.60	.74
	Percentile 75	9.60	2.98
P value	<0.001**		

**= highly statistical significant difference (p<0.001)

Table 5. CRP parameter distribution in T2DM and control group

		Study groups	
		T2DM	Control
TNF-alpha (pg/ml)	Median	90.57	26.53
	Percentile 25	65.39	18.81
	Percentile 75	129.52	41.75
P value	<0.001**		

**= highly statistical significant difference (p<0.001)

Table 6. The concentration of TNFα in T2DM and control group.

		BMI	
		non-obese	obese
TNF-alpha (pg/ml)	Median	90.71	90.44
	Percentile 25	66.64	65.39
	Percentile 75	120.54	129.52
P value	0.976 NS		

NS=none statistical significance (p>0.05)

Table 7. The concentration of TNFα between obese and non-obese patients.

		HbA1C in T2DM	HbA1C in control
TNF-alpha (pg/ml)	Median	103.63	83.24
	Percentile 25	90.17	61.84
	Percentile 75	140.07	129.52
P value	0.426NS		

No statistical significance (p>0.05)

Table 8. Association between TNF-alpha and HbA1C in T2DM.

		T2DM	
		non-obese	obese
HbA1c	Median	11.00	8.80
	Percentile 25	7.70	6.90
	Percentile 75	11.80	10.50
P value	0.162		
CRP	Median	2.80	6.45
	Percentile 25	2.40	3.20
	Percentile 75	4.90	14.80
P-value	*0.020		
TNFα	Median	90.71	90.44
	Percentile 25	66.64	65.39
	Percentile 75	120.54	129.52
P value	0.976 NS		

Table 9. Level of HbA1c, CRP, TNFα in obese and non-obese T2DM.

	TNF-alpha (pg/ml)	
	r	p
Age (years)	0.045	0.754
BMI	-0.066	0.650
FBS	-0.136	0.347
HbA1c	-0.219	0.126
TG	-0.007	0.960
Cholesterol	-0.240	0.093
HDL	-0.024	0.868
LDL	-0.061	0.675
AIP	-0.042	0.774
CRP	0.255	0.074

Table 10. Correlation of TNFα with demographic and laboratory findings among the studied groups.

to standard or near-normal following adequate and suitable diabetic control[14,15]. In concerning to LDL and HDL current study revealed a significant difference in LDL levels of T2DM compared to healthy control subjects. Even though newly diagnosed diabetic patients show a significant decrease in high-density lipoprotein (HDL) compared to the healthy control group, this is consistent with a study[16] that found a significant difference (p<0.001) in the newly diagnosed diabetic group as compared to healthy control group regarding LDL and a negative correlation of T2DM with the control group in HDL Present study revealed a significant difference between the T2DM in compared to control groups regarding AI and this agrees with (17) studies which shown also AI in T2DM group was significantly higher than in the healthy control group. Study[16] reported a significant increase in levels AI in T2DM compared to the control group. AI is a good predictor and indicator for follow-up monitoring in managing patients with high-risk type 2 diabetes because it is a clinically convenient indicator for detecting T2DM with a high risk of complications and related disorders[18]. Sugar, cholesterol, and triacylglycerol levels were more elevated in patients with type 2 diabetes than those with normal blood sugar levels and lower serum levels of high-density lipoprotein cholesterol compared to those without type 2 diabetes. Diabetic people are more prone than non-diabetic patients to develop dyslipidemia, a key risk factor for atherosclerosis and coronary heart disease[19]. This study found that the CRP concentration was substantially more significant in the newly diagnosed T2DM group than in the healthy control group. This finding is consistent with research 20, which found that the serum CRP concentration was significantly higher in the IGT and type 2 DM groups (p 0.001). Some researchers believe that type 2 diabetes is a symptom of a persistent acute-phase response defined by changes in the levels of so-called acute-phase proteins, such as the inflammatory protein C-reactive protein (CRP) 21,22 Furthermore, according to the findings of the current investigation, the blood TNF- level was considerably greater in newly diagnosed T2DM patients. Compared to obese non-diabetic patients and non-obese diabetic patients, there was no significant difference in TNF- α levels between obese diabetic patients and obese non-diabetic healthy subjects. Study (10) did not confirm this association.

In this current study, the absence of correlation concerning TNF α concentration in obese and non-obese diabetic patients could be related to the duration of the disease. All patients present in the study were selected orderly as newly diagnosed T2DM. Most of the diabetic patients in this study didn't have a BMI greater than 30kg. Could be linked to the small sample size of obese patients in the current study. Furthermore, neither the waist-to-hip ratio nor the quantity of visceral fat was measured in this research. The inability to find any link between BMI and serum TNF- α may also be explained by several sources of circulating TNF- α and the failure to account for the independent impacts of obesity and diabetes. Multiple studies have shown that blood TNF levels were linked with abdominal obesity[23] or visceral fat[24], but not with subcutaneous fat or BMI. These findings are consistent with previous findings.

Additionally, TNF- α is produced and released by muscle[25] and lymphocytes and macrophages[26]. It has been proven *in vitro*[25] People with insulin resistance, and type 2 diabetes have higher levels of TNF- α in their muscles. Also, the generation of TNF- α by monocytes and macrophages is boosted by hyperglycemia[27] and hyperinsulinemia[28]. TNF- α levels seem to rise with aging in certain studies[29]. However, It was shown that even after adjusting for age, the diabetic group's TNF-a concentration increased. Meta-analysis of 19 studies shows that elevated TNF- levels are strongly associated with an increased risk of developing Type 2 Diabetes Mellitus (T2DM)[30]. TNF- α is a crucial modulator of inflammatory responses.

The role of cytokines in promoting the production of acute-phase proteins is well-known. Increases in inflammatory biomarkers are a result of both obesity and hyperglycemia. After neutralizing TNF- α, obese rats showed increased glucose absorption in the peripheral bloodstream, indicating that TNF- α plays a critical role in developing insulin resistance and diabetes[31]. TNF- α has previously been linked to insulin resistance and type 2 diabetes (T2DM) associated with obesity[32,33]; however, another investigation did not support this link[34]. The current study demonstrated no significant association between TNFα and HbA1C in T2DM. The duration of the diseases could cause this, the limited sample size or some of those patients had insulin sensitivity. High levels of inflammatory cytokines that emerged in the early stages of T2DM could predict the development of type 2 diabetes by lowering insulin sensitivity[35]. Current findings contribute to a more significant number of studies indicating that certain inflammatory factors play a vital role in the pathophysiology of T2DM. They also provide insights on the potential clinical utility of certain inflammatory factors as biomarkers for early T2DM detection and treatments[36]. According to another study, people with diabetes were shown to have considerably raised levels of TNF-α, which was most pronounced in those with HbA1c values of more than 6.5 %[37]. In this study, HbA1c levels were not linked to TNF- α in this group of T2DM patients, whose mean HbA1c was 9.49%. Because the glycation rate and the turnover of erythrocytes aren't directly related to blood glucose levels, the amount of HbA1c might fluctuate for reasons unrelated to blood glucose levels[37]. The negative relationship between TNFα levels and insulin sensitivity in persons with Normal Glucose Tolerance (NGT) and Impaired Glucose Tolerance (IGT) or prediabetics, regardless of age, sex or obesity, supports circulating TNFα playing a role in insulin action control[15]. Activated macrophages and lymphocytes were observed to produce and release tumor necrosis factor[26] because type 2 diabetes is thought to result from a constant state of inflammation[38]. A soluble TNF receptor-IgG fusion protein has been shown to promote insulin-stimulated autophosphorylation of insulin receptor and insulin receptor substrate-1 in muscle and fat, supporting this hypothesis[39]. Similarly, Obesity-induced suppression of TNF - α activity by an adenovirus vector with impaired replication By increasing insulin receptor signaling, the Zucker rat enhances hepatic and muscular sensitivity [23,40]. Neutralizing circulating TNF- α levels did not improve glycemic control or insulin sensitivity in obese, insulin-resistant diabetic and non-diabetic adults.

Conclusions

TNF- produced by fat cells and macrophages/lymphocytes may have interfered with the paracrine effects of TNF-neutralizing antibodies on the local level.

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Informed Consent Statement

Al-Nahrain University's College of Medicine's ethics committee authorized this research. All samples were taken with informed permission to comply with the Al-Imamain Al-Kadimain Medical City's policies on informed consent and informed consent forms.

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Author conflict

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ARTICLE / INVESTIGACIÓN

Antimicrobial activity of *Saussurea costus* extracts against *Streptococcus penumoniae* and *Escherichia coli*

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Abstract: Traditionally, numerous components of medicinal plants, such as their roots, stems, flowers, fruits, and twigs, were widely employed as crude extracts to treat many human ailments. This investigation aimed to look at the effectiveness of various antimicrobial agents of *Saussurea costus* against G+ve bacteria *Streptococcus penumoniae* and G-ve bacteria *Escherichia coli*. The antibacterial activity of the plant was determined using the diffusion technique, which was used to both alcoholic and aqueous extracts generated from the plant's roots against streptococcus penumoniae and *Escherichia coli*; the inhibition zone was measured by millimeter. The antimicrobial examination revealed that the Gram-positive bacteria represented by *Streptococcus penumoniae* were susceptible to the alcoholic extracts of *S. costus* with inhibition zones (18 mm) at 200 mg/ml concentration. In contrast, the aqueous extract had fewer inhibition zones (16 mm) at a 200 mg/ml concentration. Meanwhile, *E.coli* had a weak effect on the alcoholic extracts with inhibition zones (13 mm) at 200 mg/ml concentration. Still, aqueous extract was (10 mm) at200 mg/ml concentration. The constituents of the ethanolic extract of roots were investigated using Gas chromatography-mass (GC); twenty-four compounds were identified in the extract of the roots, such as C10H14 and C10H16. The results demonstrated that the Saussurea costus extracts have antimicrobial activity against Gram-positive bacteria *S. pneumoniae* while it has a weak effect against *E.coli*.

Key words: *Saussurea costus*, Antimicrobial, Ethanolic extract, *Streptococcus penumoniae*, *Escherichia coli*.

Introduction

Plants have long been recognized as a significant source of natural compounds for the maintenance of human health, particularly in developing countries. This has been even truer in recent years as more thorough research into natural medicines has been done, notably in the past decade[1]. Studies of natural products derived from various sources, notably higher plants, have piqued researchers' interest in discovering new antimicrobial and antioxidant agents. These antimicrobial agents include compounds such as tannins and alkaloids and terpenoids and flavonoids, which have been proven to exhibit antibacterial activities *in vitro* and *in vivo*, respectively[2]. *Saussurea costus* (also known as Saussurea lappa) is a member of the Asteraceae family, which is widely spread around the world; however, a large number of species are found in India, Pakistan, and some sections of the Himalayas, where it is known as the "Indian Aster"[3]. The therapeutic effects of *S. costus* have been thoroughly documented in several medical systems. Many cultures have employed the aromatic roots of *Saussurea costus*, as medicines, incense, and ointments for thousands of years[4]. This research aimed to determine the antibacterial activity of extracts against different pathogenic bacteria in vitro represented by *Escherichia coli* and *Streptococcus penumoniae*.

Materials and methods

Sample Collection

The dried roots of *S. costus* were purchased at a herbal market and were later recognized by a taxonomist at the Desert Studies Center at the University of Anbar in Iraq.

Aqueous Extract Preparation

The plant's roots were washed "for 2-3 times with tap water, then dried significantly, using an electrical grinder, the root was crushed into small particles and subsequently fine powder. The powder of dried roots was 25 gm mixed with 250 ml of solvent (water) for 24 hours; after that, the mixtures were filtered by gauze and centrifuged at 3000 rpm for 15 minutes and then dried in a hot air oven at 45 ° C for 48 hrs. The dried extract was kept in a sterile container until used[5].

Alcoholic Extract Preparation

The roots of the plant (25g) were crushed and soaked with 90% ethanol, and the mixture was homogenized in magnetic stirrers; after that, crude was filtered twice by gauze. The filtrate was concentrated on a rotary evaporator at 45ºC[6].

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Antimicrobial activity

The antimicrobial activity of the extract was performed by the diffusion method. The selected bacterial isolates *streptococcus penumoniae* and *escherichia coli* were activated on nutrient broth and incubated for 24 hours at 37°C. Holes were made on mueller- hinton by using a 6 mm cork borer. Each hole was filled with extracts. Antibacterial activity was evaluated by measuring the area of inhibition zone[7]. The chromatograph utilized at the Iraqi Ministry of Sciences and Technology was a Shimadzu GC-2010 plus-Japan, which was purchased in Japan. The gas chromatography-mass spectrometry (GC-MS) system is an extremely significant tool for the study of bioactive chemical substances: ZB-5MS is the Capillary column number (30 m x 0.25 mm, ID 0.25 µm), Carrier Gas: UHP Helium, Injection Temperature: 280.00˚C, Detector Temperature: 280.00 °C, Injection Mode: Split, Flow Control Mode: Pressure, Injector Pressure: 100.0 kPa, Total Flow: 47.3 µl/min, Column Flow: 1.43 µl/min, Linear Velocity: 44.1 cm/min, Injection Volume: 1 µL, Run Time: 35 minutes. A freshly prepared sample (10mg/ml) was used.

Results

The results showed that S. pneumoniae (Table1) showed a sensitivity to the alcoholic extract, with the highest inhibition zone (18 mm) at 200 mg/ml concentration and the lowest inhibition zone being 16 mm at 50 mg/ml concentration. At the same time, the aqueous extract showed a sensitivity of *S. pneumonia* to the extract (16 mm) at a 200 mg/ml concentration. The lowest rate of inhibition zone was 15 mm at a concentration of 50 mg/ml.

Concerning that *E.coli*,(Table 2 below) (ethanolic extracts) showed the highest inhibition zone was (13 mm) at 200 mg/ml, while aqueous extracts of *S. costus* showed 10 mm at 200 mg/ml, which means that *E.coli* has weak susceptible to the extracts.

After a GC-analysis evaluation, *Saussurea costus* roots have been shown to be a rich source of numerous bioactive elements, which identified twenty-four biochemical substances in the roots. Table 3 shows the results of a compound analysis of an ethanolic extract of *S.Costus*.

Discussion

High-quality plant extracts with antibacterial properties have recently piqued researchers' curiosity, and various phytochemicals, such as the antimicrobial actions of several plant species, have been extensively studied. Examples of antimicrobial capabilities include the crude extracts of herbs such as cinnamon and garlic; basil and curry; ginger; sage; mustard; and other herbs. These plants also have a killing role to word various microorganisms[8]. In the current study, the results are shown in Table (1) indicated that the zone inhibition of *streptococcus pneumoniae* at the lowest concentration 50 mg/ml was (15 mm), increased by increasing the concentration of the alcoholic and aqueous extract, which indicates a direct relationship between the concentrations of the extracts and the rates of the zone of inhibition. The ethanolic extracts offered somewhat good antimicrobial activity compared with aqueous extracts; there may be some active substances soluble in alcohol and insoluble in water, where the active substances released are more. It is commonly known that ethanol at 90% concentration can have several components more effectively than at lower concentrations. The results showed that Gram-positive bacteria were more sensitive to the antibacterial activity of ethanolic extracts and aqueous extracts of *S. costus* roots. This may be attributed to the fact that the "cell wall in Gram-positive bacteria consists of a single layer, whereas Gram-negative cell wall is a multilayered structure" bounded by an outer cell membrane[9]. There has been a plethora of research undertaken using plant extracts to screen for antimicrobial activity and the identification of novel antimicrobial chemicals, all of which have proven beneficial. When tested against all serogroups of *E. coli* at (100 % concentration), garlic extract shown high antibacterial activity owing to the action of allicin, diallyl thiosulphinic acid, or diallyl disulfide, among other active ingredients[11]. On the other hand, the earlier research has looked at several ways and chemicals that may be utilized to suppress pathogenic bacteria, such as the direct impact of nanoparticles on pathogenic microorganisms such as *Proteus spp*[12]. Also, several studies have shown that applying physical forces to dangerous bacteria, such as Audible Sounds and Magnetic Fields, may help to reduce infection resistance[13].

Samples	Conc. Mg/ml	Zoon Inhibition mm.
Ethanol extract	50 mg/ml	16 mm
	100 mg/ml	17mm
	150 mg/ml	18mm
	200 mg/ml	18mm
Samples	**Conc. Mg/ml**	**Zoon Inhibition *mm***
aqueous extract	50 mg/ml	15 mm
	100 mg/ml	16mm
	150 mg/ml	16mm
	200 mg/ml	16 mm

Table 1. Inhibition zone of the alcoholic and aqueous extract against *s. pneumoniae.*

Samples	Conc. Mg/ml	Zoon Inhibition *E-Coli*
ethanolic extract	50 mg/ml	11 mm
	100 mg/ml	12 mm
	150 mg/ml	12 mm
	200 mg/ml	13 mm
Samples	**Conc. Mg/ml**	**Zoon Inhibition *E-Coli***
aqueous extract	50 mg/ml	5 mm
	100 mg/ml	5 mm
	150 mg/ml	7 mm
	200 mg/ml	10 mm

Table 2. Inhibition zone of the alcoholic and aqueous extract against *E.coli.*

	RT	Area%	Name of the Compound	Molecular Formula
1	6.540	0.02	1-Pentanone, 1-(4-methylphenyl)	$C_{10}H_{14}$
2	6.617	0.24	1,5-Cyclooctadiene, 1,5-dimethyl	$C_{10}H_{16}$
3	7.530	0.03	3,6-Diketohept-1-ene	$C_7H_{10}O2$
4	8.646	0.16	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	$C_6H_8O_4$
5	9.984	0.18	4-Hepten-3-one, 4-methyl	$C_8H_{14}O$
6	11.842	0.07	(E)-2-methoxy-5-(prop-1-en-1-yl)phenol	$C_{10}H_{12}O_2$
7	13.101	0.01	(E)-hept-4-en-2-one	$C_7H_{12}O$
8	13.610	0.02	Oxalic acid	$C_{10}H_{16}O_4$
9	14.915	0.01	1,5-Hexadiene	C_6H_{10}
10	15.101	0.02	3-Chlorohexane	$C_6H_{13}Cl$
11	15.949	5.42	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$
12	16.000	0.82	"1,3-Bis-(2-cyclopropyl,2-methylcyclopropyl)-but-2-en-1-one"	$C_{18}H_{26}O$
13	17.036	0.03	1,11-Dodecadiyne	$C_{12}H_{18}$
14	17.404	1.58	3-Ethyl-3-hydroxyandrostan-17-one	$C_{21}H_{34}O_2$
15	18.220	0.38	1H-3a,7-Methanoazulene, octahydro-1,4,9,9-tetramethyl	$C_{15}H_{26}$
16	18.611	0.66	cis-Z-.alpha.-Bisabolene epoxide	$C_{15}H_{24}O$
17	18.822	0.06	Cyclopentaneacetaldehyde, 2-formyl-3-methyl-.alpha.-methylene	$C_{10}H_{14}O_2$
18	18.972	0.05	1H-3a,7-Methanoazulene, octahydro-1,4,9,9-tetramethyl	$C_{15}H_{26}$
19	19.389	13.53	cis-4,7,10,13,16,19-Docosahexanoic acid	$C_{22}H_{32}O_2$
20	19.530	0.40	"Spiro[androst-5-ene-17,1'-cyclobutan]-2'-one, 3-hydroxy-, (3.beta.,17.beta.)"	$C_{22}H_{32}O_2$
21	20.271	76.07	1,8-Cyclopentadecadiyne	$C_{15}H_{22}$
22	21.832	0.12	1-Pentanol, 5-(methylenecyclopropyl)	$C_9H_{16}O$
23	22.158	0.11	Spiro[2.2]pentane-1-carboxylic acid, 2-cyclopropyl-2-methyl	$C_{10}H_{14}O_2$
24	22.874	0.02	4-Hepten-2-one, (E)	$C_7H_{12}O$

Table 3. Identification of biochemical compounds by GC-M S.

Conclusions

From the data presented above, we can deduce that Saussurea costus extracts have antibacterial activity against Gram-positive bacteria such as *S. pneumoniae* but only a mild effect on *E.coli*.

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Conflicts of Interest

None.

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CLINICAL REPORT / REPORTE DE CASO

AntI GABA auto-immune encephalitis as schizophrenia and status epilepticus in a female patient: A case report

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Abstract: Encephalitis is a severe neurological syndrome with a rapid progression to encephalopathy caused by brain swelling. One of its causes is antibodies against neuron surface proteins, ionic channels, or receptors so that they can start encephalitis with psychiatric symptoms. Worldwide, auto-immune encephalitis annual incidence is 12.6 per 100000 inhabitants. We present a case report of a 44-years-old female who developed behavioral changes and seizures in a time-lapse of months. She was diagnosed with anti-GABA auto-immune encephalitis, a rare type of this disease.

Key words: Auto-immune disease, Epilepsy, Mental disorders, Neurology, Critical Care

Introduction

Encephalitis is a severe neurological syndrome with a rapid progression to encephalopathy caused by brain swelling. It is characterized by fever, neurological focality, new-onset seizures, and electroencephalography (EEG) changes[1]. Infectious diseases are one of its main etiologies; nevertheless, antibodies against neuron surface proteins, ionic channels, or receptors can start encephalitis with psychiatric symptoms[2].

Worldwide, auto-immune encephalitis is present more frequently in young people, with a peak at 21 years[3]. The annual incidence is 12.6 per 100000 inhabitants. Anti N-methyl-D-aspartate receptor (NMDA) auto-immune encephalitis is the most common presentation; however, lack of identification of antibody happens in 40-50% of cases[2].

Early diagnosis and treatment are associated with a good prognosis. Initial immunotherapy has good results in the short and long term[2].

A case report of a female patient of 44 years is presented, who developed behavioral changes and seizures in a time-lapse of months. She was diagnosed with anti-GABA auto-immune encephalitis, a rare type of this disease.

Case report

Forty-four-year-old female patient with a familiar history of schizophrenia presented to the emergency room with behavioral disorders and headache, without fever, parrhesia, or speech problems, is referred to Psychiatry because of suspicion of schizophrenia. Nevertheless, she developed generalized seizures upon her arrival and is hospitalized in Internal Medicine with a probable diagnosis of encephalitis. She is started on phenytoin, and antibiotics are indicated to cover a possible neuro-infection. Meropenem, vancomycin and fluconazole are used. Head and thorax computed tomography were indicated and resulted in normal.

The patient presented other seizures episodes. It was decided to administrate midazolam to control them and protect the airway, so she was put on mechanical ventilation and transferred to Intensive Care. Acyclovir is added to treatment in case of a possible viral infection. A lumbar punction was performed, and its results were expected (table 1). An electroencephalogram was carried out, and it showed it lateralized epileptiform cortical discharges in the right frontal lobule that spread to ipsilateral and contralateral regions.

Auto-immune encephalitis was considered in this clinical picture, and a sample of cerebrospinal fluid was sent to molecular studies. Methylprednisolone was indicated, and treatment allocation procedures were started. Immunoglobulins and plasmapheresis were considered first-choice treatments, even though they are not routinely available in the care facility where she was hospitalized.

The cerebrospinal fluid molecular studies returned and showed auto-antibodies anti GABARB1/B2.

Finally, after seven days of care, a vacant was available to move the patient and administrate an appropriate treatment. The patient was transferred on mechanical ventilation and sedatives. Phenytoin and lamotrigine were used as anticonvulsants. Antibiotics were stopped as no infectious diseases were active. Steroids were administered for five days and tapered accordingly.

Discussion

It is presented the case of a young female who was initially diagnosed with schizophrenia and treated consequently. Nonetheless, she developed status epilepticus and needed sedatives and intensive care management. Auto-immune encephalitis was suspected, and an investigation for autoantibodies started. Anti GABA was positive, and treatment was allocated subsequently.

Ecuador does not have a formal registry for auto-im-

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TEST	CSF study
Appearance	Clear
pH	7
Cells	Absent
Gram stein	Normal
Red blood cells	0
Piocytes	0
White blood cells	2
Glucose	71.3
Proteins	27
LDH	44.2
Culture	Negative

Table 1. Cerebrospinal fluid test characteristics of the patient.

mune encephalitis. Worldwide is reported an incidence of 12,6 cases per 100 000 inhabitants[4], and 20% to 30% of these cases are related to autoimmunity[5]. It is described anti-NMDA auto-immune encephalitis as the most common variant[2], and anti-leucine-rich glioma-inactivated 1 (LGI1) is the second in frequency with an incidence of 0.83 cases per 1 000 000 inhabitants[6]. Latin America neither has an incidence database about this pathology; however, several cases are reported, and published literature reviews show experience with this disease[7-9].

High clinical suspicion is needed to accomplish diagnosis because non-specific symptoms are present like behavioral changes and seizures[1]. In our case, antibodies investigation was motivated by variability in the clinical picture, and anti-GABA resulted in positive.

There are two types of antibodies directed against the central nervous system: 1) antibodies against intracellular antigens (Hu type, Ri type, anti-fisine type, Ma2 type, CRMP 5-8-9-10) related to paraneoplastic syndrome with a poor response to treatment and a bad prognosis. 2) antibodies against surface and synaptic proteins (anti-AMPA-GluR3, anti-NMDA-NR1, anti-NMDA-NR2) better respond to first-line treatments[10].

Anti GABA auto-immune encephalitis has an incidence of less than 5%[11] of all cases. It happens more frequently in young female patients with refractory status epilepticus, behavioral changes, and severe psychiatric symptoms presentation[1,11]. As depicted in our case report.

To accomplish diagnosis, a cerebrospinal fluid (CSF) study is imperative. It is described with: pleocytosis (>4/ul) in 69% of cases, proteins >0.5 g/l in 44% of cases, CSF to serum glucose ratio <0.5 in 27% of cases[12]. Our patient result was similar.

Literature reports show anti-GABA auto-immune encephalitis as strongly related to small cells lung cancer as in 50% of cases[2,4,11]; despite that, there is usually no clear trigger for this type of encephalitis[1]. Image thoracic studies in our patients do not display significant alterations.

In image studies, brain magnetic resonance image (MRI) usually shows changes in the hippocampus and amygdala in FLAIR and T2 sequences, cortical changes in FLAIR sequence and even a normal study[2,11]. Our patient had a normal brain MRI.

Steroids are considered first-line therapy in anti-GABA auto-immune encephalitis cases, and plasmapheresis as second-line therapy[1]; 33% of patients have total remission to treatment and 40% with partial response[4]. When an oncologic cause is detected, treatment starts with antineoplastic therapy.

It has been reported that between 50% and 70% of auto-immune encephalitis cases require intensive care management and 41% mechanical ventilation. Intensive care length of stay has a mean of 89 days[3]. In our case, it was not possible to determine the total extension of hospitalization because the patient was transferred.

Despite a long hospital stay, recovery is encouraging; nevertheless, bad prognosis signs are central hypoventilation, movement disorder, abnormal MRI, late treatment, and lack of response to initial treatment[13,14]. Titulaer, *et al*[3] reported that early treatment and non-intensive care unit (ICU) admission are related to good prognosis (OR 0.62, CI 0.50–0.76; p<0.0001; OR 0.12, CI 0.06–0.22; p<0.0001).

Limitations of this case were difficult to diagnose by identifying antibodies because this type of test is not common in Ecuador; our hospital is a second-level care facility, so there was a limitation to delivering appropriate treatment, and the patient needed transfer and follow-up was lost.

The strength of this report was high suspicion for auto-immune diagnosis after failure to initial antibiotic treatment, taking into account psychiatric subacute symptoms.

Conclusions

Auto-immune encephalitis is a rare disease with a low worldwide prevalence, so it is necessary to maintain a high clinical suspicion to achieve prompt diagnosis and treatment. Patient prognosis is related to it and avoiding the need for mechanical ventilation and ICU admission. Steroids are first-line drugs in these cases, with a third of patients having a good initial response, so plasmapheresis availability is key to continuing appropriate care. Patients can recuperate most of their functionality despite long recovery inside the hospital, and outpatient follow-up is essential for physiotherapy and reevaluation.

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ARTICLE / INVESTIGACIÓN

Efficacy of Health Beliefs Model-Based Intervention in Changing Substance Use Beliefs among Mosul University Students: A Randomized Controlled Trial

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Abstract: Substance use disorders (SUD) are a significant health concern worldwide. Substance abuse is a persistent problem linked to high rates of illness and loss. These issues are also responsible for many healthcare workers and health bills. However, the Substance use problems are associated with capitalize health retrogradation, weakness and death due to impacts and exceed. Throughout Iraq, there is growing concern about the effects of violence and war on drug use. To see how effective HBM-based intervention is at changing college students' health beliefs about drug usage. Using a randomized controlled trial approach, experimental design is carried out throughout the current study to determine the efficacy of health beliefs model-based intervention in changing the belief related to substance use among university students in Mosul City from 26-November 2019 to 1-March 2021. The study sample consisted of 80 students who participated in the training program for behavioral change. The intervention for the experimental group involved a health education lecture about substance use. Analyzed data using SPSS, Version 23 using both descriptive statistics and inferential statistics (Means, SD, Number and percentage). These results revealed statistically significant differences among all concepts of the Health Belief Model related to substance use, adding to behavioral motivation, behavioral control and intention) overtime. On the contrary, the perceived barrier was the only belief that showed no significant changes over time. According to the findings, the design of an HBM-based study could impact students' cognition and conduct in the field of drug dependence. Given the positive association across HBM dimensions and pupils' opinions, notably in "significant advantages and regarded harshness," These beliefs seemed to have a strong connection to each other and the avoidance of alcoholism.

Key words: Efficacy, Health Beliefs Model, Belief, Substance Use, Students.

Introduction

Substance use disorders are a significant health concern worldwide. Substance use is inveterate trouble that is associated with substantial morbidity and mortality. These problems also account for essential health care employment and medical costs[1,2]. Substance use disorders and intemperance represent universal public health problems of substantial socioeconomic inclusion[3,4]. Many clinical studies signalize an association between substance use and personality troubles with the guide that personality pathology may affect both an etiology and course of substance use troubles[5,6]. Substance use problems are associated with capitalize health retrogradation, weakness and death due to impacts and exceed[7,8]. In Iraq, there is a growing issue regarding the impact of violence and war on narcotic use[9,10]. It has been discovered that alcoholism can have a consequence on civilization systems, as a sickness and as a social one, as well as other narcotics habits, which can pose health struggles and difficulties[11,12]. Students are among the groups with higher drug risk abuse because they don't know the illegal drugs' outcomes yet have no right convictions about them[13]. Substance use, "alcohol, tobacco, hookah and illicit drugs," is a big issue today[14,15]. Substance use causes clinical, practical, and significant sicknesses such as health problems, inability and defeat to meet responsibilities at work, school, house, and university[16]. Usage of substances by young adults. In a 2010 survey, it was found that approximately 27 million psychological and social disorders were triggered by "high-risk" drugs that posed a danger to their health. Around 300,000 casualties in 2015 were caused by substance use disorders[17]. In terms of drug use disorders, evidence now shows that more than (85 percent) of those who meet requirements for a drug use disturbance early in their lifetime do so during puberty. Unlike adulthood, which transmits adolescents without meeting needs for a drug use problem, it is unlikely that one will ever grow[18,19]. However, preparing for college has historically been looked a defensive factor against the development of substance use troubles; in new decades, substance use has become one of the most diffuse health troubles in universities in the United States[20,23]. In 2017, (more than 70,000 population) died from medicine overdoses, unmatching the number of deaths from HIV, vehemence or traffic accidents at their peak[24].

Smoking use is the single most blockable cause of death in the United States. Although the 2014 Surgeon General's statement on the health successive of Smoking (50 Years) of Progress demonstrated that the spread of stream smoking is on the refuse, the report confirms the need further to

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monitor types of use for all tobacco products, particularly as the disparity in use persevere. Alternative forms of smoking use are rising in publicity among young[25]. Mousawi,2014, found that Iraqi studies on secondary school and university students reported smoking use rates spreading from (3.2- to 21%). According to the World Health Organization (WHO), documentation (3million) passing's in the known universe were precipitated by heavy drinking in 2012[26]. The above quote further explained that incidents accounted for the two lion's share of vodka deaths after cvds, so a huge proportion of drinking fatalities around the globe are affected by motorized vehicles and take place in poor economies. The most common age ambit of narcotic abusers globally is (18 -25) years. Some students are at these ages, and narcotic abuse has been shown in them; among illegal drugs, consuming (3.8%) is the most common abused narcotic in the world[27,28].

Materials and methods

Experimental design using the random controlled trial approach is conducted to determine the efficacy of the HBM in changing the belief related to substance use among university students in Mosul City from the 26 of November / 2019 to the 1 of March/2021. A proportion (simple random selection) of (N=80) college students from various fields of study would really be chosen. This study sample will be selected from the Technology, Humanities, Health, and Community Colleges at the College of Nineveh. A sampling pool consisted distributed at four colleges in Mosul University, including (Political Science, Engineering, Sciences, and Nursing)The sampling will be divided into treatment and control groups at unexpected times, with (40) young adults for every category benefitted by gender and university. Such a selection is made from a source of items that fulfill the standards while including pupils who have been using them previously. The study is carried out in Iraq. The University of Mosul is a public university situated in Mosul. It's one of the largest educational and research centers in Mosul City and the second-largest in Iraq.

Contain 22 colleges in different specialties divided into four colleges in the University of Mosul's Engineering, Sciences, Medicine and Education College. Mosul University is located on the north side of the center of Mosul City at the right braid of the Tigris River. The data are collected from students at the University of Mosul of selected 22 colleges in Mosul City. There are four colleges in the University of Mosul's Political Science, Engineering, Sciences, and Nursing Colleges, from 19 of January up to the 27 of February / 2020. The (SPSS) curriculum for Pc is used to examine the facts (V:26).We calculated the mean, standard deviation, frequency and percentage to describe the entrant of the study. Pearson chi-square and t-test are used to explore the homogeneity of characteristics between experimental and control groups at baseline (Pretest)

Results

Concerning the variations in the Health Belief Model throughout the three times, Table 3 shows substantial variations in the research group participants' perceived benefits of beliefs. Concerning the control group, table 3 reveals the fluctuation of Beliefs over time. However, no exact improvement or stable continuous change in students' beliefs is observed.

Table 4 indicated that before the intervention, mean scores for all concepts of HBM were nearly comparable in terms of interest, manageability, and purchase intent of students. But, posttreatment, the study group was dissimilar, whereas group 2 was not. The difference was higher among the intervention group than the control group, and it was positive for all concepts except for perceived barriers. This issue indicates that education caused a significant increase in scores of sensitivities, severity, perceived benefits, cues to action, self-efficiency, motivation, behavioral control and intentions. It further reduced the perceived barriers of stu-

Socio-demographic	Experimental (n=40)		Control (n = 40)		Total (n = 80)		*t*
	M	SD	M	SD	M	SD	
Age	23.37	2.09	23.70	2.04	23.53	2.06	0.230
BMI	20.51	2.64	21.95	2.22	21.23	2.53	0.283
Characteristics	F	%	F	%	F	%	χ^2
Gender							0.694
Male	36	90	37	92.5	73	91.25	
Female	4	10	3	7.5	7	8.75	
Marital status							0.856
Married	10	25	11	27.5	21	26.25	
Single	29	72.5	27	67.5	56	70	
Divorced	1	2.5	2	5	3	3.75	
Residential unit							0.433
House owner	29	72.5	32	80	61	76.25	
House rent	11	27.5	8	20	19	23.75	

F: Frequency, %: Percent, M: Mean, SD: Standard deviation, t: t-test, χ2: Chi-square, BMI: Body Mass Index, All group differences p > 0.05.

Table 1. Demographical Characteristics and Homogeneity Between Experimental and Control Groups.

Beliefs	Groups					
	Experimental (n=40)		Control (n=40)			
	M	*SD*	*M*	*SD*	*t*	*p*
Perceived Susceptibility	2.42	0.76	2.39	0.48	0.217	0.829
Perceived Severity	3.12	0.71	3.17	0.67	-0.339	0.736
Perceived Benefit	2.91	0.76	2.96	0.78	-0.288	0.774
Perceived Barrier	2.75	0.59	2.91	0.56	-1.229	0.223
Cue to action	2.31	0.70	2.27	0.61	0.211	0.779
Perceived Self-Efficacy	2.27	0.91	2.41	0.99	-0.643	0.522
Motivation	2.77	0.76	2.69	0.90	0.482	0.631
Behavioral control	2.83	0.99	2.73	0.54	0.573	0.568
Behavioral Intentions	2.81	1.05	2.92	0.86	-0.443	0.604
M: mean; SD: Standard Division; t: t-test; (p): P-value; Minimum value for health belief model constructs= 1; Maximum value for health belief model constructs= 5						

Table 2. Baseline Homogeneity in the Health Belief Model Concepts, Motivation, Behavioral Control and Intensions Between Experimental and Control Groups Beliefs.

concepts	Groups	Post hoc Using Bonferroni		
		(pretest) vs (post 1)	(pretest) vs (post 2)	(Post1) vs (post 2)
HBM	Exp	**0.000**	**0.000**	**0.645**
	Con	**0.616**	**0.681**	**1.000**

Table 3. Post-hoc Test Using Bonferroni Corrections Procedure for Changing in the Students' Beliefs about Health beliefs Model among Study and Control Group over Times concepts.

HBM Concepts	Groups	*M (SD)*		
		(T 0)	(T 1)	(T 2)
Perceived Susceptibility	Exp	2.42 (0.76)	3.25 (0.41)	3.21 (0.46)
	Con	2.39 (0.48)	2.44 (0.70)	2.51(0.62)
Perceived Severity	Exp	3.12 (0.71)	3.89 (0.38)	3.82 (0.33)
	Con	3.17 (0.67)	3.28 (0.79)	3.23 (0.64)
Perceived Benefits	Exp	2.91 (0.76)	3.78 (0.41)	3.63 (0.90)
	Con	2.96 (0.78)	3.13 (0.72)	3.11 (0.88)
Perceived Barriers	Exp	2.75 (0.59)	2.69 (0.50)	2.70 (0.56)
	Con	2.91 (0.56)	2.93 (0.51)	2.87 (0.70)
Cue to action	Exp	2.31 (0.70)	3.18 (0.78)	3.35 (0.40)
	Con	2.27 (0.61)	2.61 (0.62)	2.83(0.61)
Perceived Self-Efficacy	Exp	2.27 (0.91)	3.22 (0.60)	3.26 (0.56)
	Con	2.41 (0.99)	2.56 (1.00)	2.55 (0.95)
Motivation	Exp	2.77 (0.76)	3.62 (0.36)	3.58 (0.48)
	Con	2.69 (0.90)	2.78 (0.77)	2.72 (0.91)
Behavioral Control	Exp	2.83 (0.99)	3.38 (0.30)	3.36 (0.45)
	Con	2.73 (0.54)	2.81(0.51)	2.89 (0.59)
Behavioral Intentions	Exp	2.81(1.05)	4.07 (0.70)	3.71 (0.69)
	Con	2.92(0.86)	3.08 (1.02)	3.04 (0.89)

Table 4. Descriptive Statistics Measuring Change in Health Belief Model Concepts, Motivation, Behavioral Control and Behavioral Intensions Across Study Group and Over Times.

Health Belief Model	"Repeated Measures ANOVA Tests"			
	F	P	(η2)	O.P.
Main time effects	26. 075	0.000	0.404	1.000
Between group effects	7.877	0.006	0.092	0.792
Groups interaction overtime	11.948	0.000	0.237	0.994

Table 5. Repeated Measures ANOVA Tests the health beliefs model in changing the belief related to substance use among university students.

dents in the intervention group.

The findings of Table 5 show that the interaction of time and group is significant (F= 11.948, p= 0.000) and that the key impact of time is significant (F= 26.075, p= 0.000) in the subject's test. And the test between groups shows the importance of the variable group F= (7.877, p= 0.006) there.

Discussion

The findings showed a positive influence of health education on improving student beliefs in preventive addiction based on the Health Belief Model. In particular, the participants found post-education that substance use is a serious condition for substance use if they did not participate in preventive substance use behaviors. In addition, participants' susceptibility, benefits, cues to action, motivation, control, self-efficacy, and perceived intentions to modify future substance use behaviors improved significantly after the intervention based on the health belief model. According to the results of (Table 1), the mean ± SD age of the study group and control group was (23.37 ± 2.09) and (23.70 ± 2.04) respectively. In addition, the same table demonstrated that the majority of the participant were normal body weight, and the overall mean (± SD) of the body mass index was (21.23 ± 2.53). Concerning other demographic characteristics, the majority of the participant were male (91.25 %) and house owners (76.25 %).Most of the participants were single (70 %) regarding marital status. Table (1) this study is inconsistent with (9), who found that the male participants reported a significantly higher prevalence of tobacco use, alcohol use, and drug abuse than females. This study is similar to (29,30), which found the average age of students was (21.21 ± 2.90). About 97.9% were single, 72.9% had low family income, and 81.3% lived in houses. This study agrees with (31) who found the mean age of the studied population was (23.48±2.51). 56.6% of fathers and mothers had low-level education. One explanation of this table (2) is normal in the initial testing of the two groups (Experimental and Control) that their knowledge and belief about substance use is little or weak because they are not exposed to a program, as well as the fact that the two groups are homogeneous with each other, the results are convergent and logical. This outcome is consistent with the research (Mahmood et al., 2018) that showed the homogeneity of students' awareness participants regarding the harms of substance use[32]. This study, consistent with Bonyani *et al.*, 2018[33] in Iran, also showed no substantial difference in the baseline attitude between students, and they had a relatively negative attitude toward substances abuse[33]. This result is consistent with Fadaei *et al.*,2020[34]. The mean score of prevention behaviors in substance use showed the pre-test, and their measurements were not substantially different at (p-value = 0.641 and t = -2,597) between the study and control groups[34]. These analysis findings are in line with the results of other studies by Mohammadi and Tavafian., 2020[35], who found that before implementing the software, there was no substantial difference between the mean score of the HBM constructs (P > 0.05)[35,36].The post-doc test revealed that the mean score of the changed Health Belief Model differed significantly among study group participants (p> .000). Relative to control group participants, the findings showed no significant change in the mean score of Beliefs throughout the educational program. Specifically, the Health Belief Model score among the study group at post-test-1 and post-test-2 differed significantly from the pre-test. Conversely, there is no significant change from posttest-1 to posttest-2. This result indicates that the student's beliefs are improved significantly after education. According to the results of (Table 4), there was an increase in the value of the (perceived susceptibility, perceived severity, perceived benefits, cues to action, self-efficacy, motivation, behavioral control and intentions) over time, and there was a decrease in the value of perceived barriers over time. Based on the findings, there was no substantial difference between the mean score of beliefs before the intervention of the research and control groups. Still, the difference was substantial after the experiment and indicates the positive effect of health education in changing students' health beliefs about substance use and minimizing students' perceived substance use barriers. Table (4). This means that the students' belief was low before implementing the intervention in the pre-test. It was also found that there were no statistically significant differences between the study and control groups (p > 0.05) in the score of the students' beliefs towards all concepts of the Health Belief Model related to substance use at pre-test (table 4). All students who participated in the study (study and control groups) were homogeneous and had the same information and knowledge about substance use at baseline. After that, all intervention sessions were given to study group participants only. After completion of the intervention, a posttest-1 was carried out for both groups (study and control).The results showed a clear change in mean except for perceived barriers for a study group. This indicates that the interference has become positively affected.The main purpose of this step was to evaluate the outcomes of the educational program contrary to the control group. And then, after (2) months, a posttest-2 was also performed for both groups to investigate the efficacy of the education program. The main purpose of this step was to identify the efficacy of the educational program in retaining adequate information and knowledge by the study group participants.This research is consistent with the findings of studies performed on the target group on substance use and the impact of education on the health belief model[37,38].This result is consistent with the study of Barjouny, *et al.*, 2012[39] showed that there is a significant correlation between the health belief model

parts and preventive behaviors of addiction and drug abuse and also a significant correlation between the variables of students and (HBM) in preventive behaviors of drug abuse (P=0.002)[39,40].This outcome is consistent with the (Fadaei *et al.*, 2020)[34] study that showed that there is a post-test level, the mean intervention and control groups scored substantially different; the preventive substance abuse in the intervention group, behaviors and their dimensions changed (p-value < 0.001and t = 14.57).In line with this study Mahmood *et al.*, 2018[32], after introducing a health education program, students' knoweldge of drug use has increased significantly, reflecting the effectiveness of health education programs in enhancing awareness of substance use amongst students. This study is similar to Nayak &Jose., 2018[41]; this research also aligned with our results, as students' expectations regarding drugs have increased following an education program with a (3.03) Mean gap f[41].

Conclusions

This study concludes that health education through a belief model demonstrates the importance of substance use prevention and positively impacts students' perceptions of susceptibility, severity, and perceived benefits of advised interventions to stop substance use in preventing addiction and reducing various health risks.

Cues for action to activate "readiness" to quit in substance users and self-efficacy for confidence in their ability to end substance use students' behavior on the topic of addictions could be affected by the structure of a Greater management study. Noting the positive relationship between HBM elements and peer perceptions, mainly on "benefits and considered harshness," These thoughts seem to have a clear connection and habit mitigation.

Conflicts of Interest

The author declares that there is no conflict of interest in this study.

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ARTICLE / INVESTIGACIÓN

Statistical Analysis of COVID-19 infections according to the gender and age in Najaf Province, Iraq

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Abstract: A newly discovered coronavirus, COVID-19, creates a new infectious illness. According to current clinical observations, people's age and gender appear to have a role in their vulnerability to COVID-19. This research aims to see whether there is an association between gender, age, and COVID-19 susceptibility in the public health department of Najaf. The study included 36607 subjects (evenly distributed between sexes), and their ages ranged from (<10 - 80) years. According to the study's findings, males were found to be more infected than females. Results of the research indicated that gender and Covid-19 had a strong correlation. According to the study, people between the ages of 30 and 39 were more common than people of other ages range. The study's findings revealed a significant difference between Covid-19 and the participants' ages.

Key words: Covid-19, Gender, Age, Najaf, Iraq.

Introduction

As part of the Nidovirales order, the Coronaviridae family, Coronaviruses, are classified. The term "coronavirus" refers to the virus's outer surface, which is covered with spikes that resemble a crown. Coronaviruses have genetic material consisting of a single-stranded RNA molecule ranging from 26 to 32 kilobases (kb). Coronaviruses are very tiny viruses (65-125 nm in diameter). Coronavirus family subgroups are alpha (α), beta (β), gamma (γ), and delta (δ)[1]. Due to the COVID-19 epidemic, there has been an unparalleled level of crises in contemporary times[2]. The severity of the disease varies greatly from person to person, ranging from moderate or even subclinical infection to full-blown disease[3,4].

More than 1 million COVID-19-related deaths have been recorded over the globe. There has been no viable treatment or vaccination for this infectious disease as of this writing. Several elements might affect the development of COVID-19; thus, it's essential to identify them. Potential risk factors for infection and illness progression are of interest. As a result of several medical and epidemiological studies, it has been concluded that all communities are in danger of infection. However, several risk factors have been linked to an increased incidence of COVID-19, and age may be one of these. Even though COVID-19 may affect persons of various ages, the elderly are the most vulnerable. Pediatric patients less than one year of age have been reported to have more severe clinical symptoms. COVID-19 may potentially put newborns at risk of respiratory failure, according to certain studies[5]. The severity of the condition in males was shown to be higher than in females, suggesting that gender may be a risk factor for COVID-19. When it comes to COVID-19 infections, males are generally more susceptible to death than women[6], and the study's goal is to conduct a statistical analysis of conditions by gender and age.

Materials and methods

For coronavirus infection, data on age and gender were collected from the public health department and Najaf hospital in Najaf province in 2021. As a result, to evaluate the impacts, the Statistical Analysis System program (SPSS) was utilized. The Chi-squared test was used.

Results and discussion

The study revealed males (57%) were found to be more infected than females (43%), as shown in Figures (1); this result agrees with de Souza et al.,2020. COVID-19 causes a disproportionately high number of deaths in men, prompting the hypothesis that men are more at risk than women. Males are more likely to die from heart disease, diabetes, liver disease, and cancer than females[7]. COVID-19's sex-biased mortality maybe because these illnesses have been shown to have a sex-specific incidence. Men in Italy die at a greater rate than men in China. It is assumed that the increased incidence of cardiovascular disease among Italian males is responsible, as an example[8]. Males and females differ in their susceptibility to infection, ability to withstand stress, and total lifespan. In certain nations, females outlive their male counterparts by over seven years[9]. According to Pradhan and Olsson's Biology of Sex Variations, sex-rela-

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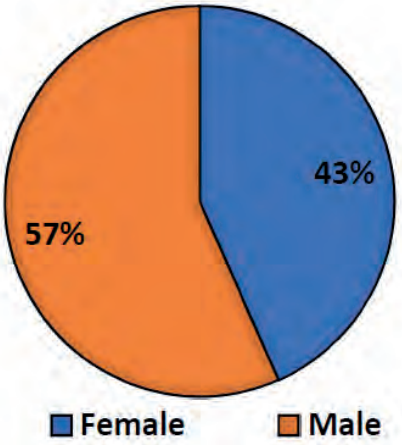


Figure 1. Rate of infection according to gender.

ted differences, including variances in hormone composition, heredity, and other physiological features, may be to blame for this discrepancy in life expectancy. Evidence suggests that females may outlast their male counterparts even under the worst conditions, such as hunger[9]. In Sweden, male life expectancy was slashed to 17.15 years during the 1772–1773 famine, while female life expectancy rose to 18.79 years. An estimated 18.7 and 22.4 years of life expectancy were slashed by the Irish famine of 1845–1849. Male life expectancy decreased from 41.58 years to 7.3 years, while female life expectancy decreased from 45.93 years to 10.9 years[9]. Other historical instances suggest that men are less able to deal with challenging conditions than women in terms of their level of endurance. In the first year of their voyage back to Africa, 43 percent of the formerly enslaved Americans died. Male's life expectancy was 1.68 years, and females' was 2.23 years[9].

As shown in Figure 2, those between the ages of 30 and 39 were older than those between the ages of 40 and 49; this finding aligns with[10]. A personal encounter with COVID-19 between 18 and 69 may have helped them estimate their risk perception of the virus[11,12]. Participants above the age of 70 did not use the heuristic to estimate the hazard presented by COVID-19 in the research, perhaps because of reduced cognitive resources. Among sociodemographic factors, only in the age range of 40–49 was being female associated with significant risk levels[13]. Moreover, in the 30-39 age range, having a job predicted a greater chance of exposure. Subjective health perceptions were detrimental in explaining risk susceptibility in the ages of 18-29 and 50-59, respectively. People in these age groups may assume they are more susceptible to COVID-19 because they have poor physical health. As for emotions, tension-anxiety and hostility-anger indicated vulnerability in the age groups of 30-39 and 50-59. In younger people aged 30–39, higher levels of tension-anxiety were linked to a lower risk susceptibility. Be aware that this POMS subscale does not reflect COVID-19-specific anxiety levels but rather general levels of stress and anxiety in general. Aggressive-hostile adults in the 50–59 age bracket showed a decreased susceptibility[14].

Conclusions

The study revealed males were found to be more infected than females. The study also mentioned that infection with COVID-19 focused on 30 -39 years old.

Author Contributions

Conceptualization, SGA and IAA; methodology, SGA and IAA; software, SRB; validation, SGA, IA.A and SRB; formal analysis, IAA; investigation, SGA; resources, S.GA.. and IAA; data curation, SGA, IA.A and SRB; writing—original draft preparation, IAA; writing—review and editing, SGA and SRB; visualization, IAA; supervision, SGA; project administration, IAA; funding acquisition, SGA, IA.A and SRB. The published version of the work has been reviewed and approved by all authors.

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Informed Consent Statement

Not applicable.

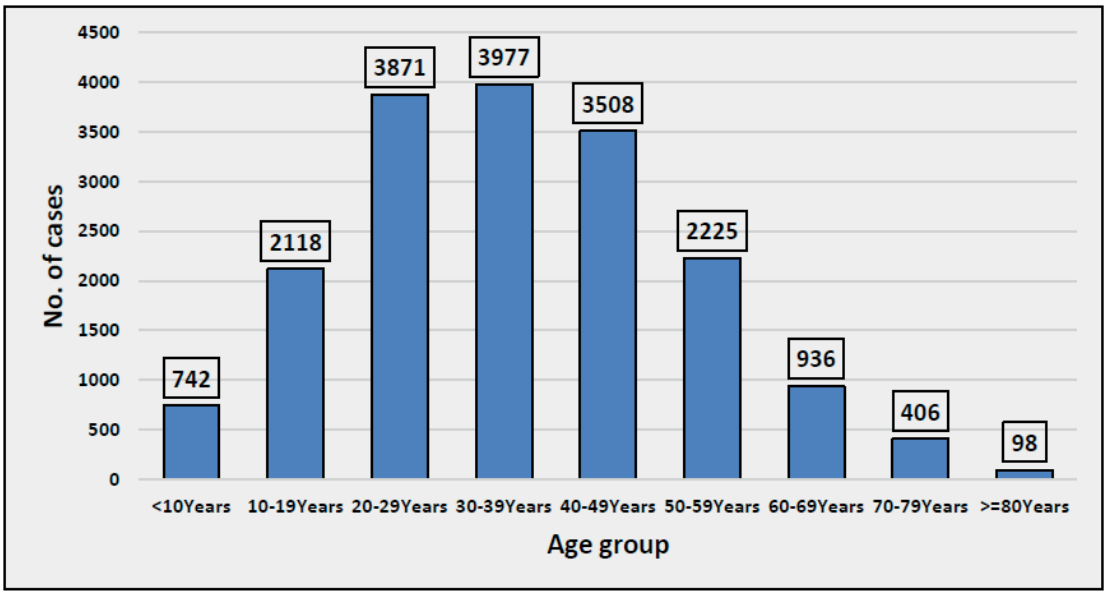


Figure 2. Distribution covid-19 infection according to age.

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Conflicts of Interest

The writers have stated that they have no conflicts of interest to disclose.

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ARTICLE / INVESTIGACIÓN

Human Resistin as a Marker to Monitor Lupus Nephritis and Atherosclerotic Changes in Females with SLE

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Abstract: Systemic lupus erythematosus (SLE) is a polygenic chronic inflammatory condition that influences several body organs. While renal and dermatological changes are among the most common presentations of the disease. The current study examines the possible association between human Resistin (h-Res) and signs of disorder activities such as inflammation, renal function, lipids, and Bone Mineral Density (BMD) in females with SLE.
We performed cross-sectional research on 50 females with SLE diagnosed using the American College of Rheumatology (ACR) criteria. Forty females were recruited as controls. They matched the following items, including gender and body mass index (BMI) with age. In SLE patients, we discovered a statistically significant positive relationship between h-Res levels and ESR, CRP, Cr, ds-DNA, C3, duration of steroid treatment, and other variables. Disorder activity as determined by systemic lupus erythematosus disease index (SLEDAI) and carotid intima-media thickness (CIMT) (p-value <0.05). The levels of h-Res and C3 are discovered to have a statistically significant negative relationship. In the case of SLE, the h-Res level can be used as a marker for Lupus Nephritis (LN) and atherosclerosis. In patients of SLE, more aggressive therapy of the underlying inflammatory process and better control of the traditional risk factors may be beneficial in reducing the risk of renal and atherosclerotic derangement.

Key words: Biomarker, lupus nephritis, systemic lupus erythematous, Atherosclerosis.

Introduction

SLE is a chronic inflammatory illness that mainly affects women and for which there is now no recognized solution. Lupus nephritis (LN) is a severe symptom of SLE that damages the kidneys and is associated with renal failure, Patients with SLE, which affects 50% of the population and causes significant morbidity and mortality, are at an elevated risk of having a premature atherosclerotic cardiovascular disease (ASCVD)[1]. Many hormones and cytokines with significant systemic metabolic effects have been discovered in adipose tissue, which has been investigated for its capacity to release them. It is commonly acknowledged that adipocytes and a variety of other cells found in adipose tissue, such as immunocompetent cells, contribute to the total endocrine production of the body[2]. Resistin is a cysteine-rich secretory peptide that has just been found, and It is thought that adipocyte is the source of its synthesis. According to animal research, h-Res is produced mainly in white adipose tissue and might represent the link between obesity and insulin resistance (IR). In humans, the function of h-Res is not yet totally elucidated. There is a hypothesis that h-Res has a pro-inflammatory impact and is plentiful in inflammatory illnesses (for example, Crohn's disease and rheumatoid arthritis (RA), and similarly, it is produced together with other inflammatory markers in many individuals[3].h-Res seems to have some role in bone metabolism, perhaps by stimulation of osteoblast and osteoclast activity, this is mediated by the nuclear factor-kappa B either directly or indirectly (NF-B) pathway. SLE is a chronic inflammatory illness that affects the kidneys and other organs throughout the body. It is characterized by a state of chronic inflammation throughout the body[4]. As a result, we wanted to determine if there was a relationship between h-Res and numerous indicators of disease activity, lipids, renal function, inflammation, and bone mineral density (BMD) in females with SLE.

Materials and methods

In this study, 50 females with SLE illness participated in a cross-sectional design. SLE was diagnosed using the American College of Rheumatology's criteria (ACR). A total of forty matched controls were included in the investigation. Because h-Res is associated with obesity, the controls and the experimental group had similar BMIs. All attendees received educational booklets as a part of the program. All participants, including the control individuals, provided written informed permission before participating in the study. The medical ethics committee at Ain Shams University approved the study.

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Exclusion criteria

The following problems have been identified: gestational diabetes, malignancy, SLE linked with other diagnoses (overlap conditions), liver diseases, intestinal inflammation, and instances with a history of angina, stroke, myocardial infarction, and renal failure on hemodialysis.

Inclusion Criteria

The other patients, however, while the study included cases of subclinical atherosclerosis (dyslipidemia) and patients with concomitant hypertension, this was done because some of the conventional hazards for atherosclerosis can be caused or worsened by SLE and/or interventions (steroid intake), hypertension alone with no dyslipidemia may be secondary to renal dysfunction rather than SLE.

Clinical evaluation

History and physical examinations were completed focusing on the duration of illness, history of medications intake, and conventional cardiovascular risk factors. The complete clinical study was done for all patients, and controls included calculating BMI (kg per m^2) and BP measurement. Hypertension has been defined as follows: SBP ≥ 140 mmHg or DBP ≥ 90 mmHg.

Laboratory assessment

Venous blood specimens were taken after fasting overnight, and sera were kept at -70 C until analyzed. Standard laboratory procedures were used to assess CRP levels, blood cell count, erythrocyte sedimentation rate (ESR), C3, C4, Cr, and HDL, total cholesterol, triglycerides (Tg), and low-density lipoprotein both were investigated employing ordinary lab methods.

Bone biomarkers

The radioimmunoassay method was used to assess the quantitative levels of the bone reabsorption indicators Cross-linked carboxyterminal telopeptide of type I collagen (ICTP) and the bone formation markers Procollagen type 1 N-terminal Propeptide (P1NP) (Orion Diagnostical, Espoo, Finland). Threshold values for CTP were 0.7-µg/L and 2-µg/L for P1NP.

Human Resistin (h-Res)

h-Res levels have been determined with sandwiched enzyme-linked immunosorbent assays (ELISA) (R & D Systems, Inc., Minneapolis, MN, USA).

Cytokines

The pro-inflammatory cytokines such as TNF- α -, IL-1β, IL-6, and soluble IL-6 receptor (sIL-6R) were quantified using a quantitative sandwich ELISA kit with a diagnostic threshold of 0.12, 0.1, 0.7, and 6.5 pg/mL, respectively.

Bone mineral densities measures

This study used the non-dominant hip (femoral neck and entire hip), Lumbar spine (L2–L4), and According to the DEXA scan, the non-dominant distal forearm has been identified (Dual energies x-ray absorptiometry) with a Lunar Prodigy densitometer 12165 (GE Healthcare, Little Chalfont, UK).

Fractures

Using a visual semi-quantitative method, we detected prominent vertebral compression fractures in the thoracic and lumbar spines (Th4-L4) on lateral x-rays of these regions. Generally speaking, compression fractures are defined as any vertebral abnormality that results in a minimum of a 20-25 % drop in height at the frontal, middle, and/or back levels. The same radiologist reviewed all X-rays throughout the study.

Normal controls

The levels of h-Res were determined in ten healthy donors and thirty healthy female staff members and Ph.D. students in the Rheumatology department.

Statistical analysis

The statistical analysis was carried out using SPSS-12.0.1 (IBM Inc., USA). Descriptive parameters were provided as median and range or as mean and standard deviation (mean SD). The Kolmogorov-Smirnov test investigated the relationships between all of the variables. The Pearson correlation was used when the variables had a normal distribution; otherwise, the Spearman correlation was used. All tests were two-tailed, and the level of statistical significance was set at $p<0.05$.

Results

According to the findings, the mean age of the patients under investigation was 25.26 yrs (±7.37 SD). The mean disorder (SLE) duration was 3.53 yrs (± 1.34 SD). The mean systolic blood pressure appeared to be 128.4 (± 15.5). Mean Diastolic blood pressure was 82.69 (±8.4 SD). The mean BMI was 28.09 (± 0.74 SD). Mean cholesterol was 225.01 (± 86.5 SD). Mean LDL was 149.05 (± 49.7 SD). Mean HDL was 40.5 (± 10.56 SD). Mean Triglycerides were 132.09 ± 57.25 SD) as in figure(1). There was a very significant difference in the levels of Cr, ESR, CRP, h-Res, or CIMT between the experimental and control groups, as in figure 2. Additionally, when comparing individuals with and those who do not have lupus nephritis, we found that those disparities were evident as well figure 3; in SLE cases, a significant positive association has been found between h-Res levels and CRP, ESR, Cr, ds-DNA, steroid use, disorder activities determined using CIMT and SLEDAI (p-value < 0.05). It was discovered that there was a significantly substantial negative relationship between h-Res levels and C3-figure 4.

Discussion

It has been shown that h-Res have pro-inflammatory and atherogenic properties related to the inflammatory response. It is widely believed to be connected with obesity and type 2 diabetes. Higher h-Res levels in the blood characterize obesity. Reduced glomerular filtration rates (GFR) and inflammation are related to high levels of h-Res in the blood. The researchers wanted to see whether h-Res might be used as a biomarker for nephritis and early atherosclerosis in SLE patients. Elshishtawy team enrolled 50 SLE females as well as 40 matching controls. All of the patients and participants in the control group were exposed to laboratory tests and carotid duplex exams. The SLE disorder Activity Index (SLEDAI) was developed to measure the severity of the disorder. A renal biopsy was conducted in

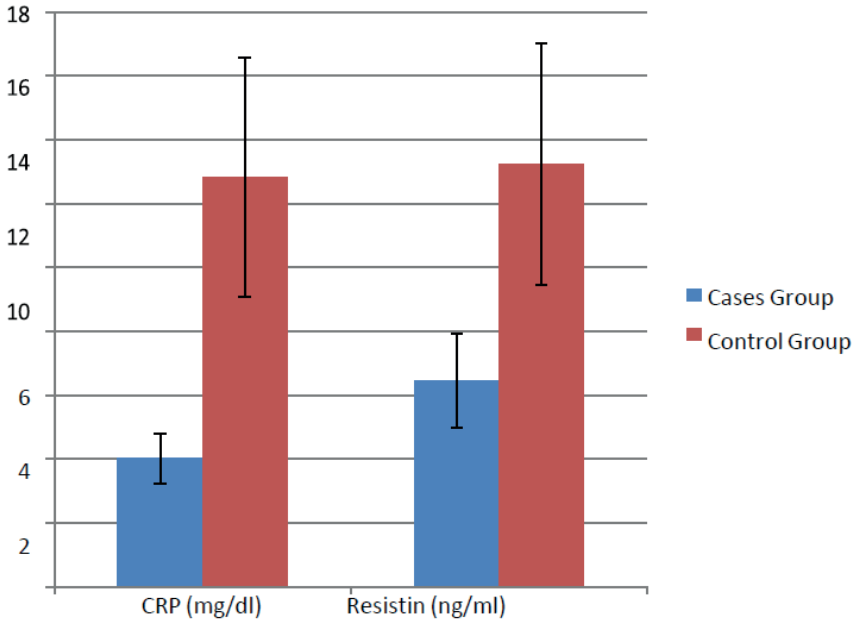


Figure 1. Comparing among the study groups regarding CRP and resistin.

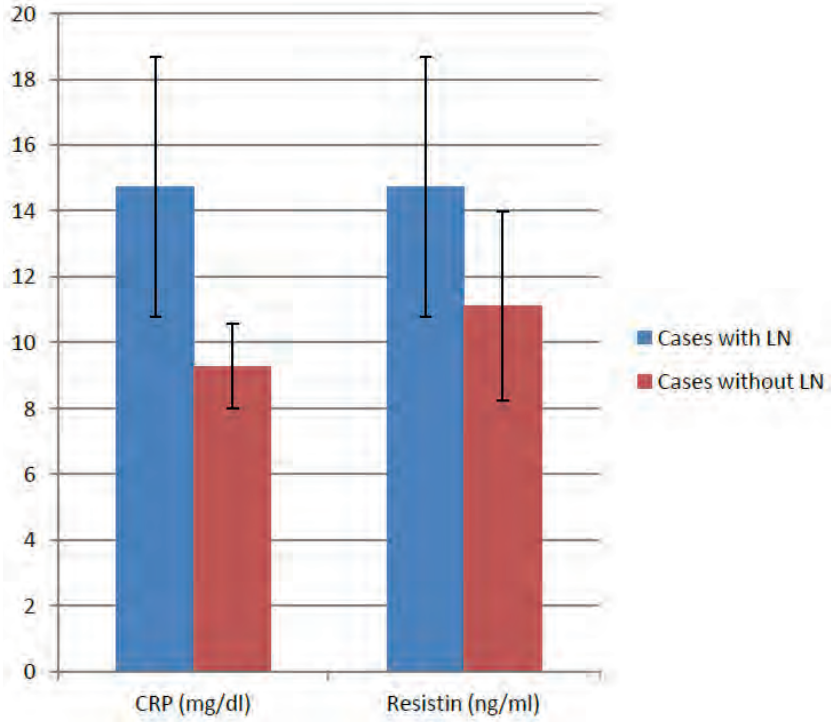


Figure 2. Comparing cases with LN and those without LN, regarding CRP with Resistin.

individuals with SLE who also had LN. He reported elevated h-Res levels in SLE cases with LN compared to SLE cases with no nephritis, in concordance with other authors who revealed that renal dysfunctions in SLE were correlated with increased levels of h-Res. Elshishtawy team discovered a significant increase in cholesterol, LDL, triglycerides, and CIMT in individuals with SLE when compared to a control group of people with no SLE. This shows that females with SLE are at higher risk of developing early atherosclerosis and chronic vascular inflammation than men.

There was a statistically significant positive correlation between h-Res levels and CIMT, cholesterol, and triglyceride levels in the study participants[5]. Almehed and his co-workers showed that 2.5% of his patients had reduced renal functions with GFR of <30-mL/min, and 14% had GFR of <50-mL/min using the projected GFR values[6]. Over time, our findings showed a significant shift in Cr, ESR, CRP, h-Res, and CIMT. Even though the exact cause of elevated h-Res in SLE has not been determined, it is believed to be connected to changes in renal function, the direct effects of inflammatory mediators on h-Res production, changes in fat distribution, or a combination of these factors and others. Histamine's role in inflammation seems to be consistent with the relationship between h-Res levels, ESR, and CRP levels that have been identified before in studies[7]. Researchers have discovered that indicators of inflammation in SLE, including higher ESR, pro-inflammatory cytokines, immunoglobulin G (IgG), CRP, and low albumin, are related

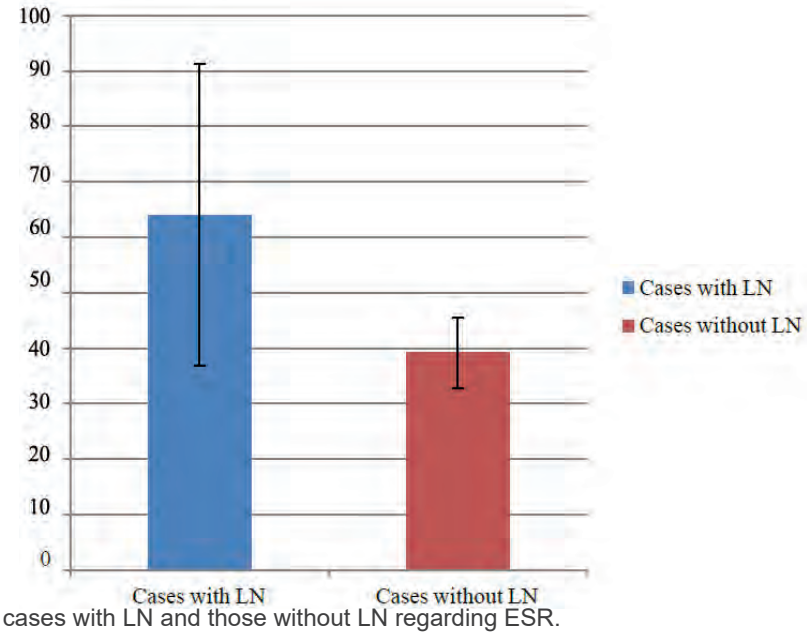


Figure 3. Comparing cases with LN and those without LN regarding ESR.

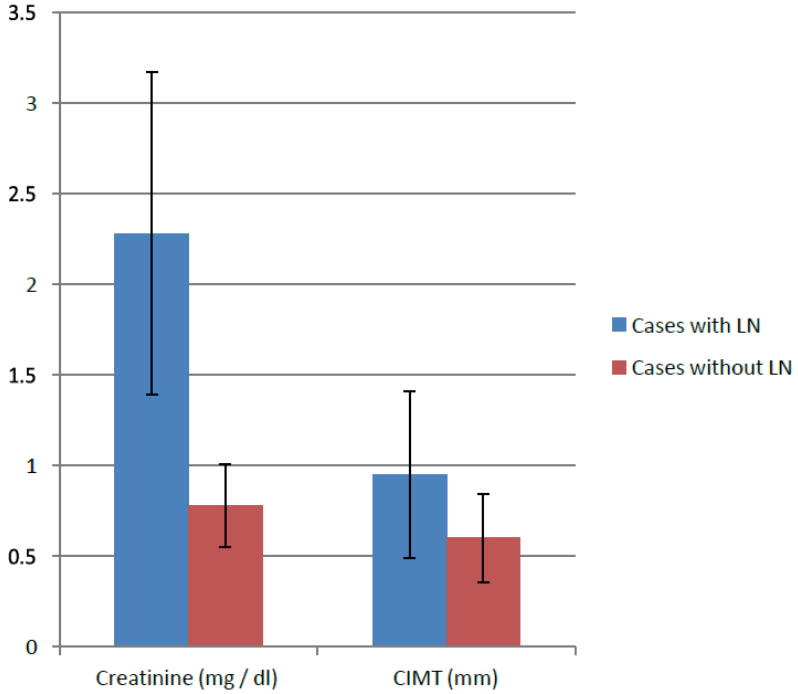


Figure 4. Comparing cases with LN and those without LN regarding creatinine and CIMT.

to high levels of h-Res in the blood[6]. But, the Chung team observed a comparably modest association between h-Res and ESR but not with CRP; this might be due to the difference in sample size and the higher levels of disorder in our SLE patients. CRP persuaded both mRNA expressions and protein secretions of h-Res in a dose and period-reliant manner in peripheral blood mononuclear cells (PBMCs)[8]. Our study demonstrated a highly significant change in SLE cases with LN and cases with non-LN in levels of Cr, ESR, CRP, h-Res or CIMT. But, Malyszko and his colleagues established a correlation between h-Res and renal functions[9], in concordance with a finding of Nu¨sken et al. in children on hemodialysis for chronic kidney disease (CKD)[10], and the limited number of patients with IgA renal dysfunction[11].

H-Res was significantly correlated with GFR and inflammatory biomarkers in CKD. Because h-Res have a low molecular weight, The kidneys are thought to be the primary secretory channel. Chronic kidney disease (CKD) is a state of chronic inflammation characterized by a considerable increase in blood levels of pro-inflammatory cytokines at various stages of renal function impairment[12]. Our findings revealed that among SLE patients, there is a statistically significant positive correlation between h-Res levels and ESR, CRP, Cr, dsDNA, steroid period, disorder activity as measured by the SLEDAI and CIMT, and a negative association between h-Res levels and ESR (p value< 0.05). A highly significant negative association was found between h-Res and C3. Our findings were also analogous to Baker[13],

who found raised levels of h-Res in SLE patients with coronary artery calcifications. The early results of Kougias and co-workers[14], from their porcine vascular research revealed that h-Res might result in endothelial dysfunctions in the porcine coronary artery via oxidative stress and downregulation of endothelial nitric oxide (e-NO), consequently, has a direct effect on vascular disorders. The study done by Yaturu[15] revealed that levels of h-Res didn't correlate with GFR. This finding aligned with our findings though it contradicted the author's results, who found a significant negative association between h-Res and GFR[11]. Still, their patients were not homogenous in the degree of CKD dysfunction. But some reports refer to a highly effective association between the level of resistance-to-insulin and TNF-a. Resistance-to-insulin was found to promote endothelially cell activations and was connected to cardiovascular disorders in metabolic disorders[15]. Inflammation of the vascular endothelial cells seems to result from insulin resistance, reduced by adiponectin[16] directly.

Conclusions

In SLE patients, the blood h-Res level may be used as a biomarker for LN and atherosclerosis, which can be measured. Controlling the underlying inflammatory process and traditional risk factors for both illnesses with more vigor may effectively lower the risk of renal and atherosclerosis dysfunction in rheumatoid arthritis and other autoimmune disorders.

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Conflicts of Interest

The authors report no conflicts of interest.

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ARTICLE / INVESTIGACIÓN

Evolution of agricultural wastes for the cultivation of edible mushroom *Pleurotus eryngii*

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Abstract: This research studies the possible use of some of Iraq's local agricultural wastes as growth substrates for producing the edible oyster mushroom (*Pleurotus eryngii*). The biological process of growing and producing edible mushrooms is a bioconversion of organic wastes to bioprotein (mycoprotein). Therefore, this study aims to investigate nine different substrates from plant residues. The three primary substrates are typical viz date palm waste (*Phoenix dactylifera*), maize cob (*Zea mays*) and cane (*Phragmites communis*), with two types of additional enrichment, i.e., rice husks (*Oryza sativa*) and corn seeds husks (*Zea mays*), and without further enrichment (control). The experiment was randomly distributed according to Randomized Complete Design (RCD), with three replicates. The less crop cycle period (day) which in Treatments T7 and T8 (30.33, 31.67 days) when T2 (100%Corncobs), T1 (100%Waste of Date palm), T7 (80%Waste of the date palm with 20%Corn husks) and T8 (80% Corncobs with 20%Corn husks) provide the crest mushrooms yield arrived 293.0 280.7, 268.7 and 264.0 g kg^{-1} substrate respectively.

Key words: Bio protein, lignocelluloses, Mushroom substrates, Oyster mushrooms.

Introduction

The mushroom is a fleshy saprophytic fungus that grows naturally on tree trunks containing rotting natural matter and moist soil. Mushroom cultivation can be used as an efficient method of harvesting and as a strategy to protect the environment[1]. Using the residues in biological processes can consider as one of the biological transformations from non-edible biomass waste solution to nutrient-packed foods protein in the form of edible mushrooms. Production is the transformation of household agricultural or industrial waste into food for people. The concepts of microorganisms, environmental technology, and fermentation of solid-state cover every form of mushroom[2-4]. The genus and species of *Agaricus spp*, *Pleurotus spp* and *Lentinus edodes* Are the most common edible mushroom cultivated worldwide. However, *Pleurotus* is a widely cultivated type of edible mushroom across a globe at a range of pillars with surroundings[5]. That genus contains greater than 200 throwing organisms spreading in tropical regions worldwide. *P. ostreatus*, *P. citrinopileatus P. djamor*, *P. citrinopileatus* and *P. eryngiii* are the most common species in the genus *Pleurotus* (oyster mushroom)[6]. In Asia, North Africa and Europe, *Pleurotus eryngii*, popularly referred to as the king oyster mushroom, is commonly used. Several primary functions, such as antioxidants (antioxidants, cancer, viruses, bacteria, and leukemia), hypolipidemia, immunity, and estrogen-like activities, include the potential therapeutic activities of edible mushrooms. Its biologically active compounds such as polysaccharides, phenolic molecules, ergothioneine and triterpenoids form the basis of this bioactive properties[7-9]. *P. eryngii* is commercially grown on several raw plant materials due to its excellent flavor, high nutritional value, and medicinal qualities. The tailings are based on the use of a powerful lignin degradation enzyme system that effectively breaks down various aromatic compounds and its usefulness in the nutrient use of lignocellulose. For the same purpose, and because of the potential in several biotechnology processes, *Pleurotus eryngii* plays a vital role in these enzymes, such as food preparation (edible fungi), biotransformation of raw plant material into forage, and biological pulp. As well as the biological treatment of soils and industrial water, pulp bleaching[10]. Oyster mushrooms have been found to grow quickly on various waste substrates (industrial, agricultural residues), such as grain straw, leaves (banana leaves), bagasse (cotton waste, coffee pulp and sugar cane), body (cottonseed husks), wood chips (sawdust and paper waste). Thus, most residues materials (lignocelluloses) containing hemicellulose, celluloses, and lignin can be used as a substrate for mushrooms[2]. Since organic carbon, nitrogen, and inorganic compounds are essential to produce mushrooms as a portion of the main food and nutrients are organic carbon origins like cellulose, hemicellulose and lignin, oyster mushrooms require less nitrogen more carbon origins. Mushroom cultivation requires an adequate balance of the carbon and nitrogen ratio in the substrate. The overall carbon quality, including refractory polysaccharides (cellulose and hemicellulose), represents the overall organic carbon (C/N) ratio[11]. This study aimed to find out the possibility of applying some of Iraq's local agricultural wastes as growing substrates for the production of the *Pleurotus eryngii* edible oyster mushroom.

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Materials and methods

Pleurotus eryngii strain 008 was used in this study. The first factor Includes three types of substrates comprising three types) date palm waste (*Phoenix dactylifera*), corncobs (*Zea mays*) and common reed (*Phragmites communis*), and the second factor is the type of supplement (20 percent dry weight) comprising (2 classes) rice husks (*Oryza sativa*), corn husks (*Zea mays*) and without accompaniment. As in table 1, the experiment contains nine treatments. Table 2 shows the chemical analysis of the components of the agricultural wastes and supplementation. The experiment was laid out with five replicates according to Completely Randomized Design (CRD).

The substrate was put and in the polyethylene bags, then it sent for heating treatment for 60 minutes, after that it was put in a metal tank containing hot water, with the change the water. Then, the substrate was left to cool and get rid of excess moisture; after that, it was mixed with two percent calcium carbonate (lime) to the wet weighing procedure with an acidity equivalent to the acidity of the treatment[15]. It had been the addition of composites per 1 kg wet weight after cooling in 24*40 cm polyethylene bags (experimental unit).

Spawning was carried out at 3% of the substrate in the aseptic state to prevent contamination. After closing the bag's opening with a thread, the spawn was inserted in the middle of the substrate bag and hanging tightly[16].

The incubation place (aseptic conditions) was prepared by providing dark conditions, temperatures between 25-27°C with air humidity 85-90%[17-18]. The temperature decreases to 14-16oC after mycelium runs on the most substrate to create cold shock by spraying the water to increase humidity to 80-90 percent. Owaid *et al.* (2015)[19] show that it is 10-15°C with the light available for 8-10 hours. The first day with 400 lux. Level 1 is the ideal temperature for inducing the fruits of the oyster mushrooms and lowers carbon dioxide and O_2 increases. Holes were made in the bags in the direction of the light at the cross shape (+) when generating the pin-head with a sterile sharp scalpel in all bags with the same number[20]. The investigator retains this environment for about 7-10 days until the end of the first harvest and then returns to the incubation cycle again. The phases of fruiting growth in the Cultivation Room are shown in Figure 1.

Results and discussion

The results of the variance analysis show that the intermediate period stage of initial growth characters (spawn run, Pin-head, fruit body formation and Production cycle) of oyster mushroom *Pleurotus eryngii* is presented in Figure 2. The spawn run of mycelium shows a statistically significant difference in T5, T6 and T8 required a longer time to complete the colonization period compared with less period in T1, T2 and T9. While the highest period of pin-head value is obtained in T8, T5 and T9 than the less period to the formation of a pin-head in T4, T1 and T3. This shows the significant difference in the period of fruit body (fruiting). The earliest period was recorded from T1, T3 and T4 than the other substrate such as T2 and T7. The results in Figure 2 show that the considerable crop cycle period (day) significantly is in T1 compared with another combination substrate that records less in T7, T8 and T6.

The observational treatments take 44 to 74 days from spawn run (complete colonization) to the completion of the mushroom fruiting body. After that, mushrooms become ready to be picked from 89 to 32 days. This variation in periods may be due to the variation in chemical and physical characteristics of substrates (Nitrogen%, Protein %, CHO%, Ash %, Fiber %, O.M %, O.C %, C: N Ratio, water absorption % and Porosity %) Table 2.

Table 3. Showed a statistically significant difference in characteristics of *Pleurotus eryngii* yield (Number of the fruit body, weight of fruit body, number of flashes, Total yield and Bio-efficiency). Total yield, is one of the essential objectives of mushroom producers; the yield as recorded in T2 (100% Corn cobs), T1 (100% Date palm waste), T7 (80% waste of date palm with 20% Corn husks) and T8 (80% Corn cobs with 20% Corn husks) Had the highest values mushroom yield reached (293.00 280.70, 268.70 and 264.00 gk g^{-1} substrate). This yield is significantly different from those found in the other substrate combinations. Bio-efficiency is Significantly different compared with another combination substrate, in general, substrates of Corncobs and give the largest yield and give the height value of Bio-efficiency. Whereas the mushroom yield of wastes of date palm and common reed substrate comes in second and the Bio-efficiency. The supplementation of the substrate did not lead

Combination of Substrate		
NO. Treatment	Main Substrate type (1st factor) %	Supplementation type (2nd factor) %
NO. Treatment	Main Substrate type (1st factor) %	Supplementation type (2nd factor) %
1	Date palm waste	lack of supplement
2	Corncobs	lack of supplement
3	Common reed	lack of supplement
4	80% (Date palm waste)	20% (Rice husks)
5	80% (Corncobs)	20% (Rice husks)
6	80% (Common reed)	20% (Rice husks)
7	80% (Date palm waste)	20% (Corn husks)
8	80% (Corncobs)	20% (Corn husks)

Table 1. Types of agricultural wastes (substrate and supplementation) and experiment treatments.

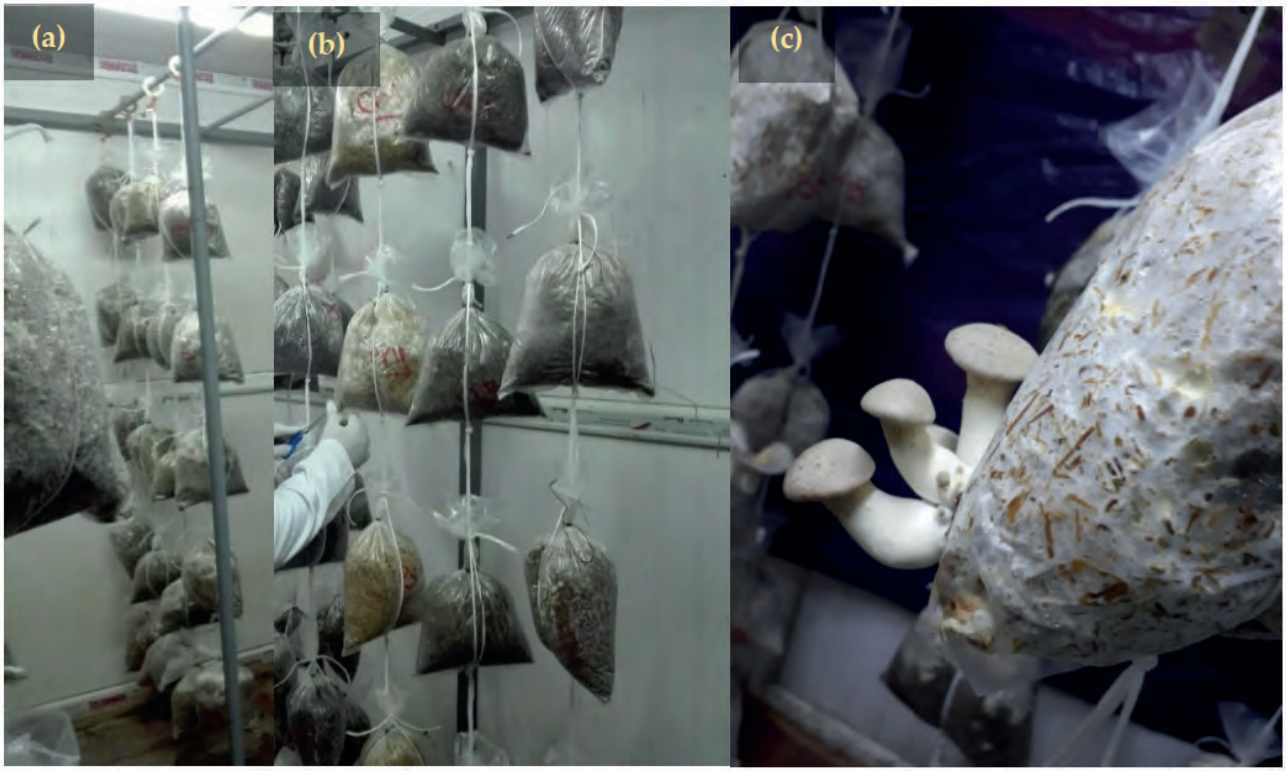


Figure 1. Fruit Body Developing from Mycelium in Cultivation Room of Oyster Mushroom *Pleurotus eryngii* (a) Post-spawning stage and mycelia growth began on the substrate; (b) Making holes in the bags for the exit of the fruiting bodies (c) The stage of formation of the fruiting bodies and their exit from the bags.

NO. Treatment	Number of fruit body	Mean of Flash. g	Number of flash	Mean of fruit body weight. g	Total yield g kg^{-1}	Bio-efficiency %
1	4.00a	93.56a	3.00a	70.17a	280.70a	61.68bcd
2	4.00a	97.67a	3.00a	73.25a	293.00a	105.78a
3	4.00a	116.25a	2.00ab	58.12a	232.50ab	65.10bcd
4	2.67ab	86.56a	2.667ab	86.56a	228.30ab	51.04cd
5	3.00 a	100.44a	2.333ab	77.00a	231.00ab	75.60abc
6	1.33b	87.00a	1.667b	118.17a	139.30b	37.77d

Different alphabets in the same column show significant differences using Turkey's HSD test.

Table 2. Analysis of chemical and physical qualities of the agricultural wastes for substrate and supplementation[1].

to the increment of profit compared to the substrates without supplements. The three types of primary substrates (wastes of date palm, Corncobs and Common reed) gave the highest values in yield the approaching result between them so that the efficiency of *Pleurotus eryngii* to grow successfully on the substrate of wastes of Date palm and Common reed substrate may be linked with the chemical composition and physical properties of selected substrates that important for the growth of this mushroom. The result is corroborative with the finding of Owaid *et al.* (2018)[19] and in harmony with the value reported by Kumar *et al.* (2020)[20] and Mkhize *et al.* (2016)[24].

The trend analysis shows that in the meantime for, the original attributes of development of the oyster mushroom *Pleurotus eryngii* (spawn running, pin-head, fruit body growth and production cycle) are shown in Figure 2. There is a statistically significant difference between ovulation. The period that takes longer to complete the T5 colonization cycle. And T6 and T8 compared to less time in T1, T2 and T9. Thus in T8, T5 and T9, the highest pin-head interval is obtained; in T4, T1 and T3, the lowest pin-head formation interval is obtained. Compared with other substrates, such as T2 and T7, the first period was recorded in T1, T3, and T4, indicating a large difference in the fruiting period of the

body. The results from Figure 2 show that at T1, the crop cycle is much longer at T1 (day) compared to another stratum of the formulation, which is lower in T7, T8 and T6. Most proposed work operations take from 44 to 74 days of ovulation (complete colonization) to ripen the fruit body of the mushroom. Then from 89 to 32 days, mushrooms become ready for harvesting.

This change in cycles might be back to the concentration in the Physicochemical effects of the substrates percentage of Nitrogen (N), Protein, Total carbohydrates, Ash, Fiber, Organic matter (O.M), Organic carbon (C), C:N ratio, water absorption and porosity Table 2. However, the results of this analysis are close to some previous studies, such as Hoa *et al.* (2015)[21] and Mkhize *et al.* (2017)[19]. Table 3. The analysis shows that there is a statistically significant difference in the characteristics of the oyster mushroom yield (number of fruits, fruit body weight, flash number, average flash yield, total yield and bio-efficiency) yield, one of the main aims for mushroom producers, yield as is Listed in T2 (100 percent corn litter), T1 (100% palm waste), T7 (80% palm waste + 20% corn husks) and T8 (20% palm waste). In general, Corncob substrates give the most significant yield and provide the height value of Bio-efficiency compared to another mix substratum. While the mushroom yield, as well as the Bio-efficiency of palm waste timeline and mutual reed substratum, comes in second. As for the substrate supplementation, there is no yield promotion compared to the substratum without supplement.

The Pleurotus eryngii mushroom yield characteristics have been grown on Agro-Waste substrates. The converging result between the three substrate forms (palm waste timeline, corn cob and ordinary cane) gives oyster mushrooms the ability to grow successfully on date palm residues and Mutual reed substrates. To relate the Physicochemical effects of the selected substrates necessary for the growth of this fungus.

The results were similar to the results reported by Mkhize *et al.* (2016)[24] and Owaid *et al.* (2018)[25].

Conclusions

This study indicates that agricultural residues of date palms and corn cobs can give an efficient yield because these wastes appear as suitable substrates for the growth of oyster mushrooms (*Pleurotus spp.*) And a nutrient medium that provides these mushrooms with the necessary elements for forming fruiting bodies.

Author Contributions: Mustafa alqaisi designed and performed the experiments, derived the models and analyzed the data. Manaf K. M. Alabtan with Mazin A. Owine measurements and Mazin A. Owine helped carry out the Manaf K. M. Alabtan simulations. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

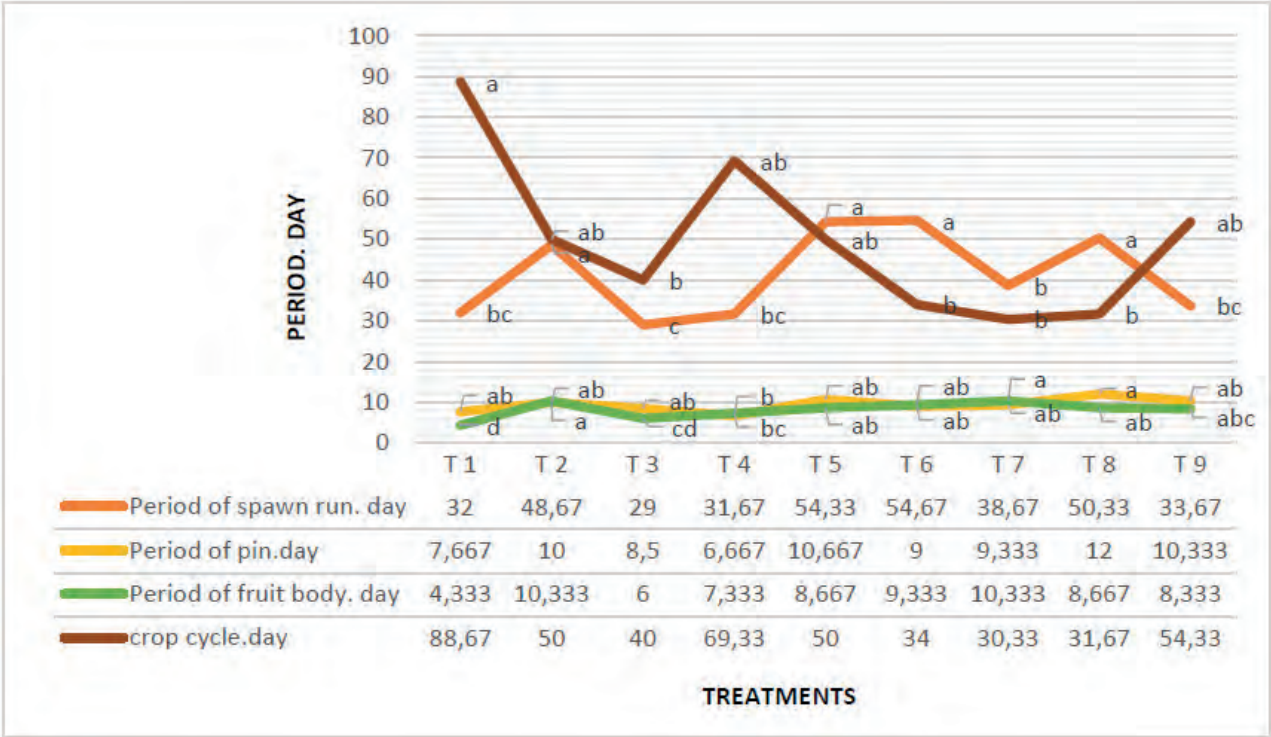
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NO. Treatment	Number of fruit body	Mean of Flash. g	Number of flash	Mean of fruit body weight. g	Total yield g kg^{-1}	Bio-efficiency %
1	4.00a	93.56a	3.00a	70.17a	280.70a	61.68bcd
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3	4.00a	116.25a	2.00ab	58.12a	232.50ab	65.10bcd
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6	1.33b	87.00a	1.667b	118.17a	139.30b	37.77d

Different alphabets in the same column show significant differences using Turkey's HSD test.

Table 3. Attributes of mushroom yield Pleurotus eryngii grown on agricultural wastes.



	T 1	T 2	T 3	T 4	T 5	T 6	T 7	T 8	T 9
Period of spawn run. day	32	48,67	29	31,67	54,33	54,67	38,67	50,33	33,67
Period of pin.day	7,667	10	8,5	6,667	10,667	9	9,333	12	10,333
Period of fruit body. day	4,333	10,333	6	7,333	8,667	9,333	10,333	8,667	8,333
crop cycle.day	88,67	50	40	69,33	50	34	30,33	31,67	54,33

Figure 2. Period stage of initial growth of oyster mushrooms (*Pleurotus eryngii*)

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ARTICLE / INVESTIGACIÓN

Vitamin D as predictor Marker of kidney disease in males with type 2 diabetes mellitus

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Abstract: The current work was done to determine the correlation between vitamin D and cystatin C as a predictor of kidney disease in males with diabetes mellitus. A total of 60 males were taken from October to December 2018. They were divided into 30 patients with type 2 diabetes and 30 healthy volunteers' as a control group. Glycated hemoglobin HbA1c, plasma glucose level, creatinine and urea were measured for all subjects. Also, levels of 25(OH) D and cystatin-c were determined using the enzyme-linked immune sorbent assay method. In this current study, as expected, patients with diabetes mellitus had significantly higher ($p< 0.001$) fasting blood glucose (FBG), body mass index (BMI), and glycated hemoglobin (HbA1c) levels as compared to the age-matched controls group. In addition, a significantly higher increase in the average level of urea, creatinine, and cystatin-c while significant higher decreases in vitamin D concentration compared to the patients group with the control group were found. A significant negative correlation was found between 25(OH) D and urea levels. On the other hand, a significant positive correlation was observed between the 25(OH) D level and HbA1c, FBG, cystatin-c and creatinine. From the results of this work, vitamin D could be a valuable predictor of nephropathy in males with diabetes mellitus. Likewise, further work is required to suggest that vitamin D may be prone to nephropathy in all patients with diabetes mellitus by estimating cystatin C as a clinical risk for kidney function.

Key words: Nephropathy, cystatin C, vitamin D.

Introduction

Nephropathy is a crucial chronic complication of type 2 and type 1 diabetes mellitus and is considered the first cause of chronic kidney disease worldwide. A third of people with diabetes will develop nephropathy after fifteen years of diagnosis[1]. Many causes such as genetic variability, diet and lifestyle have a role in developing nephropathy. Diabetes stimulates various biochemical, metabolic, and changes in the hemodynamics of kidneys. Major pathways to nephropathy include activation of polyol pathway, glomerular hyperfiltration, protein kinase C, oxidative stress by reactive oxygen species and advanced glycation end products[2]. Several markers can be an early predictor of renal damage, such as cystatin-c, nephrin, β-microglobulin and α-microglobulin in diabetes mellitus, both type 2 and type 1[3]. Vitamin D belongs to fat-soluble steroid hormones, has a protective function against diabetes and cardiovascular disease, and regulates renal function, mineral metabolism, and immunity. Calcitriol can be hydroxylated in the liver to main form 25(OH) D then converted to 1, 25(OH) 2 D active form of VD in proximal renal tubules[4]. 25(OH) D is considered a precursor of the vitamin D active form, but when there is a very high vitamin D concentration, it will bind to the vitamin D receptor (VDR). The vitamin D receptor (VDR) is a nuclear receptor for 1-α, 25-dihydroxy vitamin D, an active metabolite of vitamin D, and its receptor contributes to inflammatory pathways. VDR was founded in the glomerular parietal epithelium, proximal tubular epithelium and podocytes in kidneys[5]. They decrease the vitamin D levels associated with insulin resistance and diabetes mellitus.

Therefore glucose concentration, renal function and obesity are risk factors for deficiency of vitamin D. Oxidative stress and inflammation are related to diabetic nephropathy pathogenesis and regulated by VDR[6]. Plasma 25 (OH) D concentrations are used to evaluate VD status. The reason is that 25(OH) D, which has a long half-life (about two weeks) and 25-hydroxylation, has not been under efficient metabolic control[2]. Cystatin C is a protein that is liberated from nucleated cells at a constant rate and is not affected by age, muscle mass, or gender and is excreted exclusively by glomerular filtration. Cystatin C is a more accurate marker to detect mild levels of kidney dysfunction and glomerular filtration rate (GFR) than creatinine[7]. Some studies have shown that cystatin C levels are stronger predictors of acute renal injury; increased cystatin C concentrations indicate impairment in GFR, renal dysfunction, and pathophysiological processes[8,9]. The current study aimed to determine the correlation between vitamin D and cystatin C as predictors of kidney disease in males with diabetes mellitus.

Materials and methods

Sixty males were enrolled in "The National Diabetic Center (NDC) of Al-Mustansiriya University" from October to December 2018. They were divided into two groups 30 patients with type 2 diabetes with a mean age (of 47 ± 0.31) years and 30 healthy subjects as a control group with mean

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age (46± 2.99) years. Excluding patients with infection in the urinary tract, kidney failure, stage of heart failure, thyroid disease, and uncontrolled hypertension was done. A blood sample from all subjects was drawn to measure glycated hemoglobin HbA1c[10], plasma glucose level[11], creatinine[12] and urea[13]. Levels of 25(OH) D and cystatin-c were also determined by ELISA technique[14]. And body mass index BMI was calculated for each male in the patient and control group.

Statistical test

All study data were submitted as (mean ± SD). Student's T-test was applied to compare patient groups with the healthy group; also, Pearson's coefficient was used for correlation analysis.

Results

Results in table 1 summarized the anthropometric and metabolic characteristics of all participants. The average age of the volunteer group (46±2.99) was similar to the average age of the patient group (47±0.31); therefore, no significant difference was noticed when compared studied groups. As expected, patients with diabetes mellitus had significantly higher (p< 0.001) BMI, fasting blood glucose (FBG), and glycated hemoglobin (HbA1c) levels as compared to the controls group were found. Table 2 showed a highly significant increase in the average level of urea, creatinine, and cystatin-c and a highly significant decrease in vitamin D concentration compared to the patients group with the control group.

Discussion

Research on vitamin D's effect on decreasing the complication of diabetes mellitus on kidney function is limited. So the objective of the present work was to illustrate the vitamin D relationships with some parameters such as cystatin C, urea and creatinine that measured renal function efficiency in diabetic Mellitus patients to avoid their development of nephropathy diabetes. In this study, the duration of diabetes mellitus in the patient's group was (7 ± 0.62) years, so these patients were at risk of nephropathy diabetes a few years later. As expected, the patient's group showed a highly significant increase in urea, creatinine and cystatin C concentration when compared with the control group. Our results are in agreement with other studies[15-17]. Creatinine is the blood depends on creatinine production, tubular handling, and kidney elimination. Some tubular proteins and enzymes are detectable in rising serum creatinine even before microalbuminuria, so tubular involvement may outstrip glomerular involvement.

On the other hand, cystatin C is a biomarker for determining kidney functions. It could be another tubular factor that clarifies the renal state in patients with diabetes and is considered a tool for the early detection of diabetic nephropathy[18]. As for the vitamin D concentration in our study's diabetes mellitus male group, the results indicated a highly significant decrease in 25(OH) D levels compared to the healthy male group. Also, a recent study has proposed a positive correlation between VD and cystatin C, urea, and creatinine. There is no available data in previous studies to find a relationship between vitamin D and cystatin C to predict the status of renal functions in diabetes mellitus male penitents.

The mechanisms of relation vitamin D deficiency with diabetes mellitus remain incompletely understood; maybe high glucose levels increase the synthesis of the angiotensin II hormone, which affects the contractile arteriole of the glomerulus. So, hemodynamic changes distinct by high filtration, high pressure and hypertransfusion in the early stage of diabetes mellitus will induce diabetes nephropathy development[4]. Also, 1, 25(OH) D increases the transcription of insulin receptor genes and suppresses the renin gene, reducing hyperglycemic induced increases in renin levels

	Patient group	Control group	*P-value*
Age (Yrs)	47 ± 0.31	46± 2.99	>0.05
Duration of DM (Yrs)	7 ± 0.62	0 ±0.0	
BMI	30 ± 4.7	25± 3.4	<0.001
FBG (mg/dl)	167.87 ± 6.28	91.90 ± 3.82	<0.001
HbA1c %	8.94 ± 2.45	4.8 ± 0.21	<0.001

Table 1. Clinical characteristics of the patients and control groups.

	Patient group	Control group	*P-value*
Urea (mg/dl)	50.08±12.30	26.21 ±2.33	<0.001
Creatinine (mmol/L)	1.16 ±0.19	0.76 ± 0.54	<0.001
Cystatin-c (ng/ml)	19.04 ±1.03	6.48 ±0.46	<0.001
25(OH)D (ng/ml)	11.77 ±1.63	32.4 ±1.35	<0.001

Table 2. Kidney biomarkers levels and VD concentration in all studied groups.

	r	P-value
25(OH)D & HbA1c	0.82	<0.001
25(OH)D & FBG	0.23	<0.001
25(OH)D & cystatin-c	0.15	<0.001
25(OH)D & urea	-0.11	<0.001
25(OH)D & creatinine	0.55	<0.001

P-values <0.05 were considered statistically significant

Table 3. Correlate vitamin D with some variables.

in pancreatic β cells[9]. Vitamin D in the blood binds to serum proteins either to D-binding protein or albumin to carry out its function. In diabetes mellitus patients, when microalbumin is lost with urine, that will decrease the concentration of serum albumin and explain decreasing in protein synthesis besides the significant loss in urinary albumin, which affects serum 25 (OH) D concentrations[19].

Conclusions

Depending on the results of this work, vitamin D could be a valuable predictor of nephropathy in males with diabetes mellitus. Likewise, further work is required to suggest that vitamin D may be prone to nephropathy in all patients with diabetes by estimating cystatin C levels as a clinical risk for kidney function.

Author Contributions

Collecting samples; Dunya NA Ahmed, investigation; Sarmad A Hazzaa; review and editing; Abeer J Hassan.

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Institutional Review Board Statement

The study was approved by the ethics committee of the Iraqi minister of health, and the study followed the principles stated in the Declaration of Helsinki.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

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To all subject those participants in this study.

Conflicts of Interest

The authors declare no conflict of interest.

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ARTICLE / INVESTIGACIÓN

Molecular Detection of Some Vancomycin and Virulence Factor Genes in *Enterococcus faecium*

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Abstract: Multidrug-resistant (MDR) and pathogenic *E. faecium* is a crisis in healthcare settings. This survey aimed at antibiotic susceptibility profiling and virulence determinants of *E. faecium*. This study pooled 100 fecal *E. faecium* isolates identified by phenotypic and molecular tests. Antibiotic susceptibility and ampicillin MIC were determined according to clinical and laboratory standards institute (CLSI) 2017. Moreover, biofilm formation was assayed by a microtiter tissue plate assay. Virulence genes pattern was performed by polymerase chain reaction (PCR) method. Among 460 fecal samples, 100 isolates of *E. faecium* were identified, among which the highest resistance was related to penicillin (81%), cephalothin (78%), cefazolin (76%), tetracycline and cefepime (69%). In contrast, 83% of them were susceptible to vancomycin. Moreover, four vancomycin-resistant isolates had vancomycin MIC>32µg/mL, and 11 isolates had MIC>8µg/mL. Of 32 ampicillin-resistant isolates, 18 (56%) produced strong biofilm, and 14 isolates (44%) produced moderate biofilm. Among 17 vancomycin-resistant *E. faecium* (VREfa), 15 (88.23%) isolates produced strong biofilms, and two of them produced moderate-level biofilms, which was significantly higher than susceptible isolates (p=0.0144). The vanA (vancomycin MIC: 16-64µg/mL) and vanB (vancomycin MIC: 8-64µg/mL) genes were detected in twelve and five isolates, respectively. The rate of adhesin genes ace, esp and ebp included 68%, 97% and 82%, respectively. All the VREfa harbored the ace, esp and ebp genes. The antibiotic resistance rate of *E. faecium* was low. Biofilm formation was significantly different between VREfa and vancomycin-susceptible isolates but not between k9ampicillin-resistant and ampicillin-susceptible isolates. All the VREfa harbored the ace, esp and ebp genes. The virulence adhesin genes were not significantly associated with biofilm formation. Further studies are essential to appreciate better the relation between biofilm formation, drug non-susceptibility and adhesin genes.

Key words: *E. faecium*, antibiotic resistance, biofilm formation, virulence genes.

Introduction

In recent years, *Enterococcus* species have been identified as the major contributing pathogens in hospital infections and community-acquired infections[1,2], due to the acquisition of multiple antibiotic resistance genes, such as beta-lactams, cephalosporins, trimethoprim-sulfamethoxazole and glycopeptides. Some *Enterococci* are inherently resistant to beta-lactam, and aminoglycoside antibiotics are considered a medical crisis[3-6]. Glycopeptides like vancomycin are used as the last line of antibiotics in treating multidrug-resistant (MDR) Gram-positive bacteria[7]. Vancomycin-resistant *Enterococci* (VRE) carrying the resistance genes comprise the most common pathogens in hospitals worldwide[8,9].

In numerous studies, the adhesin and effective binding of *Enterococci* is reported on the levels of medical devices, such as intravenous and urinary catheters and ocular lenses, and lead to biofilm production[10,11]. The *Enterococci* pathogenic spp are less dependent on their virulence factors and the ability to adapt to environmental conditions[8,10]. Their resistance to antibiotics and ecological stresses, binding properties, and biofilm formation has resulted in crucial pathogenic features associated with implant infections[5,6,8]. In addition, *Enterococci* are significant contributors to nosocomial infections, and biofilm formation, which employ a wide variety of factors, including genetic factors, various environmental conditions, and signals mostly unknown and required for identification and characterization 11,12. Enterococci, development of resistance by multiple mechanisms and a series of virulence factors, are due to physical inhibition and biofilm conditions. A complete understanding of genetic and environmental factors involved in the development of biofilms leads to improved and accurate strategies for controlling biofilm formation. This study aimed to investigate some *E. faecium* antibiotic resistance, biofilm formation, and virulence factors.

Materials and methods

Bacterial isolates

In this study, 460 fecal samples were collected in Baghdad city during May 2017-October 2019. The *E. faecium* isolates were identified using conventional biochemical (VITEK 2 system, Biom' erieux, Marcy l'Etoile, France) and molecular tests. They were kept in brain heart infusion broth at -70 ° C. The bacterial isolates were cultured and stoked again for further studies.

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Antibiotic susceptibility test

The isolates were tested using Kirby Bauer disk diffusion and Muller Hinton agar medium by suspension equivalent to the half MacFarland for susceptibility to discs including penicillin 10U, ampicillin (10µg), cephalothin (30µg), vancomycin (10µg), tetracycline (30µg), erythromycin (30µg), amikacin (10µg), cefazolin (10µg), ceftriaxone 30µg) and cefepime (30µg) [MAST, UK]. After incubation, the diameter of the inhibition zone was measured according to CLSI 2017 standards[11].

The minimum inhibitory concentration

The minimum inhibitory concentration (MIC) for ampicillin and vancomycin was determined using the agar dilution method onto the medium of brain hearth infusion agar (BHI). In this study, the standard strain of *E. faecalis* (ATCC 292152) was used as a control, and the results were interpreted following the CLSI 2020 protocol[11].

Biofilm formation

At this stage, the phenotypic measurement of the biofilm formation was carried out using a 96-well microplate[13].

Extraction of total genomic DNA

For DNA extraction, the bacterium was cultured in a BHI medium (Merk, Germany) for 24 hours at 37° C in an incubator. Then according to the DNA purification kit purchased from Cinapure DNA, the DNA was extracted from isolates. Quantification of extracted DNA was conducted using a spectrophotometer[14].

Polymerase chain reaction

An investigation of the presence of a specific gene in *E. faecium* was conducted using specific primers in Table1. The molecular identification of isolates was performed by amplifying the *ddl* gene with a 658bp size.

Additionally, virulence and vancomycin resistance genes in *E. faecium*, including esp, ace, ebpA, were investigated using specific primers depicted in table1. Moreover, for vancomycin resistance genes, *van A* primers included F: ATCAACCATGTTGATGTAGC and R: AAGGGATACCGGACAATTCA. The van B primers included F: ACCCTGTCTTTGTGAAG and R: GAAATCGCTTGCTCAAT[8].

Statistical analysis

Comparisons of the prevalence of antibiotic resistance and virulence genes between groups were performed using the SPSS version 20 (Chicago, IL, USA). The statistical tests included Chi-Square or Fisher's exact test, and ANOVA, where a p value<0.05 was a significant result.

Results

E. faecium antibiotic resistance pattern

Among the isolates of *E. faecium*, the highest resistance was against the penicillin (81%), cefazolin (76%), tetracycline (69%), and cephalothin (78%), cefepime (69%) and ceftriaxone (67%) and erythromycin (68%). The lowest resistance rate was against ampicillin (32%), vancomycin (21%) and amikacin (21%). Therefore, 64% of isolates were simultaneously resistant to tetracycline, erythromycin and β-lactams and were considered as MDR *E. faecium*.

MIC of ampicillin and vancomycin

The MIC of ampicillin among 32% of *E. faecium* included ≥16 in the resistance range. The MIC in 20% of isolates for this antibiotic was < 8 µg /mL. Moreover, the vancomycin MIC ranged 2-64µg/mL. Indeed, four isolates had MIC>32µg/L, and 11 had MIC>8µg/mL (table2).

Biofilm formation

Phenotypic biofilm formation using a 96-well microplate assay is as followings:

Among 68 isolates of ampicillin-susceptible *E. faecium*, 30 isolates (44%) formed strong biofilms, and 38 isolates (56%) formed moderate-level biofilms. Furthermore, among 32 ampicillin-resistant isolates, 18 (56%) of them produced moderate-level biofilm and 14 isolates (44%) did not produce biofilm. Among 17 vancomycin-resistant *E. faecium* (VRE), 15 (88.23%) isolates produced strong biofilms and two of the produced moderate-level biofilms which was significantly higher than susceptible isolates (p=0.0144). There was no significant difference between MDR and non-MDR isolates regarding biofilm formation.

Gene: product size	Oligonucleotide sequence: 5' to 3'	References
ddl:658bp	GGCAGAGCATGAAGTGTCCACTT	4
	CTGGGTTTTCTGCTTTTGTA	
ebpA:517bp	AACTAACAAAAATGATTCGGCTCCAG	4,7
	CATCTCACGCATTTTATCTTCAACT	
esp: 419bp	AGATTTCATCTTTGATTCTTGG	3
	AATTGATTCTTTAGCATCTGG	
ace: 353bp	GGAATGACCGAGAACGATGGC	5,7
	GCTTGATGTTGGCCTGCTTCCG	
vanA: 136bp	ATCAACCATGTTGATGTAGC AAGGGATACCGGACAATTCA	8
vanB: 121bp	ACCCTGTCTTTGTGAAG GAAATCGCTTGCTCAAT	8

Table 1. Primers sequences used in this study.

Molecular tests

PCR reaction exhibited that *E. faecium* isolates amplified the *ddl* gene (Figure 2) and biofilm-associated genes (Figure 3). The prevalence of *ace*, *esp*, and *ebp* genes was 68%, 97% and 82%, respectively.

Vancomycin resistance genes

The *vanA* (MIC: 16-64µg/mL) and vanB (MIC: 8-64µg/mL) genes were detected in 12 and five isolates, respectively. Biofilm formation was significantly different between VREfa and vancomycin-susceptible isolates but not between ampicillin-resistant and ampicillin-susceptible isolates. All the VREfa harbored the ace, esp and ebp genes.

Table 3 exhibited that MDR and non-MDR isolates had no significant difference regarding the virulence rate and biofilm formation using the chi-square test.

Discussion

Although *E. faecalis* infections are ten times more frequent than those of *E. faecium*, *E. faecium* has increased recently, mainly following drug-resistant strains. *Enterococci* are the second most common opportunistic nosocomial pathogens which can spread antibiotic resistance genes[10,15-17]. The ability of *Enterococci* to colonize the intestinal tract of the individuals admitted for long periods is a critical factor affecting drug resistance. *Enterococci* act as a source of transmission cycles in the digestive system and release antibiotic resistance[1,2].

The data collected by the ICU surveillance system outlined that *E. faecium* was the third most common cause of bloodstream infections, the third leading cause of urinary excretion, the most commonly isolated of surgical site infections and the fourth most widely separated of all regions[12,14,18]. *E. faecium* are opportunistic pathogens, and the increasing severity of infections in hospital patients has been associated with *E. faecium*. Extensive and widespread use of broad-spectrum antibiotics in the hospital facilitates the spread of drug-resistant organisms. The predisposing factors are colonization of *Enterococci*, hospitalization in the intensive care unit, prolonged use of antibiotics and prolonged hospitalization. Although several antibiotics have been effective against VRE, mortality rates remain high in patients[19]. In most cases, due to the excessive use of antibiotics, there are many cases of drug resistance in pathogens, which leads to failure in treatment and the emergence of many complications, despite the high cost of treatment[20,21]. Drug resistance to antibiotics in different world regions is additional due to genetic changes in the strains and the difference in the amount of antibiotics[22].

Antibiotic resistance

Herein, the highest resistance rate was against penicillin (81%), cefazolin (76%), tetracycline (69%), cephalothin (78%), and cefepime (69%) and ceftriaxone (67%) and erythromycin (68%). These results indicate that antibiotic resistance to penicillin predisposes the development of resistance to carbapenems and other beta-lactams[23]. The lowest resistance rate was against amikacin (32%), vancomycin and ampicillin. Therefore, 64% of isolates were simultaneously resistant to tetracycline, erythromycin and β-lactams and considered as MDR- E. faecium. In a study, the rate of drug resistance was low at[21]. Therefore, aminoglycosides are still appropriate for the treatment of enterococcal infections. VREfa rate was 17% and the *vanA* (MIC:

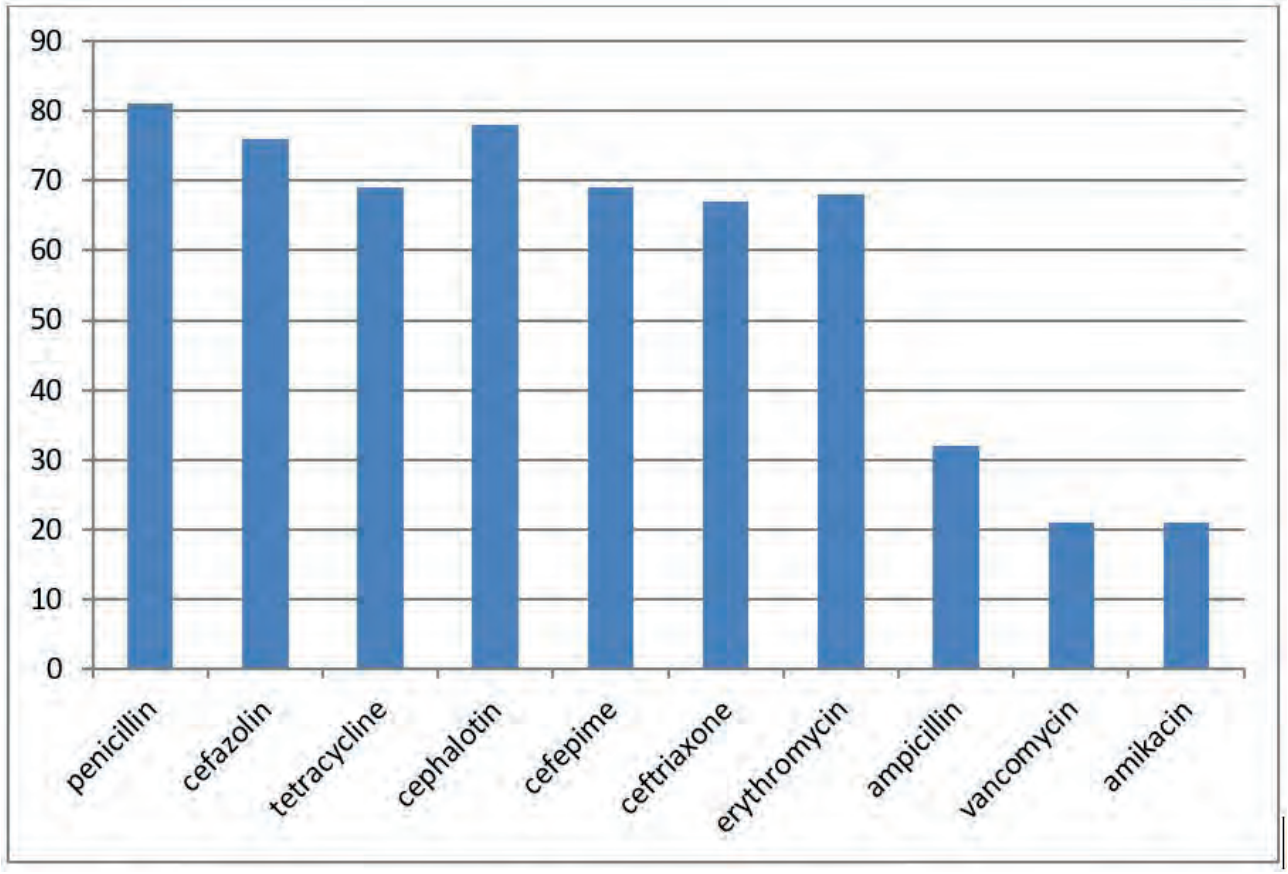


Figure 1. The antibiotic susceptibility test pattern

Antibiotic/MIC range	Susceptible	Resistant	Intermediate
Ampicillin (µg/mL)	20%	32%	48%
Vancomycin (µg/mL)	83%	15%	2%

Table 2. The MIC levels of vancomycin and ampicillin.

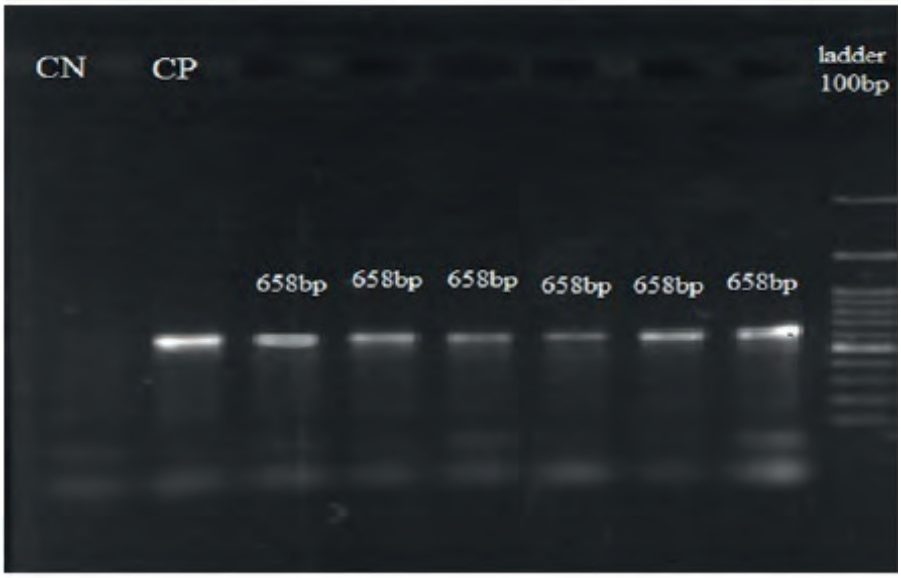


Figure 2. The electrophoresis of ddl gene (658bp), approving samples, CN: negative control, CP: positive control and 100bp DNA ladder have been exhibited.

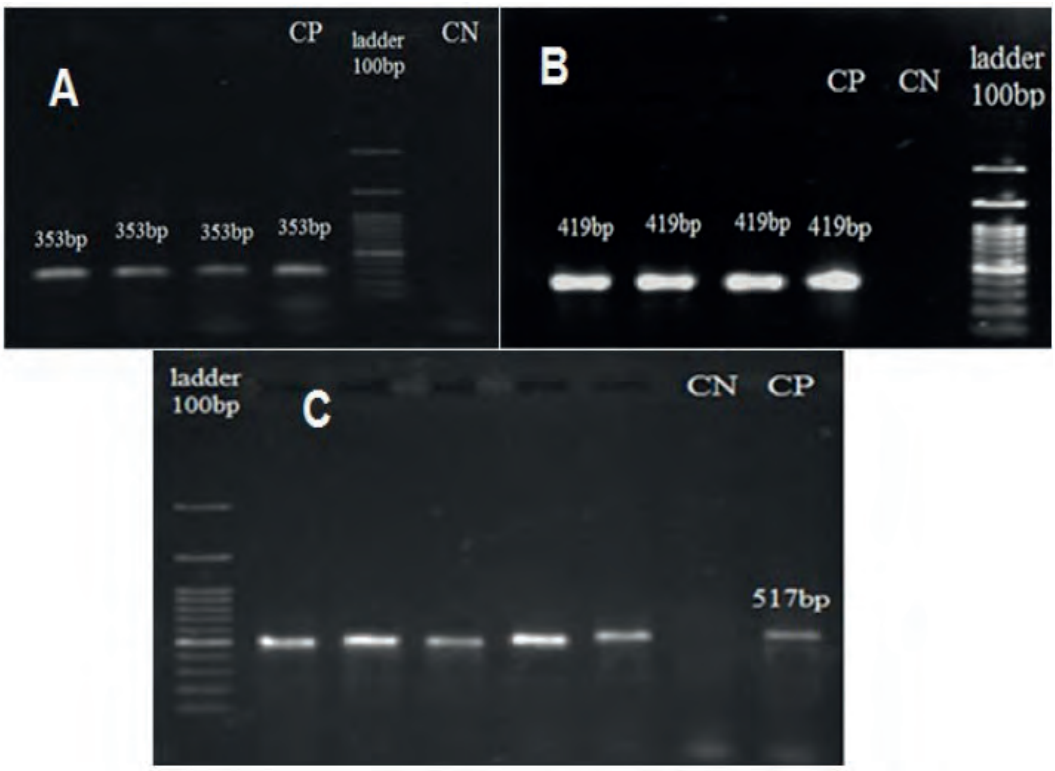


Figure 3. The electrophoresis of ace gene (353bp), in which positive samples, CN: negative control, CP: positive control and 100bp DNA ladder have been exhibited. B: The electrophoresis of esp gene (419bp), in which positive samples, CN: negative control, CP: positive control and 100bp DNA ladder have been exhibited. C: The electrophoresis of ebp gene (517bp), in which positive samples, CN: negative control, CP: positive control and 100bp DNA ladder have been exhibited.

16-64µg/mL) and *vanB* (MIC: 8-64µg/mL) genes were detected in 12 and five of them, respectively. Noticeably, the penicillin and ampicillin resistance rate of *vanA*-type VREfa included 100% for both. Likewise, 96% and 93% of *vanB*-type VREfa were resistant to penicillin and ampicillin disks, respectively, significantly higher than vancomycin-susceptible isolates.

Biofilm formation

E. faecium is a crucial agent with a vast antibiotic resistance rate and biofilm formation among *Enterococci* species. The ability to form biofilm on living surfaces is one of the key pathogenicity factors for this bacterium. *E. faecium* is associated with biofilm formation in endocarditis, urinary tract infections, and dental root and ocular infections. Various factors such as antibiotic resistance and the expression of genes involved in pathogenicity and biofilm formation contribute to the stability of this bacterium in different conditions and the spread of infection[17]. According to our study, biofilm formation was significantly different between VREfa and vancomycin-susceptible isolates but not between ampicillin-resistant and ampicillin-susceptible isolates. All the VREfa harbored the ace, esp and ebp genes. Of the studied genes, *ebp* and *esp* had the highest rate and *ace* gene was the lowest among the isolates. 79% of the isolates had the pattern *ebp* + / *esp* +. Most antibiotic-resistant isolates had virulence type *ebp* / *esp* (78.1%). Gok detected ace (83.6%), esp (66.4%), ebp (60.0%) in Turkey[24], but the rate of these genes has not been reported in this country.

Virulence factors

In numerous studies, the adhesion and effective binding of *Enterococci* have been reported on the levels of medical devices, such as intravenous and urinary catheters, ocular lenses, and ultimately biofilm production[12,16,17]. In a study by Ochoa *et al.*, the esp gene was observed in 83% of isolates, being similar to our result (97%) 12. The first beta-lactamase-producing *E. faecium* was reported in New Mexico[14]. The second report was in a study in Italy in 2011, of which eight isolates were beta-lactamase-producing *E. faecium*[18]. However, beta-lactamase-producing *E. faecium* is still in scarcity. The relationship between multidrug resistance and biofilm formation is a hypothesis that needs detailed verifications. In our study, VREfa varied adhesin genes and produced robust biofilms. In a study by Donna *et al.*, 72% of isolates carrying *esp* were in southern California, and 84% were VRE. In another study in Brazil, the esp generated was 72% which was lower than our study[19]. In a survey among VRE isolates, the *esp* gene was observed among 80% of them, nearly consistent with our study[20]. In a study, the *esp* gene was detected among *E. faecium* isolates from blood, urine, and stools with rates 46%, 38%, and 23%, respectively, significantly lower than our study[12]. In a survey by Sharifi *et al*. among VREfa in northwest Iran, the prevalence of *esp* gene was 89%, lower than our finding (97%)[21]. In Fallah *et al.*, among VREfa from Iran, 75% of them produced biofilms, being significantly higher than ours (56%), and antibiotic resistance in the biofilm formation strains was consistent with the findings of this study. Further verifications did not demonstrate any significant correlation between biofilm formation and *esp*, *asa1* and *ebpR* genes[23]. The rates of *esp*, *asa1* and *gelE* genes were also 53%, 20% and 51%, respectively, with a lower prevalence of esp and 75% biofilm production, which were more than reported in our study[25]. Selected genes involved in the formation of biofilms in other parts of the world were also present in resistant strains with higher resistant rates than biofilm non-producing strains 12,14,16,18-20. Noticeably, the relationship between biofilm formation and antibiotic resistance in *Enterococci* was confirmed in this study.

Conclusions

The antibiotic resistance rate of *E. faecium* was low. Biofilm formation was significantly different between VREfa and vancomycin-susceptible isolates but not between ampicillin-resistant and ampicillin-susceptible isolates. All the VREfa harbored the *ace*, *esp* and *ebp* genes. The virulence adhesin genes were not significantly associated with biofilm formation. However, there was a significant relation among VREfa that carried all these genes and formed robust biofilms. Further studies are essential for a better understanding of biofilm formation, drug non-susceptibility and adhesin genes.

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Informed Consent Statement

Not applicable.

Data Availability Statement

The results of this study will be found under the journal's rules and related links.

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Conflicts of Interest

None to declare.

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ARTICLE / INVESTIGACIÓN

Comparison between the presence and absence of mixing in the anaerobic biological treatment of liquid waste for the cheese industry

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Abstract: Many nations are seeing an increase in water pollution from dairy and cheese production due to the high organic and fat content in their waste products and the high temperature of their waste products, which elevates the water temperature and causes loss to ecosystem components. Reusing industrial wastewater that has been treated to guarantee no harm has been done to the environment is being hampered by a lack of water. This study compares the presence and absence of mixing in the anaerobic biological treatment of liquid waste for the cheese industry. To decrease heat exchange with the external environment, cube-shaped anaerobic reactors with dimensions of (30 x 30 x 30) cm and thick glass (10 mm) were utilized in this investigation. The temperature of the two reactors was 35°C, with the first reactor containing a mixture and the second reactor containing no mixture. Samples were obtained every five days and evaluated in the laboratory to monitor changes in the characteristics of liquid cheese waste during the treatment period, which was 30 days. The indicators (pH, EC, TDS, TSS, COD, and Alkalinity) were used, and a total of 72 tests were performed throughout the research period for each indicator. The results appeared that the first anaerobic reactor with the combination removed 95.1 % COD, whereas the second reactor that did not contain the mixture removed 94.1 % COD. The anaerobic reactors are very efficient in treating COD; the COD removal rate in the first reactor with the combination was 1.9 %, while the COD removal rate in the second reactor without mixing was 94.1 %. The increase in pH in the first and second reactors, followed by achieving the neutralization value, is proof of anaerobic reactor success. Total dissolved solids removal was achieved in anaerobic reactors in the first reactor (7.5 %) and peaked in the second reactor (17.83). The proportion of TSS removed in the first anaerobic reactor was 57.9%, and in the second reactor, it was 60.3 %. From all above can be concluded that the presence of mixing had a slight and negligible positive effect on the anaerobic reactors.

Key words: Mixture, Liquid Waste, Cheese Industry, Anaerobic reactors.

Introduction

During the previous century, pollutants from human activities have contaminated the waters of rivers and lakes, causing negative consequences and major difficulties in the aquatic environment and the living animals that live there[1]. Human-caused pollution in the aquatic environment occurs via industrial wastewater or residential wastewater, which is disposed of without treatment, causing contamination in rivers, lakes, and natural streams[2]. The dairy and cheese industry is one of the food industries whose liquid wastes contribute to water pollution because of the high levels of organic matter and fats in its waste products, as well as the high temperature of its waste products, which raises the water temperature, causing losses to ecosystem components, and this is the current trend in many countries. The reuse of industrial wastewater in general, once it has been treated in a manner that does not harm the ecology, is hampered by water constraints[3]. Temperature, pH, alkalinity, presence of toxic substances, amount of discharges entering the reactor, quality of the wastes being treated, availability of nutrients used by decomposing anaerobic organisms, rate of the final removal of toxic substances by anaerobic fermentation, the concentration of pollution inside excretions, sludge residence time (SRT), hydraulic retention time are all factors that influence the rate of anaerobic biological treatment efficiency (HRT)[4]. The anaerobic digestion process happens in three stages. First, bacteria transform organic food molecules (carbohydrates, proteins, and lipids) into (liquid polymers of fatty acids and amino acids) and monosaccharides, as shown in this equation, which illustrates the hydrolysis of complex sugars into simple sugars[5]. The second stage is (Acidogensis) The acid-producing bacteria transform the results of the first-stage reaction into a short chain of volatile acids (ketones alcohol, hydrogen, and carbon dioxide), with ethanol (C_2H_5OH), methanol (CH_3OH), and propanoic acid as the primary products (CH_3CH_2COOH). Acetic acid (CH_3COOH), butanoic acid ($CH_3CH_2CH_2COOH$), lactic acid ($C_3H_6O_3$), and formic acid ($C_3H_6O_3$) are some of the most common acids (HCOOH). The equations below show the second-stage processes in which glucose is transformed into ethanol, propanoate, and acetic acid are shown in the equations below[6]. The third stage is (Methanogenesis), the last stage during which microorganisms convert hydrogen

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and acetic acid generated from acid generators into methane and carbon dioxide[7]. The stabilization of waste is completed when methane and carbon dioxide are produced. The following equations show the reactions taking place in the last stage.

Materials and methods

Anaerobic Reactor Design

Two anaerobic treatment reactors were manufactured of thick (10) mm glass, identical in form and size, with dimensions of (30 x 30 x 30) cm and 27 liters each. Each anaerobic reactor has a heater set at (35) °C, a sampling hole, a sample entrance hole, and a cleaning aperture at the bottom of the reactor. The first reactor contained a mixer with a speed of (70) Circular/ minute, and it was connected to the (Arduino) device, which was programmed to run the mixer (10) minutes/hour, as in Figure (1). The second anaerobic reactor was without a mixer shown in Figure 2.

Anaerobic Reactor Design

The work of the anaerobic reactors that were made in this study was limited to the treatment of liquid wastes for the manufacture of cheese. The samples of liquid wastes for the dairy factory were kept in sealed plastic containers and kept away from sunlight until they were used for anaerobic treatment. These anaerobic reactors were connected to the electric current and constantly monitored to ensure treatment flow. At the end of the third week of treatment, the anaerobic fermentation process took place in the first and second reactors, and we noticed the color change from white to dark brown, as in figure 3.

Results

The qualitative characteristics of liquid waste for dairy factories

The chemical and physical tests of the raw liquid waste for the dairy industry, specifically the liquid waste for the

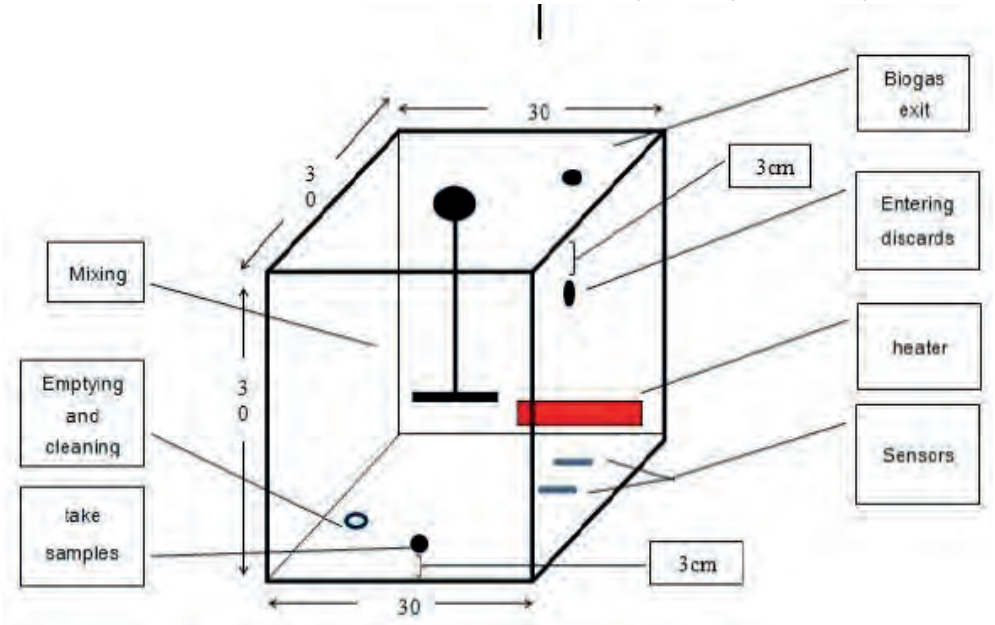


Figure 1. First Anaerobic Reactor.



Figure 2. Anaerobic reactors and the fermentation process.

cheese industry, before starting the anaerobic and aerobic treatment processes are shown in Table (1). appeared the first anaerobic reactor with the combination removed 95.1 % COD, whereas the second reactor that did not contain the mixture removed 94.1 % COD. The anaerobic reactors are very efficient in treating COD; the COD removal rate in the first reactor with the combination was 1.9 %, while the COD removal rate in the second reactor without mixing was 94.1 %. The increase in pH in the first and second reactors, followed by achieving the neutralization value, is proof of anaerobic reactor success. Total dissolved solids removal was achieved in anaerobic reactors in the first reactor (7.5 %) and peaked in the second reactor (17.83). The proportion of TSS removed in the first anaerobic reactor was 57.9%, and in the second reactor, it was 60.3 %.

Value	Unit	Indicator
00688	mg/l	COD
08668	mg/l	Alkalinity
09.8	-	pH
0.98	µS/cm	EC
0069	mg/l	TDS
0093	mg/l	TSS

Table 1. Characteristics of Raw Liquid waste for cheese.

Discussion

Chemical Oxygen Demand (COD)

The amount of (COD) was very high in this waste, and this is due to the nature of the cheese-making subtractions, which are characterized by an increase in their organic load and an increase in temperature as well, and thus will consume oxygen[8]. Anaerobic reactors achieved excellent removal of (COD) and this is due to the sufficient time for anaerobic microorganisms and their consumption of good amounts of organic matter[9] and the value of COD after the end of the treatment period in the first reactor was (3980) mg/liter and was In the second (4730) mg/liter.

Alkalinity

The tests for liquid excretions for the cheese industry before starting treatment indicated that the basal concentration was high. Its concentration reached (10660 mg/L) and the basal concentration increased with a decrease in the pH value. This increase is due to reducing the concentration of volatile fatty acids (VFA) and the collection of acidophilic bacterial masses[10]. All anaerobic processes occur together and in succession, such as hydration, decomposition, formation of acids and production of methane, and under appropriate environmental conditions for each stage within anaerobic reactors, which leads to fluctuations in the basal values. The slight increase in the basal concentration during anaerobic processes may be Because methane bacteria quickly withdraw hydrogen from the solution during the production of methane gas, which is according to what was shown. After the treatment in the first reactor, the basal values reached (5400) mg/L and arrived in the second reactor (at 6100) mg/L.

Acidity (pH)

Its value was low (3.9), and this is due to the conversion of milk lactose to lactic acid and the use of detergents that contain caustic soda and nitric acid[11]. The values of the acidity after the completion of the treatment in the first reactor reached (7.95) and reached (7.99) in the second reactor.

Electrical conductivity (Ec)

The liquid wastes have high electrical conductivity, and their value before treatment (4950 micro Siemens/cm), and this rise is due to the presence of inorganic salts. After treat-

TSS (mg/l)	TDS(mg/l)	EC (µS/cm)	pH	Alkalinity mg/l	COD(mg/l)
9093	9069	0398	9.3	08668	00688
9800	9038	0003	0.30	3598	63908
3000	9938	0009	0.03	5608	96908
3939	9999	0593	9.95	0358	38958
3950	9989	0650	6.00	0868	00068
0356	9308	0936	5.99	6308	0008
0090	9389	0999	5.39	9088	9308

Table 2. Results of the first anaerobic reactor (with mixer).

TSS mg/l	TDS mg/l	EC (µS/cm)	PH	Alkalinity mg/l	COD mg/l
9093	9069	0398	9.3	08668	00688
9095	9399	0033	0.80	3558	60008
3589	9886	0530	0.03	5538	95698
3005	3353	0533	9.06	0538	33908
3809	3909	0600	6.00	0968	03908
0093	3308	0686	5.99	5808	5008
0963	3005	0908	5.33	6088	0598

Table 3. Results of the second anaerobic reactor (without mixing).

ment, the electrical conductivity value in the first reactor reached (4533) mg/liter and in the second reactor (4580) mg/liter.

Total Dissolved Solids (TDS)

In most cases, the concentrations of total dissolved solids (TDS) of liquid wastes for cheese making are relatively high due to the liquids containing dissolved salts and organic materials[12]. The total dissolved solids value after treatment in the first reactor was (3205) mg/L, and in the second, it amounted to (2847) mg/L; this decrease is due to the anaerobic decomposition process[13].

Total Suspended Solids (TSS)

The presence of high concentrations of degradable organic matter in the liquid wastes for the manufacture of cheese will lead to an increase in the concentration of total suspended solids, and the TSS value before treatment was (3452) mg/liter. The value of the total suspended solids after treatment in the first reactor was (1451) mg/liter, and in the second, it amounted to (1369) mg/liter. The following tables show the results of tests during the research period for the first and second anaerobic reactors.

Conclusions

We conclude that processing milk wastes is booming and requires only an expansion in the size of the treatment plant, according to what we have found. In addition, the second reactor without the mixture is the most efficient where they worked; the COD removal and the rise in the pH value reaching the neutralization value is evidence of the success of the second reactor. And also TSS removal by the second reactor.

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Author conflict

No conflict.

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ARTICLE / INVESTIGACIÓN

Nonalcoholic Fatty Liver Disease Among Obese Patients in Oman

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Abstract: Studies characterizing Nonalcoholic Fatty Liver Disease (NAFLD) are limited in Oman. This study aims to assess the prevalence of NAFLD in obese Omani patients and find its relationship with other obesity-related medical conditions. Fifty-five adult obese patients were evaluated via medical history, clinical examination, and laboratory tests. Patients with any possible risk of liver injury were excluded. Diagnosis of NAFLD relied on ultrasonography criteria. Data were entered and analyzed in SPSS (version 22). The prevalence of NAFLD was calculated using frequency and percentages. Out of the total patient population, 37 (67.3%) have had NAFLD. Most of the patients (81%) were below 50 years of age. Systolic hypertension was present in 45.9%, while diastolic hypertension was present in 43.2%. AST and ALT levels were significantly increased ($p<0.001$) in most patients; 81.1% and 73%, respectively. Fasting blood sugar (FBS) and glycosylated hemoglobin (HbA1c) levels were higher in NAFLD patients ($p<0.01$). Mean levels of LDL, uric acid, and total cholesterol were significantly higher in the NAFLD patients ($p<0.01$). In conclusion, NAFLD and related metabolic complications are prevalent in obese Omani individuals.

Key words: NAFLD, Obesity, Oman.

Introduction

Obesity has become an increasing medical concern worldwide since its rate is steadily rising. The WHO reported that the obese population (defined as body mass index BMI equal to or more than 30 kg/m^2) exceeds 500 million globally[1]. Among all Arab Gulf countries, the prevalence of obesity and overweight is increasing, where more than 30% of the population is obese[2]. Such higher percentages of obesity and overweight are also seen in Middle Eastern, United States, New Zealand, Canada, Australia[3].

Obesity is a well-known risk factor for many medical conditions like type 2 diabetes, hypertension, dyslipidemia, and Nonalcoholic Fatty Liver Disease (NAFLD) among adults[4].

It has been reported that all ranges of obesity, from overweight to obese and severely obese, are associated with NAFLD[5].

The definition and diagnostic modalities for NAFLD depend on imaging or histopathological evidence of hepatic steatosis after excluding all possible secondary causes like alcohol consumption, steatogenic drugs, or genetic disorders[6].

The overall prevalence of NAFLD diagnosed by imaging is between 14-31%[7]. The highest prevalence is reported in the Middle East and the United States, whereas the lowest prevalence is reported in Africa[8]. Increasing incidence of obesity, insulin resistance and Type 2 diabetes in Oman sensibly predict an associated increase in NAFLD incidence. Despite this, the exact figure of NAFLD prevalence is still obscured due to a lack of data.

NAFLD can be classified histologically into the nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH); such classification depends on the percentage of hepatic steatosis and the presence or absence of hepatic injuries with or without the element of fibrosis[6].

Clinically, NAFLD presentation varies according to its histological changes. Patients with only hepatic steatosis are commonly asymptomatic; however, others might present with features related to advanced cirrhosis or hepatocellular carcinoma in its most severe form. After a speedily growing number of obese individuals in society, NAFLD has become one of the most common causes of chronic liver disease[9]. NAFLD with associated cirrhosis of the liver curries considerable patient morbidity and mortality, representing a real encumbrance on health care providers[10,11]. It has been found that hepatic mortality encounters the second or third cause of death among NAFLD[12].

This study aims to find the prevalence of NAFLD amongst obese Omani patients and show its relationship with other components of metabolic syndrome. This will help increase healthcare providers' orientation toward NAFLD with possible early intervention and prevention of subsequent serious complications.

Materials and methods

Obese patients who attended Sohar polyclinic for regular check-ups or mild acute illness were offered to be enro-

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lled in this study between August 2018 and December 2019. All participants have explained the nature and the objectives of the study comprehensively and have given their consent to be part of it. Individuals who initially indicated known liver disease, alcohol consumption, usage of medications with possible liver damage as a side effect (Chronic use of NSAID/Acetaminophen, Methotrexate, Corticosteroid, Amiodarone, Antidepressant, Antipsychotic and Tamoxifen), those with known history of diabetes, hypertension or dyslipidemia, and those who later showed positive markers for hepatitis B or C were excluded from the study.

Obesity was diagnosed according to the WHO definition. BMI ranging between 30 and 39.9 kg/m^2 was defined as Grade 2 obesity, whereas patients with a BMI of 40 kg/m^2 or more were defined as having Grade 3 obesity. To obtain BMI, height and weight were measured in the OPD using the same scaling device. We followed The Eighth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC VIII) recommendations in assessing blood pressure and diagnosing hypertension. Hypertension is diagnosed if the systolic blood pressure is more than 140 mmHg and the diastolic one of more than 90 mmHg.

While fasting for 12 hours, a blood sample was collected from each participant and sent for the following tests: lipid profile (total cholesterol, HDL, LDL, TG and VLDL), liver function (total bilirubin, AST, ALT, alkaline phosphatase and albumin), fasting glucose, glycosylated Hb, uric acid and hepatitis B and C markers.

To diagnose fatty liver changes, candidates who achieved inclusion criteria were examined ultrasonographically by a single qualified radiologist using the same device (multifrequency 2-5 MHz convex transducer of Toshiba Aplio 500 ultrasound machine) each time. Normal liver parenchyma displays homogeneous echogenicity equal to that of the renal cortex and spleen. In patients with fatty infiltration, the liver appears hyperechoic as the echogenicity is higher than the renal cortex and spleen with increased brightness.

Data was entered and analyzed in SPSS (version 22). The prevalence of NAFLD was calculated using frequency and percentages. Two-by-two table comparisons were made to study the covariates of NAFLD, and results were presented using descriptive statistics. Mean and standard deviations were calculated to compare continuous variables. A test of significance in a t-test was used to compare the means. A p-value of less than 0.05 was considered for statistical significance.

Results

There were 55 study participants, out of which there were 28 (50.9%) males and 27 (49.1%) females. The mean age of the participants was 38.22 ± 12.62years. Out of the total study population of 55 patients, Nonalcoholic Fatty Liver Disease (NAFLD) was found in 37 patients, thus making the prevalence of NAFLD in this study 67.3%. [Table 1].

NAFLD grading	Frequency	Percent
No NAFLD	18	32.7
NAFLD	37	67.3
Total	55	100.0

Table 1. Prevalence of NAFLD.

The mean age of the NAFLD patients was 39.43 ± 2.01 years. The age distribution showed that most of them were less than 50 years of age (81.1%), and only 18.9% were more than 50 years of age. Most of the NAFLD patients were females. However, age and gender differences were not found to be statistically significant (p>0.05). Grade 2 obesity with BMI classification was found in most patients (78.3%). Mean BMI was higher in NAFLD patients (37 ± 0.34) compared to the control group, and this difference was statistically significant with a p-value <0.01.

Systolic hypertension was present only in 17 patients (45.9%), while diastolic hypertension was present in 16 patients (43.2%). Total serum bilirubin, alkaline phosphatase and albumin levels were normal in all the patients. However, AST and ALT levels were increased in most patients; 81.1% and 73%, respectively. [Table 2]

The mean values of AST and ALT levels differed significantly from the patients without NAFLD (p<0.001). According to fasting blood sugar (FBS) levels, most of the patients had diabetes (59.5%), and a few (18.9%) had prediabetes. The HbA1c levels correlated with the FBS levels, and thus in 81.1% of patients; it was higher than the normal range. Both FBS and HbA1c mean levels were higher in the NAFLD group compared to a non-NAFLD group of patients, and this difference was statistically significant (p<0.01) [Table 3]. Serum uric acid levels were high in 64.9% of patients, while total cholesterol was high in most patients (89.2%). Similarly, LDL was increased in 81.1% of patients while VLDL levels were normal. Significantly low HDL levels were found in 67.6% of patients compared to the non-NAFLD group. Mean levels of LDL, uric acid, and total cholesterol were significantly higher in the NAFLD patients (p<0.01).

Discussion

Among the six Arab Gulf countries, obesity and overweight are very high. Assessing obesity by measuring the BMI, which is linked to increased fat in the body and subsequently insulin resistance, is considered a good tool to predict possible complications. Thirty percent or more of the individuals in these countries are obese (BMI equal to or more than 30 kg/m^2), and more than 60% have a weight range higher than usual (BMI equal to or more than 25 kg/m^2). Similar percentages of obesity and overweight are found in other Middle Eastern countries, Maghreb countries, the United States, New Zealand, Canada, Australia, and some European countries[3]. In Oman, the prevalence of obesity and overweight for males and females is 61% and 66%, respectively[3]. Liver diseases in obese individuals have become a recognized medical problem worldwide. Among them, NAFLD represents the primary etiology.

In this study, we found that the overall prevalence of NAFLD is 67.3% (37 patients). The global prevalence of NAFLD diagnosed by imaging is around 25%, where the highest majority is found in the Middle East (31%), and South America (30%) and the lowest is found in Africa (13%)[8]. The increased prevalence of NAFLD among diabetes mellitus is well documented in many studies[13,14]. Given that the prevalence of diabetes mellitus in Oman is on the rise (15.7%)[15], is anticipated to find a high rate of NAFLD in Omanis, as shown in this study. Despite this, we could not find adequate data on the prevalence of NAFLD in the general population (obese or non-obese) in this country.

This study shows that NAFLD patients are slightly

Variable		Frequency (n=37)	Percent
Age	Less than 30	9	24.3
	31 to 40	11	29.7
	41 to 50	10	27.0
	More than 50	7	18.9
Gender	Male	17	45.9
	Female	20	54.1
Obesity	Grade 2 Obesity	29	78.3
	Grade 3 obesity	8	21.7
SBP hypertension	Yes	17	45.9
	No	20	54.1
DBP hypertension	Yes	16	43.2
	No	21	56.8
Total serum bilirubin	Increased	0	0.0
	Normal	37	100.0
AST	Increased	30	81.1
	Normal	7	18.9
ALT	Increased	27	73.0
	Normal	10	27.0
Alkaline phosphatase	Yes	0	0.0
	No	37	100.0
Albumin	Low	2	5.4
	Normal	35	94.6
FBS	Increased	29	78.4
	Normal	8	21.6
FBS grading	Normal	8	21.6
	Pre diabetes	7	18.9
	Diabetes	22	59.5
HbA1c	Increased	30	81.1
	Normal	7	18.9
Uric acid	Increased	24	64.9
	Normal	13	35.1
Total Cholesterol	Increased	33	89.2
	Normal	4	10.8
HDL	Low	25	67.6
	Normal	12	32.4
Triglycerides	Increased	18	48.6
	Normal	19	51.4
LDL	Increased	30	81.1
	Normal	7	18.9
VLDL	Yes	0	0.0
	No	37	100.0

Table 2. Covariates of NAFLD in the study population.

younger than similar studies, with a mean age of 39.43 ± 2.01 years and the majority of the patients' age was less than 50 years. Most western studies have reported the mean age of NAFLD to be between 41-45 years. In Indian studies, the mean age was said to be (49.14±9.65) years by Mahaling *et al.*[16] and (42.90±10.54) years by Agarwal *et al.*[17] This earlier development of NAFLD in the examined sample may be attributed to the early onset of obesity among young adults in the Gulf countries[18].

We found that most NAFLD patients were female (81.1%). Most studies in a meta-analysis also showed a female predominance, with the mean proportion of females at 73%[19]. Such females' predominance may be due to the high prevalence of obesity. Additionally, men were less included in the study due to a history of significant alcohol intake among them compared to women in this part of the world. In controversy to other studies, male sex has been considered a risk factor for NAFLD with two times higher

Variable	NAFLD present (n=37)	NAFLD absent (n=18)	p value
	Mean ± S.E	*Mean ± S.E*	
Age (years)	39.43 ± 2.01	35.72 ± 3.17	0.330
Body mass index (kg/m^2)	37.00 ± 0.34	33.55 ± 0.25	0.000
SBP hypertension (mmHg)	142.27±3.49	142.5 ± 3.43	0.967
DBP hypertension (mmHg)	88.7 ± 2.14	90.56 ± 2.64	0.607
Total serum bilirubin (µmol/L)	10.95 ± 0.66	10.28 ± 0.75	0.542
AST (U/L)	70.95 ± 4.20	29.44 ± 1.59	0.000
ALT (U/L)	62.92 ± 3.63	27.94 ± 1.35	0.000
Alkaline phosphatase (U/L)	91.73 ± 4.72	79.00 ± 5.28	0.105
Albumin (g/L)	38.51 ± 0.90	40.89 ± 1.19	0.478
FBS (mmol/L)	7.61 ± 0.35	4.82 ± 0.20	0.000
HbA1c (%)	7.77 ± 0.27	5.97 ± 0.21	0.000
Uric acid (mg/dl)	7.91 ± 0.33	6.23 ± 0.31	0.002
Total Cholesterol (mmol/L)	5.97 ± 0.15	4.21 ± 0.15	0.000
HDL (mmol/L)	0.90 ± 0.04	1.30 ± 0.03	0.000
Triglycerides (mmol/L)	1.92 ± 0.16	0.86 ± 0.10	0.000
LDL (mmol/L)	4.20 ± 0.16	2.52 ± 0.15	0.000
VLDL (mmol/L)	0.87 ± 0.07	0.39 ± 0.04	0.000
Gender Male (no. & %)	17 (60.7%)	11 (39.3%)	.291
Female (no. & %)	20 (74.1%)	7 (25.9%)	

Table 3. Comparison of covariates of NAFLD in the study population.

than females[20-22]. Exploring the possible etiology behind this gender-wise discrepancy in the prevalence of NAFLD necessitates further dedicated studies.

The prevalence of hypertension, diabetes mellitus, hyperuricemia and dyslipidemia was significantly higher among obese patients (P<0.000). This obviously, could be attributed to the associated metabolic syndrome. This finding is consistent with the majority of comparable studies[16,17,23-27].

NAFLD is usually symptomless in most patients due to its slowly progressing course despite possible alterations of liver functions. All patients included in our study were asymptomatic concerning the liver disease. However, deranged liver function was seen as significantly raised AST and ALT among the majority of NAFLD patients, and such finding is consistent with Marchesini et al.[28], Angulo *et al.*[29], Rushad Patell *et al.*[9], were ALT and or AST often the only laboratory abnormality found in NAFLD patients.

Conclusions

The increasing number of obese individuals in developing countries, including Oman, heralds the rising associated metabolic problems, including NAFLD. Our study documented this, along with the increasing prevalence of other related medical complications like hypertension, diabetes, and dyslipidemia.

Limitations

Although it reflects a significant prevalence, the number of patients included in the study is relatively small, and further, more exhaustive studies are recommended in the future.

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Conflicts of Interest

The author(s) declares no conflict of interest.

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ARTICLE / INVESTIGACIÓN

Statistical Analysis of Mortality by Non-Communicable Diseases (NCDs) and food supply in Ecuador, 1990-2017

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Abstract: An unhealthy diet is a risk factor for non-communicable diseases (NCDs). Data on the average protein supply and meat products between 1990-2017 consumed by the Ecuadorian population are collected. The HJ-Biplot method is used to identify clusters with MultBiplot software to obtain a two-dimensional representation of the data in the plane along with the quality of representation of the variables and the total variance explained. This work aims to determine how much the source of protein, animal or vegetal, influences the amount of death caused by non-communicable diseases such as Cardiovascular diseases and Diabetes Mellitus. Finally, a Hierarchical Cluster Analysis is carried out with the food supply related to those diseases. It can be concluded that NCD mortality is primarily explained by the average protein supply of animal and meat products in Ecuador.

Key words: Ecuador, Non-Communicable Diseases, Cardiovascular disease, Diabetes, HJ-Biplot, food, proteins

Introduction

By 2030, the UN Sustainable Development Goals aim to reduce premature mortality from non-communicable diseases (NCDs) by a third[1]. The most common is cardiovascular disease (CVD) which is responsible for an estimated 17.8 million deaths in 2017, and more than three quarters were in low-income and middle-income countries[2,3]. While, Diabetes mellitus (DM) has become one of the most pressing and prevalent diseases in the last few decades and now is the seventh leading cause of death worldwide, with 5.2 million deaths globally and a mortality rate of 82.4 per 100,000[4].

Furthermore, tobacco, harmful alcohol use, unhealthy diet, and physical inactivity are the four behavioral risk or lifestyle factors that drive CVD epidemic[3,5,6]. An unhealthy diet is a modifiable risk factor for CVD, as well as DM, overweight, and obesity, are essential risk factors[7].

There are strategies to reduce the high mortality rate, including accelerated tobacco control, salt reduction, healthy diets and physical activity, reduction of harmful alcohol consumption, and access to essential drugs and technologies[8,9]. These strategies aim to reduce the number of premature deaths associated with chronic non-communicable diseases by 25%[2,10]. Consequently, CVD death rates have decreased over the past few decades due to the improved prevention and treatment in high-income countries, but in low- and middle-income countries, it has increased between 2007 and 2017[11].

In Ecuador, for instance, Ischemic Heart Disease (IHD) occupied first place among the principal causes of death for several years. So by 2016, IHDs were the first cause of mortality in men and women[12,13]. Similarly, DM caused 5064 deaths in 2015, ranking as the second cause of general mortality, the first cause of death in women, and the third in men[13]. Besides, cardiovascular deaths account for 44% of death in those with type 1 DM and 52% of deaths in type 2 DM[4].

Moreover, related to diets, dietary protein is integral to human health. However, there are controversies around the cardiovascular health effects of consuming animal-based and plant-based protein-rich foods[14]. Besides, increasing dietary protein may benefit cardiovascular health by aiding in weight loss/maintenance, improving the lipid/lipoprotein profile, and reducing blood pressure. In addition, the health benefits of dietary protein should interpret with caution since increasing the consumption of protein/protein-rich food typically results in other changes in diet, such as energy, nutrients, or food intake, depending on the source of the protein. Therefore, the specific protein food source determines the effect of increased protein consumption[14,15].

Plant-based intake pattern does not exclude all animal products; instead, the primary source of protein relies on plant foods like fruit and vegetables, plant proteins, oils, nuts and seeds, and wholegrain carbohydrates[16,17]. Plant-based diets can improve blood lipid profiles, blood pressure, and weight management[16,18], which reduces the risk of developing CVD and diabetes[7,11]. However, on the intake outcomes comparison, studies do not specify the type of diet or food given to subjects, whether animal-based or plant-based protein. And the plant protein sources (grains, legumes, soy, nuts, or whether they are low or high quality) or meats (red or white meat) are not specified.

This study analyses different food supplies across the population from 1990 to 2017 with data published by United Nations Food and Agricultural Organization (FAO)[19]. In addition, provided by Amnesty International, the annual number of death by cause in Ecuador during that period is also

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analyzed as a variable through the years. To determine a relationship between the food supply of Ecuadorians and the mortality rate of NCDs such as DM and CVD, HJ-Biplot and hierarchical Cluster Analysis is used to analyze the years when the animal-protein or meat supply plant-based proteins affected the number of death by NCDs.

Materials and methods

Data Collection

For this study, the variables are described in Table 1. The average supply of protein from animal products (g/ capita/ day), the average supply of plant-based protein products (g/ capita/ day), and the average supply of meat (kg/ capita/ year) are from data published by the United Nations Food and Agricultural Organization (FAO)[19] where food supply defines as food available for human consumption. In addition, at the country level, in this case, Ecuador, it is calculated as the food remaining for human use after deduction of all non-food utilization. However, such values do not include consumption-level waste and therefore overestimate the average amount of food consumed[19].

The annual number of deaths by cause is from data published by Amnesty International[20] that consists of executions, death sentences, and commutations and exonerations, where there is reasonable confirmation[20].

RStudio Software

The multivariate statistical analysis used RStudio, an Integrated Development Environment (IDE) for the R programming language. RStudio combines various components of R into one seamless and productive workbench[21]. In addition, the MultBiplot[22] and the SparseBiplot R-packages[23] performed the HJ-Biplot and Hierarchical Cluster analysis.

The 'MultBiplot' package offers Several Multivariate Techniques from a biplot perspective[22]. While the 'SparseBiplot' Package implements three new techniques and constructs in each case an 'HJ-Biplot'. Based on the regularization theories, it adapts restrictions to reduce weights and/or produce zero weight in the dimensions. In addition, it implements three methods of regularization: Ridge, LASSO, and Elastic Net[23].

HJ-Biplot analysis

The HJ-Biplot originally proposed by Galindo-Villardon[24] presents the

advantages of Correspondence Analysis and applies to any data matrix, not just frequencies. It is a technique for inspecting multivariate data matrices with fewer restrictions than Correspondence or Factor Analysis. It has been applied to many fields of science. For instance, Cárdenas & Galindo[25] cite applications in medicine, economics, biology, or environmental technology.

In this study, HJ-Biplot analysis was done using the 'SparseBiplot' Package, with the years as labels for the rows shown in Figure 1. The HJ-Biplot command returns values such as an eigenvalues vector, a vector containing the proportion of variance explained by the first principal components obtained, a matrix with the coordinates of individuals, matrix with the coordinates of variables. While the HJ-Biplot command of MultBiplot returns more values such as inertia and column contributions.

Furthermore, the Biplot method, developed by Gabriel[26], allows the representation of the data matrix (X(I*J)) in low dimensional space, facilitating interpretation. It uses vectors as markers i_1, i_2, …, ii for each row, and j_1, j_2, …, jj for each column, which are obtained from the usual singular value decomposition (SVD) of the data matrix[27].

For this paper, the goal of the HJ-Biplot is to describe the relations between the row markers (years 1990-2017)

Variable	Description
AnimalPS	Average supply of protein from animal products (grams per person per day)
VegetalPS	Average supply of plant-based protein products (grams per person per day)
MeatFS	Average supply of meat across the population (kilograms per person per year)
DeathsDM	Annual number of death by Diabetes Mellitus
DeathsCD	Annual number of death by Cardiovascular Disease

Table 1. Variable Description.

and column markers (NCDs and food supplies, variables). The analysis of the HJ-Biplot is explained as follows: two variables separated by a minimal angle are strongly correlated; in addition, a group of years next to a variable indicates that the years have taken preponderant values for this variable[27,28].

Cluster Analysis

The Hierarchical cluster analysis of n objects is a stepwise algorithm that merges two things with a high similarity at each step[29,30]. In this study, these differences between clusters of objects are defined by average linkage. The cluster analysis results are a binary tree, or dendrogram, with n–1 nodes[31].

In this study, cluster analysis was performed using a hierarchical cluster using the HJ-Biplot (principal components) scores, Euclidean distances, and Ward's method. The linkage process used the MultBiplot Package as shown, for instance, in Figure 2.

Results and discussion

Figure 1, the total absorption of information by the axes is 96.82%, and the vectors have similar vector lengths showing the same variability. Besides, the columns that contribute the most to dimension 1 (73.18%) are the average supply of protein from animal products, meat supply, and DM's deaths (see Table 2). Similarly, the years when the animal products have higher contributions to dimension 1 than the plant-based products. While the years (2003-2015,2017) dominated by plant-based products, contribute the most to dimension 2 (23.6%).

In figure 2, there are 3 groups, where the labels are from I1 to I28, which correspond to 1990 to 2017, respectively. In blue, the years 2014-2017 have similarities in the number of plant-based and animal protein products but higher deaths by DM. During these years, there were patterns of obesity and overweight because of the rapid economic and demographic changes that Ecuador, as a developing country, was facing[13]. These years are marked by an easy replacement of predominantly plant-based low-calorie diets with predominantly animal-based high-calorie diets high in fat, sugar, and refined grains[32].

While in red (1900 – 2005), the supply of plant-based protein started to decrease as animal products and meat started to increase, and it was reported that between 1990 and 2000, DM's deaths rose from 8% to 18% for men; and for women rose from 11% to 22%[32].

Similarly, for the CVDs, the HJ-Biplot showed a total absorption of information by the axes of 94.7% (see Figure 3 and Figure 4). The animal products and meat supply contribute the most to dimension 1 (see Table 3). There are three clusters (figure 4), where the green cluster confirms a study done in 2018 about ischemic heart diseases (IHDs)[13], which states that mortality from ischemic heart diseases increased in Ecuador from 2001 to 2016 (Figure 3). Researchers have found that a diet with high contents of red meat, mainly processed red meat, is primarily to heart disease and stroke[33–35]. Moreover, a diet with high contents of plant-based protein could have benefits like lower risk of cardiovascular disease because these are mostly unsaturated, which lowers LDL cholesterol—an established risk factor for heart disease[16,36,37].

Moreover, countries and international agencies for the UN High-Level Meeting on NCDs have recommended aligning national policies on agriculture, trade, industry, and transport to promote improved diets and increase physical activity to reduce obesity, unhealthy diet, and physical inactivity as an intervention to tackle the NCDs crisis[8]. Also, mass-media campaigns, food taxes, subsidies, labeling, and marketing restrictions could raise awareness to maintain a good lifestyle[38].

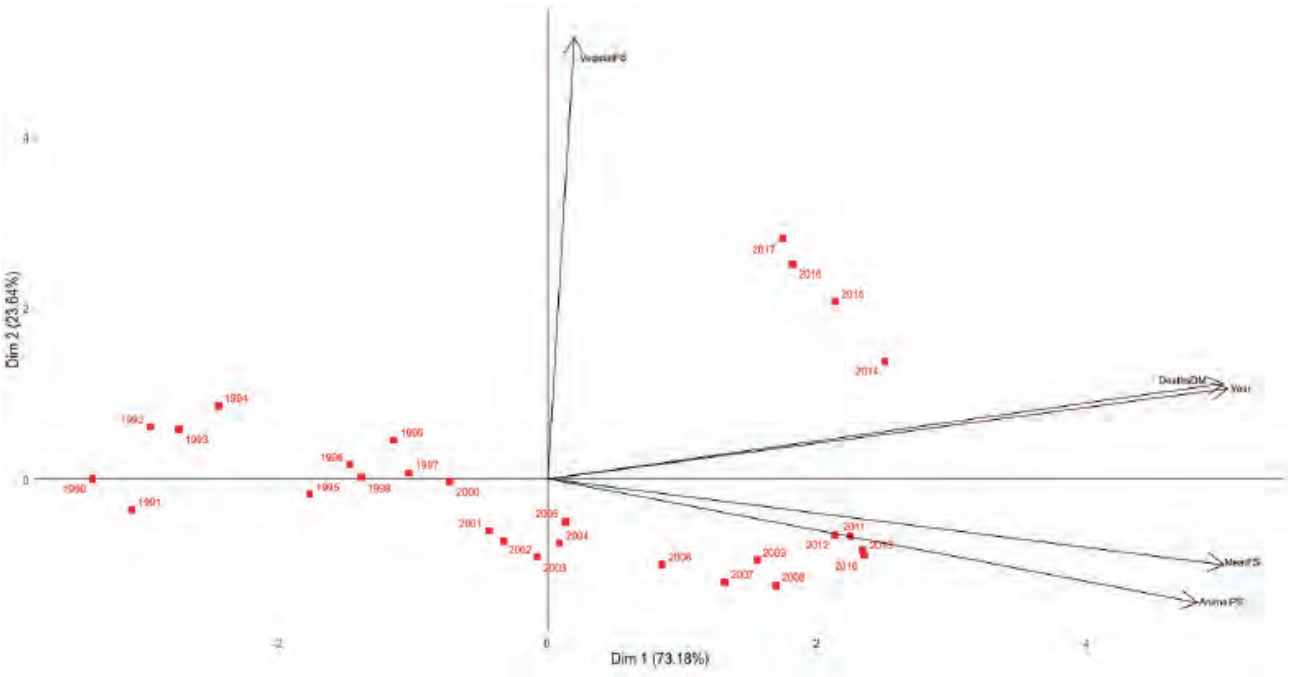


Figure 1. HJ-Biplot with SparseBiplot Package of Food supply and deaths by DM,1990-2017.

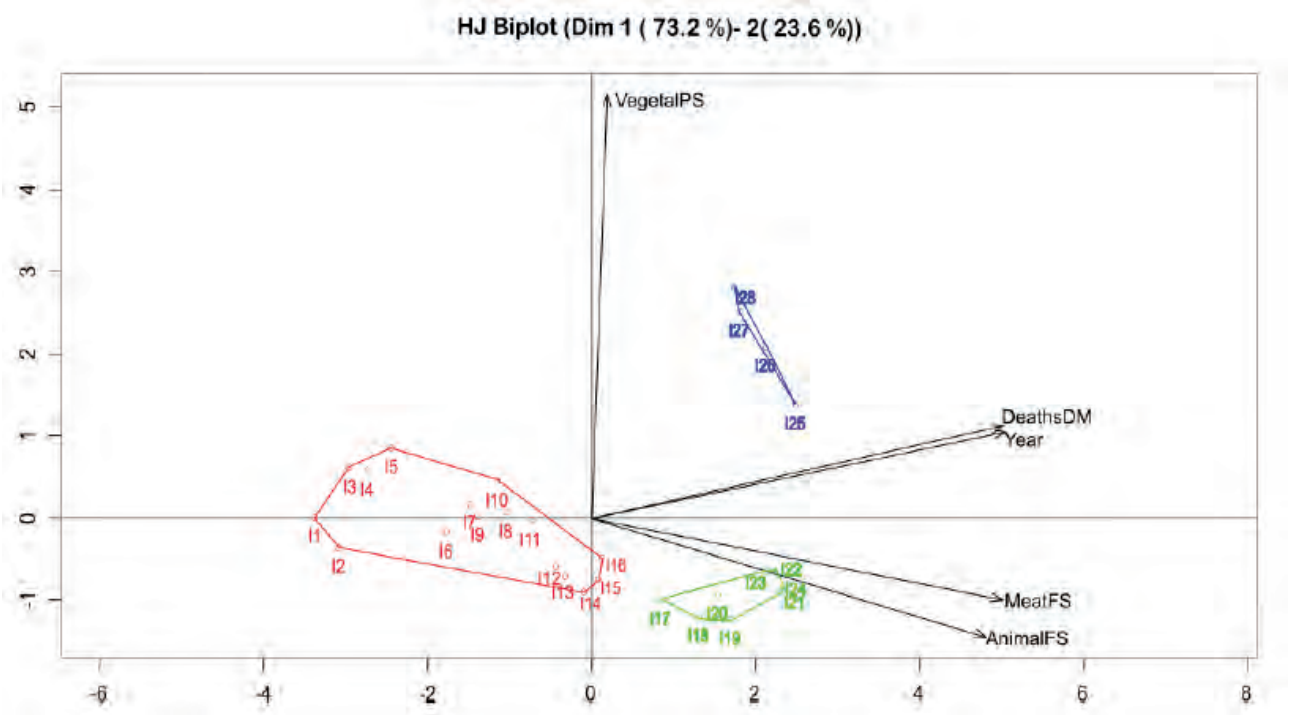


Figure 2. HJ-Biplot and Clusters formation with MultBiplot Package of Food supply and deaths by DM, 1990-2017.

	Dimension 1	Dimension 2
Year	94.03	4.11
Supply of protein from animal product	85.85	7.69
Supply of meat	93.04	3.65
Supply of protein from plant-based product	0.14	98.14
Deaths by Diabetes Mellitus	92.83	4.61

Table 2. Column Contributions for HJ-Biplot analysis of food supply and deaths by DM.

	Dimension 1	Dimension 2
Year	75.69	20.74
Supply of protein from animal product	92.10	0.03
Supply of meat	95.83	0.91
Supply of protein from plant-based product	5.34	92.56
Deaths by cardiovascular diseases	79.88	10.04

Table 3. Column Contributions for HJ-Biplot analysis of food supply and deaths by CVDs.

Conclusions

According to the HJ-Biplots analysis, the average amount of proteins from animal products and meat supply in the diet of Ecuadorians is highly and positively correlated with mortality by CVDs and Diabetes Mellitus.

From the Cluster Analysis, the years when the diet of Ecuadorians included more proteins from animal products and meat align with the years when the deaths by CVD and DM were high in numbers.

Since 2015, when the 2030 Agenda for Sustainable development[2] started, the awareness of the death risk by NCDs rose. Nevertheless, Diabetes Mellitus has not decreased as CVDs have because DM cases and deaths keep rising at dangerous levels globally (including in Ecuador); it is crucial to raise awareness in public health about this disease using the recommended guidelines by the UN[8].

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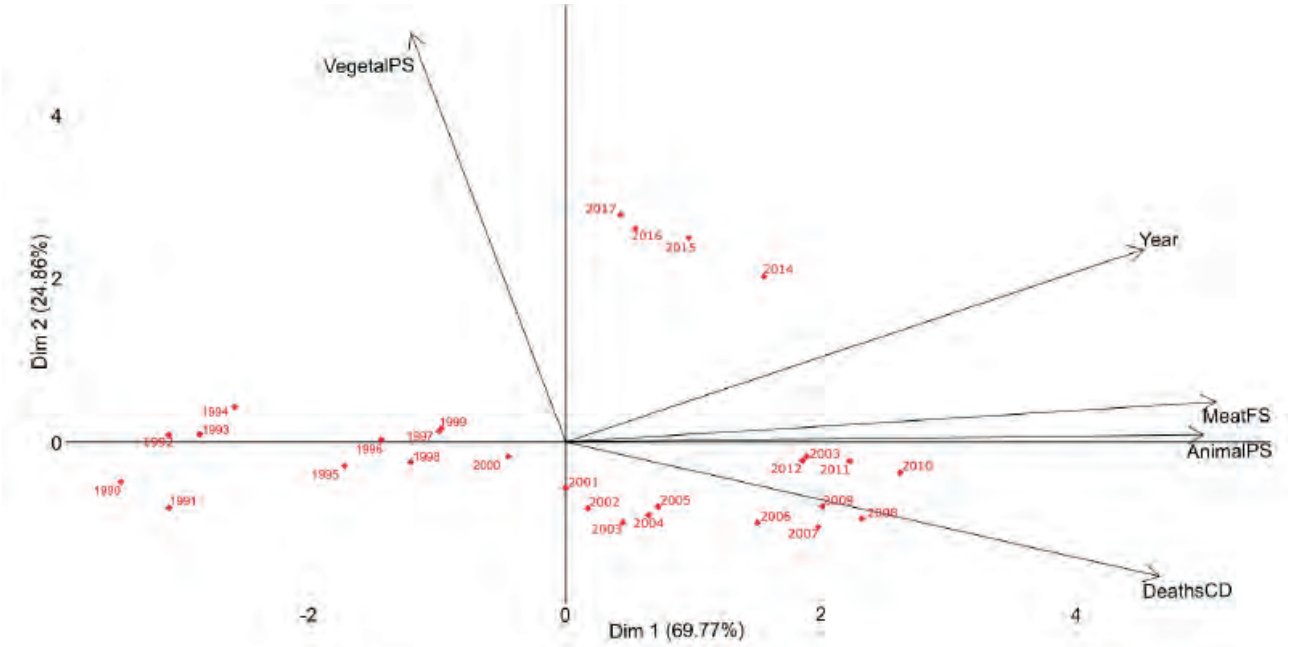


Figure 3. HJ-Biplot with SparseBiplot Package of Food supply and deaths by CVDs,1990-2017.

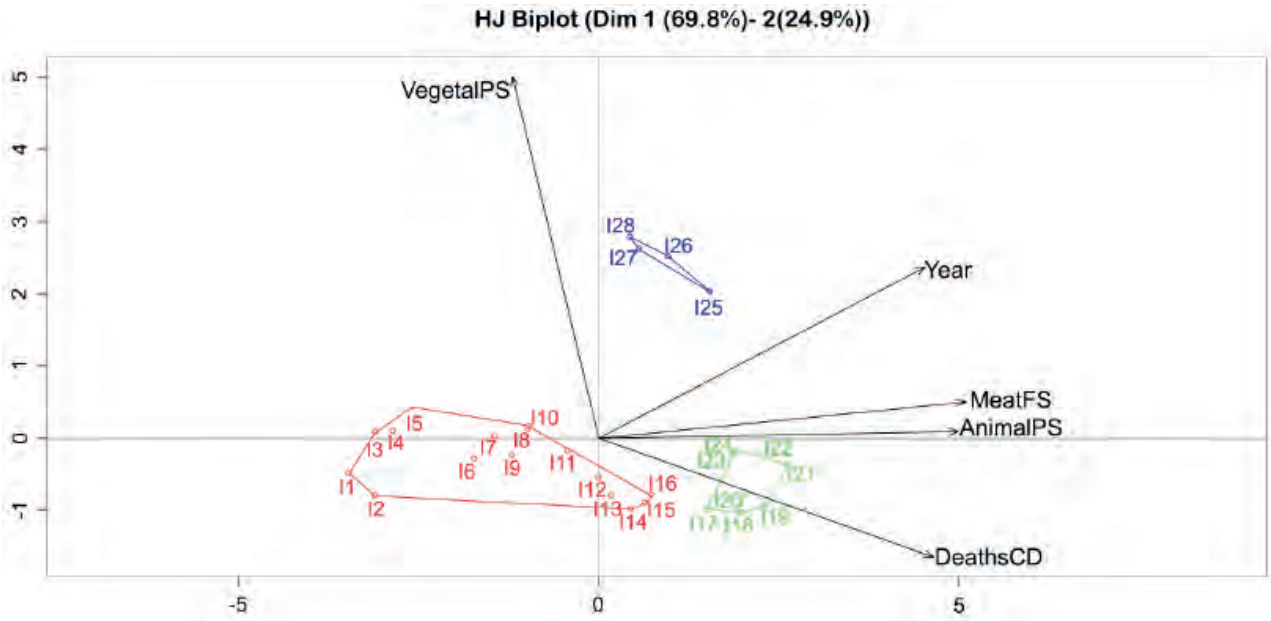


Figure 4. HJ-Biplot and Clusters formation with MultBiplot Package of Food supply and deaths by CVDs, 1990-2017.

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ARTICLE / INVESTIGACIÓN

Identification of methicillin-resistant strains of *Staphylococcus aureus* isolated from humans and food sources by use *mecA* 1 and *mecA* 2 genes in Pulsed-field gel electrophoresis technique

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Abstract: *Staphylococcus aureus* causes mastitis in dairy cows, lambs, goats, and skin disorders in pigs and other animals. *S. aureus* causes localized purulent infections that affect soft tissues, bones, and other organs in humans. Using restriction patterns, the researchers want to isolate and identify methicillin-resistant *Staphylococcus aureus* (MRSA) strains from cattle and humans. They also hope to assess their genetic relatedness by comparing the *mecA*1 and *mecA*2 gene sequence discrepancies. Animals (223 strains) and people have been used to acquire *S. aureus* strains for study (83). The E-test was used to assess whether or not the bacteria were resistant to methicillin. The *mecA*1 and *mecA*2 genes were identified by using pulsed-field gel electrophoresis (PFGE) to analyze DNA restriction patterns. The results were shown. S. aureus strains from animals and men were resistant to methicillin in 32 (14.34 %) and 53 (63.8 %), respectively. PFGE was used to determine the differences between human and veterinary pathology strains. Two strains of bacteria collected from animals were discovered to be identical; nevertheless, microorganisms recovered from humans were found to be significantly similar to the bacteria recovered from animals. Both human and veterinary pathology were implicated in the development of methicillin resistance. The MRSA strains found in humans were much more significant than those found in animals. The strains recovered from animals exhibited a high degree of genetic heterogeneity. Still, the enormous number of indistinguishable bacteria in humans leads one to believe that a dominant clone is present. When it comes to the molecular characterization of MRSA isolates, PFGE might be regarded as the gold standard.

Key words: Animals, Human, MRSA, PFGE, Staphylococcus, *mecA* genes.

Introduction

A report on the occurrence of methicillin-resistant *S. aureus* (MRSA) in medicine was published soon after the antibiotic methicillin was introduced into clinical practice to treat infections caused by these germs[1]. Characteristics of staphylococci that increase their pathogenicity include their propensity to colonize and multiply actively. When the infection takes hold, contributing risk factors include the slow healing of certain recurring infections, the use of lengthy antibiotic treatments, the use of catheters, and, once again, the presence of open wounds[2]. In dairy cows, *S.aureus* is one of the most common agents responsible for mastitis[3, 4], sheep and goats and is often involved in skin infections in pigs and other animal species[5]. S. aureus is the leading etiological agent of localized purulent infections affecting soft tissues, bones, and other organs in humans. It is mainly responsible for infections of natural exogenous according to its ability to spread in the environment, thus causing epidemics in closed communities such as hospitals, homes in care and schools[6-8]. Under some situations, the microbe demonstrates a high virulence capability, capable of exacerbating the inflammatory process till the host dies. Methicillin-resistant strains have been recorded in dogs[9], horses, and dairy cows after treating staphylococcal infections with methicillin and -lactams in general[4]. In humans, the MRSA strains are fearsome pathogens, especially in the hospital field, and countless epidemics are reported in the bibliography[7]. It has recently been proven that the transmission routes can be mainly two, one direct from patient to patient and the other, indirect, through healthcare professionals[7,10]. Methicillin resistance arises due to the presence of the *mecA* genes those code for a Penicillin Binding Protein (PBP2a), which has a low affinity for β-lactam compounds[11]. Veterinary pathology has long recognized that resistance to methicillin and oxacillin, a closely related antibiotic extensively used in veterinary pathology, is often linked with analogous behavior against a broad spectrum of antibiotics[12] to compromise therapeutic interventions, which, concerning bovine mastitis, represent the most effective method for controlling

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the disease. Now the 1960s, MRSA isolates in people have been recorded in European hospitals, and the disease has since expanded to non-hospital settings as well[13] so much so that, At the moment, MRSA jambs account for 25 percent of the strains of *S. aureus* that cause nosocomial infections in hospitals. In this work, the goal was to determine the existence of isolated MRSA strains in both human and animal samples and submit these jambs for molecular examination of chromosomal DNA through PFGE.

Materials and methods

Bacterial isolates

From the mammary secretion of cows with mastitis subclinical, 223 *S. aureus* isolates have been obtained. Of patients admitted to different wards of the hospital, 83 were selected from the isolates of *S. aureus*. The pathological material was sown in Nutrient Agar with the addition of 5% sheep blood and in Mannitol Salt Agar. The isolated microorganisms were identified by coagulase and Dnase tests, API Staph.

Methicillin-resistance

Mueller Hinton Agar was used to cultivate the isolates. Following that, 4-5 isolated colonies were collected and planted in Nutrient Broth up to a turbidity of 0.5 McFarland in 2-8 hours at 37°C till the turbidity of 0.5 McFarland was reached. For the determination of the Minimum Inhibiting Concentration (MIC) expressed in micrograms per milliliter (g/ml), an aliquot of 0.01 ml was diluted in 10 ml of saline solution, and 3 ml were distributed on the surface of a plate Mueller Hinton Agar[14] on which they have placed the nitrocellulose strips containing Methicillin E-test for the determination of the Minimum Inhibiting Concentration. Following incubation at 35°C for 24 hours, *S. aureus* jambs were tested for resistance (MRSA). Those that showed an equal or more significant inhibition at µg / ml were designated as resistant (MRSA). As a result of PCR[15], the existence of the mecA gene has been identified. The QIAmp tissue Kit was used to perform the DNA purification.The primers *mecA*1 (5'-AAA ATC GAT GGT AAA GGTTGG C) and *mecA*2 (5'-AGT TCT GCA GTA CCGGAT TTG C) highlight a product of PCR of 533bp[16]. Ungeheuer's method of DNA amplification was used[17]. DNA electrophoresis was carried out on a gel containing 2 % agarose, after which it was colored with ethidium bromide and photographed under the influence of ultraviolet light.

Pulsed-field gel electrophoresis (PFGE)

The macro-restriction approach with Sma I described the MRSA jambs, which were then resolved by pulsed electrophoresis[18]. In 10 ml of Trypticase Soy Broth at 37°C for 24 hours, the bacteria were grown before being centrifuged at 2000 g for 10 minutes, and the pellet was resuspended in PIV buffer. To manufacture the plugs, an aliquot of 0.5 ml of the latter suspension was combined with an equal quantity of Incert Agarose (low melting) 1 % to form a homogeneous mixture. Each plug received one milliliter of lysis solution, and they were incubated at 37°C for 24 hours in agitation. The lysis solution was replaced with a protein digestion buffer and then incubated at 50°C for 48 hours under agitation. The plugs were washed with TE and PMSF and three more times with TE. Later iplugs were subjected to enzymatic digestion in a mixture of H2O sterile, spermidine (1 mM), Smal 100U. After boiling for around 24 hours at 25°C in a final volume of 250 mL, the solution was utilized for pulsed electrophoresis on a 1 percent Fast Lane agarose gel at 14°C in TBE buffer auto-algorithm for 24 hours. Lambda Ladder was the standard that was utilized. The PFGE was done out using the CHEF Mapper system, which may be found here. To build a similarity dendrogram[18], the patterns electrophoretic were captured using a camera in the Molecular program Analyst Finger Printing and analyzed using the MA Finger Printing. The similarity coefficients (SAB) were estimated using software that was not modified in any way. From 0 to 1, the SAB indicated whether or not the patterns were connected, with 0 indicating that they were not related and one suggesting that they were tightly associated.

Results

The chemical and metabolic properties of the 306 *S. aureus* isolates were consistent throughout the group. Of the 223 jambs studied, 32 (14.34 percent) were found to be resistant to methicillin, but of the 83 jambs investigated that were of human origin, 53 (63.8 percent) were found to be MRSA. Forty MRSA and twenty MSSA jambs, derived in equal parts from human and veterinary illness, in which the presence of the mecA gene has been validated by PCR to verify the findings obtained with the E-test and Sceptor System, have been subjected to PFGE. As a consequence of the PFGE data, the investigation of the genetic similarity of the MRSA jambs, which was shown using a dendrogram (Figs. 1 and 2), revealed that the strains of human and veterinary origin were distinct. The blocks that arrived showed a significant degree of resemblance among themselves; however, just two strains of animal origin showed identical results compared to the unions that came (Table 1).

Discussion

Staphylococcus aureus is one of the most prevalent germs found in nature and one of the most responsible for illness, both in people and in animals. It can produce infections that are difficult to treat with conventional therapy[19].

Resource	Indistinguishable	Closely related	Probably related	Not related	Total
MRSA Human isolates	6	6	6	2	20
Animal isolates	2	2	0	16	20
MSSA Human isolates	2	3	2	3	10
Animal isolates	0	2	2	6	10

Table 1. *Staphylococcus aureus*: genomic similarity of the isolates from human and animal pathology.

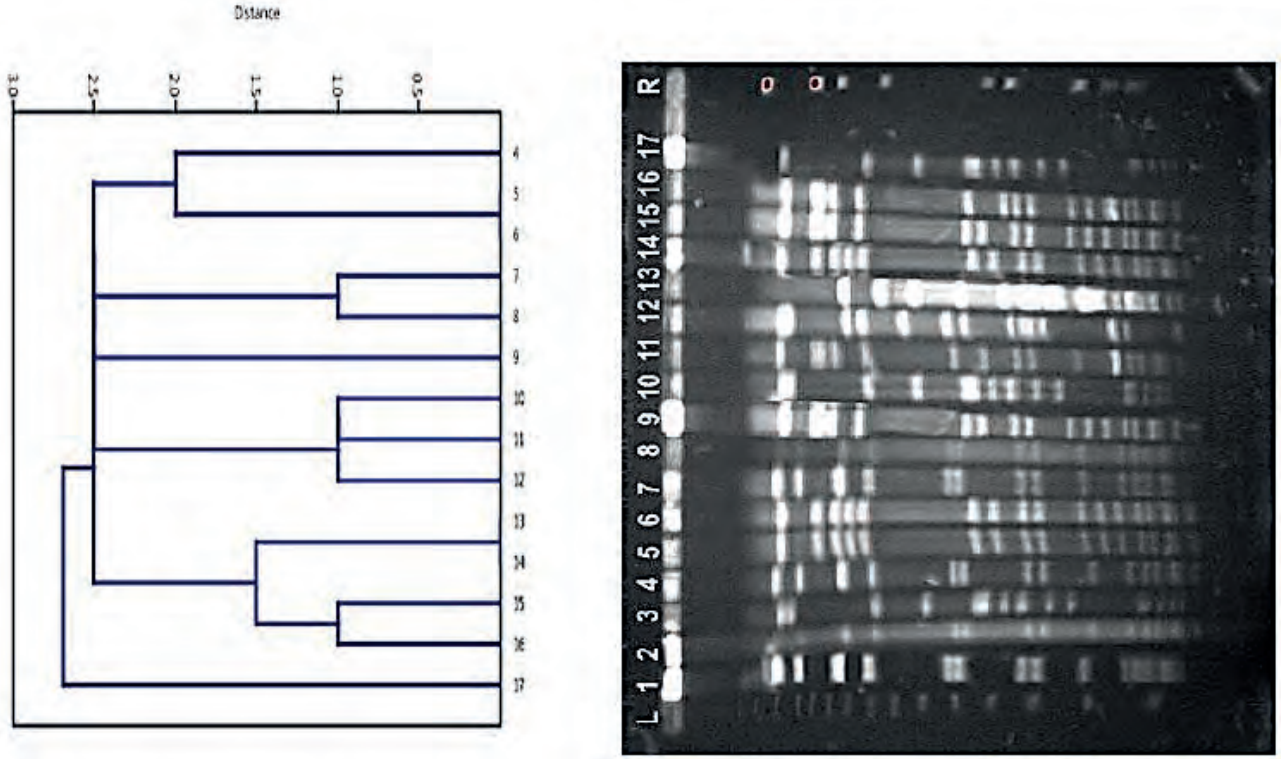


Figure 1. *Staphylococcus aureus* (MRSA) of human origin: similarity dendrogram.

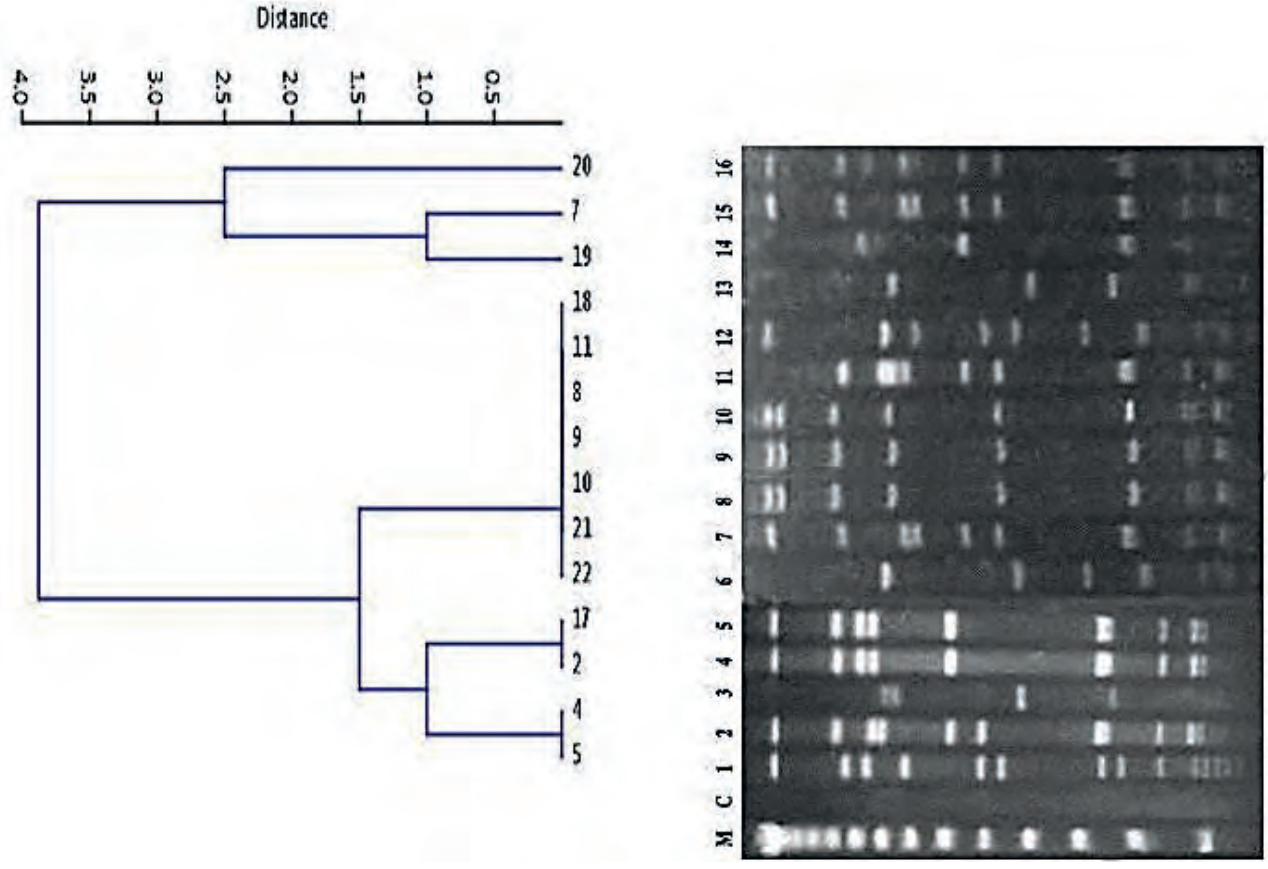


Figure 2. *Staphylococcus aureus* (MRSA) of animal origin: similarity dendrogram.

To adapt to the increased use of antibiotics, *S. aureus* has modified its genomic structure and made the PBP2a protein via the mecA genes, which has a low affinity for -lactam antibiotics, leading in the appearance of methicillin-resistant jambs[18,20]. One of the issues that must be addressed is selecting a technique for identifying staphylococcal jambs. The identification of MRSA strains is difficult, even with the international set of phages, and it was therefore deemed appropriate to replace conventional methods with molecular biology techniques, such as PFGE or polymerase chain reaction, which, when performed with the primers *mecA*1 and *mecA*2, results in the identification of a sequence of 533 base pairs, specific for MRSA jambs[14]. Since it has been demonstrated in human pathology that MRSA jambs from different continents are classified as indistinguishable, it was deemed appropriate to compare jambs of human and veterinary origin, in the same territorial context, to verify if they had been realized under the same conditions. Although the jambs of animal origin originated from farms in different geographies, this does not excuse the lack of genetic similarity between them, as MRSA jambs considered unrecognizable have a genetic similarity of 80% or greater[21,22]. When a comparison analysis was conducted between the 40 MRSA jambs and the 20 MSSA jambs, it was discovered that there is no genetic similarity between them, just as there were no commonalities between the MRSA jambs from veterinary and human pathology. However, only two MRSA jambs of veterinary origin could be identified as genetically identical and two other closely related ones.

In contrast, in human pathology, the genetically similar jambs (n.6) and closely related jambs (n. 6) suggested the presence of a dominant strain in the degree to which there is a close correlation between strains isolated over time. Aside from the genetic similarities, the difficulties associated with methicillin resistance impact veterinary and human illnesses similarly. Pulsed electrophoresis is widely recognized as the gold standard for molecular typing of a broad variety of microorganisms. It has been shown to be highly selective and equivalent, if not better, to other approaches for a large number of harmful bacteria[4,16,18]. The findings of the investigation, despite being constrained by a small number of jambs, confirm the presence of MRSA in cattle and demonstrate the high level of genomic variability found within methicillin-resistant strains, which suggests that these bacteria could spread from animals to the environment through contact with other animals other than humans[20]. As an additional indication of the complex somatic structure of *S. aureus*, there is an inverse relationship between the homogeneity shown by chemical-metabolic analysis and genomic inequality, which is the element that best defines the bacterium.

Conclusions

MRSA isolates from several hospital wards have been studied. The results indicate a significant degree of similarity between certain jambs, which suggests the presence of a typical clone from which they might originate the isolates. This clone has been present at the hospital for at least 15 years. It has caused periodic epidemics (data not published), leading some to speculate that it has colonized the healthcare workers, who would then act from tanks and vehicles due to the outbreak. The genetic analysis of MRSA strains highlights the necessity for adequate procedures to be taken to monitor clinical isolates so that they may act effectively to stop the epidemiological cycle, as shown by the research.

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Conflicts of Interest

No conflict.

Funds

Self by authors

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ARTICLE / INVESTIGACIÓN

Investigated of the Hydrogen peroxide's efficacy in sterilizing various substrates for the formation of the oyster mushroom *Pleurotus sapidus*

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Abstract: This study was conducted in order to test some agricultural wastes from fruits and sterilization method in the production of oyster mushroom *Pleurotus sapidus*. This study included two factors, the first is the type of substrate which consisting of seven combinations: A0 (wheat straw 80% + flour bran 20%), A1 (pomegranate peel 25% + wheat straw 75%), A2 (pomegranate peel 50% + wheat straw 50%), A3 (pomegranate peel 75% + wheat straw 25%), A4 (date fruit residues 25% + wheat straw 75%), A5 (date fruit residues 50% + wheat straw 50%), A6 (date fruit residues 75% + straw wheat 25%). The second factor is the sterilization method, represented by two methods of sterilization, the first is sterilization by Autoclave (P0) and the second is sterilization using hydrogen peroxide H_2O_2 at two concentrations of 3% (P1) and 5% (P2). A2 achieved the shortest period of growth and spread of mycelium which was 51.67 days and the highest amount of total wet yield was 199.2 g.kg^{-1}. A0 and A6 had the shortest period for primordia formation which was 61.0 and 62.8 days, respectively. The mixture of A6 substrate gave the longest production cycle duration of 40.8 days and the highest biological efficiency rate was 44.78%. A1 and A2 gave the shortest fruiting period of 6.22 days. The P0 sterilization method recorded the shortest period for the growth and spread of mycelium, shortest period for the formation of primordia and the longest production cycle resulting in 48.88, 47.4 and 43.6 days, respectively, while the P1 sterilization method recorded highest wet yield of 175.6 g.kg^{-1} and the highest biological efficiency rate of 39.85%.

Key words: *Pleurotus sapidus*, hydrogen peroxide, sterilization, substrate.

Introduction

Mushrooms are large, heterotrophic, spore-producing fungi belonging to the kingdom of fungi[1]. Most of the cultivated edible mushrooms belong to the phylum Basidiomycota, although some genera of the phylum Ascomycota such as species of the genus *Morchella* and *Tuber* have been successfully cultivated and commercially exploited[2]. Mushroom usually grows either above the ground on the soil or on a substrate prepared industrially and used as a substrate[3]. The species belonging to the genus *Pleurotus* are economically important and its importance comes after the white button mushroom. Its importance is due to the fact that it is rich in proteins, essential amino and fatty acids, sugars, vitamins and important minerals such as calcium, iron, manganese and copper, as well as this mushroom had several medicinal properties in resisting cancer diseases, hypersensitivity and atherosclerosis[4,5]. Oyster mushroom contains protein in abundant quantities, it is also rich in vitamin B, oyster mushrooms do not contain cholesterol that causes hypertention, in addition to the fact that it contains high percentage of lovastatin, which lowers cholesterol levels, this led to encourage the study of oyster mushrooms for their benefits in adjusting cholesterol levels in the blood[6]. Oyster mushroom was grow on dead wood and other substrates rich in cellulose and lignin, it can be grown on agricultural and industrial wastes containing lignocellulosic biomass, such as wheat straw, soybean straw, cotton stalks, corn cobs, etc[7]. Wheat straw was used as an ideal substrate for the production of oyster mushroom, but it is not available at all times of the year, in addition to its use as animal feed at high prices, which prompted researchers to find other substrates as alternatives to the substrate of wheat straw, such as wild plants, agricultural residues and date palm waste[8]. The productivity and biological efficiency of oyster mushroom were increased in some mixtures when compared to wheat straw alone, due to the difference in the ability of these substrates to provide nutritional and environmental requirements, in addition to the difference in the contents of cellulose, hemicellulose and lignin[9]. Several methods have been used to sterilize oyster mushroom substrate such as pasteurization, steam sterilization, hot water sterilization, chemical sterilization and sterilization with plant extracts in order to get rid of unwanted microorganisms present in the substrate[10,11].

Materials and methods

Treatments of experiment

This experiment was carried out in Department of Horticulture and Landscape design/College of Agriculture/ Tikrit University in Iraq for production of oyster mushrooms *Pleurotus sapidus*, was conducted to test seven different mixtures of substrates (wheat straw 80% + 20% wheat bran,

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pomegranate peel 25% + wheat straw 75%, pomegranate peel 50% + wheat straw 50%, pomegranate peel 75% + wheat straw 25%, molasses factory wastes (date husk only) 25% + wheat straw 75%, molasses factory waste (date husk only) 50% + wheat straw 50%, molasses factory waste (date husk only) 75% + wheat straw 25%), with two types of substrates sterilization, the first is thermal by Autoclave and the second is chemical using hydrogen peroxide (H2O2) at concentrations of 3% and 5% .

Substrates sterilization

In the first sterilization method, the substrates were prepared and placed in mesh bags, they were soaked in tap water for 4-5 hours, then lifted and suspended to get rid of excess water for 24 hours, then they were mixed well with 2% calcium carbonate (CaCO3) on the basis of wet weight[12] in order to ensure an even distribution of calcium carbonate in the substrate and adjust the acidity of the substrate to approximately pH 6.5, then the substrates (each substrate weight 1 kg) was placed in polypropylene plastic bags resistant to heat and pressure and sealed with heat-resistant thread, then placed in Autoclave device in the Plant Tissue Culture Laboratory/ Department of Horticulture and Landscape design / College of Agriculture/ Tikrit University, the sterilization was carried out under pressure of 1.5 bar and temperature of 121°C for 20-30 minutes[13]. Polypropylene plastic bags were taken out and left until its temperature decreased, then 2% of *Pleurotus sapidus* spawn was added to each bags by making a small hole in the bag and then closed well to prevent contamination of the substrate. In the second sterilization method, commercial hydrogen peroxide (50%) was used after diluting it to two concentrations, the first 3% and the other 5%[14], after the substrates was prepared and placed in perforated nylon bags, the bags were immersed in a plastic container with a diameter of 40 cm and a height of 80 cm containing dilute hydrogen peroxide for 5-6 hours, then lifted and placed on an iron shelf for 24 hours to get rid of excess water and hydrogen peroxide, then mixed well with calcium carbonate (2% on a wet weight basis) as it was found to be more suitable[12] to ensure equal distribution of calcium carbonate in the substrate and adjusting the acidity of the substrate to pH 6.5, then the sterile substrates were filled with both concentrations in transparent plastic bags of the polyethylene 35 × 50 cm in dimensions and weighed so that each bag contains 1 kg of the wet substrate, then 2% (approximately 20 g.bag^{-1}) of the fungal spawn was added to the substrate[15].

Studied traits

A different set of traits were studied, including:

Time period for the mycelium run: which represented the number of days from the beginning of the spawning substrate until the completion of the mycelium full growth in the substrates.

Primordia formation period: The number of days from the beginning of the spawning until the beginning of the emergence of the first primordia outside the bag in the form of pinheads.

Formation of the fruiting bodies: which is the number of days from the emergence of the primordia until the fruiting bodies are ready to be harvested[16].

Number of fruit bodies: represented the number of fruit bodies resulting from one bag containing 1 kg of wet substrate for all flashes.

Total yield: total yield was estimated on the basis of substrate wet weight where the sum of all the yields produced from one bag containing 1 kg of substrate, and expressed on the basis of g. kg^{-1} substrate[16].

Biological efficiency: the ability of a substrate to produce the largest amount of fruit bodies and is expressed in terms of the percentage of substrate production.

Production cycle: the number of days from the beginning of the harvesting stage to the last harvest.

Statistical analysis

All experiments units were performed at least in triplicate according to the Randomized Complete Block Design (RCBD). The obtained data were evaluated by two-factor analysis of variance, and significant differences were determined by Fisher's test (L.S.D) at significance level of 0.05.

Results

Effect of the substrates combination and the sterilization method and the interaction between them on the mycelium run of the of the oyster mushroom *Pleurotus sapidus*

The results of Table 2. showed that there were significant differences between the averages of the substrates combination, where the A2 substrate combination (50% pomegranate peel and 50% wheat straw) was superior on the most other mixtures with the shortest period of mycelium spread of 51.67 days. For the sterilization method, P0 sterilization method (autoclave sterilization) gave the shortest period for the spread of mycelium, reaching 76.45 days, superior on the sterilization method using hydrogen peroxide. In the interaction between combinations and sterilization methods, A0P0 treatment (80% wheat straw and 20% flour bran with autoclave sterilization) gave the shortest mycelium completion time of 35.67 days, superior on the most of the other treatments.

No.	Substrate	N %	Protein %	Fiber %	Ash %	O.M %	O.C %	C:N Ratio
1	A0	1.47	46.4	30.26	9.81	90.18	52.30	36:1
2	A1	0.93	84.0	32.54	5.47	94.52	54.82	59:1
3	A2	0.98	4.29	23.68	6.01	93.98	54.51	56:1
4	A3	0.98	4.29	16.33	5.11	94.88	55.03	57:1
5	A4	40.7	33.2	29.37	9.70	90.29	52.36	71:1
6	A5	1.27	5.58	17.25	5.39	94.60	54.87	43:1
7	A6	1.37	16.0	16.68	5.32	94.67	54.91	40:1

Table 1. Some laboratory test to substrates combination according to Al-Amiri[16].

Effect of the substrates combination and the sterilization method and the interaction between them on the time period for the primordia formation of the oyster mushroom *Pleurotus sapidus*

Statistical analysis of the data in Table 3 showed there are significant differences between the combination of the substrates, the two factors of substrates combinations A0 (wheat straw 80% and flour bran 20%) and A6 (date fruit residues 75% and wheat straw 25%) gave the shortest period for primordial formation reached 61.00 and 62.80 days, respectively, significantly superior on the factors of substrates combination A3 (75% pomegranate peel and 25% wheat straw) and A4 (25% date residues and 75% wheat straw). For the sterilization method, P0 (autoclave sterilization) outperformed the sterilization method using hydrogen peroxide with both concentrations, which gave the shortest period for primordial formation reached 47.40 days. In interaction between substrates combination and sterilization methods, the treatment A0P0 (wheat straw 80% and flour bran 20% with autoclave sterilization) gave the shortest duration of primordial formation reaching 34.00 days, superior on the most other treatments.

Effect of substrates combination and the sterilization method and the interaction between them on the formation of the *Pleurotus sapidus* fruit bodies

It was shown through the statistical indications in Table 4. there were significant differences between the substrates combination, as the substrates combination A0 (wheat straw 80% and flour bran 20%) and A4 (date fruit residues 25% and wheat straw 75%) recorded the longest period for the fruit bodies formation reached 7.89 days, superior on the substrate combination A1 (pomegranate peel 25%, wheat straw 75%) and A2 mixture (pomegranate peel 50% and wheat straw 50%), which recorded the shortest period for the formation of the fruit bodies reached 6.22 days, while there were no significant differences between the other substrates. For the sterilization method, there were no significant differences between the means of sterilization methods. The interaction between the substrates combination and the sterilization method had significant differences, as the two treatments A0P0 (wheat straw 80% and flour bran 20% with autoclave sterilization) and A1P2 (pomegranate peel 25% and flour bran 75% with sterilization using hydrogen peroxide at 5%) showed superiority over most of the other treatments, where these treatments recorded the longest period for the formation of fruit bodies, which was 8.33 days.

Effect of substrates combination and sterilization method and their interaction on the total fresh weight of oyster mushroom *Pleurotus sapidus*

The results of Table 5. Showed there are significant differences between the substrates combination, where the mixture of substrate A2 (pomegranate peel 50% and wheat straw 50%) significantly outperformed most of the treatments with the highest wet yield of 199.2 $g.kg^{-1}$. As for the sterilization methods, the sterilization method P1 (hydrogen peroxide at 3%) outperformed the sterilization method P2 (hydrogen peroxide at 5%) with the highest wet yield

Sterilization method / Substrates combination	Mycelium run. Day			Mean substrates combination
	P0	P1	P2	
A0	35.67	74.00	74.00	61.22
A1	36.33	39.00	89.00	54.78
A2	74.00	42.33	38.67	51.67
A3	39.00	97.00	58.50	64.83
A4	45.67	65.33	107.33	72.78
A5	37.50	70.67	77.67	61.94
A6	47.00	43.33	90.00	69.11
Mean Sterilization method	48.88	61.67	76.45	
L.S.D	Interaction		Sterilization method	Substrates combination
	9.60		3.63	5.54

Table 2. Effect of the substrates combination and the sterilization method and the interaction between them on the mycelium run of the of the oyster mushroom *Pleurotus sapidus* (day)

Sterilization method / Substrates combination	Primordia formation. Day			Mean substrates combination
	P0	P1	P2	
A0	34.00	75.30	73.70	61.00
A1	39.00	66.30	99.70	68.30
A2	84.70	48.00	63.30	65.30
A3	42.30	111.50	59.50	71.10
A4	39.00	71.30	114.00	74.80
A5	43.50	68.00	82.00	64.50
A6	49.00	61.30	78.00	62.80
Mean Sterilization method	47.40	71.70	81.50	
L.S.D	Interaction		Sterilization method	Substrates combination
	13.36		5.05	7.71

Table 3. Effect of substrates combination and sterilization method and the interaction between them on the time for the primordial formation of the oyster mushroom *Pleurotus sapidus* (day).

Sterilization method / Substrates combination	Fruit body formation. Day			Mean substrates combination
	P0	P1	P2	
A0	8.33	7.67	7.67	7.89
A1	6.33	4.00	8.33	6.22
A2	5.67	7.00	6.00	6.22
A3	6.67	7.00	6.00	6.56
A4	9.00	7.33	7.33	7.89
A5	5.50	6.67	9.00	7.06
A6	7.67	7.33	7.00	7.33
Mean Sterilization method	7.02	6.71	7.33	
L.S.D	Interaction		Sterilization method	Substrates combination
	2.56		n.s	1.48

Table 4. Effect of substrates combination and the sterilization method and the interaction between them on the formation of the *Pleurotus sapidus* fruit bodies (day).

Sterilization method / Substrates combination	Total wet weight g.kg wet $^{-1}$			Mean substrates combination
	P0	P1	P2	
A0	188.60	156.40	147.00	164.00
A1	149.30	152.90	162.70	155.00
A2	188.70	200.40	208.50	199.20
A3	173.10	131.10	142.30	148.80
A4	165.30	164.70	85.60	138.50
A5	159.20	190.50	120.80	156.80
A6	170.90	233.10	174.20	192.70
Mean Sterilization method	170.70	175.60	148.70	
L.S.D	Interaction		Sterilization method	Substrates combination
	42.78		16.17	24.70

Table 5. Effect of substrates combination and sterilization method and their interaction on the total fresh weight of oyster mushroom *Pleurotus sapidus* (g.kg^{-1}).

of 175.6 g. The results of the interaction between the two factors (substrates combination and sterilization method) showed that there were significant differences between the treatments, whereby A5P1 treatment (date fruit residues 75% and wheat straw 25% sterilized with 3% hydrogen peroxide) significantly outperformed most of the other treatments with the highest wet yield of 233.1 g. kg^{-1}.

Effect of substrates combination and sterilization method and their interaction on the number of *Pleurotus sapidus* fruit bodies

The results of the statistical analysis in Table 6. indicate that there are no significant differences between the substrates combination factor in the characteristic of the number of fruit bodies, and the sterilization method used did not affect the number of fruit bodies, and thus no significant differences appeared between the sterilization methods. The results of the interaction between the two factors (substrates combinations and sterilization method) indicate that there are significant differences between the treatments, as the A4P0 treatment (date fruit residues 25%, wheat straw 75% with autoclave sterilization) outperformed most of the other treatments with the highest number of fruit bodies reaching to 6.33 fruit bodies.

Effect of substrates combination and the sterilization method and the interaction between them on the production cycle (days) for the oyster mushroom *Pleurotus sapidus*

It is clear from table 7. presence of significant differences between the substrates combination, where the mixture of A6 substrate (date fruit residues 75% and wheat straw 25%) outperformed the mixture of substrate A4 (date fruit residues 25% and wheat straw 75%) with the highest production cycle duration of 40.80 days. In case of the sterilization method, P0 (Autoclave sterilization) outperformed all other sterilization methods, as it recorded the highest period of 43.60 days. The interaction, A6P0 (date fruit residues 75% and wheat straw 25% sterilized by Autoclave) outperformed most of the other treatments with a production period of 68.70 days.

Effect of substrates combination and the sterilization method and the interaction between them on the biological efficiency (%) of the oyster mushroom *Pleurotus sapidus*

In table 8, it is clear that there are significant differences between the factor of substrates combination, where the mixture of substrate A6 (date fruit residues 75% and wheat straw 25%) significantly outperformed most of the other substrates combination with the highest biological efficiency rate of 44.78%. However, a significant difference was found between the sterilization methods, where the sterilization method P1 (3% hydrogen peroxide) recorded the highest biological efficiency rate of 39.85%, significantly superior on the sterilization method P2 (5% hydrogen peroxide). In the interaction between the substrates combination and sterilization method, A6P1 (date fruit residues 75% and wheat straw 25% sterilized with 3% hydrogen peroxide) significantly outperformed all other treatments, recording the highest biological efficiency rate of 61.34%.

Sterilization method / Substrates combination	Total wet weight g.kg wet $^{-1}$			Mean substrates combination
	P0	P1	P2	
A0	188.60	156.40	147.00	164.00
A1	149.30	152.90	162.70	155.00
A2	188.70	200.40	208.50	199.20
A3	173.10	131.10	142.30	148.80
A4	165.30	164.70	85.60	138.50
A5	159.20	190.50	120.80	156.80
A6	170.90	233.10	174.20	192.70
Mean Sterilization method	170.70	175.60	148.70	
L.S.D	Interaction		Sterilization method	Substrates combination
	42.78		16.17	24.70

Table 6. Effect of substrates combination and sterilization method and their interaction on the number of *Pleurotus sapidus* fruit bodies.

Sterilization method / Substrates combination	Production cycle. Day			Mean substrates combination
	P0	P1	P2	
A0	36.00	37.30	32.00	35.10
A1	44.70	55.30	13.00	37.70
A2	34.30	28.30	45.70	36.10
A3	52.70	14.00	36.50	34.40
A4	36.00	28.00	9.00	24.30
A5	33.00	30.70	24.30	29.30
A6	68.70	39.30	14.30	40.80
Mean Sterilization method	43.60	33.30	25.00	
L.S.D	Interaction		Sterilization method	Substrates combination
	23.68		8.95	13.67

Table 7. Effect of substrates combination and the sterilization method and the interaction between them on the production cycle (days) for the oyster mushroom *Pleurotus sapidus*.

Sterilization method / Substrates combination	Biological efficiency (%)			Mean substrates combination
	P0	P1	P2	
A0	43.85	35.55	38.70	39.37
A1	27.64	35.56	36.97	33.39
A2	42.89	37.11	48.49	42.83
A3	44.38	29.79	26.35	33.51
A4	32.41	42.24	19.45	31.36
A5	41.89	37.36	30.97	36.74
A6	38.84	61.34	34.17	44.78
Mean Sterilization method	38.84	39.85	33.58	
L.S.D	Interaction		Sterilization method	Substrates combination
	10.25		3.87	5.92

Table 8. Effect of substrates combination and sterilization method and the interaction between them on the biological efficiency (%) of the oyster mushroom *Pleurotus sapidus*.

Discussion

The difference in the period of completion of mycelium growth may be due to several reasons such as the method of sterilization, type of material used for sterilization, moisture of the culture substrate, amount and method of adding the spawn to the mushroom substrate and requirements for the growth of the fungal strain used and its characteristics[17]. The reason for the variation in the growth period of the mycelium and the primordial formation as well as the completion of the fruit bodies growth may be due to the different substrates and their components, this is consistent with[18,19].

The growth stages of oyster mushrooms are affected by the type of substrate and its components. The variation in the amount of the total mushroom yield (on the basis of wet weight) may be due to the different substrates in their ability to provide the mycelium with the nutrients, with different substrates content of cellulose and hemicellulose compounds, hemicellulose degrades faster than cellulose compounds due to their low polymerization and amorphous nature[20]. The biological efficiency and production cycle differ according to the different components of the substrate itself from cellulose and hemicellulose in addition to the strength of the mushroom spawn and the generation and the strain used of

the spawn[21], the additives to the substrate and the amount of the mushroom spawn[22]. On the other hand, research did not agree on a specific efficacy and vitality for a particular species of mushroom or a substrate[8,19].

Conclusions

The growth and production of oyster mushrooms was affected by different substrates and their combinations, as well as the sterilization method. Oyster mushrooms can be grown on substrates with pomegranate peels in their combinations, and they may be effective in producing mushrooms if some additives were added to them. The method of sterilization with hydrogen peroxide H_2O_2 can be used as a successful method for sterilization of cultivation substrates for oyster mushrooms, taking into account the soaking for a longer period and increasing the period of time before adding the mushroom spawn to the substrates to get rid of the largest possible amount of hydrogen peroxide.

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Conflicts of Interest

There are no conflicts of interest reported by the authors.

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ARTICLE / INVESTIGACIÓN

Laboratory diagnosis of urinary tract infections in patients with resistance genes towards antibiotics

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Abstract: *Escherichia coli* are gram-negative bacteria that cause urinary tract infections (UTIs). UTIs have affected a significant percentage of humans yearly due to bacterial infection. Our study aims to determine the prevalence of resistance genes in *E. coli* towards sulfamethoxazole. This study included (490) patients with UTIs, and the urine samples were cultured on media. The patients were admitted to the Medical City in Baghdad to treat UTIs. 116 *E.coli* isolates were isolated from urine specimens, 35 isolates of them were resistant to trimethoprim/sulfamethoxazole, and 81 isolates were sensitive to trimethoprim/sulfamethoxazole; the *E. coli* isolates were submitted to multiplex PCR to detection some resistance genes (Sul1, sul2) after detected the isolates by PCR depending on 16S rRNA. Our study showed that identified *E. coli* was (91-99%) depending on the number of the examined samples by the Vitek 2 system. The molecular study included extraction of chromosomal DNA from (53) *E. coli* isolates; 35 samples were taken resistant to antibiotics, while from the total of 81 sensitive isolates, only 18 sensitive samples were taken from that are the most sensitive to Timethprime/sulfamethoxazole, then identification by 16S rRNA gene. Detection of Sulfonamides resistance genes included sul1 and sul2. The results showed the 16S rRNA gene identification found in all *E. coli* isolates and the detection of antibiotic resistance genes. The resistant isolates with the Sul1 gene prevalence were 11(31%), while the sensitive isolates with Sul1gene were 1(6%). Moreover, the resisted isolates with Sul2 gene prevalence was 8(23%), while the sensitive isolates with the Sul1 gene were 0(0%). The numbers of the resistant isolates were (11) and (8) that carry the Sul1 gene and Sul2 gene, respectively, while the numbers of the sensitive isolates were (1) and (0), respectively. We can conclude that a high percentage of Sul1 gene and Sul2 genes in *E. coili* isolated from UTIs were high.

Key words: UTI, Sul1, Sul2, resistant gene, trimethoprim-sulfamethoxazole.

Introduction

Urinary Tract Infection (UTI) is a widespread disease in males and females. The occurrence percentage of Urinary Tract infections was 35% of healthy women with clinical signs of UTIs[1]. UTI was more incidence in females than males because of easy contamination with fecal flora, the squatness of female urethra dearth, and pregnancy2. Many neonates, young females, infants, children and older men are infected with UTIs[3]. Uncomplicated UTIs include bacterial entrance infection and bacterial proliferation in the urinary tract system[4]. UTIs are mostly the second most common infections bacterial after respiratory tract infections. UTIs are inflammatory conditions in the urinary system leading to pyuria and bacteriuria[5]. Antibiotic resistance has become a significant problem that needs coordinated action to reduce and prevent antibiotic resistance. The broad use of antibiotics for resistant bacteria may result in illness in humans that is less responsive to treatment with conventional antibiotics[6]. Drug efflux pumps and the genes that respond to antibiotic resistance (by conjugation) are two mechanisms by which bacteria develop antibiotic resistance in large numbers[7]. Trimethoprim and Sulphonamides are inexpensive antibiotics that work together to provide a synergistic effect. Since 1968, it has been used together as (co-trimoxazole) to treat some clinical cases, such as urinary tract infections. Trimethoprim sulphonamides resistance carries on the plasmids that generate the target enzymes that have a role in the resistance, such as dihydropteroate synthases against the sulphonamides and dihydrofolate reductases against the trimethoprim. Several genes, such as (*sul*1 and *sul*2) encode dihydropteroate synthases[8,9]. Because of the importance and riskiness of *E.coli*, the increased incidence of infection and the possibility of the epidemic of infection as well as the riskiness of the disease and the lack of treatment, have led to the focus of research in the world on improving the efficiency of molecular detection using advanced technologies and reduce time and effort, the best diagnostic methods, such as PCR technique, are characterized by the technique of specificity and high speed in the detection of the genes encoding for the virulence and antibiotic resistance factors in isolates of *E.coli* isolated from the clinical specimens[10]. This study aims to determine the prevalence and the resistance profile of *E. coli* in UTI, which resist Timethprime and sulfonamide such as sul1 and sul2 genes.

Materials and methods

Patient samples

This study included (490) patients suffering from uri-

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nary tract infections, the samples were acquired by culturing mid-stream urine.116 *Escherichia coli* sample isolate resistance trimethoprim-sulfamethoxazole 35 & sensitivity trimethoprim-sulfamethoxazole 81. 53 isolates were selected for genetic studies. The patients were admitted to the Medical City in Baghdad to treat UTIs between November 2020 and February 2021. Samples were collected in sterile cups using the mid-stream urine technique. The urine samples are collected into the sterile tubes; then, the urine is transported to the lab directly[11].

Instruments and Equipment

Many instruments and many types of equipment have been used to process the collected samples, such as Autoclave, Centrifuge, Deep-freezer, Oven, Gel electrophoresis apparatus and power supply, Centrifuge, Eppendorf tube, PCR tube (0.2 µl), Incubator, Magpurix, Micro Centrifuge tubes, Micropipette, microscope, Nano-drop, Refrigerator, RT-PCR, Vortex mixer, Water bath.

The used Media

It is used throughout the study; the name of basic like nutrient agar & enrichment media.

The used primers

The source of all primers used in this study was Macrogen® (Korea). The name, sequence and product size are shown in table (1).

Isolation and Identification of Bacteria

Bacteria were isolated as pure colonies on MacConkey agar, Eosin methylene blue agar, blood agar, then bacterial isolates were examined and identified by microscopic, cultural, biochemical test and Vitek2 system[12].

Microscopical Examination (Gram stain)

Bacterial isolates are examined for Gram stainability; shape and arrangement were observed[12]. The appearance of colonies on the MacConkey agar and Eosin methylene blue agar and blood agar are studied concerning the shape, color and other characteristics[13]. Some drops of 3% hydrogen peroxide reagent were added. The release of gas bubbles within (20–30) seconds indicated a positive result[14].

Confirmation of *E. coli* using API 20 E System

The API, 20 E strip system is a standardized identification technique for non-fastidious, enteric Gram-negative rods belonging to Enterobacteriaceae. The system comprises 20 microtubes that are packed along with dehydrated substrates. These tiny tubes contained the pure microbe that was incubated at (37) C for 24hour. The metabolism results in color changes; the test results are read, and the bacteria is detected. Each test's positive and negative findings are utilized to create a 7 code number to determine the microorganism identification.

Identification and Antibiotic Susceptibility Test

Genomic DNA extraction

gDNA was extracted from the blood according to the company's instructions.

Agarose Gel Electrophoresis

After extraction of gDNA, the electrophoresis is adopted to confirm the DNA[15].

The electrophoresis agarose gel

After sealing the tray's edges with adhesive tape and positioning the comb, the agarose is put into the gel tray at (18-22 C) for a half-hour, then removed from the comb; after that, the gel tray is put in the gel tank, which is filled with 1x TBE buffer until the gel tray is fully immersed[15].

DNA loading and electrophoresis

7 DNA was combined with 3l of 6X loading dye. (Save stain) were put into the wells, then electrical power at 70V for half an hour, resulting in DNA migration from (-) to (+) poles. The save stain are stained bands were photographed and visualized using a UV light transilluminator device (Cleaver Scientific, USA).

Estimation of DNA concentration and purity

Nano-drop NAS-99 spectrophotometer was used to evaluate the concentration and purity of DNA in samples. The Nano-drop was first blanked with 2 µl of DNA rehydration solution (same elution material). The DNA samples were measured one by one, Estimating DNA concentration: reading A at 260nm.

Detection of 16s *rRNA* for *E coli* and sul1 gene And *sul* 2 gene

The primers in this study were used to amplify specific regions of the *sul*1 gene and *sul*2 gene. These primers were shown in (Table 2) as a lyophilized product of different Pico moles concentrations. According to the instructions of the manufacturing company, the primers are added to the water without nucleated at a concentration of 100 pmol /µl; from this solution (10 µl) was added to (90 µl) of water without nucleated to result in primer at (10 pmol/µl) as a table (2).

Name of Primer	Sequence	Product size
SUL1	F: 5 ' CCGCTTCTACAATCAAGTC 3' R: 5' CTGAGATTGGCATTGCTC 3'	144 bp
SUL2	F: 5' CGTTCTATCCGCAATTGG 3' R: 5' CGCAATGTGATCCATGATG 3'	116 bp
16S RNA	F: 5' CAAGGTTAAAACTCAAATGAATTG 3' R: 5' AAGGCACATTCTCATCTC 3'	131 bp

Table 1. The sequence of the used Primer with Product size.

Polymerase chain Reaction components and programs

Polymerase – Chain reaction was carried out after several attempts of optimization to detect the best temperature for annealing with a total volume of 20 µl using LM 2012 Thermal cycler (Wizbio, South Korea). PCR Amplification Program is shown in table (3).

Dye detection (Filter HRM) for EVA green

HRM analysis is an effective method in molecular science for mutation detection, epigenetic and polymorphism differences in DNA samples[16].

Results

Isolation of *Escherichia coli*

Four hundred ninety urine samples were collected from patients with a urinary tract infection (UTI). At the same time, 116 isolates were *Escherichia coli*, from many hospitals in Medical City in Baghdad (National Center for Educational Laboratories, Baghdad Teaching Hospital and Specialized Surgery Hospital) with different gender and ages, during the period between November 2020 and February 2021.

Identification of *Escherichia coli*

Microscopic examination

Escherichia coli isolates are examined under the light compound microscope shown as Gram-negative bacteria, rod-shaped, arranged in single or aggregated in pairs and non–spore-forming according to described[17].

Cultural Characteristics

Cultural characteristics for *E. coli* isolates appeared in the selective media. Morphology of the colony on the MacConkey are included large bright pink colonies due to lactose fermentation, circular, raised low convex with an entire edge, smooth surface and *E. coli* forming green metallic sheen colonies when grown on Eosin methylene blue.

Biochemical Tests

Escherichia coli isolates give results the biochemical tests wherever, a positive result for catalase test, Indole test, Methyl red, and Motility, but they have given negative results to oxidase, Simmons Citrate, Voges Proskauer and Hydrogen Sulfide (H2S) production and give for Triple sugar agar test.

Identification using Vitek 2 system

According to Table (4), the result shows that the identification probability of *E. coli* was (91-93%) for 8 isolates, while it was (93-96%) for 38 isolates and (96-99%) for 70 isolates by using the Vitek 2 system.

Distribution of *E. coli* isolated according to hospital

The overall number of collected specimens was 490, whereas the number of *E. col*i isolates was (116). The prevalence of the identified *E. coli* from National Center for Educational Laboratories was 75) 64.7 % (Baghdad Teaching was 29) 25 % (, and Specialized Surgery was 12)10.3% (, as shown in Table (5).

Escherichia coli antibiotic susceptibility

The present study showed the percentage of resistance *E. coli* isolates against antibiotics as a table (6).

Component	Volume (µl)
PCR Re Mix (Ready to use) EVA Green	10
Forward primer	0.75
Reverse primer	0.75
DNA template	3.5
D.W.	5
Final volume	20

Table 2. Monoplex PCR reaction components for amplifying the targeted fragments to detect *E coli* (16 sRNA).

Steps	Temperature	Time	Cycles
Hold	95	15 min.	1 cycle
Denaturation	95	15 sec.	40 cycle
Annealing	60	20sec.	
Extension	72	20 sec.	
Hold	55	50 sec.	1 cycle
Melting Temperature	65-95	Rising by 0.50 degrees, hold for 1 second for each step< Acquire to [HRM]	

Table 3. Shows PCR Amplification Program.

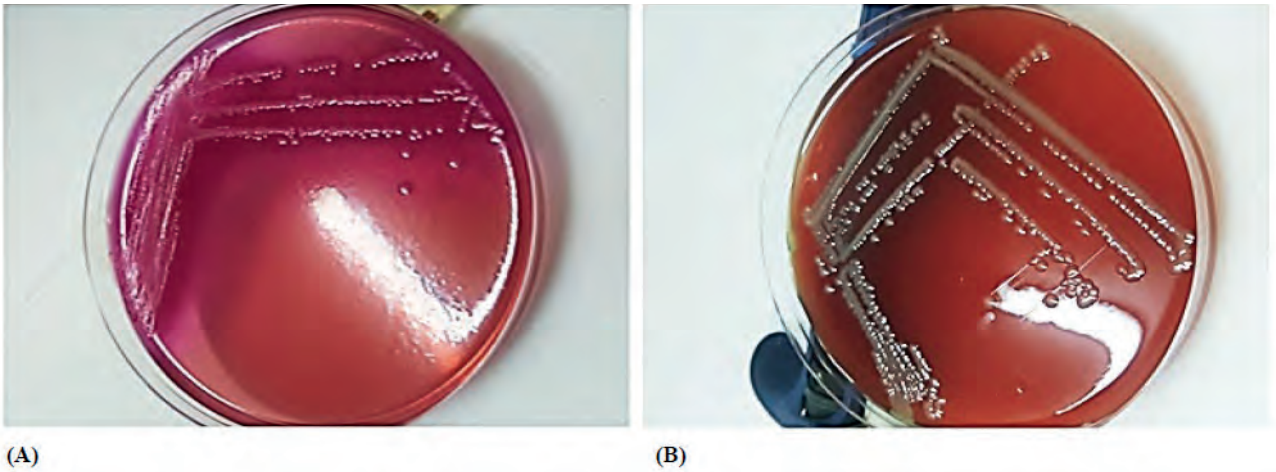


Figure 1. Growth Culture for *E. coli* on (A) MacConkey agar and (B) blood agar showed colony morphology.

Isolate	Identification Probability			Total
	91-93%	93-96%	96-99%	
	Number of isolates			
E. coli	8	38	70	116

Table 4. Species identification Probability of isolates by the Vitek 2 system.

Genomic DNA Extraction

To genomic DNA was extracted from urine samples of patients and healthy controls by automated genomic DNA according to the standard protocol recommended by the manufactured company and manual extraction. Results of extraction showed that there are high concentrations and purity of DNA. The DNA concentration ranged between (110-120) ng/ µl, while the purity ranged between (1.8-2.0), calculated by the Nanodrop NAS-99 spectrophotometer. Genomic DNA extracted from each sample was analyzed on 1% agarose gel. Results of electrophoresis showed clear bands for genomic DNA extracted.

Relationship between antibiotics susceptibility and resistance genes

Percentage of antibiotic resistant isolates and antibiotic sensitive isolates that carry the Sul1 gene and the Sul2 gene. Sul1 gene was found in 11(31%) of Trimethoprim/ sulfamethoxazole resistant isolates. Sul2 gene was found in 8(23%) of Trimethoprim/sulfamethoxazole resistant isolates. The percentage of the antibiotics resistant isolates that have Sul1 gene was 11(31%), while the antibiotics sensitive isolates that have Sul1gene was 1(6%). The percentage of the antibiotics resistant isolates that have Sul2 gene was 8(23%), while the antibiotics sensitive isolates that have Sul1 gene was 0(0%), as shown in Table (7) and figure (2).

Discussion

Distribution of age & sex among infected patients with *E. coli* Isolates

The current study found that the highest prevalence of *E. coli* isolates was at age (21-40) years in 15(51.7%) males and 40(46%) females, while the lowest prevalence was at age >=20 years at 1(3.4%) in the males and 12(13.8%) in the females. According to Al-Saadi (2018), distribution of the infected patients with four *E. coli*, three isolates was obtained from the female and one isolate from male according to within two age groups (41-50) and (61-70) years, respectively. Urinary tract infection was in age > 18 years was (12.9%) in females, while in the male (24.6%), the same study found that UTI was the percentage of the female at age (19–40) years was (32.4%), while the percentage of the male was (17. %) (16). the UTI percentage in males was (52%) while in females was (48%). Some reports were shown that the UTI rate was (23.3%) in males and was (76.7%) in females[18]. While the previous stud showed that UTI rates were (44.24%) and (55.75%) in males and females, respectively[19]. the percentage of UTs by *E. coli* was (35.3%) in males and (64.7%) in females[20].

UTI Occurrence is associated with changes in the host's immunity, like immune suppression, and the diseases which cause immune inhibition such as hyperadrenocorticism, diabetes mellitus, tumors, uroliths, and indwelling catheters. *E. coli* are normal flora in the urethra and could be transmitted to all urinary tract parts. The virulence factors of *E. coli* help invade the cells and then generate toxins to inhibit immune system activity. Causes of UTI in females are common. Therefore it occurs in a high percentage. Short urethra and complex physiology during pregnancy are common causes of UTI in young females. Additionally, contraceptives could increase the risk factors of UTIs[7].

The highest percentage of isolates, 261(73.1%), were obtained from females and 96(26.7%) from males[21]. The patient's age (2 months-90 years). The age (21-30) years group showed that *E. coli* was most common (23.53)%, followed by the group more than ten years (12.61)%, and the lowest rate was among the group (81-90) years (3.36)%. Eighty-one positive *E. coli*, the males were 17(21%) isolates, and the females were 64(79%) isolates. The age category (31-45) years is the most susceptible group for UTI was (41.98) %, followed by the age category (16-30) years was (27.16)%, the age category (46-60) years was (19.75)%, the age category (>60) years was 7.4% and the age category (0-15) years was (3.7)%. These results may suggest that UTI are common in the age category (16-60) years[22].

Hospital Name	Total number of isolates	Number and Percentage of *E. coli* isolates
National Center for Educational Laboratories	310	75 (64.7 %)
Baghdad education	125	29 (25 %)
Specialized Surgery	55	12(10.3%)
Total	490	116(100%)

Table 5. Prevalence of *E. coli* isolates according to hospital.

	Resistant		Sensitive		Intermediate		Total		<P-value	
	N.	%	N.	%	N.	%	N.	%		
Trimethoprim/ ulfamethoxazole	**35**	**30.2**	**81**	**69.8**			**116**	**100**	**0.00**	**significant**

Table 6. Number and percentage of resistance, sensitivity, and intermediate of *E. coli* isolates.

	Resistant		Sensitive		Total		<P value	
	Count	%	Count	%	Count	%		
***Sul*1**	11		1		12		0.02	significant
Total	35	31	18	6	53	23		

	Resistant		Sensitive		Total		<P value	
	Count	%	Count	%	Count	%		
***Sul*2**	8		0		8		0.00	significant
Total	35	23	18	0	53	15		

Table 7. Shows the number and percentage of resistance, sensitivity, and total isolates with *Sul*1 and *SuL*2 gene.

Antimicrobial Susceptibility

The current study demonstrated that the percentage of resistance, sensitivity, and intermediate isolates toward the trimethoprim-sulfamethoxazole were 30.2%, 69.8%, and 0%, respectively. Many studies survey the percentage of Trimethoprim-sulfamethoxazole resistance in *E. coli* isolated from UTIs. The percentage of Trimethoprim-sulfamethoxazole's resistance was (91.1%) by (23), (26.9%) by (24), (32.25%) by (25), and (57.3%) by (26). In contrast, the prevalence of sensitivity to trimethoprim-sulfamethoxazole in *E. coli* isolates was (31.3%) by (27), (70.2%) by (28), (67.74%) by (24), and (20.0%) by (29). The resistance of bacteria to trimethoprim occurs due to higher production of the enzyme (DHFR) targeted promoter for the antibiotic by promoter mutation[29], and the resisted *E. coli* to Sulfonamides antibiotic by the massive production of the enzyme (PABA), the sulfonamides mimic PABA. Therefore, the antimicrobials have difficulty reaching the target[30]. At the same time, the resistance of *E. coli* to Trimethoprim/sulfamethoxazole is due to chromosomal mutations in the *dhfr* or *dhps* genes that cause antibiotic resistance[31]. Many studies have dealt with the issue of the emergence of the phenomenon of resistance to antibiotics by many bacteria. Antibiotic resistance is common among bacteria, especially bacteria that cause disease states and lead to treatment failure or unresponsiveness[32]. Antibiotic resistance is activated by several mechanisms, either through genetic resistance, including the efflux mechanism or through acquired mechanisms and mutations in the genetic material and plasmids[33]. There are six causes of antibiotic resistance: the high concentration of antibiotics, not finishing the therapeutics course, common use of antimicrobials in veterinary products, Poor control of the infection, and poor hygiene and sanitation. Antimicrobial resistance increases due to overuse and misuse of antibiotics and poor infection control. Therefore, the prevalence of antibiotic resistance depends on the risk factors. The absence of step factors or the presence of one or more of the above is the leading and only reason for the phenomenon of variation in the incidence of antibiotic resistance[34].

Prevalence of Sul1 gene in Tri / sulpha resistant *E. coli* isolates

Sul1gene was (31%) in (trimethoprim/ sulfamethoxazole) resistant isolates. This is similar to other previous studies that found the distribution for the sul1gene in sulfamethoxazole-resistant isolates was 36.58%, 22.7%, 34%, 31.4%, and 32%, respectively[35-39]. However, lower results were recorded, Sul1 was found in (10.3%) of sulfamethoxazole-resistant isolates (40), while higher prevalence was shown by previous studies, 53.0%, 45.9%, 41.4% 41-43. Cotrimoxazole Resistance sul1 Genes in *E. coli* isolates were 81.6 %[44].

Prevalence of Tri-sulpha resistant *E. coli* isolates with *Sul*2 gene

The prevalence of the *Sul*2 gene was (23%) in studied

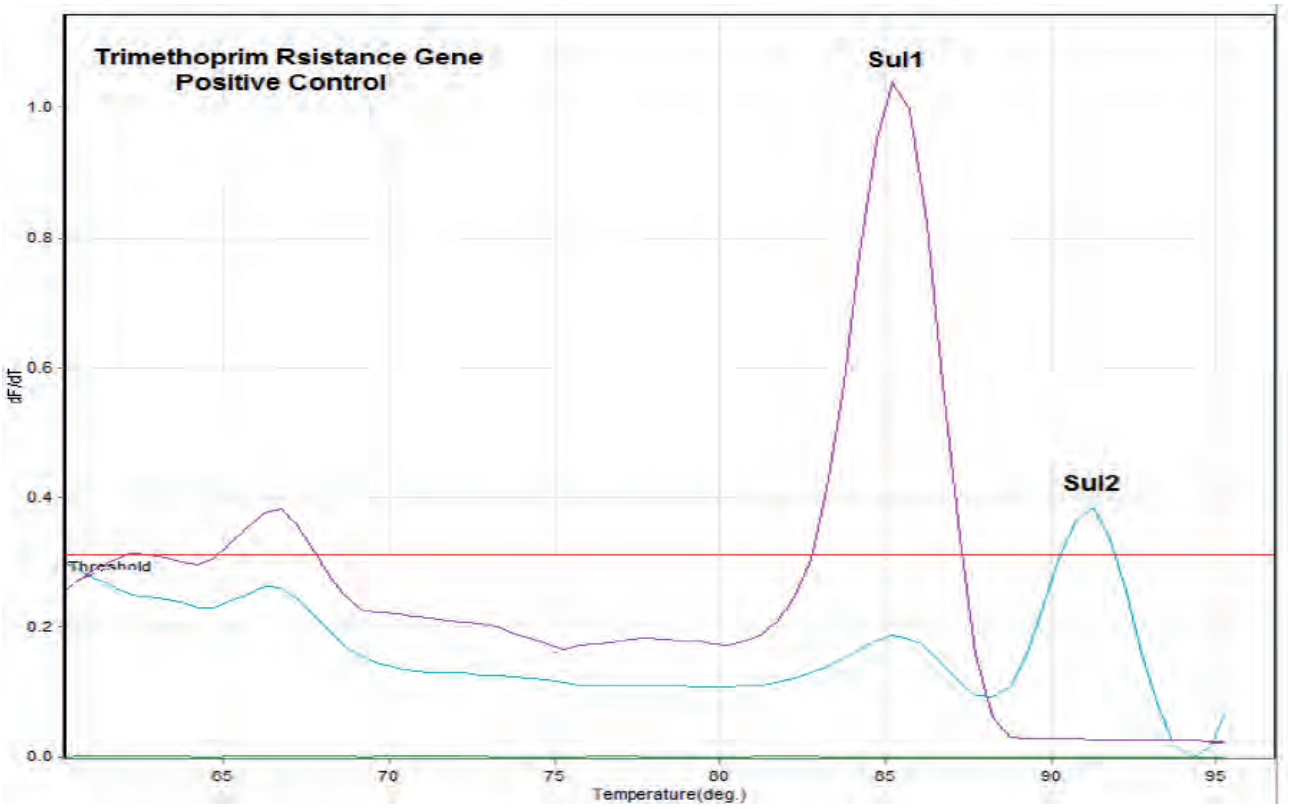


Figure 2. Showed trimethoprim resistance genes wherever the horizontal line represents the temperature while the vertical line represents the df / dt.

E. coli. It is similar to what was found by (45), who reported that *Sul*2 genes and sulfamethoxazole resistance genes were found in the isolates at (18.5%). While it was lower than revealed by other studies, it reported that *Sul*2 genes, the most common sulfamethoxazole resistance gene, were (77.9%) isolates[46]. On the other hand, Cotrimoxazole Resistance *sul* 2 Genes in.

Escherichia coli

Isolates (66.4 %) has appeared in another report[43]. Genes of *sul* 2 were found in 40% of sulfamethoxazole-resistant isolates[45], but, it was shown (81.0%) in another article[46]. The difference in the prevalence of resistance genes may be due to the difference between countries in the type of treatment used for urinary tract infections that might encourage antibiotic resistance mechanisms development due to high exposure for Trimethoprim/sulphamethoxazole, which is used as the first antibiotic against UTIs, the type of isolates present in each country and the rates of resistance genes in the bacterial isolates.

Conclusions

Escherichia coli isolates resistant to trimethoprim/sulfamethoxazole were high, and most of these isolates have a high percentage of resistance genes *Sul*1 gene and *Sul*2.

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Conflicts of Interest

No conflict.

Funds

Self by authors

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ARTICLE / INVESTIGACIÓN

Effect biological life factors on pregnant woman

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Abstract: Define anemia is the low concentration in the amount of hemoglobin according to age, sex and trimester pregnancy. The pregnant has three trimesters and has an exceptional hemoglobin range in every trimester. The pregnant has 124-135 g/dl in the 1st trimester, 110-117 g/dl in the 2nd trimester and 106-109 g/dl in the 3rd trimester. Many pregnant females had been found with anemia because of a low level of Iron. This leads to megaloblastic anemia because of iron deficiency anemia and B12 Vitamin, diet, gastrectomy, malabsorption, blood loss and physiological demand (pregnancy infancy and adolescence). The blood consists of Hemoglobin (Heam + globin) Heam and Iron protoporphyrin ring. Globin is the type of protein with a specific length of amino acid and a specific type of A, maybe 2 Alpha 2 Beta called A1 or 2 Alpha and 2 Bata called A2 or 2Alpha and Gamma called F Fetal. The production of hemoglobin depends on the previously mentioned factors. In this study, we focused on some factors associated with the amount of blood and how these factors affected anemia, which is the most important in Iron treatments, with considering other factors such as food, diversity, Economic situation, social status, number of the fetus, and number of pregnancies. Statistical analysis was used to detect the effect factors of anemia in pregnant. This study refers to 23.2% of anemia because of Iron deficiency during pregnancy. So, treatment of Iron deficiency reduces anemia during pregnancy time.

Key words: Anemia, Hemoglobin, Pregnant Woman.

Introduction

During pregnancy, anemia is a disease that is still one of the significant public health concerns worldwide because of its highly prevalent, especially in developed countries such as Sub-Saharan Africa and Southeast Asia[1]. In addition to causing maternal morbidity and death, it is a significant factor in poor pregnancy outcomes. A woman's risk of postpartum hemorrhage, pregnancy-induced hypertension, postnatal sepsis, a greater chance of preterm delivery, small-for-gestational-age and low birth weight infants, stillbirth, and other adverse perinatal outcomes increases if she has anemia during pregnancy[2]. For developing nations with weak health systems, such as those in Sub-Saharan Africa and Southeast Asia, the economic cost of an anemic pregnancy and its repercussions are tremendous. This scenario has lasted for many years despite the availability of low-cost but very effective interventions, such as iron-foliate supplementation in pregnancy[3].

According to the World Health Organization, about 38 percent of pregnant women worldwide were anemic in 2011[4].

Several researchers have investigated anemia in pregnancy throughout the globe with varying results. According to research, anemia was detected in 42.6 % of pregnant women in Kathmandu, Nepal. The majority (90.8 %) had mild anemia, 7.1 % had moderate anemia, and 2.7 % had severe anemia[5]. In a study of the prevalence and risk factors for anemia in pregnancy, researchers in urban Pakistan discovered that 90.5 % of the study participants were anemic. The majority (75%) had mild anemia, 14.8 % had moderate anemia, and the rest had severe anemia[6]. Institution-based cross-sectional research evaluating the prevalence and determinants of maternal anemia in Northwest Ethiopia found that 16.6% of pregnant women had anemia, with 64% having mild anemia[7]. In a study of anemia in pregnancy and its related variables in Southern Ethiopia, researchers discovered that 39.9% of study participants were anemic. 60 % of anemic women had moderate anemia, 30.3 % had mild anemia, and 9.7% were severely anemic[8]. Anemia was prevalent among booked antenatal customers in Puducherry, India, with 83 % of pregnant women anemic[9]. According to research, anemia was shared among pregnant women in Northern Tanzania, with 74.5 % having mild anemic, 20.9 % having moderate anemic, and 4.5 % having severe anemic[10]. Anemia was prevalent among pregnant women in Gombe, Nigeria, with a frequency of 51.8 %. Mild anemic was discovered in 67.4 % of research participants, moderate anemic in 30.5 %, and severe anemic in 2.1 %[11]. In Zaria, anemia and iron deficiency were tested among pregnant women, and 12.2% of the women were anemic[12]. Anemia in pregnancy was prevalent in 32.3 % of South Nigerian pregnant women seeking prenatal care in a missionary hospital; according to retrospective research, researchers in Enugu, Southeastern Nigeria, discovered that the prevalence of anemic in pregnancy was 40.4 % at the time of booking. Anemia was mild in 90.7 % of these women, with severe anemic accounting for 9.3% of the cases.

There were no cases of severe anemic recorded[13]. In cross-sectional research of hematocrit and arm preference for blood collection among pregnant women in Southeastern Nigeria, it was discovered that 28% of the women were

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anemic, with 94.6 % mildly anemic[14]. Cross-sectional descriptive research in Ilesha, Southwest Nigeria, found that 62.2 % of the women examined were anemic, with 2% having severe anemic[15]. In a study of the sociodemographic variables of anemic in pregnancy, researchers discovered a frequency of 32.8 % in Oyo State, Southwestern Nigeria[16]. In Ibadan, Southwest Nigeria, researchers discovered that 30 % of their study participants — pregnant women – were anemic.

A study of pregnant women in Abeokuta, Southwest Nigeria, found that 76.5 % were anemic, with 40.3 % having mild anemic, 57.8% having moderate anemic, and just 1.9 % having severe anemic[17]. Anemia in pregnancy was prevalent in 35.3 % of pregnant women in cross-sectional research conducted at a tertiary hospital in Lagos, Southwest Nigeria[18]. According to medical and allied literature, anemia in pregnancy has been linked to several variables. Age, parity, gravidity, personal and family income, household size, level of education, poor socioeconomic position, short birth intervals, and greater gestational age at booking, especially booking in the third trimester, are all recognized factors linked to anemic[19-22].

Anemia in pregnant women has also been linked to maternal and fetal anthropometry. Ethiopian researchers showed low body mass index (BMI) as an independent predictor of anemic in pregnancy. In a study of pregnant urban women in Pakistan, non-anemic women's height, weight, and BMI were substantially more significant than their anemic counterparts. Anemia in pregnancy has been linked to dietary variables such as intake of fruit, red meat, eggs, and tea before and during pregnancy and ingestion of nonfood elements such as clay, dirt, and so on (pica)[23]. Anemia has also been linked to anthropometry, nutrition, and socioeconomic position among women who are not pregnant[24,25]. In China, women from higher and medium-income categories were more likely to be anemic than those from lower socioeconomic levels. Many studies have investigated the relations between family food security and different nutritional characteristics in women of reproductive age, such as anemic, dietary consumption, and anthropometry[26-28]. In Ogun State, Nigeria, primary health-care facilities (PHCs) are the initial point of contact for most patients with the organized health system. PHCs provide a broad range of primary care services, including illness prevention, health promotion, and curative treatment. Antenatal care is an essential part of Sagamu's mother and child health services. As a result, the prevalence of anemic and its related variables among pregnant primary care customers in Sagamu, Southwest Nigeria, was evaluated in this research.

According to medical and allied literature, anemia in pregnancy has been linked to several variables. Some recognized characteristics linked with anemic include age, parity, gravidity, personal and family income, household size, level of education, poor socioeconomic position, short birth intervals, and greater gestational age at booking, especially booking in the third trimester[29-33]. Anemia in pregnant women has also been linked to maternal and fetal anthropometry. Ethiopian researchers showed low body mass index (BMI) as an independent predictor of anemic in pregnancy. In a study of pregnant urban women in Pakistan, the height, weight, and BMI of non-anemic women were substantially more significant than those of anemic women. Anemia in pregnancy has been linked to dietary variables such as intake of fruit, red meat, eggs, and tea before and during pregnancy and ingestion of nonfood elements such as clay, dirt, and so on (pica)[34,35]. Anemia has also been linked to anthropometry, nutrition, and socioeconomic position among women of reproductive age who are not pregnant[36-38]. In China, women in upper and medium socioeconomic categories were more likely to be anemic than their low-income counterparts. Many studies have investigated the relations between family food security and different nutritional characteristics in women of reproductive age, such as anemic, dietary consumption, and anthropometry. In Ogun State, Nigeria, primary health-care facilities (PHCs) are the initial point of contact for most patients with the organized health system. PHCs provide a broad range of primary care services, including illness prevention, health promotion, and curative treatment. Antenatal care is an integral part of Sagamu's mother and child health services. As a result, the frequency of anemic and its related variables among pregnant primary care customers in Mosul, Iraq, was evaluated in this research.

Materials and methods

Data collection tool

Data were collected through direct interviews with the pregnant women; the questionnaire will involve information's regarding:

1- Sociodemographic characteristics like age, educational state, occupation, and house condition.

2- Obstetric history: para, gravida, single, twin, spacing between pregnancies, any complications during pregnancy like placenta prevail, abruption placentae, preeclampsia, morning sickness in the 1sttrimester, antenatal care visits and iron supplements.

3- Nutritional history: daily tea intake, fresh fruits, vegetables, meat and fish.

4- Parameters: hemoglobin level before pregnancy.

5- Hemoglobin level in the 3rd trimester of pregnancy

Individual blood sample collection and analysis

Laboratory technicians performed hemoglobin analysis for 300 pregnant women at each center's health post. Each pregnant woman's hemoglobin concentration was measured by taking a finger-prick blood sample and testing it using a HemoCue Hb 301 device (HemoCue AB, Angelholm, Sweden). After cleaning the location with disinfectant, a puncture was performed on the tip of the middle finger. The first drop of blood was washed off, and the second drop was collected to fill the micro cuvette, which was then put in the cuvette holder of the hemoglobin concentration measurement instrument. The meter's performance was verified regularly using a control standard to improve test reliability. When the meter was ready to utilize capillary blood, the micro cuvette container was moved to its loading position, and the sample for analysis was loaded in one continuous operation. It was put in the holder and pressed into its measurement position within 10 minutes after being filled. Finally, utilizing WHO's field survey recommended techniques[39], the result was presented and recorded after 15–60 seconds. Hemoglobin levels were classified into three categories: mild anemic (11–13 g/dl), moderate anemic (9-10.9 g/dl), and severe anemic (7 g/dl). The following formula was used to modify the hemoglobin level for altitude—assessment of dietary, food security, and wealth. Dietary variety scores for

women were calculated using FAO standards[40]. The score was computed, and the tertiles were assigned (low, medium and high). Dark green leafy vegetables, cereals/starchy staples, vitamin A-rich fruits and vegetables, oils/fat, seeds, nuts, legumes and other fruits and vegetables, organ meat, meat and fish, milk and products, and egg were the nine food groups that the pregnant women reported eating the day before the survey. The first level, second level 1, and third-level 2 were assigned to participants who had eaten (at least once) the meal in each subgroup.

The wealth index was built using multi variables related to ownership of selected household assets using a principal components analysis adapted from (41). Some of them are the source of drinking water, household facility(such as TV, Radio, Cell phone, etc.), type of toilet facility, fuel types, building materials and designs, owning a car, and owning a house.

Data analysis and processing

For analysis, the data was coded and put into SPSS version 25. Descriptive statistics (frequency and percentage) were employed to analyze categorical data, while range, mean, and standard deviation displayed continuous variables. The criteria p> 0.05 were used to create a single multivariate model. Before and after pregnancy, a T-test was performed to investigate the connection between test and anemia factors.

Results

The sample analysis result produced the statue of the pregnant women who agreed to participate in the interviews. Their answers were classified and arranged according to the factors (Table1). 23% of the test sample has a good economic condition. 52% have meddled economic status, while 25 % of the pregnant women low economic status. The homemaker's women pregnancy was (69%) and (31%) were employees. (Table1) The nutrition of pregnant women was excellent. The percentage of women eating fruits, vegetables, fish, etc. was very high, 51.5% of them ate daily, while 27% of the women ate fruits weekly and 21.5% of them ate it monthly (Table1) Sociodemographic and economic characteristics, 150 pregnant women had participated in the study. The mean of age ± calculated standard deviation of the participants women was 26 ± (8) years. (38.7%) were 18–24 years old, and (34.6%) were 25–28. (22%) were in the age range of 29–33 and 4.7 from 34 years and above women. Table (2).

The women who took Iron in the first part of pregnancy were 69.1% of all pregnant women who entered the test,20.6% were mild, 38.1% were moderate, and 9.8% were severe. While those who did not take Iron were 30.1%, 10.8% were mild, 10.6% moderate and 4.4% severed and in the third trimester of pregnancy, 74.8% took Iron, 23.8% were mild, 41.4% were moderate, and 9.6% were severe. While those who did not take Iron were 25.2 %, 10.2% were mild, 10.6% moderate, and 4.4% were severe. As shown in Figure (1).

While The women who took on ANC in the first part of pregnancy were 71.5% of all pregnant women who entered the test. 38.2% were mild, 26.5% moderate, and 6.8% severe. While those who did not take Iron were 28.5%, 11.2% were mild, 12.3% moderate, and 5.0% were severe. And in the third trimester of pregnancy, 75.6% who take on ANC, 40.8% were mild, 29.8% were moderate, and 5% were severe. While those who did not take Iron were 24.4%, 40.8% were mild, 29.8% moderate, and 4.7% were severe. As shown in Figure (2).

The economic status is divided into three-part good, middle and low; the women who had good status in the first part of pregnancy were 23 of all pregnant women who entered the test. 12% were mild, 8% were moderate, and 3% were severing. While those with middle status were 52%, 15.6% were mild, 34.3% moderate, and 2.1% were severe. And the women have low status were 25%. Those with mild were 5% and 6.2% moderate, while 5% were low.

The women who had good status in the third part of pregnancy were 23 % of all pregnant women who entered the test. 12% were mild, 8.3% were moderate, and 2.7% were severing. While those with meddle status were 52%, 15% were mild, 33.8% moderate, and 5.8% were severe. And the women have low status were 25%. Those with mild were 2.7% and 2.2% moderate, while 14% were low. As shown in Figure (3).

The visited doctor in the first trimester is divided into three parts weekly, monthly, and no visiting, the women who visited the doctor weekly in the first part of pregnancy were 14 % of all pregnant women who entered the test. 2.2% were mild, 2% were moderate, and 9.8% were severe. While those who visited the doctor monthly were 63.5%, 8% were mild, 14.2% moderate, and 41.3% were severe, and the women no visited were 22.5%, those who had mild were 3.7%, and 2.3% were moderate, while 5% were low.

In the third trimester, the women who visited the doctor weekly in the first part of pregnancy were 6.5 % of all pregnant women who entered the test. 1.3% were mild, 1.9% were moderate, and 6.5% were severe. While those who visited the doctor monthly were 56.8%, 4.7% were mild, 12.6 % moderate, and 3.7 % were severe, and the women no visited were 34% those who had mild were 10.5%, and 19.8% were moderate, while 3.7% were low. As shown in Figure (4).

The complication in the first part of pregnancy was 31.9% of all pregnant women who entered the test. 4.8% were mild, 9.5% were moderate, and 17.6% were severe. While women in 1st trimester who had no compilation were 68.1%, 46.5% were mild, 19.3% moderate, and 2.3% were severe. In the third trimester of pregnancy, 47.1% had complications, 2% were mild, 12.6% were moderate, and 32.5% were severe. While those who had no complications were 56.1 %, 35.6% were mild, 13.7% moderate, and 6.8% were severe. As shown in Figure (5).

The non-pregnants in the first trimester were divided into three parts singles, twins and three & above, the women who have single in the first part of pregnancy were 73.5 % of all pregnant women who entered the test. 27.7% were mild, 45% were moderate, and 1% were severe. While those who have twins were 25.5%, 4.5% were mild, 10% moderate, and 11% were severe, and the women have three & above were 1% .those who had mild were 0%, and 0% were moderate, while 1% were low.

In the third trimester, the women who have single in the first part of pregnancy were 73.5 % of all pregnant women who entered the test. 23% were mild, 48% were moderate, and 2.5% were severe. While those who have twins were 25.5%, 1.5% were mild, 8% moderate, and 16% were severe, and the women have three & above were 1% .those who had mild were 0%, and 0% were moderate, while 1% were low. As shown in Figure (6).

Factor	Answers	First trimester			Third trimester		
		mild	Moderate	Sever	Mild	moderate	Sever
Iron supplement	Yes	20.6%	38.7%	9.8%	23.8%	41.4%	**9.6%**
	No	10.8%	11.5%	8.6%	10.2%	10.6%	**4.4%**
*ANC	Yes	38.2%	26.5%	6.8%	40.8%	29.8%	**5.0%**
	No	11.2%	12.3%	5.0%	9.7%	8.7%	**4.7%**
Economic status	Good	12.0%	8.0%	3.0%	12.0%	8.3%	**2.7%**
	Meddle	15.6%	34.3%	2.1%	15.0%	33.8%	**5.8%**
	Low	5.0%	6.2%	13.8%	2.7%	2.2%	**14.0%**
Visit doctor	Weekly	2.2%	2.0%	9.8%	1.3%	1.9%	**6.5%**
	Month	8.0%	14.2%	41.3%	4.7%	12.6%	**39.5%**
	no visited	16.5%	3.7%	2.3%	10.5%	19.8%	**3.7%**
Complications	No	46.5%	19.3%	2.3%	35.6%	13.7%	**6.8%**
	Yes	4.8%	9.5%	17.6%	2.0%	12.6%	**32.5%**
No. pregnant	Single	27.5%	45.0%	1.0%	23.0%	48.0%	**2.5%**
	Twin	4.5%	10.0%	11.0%	1.5%	8.0%	**16.0%**
	Third	0.0%	0.0%	1.0%	0.0%	0.0%	**0.0%**
Sickness	No	38.5%	13.6%	4.3%	42.4%	15.3%	**3.8%**
	Yes	24.2%	6.8%	12.6%	20.4%	5.6%	**12.5%**
**UTI	No	15%	17%	21.7%	13%	15%	**26.4%**
	Yes	15.6%	23.5%	8.3%	16.5%	20%	**9.1%**
Education	Illertral	5.0%	11.5%	5.0%	5.0%	11.4%	**5.1%**
	Primary	11.2%	33.7%	1.3%	11.0%	33.9%	**1.3%**
	Secondary	18.8%	33.6%	2.1%	18.5%	34.0%	**2.0%**
	University & above	2.3%	11.2%	1.6%	2.1%	11.5%	**1.5%**
Occupation	Employee	11.0%	14.0%	6.0%	11.3%	13.8%	**6.1%**
	nonemployee	20.0%	46.0%	3.0%	19.0%	46.0%	**4.0%**
how often you eat fresh fruits, vegetables, meat & fish	Day	17.0%	32.0%	2.5%	17.0%	31.8%	**2.7%**
	Weekly	10.0%	11.0%	6.0%	10.0%	10.7%	**6.3%**
	Month	6.0%	4.0%	11.5%	5.3%	3.6%	**12.6%**
Daily tea intake	Yes	35.7%	27.5%	25.4%	26.7%	21.0%	**10.1%**
	No	6.8%	3.2%	1.4%	22.7%	12.6%	**6.9%**
Marital status	Married	11.2%	65.0%	13.9%	11.2%	63.0%	**14.9%**
	Widow	3.8%	3.0%	1.1%	3.6%	3.2%	**1.1%**
	Divorced	**0.0%**	**1.0%**	**1.0%**	**0.0%**	**0.0%**	**2.0%**

***(ANC) Active Nose Control**

****(UTI) Urinary Tract Infections**

Table 1. Shows the percentage the statue of pregnant women entering the test with the element factor.

Age	%
18 – 24	38.7
25 – 28	34.6
29 – 33	22
34 and above	4.7
Total	100.0

Table 2. Age of the pregnancy women.

The sickness in the first part of pregnancy was 43.6% of all pregnant women who entered the test. 24.2% were mild, 6.8% moderate, and 12.6% severe. While those who did not have sickness were 56.4%, 38.5% were mild, 13.6% moderate, and 4.3% were severe in the third trimester of pregnancy, 38.5% had sickness, 20.4% were mild, 5.6% were moderate, and 12.5% were severe. While those who did not have sickness were 56.4 %, 42.4% were mild, 15.3% moderate, and 3.8% severe. As shown in Figure (7).

In the first part of pregnancy, the UTI was 47.4% of all pregnant women who entered the test. 15.6% were mild, 23.5% were moderate, and 8.3% were severe. While those who did not have UTI were 52.6%, 15% were mild, 17% moderate, and 21.7% were severe. In the third trimester of pregnancy, 45.6 % suffered from UTI, 16.5% were mild, 20% were moderate, and 9.1% were severe. While those who did not have UTI were 54.4 %, 13% were mild, 15% moderate, and 26.4% were severe. As shown in Figure (8).

The education in the first trimester is divided into four parts illiterate, primary, secondary and university & above; the women illiterate in the first part of pregnancy were 21.5 % of all pregnant women who entered the test. 5% were mild, 11.5% were moderate, and 5% were severe. While those with primary education were 46.2%, 11.2% were mild, 33.7% moderate and 1.3% were severe, and the women with secondary education were 54.5%. Those with mild were 18.8% and 33.6% moderate, while 2.1% were low, while the women with university level of education and above were 15.1%. Those with mild were 2.3% and 11.2% moderate, while 1.6% were low. During the third trimester, 21.5 percent of all pregnant women who entered the test were illiterate women in the early phase of pregnancy. 5% were mild, 11.4% were moderate, and 5.1% were severe. While those who were a primary education were 46.2%, 11% were mild, 33.9% moderate, and 1.3% were severe, and the women are secondary education were 54.5%. Those who have mild were 18.5%, and 34% are moderate while 2% were low, while the women With university level of education & above were 15.1%. Those with mild were 2.1% and 11.5% moderate, while 1.5% were low, as shown in Figure (9).

The occupation was divided into two employees and a nonemployee; the employer in the first part of pregnancy was 31 % of all pregnant women who entered the test. 11% were mild, 14% were moderate, and 6% were severe. While nonemployees were 69%, 20% were mild, 46% moderate, and 3% severe. In the third trimester of pregnancy, 31.2% were nonemployees, 11.3% were mild, 13.8% moderate, and 6.1% severe. Nonemployee pregnant women were 68.8 %, 19% mild, 46% moderate, and 4% severe. As shown in Figure (10).

They feed on fresh fruit, etc., in the first trimester, divided into three classifications daily, weekly and monthly; the women who had it daily in the first part of pregnancy were 51.5% of all pregnant women who entered the test. 17% were mild, 32% were moderate, and 2.5% were severing. While those eating weekly were 27%, 10% were mild, 11% moderate, and 6% were severe, and the women who ate it monthly were 21.5%. The mild was 6%, and 4% were moderate, while 11.5% were severe.

The women who had fresh food daily in the third part of pregnancy were 51.5% of all pregnant women who entered the test. 17% were mild, 31.8% were moderate, and 2.7% were severe. While those eating weekly were 21.5%, 10% were mild, 10.7% moderate, and 6.3% were severe, and the women who ate it monthly were 21.5% .those who had mild were 5.3%, and 3.6% were moderate, while 12.6% severed. As shown in Figure (11).

The number of women drinking tea in the first part of pregnancy was 88.6% of all pregnant women who entered the test. 35.7% were mild, 27.5% were moderate, and 25.4% were severe. While those who did not drink tea were 11.4%, 6.8% were mild, 3.2% moderate, and 1.4% were severed in the third trimester of pregnancy, 57.8 % were drinking, 26.7% were mild, 21% were moderate, and 10.1% severed. While those who did not drink were 42.2 %, 22.7% were mild, 12.6% moderate, and 6.9% were severe. As shown in Figure (12).

The marital status of the women pregnant in the first trimester was divided into three classes married, widow and divorced; the women who married in the first part of pregnancy were 90.1% of all pregnant women who entered the test. 11.2% were mild, 65% were moderate, and 13.9% were severe. While those who are widows were 7.9%, 3.8% were mild, 3% moderate, and 1.1% were severe, and the women who are divorced were 2%. The mild was 0%, and 1% was moderate, while 1% was severe.

In the third part of pregnancy, the married women were 89.1% of all pregnant women who entered the test. 11.2% were mild, 64% were moderate, and 14.9% were severing. While those who are widows were 7.9%, 3.6% were mild, 3.2% moderate, and 1.1% were severe, and the women who are divorced were 2%. The mild was 0%, and % were moderate while 2% were severe. As shown in Figure (13).

When comparing patients' outcomes with the control group, the effects of the variables were highly close. The most significant effect was on the iron supplementation factor ($p<00.5$) and the same effect in the control group. The feeding factor had the most excellent effect on the sample where ($p<00.5$) Health has moderately affected the patient sample and the control group sample. Also, frequent doctor visits and maintaining good health had a positive effect by lowering the incidence of anemia among patients ($p<00.5$).

Discussion

Anemia was found to be prevalent in 23.2 percent of the research participants. In the first trimester, 57.3 percent of women had mild anemic, 29.3% had moderate anemic, and 13.3% had severe anemic, whereas the total incidence of anemic was 24.5 percent in the third trimester. 58 percent of those had mild anemic, 32 percent had moderate anemic, and ten percent had severe anemic. The prevalence of anemic among pregnant women in this research was classified as a moderate public health concern by the WHO[42]. The prevalence found in this research was similar to that

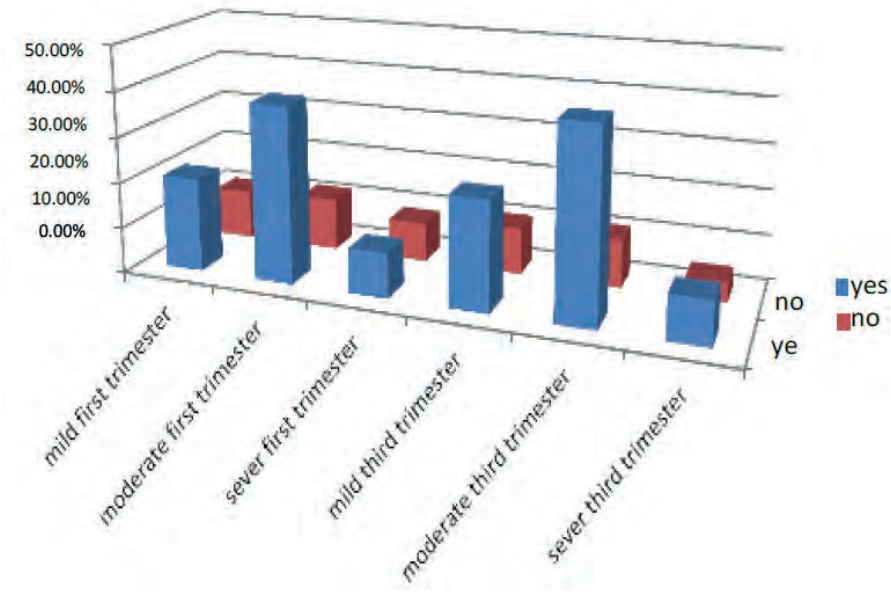


Figure 1. Shows the treatment of pregnant women with Iron.

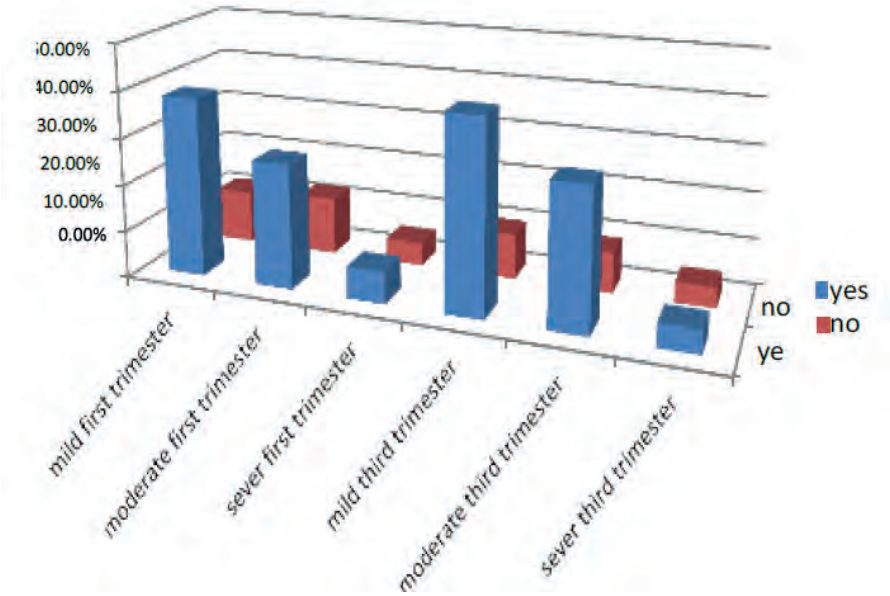


Figure 2. Illustrates how pregnant women are treated with ANC.

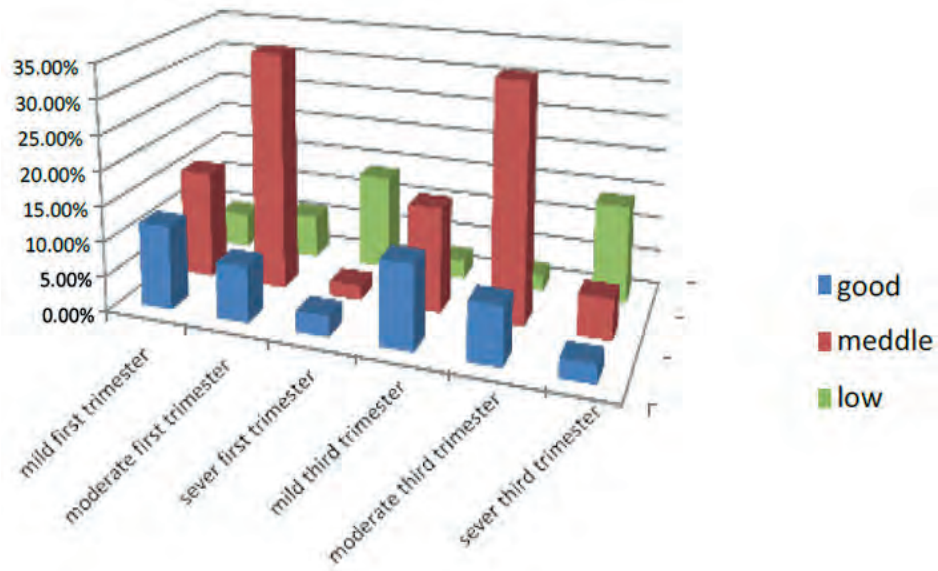


Figure 3. Illustrates pregnant women's economic status.

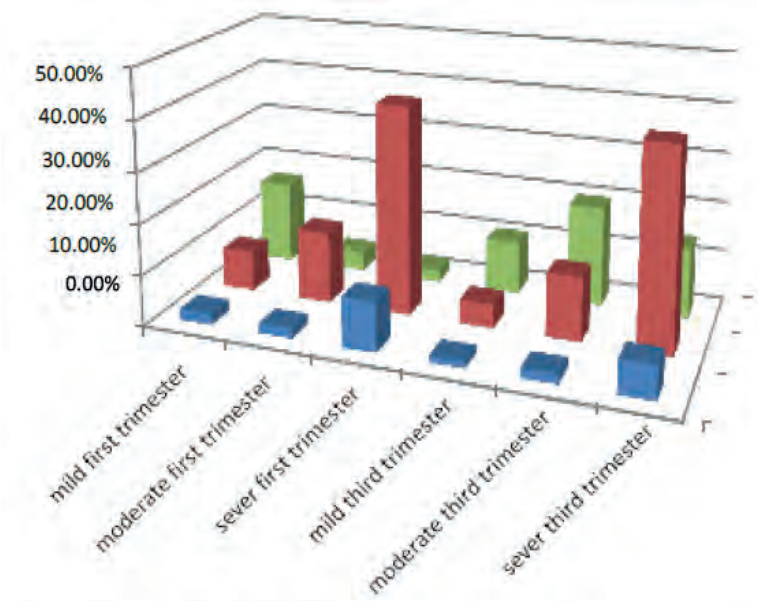


Figure 4. Shows who have visited the doctor the pregnant women.

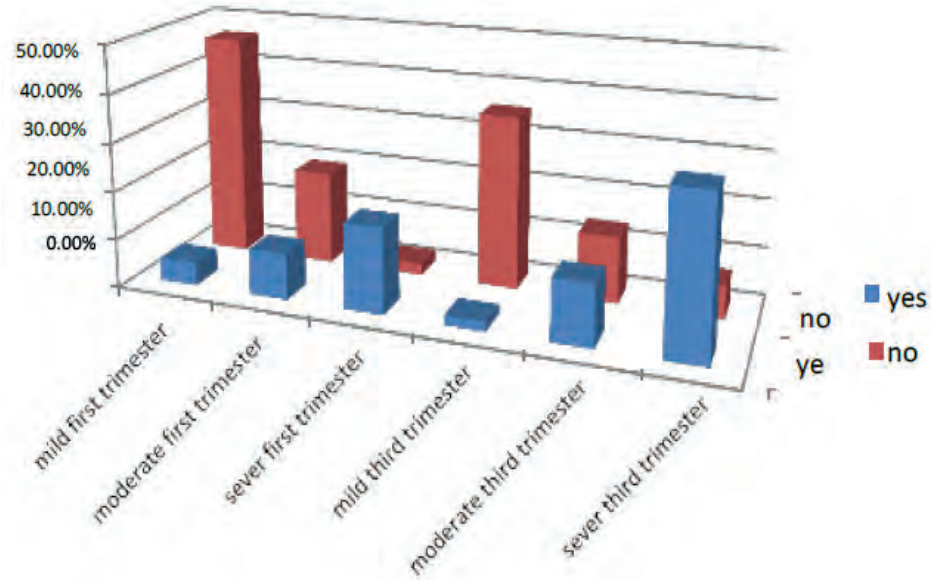


Figure 5. Shows the treatment of pregnant women with complication factor.

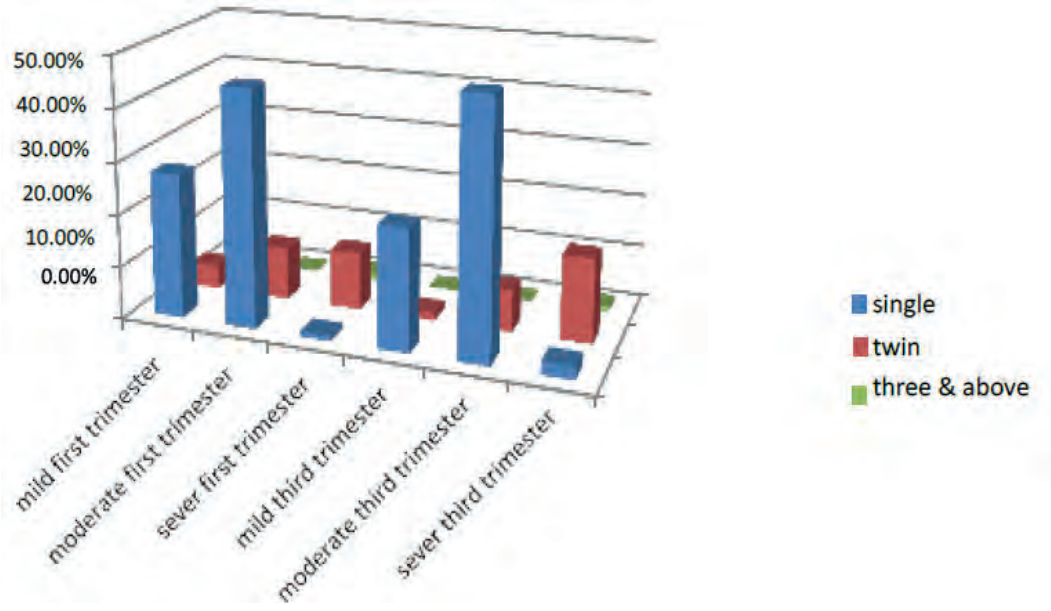


Figure 6. Shows the No. pregnant of the pregnant women.

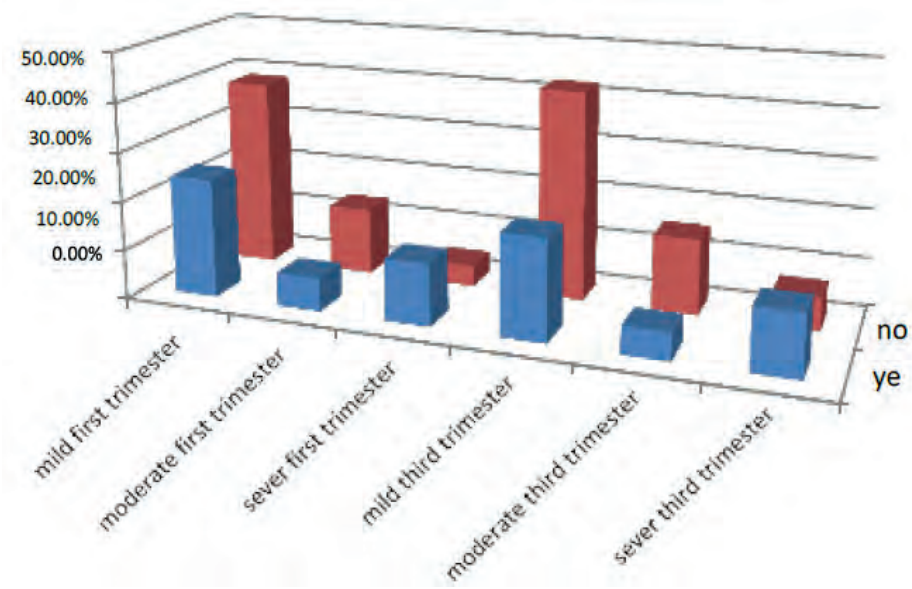


Figure 7. Shows the treatment of pregnant women with sickness Factor.

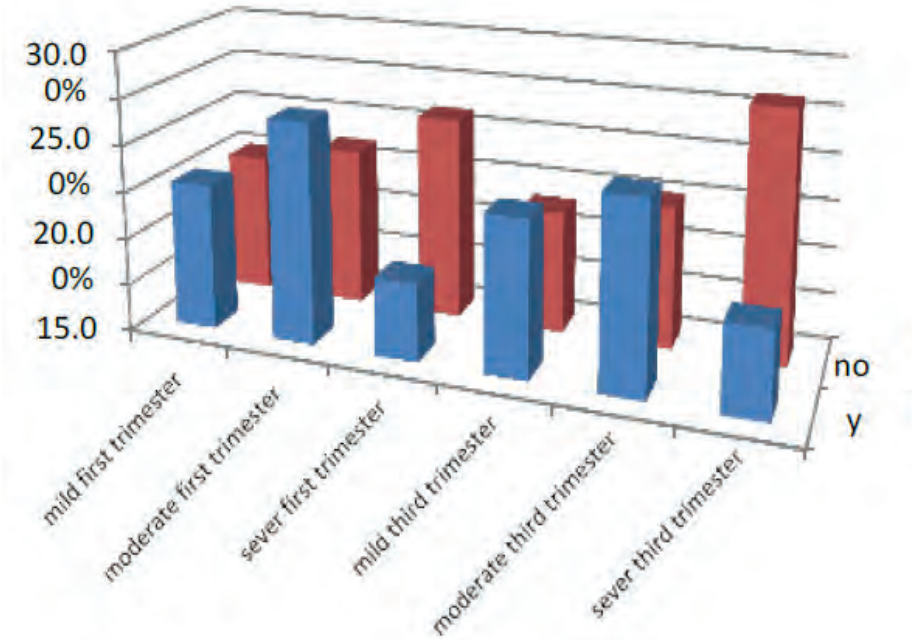


Figure 8. Shows the treatment of pregnant women with UTI factor.

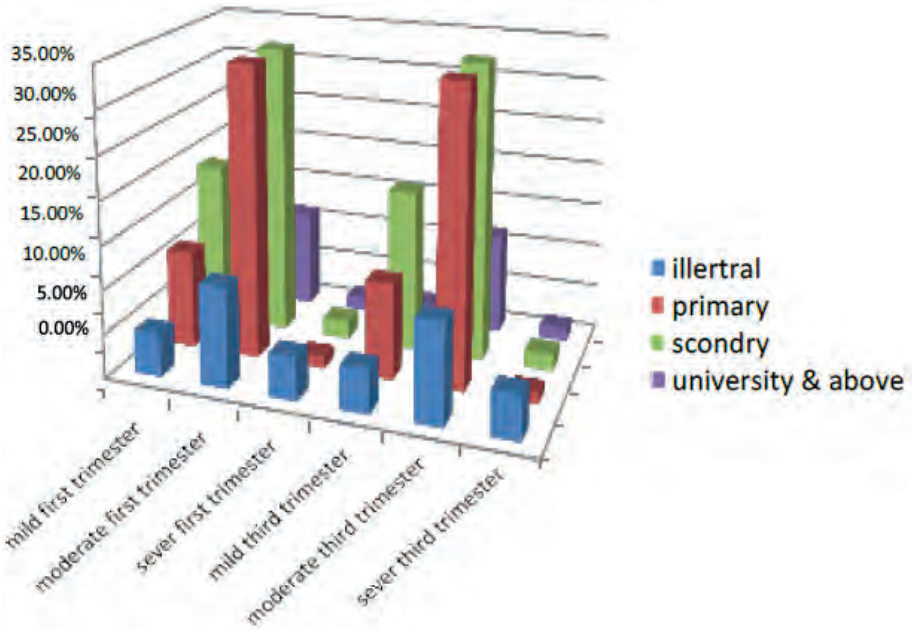


Figure 9. Illustrates the degree of pregnant women's education.

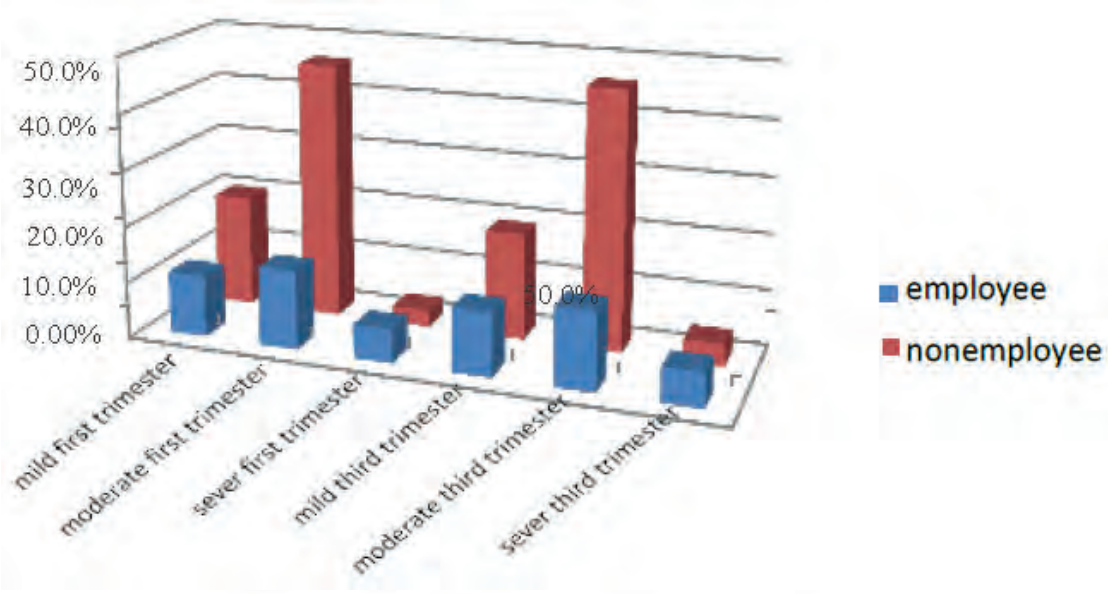


Figure 10. Shows the treatment of pregnant women with sickness factor.

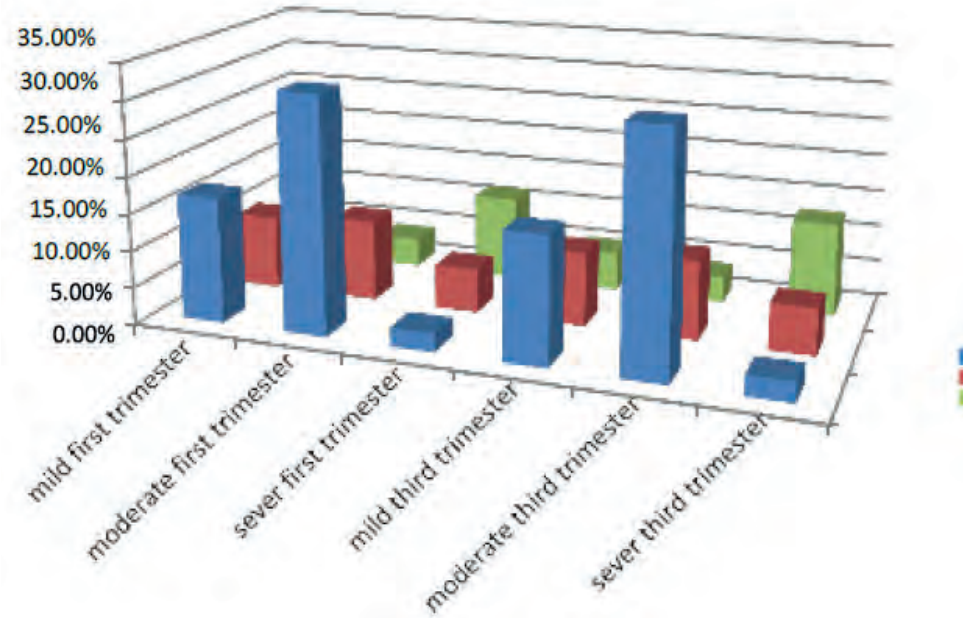


Figure 11. Shows the feed factor of the pregnant women.

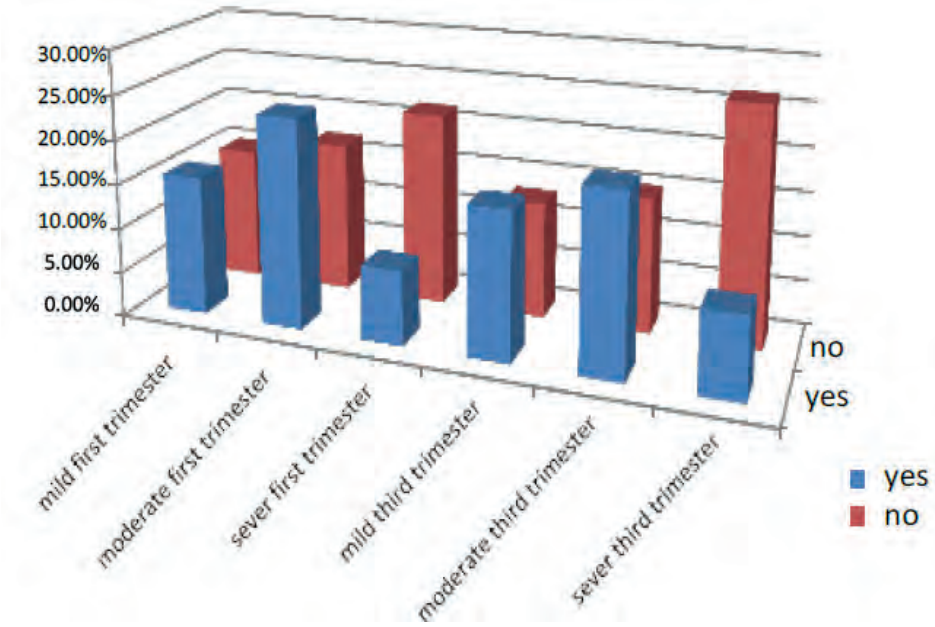


Figure 12. Shows the treatment of pregnant women who are drinking tea.

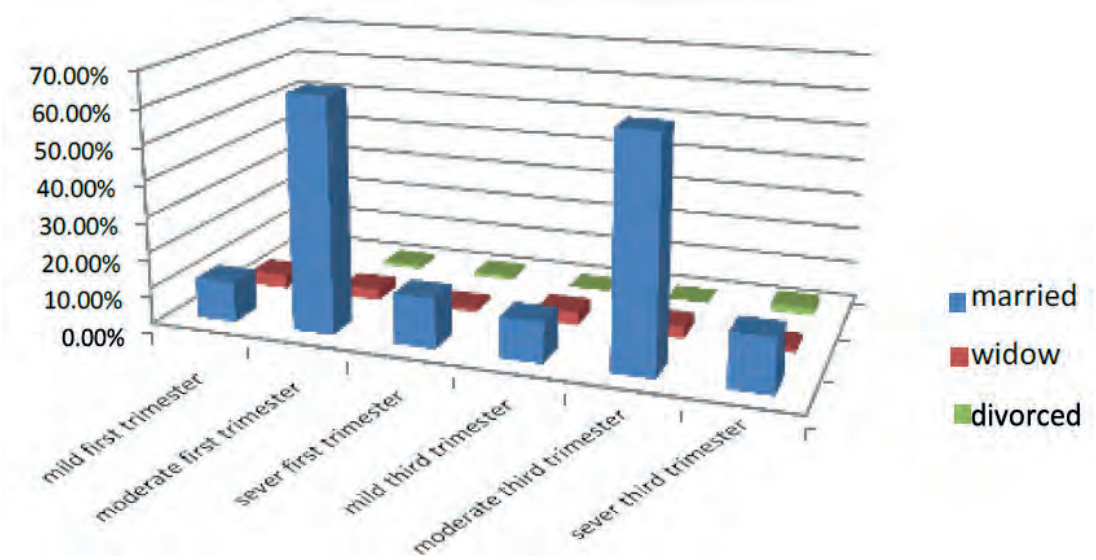


Figure 13. Shows the marital status factor of the pregnant women Factors associated with anemia.

seen in previous studies performed across the globe[43]. [2] As well as the national prevalence[44], which indicated 21% and 22%, respectively. However, it was lower than previous studies conducted in other areas of Iraq that were reported (32.8 percent)[45], 36.6 percent[46] [as well as (39.9%)[47] These disparities in anemic prevalence may be attributable to socioeconomic, cultural, and nutritional variances across areas within the same nation.

Anemia in pregnant women was linked to the household's socioeconomic level. Anemia was more common in women from lower socioeconomic classes than in those from higher socioeconomic classes. Our findings were in line with those of previous research performed in Nigeria and India, which found a greater incidence of anemic among lower socioeconomic groups[48,49]. This may be because low-income women cannot afford high-quality meals in sufficient quantities.

The second component in this research that revealed a significant relationship with anemia was gestational age: women in their third trimester were 3.09 and 3.68 times more likely to be anemic than those in their first trimester. According to research performed in various regions of Iraq, women in the third trimester had a higher chance of getting anemic than those in the first trimester[50,51].

Similarly, research in India showed that the incidence of anemic was greater in third-trimester pregnant women[52,53]. This may be because the need for calories and nutrients rises during pregnancy to support increased maternal metabolism, blood volume, and nutrition delivery to the fetus[54], and this demand increases even more in the second and third trimesters of pregnancy. The absorption of Iron decreases dramatically in the first trimester, owing to reduced iron needs and the cessation of menstruation, saving a median of 0.56 mg Fe/day (160 mg/pregnancy)[55] However, iron absorption from a diet with high iron bioavailability rises by 1.9 mg/day in the third trimester and up to 5.0 mg/day in the final trimester[56,57].

As a result, in the research region, effective intervention packages must decrease anemic among pregnant women via iron supplementation and dietary diversity. The research found that supplementing with Iron had a significant effect on hemoglobin and should be used in conjunction with iron supplementation, particularly in populations with poor iron bioavailability in their diets[58,59].

Furthermore, women with a low dietary variety score were 3.18 times more likely than those with a higher dietary diversity score to develop an anemic. This result was in line with earlier research in idea[60,61] which found that women with a low dietary variety score and who did not eat iron-rich foods were more likely to develop anemic than their counterparts. Similarly, in Ethiopia, women who followed a stringent dietary pattern were more likely to be anemic than those who did not[62]. Fruit intake two or more times per week was similarly linked to a lower incidence of anemic in studies performed in Pakistan and Turkey[63]. Furthermore, another research from Ghana found that a high level of maternal dietary variety was linked to a lower incidence of anemic, indicating that nutritional variables are relevant. This may be evidence that an increase in individual dietary variety score is linked to improved nutritional adequacy and that it can be utilized as a proxy indicator for determining nutrient adequacy in pregnant women[64,65].

Cross-sectional surveys are the most common way to assess the severity of health issues, but they have limitations in showing the time sequence of causes and outcomes. Furthermore, this research did not examine micronutrients such as folic acid, vitamin B12, and vitamin A.

Finally, iron deficiency anemic in pregnant women in the Middle East is a moderate public health issue. This research group linked socioeconomic level, gravidity, trimester, iron supplementation, and dietary variety score were all linked with anemic. The prevalence of an anemic may influence pregnant women's delivery outcomes. Improvements in eating habits, iron supplementation, and family planning promotion conclude a modest public health concern about iron deficiency anemic among pregnant women in the Middle East. The anemic variables in the study population were socioeconomic level, gravity, quarter, iron supplementation, and dietary variety. The frequency of anemic detected may influence the birth rate of pregnant mothers. Improving dietary habits, iron supplements, and family planning promotion, the study will be connected to four primary health care centers in Mosul, two of them on the right side of the river Tigris (Tamooaz and al Nahrwoan) and 2 centers on the left side (al Zahoor and alqramia).

Sample size: the study included 150 cases of pregnant women in the 3rd trimester diagnosed with anemia according to hemoglobin level and 150 control women in the 3rd trimester without anemia.

Factor	Time	P-value
Age	First trimester	.445
	Third trimester	.422
marital status	First trimester	.772
	Third trimester	.899
Education	First trimester	.039
	Third trimester	.022
Occupation	First trimester	.000
	Third trimester	.000
how often you eat fresh fruit, vegetables, meat, & fish	First trimester	.001
	Third trimester	.001
daily tea intake	First trimester	.564
	Third trimester	.742
iron supplement	First trimester	.001
	Third trimester	.004
UTI	First trimester	.049
	Third trimester	.596
Sickness	First trimester	.009
	Third trimester	.011
ANC	First trimester	.001
	Third trimester	.001
economic status	First trimester	.559
	Third trimester	.363
time between pregnant	First trimester	.000
	Third trimester	.000
no. pregnant	First trimester	.504
	Third trimester	.455
Complications	First trimester	.193
	Third trimester	.786
visit doctor	First trimester	.000
	Third trimester	.000

P-value < 0.05 significance

Table 3. Shows the P-value for all factors of deferent between element factor and first trimester, third trimester.

Conclusions

The blood consists of Hemoglobin (Heam + globin) Heam: Iron protoporphyrin ring Globin: it is the type of protein with a specific length of amino acid and specific type of A is maybe 2 Alpha 2 Beta called A1 or 2 Alpha and 2Bata called A2 or 2Alpha and Gamma called F Fetal.

In this study, we focused on different factors associated with the amount of blood and how these factors affected anemia; the most important is Iron treatment, but we have to remember the other factor such as food, diversity, Economic situation, Social status, number of fetus and number of pregnancies.

Statistical analysis was used to detect the affecting factors of anemia in pregnant women; this study refers to

23.2% of anemia because of Iron deficiency. So, the study proved a relation and effect between Iron deficiency and anemia in pregnancy.

Author Contributions

I've been working on my own

Funding

There is no funding from any party. The work was done at the researcher's expense.

Ethical considerations

Verbal consent should be taken from each patient before data collection for ethical concerns.

Data Availability Statement

University of Mosul / College of Environmental sciences and technologies.

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Conflicts of Interest

There is no conflict of interest in the research.

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ARTICLE / INVESTIGACIÓN

In vitro study of the antibacterial effect of plasma surface treatment using Argon gas on orthodontic stainless steel brackets against *Streptococcus mutans* and *Lactobacillus acidophilus*

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Abstract: Orthodontic treatment improves dental aesthetics. However, the prolonged duration of treatment and patient's oral hygiene results in more significant biofilm buildup and boosting concentrations of acidogenic bacteria, resulting in enamel demineralization. Plasma treatment technology has developed an interest in enhancing appliances' surface properties. The present study aimed to investigate cytotoxicity and antibacterial activity represented by anti-adhesion and anti-biofilm formation of Argon plasma treatment on orthodontic brackets against *Streptococcus mutans* and *Lactobacillus acidophilus* and evaluation the effect of change in plasma exposure duration on these properties in vitro. The study included three groups, group 1: untreated stainless steel brackets, group 2: brackets treated for 15 min, and group 3: brackets treated for 30 min. S.mutans and L.acidophilus were isolated from patients undergoing fixed orthodontic treatment. Microbiological tests included adhesion assay using plate counting method and biofilm formation assay using ELISA plate reader. S. mutans& L. acidophilus were cultivated in three groups, and their adherence and biofilm formation were measured and compared. The biocompatibility of treated stainless steel brackets was evaluated by MTT assay. Results showed that groups 2 & 3 had better anti-adhesion and anti-biofilm activity when compared with group 1, with a significant difference between group 1 &group 3, in which group 3 recorded the lowest biofilm formation and the highest anti- anti-A adhesion activity. MTT showed that groups 2 and 3 exhibited more than 80% cell viability after 24, 48, and 72 hours. Plasma surface treatment of metal brackets possesses antibacterial activity against S.mutans and L.acidophilus; alteration exposure time impacts the development of these properties.

Key words: Plasma treatment, Argon gas, orthodontic brackets, biofilm, antimicrobial.

Introduction

Recently, the demand for orthodontic treatment has risen due to increased population awareness about the role of orthodontic treatment in improving dental and facial aesthetics and, consequently, their popularity and social acceptance[1]. Fixed orthodontic appliance parts like brackets, bands and archwires make tooth brushing difficult and create ideal circumstances for the formation of dental biofilm and the colonization of bacteria. The biofilm formation can cause periodontal problems and enamel decalcification, which affect nearly half of all orthodontic patients[2]. In orthodontic-induced enamel decalcification, the initial bacterial adherence to the bracket-adhesive-enamel interface is crucial. The continued growth of these initially adhering bacteria that were only partially removed by tooth brushing might contribute to the establishment of a pathological oral biofilm. The organic acids generated by adhering bacteria such as *S.thoraltensis*, *S.epidermidis*, *Bacillus spp.*, *Neisseria spp.*, *S. aureus* and *M. luteus* when they come in contact with the tooth surface and left for enough time can cause demineralization of the enamel and the formation of white spot lesions (WSL), which are usually apparent clinically in the first month after orthodontic appliances are placed[3]. *Streptococcus mutans* and *Lactobacillus acidophilius* have significant components of the pathogenic bacterial flora in WSLs and dental caries[4]. While *S. mutans* has been linked to the onset of dental caries, studies suggest that *lactobacilli* may have a more crucial role in the progression of caries[5]. The surface free energy and surface roughness of the implant surfaces play a crucial role in bacterial adherence and colonization. In research conducted by Eliades and h[6], they found that stainless steel has greater critical surface tension and energy, which can be thought to have a more excellent plaque retaining ability.

Furthermore, metallic orthodontic brackets altered the oral environment, such as pH level reduction and increased colonization of *Streptococcus mutans* and *Lactobacillus acidophilus*[7]. Various methods have been utilized to reduce the development of white spot lesions during orthodontic treatment: altering dietary habits, providing good oral hygiene and the topical fluoride application (toothpaste, mouth rinse)[8]. These approaches, however, rely on patient cooperation, which is unreliable. Furthermore, professional application

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of fluoride-release agents (fluoride varnish, luting cement and bonding adhesive containing fluoride) can be utilized to minimize biofilm metabolic activity[9]. Despite topical fluoride application and brushing technique reinforcement resulting in the alleviation of these lesions, they do not lead to their complete eradication. As a result, research has been conducted to identify new alternative techniques for dealing with similar issues. Adjusting the antibacterial characteristics of a metal surface before appliance installation can effectively deter bacterial infection; many techniques have been used, including direct impregnation with antibiotics, coating and plasma-surface modification. Plasma surface modification (PSM) is a versatile and cost-effective surface treatment method that is gaining popularity in the biomedical industry by the interaction of highly reactive plasma species with the material surfaces, resulting in selectively enhancing surface characteristics while leaving the bulk properties of the materials unaltered. Existing materials may therefore be utilized, and the requirement for new materials can be avoided, reducing the time it takes to build new and better biomedical devices[10]. Various gases can be used in plasma activation in plasma surface treatment procedures, such as Ar, CO_2, O_2, He, H_2, and N_2. Argon gas has a growing interest in the biomedical field because it is biochemically inert, has a low breakdown voltage, is available and is relatively inexpensive[11]. In dentistry, plasma surface modification using Argon gas has many applications, including sterilization[12], increase in bond strength at the dentin/composite interface[13], and improved osseointegration of dental implant[14]. the present study is the assessment of antibacterial properties of Argon plasma-treated orthodontic stainless steel brackets against *Streptococcus mutans* and *Lactobacillus acidophilus* represented by anti-biofilm and anti-adherence activity and evaluation the effect of change in the duration of plasma treatment exposure on the development of these properties among the three groups of brackets.

Materials and methods

A total of 70 orthodontic bicuspid brackets made of stainless steel alloy 316L (International Orthodontic Service IOS™ Roth .022 slot size) were used in the current study. The experimental design of the current research included three main groups, as shown in table (1) &figure (1). Each group contains 20 brackets divided equally into two subgroups for *Streptococcus mutans* and *Lactobacillus acidophilus*, respectively. Another group containing 10 treated brackets was used to assess cytotoxicity and biocompatibility, as shown in table (2). At first, to eliminate visible impurities, all brackets were ultrasonicated for 30 minutes in 99 % ethanol and 99.5 % acetone, then dried in a desiccator.

Groups	Number of orthodontic stainless steel brackets	Duration of plasma Treatment (minutes)
(control)	20	Nil
Group 2	20	15 min.
Group 3	20	30 min.

Table 1. The experimental design of the study.

Time of treatment	Number
15 min. (Group 2)	5
30 min.(Group 3)	5
Total	10

Table 2. The number of brackets and duration of treatment for cytotoxicity assessment.

Plasma surface treatment procedure

The plasma surface treatment procedure is done at the Iraqi Ministry of Science and Technology/ Material Research Directorate using the DC magnetron sputtering system. The plasma treatment system used in this study consist of a cylindrical plasma chamber containing two electrodes, the cathode and the anode, separated by 4 cm, providing an electric field for gas discharge. The discharge is carried out with Argon gas at a flow rate of 40 SCCM under the pressure of 5×10^{-1} mbar. The chamber was evacuated by using a rotary pump (Edward of 12 m^3 /h) to vacuum pressure 3×10^{-1} mbar. The amount of gas inside the chamber is adjusted by using a flow meter, and the pressure inside the chamber was observed by Perani gauge with Edward's controller 1105. The applied voltage was 500 V which was controlled by a DC power supply. Cathode potential and discharge currents were measured by digital multimeter[15].

In the plasma treatment procedure, the brackets were placed in the magnetron sputtering chamber and distributed in a way that the bonding side faced the anode electrode. High purity argon gas was employed as the ambient gas. The brackets' bonding side was not treated. The plasma treatment was conducted for 15 min. & 30 min. to produce two main groups of treated brackets with two different periods of exposure, as shown in figure (2).

Cytotoxicity test

The MTT test is used to assess the biocompatibility of treated stainless steel brackets depending on the capacity of living fibroblasts to produce formazan crystals from (3-(4,5-dimethylthiazote-2yl)-2,5-diphenyl tetrasodium bromide). MTT test was used to evaluate cell viability in response to a treated stainless steel bracket (for three immersion times at 24h, 48h, and 72h). According to the International Standard ISO 10993-part 5 in 2009, At a density of 4×10^3 cells per well, Primary human dermal fibroblasts (HDFn) were injected into 96-well plates (Falcon, Teterboro, New Jersey, USA), and incubated for two days at 37°C, in 5% CO_2 in air. After incubation, the culture media was removed, and 200µL of the culture media containing the metal bracket extracts of each immersion period was added. Each well was then dyed with MTT (50 µg/l). The plates were incubated for 4 hours at 37°C with 5% CO_2 in the air. An enzyme-linked immunosorbent assay reader was utilized to determine the degree of light absorption at 570 nm in each well (U2000, Hitachi, Tokyo, Japan). The cell viability tests' results were given in percentage, the experimental wells' absorbance at 570 nm compared to the control wells' at 570 nm. For each subgroup, six replicates were performed[16].

Microbiological aspect

Collection of bacterial samples and culture procedures

Saliva samples were taken from 20 patients undergoing fixed orthodontic treatment (stainless steel brackets) with a duration from starting the treatment not less than two months by spitting into a glass flask; the saliva was then mixed with 1 ml of phosphate-buffered saline (PBS). The samples were sent for microbiological analysis within two hours. In this research, we selected *S. mutans* and *L. acidophilus* from the microbial collection because of their significant role in the development of WSL and dental caries; studies suggest that *S. mutans* has been linked to the onset of dental caries while lactobacilli have a more crucial role in the pro-

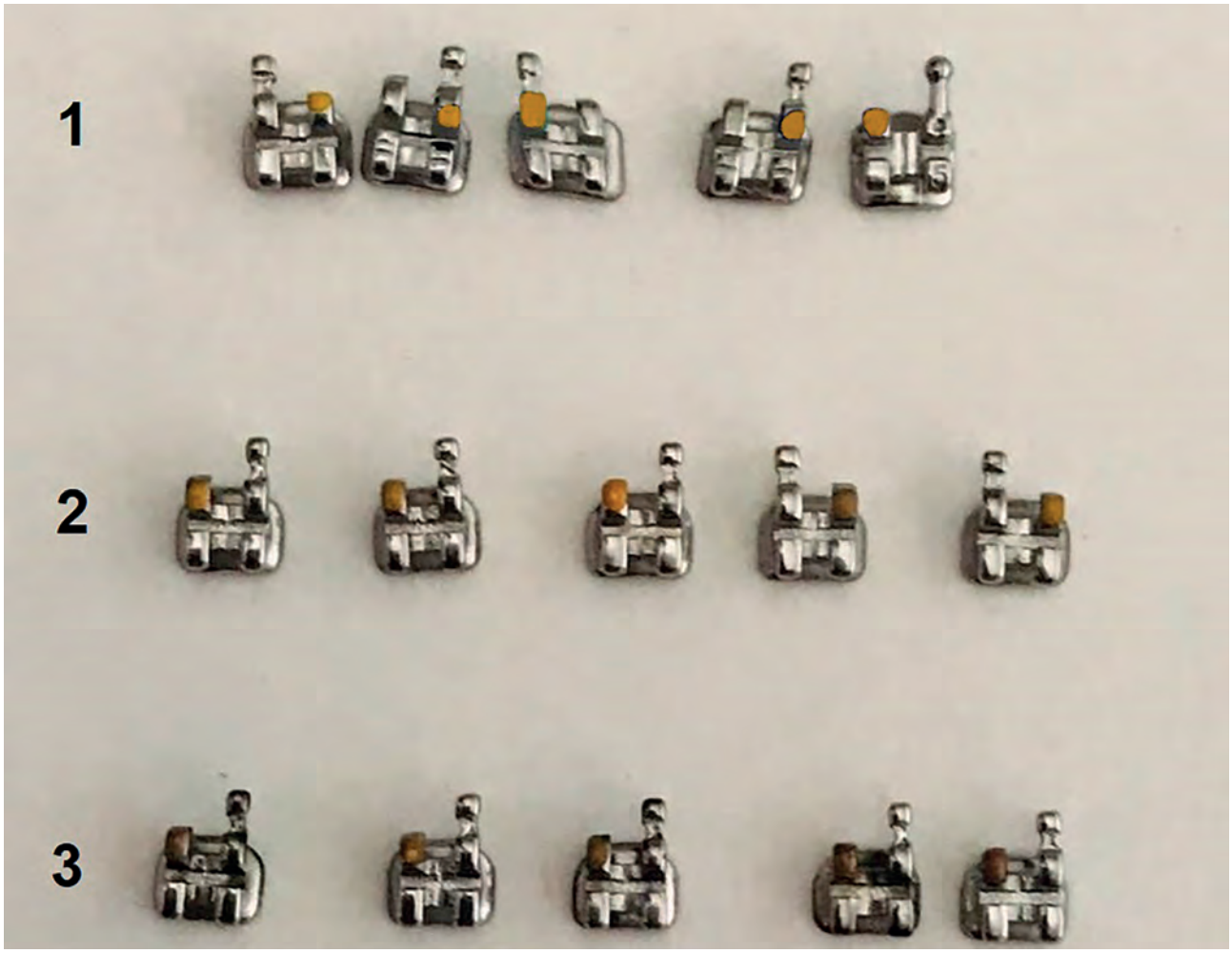


Figure 1. 1: Untreated stainless steel bracket. 2: stainless steel bracket treated for 15 min. 3: Stainless steel bracket treated for 30 min.

gression of caries. For *S. mutans* culturing, 100 ul of the sample was distributed on *Mitis Salivarius* agar with 0.5 IU/ml bacitracin (Sigma, USA) and then incubated for 48 hours at 37°C in a microaerophilic environment. While *L. acidophilus* was cultured on Man, Rogosa and Sharpe (MRS) agar for 24h at 37 ºC under 95% air and 5% CO_2, the isolates were identified based on the colonies' morphological and cultural features, finally confirmed by Vitek 2.

Adhesion Assay of bacteria to orthodontic brackets

The adhesion ability of *S. mutans* & *L. acidophilius* to the brackets was measured by using the plate counting method[17]. In this assay, stainless steel bracket segments roughly (1 cm²) were put in tubes containing 5 ml suspension of tested microorganisms which is (1X10^⁸ CFU/mL) according to (0.5 McFarland turbidity standard), then incubated for 1 hour at 37°C. Following incubation, Phosphate buffer saline was used to wash stainless steel brackets three times. The brackets were put in 10 ml fresh PBS and sonicated for 5 minutes at 40 kHz to remove adherent cells. The sonicated PBS was serially diluted and cultivated on tryptic soy agar plates. The viable colony count approach was used to determine the number of adherent bacteria in sonicated PBS (pH= 7.2, 0.1 M), which refers to the degree of adherence of bacteria; this procedure was repeated for each group. The biofilm formation on the orthodontic bracket was tested by using an ELISA plate reader[18]. The following steps were applied for six groups in which Serial dilutions of *S.mutans* and *L. acidophilus* bacterial cultures were made from nutrient broth to exhibit a cell density of 5×10^6 cells/ ml, and stainless steel bracket (1 cm long) were submerged in 5 ml culture in culture tubes (for each group), the culture tubes were incubated at 37 °C for 24 hours.

The stainless steel brackets were removed, then rinsed in sterile water, put into culture tubes within 2 ml of nutrient broth, and incubated for 24 hours at 37 °C and 30 °C for *S.mutans* and *L. acidophilus*, respectively. Stainless steel brackets segments were removed and rinsed in sterile water before being submerged in tubes within a 0.1% crystal violet solution for 5 min. Stainless steel brackets segments dyed with crystal violet were rinsed in sterile water and moved to separate wells of a 96-well microtiter plate. 25 µl of 30% acetic acid was dropped onto the surface of each catheter to allow solubilization of the adhered biofilm into their respective wells. Absorbance was carried out at 550 nm, and the optical densities of *S. mutans* and *L.acidophilus* biofilms on U-C and BCN-C were measured. The optical density was recorded by using 30% acetic acid as a blank. The orthodontic brackets' adhesion and biofilm formation assays were performed two months after subjecting the brackets to the plasma surface treatment procedure.

Statistical Analysis

The adhesion test results were expressed in viable colonies count attached to bracket surface (CFU), while the biofilm formation test's results were expressed in optical

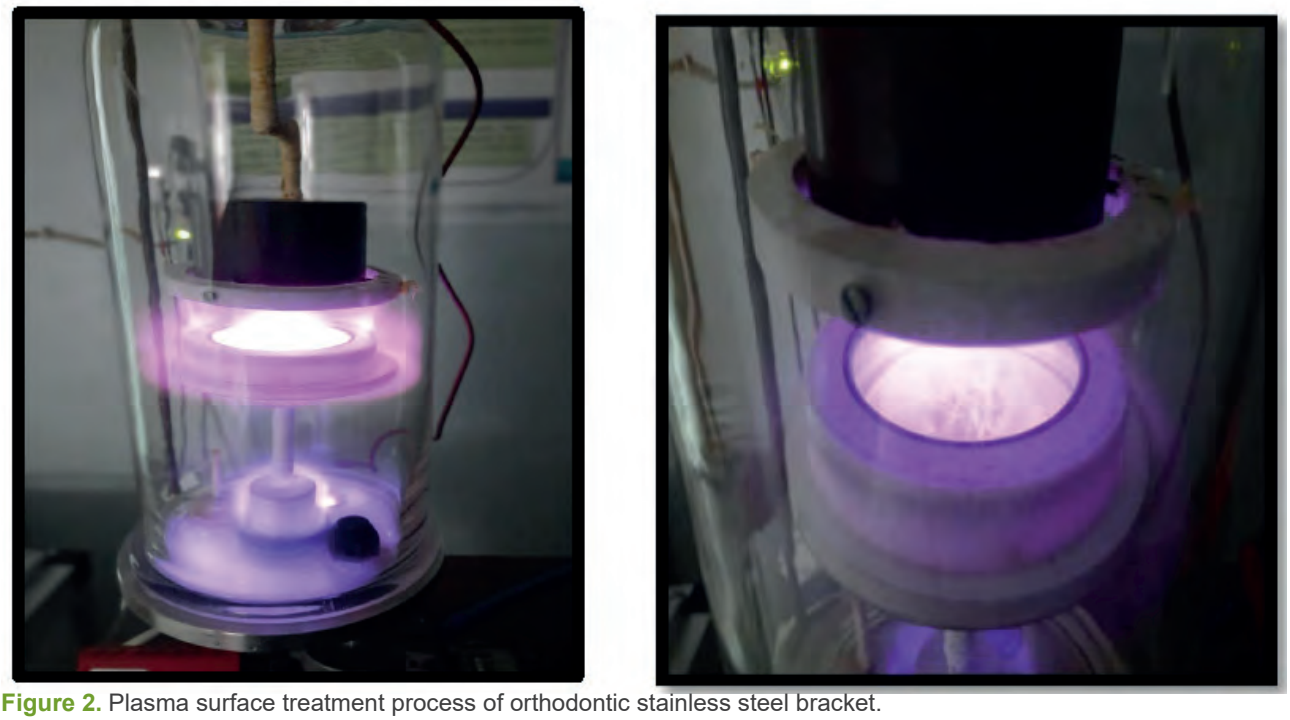


Figure 2. Plasma surface treatment process of orthodontic stainless steel bracket.

density (OD). Standard statistical analysis software was used for data assessment (version 20.0, Statistical Package for the Social Sciences, IBM Corporation, Armonk, NY, USA). Descriptive statistics, including mean and standard deviation values, were calculated for the variables. The statistical significance cut-off was set at $p \leq 0.05$.

Results

In the current study, four isolates for *S. mutans* & four isolates for *L. acidophilus* were obtained to perform microbial and statistical analysis as follows:

Adhesion assay

In the present study, adhesion assay results recorded the highest viable colonies numbers in group 1 in which *S. mutans* (186±2.8) and *L.acidophilus* (335± 0.68) when compared with group2 in which the colonies number of *S.mutans* (168.5±0.70) and *L. acidophilus* (226±0.01) while the lowest colonies number appeared in group3 in which *S.mutans* (133±1.00) and *L.acidophilus* (187±1.00) as shown in figure (3). T-test was used to compare groups 1 &2; groups 1&3 showed a significant difference between group 1 & group 3 at ($p \leq 0.05$) as shown in table (3).

Biofilm formation assay

The results of biofilm formation revealed that the lowest value of biofilm formation was recorded in group 3 in which *S.mutans* (0.274±0.00) and *L.acidophilus* (0.509±0.00), followed by group2 in which *S.mutans* (0.443±0.00) and *L.acidophilus* (0.626±0.01) while the highest value was reported in group1 in which *S.mutans* (0.495± 0.01) and *L.acidophilus* (0.688±0.00) as shown in figure (4). T-test was used to compare groups 1 &2, with group 1&3 showing significant differences between groups 1 & 3 at $p \leq 0.05$, as shown in table (4).

Cytotoxicity test

MTT test on human gingival fibroblast cells *in vitro* was used to assess the cytotoxicity of the treated brackets. The dose-response viability of fibroblast-like cells treated with the culture media containing treated stainless steel bracket of different immersion periods at (24h, 48h, and 72 h) is expressed in percentage and presented in table (5). MTT test reported more than 80% in both group 2 &group 3 for the three different periods. T-test was done to compare every two groups and revealed a significant difference between 24 and 72 hr, but there is no significant difference between 24 and 48 and between 48 and 72 hr in both group 2 and group 3. However, there is no significant difference between groups 2 &3 during the same periods ($p \leq 0.05$).

Discussion

Plasma technologies have become more critical in the medical industry for enhancing the surface characteristics of biomaterials which ultimately affect the cell's response to the material surface, providing environment-friendly and cost-effectively measures to address the bacterial infection. The present study results indicated reduced bacterial adhesion with lower viable colonies count and higher anti-biofilm activity in plasma-treated stainless steel brackets (group 2 & 3) compared with untreated brackets (group 1) with a statistically significant difference between group1 & 3. These results agree with Shah AG, *et al.*[19] & Chhattani S *et al.*[20]. The present study's findings could be attributed to the influence of the plasma treatment on surface chemistry, crystallinity, and ion release. The plasma treatment can induce the development of an oxide layer on the metal surface which can also cause crystallization of amorphous oxide; The anatase crystalline structure has a larger bandgap when compared to the rutile crystalline structure, which increases its surface redox potential to oxidize water molecules to form hydroxyl radicals which play a crucial role in cell interaction[21]. Komasa S, *et al*. showed that Argon plasma treatment of titanium surfaces was active enough to cleave C-H bond in the surface layer to form radicals and generate various ROS on the surface, including oxygen-binding compounds and nitrogen;

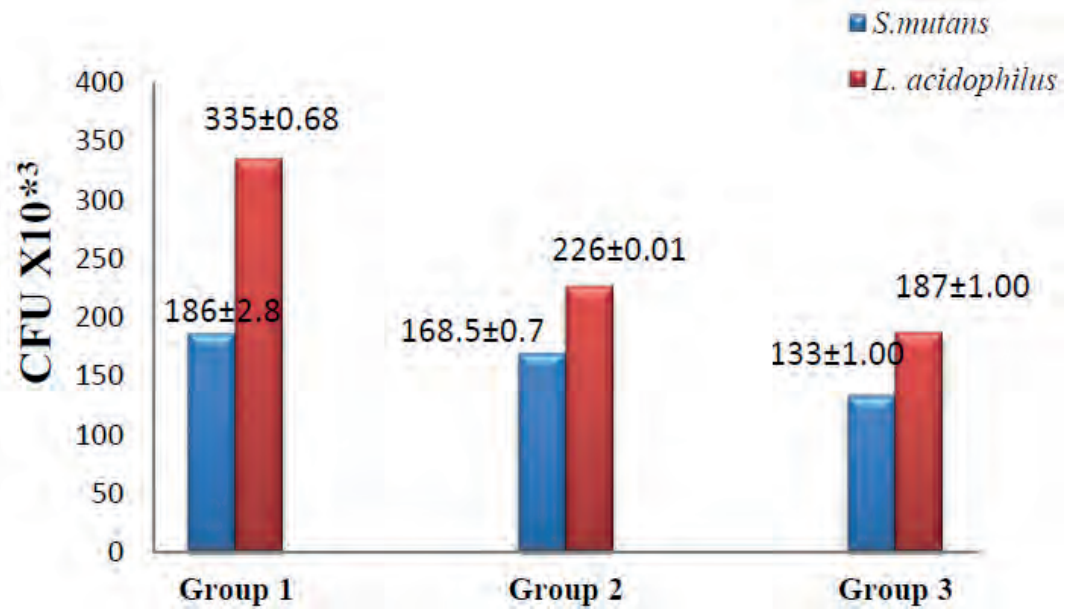


Figure 3. Adhesion assay of S.mutans & L.acidophilus to the brackets in the studied group.

Microorganism	Groups		T-test	P-value
S.Mutans	Group 1	Group 2	1.258503	0.427
	186± 2.828427	168.5±0.70		
	Group 1	Group 3	17.666667	0.035*
	186± 2.828427	133±1.00		
L.Acidophilus	Group 1	Group 2	4.360000	0.143
	335±0.698	226±0.01		
	Group 1	Group 3	7.400000	0.045*
	335±0.698	187±1.00		

(*): Significant Difference ($p \leq 0.05$). The values are given as mean and Standard Deviation (SD).

Table 3. Adhesion assay of S.mutans & L.acidophilus to the brackets in the studied groups (CFU X10*³).

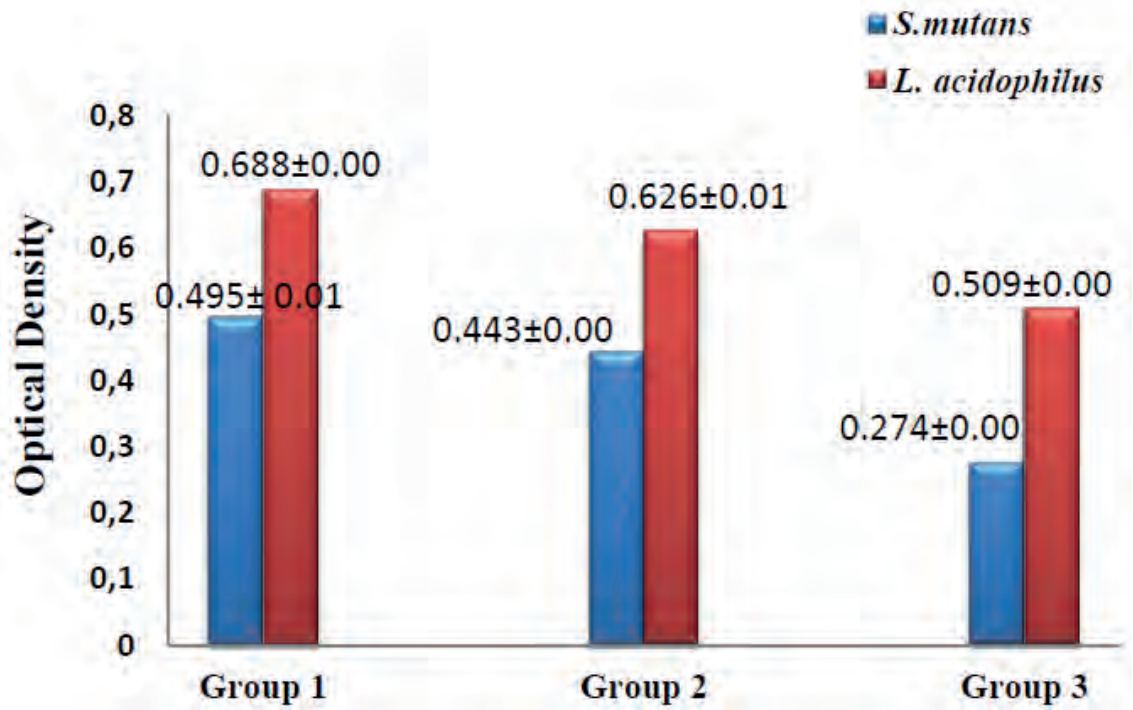


Figure 4. Biofilm formation assay of S.mutans and L.acidophilus in the studied groups.

Microorganism	Groups		T-test	P-value
S.Mutans	Group 1	Group 2	4.000	0.155
	0.495±0.014	0.443±0.003		
	Group 1	Group 3	23.315	0.027*
	0.495±0.014	0.274±0.001		
L.Acidophilus	Group 1	Group 2	4.428	0.141
	0.688±0.002	0.626±0.017		
	Group 1	Group 3	23.933	0.026*
	0.688±0.002	0.509±0.002		

(*): Significant Difference ($p \leq 0.05$). The values are given as mean and Standard Deviation (SD).

Table 4. The biofilm formation assay of S.mutans and L.acidophilus in the studied groups.

	Time	Cell viability %	P-value		
			(24 -48) hr	(48 -72) hr	(24 -72) hr
Group 2	24 hr	85.90 ± 2.805	0.1711	0.087	0.0173*
	48 hr	83.66 ± 1.797			
	72 hr	81.50 ± 1.716			
Group 3	24 hr	83.60 ±2.755	0.3184	0.01152	0.0449*
	48 hr	82.06 ±1.6987			
	72 hr	80.21 ±1.611			

(*): Significant Difference ($p \leq 0.05$). The values are given as mean and Standard Deviation (SD).

Table 5. MTT assay (cell viability results in percentage at the three immersions time periods (24 hours, 48 hours, and 72 hours).

Hydrogen peroxide and hydroxyl radicals are examples of reactive oxygen species (ROS) which are well recognized for causing oxidative damage to cell membranes. The disrupted cell membrane does not have sufficient ability to control substances' movement through the bacterial wall, which eventually causes cell death[22]. For their part, researchers have investigated a range of methods and chemicals that may be used to suppress pathogenic bacteria in the dentistry profession of medicine, including the direct action of Ag and TiO2 nanoparticles on pathogenic bacteria[23].

The release of bactericidal ions from plasma-treated surfaces is another approach to prevent bacterial adhesion. Ions can inhibit bacteria by destroying their envelope and cytoplasmic components, limiting the capacity of peptidoglycans to transport oxygen, inactivating protein enzymatic activities, and/or interrupting DNA replication[24]. In addition, Plasma treatment can alter the surface hydrophobicity, which significantly affects bacterial attachment and biofilm formation. The Argon plasma treatment of titanium surfaces improved wettability and produced super hydrophilic surfaces[22]. The *S. mutans* & *L. acidophilus* exhibited membrane's hydrophobic nature[25,26], and the bacteria with the membrane's hydrophobic nature readily colonize hydrophobic surfaces and vice versa.

Furthermore, plasma treatment has a physical antibacterial effect. Plasma treatment can induce the formation of nanotopography on metal surfaces[27]. Nanostructured surfaces or biomimetic surfaces that enable the killing of bacteria by the bacterial membrane's rupturing arises from the penetration of high aspect ratio nano-features. Hirano M, *et al*. found that Argon plasma treatment is an effective method for rapidly fabricating antibacterial nanopillars on AISI 316 stainless steel surfaces[28]. To evaluate the effect of change in the duration of plasma exposure, two groups of stainless steel brackets were subjected to plasma surface treatment for 15 min. & 30 min. The results indicated reduced bacterial adhesion and higher anti-biofilm activity in plasma-treated stainless steel brackets for 30 minutes (group 3) with a statistically significant difference when compared with group 1, while plasma-treated stainless steel brackets for 15 minutes (group 2) statistically showed no significant difference when compared with group 1 at $p \leq 0.05$. These findings could be explained by alteration in plasma discharge parameters (in our study, increasing exposure time will further reduce water contact angle, which ultimately improves surface hydrophilicity and incorporation of more oxygen-containing functional

groups onto the treated surface for a longer duration, which strengthens antibacterial properties. These results come in agreement with Lee MJ, *et al.*[29], who investigated antibacterial properties of titanium surfaces modified by nonthermal atmospheric pressure plasma and reported that a biofilm formation was shown to be lower in samples exposed to plasma for more extended periods when compared with those exposed for shorter periods. The current study evaluated the antimicrobial effects of Argon plasma surface treatment on stainless steel brackets in vitro settings. The limitation of our study is that the test findings may be different in clinically relevant oral circumstances. Seo SH, *et al.* investigated the antibacterial effect of nonthermal atmospheric pressure plasma in the artificial saliva to imitate some of the in vivo circumstances; the obtained findings suggested that plasma treatment in a natural oral environment with saliva could be more efficient than in some experimental settings[30].

Despite its effectiveness in reducing bacterial adherence and destroying biofilms, the plasma surface treatment can also exhibit a cytotoxicity concern for mammalian cells. In the present study, the MTT test reported cell viability of more than 80% in both group 2 &group 3, as shown in table (5), indicating that the remaining percentage represents the cytotoxic effect of plasma treatment. The cell death could be attributed to reactive oxygen and nitrogen species (RONS) as well as hydrogen peroxide (H2O2) produced by plasma treatment[31]. The Increased intracellular RONS is found in both microbial and mammalian cells; however, the antioxidant activity is more significant in specific cell types, which serve to ensure their survival and stimulate cellular proliferation to replenish their numbers throughout time, resulting in differences between cytotoxic effect and antimicrobial effect of plasma treatment[32]. Many types of research support minimal tissue damage that can be obtained at specific technical settings (power, gas flow rate). The potential for cell proliferation is enhanced to eliminate biofilm bacteria while minimizing or maintaining a "tolerable" level of tissue cytotoxicity[33]. In the present study, an MTT assay measuring the cytotoxicity of orthodontic stainless steel brackets treated with Argon plasma was conducted for three days; depending upon the duration of the orthodontic treatment & the time that stainless steel brackets remain inside the oral cavity, further investigations are required to determine the long-term impact of cellular damage and tissue viability recovery.

Conclusions

Plasma surface treatment for orthodontic stainless steel brackets using Argon gas possessed antimicrobial properties against *S. mutans* and *L. acidophilus* represented by anti-biofilm and anti-adherence activity with a significant difference between untreated brackets and brackets treated for 30 minutes, indicating that alteration of the time of exposure has an impact on the development of antimicrobial properties which could provide a new preventive measure against white spot lesions development and periodontal problems frequently associated with orthodontic treatment. Further research is needed to assess long-term cellular damage and recovery of cellular viability and determine the most suitable technical parameters of plasma exposure, optimizing antimicrobial properties and making its cytotoxic effect at a negligible level and enhancing tissue viability recovery.

Data Availability

The data used to support this study's findings are available from the corresponding author.

Conflict of interest

No conflicts of interest between us or anyone.

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ARTICLE / INVESTIGACIÓN

Histological Study for Median lethal Dose (LD_{50}) of Eucalyptus Oil Administrated Orally in (*Mice mus musculus*)

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Abstract: Eucalyptus oil is used in a wide range of medicinal and other uses, and the current study aims to estimate the median lethal dose LD_{50} of eucalyptus oil of *Eucalyptus camaldulensis* and estimate the pathological effect of eucalyptus oil in mice. Eucalyptus oil was obtained in the study by extracting the leaves of the plant *Eucalyptus camaldulensis* using hydrodistillation. Toxicity testing was performed by dividing mice into groups that you treated with different doses of eucalyptus oil 1200, 1600, 2000, and 2400 mg/kg. The current study showed that the average lethal dose, the LD_{50}, was 1820 mg/kg. The study suggested the emergence of clinical signs in mice when consuming eucalyptus oil in high doses, such as slow movement, suffocation, and convulsion, compared with the control group and the group that administered a lower dose, It was observed that high doses of eucalyptus oil could cause damage to the lung, liver, kidneys, and heart, as the lung was the organ most affected by the oil, followed by the liver, kidneys, and then the heart, and it was less affected by eucalyptus oil, and this indicates that eucalyptus oil, when administered orally in high doses, causes clear toxicity effects. Eucalyptus oil is a safe medicine for external use.

Key words: Eucalyptus oil, LD_{50}, Administrated, Lung, Liver, Heart; Kidney, Mice.

Introduction

The eucalyptus plant is a tall plant native to Australia and includes nearly 700 species[1]. Eucalyptus is a genus belonging to the *Myrtaceae family*. It is a fragrant evergreen flowering tree cultivated in all regions, including Iraq[2]. The common consensus was that all herbal treatments and natural items were safe to use[3], that essential oils are safe for human usage since they are maintained as natural as possible, are risk-free, and have no side effects[4]. Essential oils are extracted using various physical and chemical methods, including solvent extraction (hydrodistillation, pressing)[5]. Aldehydes, alcohols, ketones, saturated and unsaturated hydrocarbons, terpenes, phenolic oxides, and esters are the components that make up essential oils[6]. Essential oils are simple to extract and degrade in the environment, making them environmentally beneficial[7]. Essential oils have gained widespread popularity due to their widespread use, and they have been dubbed "aromatherapy."[8]. Eucalyptus essential oil has been utilized in the culinary business as a flavoring agent in the food industry[9]. Eucalyptus oil is an essential oil derived from the eucalyptus tree that contains 1,8-cineole[10] and α-pinene[11]. It's also employed in the fragrance and medicinal industries[12]. It is also used to treat respiratory infection; the eucalyptus leaf extract is inhaled[13]. Expel sputum in case of chest infection[14] and treat sore throat and cough[15]. It has antispasmodic, anti-stress, and antispasmodic properties and relieves labor pains during pregnancy[16]. Eucalyptus is used in arthritis, abscess, burns, and diabetes[17]. It can also be used as an anti-insecticide to protect people from parasites like head lice[10] and mosquito bites[18], and represents many analgesics, antiinflammatory[19], anti-oxidant[20], and is effective against cancer[21]. Eucalyptus oil is used in various methods, including applying it to the skin's surface, massaging it, and inhaling it. It is rarely taken orally[22]. This study aimed to measure the LD50 of Eucalyptus oil in mice and determine the organs will be significantly affected by the toxicity of Eucalyptus oil.

Materials and methods

Plant material collection

The leaves of *Eucalyptus camaldulensis* were collected during October, November 2020, from the governorate of Misan / awash garden.

Extraction Essential Oil

Freshly collected leaves of *Eucalyptus camaldulensis* were cleaned with distilled water to remove mud and debris from their surfaces, then air-dried in the shade at room temperature and that slice into smaller pieces[23]. and the essential oil extraction was carried out by using the hydrodistillation technique Clevenger-type apparatus, the weight of 100 grams of leaves were separately mixed with 500 ml of distilled water in a round one-liter flask[24] as well as being subjected to hydrodistillation Three hours had been spent co-distilling the essential oil with water. N-hexane was used to separate the volatile oils, then stored in bottles in the refrigerator until needed.

ExperimentalAnimals

Male BALB/c mice are aged 6–8 weeks; their weight

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was from 25 g, pathogen-free; mice were obtained from the animal house of the department of biology of the College of Sciences University Misan. The study included 50 animals and housed 5 animals per cage with food and tap water accessible ad libitum in a 12/12 h light/dark cycle. The local animal ethics commission accepted all experimental techniques, and they all met German standards.

All experimental procedures were approved by the local animal ethics committee and met German and international guidelines. The study was conducted using 50 adult mice; at least 10 animals were used per group. The handling and use of animals were following the institutional guidelines.

Acute toxicity

The experiment was conducted to determine the average lethal dose, LD_{50}, in five groups in each group of 10 animals (n=10/group) of male mice; the following control group was administered normal saline. The animals were administered eucalyptus oil once each time. If the animal survived from the first dose, the next dose animal would be increased. The next dose is reduced if the animal died from the first dose; the animals fasted for three hours before receiving the eucalyptus oil. One hour after receiving the eucalyptus oil, The four groups were administered by oral gavage using eucalyptus oil at doses of 1200, 1600, 2000, 2400 mg/kg of body weight; the dose was calculated by µl, and the average weight was 25g, animal administered was continued for 14 days. Neurobehavioral and involuntary effects were observed in animals for 24 hours and registration of deaths and survival. The toxicological effect was evaluated according to the mortality rate expressed by LD_{50}, expired animals were counted for the calculation of LD_{50} (Table 1) . In its arithmetic method, Karber has used the determination of LD50 According to the following equation[25].

$$LD_{50} = LD_{100} - \sum \frac{(a \times b)}{N}$$

LD100 = Lethal dose causing 100% death.

N = total number of animals in a group

a = the difference between two successive doses administered.

b = the average number of dead animals in two successive doses (Table 2)

Histological study

Euthanasia to mice by placing 1 ml of chloroform ($CHCl_3$) on cotton and then placing it on the animal's nose[26] after animal dissection, the lung, heart, liver, and kidneys were collected from all mice and washed with standard salt solution and then fixation[27] in a neutral buffer formalin (NBF) solution 10% after that fixation process is washed with running water and then dehydration through the concentrations of alcohol. There's the clearing, infiltrating, embedding, and sectioning in 5 µm thick sections, then staining with hematoxylin and eosin, finally examining the tissue sections under a microscope with a force of magnification 400X.

Results

Clinical signs of acute toxicity

After administering the animals with eucalyptus oil for 10 minutes, mice that were administered a high dose of 2,400 and 2,000 mg/kg of eucalyptus oil developed toxic symptoms in terms of convulsion, dizziness, slow movement, and high breathing (suffocation), as compared with the control group as shown in (Table 2). As for the mice in the lower dose groups of 1200 mg/kg of eucalyptus oil, they did not show toxic symptoms, with good health, and no deaths recorded was, as shown in Table 2, and consequently, the body weight was constantly growing, and food consumed, unlike animals that were given a high dose. The The karber method calculated the value at 1820 mg/kg, as shown in (Table 3). Mortality was recorded only at 1600,2000and 2400mg/kg body weight doses, three mice out of ten animals had died in the group (2) that received 1600mg/kg body weight test dose, was died overnight following the administration, and seven mice out of ten animals in group(3) died within 24 hours that received 2000mg/kg body weight test does, and nine mice out of ten animals in group (4) died within 24 hours that received 2400mg/kg body weight test dose, was as showed in (Table 2).

$$LD_{50} = LD_{100} - \sum \frac{(a \times b)}{N}$$

Sum of the product = 5800

=2400-580

=1820 mg/Kg

Results of Histological changes

The results of this study indicate the occurrence of tissue changes in the organs (lung, heart, liver, and kidneys) as a result of administering male mice to different doses of eucalyptus oil, and the pathological changes were as follows.

Histopathological changes in the liver

Sections obtained from the livers of mice for the control group clarified that sinusoids, hepatocytes are large cuboidal or polyhedral epithelial cells with large round central nuclei and eosinophilic cytoplasm, bile ducts, central vein, portal vein, hepatic artery, and immune cells within sinusoid called Kupffer cells (Figure: 1A). The cross-section of liver mice in the group treated with Eucalyptus oil toxic dose shows congestion in the central vein; small vacuoles were shown in hepatocytes, Focal necrosis, pyknosis, narrowing of the sinusoids (Figure:1 B).

Histopathological changes in the heart

A cross-section of heart mice shows the control group's normal appearance: cardiac muscle cells, nuclei of the cardiac muscle cells, intercalated disk, and branched cardiac muscle (Figure: 2 A). The cross-section of heart mice in the group treated with eucalyptus oil showed congestion, cytoplasmic vacuolization of cardiac muscle cells (v), and disarrangement of fibers (Figure:2 B).

Histopathological changes in the kidney

In the kidney mice sections of the control group appeared the renal capsule and renal tubular epithelial cell (RTEC), Bowman's space, Glomerulus, Distal convoluted tubule, Proximal convoluted tubule, Macula dense (Figure: 3 A). The cross-section of kidney mice in the group treated with eucalyptus oil is toxic does show congestion or hyperemia, granular degeneration, widening of the tubular lumen was observed in sections of the kidney, pyknosis of renal tubular epithelial cells, increased the area of bowman's space, desquamation of the epithelial cells of the renal tubules, and perivascular lymphocytic infiltrations were also observed in small areas of the kidney (Figure: 3 B).

Group	dose mg/kg	clinical signs	mortality	Period
Control	normal saline	not observed	negative	_
Group 1	1200	not observed	negative	_
Group 2	1600	the slow movement, dizziness.	positive	25 min
Group 3	2000	convulsion, slow movement, dizziness, suffocation.	positive	35 min
Group 4	2400	convulsion, slow movement, suffocation, dizziness.	positive	1 hour

Table 1. Results of observed clinical signs.

Group	mice number	dose mg/kg	death of number	Survived of number	mortality %	Livability %
Control	10	normal saline	0	10	0	**100**
Group 1	10	1200	0	10	0	**100**
Group 2	10	1600	3	7	40	**70**
Group 3	10	2000	7	3	70	**30**
Group 4	10	2400	9	1	90	**10**

Table 2. The result of the oral acute toxicity test of eucalyptus oil in mice.

Group	Dose mg/kg	No. of animals dead	Dose difference(a)	Mean mortality(b)	Probit (a× b)
Group 1	1200	0	400	0	**0**
Group 2	1600	3	400	1.5	**600**
Group 3	2000	7	400	5	**2000**
Group 4	2400	9	400	8	**3200**

Table 3. Calculation of by the method of karber.

Histopathological changes in the lung

The histological structure of mice lungs in the control group appeared to be the alveoli, alveolar sac, bronchioles, the lumen of a bronchus, blood vessels, and the alveolar epithelium (Figure: 4 A). Microscopic examination of the lungs of mice treated with eucalyptus oil showed different tissue changes; the changes represented the occurrence of emphysema, severe congestion in the blood vessels; also the expansion of the blood vessel, as well as the emergence of edema and hyperplasia of the epithelium lining the bronchi, which led to hemorrhage in the interstitial tissue of the lung, the degeneration of the alveoli, destruction of alveolar structures. The epithelial cells in this bronchiole are sloughing into the lumen and have lost their cilia, enlargement of the alveolar airspaces distal to the terminal bronchioles, and loss of elasticity of the lung tissue (Figure:4 B).

Discussion

Little is known regarding the toxic quality of eucalyptus oil; however, they have been separated and labeled as GRAS by USEPA[28]; due to the potential health risk, eucalyptus oil requires a quality assessment for its effectiveness and safety due to its growing demand for drugs.

Clinical signs of eucalyptus oil toxicity developed when administered at 2400 and 2000 mg/kg, such as convulsions, choking, slow motion, dizziness, and not consuming food and water (Table 1), and this is consistent with report[29], which talks about the appearance of toxic effects such as insomnia, followed by fatigue and reduction of food and water consumption, as well as the similar result with the report[30], which speak of the appearance of toxic symptoms in rats, such as slow movements and convulsions. The cause of death is respiratory failure.

The study's objective was to assess the toxicity and safety of eucalyptus oil using oral toxicity in mice. In this study, the acute toxicity was shown where the value was 1820 mg / kg (Table 3). The value calculated during this study corresponds to report[31], which addresses the acute toxicity of eucalyptus oil in mice because the value is 1824.01 mg/kg. The output also disagreed with report[30], where the LD_{50} eucalyptus oil emulsion derived from water and oil was 3500 mg/kg.

The liver is a significant site for plasma protein synthe-

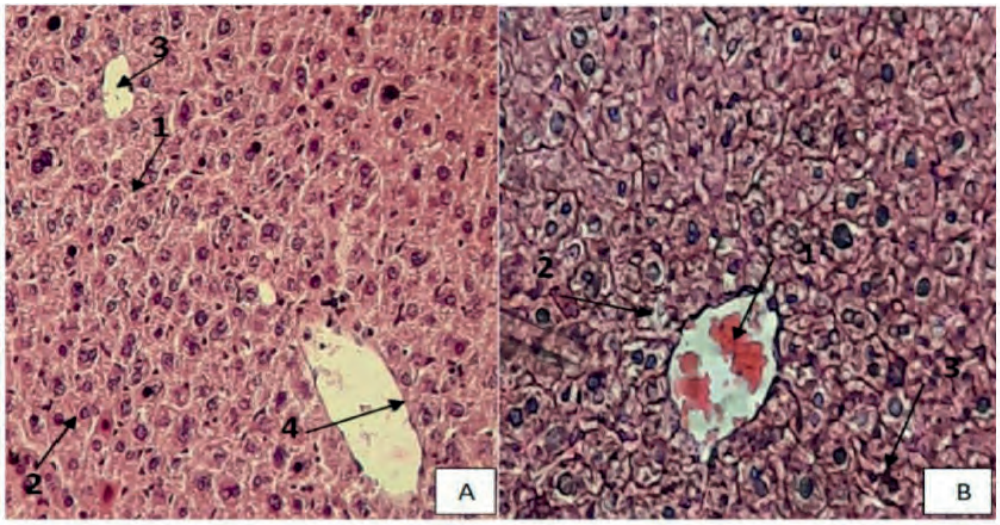


Figure 1. (A). section of the control group of liver mice showing the normal structure of the liver, Sinusoids (1), Hepatocytes (2), Central vein(3), Endothelial cells (4), (H & E stain, 400X). (B) section of liver mice treated by 2400 mg /kg showing, Congestion central venous (1) , degeneration hepatocytes (2) , Focal necrosis(3) , (H & E stain, 400X).

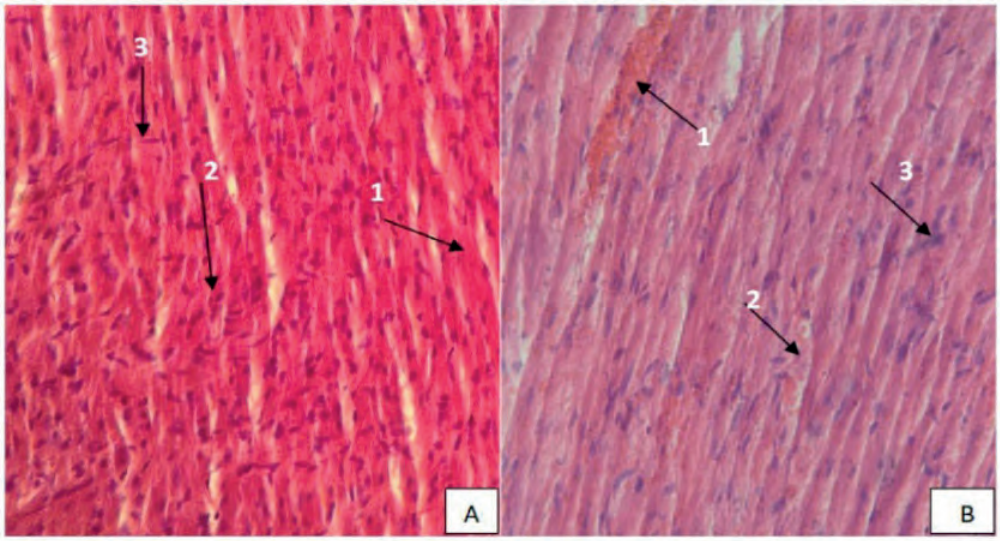


Figure 2. A Section of the control group of Heart mice showing the typical structure of the Heart, cardiac muscle cells(1), nuclei of the cardiac muscle cells(2), intercalated disk(3), (H & E stain, 400X). (B). Section of Heart mice treated by 2400 mg /kg showing hyperemia(1), coagulation necrosis(2), formation of a dense collagenous scar(3), (H & E stain, 400X).

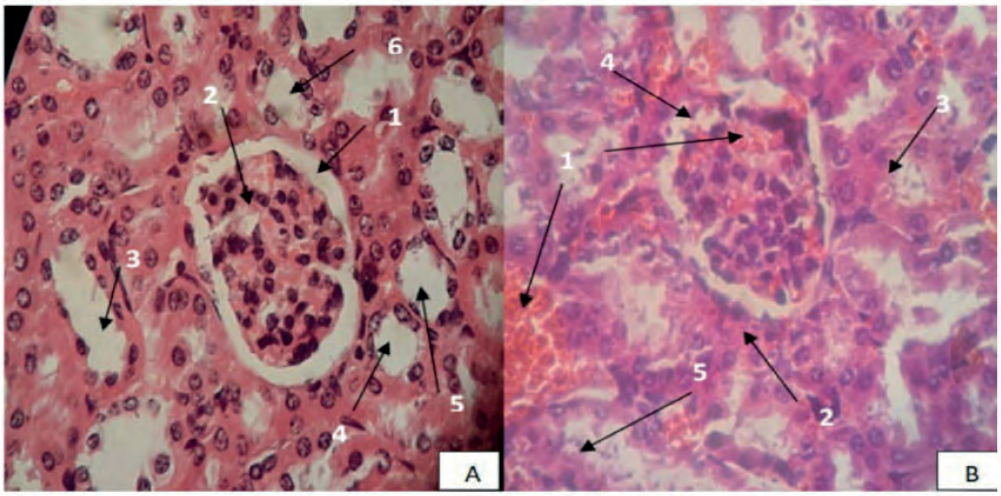


Figure 3. (A). Section of the control group of Kidney mice showing the typical structure of Kidney, Bowman's space (1), Glomerulus (2), Collection duct(3), Distal convoluted tubule(4), Proximal convoluted tubule(5), Macula dense(6), (H & E stain, 400X). (B) section of Kidney mice treated by 2400 mg /kg showing hyperemia (1), granular degeneration (2), pyknosis of renal tubular epithelial cells (3), lobulated renal capsules (4), desquamation epithelial cells(5) (H & E stain, 400X).

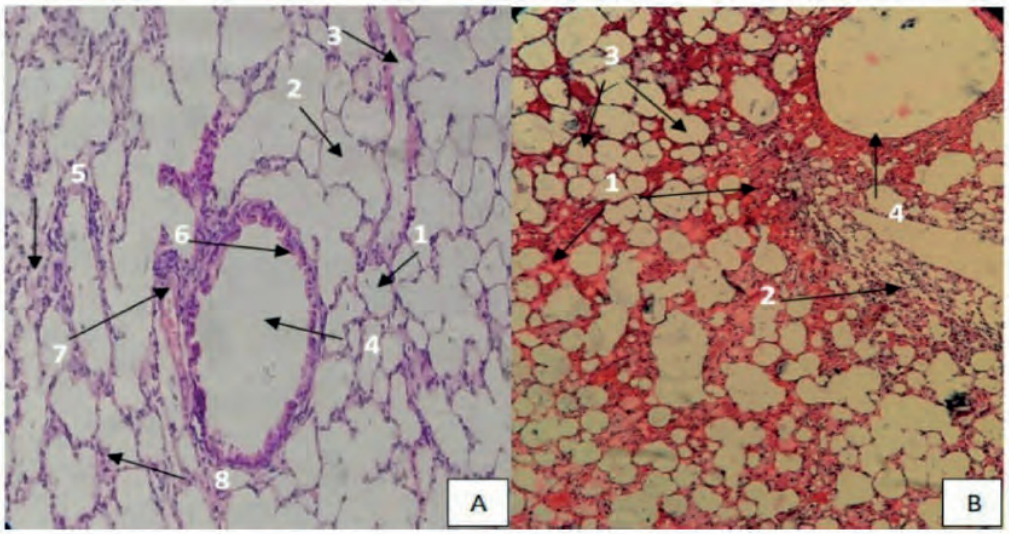


Figure 4. (A). Section of the control group of Lung mice showing the standard structure of Lung, Alveoli(1), alveolar sac(2), alveolar duct(3), bronchiole(4), smooth muscle(5), club cells(6), blood vessels(7), the alveolar epithelium(8) (H & E stain, 400X). (B). section of Lung mice treated by 2400 mg /kg showing hyperemia(1), destruction of alveolar structures (2), expansion alveoli(3), epithelial cell bronchiole sloughing (4), (H & E stain, 400X).

sis, and any strain attached to the liver produces high levels of AST and ALT[32]. This is the first sign of damage to liver cells and the presence of tiredness, necrosis, toxicosis, or biliary liver disease[33].

Histopathologic changes have occurred mainly in the liver, heart, lungs, and kidneys. The toxic dose has been shown with eucalyptus oil in focal hepatic necrosis, pyknosis, vacuolar degeneration, and distorted general architecture (Figure 1). Pyknoses occur when a cell receives an apoptosis signal[34]. The presence of pyknosis and protoplasmic changes such as vacuolation in swollen hepatocytes may indicate possible morphological signs of necrosis or degeneration, The distorted general hepatic architecture in the eucalyptus oil treatment experimental group was probably due to abnormal changes in hepatocytes. Abnormal changes in hepatocytic structure also alter the overall microscopic architecture of the liver, including the eradication of specific sinusoidal spaces[35], caused by eucalyptus oil the congestion of blood vessels into the area of the door associated with inflammatory infiltration. Also, small vacuolar were found in hepatocytes. Our outcomes are consistent with those achieved by (36). This study identified an increase in the number of Kupffer cells in the liver.

In terms of histopathological examination of mice kidney tissue shown in (Figure 3) showed dilated congested renal vein surrounded by infiltrated renal glomeruli and renal tubules and aggregation of inflammatory cells; the results of the present study on the mice kidney showed hyperemia in the Glomerulus, the epithelial cells lining the renal tubules, this result is similar to the report[5], these tubular-related changes could contribute to the increased concentration of potentially toxic essential oil components in the urine filtrate[37], The lobulated renal corpuscles and desquamation of the epithelial cells of the renal tubules were seen in the kidneys of Eucalyptus oil-treated mice (Figure 3). In a similar trend, the data was obtained by (36) peritubular and peri corpuscular mononuclear cellular infiltration into the kidneys. Previously, eucalyptus oil therapy was suggested to enhance prostaglandin synthesis, which is a critical factor in inflammation, apoptosis, and cell growth processes[38].

Eucalyptus oil in the lung of mice led to a state of alveolar emphysema, which is known as obstruction of the small respiratory tracts, which causes pressure on the alveolar walls and their rupture. Pathological alterations are congestion of the pulmonary vessels (Figure 4), that the results of this study are compatible with what he found[39], where has arisen congestion of blood vessels in the lungs of mice, loss of normal lung architecture with partial obliteration of bronchiole light by excretion of epithelial cells or occasionally causing complete bronchial deformation[40], that the inflow of inflammatory cells into the interstitial and broncho-alveolar space is considered an essential role in the progression of lung injury[41].

Our histopathological analysis revealed that this structural change induced by eucalyptus oil was characterized by lymphoid infiltration around blood vessels, perinuclear vacuolation of cardiac muscle cells (Figure 2), and interfibrillar hemorrhaging, and misarrangement of myocardial beams; this result was similar to report[42].

The organs affected by eucalyptus oil toxicity include the liver, kidneys, lungs, and heart, where the organs most affected by eucalyptus oil are the lungs, and the least affected is the heart. This result is inconsistent with report[29], which talks about the lack of side effects of eucalyptus oil emulsion on the cardiovascular and respiratory systems.

Conclusions

It can be concluded from the results of the current study that the value of eucalyptus oil for *Eucalyptus camaldulensis* was 1820 mg/kg, and it produces a severe toxic effect after oral administration in doses of up to 2400 mg/kg, and the high dose causes clinical signs such as slow movement, convulsion, suffocation, and target organs are lung, liver, kidney, and heart. It depends on the dose and the results of this study. Eucalyptus oil is a safe drug for external use.

Author Contributions

Conceptualization, AKA and ZMJ; methodology, AKA; software, ZMJ; validation, ZMJ and AKA; formal analysis, ZMJ; investigation, AKA; resources, ZMJ; data curation, AKA; writing—original draft preparation, ZMJ; writing review

and editing, AKA; visualization, ZMJ; supervision, AKA; project administration, AKA. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

All experimental procedures were approved by the local animal ethics committee in the department of biology of the College of Sciences University Misan and met German and international guidelines (30/8/2020). The study follows the rules of scientific research at Misan University, Iraq.

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Conflicts of Interest

The authors declare no conflicts of interest.

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ARTICLE / INVESTIGACIÓN

Diagnosis of *Mycobacterium tuberculosis* using GeneXpert MTB / RIF and TB-LAMP techniques from pulmonary and extra-pulmonary TB patients in Iraq

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Abstract: Tuberculosis is considered one globally public health problem, particularly in unindustrialized countries. The disease has an increasing threat to public health, causing high morbidity and mortality rates. So, rapid diagnosis is critical to diminish the death and interfere with transmission. In this study, GeneXpert MTB/RIF and Loop-Mediated Isothermal Amplification (TB-LAMP) assays were utilized for the rapid molecular diagnosis of Mycobacterium tuberculosis bacteria and compared sensitivity and specificity of Mycobacterium tuberculosis bacteria; these tests with acid-fast bacilli staining in pulmonary and extra-pulmonary clinical specimens from Iraqi patients in Baghdad capital. 141 pulmonary and extra-pulmonary samples were collected and tested for tuberculosis detection. Acid-fast bacilli, TB-LAMP and GeneXpert MTB/RIF methods were used to diagnose tuberculosis. Of 141 tuberculosis specimens, 58 were acid-fast bacilli smear-positive, while 90 samples were positive for *Mycobacterium tuberculosis* by GeneXpert MTB/RIF assay and 78 by TB-LAMP assay. The results reported a high sensitivity rate of GeneXpert MTB/RIF assay compared to the TB-LAMP and bacilli staining method. The sensitivity of GeneXpert was 63.83%, specificity 50%; positive and negative predictive values were 58.36% to 68.96% and 42.73% to 57.27%, respectively. TB-LAMP sensitivity was 55.32%, specificity 50%; positive and negative predictive values were 49.61% to 60.89% and 43.70% to 56.30%, respectively, while the sensitivity of bacilli staining was 41.13%, specificity 50%; positive and negative predictive values were 35.26% to 47.27% and 44.88% to 55.12%, respectively, these values are dependent on disease prevalence. We concluded that the GeneXpert MTB/RIF test is more sensitive and specific than the TB-LAMP and both techniques are more sensitive and specific than the conventional acid-fast staining method for both pulmonary and extra-pulmonary specimens. Despite culture remaining the gold standard for laboratory confirmation of TB, GeneXpert MTB/RIF and TB-LAMP should become standard practice for patients with suspected TB, and all clinicians and public health programs for TB control should have access to molecular testing for TB to shorten diagnosis time.

Key words: *Mycobacterium tuberculosis*, Pulmonary TB, Extrapulmonary TB, GeneXpert MTB/RIF, TB-LAMP, A.F.B.

Introduction

Tuberculosis (TB) remains a primary infectious disease, particularly in developing countries, and it is a global public health concern[1].In 2019, there were approximately 10.4 million cases of TB worldwide. 90% of them were adults[2]. Tuberculosis is a significant cause of death among infectious diseases, with about 25% of the world's population being infected[3]. According to the estimations of the Iraqi ministry of planning for 2020, the population of Iraq is approximately 40 million. In Iraq, an incidence of 42 TB per 100,000 and the case detection rate in Iraq is 54% for all forms of TB cases[2], mainly due to the poor security situation in some governorates, which report fewer cases as many cases are displaced or lost to follow up. TB clinics in these governorates cannot function normally and staffs are unable to report to their clinics[4]. It is estimated that Iraq is one of the seven countries with the highest TB burden, accounting for 3% of TB patients in the WHO Eastern Mediterranean region[5]. In Iraq, the total TB incidence in 2019 was 41.000 (36.000 - 46.000)[2].

The pathogen that causes TB disease is *Mycobacterium tuberculosis* which continuously develops strains containing resistance genes, increasing the challenge of TB treatment and prevention[6]. Initially, TB targets the lungs; however, the pathogen can invade other organs, including the skin, lymphatic, circulatory, central nervous, and digestive system[7]. Although speedy and inexpensive, both the radiographic microscopy and the smear had poor sensitivity and positive predictive values; however, they remained a valuable prognosis in most resource-limited settings, from smear microscopy, which has low sensitivity and specificity compared to culture8. The culture method is still the golden tool for final determination; nevertheless, it is slow and might take up to eight weeks due to the long incubation time of the pathogen[9]. Thus, rapid and accurate methods of diagnosing *Mycobacterium tuberculosis* are essential for early initiation of treatment, prevention and improvement of patient outcomes. With the demand and urgency for rapid and simple diagnostic tests, especially in developing coun-

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tries, polymerase chain reaction-based techniques have been developed for tuberculosis detection and antibiotic resistance testing[10].GeneXpert MTB / RIF and TB-LAMP assays are two modern molecular technologies that are widely used worldwide[11].

GeneXpert MTB / RIF is a nucleic acid test established by Xpert, Cepheid, Sunnyvale, CA, USA, developed to simultaneously diagnose TB and detect RIF resistance by detecting mutations in the RIF resistance determining region RRDR of the rpoB gene[2,12]. The Xpert MTB/RIF assay employs a molecular beacon to detect DNA sequences amplified in a hemi-nested rt-PCR assay with the ability to utilize 5 different nucleic acid hybridization probes performed in the same multiplex reaction. Every probe matches a different target sequence in the rpoB gene of rifampicin-susceptible M. tuberculosis, labeled with a different colored fluorophore, and these overlapping probes extend along the entire 81 bp core region of the rpoB gene[13].

Xpert results can be obtained in 2 hours, compared to the 8 weeks required to complete the traditional TB diagnosis by culture[14].

Loop-mediated isothermal amplification (LAMP) is the technique developed in Japan (Eiken Chemical Co., Ltd.)[15], which amplifies DNA under isothermal conditions[16].

The LAMP technique can increase small amounts of DNA to 10^9 copies in less than an hour using four to six primers that recognize six to eight regions of the target DNA. It initiates DNA amplification using different oligonucleotides simultaneously. As a result, the technique is particular, precise and fast. Reaction intermediates include several inverted repetitive loop structures. The large amounts of DNA produced, turbidity or fluorescence are observed, allowing the result to be determined visually. It prevents detection by gel electrophoresis or labeled probes. The LAMP technique is considered superior to other nucleic acid amplification methods because it is much faster, more efficient and economical than other NAATs[17,18].

Materials and methods

Study design and samples collection

This study was conducted from September 3 to December 23 in 2020 on patients suspected of having pulmonary and extra-pulmonary tuberculosis at the specialized center for chest and respiratory diseases in Baghdad's capital. A total of 141 samples (107 pulmonary and 34 extra-pulmonary) were collected from patients with symptoms suggestive of tuberculosis. In this study, suspected tuberculosis patients were assigned to sample collection based on the presentation of symptoms and chest radiographic findings.

The pulmonary samples were taken from sputum, bronchial lavage, Broncho-alveolar lavage (BAL), and gastric lavage. Patients with clinical fitness criteria were requested to provide three sputum specimens within two days, two spot specimens furthermore one gotten in the morning.

Extra-pulmonary samples of pleural fluid, lymph node biopsy, cerebrospinal fluid, skin, ascitic fluid, sinus swab, and pus were taken.

The following steps are taken according to the type and location of the sample taken:

1-Sputum samples: Instruct each patient to cough deeply from the chest and spit sputum into the sterile container, avoiding nasal secretions or saliva, and three samples were collected. The first one was taken from the patient after arriving at the center; the second sample was collected early in the morning before breakfast; the third was collected at any time of the day.

2- Body fluids and other samples: Each body fluid was concentrated by centrifuging the samples at 3000 rpm for 15 min. The sediment has been processed. All of the above samples were digested and purified with 4% sodium hydroxide (NaOH); Samples were mixed with equal volumes of NaOH in a sterile falcon tube and mixed with a vortex mixer for a few seconds. The tube was then left at room temperature for 15 minutes. The mixture was then centrifuged at 3000 rpm for 15 min. Samples were neutralized by drops of HCL solution containing phenol red as an indicator and shaken until the color changed from red to yellow. The third sputum sample was directly tested by Ziehl-Neelsen staining and the TB-LAMP and GeneXpert MTB/RIF assays. Non-respiratory specimens from closed, sterile sites were not naturally cleared before smear preparation but were centrifuged at 3000 g for 20 min[19].

Tuberculosis detection

Ziehl-Neelsen's stain (ZNS)

According to the guidelines of manual laboratory work, clinical decontaminated specimens were examined by Ziehl-Neelsen's stain[20] as follows.

Using a sterile bacteriological loop, a smear was prepared on a new and clean slide and allowed to dry by air. Heat-fixed smears were flooded with carbon fuchsin stain, and the slides were heated slowly without boiling the stain. The stain was left for 5min., then rinsed the slides with tap water. Decolorization was performed by adding 25% sulfuric acid solution (H2SO4) to each slide and left for 2 min. Then rinsed the slides with tap water. Methylene blue was added to each slide for 1 min. As a counterstain, then rinsed the slides with tap water. Smears were allowed to dry in the air and then examined under a light microscope (Model CH 30, Olympus, Tokyo, Japan); the results of direct examination appear within fewer than 1 an hour. Smear results were reported for the presence or absence of AFB using the World Health Organization/International Union against Tuberculosis and Lung Disease scale, with a positive result corresponding to ≥ 1 AFB per 100 high-power fields.

GeneXpert MTB / RIF assay

The GeneXpert MTB/RIF (Cepheid Inc. /USA) assay consists of a single-use multi-chamber plastic cartridge with liquid buffers and lyophilized reagent beads required for sample processing and DNA extraction, and real-time polymerase chain reaction interference. Clinical samples or disinfected sputum pellets are treated with sodium hydroxide and a sample reagent containing isopropanol (SR). SR is added in a 2:1 ratio to the sample or sputum pellets and incubated for 15 minutes at room temperature. The treated sample is transferred to the cartridge, the cartridge is loaded into the GeneXpert instrument, and the automatic process completes the remaining assay steps. The assay cartridge also contains lyophilized *Bacillus globigii*, internal sample treatment and PCR control. Spores are automatically resuspended and processed during the sample processing step, and *B. globigii* DNA is amplified during the polymerase chain reaction step. The standard user interface indicates the presence or absence of M*ycobacterium*

tuberculosis, the presence or absence of resistance to RIF, and a semi-quantitative estimation of the concentration of *Mycobacterium tuberculosis* (high, medium, low, very low). Negative tests for *Mycobacterium tuberculosis* and negative internal control with *B. globigii* were reported as invalid[21], GeneXpert MTB/RIF kit containing; cartridge (disposable), reagent 1, reagent 2, sample reagent and disposable transfer pipettes, the same company makes the kits.

The TB-LAMP technique

The TB-LAMP was performed on clinical decontaminated specimens following the manufacturer's recommendations (Eiken Chemical Co., Ltd., Tokyo, Japan). The Loopamp™ PURE DNA Extraction Kit extracted DNA from the sputum specimens. In a heating tube, a sixty microliter aliquot of purulent sputum was mixed with the DNA extraction solution and incubated at 90°C in an LF-160 incubator for 5 minutes to let bacterial cells lysis and DNA release into the solution. The process of DNA solution purification was conducted using porous absorbent powder in an adsorbent tube. A thirty microliter aliquot of purified DNA was distributed in the LAMP reaction tube comprising the lyophilized LAMP components (Bst DNA polymerase, dNTPs, calcein, and MTBC-specific primers targeting DNA gyrase subunit B and insertion sequence 6110). Reconstitution of the dried LAMP reagents (stored in the cap of the reaction tube) was performed by mixing it with the DNA solution. Then the mixture tube was incubated at 67.0°C for 40 minutes to permit DNA amplification by the LAMP reaction. The LAMP amplified products were visually detected based on their fluorescence under ultraviolet light.

Results

The study included 141 patients (102 male, 39 female) with symptoms suggestive of TB based on clinical, pathological, or radiological evidence of tuberculosis. The age groups included in this study are between 6 and 65 years old. Male cases were 70 (78.77%), and female cases were 20 (22.3%). Most of the patients were in the 25-35 age group, while the lowest were under 15 years.

As the results appeared in table 1 and figure 1 the distribution of positive results for 54 lung samples tested by microscope (sputum 47, bronchial lavage 5, alveolar Broncho-alveolar lavage BAL 1, gastric lavage 1) and distribution of 4 positive results for extra-pulmonary samples diagnosed with Microscope (pleural fluid 3, Lymph node 1).

At the same time as the distribution of positive results for 76 lung samples tested with GeneXpert MTB / RIF (sputum 63, bronchial lavage 8, alveolar bronchial lavage BAL 2, gastric lavage 3) and distribution of 14 positive results for extra-pulmonary specimens diagnosed with GeneXpert MTB / RIF (pleural fluid 8, skin swab 4, lymph node swab 2). Distribution of positive results for 70 lung samples tested with TB-LAMP (sputum 57, bronchial lavage 8, alveolar broncho-alveolar lavage BAL 2, gastric lavage 3) and distribution of 8 positive results for extra-pulmonary samples diagnosed with TB-LAMP (pleural fluid 6, Lymph node 2).

As shown in table 1 and figure 2, the results of the Ziehl-Neelsen stain showed that the sum of the positive results was 58 (54 pulmonary and 4 extra-pulmonary).

Molecular diagnosis of *Mycobacterium tuberculosis* bacteria by GeneXpert MTB / RIF test revealed that 90 sam-

	GeneXpert MTB/RIF			Loop-mediated isothermal amplification TB-LAMP			Z.N.S
	Sample type	**Total No.**	**Detect**	**Not Detected**	**Total No.**	**+Ve**	**- Ve**	**(+)**
1	**Pulmonary TB (PTB)**	107	76	31	107	70	37	**54**
a	Sputum	92	63	29	92	57	35	**47**
b	Bronchial Washing	10	8	2	10	8	2	**5**
c	Bronchial alveolar lavage (BAL)	2	2	0	2	2	0	**1**
d	Gastric lavage	3	3	0	3	3	0	**1**
2	**Extra - Pulmonary TB (EPTB)**	34	14	20	34	8	26	**4**
a	Pleural fluid	16	8	8	16	6	10	**3**
b	Ascitic fluid	4	0	4	4	0	4	
c	Skin swab	4	4	0	4	0	4	
d	Cerebrospinal fluid (CSF)	2	0	2	2	0	2	
e	Lymph node swab	4	2	2	4	2	2	**1**
f	PUS	2	0	2	2	0	2	
g	Sinus swab	2	0	2	2	0	2	

Table 1. Results by GeneXpert MTB / RIF, TB-LAMP and Z.N.S.

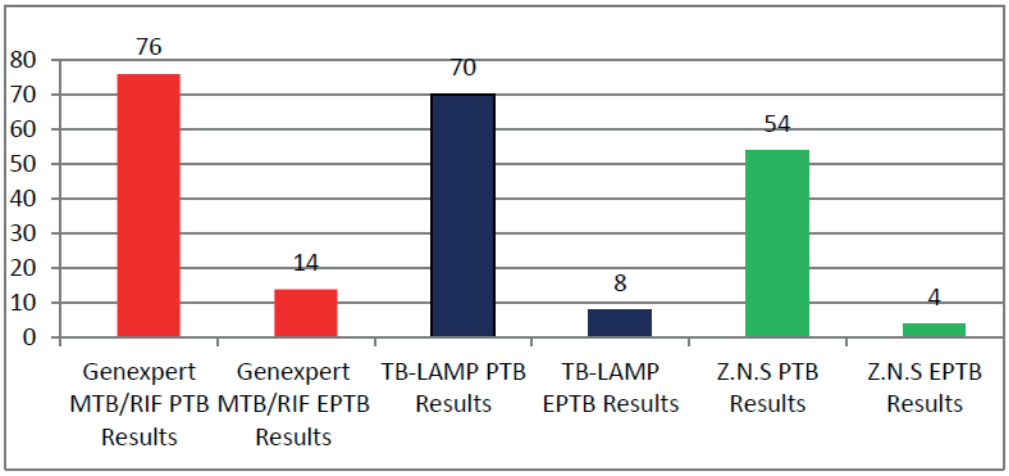


Figure 1. Distribution of PTB and EPTB samples diagnosed by the three methods.



Figure 2. *Mycobacterium tuberculosis* bacteria stained by Ziehl-Neelsen stain, under light microscope (magnification power is 1000X).

ples were positive (76 pulmonary and 14 extra-pulmonary). The results appeared on the computer screen according to the number of TB bacilli isolated by the GeneXpert MTB / RIF system, and they were (very low, low, medium or high, very high)(table 1 figure 3).

Only two cases of rifampicin resistance were diagnosed due to therapeutic relapse and referred to a respiratory consultant for evaluation.

Table 1 and figure 4 showed that molecular diagnosis of *Mycobacterium tuberculosis* using the TB-LAMP test showed 78 samples were positive (70 pulmonary and 8 extra-pulmonary). The study results appeared in the fluorescence detection unit of the TB-LAMP device, and when the UV light was turned on, the color of the pattern was green in case of a positive result, while negative results remained unchanged in color.

As shown in table 2, GeneXpert MTB / RIF sensitivity was 63.83% while 50% was specificity; the positive and negative predictive values were 58.36% to 68.96% and 42.73% to 57.27%, respectively whereas the sensitivity and specificity of TB-LAMP was 55.32% for sensitivity and 50% was the specificity ; the positive and negative predictive values were 49.61% - 60.89% and 43.70% - 56.30 respectively, while the acid-fast bacilli (AFB) sensitivity was 41.13%, and the specificity 50%; The positive and negative predictive values are 35.26% to 47.27% and 44.88% to 55.12%, respectively. These values depend on disease prevalence; statistical analysis data were analyzed using SPSS version 24. Percentages were calculated when the results of the AFB test, GeneXpert test and TB-LAMP test of the same patients as shown in table 2.

Discussion

The availability of affordable, fast and reliable diagnostic tests that can be used in resource-limited settings is a critical gap in the battle against TB. The production of nucleic acid-based tests has created new avenues for producing susceptible point-of-care tests. The GeneXpert MTB/RIF, which has turned the TB diagnostic landscape into well-fitted environments, is the latest model that has been rolled out worldwide, but it has major resource-poor setting limitations[22], while the TB-LAMP test needs fewer infrastructures as an alternative and may therefore be applicable for use in second-tier diagnostic laboratories[23].

In this study, the age groups included are between 6 and 65. Male TB positive cases were 70 (78.77%), and female TB positive cases were 20 (22.2%) from 141 pulmonary and extra-pulmonary samples. The higher percent of the patients were recorded in the 25-35 age groups, whereas

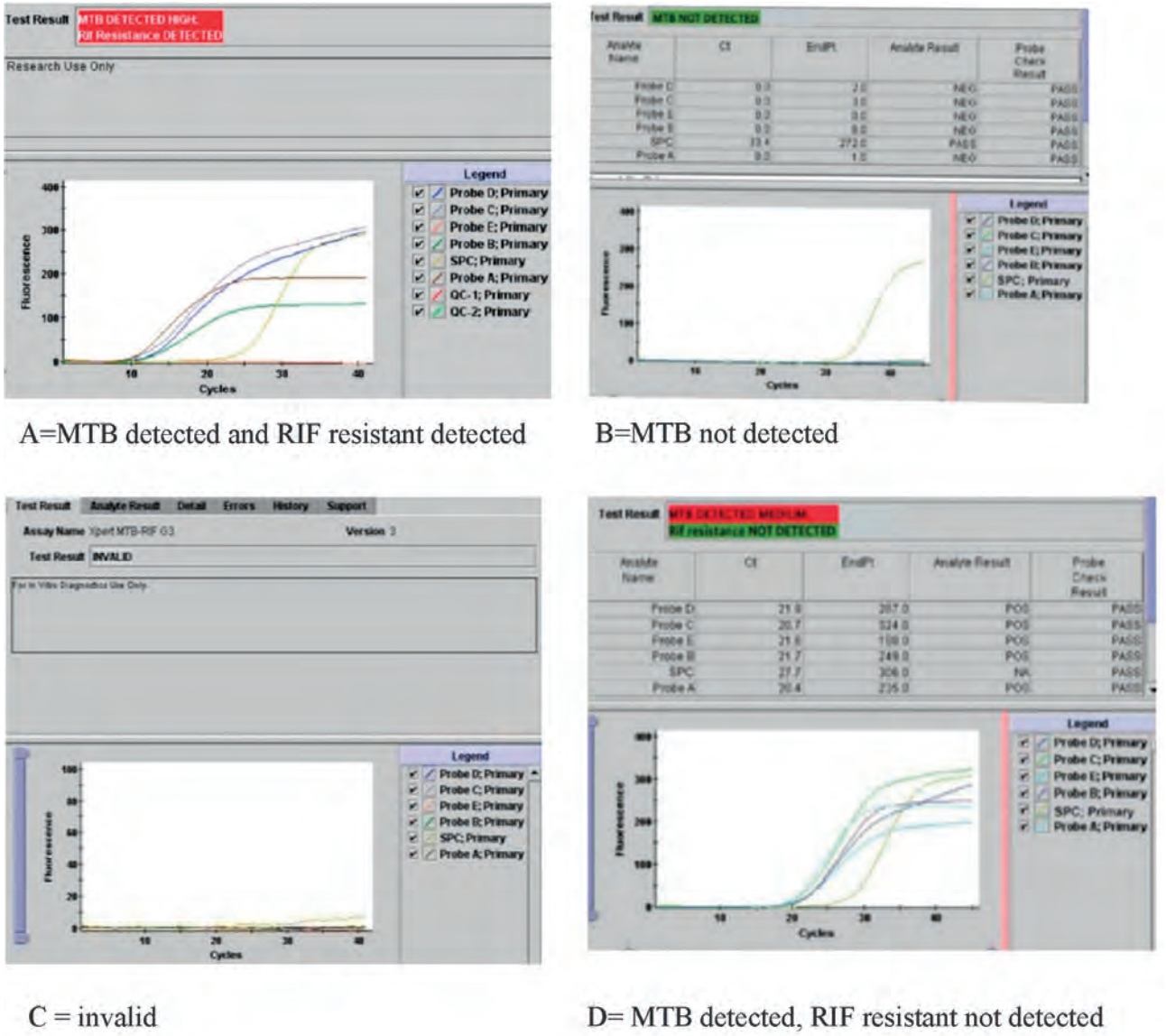


Figure 3. GeneXpert MTB / RIF test results.

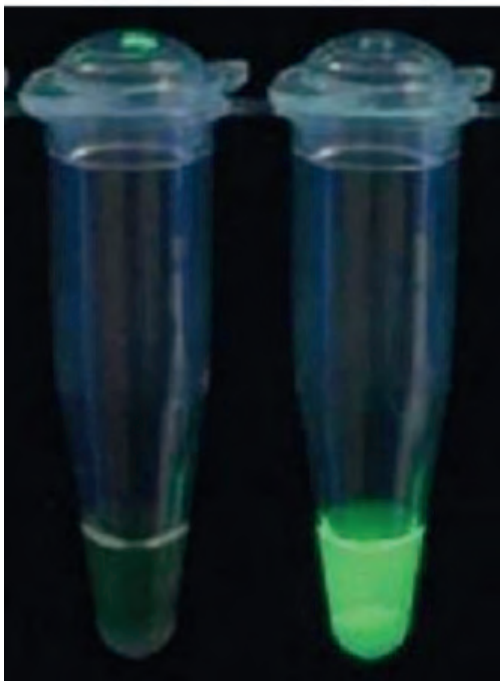


Figure 4. TB-LAMP test results, - means negative result while + means a positive result.

Statistic	Z.N.S		TB-LAMP		GeneXpert MTB/RIF	
	value	95% CI	value	95% CI	value	95% CI
Sensitivity	41.13%	32.92% to 49.37%	55,32%	46.72% to 63.69%	63,83 %	55.32% to 71.75%
Specificity	50.00%	42.15% to 57.85	50.00%	40.97% to 59.03%	50.00 %	39.93% to 60.07%
Positive Likelihood Ratio	0,82	0.64 to 1.06	1,11	0.88 to 1.39	1,28	1.01 to 1.16
Negative Likelihood Ratio	1,18	0.96 to 1.45	0,89	0.69 to 1.15	0,72	0.54 to 0.97
Disease prevalence	45,93	40.25% to 51.68%	52,81%	46.63% to 58.92%	58,02 %	51.55% to 64.30%
Positive predictive value	41.13	35.26% to 47.27%	55.32%	49.61% to 60.89%	63.83 %	58.36% to 68.96%
Negative predictive value	50.00%	44.88% to 55.12%	50.00%	43.70% to 56.30%	50.00 %	42.73% to 57.27%
Accuracy	45,93	40.25% to 51.68%	52,81	46.63% to 64.30%	58,02	51.55% to 64.30%

Table 2. Statistical analysis Z.N.S, GeneXpert MTB/RIF and TB-LAMP.

the lowest was below 15 years. A similar study described different percentages in different places around the world. In this respect, our data were in the same line with a study conducted in Turkey in 2015 on pulmonary and extra-pulmonary TB Established that positive results for 372 patients were 227 (61%) men, 145 (39%) women, 168 (45.2%) EPTB and 204 (54.8%) PTB[24], however, our study conflicts with the study conducted by Al-Otaibi and Al-Hazmi (2010) in Saudi Arabia, which reported that the rate of EPTB was 57.5% versus 42.5% according to PTB. The most dominant age group impacted was 20-29 years (n=38; 33%) followed by age 60 and more (n=25; 21.7%)[25]; these findings were different from data obtained by (Sharma et al., 2017), who reported that 41-60 age group was the most affected one in their study[26].

A study by Rakotosamimanana in Madagascar found the high sensitivity and specificity was confirmed for both GeneXpert MTB/RIF® (86.6% (95% CI 81.1-90.7%) and 97.4% (95% CI 94.9-98.8%)) and TB-LAMP (84.6% (95% CI 78.9-89.0%) and 98.4% (95% CI 96.2-99.4%)). Implementation of GeneXpert MTB/RIF and TB-LAMP increased tuberculosis diagnostic algorithms sensitivity from 73.6% (95% CI 67.1-79.3%) up to 88.1% (95% CI 82.8-91.9%). This increase was highest when molecular assays were used as add-on testing following negative smear microscopy, as add-on testing, GeneXpert MTB/RIF and TB-LAMP respectively improved case detection by 23.8 and 21.2% ($p < 0.05$)[27].

The results of a study in Gambia (2016) demonstrate the diagnostic utility of both GeneXpert and TB-LAMP. While the TB-LAMP program requires fewer infrastructures, it cannot detect drug resistance patterns and is thus more suitable as a screening test for new TB cases in peripheral health clinics. When using culture as the reference standard, it was found to have an overall sensitivity of 99% (95% CI: 94.5-99.8) and a specificity of 94% (95% CI: 89.3-96.7) for TB-LAMP. When performing a latent class analysis, TB-LAMP has a sensitivity of 98.6% (95% CI: 95.9-100) and a sensitivity of 99% (95% CI: 98 compared to 91.1% (95% CI: 86.1-96). 2-100) and 100% (95% CI: 98.2-100) specificity to MGIT culture. GeneXpert had the highest sensitivity of 99.1% (95% CI: 97.1-100), but the lowest specificity of 96% (95% CI: 92.6-98.3). TB-LAMP and GeneXpert showed high sensitivity and specificity regardless of age or type of infection[28].

GeneXpert test with smear microscopy and the gold standard of Mycobacterial culture and TB-LAMP were compared to a diagnosis of TB. Sputum Specimens were collected from patients with pulmonary TB symptoms who attended a primary health care clinic. The results showed that the TB-LAMP test had significantly higher sensitivity than smear microscopy (72.6% versus 45.4%, P, 0.001) but was significantly higher than GeneXpert (92.9%, P¼0.004). There was no significant difference in the sensitivity of smear-positive, culture-positive and smear-negative, culture-positive sputum samples using GeneXpert versus TB-LAMP (95.9% / 55.9% versus 97.6% / 66.1%, respectively; P ¼0.65, P ¼ 0.27). The positive predictive value of TB LAMP was significantly higher than that of GeneXpert (87.5% versus 77.0%; P 0.02), but similar to that of the smear microscope (94.2%; P 0.18). The negative predictive value was 91.9%, 92.5% (P ¼ 0.73) and 83.1% (P ¼ 0.0001 respectively[29].

A study conducted in North India (2017) stated that 530 patients with PTB symptoms with one sputum sample was collected from each patient. The TB-LAMP assay was performed. The rest of the sample was used for microscopy and culture. Xpert® MTB/RIF assay was also enrolled in this study; the findings of this study show the sensitivity and specificity of the TB-LAMP assay in culture-positive samples obtained from 453 patients presenting with PTB symptoms were respectively 100% (95%CI 94.7-100) and 99.2% (95%CI 97.8-99.8). The sensitivity and specificity of Xpert in culture-positive samples were respectively 82.6% (95%CI 71.5-90.6) and 94.9% (95%CI 92.2-96.8). The study concludes that the TB-LAMP assay showed high sensitivity and specificity with the limited requirement of testing infrastructure and is thus a promising diagnostic tool for TB diagnosis in resource-poor settings[30].

GeneXpert MTB/RIF and TB-LAMP provide a definitive diagnostic test for identifying MTB with the advantage of GeneXpert MTB/RIF over TB-LAMP contrasting conventional methods. The main disadvantage of AFB smear is low

sensitivity, and essential benefits of GeneXpert MTB/RIF and TB-LAMP are the early diagnoses of TB and early initiation of treatment; Thus. They were improving patient consequences. Although culture remains the necessary standard test for laboratory confirmation of TB, GeneXpert MTB/RIF should become the standard first and then TB-LAMP. This study is for patients suspected of having TB, and all physicians and public health programs must obtain molecular TB tests to shorten the diagnostic time. Through this study, we found some advantages and disadvantages in both techniques, which are as follows, advantages of GeneXpert MTB / RIF; fast completion time (two hours), the ability to detect very few living tubercles, detection of rifampicin-resistant and full automation is possible to eliminate personal errors and cross-contamination of samples, while the disadvantages were; Incorrect results due to the presence of amplification inhibitors in samples, high cost and technical skill requirement, Nucleic acid contamination - false results, amplification from dead bacillus and both Cartridges and the machine must be stored appropriately (at optimum temperature), connection with a computer and a continuous optimum power supply are the absolute fundamental. On the other hand, the advantages of TB-LAMP were; fast technique (one hour), low cost, simple skills, and cheaper than GeneXpert kits, while the disadvantages were; It is not automatic; all processes (DNA extraction, amplification and result interpretation) are manual, it is challenging to run multiplex LAMP reactions in one tube, and Unlike the GeneXpert device, the resistance of *Mycobacterium tuberculosis* bacteria to rifampicin is not detected.

Conclusions

This study firmly concluded the high sensitivity and specificity of molecular tests as compared with conventional methods, which need more time and heavy labor. Also, this study confirmed that the GeneXpert MTB/RIF test is more sensitive and specific than the TB-LAMP, and both are more sensitive and specific than the conventional acid-fast staining method for both pulmonary and extra-pulmonary specimens and recommended to use of these two techniques especially suspected cases of TB.

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Conflicts of Interest

The authors declare no conflict of interest.

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ARTICLE / INVESTIGACIÓN

The Impact of Vaccination on Severity of COVID-19 Illness in Hematologic Malignancies Patients

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Abstract: Covid 19 in hematological cancer patients can lead to a deterioration in the clinical course and a reduction in life expectancy leading high fatality rate. Vaccines developed against the causative virus may reduce the severity of the disease and a decline in mortality rate. This study aims to determine the impact of vaccination on the severity of COVID-19 illness in patients with hematologic malignancies. A multicenter retrospective observational study was conducted on 60 hospitalized patients admitted to intensive care units (ICUs). All patients were previously diagnosed with leukemia or lymphoma and tested positive for COVID-19 by real-time polymerase chain reaction (RT-PCR). Protocol version 8 was used to determine the patients' results, including improvement to discharge criteria or deterioration to death. The most results in this study; The mean age of the patients was 33.63± 16.5 years (range 6-72 years). Diabetes was the most common comorbid illness (10%). The Oxygen saturation range was (70-95%). Among (60) patients enrolled in this study;29(48.33%) was not received any Covid-19 vaccine while 31(51.67%) were fully vaccinated; concerning patients with positive COVID19; the underlying hematological malignancy was leukemia (63.3%) while patients with lymphoma were (36.7%); after 30-day follow up, depending to the outcome. The number of deaths was 14 cases (23.3%) of patients while the other 46 patients (74.7%) survived and were discharged from the hospital. Our study concludes that vaccinating hematological malignancy patients against Covid19 may provide clinical protection from this illness compared to no vaccination patients.

Key words: COVID-19 disease, Hematological Malignancy, Vaccine, intensive care units.

Introduction

World Health Organization (WHO) in March 2020 stated that Coronavirus disease 19 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was a pandemic[1]. Within a year, the mortality rate of this disease increased over the world; some groups of individuals are at high risk of developing severe and life-threatening infections. One of them is patients with hematological disorders due to immunocompromised status as chemotherapy treatments. Covid 19 disease in hematological cancer patients can lead to a deterioration of the clinical result and a reduction in life expectancy leading high fatality rate[2].

Patients with hematological malignancies deal more with the healthcare system, which puts them at a higher risk of contracting COVID-19. Furthermore, preliminary trials in China revealed that severe COVID-19 infection and death were common in all types of cancer patients compared to the general population[3].

Another study in this regard reported that patients suffering from blood malignancy are more susceptible to developing more severe infection and a high death rate of Covid-19 among all cancer patients. This could be because hematological malignancy patients frequently exhibit innate and adaptive immunological abnormalities, which is a critical risk factor for increased mortality in older age when it's associated with cancer disease[4].

After the scientist develops many types of Covid19 vaccines, professional oncology societies declare an urgent need for vaccination in patients with cancer, including hematological malignancies[5].

In people who have been vaccinated, the rate of hospital admissions and death from Covid 19 remained stable. According to Public Health England's latest surveillance report, unvaccinated people have a "significantly higher" risk than vaccinated people[6]. Some studies reported that patients with immunocompromise might be less likely to produce a robust immune response to the vaccine, putting them at a higher risk of moderate to severe covid-19[7].

This study was conducted to determine the impact of vaccination on the severity of COVID-19 illness in Hematologic malignancy patients.

Materials and methods

A Multicenter retrospective observational study was conducted on 60 hospitalized patients who were admitted to intensive care units (ICUs). All patients were previously diagnosed with leukemia or lymphoma from January 2021 to the end of December 2021. All patients were tested positive for COVID-19 by real-time polymerase chain reaction (RT-PCR) assay, which was achieved in the hospital-based clinical laboratory.

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All patients under investigation in this study were referred by consultation of a hematological specialist and verification of the information in the medical record. Patients with incomplete data, information or presented with non-malignant hematological diseases or solid cancer were excluded from this study.

Demographics, clinical and laboratory data were accumulated from a medical record for all participants; patients who are fully vaccinated have positive COVID-19 laboratory testing and symptoms that appear >14 days after the second dosage; while Individuals who have positive laboratory COVID-19 tests but no record of receiving any type of COVID-19 immunization or first-dose vaccination after symptom start are considered unvaccinated.

Outcomes

To observe the principles of early detection, reporting, quarantine and treatment of COVID-19 patients to increase recovery rates and lower fatality rates. Protocol version 8 was used to determine the patients' results, including improvement to discharge criteria or deterioration to death[8]. COVID-19 death was defined as the death of COVID19 patients who died as a direct result of coronavirus infection but not due to the pandemic's indirect effects[9].

Statistical Analysis

The quantitative data were expressed as mean ± standard deviation. Binomial data were presented as frequency percentages. Comparisons between quantitative were performed by the parametric Student t-test, while the Chi-square test made the comparison between binomial data. Multivariate analysis was performed with a binary logistic regression test. The odds ratio (OR) and its corresponding 9% confidence interval (CI) were calculated from this test. All data were analyzed with SPSS for Windows, v.25.0; IBM Corp, Armonk, USA.

Results

Demographic Characteristics of the Patients

The mean age of the patients was 33.63± 16.5years (range 6-72 years); about two-thirds of them (60%) were females. Only a small percentage (15%) of the patients were ex/current smokers. Diabetes was the most common comorbid illness (10%). Many patients had more than one comorbidity (Table 1).

Clinical Characteristics of the patients

During the sickness, all patients underwent respiratory support, and the oxygen saturation (SpO2) was measured using pulse oximetry; the saturation range (was 70-95%); among (60) patients enrolled in this study; 29 (48.33%) was not received any COVID-19 vaccine while 31 (51.67%) was fully vaccinated; Headache is the most prominent complaint in 39 (65%) of patients followed by cough in 38 (63.33%) in addition to that fatigue and loss of smell and taste was also reported as frequent symptoms and was 27 (45%) and 22 (36.67%) respectively. The other complaints include three cases of abdominal pain and two cases of vomiting and sore throat were much less frequent symptoms 7 (11.67%) table (2).

Type of Malignancies

Concerning patients positive for COVID-19, the underlying hematological malignancy was leukemia at 63.3%, while patients with lymphoma were 36.7%. After a 30-day follow-up, depending on the outcome, death was reported in 14 patients (23.3%), while the other 46 patients (74.7%) survived and were discharged from the hospital figure (1) and (2).

Association of Clinical Characteristics with a Survival rate

Patients were classified into two groups according to outcomes (survival and dead patients); patients in the survived group reported SPO2% (88.9±4.1%) compared to (81.79±7.56) in dead patients with significance differences (*P-value* <0.001). Many patients in the survived group underwent fully COVID 19 vaccination 31 (67.39%). On the other hand, no patients in the dead group received such vaccines with a significant difference (*P-value* <0.001). Among clinical symptoms, non -significant differences were obtained between the two groups. Concerning the type of hematological malignancies, the leukemic patients were more common among survival (33 patients, 71.74%) than deceased patients (5 patients, 35.71%) with a significant difference (Table 3).

Multivariate Analysis

All variables that demonstrated significant association with the patient's outcome in univariate analyses were entered into multivariate analysis. The results are shown in Table 4. Two variables remained significantly associated with the patients' outcomes. More than half of deceased patients (57.14%) had SPO^2 <85% compared with 23.91% of surviving patients who had such percent with a significant difference (OR= 2.62, 95% CI 1.21-3.48 P=0.042). Furthermore, 76.39% of surviving patients were vaccinated versus none of the deceased patients who had a vaccine, with a highly significant difference (OR=15.6, 95% CI 1.75-18.17, P=0.003).

Discussion

The rapid spread of SARS-CoV-2 worldwide has created a unique medical situation for individuals with chronic diseases, including patients with hematological malignancies who are at the most significant risk of severe COVID-19. As a result, incremental vaccines have been a top goal; and highly protective vaccinations are already being distributed. The current study looked at the crucial role of COVID-19 vaccination in the survival of patients with hematological malignancies who were admitted to the hospital.

Among sixty patients with hematological malignancy confirmed to COVID-19 infection in this study, the mean age of the patients suffering from this infection was 33.63± 16.5 with a range of 6-72 years. However, this observation disagreed with many studies that reported severe cases of COVID-19 were more common in older patients 65 years old[10,11].

The difference in this study from other studies can be attributed to the fact that the selection of patients in this study is restricted to people who suffer from hematological diseases, and it is known that blood malignancy affects different ages.

In the current study,60% of patients were female, and (40%) of them were male and male to female (1:1.5). Much

Variables	Values
Age, years	
Mean±SD	33.63± 16.5
Range	6-72
Gender	
Male	24(40%)
Female	36(60%)
Smoking	
Never	51(85%)
Ex/current	9(15%)
Comorbidities	
No	50(83.33%)
DM	6(10%)
HTN	3(5%)
IHD	3(5%)
Others	3(5%)

Table 1. Patients' characteristics and demographic data (n=60).

Variables	Values
SPO_2 (%)	
Mean±SD	87.02±6.05
Range	70-95
Vaccination	
No	29(48.33%)
Yes	31(51.67%)
Sinopharm	11(18.33%)
Pfizer	16(26.67%)
AstraZeneca	4(6.67%)
Clinical presentation	
Cough	38(63.33%)
Fever	15(25%)
Fatigue	27(45%)
Running nose	11(18.33%)
Loss of smell and taste	22(36.67%)
Diarrhea	18(30%)
Headache	39(65%)
Others	7(11.67%)

Table 2. Clinical presentation (n=60).

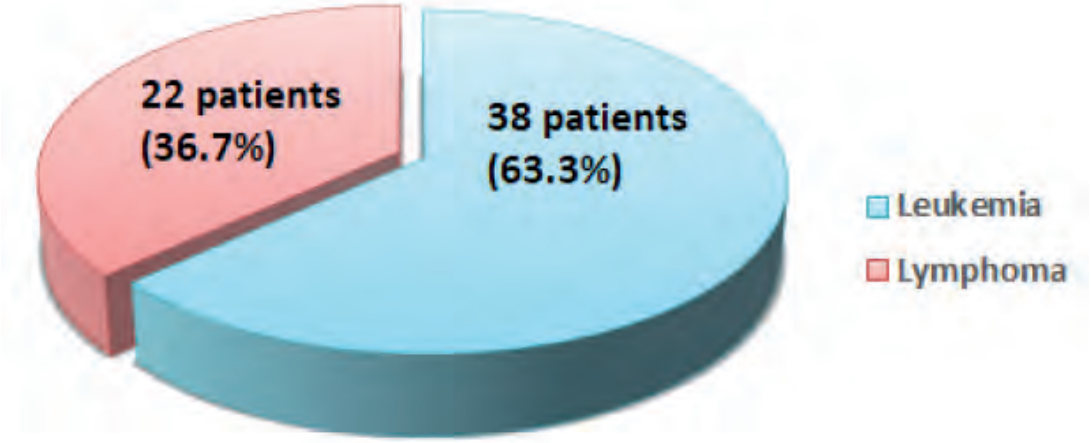


Figure 1. The proportion of leukemia and lymphoma in patients with COVID1-9.

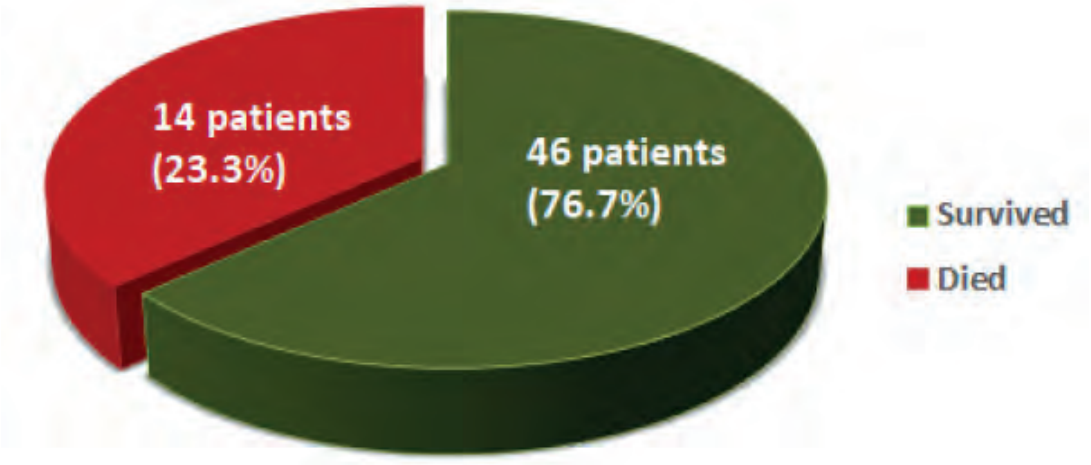


Figure 2. Mortality and Survived Percentage.

Variables	Survived patients (n=46)	Died patients (n=14)	p-value
SPO_2 (%)	88.9±4.1	81.79±7.56	<0.001
Vaccination			
No	15(32.61%)	14(100%)	<0.001
Yes	31(67.39%)	0(0.00%)	
Clinical presentations			
Cough	28(60.87%)	10(71.43%)	0.473
Fever & Fatigue	11(23.91%)	4(28.26%)	0.724
Running nose	10(21.74%)	1(7.14%)	0.217
Loss of smell and taste	16(34.78%)	6(42.86%)	0.583
Diarrhea	14(30.43%)	4(28.57%)	0.894
Headache	28(60.87%)	11(78.57%)	0.224
Others	5(10.87%)	2(14.29%)	0.660
Type of Malignancy			
Leukemia	33(71.74%)	5(35.71%)	0.014
Lymphoma	13(28.26%)	9(64.29%)	

Table 3. Association of clinical characteristics with outcomes.

Variables	Survived (n=46)	Died (n=14)	p-value	OR (95%CI)
SPO_2 (%)				
≤85	11(23.91%)	8(57.14%)	0.042	1.0
>85	35(76.09%)	6(42.86%)		2.62(1.21-3.48)
IHD				
No	45(97.83%)	12(85.71%)	0.129	1.0
Yes	1(2.17%)	2(14.29%)		0.15(0.02-1.17)
Vaccination				
Non -vaccinated	15(32.61%)	14(100%)	0.003	1.0
Vaccinated	31(67.39%)	0(0.00%)		15.6(1.75-18.17)
Malignancy				
Leukemia	33(71.74%)	5(35.71%)	0.086	1.0
Lymphoma	13(28.26%)	9(64.29%)		3.45(0.82-12.84)

Table 4. Multivariate Analysis.

literature on gender as a risk of in-hospital mortality provides mixed results. According to a few studies, sex differences may exist in COVID-19 individuals with severe illnesses, with men being more likely than women and recovery is more challenging[12,13].

This result is incompatible with those obtained in the above studies. However, many researchers in Middle Eastern countries, including Iraq, mentioned that hematological cancer affects females more than males[14,15].

The majority of patients in the current study (85%) were nonsmokers, and it was higher than the results obtained by Prats-Uribe *et al.*, Who mentioned that (56.2%) of COVID-19 patients were nonsmoker[16]; Conversely, it was less than the results reported by Heydari *et al.* (77.8%)[17].

The explanation for these variations in smokers and nonsmoker COVID-19 patients could be attributed to differences in the socioeconomic and geographical aspects of the studied locations and differences in the study methods. The size of the sample and the distinction in the mode of life factors.

The data reported that most patients had no comorbidities (83.33%) while (16.67%) of the patients had comorbid diseases. Furthermore, diabetes mellitus is the most frequent comorbidity in the current study but less than the finding of other studies conducted in Myanmar 37.8%[18]. Singapore 28.3%[19]. But it's higher than the finding reported in China, 15.8%[20]. This disparity in such results could be explained by differences in the prevalence of chronic diseases by age, gender, and geographic region.

In the current study, headache (65%) and cough (63.33%) are the predominant clinical impression associated with COVID 19 infection, followed by fatigue (45%) then smell and taste (36.67%); this result is inconsistent with prior studies stated that fatigue, sore throat, shortness of

breath and rhinorrhea were the most common presenting symptoms of COVID 19 patients[21,22].

In this study, patients were classified into two groups according to outcomes (survival and dead patients); overall results in the current study showed a considerable decline in Oxygen saturation SPO_2% in Died (81.79%). Such results are in line with other studies that stated decreasing in SPO_2% is closely related to an increased mortality rate in COVID19 patients in which pulmonary infection may affect the oxygenation state, resulting in a decrease in peripheral SpO_2, which subsequently leads to acute respiratory distress syndrome (ARDS) and acute hypoxemic respiratory failure[23,24].

In the current study, out of 14 deceased patients, 2 (14.29%) were presented with IHD. Such a finding is lower than the result by Shi *et al.*[25]. Who reported that 19.7% of COVID 19 patients displayed heart disease. In another study conducted in China, the authors displayed that the SARS-CoV-2 infection has a more significant clinical impact in people who have had previous cardiovascular illness[26].

This study reported a higher mortality rate in non-vaccinated patients than surviving. Many studies mentioned that vaccination against COVID-19 is a cornerstone for reversing the COVID-19 pandemic; all vaccines have reduced disease severity and mortality, particularly in immunocompromised individuals[27].

Our data confirm that a more significant number of COVID-19 cases were diagnosed with leukemia 63.3%; most of the patients recorded 33 (71.74%) in a survived group with leukemia, however; 13 (28.26%) of the survived patients presented with lymphoma comparatives for 9 (64.29%) in the dead group. This result contradicts the finding of Xuejun *et al.*, Who found that most patients with COVID-19 had lymphoid malignancies[28]. This discrepancy may be due to our regional patient population distribution, which has higher rates of leukemia malignancies. Pagano *et al.* mentioned that a clear correlation between the type of hematological malignancy and the incidence of COVID-19 had not been described in the literature[29].

Furthermore, COVID-19 and Cancer Consortium (CCC19), the UK Coronavirus Cancer Monitoring Project (UKCCMP), and the American Society of Hematology (ASH) Research Collaborative are all larger cohort and registry studies that will give insight to the present questions about hematological malignancies and COVID-19. However, it is evident that patients with such malignancies are a susceptible group, and further research is needed to comprehend COVID-19's influence on these individuals 30 fully.

Conclusions

The current study results suggest that; vaccinating hematological malignancy patients against COVID19 may provide clinical protection from this illness compared to no vaccination, patients which supports national and international public health policy suggestions for the targeting of immunocompromised patients for such vaccination.

Author Contributions

(1) Design and acquisition of data.

(2) Writing the article and revising it critically for important intellectual content analysis and interpretation of results.

(3) & (4): Samples collection from patients and Statistical analysis.

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Institutional Review Board Statement

The ethical aspects of this study have been approved by the ethical council in Medical College, Al- Nahrain University.

Informed Consent Statement

Informed consent was obtained from all patients involved in this study.

Conflicts of Interest

The author declares that they have no competing interests.

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ARTICLE / INVESTIGACIÓN

Survey the Microbial Load in the Tigris River in South of Baghdad City and Some of the Physiochemical Parameters

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Abstract: This study included an analysis of three stations (Al Dora, Al Za'franiya, and Arab Ejbur) chosen to study the Physiochemical and microorganism (Fungi and Bacteria) loud of the Tigris River in the southern section of Baghdad city. The result of this research shows that the highest temperature recorded in summer in Al Za'franiya was 37Co, while the lowest temperature recorded in winter in Al Dora was 9Co. and the value of pH recorded the highest in summer it was 7.9 in Arab Ejbur, and the lowest value was in winter 7.1 in Al Dora regions, While Total Organic Carbon (TOC) shows the highest values found in the summer was 6.7 Mg L^{-1} in Al Za'franiya Samples, and the lowest values were 2.0 Mg L-1in Arab Ejbur during the winter. The more frequently isolated fungi were *Aspergillus niger*, and the lowest frequently was *Chrysosporium was* observed during the survey period. At the same time, Bacteriological analysis of water resources showed that *Escherichia coli* and *Pseudomonas aeruginosa* were more frequently isolated bacteria and the lowest frequently bacteria were *Klebsiella ornithinolytica* and *Proteus mirabilis*.

Key words: Fungi, Bacteria, Temperature, TOC, pH, Tigris River.

Introduction

Over the last few decades, understanding the mechanisms that determine microbial biodiversity in aquatic environments has been a significant focus of freshwater ecology research. Migratory microbial communities, which are found in freshwater environments, are made up of a diverse group of bacteria, fungi, and archaea responsible for various processes that are critical to ecosystem functioning. These processes include carbon cycling and biological nitrogen fixation as well as denitrification and methane production, sulfate reduction, and the transformation of metals and various molecules that are found in the environment[1]. In the absence of treatment, the release of untreated effluents can limit the microbiological diversity of natural environments, which may have a detrimental impact on the functioning and health of aquatic ecosystems. According to the findings of many research, both changes in local circumstances and the presence or absence of biotic interactions may impact the formation of microbial communities in fluvial networks. To measure the health of an ecosystem, it has been proposed that microbial diversity and community composition be used as sensitive indicators of ecosystem health since they may be impacted by water pollutants[2]. The contamination of natural water bodies by industrial, agricultural, and urban wastewater can affect the composition, structure, and microbial activity of the water body on a local and global scale, resulting in a decline in aquatic life and soil fertility; as a result consequence.

As a consequence of drastic changes in the structure and composition of these communities, unanticipated alterations in nutrient flow may occur[3]. The diversity and composition patterns of aquatic fungal communities have been influenced by pollution. The amount of nutrients dissolved in water, for example, has a significant influence on the diversity and composition patterns of aquatic fungal communities[4]. Despite this, most environmental microbiology sequencing investigations have concentrated on prokaryotic species, with microbial eukaryotic communities being generally ignored in the process[5]. Apart from their contribution to aquatic environments, bacterial populations play an essential role in transforming and demineralizing nutrients[6]. To keep the energy flow going. A significant role in the biodegradation of allochthonous compounds, such as pollution originating from human activities, is played by surface-associated bacterial communities, which are especially prevalent in rivers[7]. A river's importance to civilization is shown by the fact that it provides water for consumption, agriculture, and the removal of human waste. However, in contrast to our understanding of marine and freshwater lake microbiology, the ecological influence of planktonic and attached bacteria within river ecosystems is still understudied and underappreciated[8]. As the primary supply of irrigation for Iraqi land, the Tigris River has significant significance for Iraqi environmental researchers due to the negative impact of pollutants arising from treated and untreated home waste and agricultural pollutants[9]. However, according to a previous study conducted on the Tigris River in Baghdad city, the quality of water becomes worse when it reaches the city's southern cross because the study area is densely populated along its banks, as well as having some industrial activities present, as well as having some wastewater fallings that are distributed on both sides of the river and discharge the waste into a stream of Tigris River[10]. As a result, this research aims to

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survey the microorganisms (fungi and bacteria) in the south Tigris River in Baghdad city and examine their appearances and characteristics.

Materials and methods

Study area

This study was included an analysis of three stations (Al Dora, Al Za'franiya, and Arab Ejbur) that were chosen to study the Physiochemical and microorganism (Fungi and Bacteria) loud of the Tigris river in the southern section of Baghdad city figure (1), for a period of twelve months from November 2017 to October 2018. Obtaining representative samples has proven to be problematic due to the heavily populated nature of the study location along the river's banks, as well as the presence of diverse industrial activities, making it necessary to get these samples, in addition to several fallings the wastewater, which are distributed on both sides of the river and throw the waste to the Stream of Tigris River.

Collection of water samples

The water samples taken from south of Tigris River from three regions(Al Dora, Al Za'franiya, and Arab Ejbur) were collected in the morning every month using sterile polyethylene containers to (10).

Isolation and Identification of Fungi

Isolation of fungi was carried out by standard isolation techniques[11]. Some drops of water samples were separated using a spreader on Potato Dextrose agar (PDA) and Sabrouid Dextrose agar (SDA) and then incubated at room temperature for one week in a dark place. After one week, the fungi appeared on the surface of the agar. Fungi colonies were identified according to the shape of the colony and microscopically identified according to their spores and fungal filaments according to the fungi manual[12] with support of various standard references and monographs[13].

The Percentage of Occurrence and Frequency

The percentage of occurrence and frequency is defined as follows in equation[14]:

$$\text{Occurence\%} = \frac{\text{No. of positive samples}}{\text{Total number of samples}} \text{ X } 100$$

Isolation and Identification of Bacteria

Bacterial surveys of water samples, including total viable bacterial count, were carried out in accordance with the general standard methodology for the study of water and wastewater, as previously described[15]. A conventional coliform fermentation procedure, comprising presumptive, confirmed, and finished tests, was used to detect total coliform, fecal *E. coli*, and *Pseudomonas aeruginosa*[16]. A variety of dilutions of water samples from various sources were distributed over a variety of agars, including Eosin-Methylene Blue Agar (EMB), Nutrient agar, Thiosulfate citrate bile sucrose Agar (TCBS) medium, Macconkey agar, and Blood Agar, to identify additional harmful enteric bacteria at 37°C, the plates were incubated overnight, and the cultures were inspected for different colonies the next day after incubation. After being streaked on nutrient agar slant and cultured for 24 hours at 30°C, these colonies were retained as stock cultures. Bacteriological techniques that are currently in use[17] and API_{20E} were employed to determine the identity of each isolate.



Figure 1. Shows the selected study areas within the city of Baghdad.

Results

Temperature

The obtained results showed that the highest temperature was 37Co in summer in Al Za'franiya, while the lowest temperature was 9 Co in Al Dora in winter, as in Table (1).

Although there were statistically significant differences between the stations, no statistically significant differences existed between the seasons except the spring season, which had significant differences that reached 3.97 at the level of (P≤0.05) and no statistically significant differences between the seasons.

Hydrogen Ion (pH)

The maximum (7.9) and minimum (7.1) values of pH were recorded in the Arab Ejbur and Al Dora region during Summer and Winter, respectively these values agree with Iraqi standards for maintenance of rivers No. 25 of 1967, which was 6.5-8.5[18] as shown in Table (2).

According to the statistical study, there were no statistically significant differences across the seasons, but that there were statistically significant differences between the two stations of Al-Dora and Arab Al-Jabour, with differences reaching up to 0.613 and 0.627 respectively at the level of (P≤0.05).

Total Organic Carbon (TOC)

The highest values for drinking water found in summer were 6.7 Mg L-1in Al Za'franiya.

Samples and the lowest values were with 2.0 Mg L-1for Arab Ejbur in winter. Table (3).

Following the statistical analysis, it was discovered that there were no statistically significant variations between the seasons, and that there were no statistically significant differences between the stations at the level of (P≤0.05).

Isolation and Identification of microorganisms

The obtained results revealed that 18 fungal species were identified in the (Al Dora, Al Za'franiya, and Arab Ejbur) stations and their appearances threw the study season as explained in Table (4). While the number of bacteria species was 14 in the study station and study season Table (5).

Fungus isolation and Identification

The fungus species were isolated throughout four seasons. *Aspergillus niger* was the fungus that was isolated the most often. *Rhizopus sp.*, *Fusarium sp.*, *Trichophyton megnini*, and *Mycelia sterilia* are among the pathogens that have been identified, Table (4).

18 fungal genera are isolated; *Aspergillus niger* is the most common genus and is always represented during the study period, followed by the *Rhizopus genus*. The fungal genera *Aspergillus*, *Rhizopus*, *Fusarium*, *Trichophyton*, *Mycelia*, *Mucor*. *Penicillium*, *Microsporum*, *Trichoderma*, *Alternaria*, *Ulocladium*, *Phialophora*, *Epidermophyton* and *Chrysosporium* were observed during the survey period.

Isolation and Identification of bacteria

The total viable bacterial counts, total coliforms, total *E. coli*, and total *Pseudomonas aeruginosa* were found to be the most prevalent bacteria in water resources, according to a bacterial investigation, Table (6).

According to the data in the paper, at least 14 species

Season	Al Dora	Al Za'franiya	Arab Ejbur	LSD value
Autumn	27	24	25	3.02 NS
Winter	9	11	12	3.17 NS
Spring	27	23	25	3.97 *
Summer	35	37	34	3.26 NS
LSD value	6.49 *	6.07 *	5.92 *	---
* (P≤0.05), NS: Non-Significant.				

Table 1. Effect of Station and Season on Temperature.

Season	Al Dora	Al Za'franiya	Arab Ejbur	LSD value
Winter	7.1	7.3	7.2	0.419 NS
Spring	7.7	7.6	7.7	0.335 NS
Summer	7.8	7.8	7.9	0.296 NS
Autumn	7.5	7.5	7.4	0.301 NS
LSD value	0.613 *	0.548 NS	0.627 *	--
* (P≤0.05), NS: Non-Significant.				

Table 2. Effect of Station and Month on pH.

Season	Al Dora Mg L^{-1}	Al Za'franiya Mg L^{-1}	Arab Ejbur Mg L^{-1}	LSD value
Autumn	4.7	4.9	4.6	1.06 NS
Winter	2.2	2.7	2	0.893 NS
Spring	4.9	5.2	4.8	0.904 NS
Summer	6.2	6.7	6	0.822 NS
LSD value	2.371 *	2.084 *	2.177 *	--
* (P≤0.05), NS: Non-Significant.				

Table 3. Effect of Station and Month in (TOC).

Fungi	Winter			Spring			Summer			Autumn		
	Al Dora	Al Za' franiya	Arab Ejbur	Al Dora	Al Za' franiya	Arab Ejbur	Al Dora	Al Za' franiya	Arab Ejbur	Al Dora	Al Za' franiya	Arab Ejbur
Alternaria sp.	-	+	-	+	+	+	+	+	+	+	+	-
Aspergillus versicolor	-	-	+	-	+	+	+	+	+	-	+	+
Aspergillus niger	+	+	-	+	+	+	+	+	+	+	+	-
Chrysosporium sp.	-	-	+	+	+	+	+	+	+	-	+	+
Cladosporium sp.	-	-	-	-	-	+	-	+	+	-	+	-
Epidermophyton	-	-	-	+	-	+	-	-	+	-	-	-
Fusarium sp.	-	+	+	-	+	+	+	+	+	-	+	+
Microsporium ferrugineum	-	+	-	-	-	-	+	-	+	+	-	-
Microsporium nanum	-	-	-	-	-	+	-	-	+	-	-	-
Mucor sp.	-	-	-	-	-	+	-	+	+	-	-	+
Mycelia sterilia	-	-	-	+	+	+	+	+	+	-	+	
Penicillium sp.	+	+	+	+	-	+	+	+	+	+	+	+
Phialophora sp.	-	-	-	-	-	-	-	+	+	-	-	-
Rhizopus sp.	-	+	+	+	+	+	+	+	+	+	+	-
Trichoderma sp.	-	-	+	-	+	+	+	+	+	-	-	+
Trichophyton megnini	-	+	-	+	+	+	+	+	+	+	+	-
Trichophyton Vubrum	-	+	-	+	+	+	+	-	-	-	-	-
Ulocladium sp.	-	-	-	-	+	-	-	+	+	-	-	+

+ = Fungi found in the station
- = Fungi not found in the station

Table 4. Prevalence of fungal species isolated during the study period from (Al Dora, Al Za'franiya, and Arab Ejbur) stations.

of bacteria belonging to the family Enterobacteriaceae are present and several additional species belonging to the family Pseudomonadaceae Table (7). The bacteria *E. coli* and *Pseudomonas aeruginosa* were the most often seen.

Discussion

The main reason for the lower degree measured during the winter and higher degree in the summer months is linked directly with air temperature[19]. The relatively low pH value obtained in the river during winter may be due to the high levels of free CO_2 that is dissolved in water by diffusion from the atmosphere and may be due to the dilution effect[20], while in the summer, CO_2 levels are decreased by moving to the atmosphere or consumed via the photosynthesis process of algae or water plants and so, the pH value is raised[21]. The research findings indicate that the most considerable fungus abundance was found in a river with a high concentration of organic matter, while the highest microorganism taxonomic biodiversity was found in waters with a high concentration of organic matter. The majority of potentially harmful fungus is found in the water of pollu-

Fungi	Station frequency and they are average				Occurrence%
	Al Dora	Al Za' franiya	Arab Ejbur	Average	
Alternaria sp.	6	9	7	7.3	4.6
Aspergillus versicolor	4	4	6	4.7	3.0
Aspergillus niger	38	33	41	37.3	23.6
Chrysosporium sp.	2	0	3	1.7	1.1
Cladosporium sp.	8	8	6	7.3	4.6
Epidermophyton	2	2	1	1.7	1.1
Fusarium sp.	14	18	13	15.0	9.5
Microsporium ferrugineum	7	7	6	6.7	4.2
Microsporium nanum	4	6	5	5.0	3.2
Mucor sp.	9	10	12	10.3	6.5
Mycelia sterilia	12	9	12	11.0	7.0
Penicillium sp.	7	12	8	9.0	5.7
Phialophora sp.	3	3	3	3.0	1.9
Rhizopus sp.	16	14	14	14.7	9.3
Trichoderma sp.	6	8	8	7.3	4.6
Trichophyton megnini	12	10	10	10.7	6.8
Trichophyton vubrum	1	0	0	0.3	0.2
Ulocladium sp.	6	5	4	5.0	3.2

Table 5. Occurrences and frequency of fungal genera isolated from (Al Dora, Al Za'franiya, and Arab Ejbur) stations.

ted rivers. Furthermore, elements impacting the structure of microbes include the pH, temperature, and total organic carbon (TOC) of the water[20].

Conclusions

These microbial counts were far higher than the maximum limits permitted by international standards, particularly at the Al Za'franiya station. According to the water quality in (Al Dora, Al Za'franiya, and Arab Ejbur), a significant quantity of bacteria was found in the water in the southern Tigris River basin. For their part, the physical-chemical parameters were within acceptable ranges of values. This may be due to the contamination of raw water by urban garbage and other sources.

Conflict of interest

No conflict of interest.

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Funds

Self.

Bacteria	Winter			Spring				Summer			Autumn		
	Al Dora	Al Za' franiya	Arab Ejbur		Al Dora	Al Za' franiya	Arab Ejbur	Al Dora	Al Za' franiya	Arab Ejbur	Al Dora	Al Za' franiya	Arab Ejbur
Aeromonas hydrophila	-	-	-	-		-	+	+	-	+	-	-	-
Citrobacter freundii	-	+	-	-		+	+	+	+	+	-	-	-
Enterobacter cloacae	–	–	+	+		+	+	+	+	+	–	–	+
Escherichia coli	–	–	–	–		–	+	–	+	+	–	–	–
Klebsiella ornithiolytica.	-	-	-	-		+	-	+	+	+	-	-	-
Klebsiella pneumonia	+	+	+	+		+	+	+	+	+	+	+	+
Pantoea spp.	–	–	–	–		–	–	–	–	–	–	–	–
Proteus mirabilis	-	-	+	+		+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	-	-	-	-		-	-	-	+	+	-	-	-
Pseudomonas fluorescens	–	–	–	–		–	+	–	–	+	–	–	+
Rahnella aquatilis	-	-	-	-		-	-	-	+	-	-	-	-
Serratia ficaria	–	–	–	–		–	+	–	+	+	–	–	+
Vibrio cholera	-	-	-	-		-	-	-	-	+	-	-	-
Vibrio fluvials	-	-	-	-		-	-	-	+	+	-	-	-

+ = Bacteria found in the station
- = Bacteria not found in the station

Table 6. Prevalence of bacteria species isolated during the study period from (Al Dora, Al Za'franiya, and Arab Ejbur) stations.

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Bacteria	Station frequency and they are average				Occurrence %
	Al Dora	Al Za' franiya	Arab Ejbur	Average	
Aeromonas hydrophila	2	0	2	1.3	1.3
Citrobacter freundii	5	7	6	6	5.8
Enterobacter cloacae	4	4	5	4.3	4.2
Escherichia coli	30	33	32	31.7	30.7
Klebsiella ornithiolytica.	1	1	1	1	1.0
Klebsiella pneumonia	14	12	14	13.3	12.9
Pantoea spp.	2	3	1	2	1.9
Proteus mirabilis	1	1	1	1	1.0
Pseudomonas aeruginosa	30	30	34	31.3	30.3
Pseudomonas fluorescens	2	5	1	2.7	2.6
Rahnella aquatilis	2	2	2	2	1.9
Serratia ficaria	1	0	1	0.7	0.7
Vibrio cholera	3	3	3	3	2.9
Vibrio fluvials	3	2	4	3	2.9

Table 7. Occurrence and frequency of bacterial isolated from (Al Dora, Al Za'franiya, and Arab Ejbur) stations.

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ARTICLE / INVESTIGACIÓN

Prognostic Value of C-Reactive Protein and Platelet Lymphocyte Ratio in Coronavirus Disease 19

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Abstract: The coronavirus (SARS-CoV-2) that causes Coronavirus disease 19 (COVID-19) has recently emerged as a cause of severe infection in a considerable percentage of infected persons. Predicting the risk factors for severe disease can greatly help manage critical cases and save lives. This study aimed to assess the prognostic value of the platelet-lymphocyte ratio (PLR) and C-reactive protein (CRP) in patients with COVID-19. This cross-sectional study enrolled 160 confirmed cases with COVID-19 by real-time polymerase chain reaction. Demographic data (age, gender, smoking status, body mass index (BMI)) and comorbidity were collected through direct interviews. Laboratory investigations, including total leukocyte count, absolute neutrophil, lymphocyte, platelet count, serum level of C-reactive protein, and hemoglobin, were obtained from the patient's records. The platelets-lymphocyte ratio was calculated by dividing absolute platelet count by absolute lymphocyte count. According to their outcome, patients were categorized into two groups: those discharged well and those who required intensive care unit (ICU) admission. Out of 160 included patients, 32 (20%) needed ICU admission due to the deterioration of their status. Age (64.28±13.08 years versus 57.43±13.15 years), hypertension (40.63% versus 20.31%) absolute neutrophil count (median = 12.9×10^3/ml, range 3.83-22.8×10^3/ml versus median=6×10^3/ml, range 2.17-22.8×10^3/ml) and PLR ((median= 257.27, range= 62.72-1072 versus median= 191.54, range= 17.85-919.12) were significantly higher in patients required ICU admission than those discharged well, and associated significantly with the severity of the disease. Advanced age, hypertension, neutrophilia, and PLR at admission are predictors of severity and need for ICU admission in patients with COVID-19. PLR is an inexpensive, easy-to-be-calculated parameter that can be used routinely to predict the severity of COVID-19.

Key words: COVID-19, intensive care unit, platelet-lymphocyte ratio.

Introduction

Coronavirus disease 2019 (COVID-19) is caused by SARS-CoV-2 with a wide range of symptoms, from asymptomatic to severe pneumonia and acute respiratory distress syndrome (ARDS) or even severe symptoms of death[1]. Immunological investigations have demonstrated that high concentrations of proinflammatory cytokines (cytokine storm) are the hallmark characteristic of severe COVID-19 cases. This unusual rise of cytokines associated with a tremendous proinflammatory response leading to Multiple Organ Dysfunction Syndrome (MODS) and ARDS, which are the leading causes of death in patients with COVID-19[2]. Thus, theoretically, inflammatory markers could be used to measure the severity and death risk of COVID-19 patients. Platelet-to-lymphocyte ratio (PLR) is a new marker of inflammation, which is cheap and easily obtained from resource-limited settings. This ratio has been used to diagnose various diseases, such as autoimmune diseases and heart diseases[3]. Previous studies disclose that severe COVID-19 patients had a rise in PLR at admission[4]. This proposes the potential prognostic value of this ratio in COVID-19 patients, especially in poor settings. C-reactive protein is a protein formed by the liver[5]. Usually, the serum level of CRP is less than 10 mg/L; however, it rises rapidly when there is an inflammatory process. Its half□life is about 19 hours[6], and its concentration drops when the inflammatory stages end. CRP preferably links to phosphocholine, which is expressed on the surface of injured cells[7]. This linking activates the classical complement pathway of the immune system. Therefore, CRP could be a valuable marker for monitoring COVID-19 severity[8]. The present study aimed to assess the prognostic value of PLR and CRP in patients with COVID-19.

Materials and methods

Patients and Methods

This was a prospective cross-sectional study including 212 patients suspected of having an infection with SARS-CoV-2 who were admitted and treated at Al-Shifaa Hospital/ Baghdad from 1st May to 1st October 2020. Patients were diagnosed after nasopharyngeal swab examination of SARS-CoV-2 RNA by real-time polymerase chain reaction (RT-PCR). Patients with asthma or COPD, and those with known coagulopathies and autoimmune diseases, were excluded from the study. Thus, according to RT-PCR and exclusion criteria, the eligible patients were 160 patients who

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represented the study population. The review board approved this study at the University of Mashreq. Written consent from each participant was obtained before sample collection after explaining the aim of the study. Each patient was given the complete unconditioned choice to withdraw anytime. The confidentiality of data throughout the study was guaranteed, and the patients were assured that data would be used for research purposes only. Socio-demographic and clinical data, including age, sex, body mass index (BMI), and smoking, were collected through direct interviews.

Sample Collection and Laboratory Investigations

Three mL of peripheral blood were collected from each participant. A hematology auto-analyzer (Huroba ABX/India) measured blood parameters. PLR was calculated by dividing the absolute platelet count by the absolute lymphocyte.

Follow-up

Patients were followed up for one month after hospital admission. The need for ICU admission or death was reported. Accordingly, patients were categorized into two groups: those who were discharged well and those who required ICU admission or died.

Statistical analysis

All statistical analyses were performed using SPSS statistical software, version 25 (SPSS, IBM Company, Chicago, USA). The normal distribution of continuous data was tested with the Shapiro Wilk test. Normally distributed variables were presented as mean ± SD and analyzed with the Student t-test. Non-normally distributed variables were presented as median and range and analyzed with a nonparametric Mann Whitney U test. Categorical variables were expressed as counts and percentages and analyzed with the Chi-square test. Receiver operating characteristic (ROC) curve analyses were used to evaluate the prognostic value of PLR. A significant level of statistics was considered for all tests when $p<0.05$.

Results

Demographic Characteristics of the Patients

The mean age of the patients was 61.05±12.57 years; among them were 96 males (60%). Smoking habit was encountered in 13.75% of the patients. The mean BMI was 25.34±3.85 kg/m^2. Hypertension was the most common comorbidity affecting about a quarter of the patients (24.38%), followed by T2DM (22.5%). The mean total WBC, neutrophil, lymphocyte and platelet count was 14.1±5.06×10^3/ml, 11.34±4.82×10^3/ml, 1.33±0.79×10^3/ml and 261.5±111.17×10^3/ml, respectively. Hemoglobin concentration ranged from 7.8-17.0 g/dl with a mean of 12.18 ±1.5 g/dl. The mean PLR and CRP were 297.16±215.3 and 50.11±36.56 ng/ml, respectively (Table 1).

Outcomes

Out of 160 included patients, 32 patients (20%) required ICU admission and 8 patients (5%) of those died during the follow-up period. The other 128 patients (80%) were discharged well.

Association of Demographic Factors with the Outcome

Four demographic and clinical factors were significantly

Variables	Values
Age, years	
Mean±SD	61.05±12.57
Range	35-85
Gender	
Male	96(60%)
Female	64(40%)
Smoking	
Never	138(86.25%)
Ex/current	22(13.75%)
BMI, kg/m^2	
Mean±SD	25.34±3.85
Range	18.2-40.11
Comorbidities	
Hypertension	39(24.38%)
T2DM	36(22.5%)
IHD	17(10.63%)
Malignancy	8(5%)
Total WBC count ×10^3/ml	
Mean±SD	14.1±5.06
Range	3.0-29.8
Neutrophil count×10^3/ml	
Mean±SD	11.34±4.82
Range	2.17-22.8
Lymphocyte count×10^3/ml	
Mean±SD	1.33±0.79
Range	0.27-4.64
Platelet count×10^3/ml	
Mean±SD	261.5±111.17
Range	85.0-558.0
PLR	
Mean±SD	297.16±215.3
Range	17.85-1072
Hb, g/dl	
Mean±SD	12.18 ±1.5
Range	7.8-17.0
CRP, ng/ml	
Mean±SD	50.11±36.56
Range	6.0-215.0

Table 1. Patients' characteristics and demographic data (n=160). BMI: body mass index, WBC: white blood cells, T2DM: type 2 diabetes mellitus, IHD: ischemic heart disease, PLR: platelet- lymphocyte ratio, Hb: hemoglobin, CRP: C-reactive protein.

associated with ICU admission in patients with COVID-19. The mean age of the ICU group was 64.28±13.08 years which was higher than those discharged well (57.43±13.15 years), with a highly significant difference. The frequency of hypertension in the ICU group and the well-discharged group was 40.63% and 20.31%, respectively, with a significant difference. The median neutrophil count in the ICU group was 12.9×10³/ml (range 3.83-22.8×10³/ml) compared with 11.6×10³/ml (range 2.17-22.8×10³/ml) in the well-discharged group with a significant difference. Finally, ICU group had significantly higher PLR than well-discharged group (median= 257.27, range= 62.72-1072 versus median= 191.54, range= 17.85-919.12) as shown in table 2.

Prognostic Value of PLR

The receiver operating characteristic (ROC) curve was used to evaluate the prognostic value of the platelet-lymphocyte ratio in COVID-19. The area under the curve (AUC) was 0.617, 95%CI= 0.510-0.725, p= 0.040. The sensitivity and specificity of the test at cut-off values of PLR= 235.28 were 0.63 and 0.60, respectively (Figure 1).

Discussion

The present study aimed to assess the prognostic value of CRP and PLR in patients with COVID-19. According

Variables	Well discharged (n=128)	Need ICU admission (n=32)	p-value
Age, years	57.43±13.15	64.28±13.08	**0.009†**
Gender			
Male	54(42.19%)	10(31.25%)	0.259
Female	74(57.81%)	22(68.75%)	
Smoking			
Never	109(85.16%)	29(90.63%)	0.422
Ex/current	19(14.84%)	3(9.37%)	
BMI, kg/m²	25.63±3.81	25.67±2.66	0.956
Comorbidities			
Hypertension	26(20.31%)	13(40.63%)	**0.017**
T2DM	25(19.53%)	11(34.37%)	0.063
IHD	11(8.59%)	6(18.75%)	0.099
Malignancy	7(5.47%)	1(3.13%)	0.581
Total WBC count ×10³/ml			
Median	12.2	12.8	0.148‡
Range	2.6-29.8	6.7-29.8	
Neutrophil count×10³/ml			
Median	11.6	12.9	**0.012‡**
Range	2.17-22.8	3.83-22.8	
Lymphocyte count×10³/ml			0.058‡
Median	1.29	0.97	
Range	0.17-6.5	0.28-4.64	
Platelet count×10³/ml			
Median	249.5	239.5	0.238‡
Range	64.1-558.0	94.0-536.0	
PLR			
Median	191.54	257.27	**0.040‡**
Range	17.85-919.12	62.72-1072	
Hb, g/dl	13.03±6.1	12.4±1.72	0.546†
CRP, ng/ml			
Median	37	48.0	0.088‡
Range	6.0-130	12.0-215.0	

Table 2. Association of demographic factors with the outcome. BMI: body mass index, WBC: white blood cells, T2DM: type 2 diabetes mellitus, IHD: ischemic heart disease, PLR: platelet- lymphocyte ratio, Hb: hemoglobin, CRP: C-reactive protein.
† Student t-test, ‡ Mann Whitney test, Values were expressed as mean±SD unless indicated otherwise.

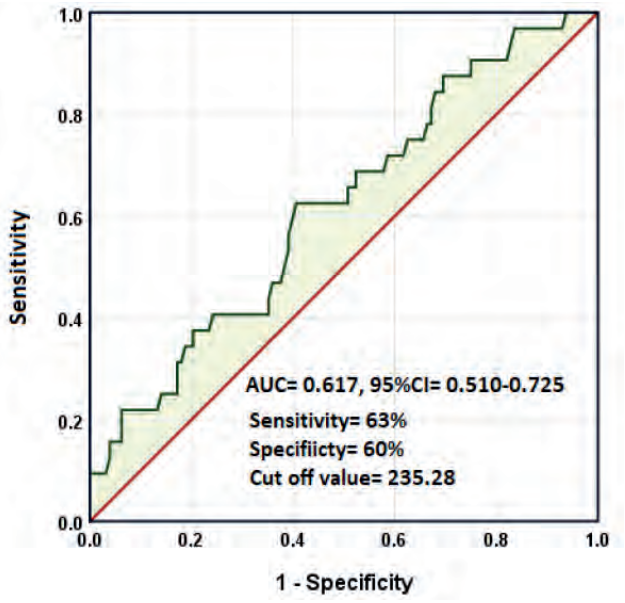


Figure 1. Receiver operating characteristic curve for PLR in predicting worse outcomes in patients with COVID-19.

to the result of the study, 20% of patients needed ICU admission, among whom 5 patients (25%) died. These figures are slightly lower than in the global context. In a large meta-analysis including 646 studies with 24983 patients, Abate *et al.*[9] demonstrated that 32% of the patients required ICU admission, and the mortality rate among those was 39%. The variation may explain this discrepancy in the demographic and clinical features of the patients, especially age and the presence of comorbidities.

In the present study, advanced age, hypertension, leukocytosis, ad admission were predictors for worse outcomes in COD-19 patients. Advanced age may be the most risk factor confirmed as a predictor of mortality in different studies worldwide. In a large Spanish cohort involving 2226 patients, the worse outcome for younger patients was 0.5 % for those below 40 years, 1.5 % for those 40–49 years, and 3.8 % for those 50–59 years[10]. In another study, Lei *et al.*[11] investigate the outcome of 288 Chinese patients in a retrospective study. The authors found that each 1-year increase in age increases the chance of ICU admission by 1.07-time. Although the pathophysiological mechanisms are still not understood, they may be explained by the dysfunction of the immune system with aging[12]. In addition, a study reported that older age was related to defects in T-cell and B-cell function and excess inflammation markers, which could be detrimental to the control of viremia and inflammation, aggravating morbidity and mortality in older patients[13]. Also, in line with the present study about the role of hypertension are many studies worldwide. Wang *et al.*[14] reported 138 COVID-19 patients hospitalized in Wuhan, China: 31% had hypertension. Of those requiring ICU admission, 58% had hypertension compared with 22% who did not. Guan *et al.*[15] reported 1099 COVID-19 cases across China: 15% of all patients had hypertension, including 24% in severe cases and 13% in mild cases. Among patients admitted to an intensive care unit (ICU) who required tracheal intubation or died, 36% had hypertension. This association between hypertension and disease severity may be explained based on two facts: firstly, hypertension is associated with more severe COVI-19. Supporting this fact are many studies worldwide. In a cohort of 1389 patients, a history of hypertension was more common among those who had severe than non-severe COVID-19[16]. Similarly, in a separate cohort of 1590 hospitalized patients in China, underlying hypertension was independently associated with severe COVID-19[17].

There is no precise mechanism(s) that explains this association between hypertension and the severity of COVID-19. However, antihypertensive medications based on Renin-Angiotensin-Aldosterone System (RAAS) inhibition, such as angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs), that use in the treatment of hypertension can upregulate the expression of ACE2 in the kidney tubules[18]. Therefore, they could hypothetically increase the virus's targeting of some vital organs like kidneys. This explains why hypertension but not diabetes was significantly associated with disease severity in those patients. In most studies, neutrophilia was a common finding in severe patients. A Singaporean study including 138 hospitalized patients showed that neutrophilia was significantly higher in patients requiring admission to ICU (11.6×103/ml vs. 3.5×103/ml)[19].

Similarly, an Ethiopian study showed that neutrophilia was more prominent in severe and critical than moderate patients[20]. Qin *et al.*[12] and Gong *et al.*[21] reported significantly higher neutrophil count in severe than non-severe patients. Another study by Zhang *et al.*[22] revealed that 74.3% of dead patients had neutrophilia on admission, increasing to 100% 24 hours before death. The presence of neutrophilia could be related to the cytokine storm that characterizes COVID-19 disease. However, careful interpretation is required as neutrophilia could be due to bacterial co-infections[20]. Another important finding in the present study was that high PLR at admission was significantly associated with ICU admission despite that platelet count was higher in patients who did not need ICU admission. Following this result is a meta-analysis of seven studies with 998 participants. The study showed that severe COVID-19 patients had higher PLR levels on admission[23]. Yang *et al.*[24] reported the optimal cut-off PLR value as 180 with an AUC of 0.784, specificity of 44%, and sensitivity of 77%.

Meanwhile, Sun *et al.*[25] suggested a cut-off PLR value of 226.67 with an AUC of 0.746, specificity of 80.90%, and sensitivity of 59.26%. This discrepancy warrants further research to determine the most appropriate PLR cut-off value in determining the severity of COVID-19 patients. PLR was initially suggested as an excellent candidate marker for determining the severity and mortality of COVID-19. First, PLR is an established marker of inflammation[26].

Inflammation plays a considerable role in the pathophysiology of COVID-19, with cytokine storm as a hallmark condition in severe disease and poorer prognosis[27]. Thus, a high PLR value suggests an overactive inflammatory response and a worse prognosis. Second, PLR is sensitive to natural and acquired immune responses because it includes the absolute number of lymphocytes, the leading player in immune response[28]. Third, PLR is an inexpensive and readily available measurement used in resource-limited settings. The underlying mechanism for the decreased absolute lymphocyte count is thought to be due to induction by SARS-CoV-2 of pyroptosis in lymphocytes through the activation of nod-like receptor Family Pyrin Domain Containing 3 (NLRP3) inflammasome[24]. The present study showed no significant association of CRP with the severity of COVID-19. This result agrees with a Serbian study involving 128 patients, in which there was no significant difference in

CRP concentration between patients with different severity of the disease[29]. However, many other studies do find such differences. For example, elevated CRP values have been reported in viral respiratory illnesses such as SARS, MERS-CoV, and H1N1 and have been reported to correlate with disease severity and predictors of disease progression[30,31]. Similarly, CRP levels have been elevated in hospitalized patients with COVID-19 and correlate with the severity of the disease and mortality[32]. This discrepancy between different studies is mainly attributed to the underlying comorbidities that may enhance CRP production.

Conclusions

These data suggest that advanced age, hypertension as a comorbidity, higher neutrophil absolute count, and PLR ad admission are predictors of severity and need for ICU admission in patients with COVID-19. PLR is an inexpressive, easy-to-be-calculated parameter that can be used routinely to predict the severity of COVID-19. However, a more reliable conclusion requires further studies with larger sample size.

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Conflict of Interest

The author declares that she has no competing interests.

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ARTICLE / INVESTIGACIÓN

Management of Incidental Rupture of Abdominal Hydatid Cyst

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Abstract: Abdominal hydatid cyst rupture is one of the critical situations which challenged the surgeon incidentally during explorative lobotomy for acute abdominal conditions. It is considered an endemic area for hydatid disease. To study and evaluate different modalities of surgical treatments used to deal with the incidental rupture of abdominal hydatid cyst. Patient and methodology: Patients (n=200) underwent surgical treatment for abdominal hydatid cysts in Al -Kindy teaching Hospital and Diala private hospital. A review was carried out from January 1996 to January 2006. About 18 patients (6%) had a rupture of abdominal hydatid cyst; in 15 patients (83.3%), the rupture was discovered incidentally during surgery for urgent acute abdomen, and 3 patients (16.7%) were detected clinically and using ultrasound. The median age was 32 years, and the female to male ratio was 2:1. 17 patients presented with signs & symptoms of acute abdomen. 11 patients presented with hypovolaemic shock after trauma to the liver (RTA in 9 patients, anaphylactic shock in 1 patient, allergic reaction in 1 patient). 4 patients presented with spontaneous rupture, 2 presented with secondary hydatidosis, and 1 presented with haematuria. Results: Twelve females and six males were affected. Rupture of abdominal hydatid cyst was diagnosed in only three patients by history, clinical examination, and ultrasound. At the same time, the other 15 patients were diagnosed during surgery. The mean hospital stay was 14 days. About 5 patients developed post-operative complications, i.e., wound infection (n=1), stress ulceration in the duodenum (n=1), subphrenic abscess (n=1), an infected remnant of hepatic hydatid cyst cavity (n=1), and recurrence of the hydatid cyst (n=1). Unfortunately, intra-operative mortality was recorded in 1 patient. The clinical diagnosis is usually tricky, but ultrasonography is an excellent initial diagnosis in suspected cases.

Key words: Surgical treatment, Incidental Ruptured Abdominal hydatid cyst, Traumatic rupture.

Introduction

Hydatid disease is caused by *Echinococcus granulosus*, a common parasitic infestation in Iraq and worldwide. It is still an important and challenging medico-surgical problem in endemic countries like Iraq, Iran, Turkey, Australia, South America, and Europe[1]. Generally, hydatid cysts are incidentally diagnosed via radiological assessment for another symptom[2]. Nevertheless, a few patients may show jaundice, vomiting, nausea, and abdominal pain depending on the hydatid cyst's localization and dimensions of the hydatid cyst[2]. Internal rupture perforation (intraperitoneal rupture, intrapleural rupture, intrapericardial rupture, bronchopleural fistula, broncho-biliary fistula, rupture into hollow viscous or cysto-biliary fistula), and neighboring organ compression, biliary compression, vascular compression, anaphylactoid reaction, bacterial superinfection or external rupture like cysto-cutaneous fistula are some of the hydatid disease-related complications[3-5]. The hydatid cyst intraperitoneal rupture is a low and lethal complexity of hydatid disease for which quick treatment and diagnosis are necessary to lower the death rate[6,7].

The primary carriers of hydatid disease in human beings are dogs. Infection can be through direct contact or ingesting ova of the parasite while consuming unwashed and uncooked vegetables or after the digestion of contaminated food. The embryo is released in the intestinal tract and carried to the liver by portal circulation[8]. The disease may attack every organ in the human body, but the most standard site of infection is the liver, with a rate of 50-70%[1]. A typical hydatid cyst of the liver is composed of host and parasite tissue. The former (pericyst), formed by the reaction of the liver to the presence of the parasite, is gray in color and thick. The latter (endocyst) is formed of two layers, and an outer laminated layer is formed of the parasite itself. The outer layer is whitish & elastic, and an inner germinal layer consists of the living part of a hydatid cyst. This inner layer internally secretes hydatid fluid, broods capsules scolices and externally consists of the laminated membrane.

Many cysts are asymptomatic or present as a painful mass in the abdomen. The patient may discover cysts during self-examination or by the routine investigation done by the physician. Urgent surgical interventions are required for unexpected problems occurred during laparotomy. Hydatid disease patients used different modes of treatment like medical treatment with albendazole[9], ultrasound-guided PAIR (puncture, aspiration, injection, respiration), and albendazol treatment[10] Laparoscopic (cystectomy, deroofing and omentoplasty)[11]. Surgical treatment is the standard treatment of hydatid disease. However, controversy still exists about the selection of different surgical procedures[12]. In the present study, we have evaluated the efficiency and complications of different modes of surgical treatments used to deal with rupture of abdominal hydatid cyst, which was discovered incidentally.

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Materials and methods

Planing of a retrospective analytic study

It is a retrospective analytic study. Patients (n=200) who underwent surgical treatment for abdominal hydatid cysts in Al -Kindy teaching Hospital and Diala private hospital were screened. A review was carried out from January 1996 to January 2006.

Patient distribution

Out of 200, about 18 patients (6%) had rupture of abdominal hydatid cyst, in 15 patients (83.3%) rupture was discovered incidentally during surgery for urgent acute abdomen, and 3 patients (16.7%) were detected clinically and using ultrasound. The median age was 32 years, and the female to male ratio was 2:1 [Twelve females (64.6%) and six males (43.4%)]. About 17 patients presented symptoms of acute abdomen. About 11 patients presented with hypovolaemic shock after trauma to the liver (RTA in 9 patients, anaphylactic shock in 1 patient, allergic reaction in 1 patient). 4 patients presented with spontaneous rupture, 2 presented with secondary hydatidosis, and 1 presented with haematuria.

Operative procedure for patients

There are free intraperitoneal fluids mixed with hydatid cyst material (daughter cysts scolices sucked the fluid, and cystic material was removed). The peritoneal cavity was washed with normal saline followed by a 1% povidone-iodine solution (Figure 1A). Around four patients showed spontaneous intraabdominal rupture due to cyst infection forming, subdiaphragmatic abscess and intraperitoneal abscess represented as peritonitis. In this condition, the cystic rupture cavity was cleaned from infected contents and aspirate of the abscess from the intra-abdominal and sub-hepatic abscess. The peritoneal cavity was washed with normal saline followed by a 1% povidone-iodine solution. The tube drain was inserted inside the cystic cavity and the near hepatic cystic cavity.

Two patients noted secondary hydatidosis (Figures 1B and 1C). In one patient (Figure 1B), around 13 cysts were observed. Unfortunately, one cyst was ruptured due to some trauma before the cystectomy procedure (before hospitalization). The second patient showed four cysts in the intraperitoneal (3 cysts) and pancreas (1 cyst). The cyst was ruptured during the operation. The patient developed uncorrectable anaphylactic shock and died during the operation. Out of 18 patients, one patient showed haematuria due to a football hit on the abdomen. On the right loin, daughter cysts were clinically diagnosed by ultrasound. The patient reported the presence of blood in the urine. The hydatid occupies most renal parenchyma (Figure 1C). Transabdominal nephrectomy was done using the right para-median incision.

Fifteen cases were diagnosed during explorative laparotomy, and three cases were diagnosed by ultrasound. One patient showed a cyst in the right kidney in these three cases. The second patient showed an allergic reaction after trauma manifested by rash, urticaria and visible swelling on the abdomen (Figure 2A). The ultrasound demonstrated two cysts in the liver, multiple cysts in the peritoneal cavity, and free peritoneal fluid (Figures 2B and 2C). The third patient had a history of surgery for hepatic disease before 3 years. All cystic rupture cavities were operated on according to their size, site and condition.

An omentoplasty was carried out for clear fluid cysts (Figure 3A). The bile stain containing cysts was operated with tube drainage (Figure 3B). The infected cyst contents were removed carefully if cystic-biliary communication was observed (Figure 3C). The opening was secured by sutured (synthetic absorbable 2/0 Dexon suture).

Partial cystectomy was done for a large hydatid cystic cavity. Total cystectomy was done for extrahepatic or peripheral located hydatid and pedunculated cysts. Non-rupture hydatid cysts were operated on surgically except for one patient. This patient had urticarial. During this patient's surgery, gauze soaked in scolicida agent (10% povidone-iodine) was used to avoid dissemination of the parasite. Around 10-20ml hydatid fluid was aspirated, followed by a 10% povidone iodine injection into the hydatid cyst cavity for 10 min. The pseudocyst (pericyst) was opened. The laminated membrane and daughter cyst was removed by suction and sponging. The cystic cavity was incised between the suture. All cystic elements were evacuated by suction. Before the closure of the abdomen, the peritoneal cavity is clean with povidone-iodine (1%) to kill all scolices and prevent their recurrence. The tube drain was inserted in the peritoneal cavity beside the cyst (Figures 4A and 4B).

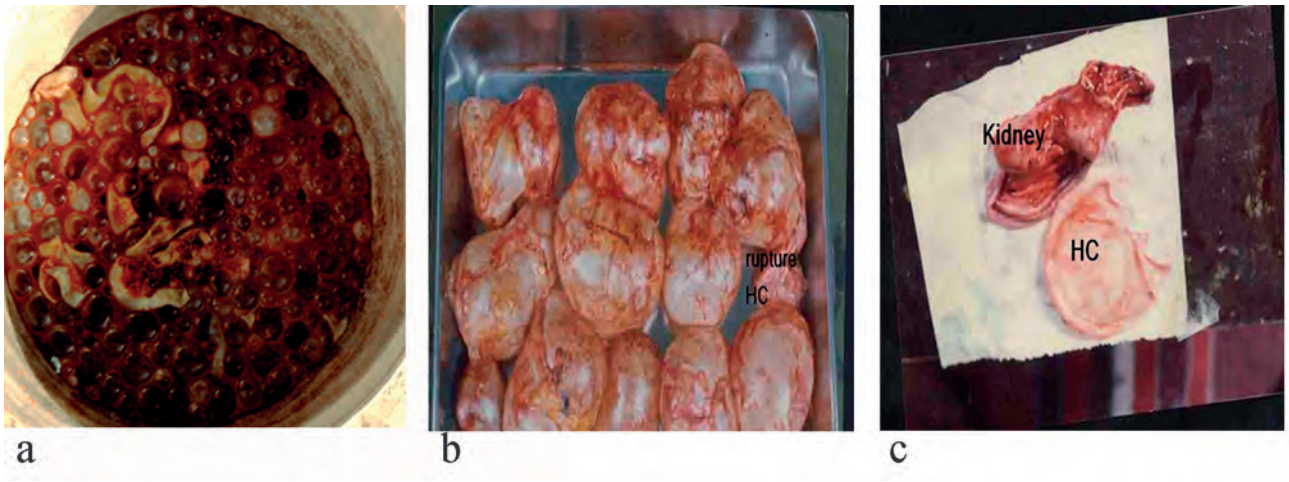


Figure 1. (a) Daughter cyst, scoliosis; (b) Secondary hydatidosis; (c) Nephrectomy.

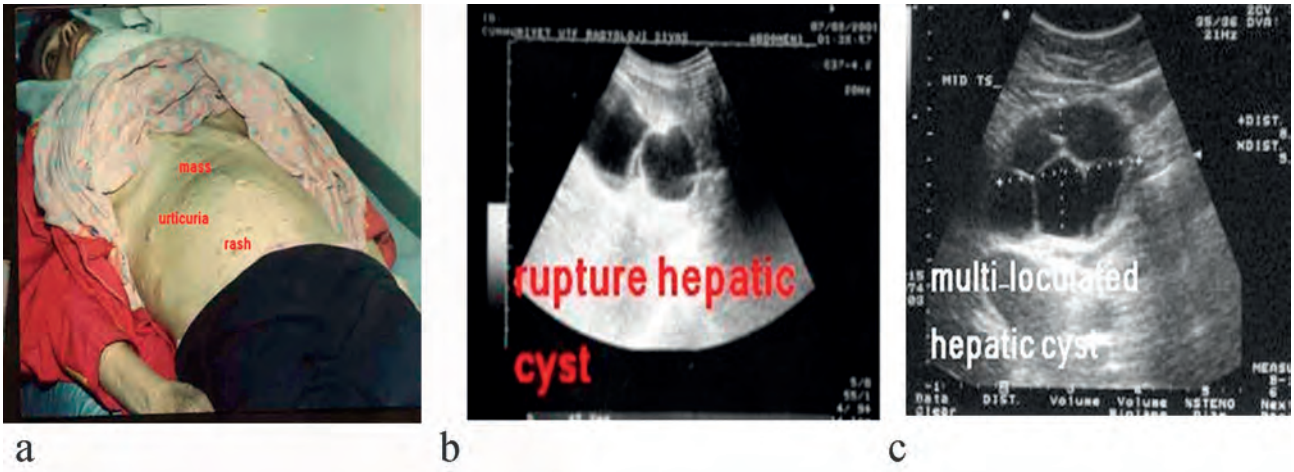


Figure 2. (a) Allergic reaction; (b) Ultrasound showing hepatic rupture cyst; (c) Ultrasound showing the multi-loculated hepatic cyst.

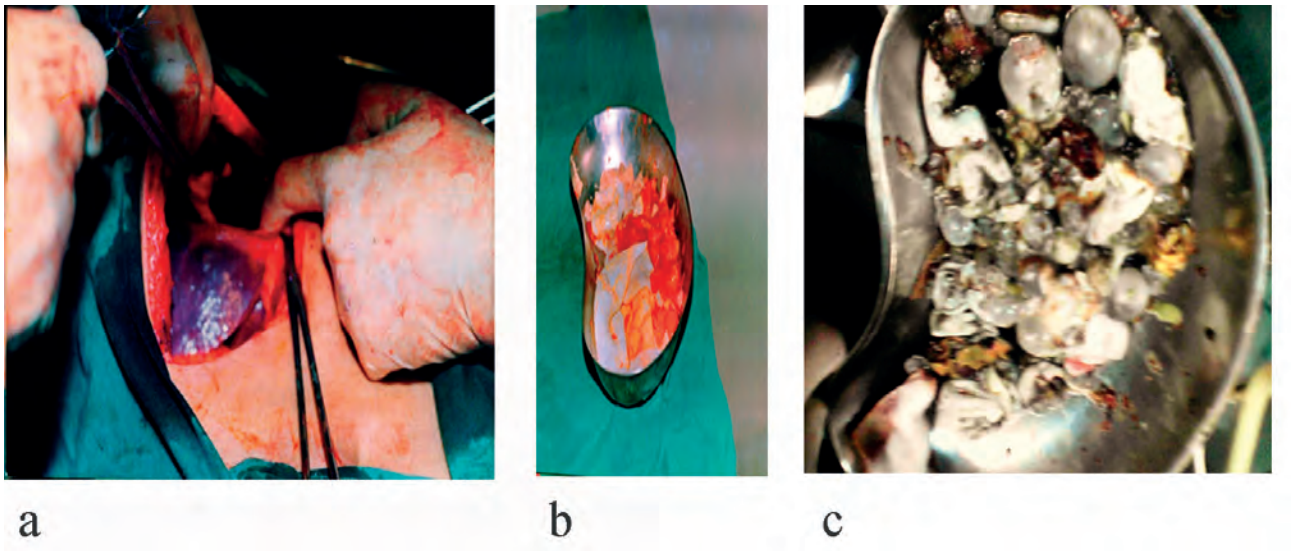


Figure 3. (a) Remnant huge cystic cavity; (b) Bile stain contents; (c) Infected contents.

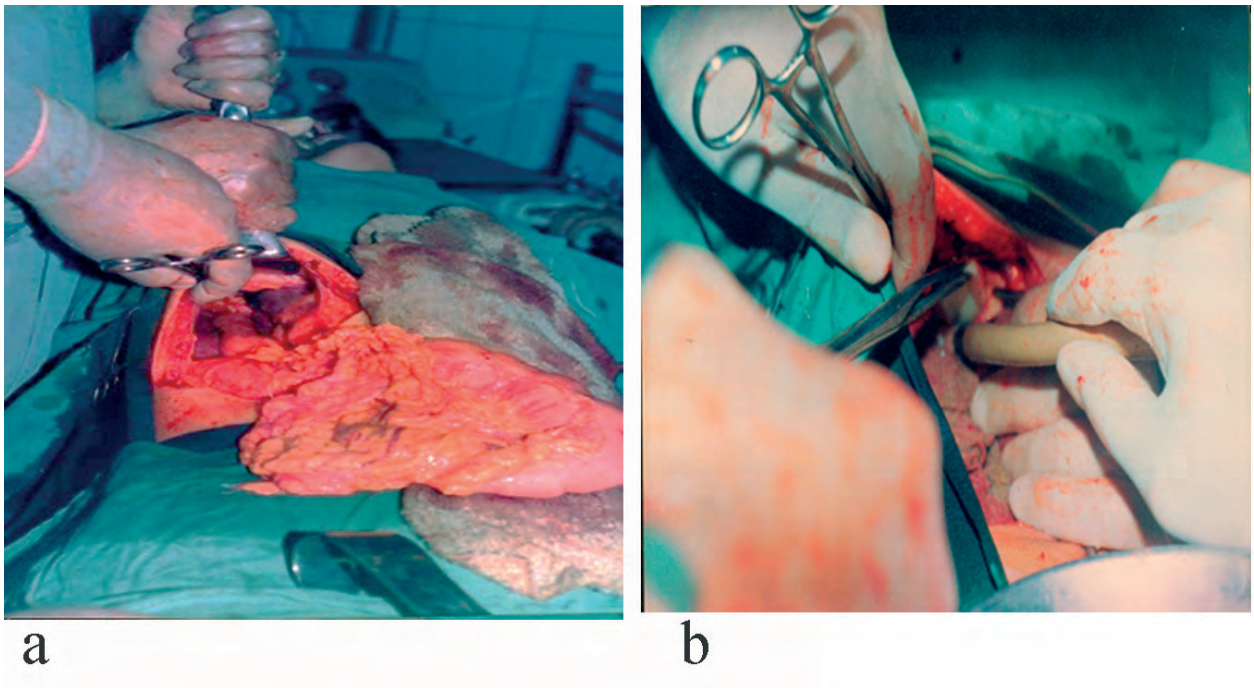


Figure 4. (a) Omentoplasty; (b) Tube drainage.

Post-operative care

All patients were given Albendazole (200 mg) twice a day. Three treatment cycle courses of 112 days were done. Each cycle was about 28 days, followed by 14 days in drug-free intervals.

Patient followup

All patients were followed up for 5 years and invited to attend every 3 months at which clinical examination, ultrasonography, and serological tests (indirect haemagglutinin reaction were performed), which is the only available test in Iraq.

Three patients need urgent reoperation, cyst abscess in one patient, treated by aspiration of the cyst under ultrasound guidance and antibiotics, peritonitis in one patient treated by re-exploration, suction of the peritoneal abscess and toilet with normal saline, and subdiaphragmatic abscess and right pleural effusion in other patient treated by suction of the abscess and chest tube.

Results

Out of 18 patients who presented with symptoms and signs of acute abdomen, only 3 cases were diagnosed preoperatively by ultrasound. The first patient had a history of surgery for hepatic hydatid disease 3 years ago. The second patient presented with haematuria after hitting a football on his abdomen. The third patient presented with rash and urticaria with multiple masses in the abdomen after trauma. Fifteen cases (83.3%) were diagnosed incidentally during explorative laparotomy, and three cases (16.7%) were diagnosed clinically by ultrasound (Table 1).

In this study period, a total of 48 cysts were treated. Around 30 cysts (62.5%) were found in the liver, while the remaining 18 cysts (37.5%) were found in the extrahepatic region (17 cysts in the intraperitoneal cavity and 1 cyst in the right kidney). In the liver, around 24 cysts (80%) were in the right lobe (size 6-10cm), and 6 cysts (20%) were in the left lobe (size 4-6cm). In the right lobe cysts, 15 cysts (2 were cystic-biliary communication) were ruptured, and 9 cysts were non-ruptured. While, in the left lobe cysts, 1 cyst was ruptured, and 5 cysts were non-ruptured. The localization of the cysts is depicted in Figure 5.

Omentoplasty was done to operate on 14 cysts that contain clean fluid. Tube drainage of the cystic cavity was carried out in 10 patients (2 bile stain content with cysto-biliary communication and infected cyst in 8 patients). Extrahepatic, peripheral located or pedunculated cyst was operated on with total cystectomy (23 cysts). These cysts were in the peritoneal cavity (17 cysts), in the liver (right lobe 1 cyst and left lobe 4 cysts), and in the right kidney (1 cyst). Hepatomy with selective vascular ligation suture of the inferior vena-cava was carried out in 1 cyst.

Out of 200, around 18 patients (09%) showed spontaneous rupture of hydatid cysts (Table 2). Of these 18 patients, around 12 were female (64.6%), and 6 were male (43.4%). The female to male ratio was 2:1. The mean age group was 36 years and ranged from 12-52 years. Around 16 days of hospital stay were observed.

Management was done according to the site, cyst size, and type of complications. Urgent surgical treatment is done after appropriate correction of shock having fluid and electrolyte upsets. Before the closure of the abdomen, irrigation of the peritoneal cavity was done with a povidone-iodine solution of 1%, and post-operative medical treat-

Parameters	Condition / Cause	No of patients	Total patients
Presentation	Acute abdomen	17	18
	Haematuria	01	
Causes	Traumatic rupture (HC)	11	18
	Spontaneous rupture	04	
	Secondary peritoneal hydatidosis	02	
	Cyst in the Right kidney	01	
Other	RTA	09	18
	Anaphylactic shock	01	
	Allergic reaction	01	
	Intra biliary fistula	02	
	Subdiaphragmatic abscess	01	
	Peritonitis	01	
	Intra peritoneal	01	
	Intra peritoneal with one on the	01	
	Hit on the right loin	01	

Table 1. Type of Presentation of 18 patients.

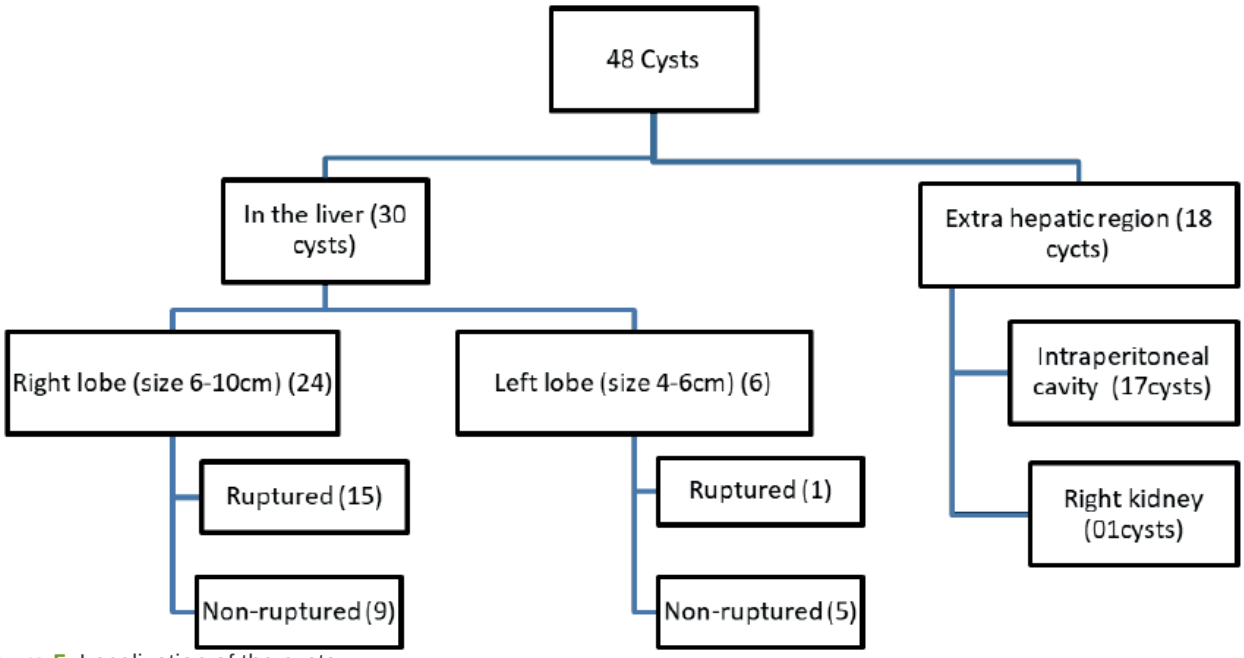


Figure 5. Localization of the cysts.

	Parameter	No of patients	Percentage
Gender	Female	12	66.6%
	Male	06	34.3%
Presentation	Acute abdomen	17	94.4%
	Heamaturia	01	5.6%
Morbidty	--	06	33.3%
Mortality	--	01	5.6%
Diagnosis	Diagnosed	03	16.6%
	Un-diagnosed	15	83.4%

Table 2. The main character of the patients with incidental ruptured hydatid cysts (n=18).

ment was done with Albendazole. Physical examination, ultrasonography, and serological tests were carried out to evaluate the recurrence and symptoms. The recurrence rate was in one case (5.6%), and the duration of follow-up has ranged 2-5 years. Post-operative morbidity occurred in 6 patients (33.3%). These patients showed wound infection (n=1), bleeding on stress ulcer (n=1), pleural effusion with hepatic abscess (n=1), infected cystic cavity (n=1, with tube drain), and peritonitis (n=1) and recurrence of the hepatic cysts (n=1). Out of these 6 patients, 3 patients (with hepatic abscess, infection) required urgently reoperated. The ultrasonography showed a 100% success rate. *Echinococcus granulosus* was confirmed histologically in all cases.

Discussion

Rupture of hydatid cyst was an urgent surgical condition that needed prompt surgical treatment after resuscitation of the patients. It is a rare complication. The present study shows that female gets more affected than males, and the mean age of patients was 36 years, which was lower than the mean age mentioned by (13). This may occur when female and young patients contact dogs or consume contaminated un-cooked vegetables with the parasite's eggs on the farm and houses in the rural area.

The clinical features of the hydatid disease depend on the site and size and may produce very few symptoms until complications occur. Complications of hydatid disease are traumatic rupture of hydatid cyst, the intraperitoneal release of contents of hydatid cyst (fluid, scolices daughter cyst) into the peritoneum[14]. Hydatid disease may lead to allergic reactions[15], anaphylactic shock and form new cysts (secondary hydatidosis) independent of hepatic cyst[16]. This also occurs during a previous surgery. This cycle takes several years. Intra biliary spontaneous rupture causes obstructive jaundice and cholangitis[17,18]. Sub diaphragmatic and hepatic abscess or spontaneous rupture occurs due to cyst infection. Fever supervenes if there is a secondary bacterial infection, post-operative medical treatment is mandatory in rupture of cysts, and Albendazole is currently the drug of choice[19].

Acute abdomen constitutes (94.4%) which is more than

explained in a study by Gunay *et al.*[20]. Anaphylactic shock (5.6%) and allergic reaction (5.6%) have the same results as another author (27%). Haematuria in 1 patient (5.6%) is a rare presentation. Spontaneous rupture in four patients (27%) is more than explained by Becker *et al.*[21]. Patients in rural areas have no facilities to do investigations. Urgent surgical treatment remains the primary treatment of choice in managing complicated hydatid disease. Total cystectomy is the best treatment if possible depending on the location of the cyst and clinical condition of the patient. Tube drainage of the cystic cavity after the evacuation of the cystic contents had complications more than obliteration by omentum, like hepatic abscess and a more extended hospital stay, and this result corresponds with Erguney *et al.*[22]. In our opinion, irrigation of the peritoneum with 10% povidone-iodine solution and post-operative medical treatment with Albendazole is the treatment of choice to decrease the incidence of recurrence, which is 5.6% less than explained by Mottaghian and Saidi.[23]. Trauma causes rupture to the right lobe of the liver and a large cyst more than the left lobe. Spontaneous rupture occurs due to infection of the cyst.

Secondary hydatidosis occurs after the first operation due to the spillage of hydatid fluid. Broods scolices become more extensive in the peritoneum and are usually multiple[8]. The first operation for hydatid disease is essential. If all cyst material is not cleaned correctly, secondary cyst formation in the liver & peritoneum will be unavoidable.

Pre-operative diagnosis is difficult because patients are generally admitted urgently. For acute abdomen and hypotension, diagnostic ultrasound should be performed initially in the evaluation process as it is fast and has high accuracy. Laboratory tests should be limited as they consume time.

Conclusions

Spontaneous or traumatic rupture of the liver was found to be rare. It is considered the most severe complication. In the case of acute abdomen, anaphylaxis, or allergic reaction in the endemic region, the diagnosis is tricky, and ultrasonography is suitable for initial investigation in the suspected case. Early diagnosis and urgent surgical treatment reduce the morbidity and mortality associated with this disease. Before the closure of the abdomen, it is irrigated with a sporicidal agent. Surgical treatment of rupture of hydatid cyst is still the treatment of choice. Total cystectomy and omentoplasty are preferred methods with the lowest morbidity rate and acceptable rate of recurrence. The cystic cavity should be managed according to the site, size and contents before the closure of the abdomen. The peritoneal cavity should be irrigated with 1% povidone-iodine solution, and post-operative Albendazole would decrease the incidence of cyst implantation after rupture and secondary hydatidosis development.

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ARTICLE / INVESTIGACIÓN

Studying the efficiency of the multiple biofilters in the reduction of pollutants from wastewater

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Abstract: The Microorganisms: yeast *Sporobolomyces yunnanensis*, *Rhodotorula mucilaginosa* and *Kluyveromyces marxianusin* growth of 184× 10^6 ± 15 cell\ml, and bacteria *Pseudomonas aeruginosa* and *Bacillus cereus* in the growth of 9 – 15× 10^8 ± 5 cell\ml, and algae *Chlorella vulgaris* in the growth of 45 × 10^5 ± 5 cell\ ml were selected as a bio filter to reduce organic and nonorganic pollutants from wastewater by COD, TOC, TN and TP concentrations of 455, 151, 9.5 and 31 ppm in treatment period 4, 6 and 18 hr. and pH of 7-8 in 25 Co as a Batch culture system. The yeasts showed ability to reduce COD concentration by 50-64% in 18 hr. treatment period, and the bacteria showed the ability to reduce COD, TOC, TN and TP concentrations by 59-69%, 57-66%, 53-63% and 40-55% in 18 hr. treatment period. At the same time, algae showed efficiency in reducing TN and TP by 81, 82%. The selective microorganisms showed high efficiency as a biofilter to reduce pollutants concentrations from wastewater in three serial steps by a treatment period of 6 hr in each step with a high ability to reduce COD, TOC, TN , and TP concentrations by 83, 91, 100, and 100% in 18 hr. treatment period. Ultimately, this study demonstrated the high efficiency of the multiple biofilters consisting of bacteria, yeasts, and algae in reducing the concentration of pollutants in sewage wastewater.

Key words: Multiple biofilters, Bacteria, Yeast, Algae, COD, TOC, TN.

Introduction

Industrial, agricultural and residential wastewater is one of the main sources of pollution of water bodies after being discharged into rivers without adequate treatment or partial treatment. These wastes contain various organic materials and pathogens that lead to a decrease in the status of natural waters and to the alteration of the balance of the aquatic environment. They also lead to pollution, as the characteristics and composition of the biodegradability of a pollutant are affected by the type of resulting industry and the presence of other pollutants. Environmental conditions such as temperature, humidity and the degree of concentration of the pollutant affect the waters. Taking into account the ability of these organisms to consume nitrogen and phosphorus compounds and carbon and methane compounds[1].

Wastewater treatment processes have evolved in recent years and have been developed with highly efficient technologies to reduce pollutants, such as Biofilteration, Bioshaft, and Moving Bed Biofilm Reactor (MBBR) technology, as international companies and research centers compete to find low-cost and high-efficiency methods for treating industrial and domestic wastewater and recycling this water for agricultural, industrial and other uses. The bio-filter technology is one of the efficient techniques for reducing pollutant concentrations from wastewater. These techniques depend on the metabolic activity of microorganisms to reduce the organic and inorganic pollutants in wastewater and select a group of efficient species in biological treatments according to the type of pollutants[2]. Also, (3) in their study showed that biofilters are highly effective in the biodegradation of organic and inorganic pollutants in wastewater. This effect depends on the type of pollutant and the microorganisms used as biofilters and the ability of these organisms to coexist within specific conditions in the waste sample, and the exploitation of pollutants as a source of energy liberation for its permanence and reproduction.

Bacteria are among the efficient microorganisms in the biological treatment of wastewater, as they can secrete a wide range of decomposing enzymes that break down organic and hydrocarbon materials and convert them into more explicit materials and adsorb many heavy elements on their cell wall; in addition to their spread throughout the water body and their rapid growth. *Bacillus*, *Pseudomonas*, and some *Micrococcus* strains are highly efficient in breaking down dissolved organic matter by their decomposing enzymes[3], and (4); in their study, it was proved that the ability of *Chromatium* and *Bacillus* bacteria to consume sulfates significantly in addition to nitrate and nitrite from polluted water and reduce their concentrations significantly. (5) their study showed the ability of bacteria to reduce the concentration level of phosphates, nitrates, and nitrites from polluted water and convert them into food granules stored inside their cells.

Bacteria are divided into several classes according to their metabolism and breakdown abilities: (1) hydrolytic bacteria that secrete a group of enzymes that break down polysaccharides, fats, and proteins and convert them into organic acids, amino acids, and other simpler compounds, and (2) fermentative bacteria that work on converting the-

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se compounds into alcohol and fatty acids. Consequently, acetogenic bacteria convert it into acetate and hydrogen, then degradable bacteria break down these compounds and convert them into carbon dioxide and water[6].

Yeasts are among the efficient microorganisms in the biodegradation of organic pollutants in wastewater; as (7) showed in their study, the efficiency of *Candida* and *Rhodotorula* yeasts in reducing pollutant concentrations and using this yeast within a biofilter with an efficiency of more than 40% in reducing pollutants. As well as (8) demonstrated the efficiency of some species of black yeast isolated from domestic sewage wastewater in breaking down organic pollutants because they possess an effective and highly efficient enzymatic system to break down a wide range of hydrocarbons, aromatic and aliphatic substances.

The current study aims to use the yeast *Sporobolomyces yunnanensis*, *Rhodotorula mucilaginosa* and *Kluyveromyces marxianus*, *Bacillus cereus*, *Pseudomonas aeruginosa* bacteria and *Chlorella Vulgaris* as biofilters for multiple and sequentially to reduce the concentrations of organic and inorganic pollutants from wastewater.

Materials and methods

Collection and preparation of samples

Wastewater samples were collected from the secondary sedimentation stage of the Al-Rustumiyah wastewater treatment plant using polyethylene plastic bottles (5 liters) and transferred to the laboratory. Other wastewater samples were collected using sterile glass bottles (100ml) to isolate and identify bacteria and yeasts.

Isolation, culturing, and identification of yeasts

Yeasts were isolated from sewage water. Potato dextrose agar (PDA) medium was prepared with the addition of the antibiotic chloramphenicol prepared by dissolving 250 mg of chloramphenicol in 250 ml of distilled water, one ml of the sample placed in sterile glass plates, and adding the sterilized solid media then was shaken well, and the plates were incubated at 27 °C for 48 hr. A smear was taken from each colony using a sterile loop and transferred to a new sterile plate containing PDA media by streak plate method and incubated at 27 °C for 48 hr, after which the resulting yeasts were diagnosed by conducting biochemical tests and based on the key of taxonomy[9], *Sporobolomyces yunnanensis*, *Rhodotorula mucilaginosa* and *Kluyveromyces marxianus* were diagnosed.

Isolation and identification of bacteria

One milliliter of sewage was taken from a sewage water sample and planted by spreading a nutrient medium on the plates. The plates were incubated at 37 °C for 24 hours. A smear from each bacterial colony was transferred to the diagnostic media (differential and selective) to differentiate between different bacterial species and obtain pure bacterial colonies, and biochemical tests were used to diagnose the bacteria. *Bacillus cereus* was isolated and identified according to (10), and a pure isolate of *Pseudomonas aeruginosa* was used, diagnosed by VITEK 2 compact system in the Bacteriological Laboratory/Water Research Center.

Algae culturing and diagnosis

Suitable biomass for *Chlorella vulgaris* cells was obtained within liquid cultures based on (11) through the availability of nutrients in sufficient quantities and the use of culturing with the continuous movement to eliminate local environments at a temperature of (25 ± 2 C) and a light intensity of (250 microeinstein /m^2 / s), green algae were diagnosed by preparing slides and examining them at a power of 400 X using a compound light microscope (12) and (13).

Chemical tests

The chemical factors (the chemical oxygen requirement (COD), total organic carbon (TOC), total phosphorous (TP), and total nitrogen (TN)) were measured before and after treatment according to (14).

Counting of bacterial cells number

Cell numbers of *Pseudomonas aeruginosa* and *Bacillus cereus* were calculated by preparing McFarland 0.5 solution according to (15).

Counting of yeast and algae cells number

The numbers of yeast and algae cells were calculated in 1 ml of broth using the Hemocytometer (Neubauer counting chamber)[16] and depending on the equation:

Number (cells/ml) = Number of cells in 4 squares x 4 x 10

The multiple biofilters

Three isolates of yeast *Sporobolomyces yunnanensis*, *Rhodotorula mucilaginosa* and *Kluyveromyces marxianus* were used as a mixture of the first stage of the biofilter to reduce the concentration of pollutants from sewage wastewater in pH 5-6 and covering the bottles with foil aluminum to prevent the light. *Bacillus cereus* and *Pseudomonas aeruginosa* were used in the second stage as a mixture in pH conditions 7-8, and *Chlorella vulgariswas* used in the last stage of the biofilter in pH 7-8.

Experiments

Yeasts *Sporobolomyces yunnanensis*, *Rhodotorula mucilaginosa* and *Kluyveromyces marxianus* were used with a growth density of 184 x 10^6 ± 15 cells/ml, while *Bacillus* and *Pseudomonas* were used at a growth density ranging 9-15 x 10^8 ± 5 cells/ml and *Chlorella vulgaris* at a growth density of 45 x 10^5 ± 5 cells/ml to reduce pollutant concentration in a sample of sewage water in terms factors the chemical requirements for oxygen (COD), total organic carbon (TOC), total phosphorous (TP), and total nitrogen (TN) using of the glass cylinders, which are contained a sterile wastewater sample (1 liter) each separately, and 20 ml of the broth of the selected microorganisms were added for each cylinder with the period treatment of 4, 6 and 18 hrs.; also it was used a wastewater sample without any addition as the control. The factors COD, TOC, TN, and TP were measured before and after treatment and for each cylinder to know the efficiency of these selected microorganisms in the treatment processes.

The selected microorganisms were used as multiple biofilters using graduated glass cylinders containing a sterile sewage wastewater sample (1 L), and 20 ml of broth for each yeast were added to them at a treatment period of 6 hrs. The treated samples were taken and filtered with filter paper (0.45 µ) and added 20 ml of each bacteria to them at a treatment period of 6 hrs. The samples were filtered and 20 ml of broth algae were added at a treatment period of 6

hrs. The factors COD, TOC, TN, and TP were measured before and after the treatment, and a wastewater sample was used as a control.

Calculations

The percentage of concentration reduction % = initial concentration- final concentration / initial concentration x 100.

Results and discussion

Efficiency of yeasts *Sporobolomyces yunnanensis*, *Rhodotorula mucilaginosa* and *Kluyveromyces marxianus* in reducing pollutants concentration from sewage wastewater

Table (1) shows the reduction of pollutant concentration from sewage wastewater for the control in terms of the factors COD, TOC, TP, and TN at a treatment period of 4, 6, and 18 hrs., the concentration of the factors before treatment was 455, 151, 9.5 and 31 ppm. The concentration of the factors became 454, 151, 9.2 and 29, respectively, after 4 hrs. After 6 hrs. of the treatment period, the concentration of the factors became 452, 144, 8.7 and 29.5 ppm, respectively, and after 18 hrs. The concentration of the factors COD, TOC, TP, and TN became 440, 128, 8.5 and 26 ppm with a reduction percentage of 3, 15, 10 and 16%, respectively.

Table (2) shows the reduction of pollutants concentration from wastewater in terms of factors COD, TOC, TP, and TN using *Sporobolomyces yunnanensis* yeast for a treatment period of 4, 6, and 18 hours, as the concentration of factors before treatment was 455, 151, 9.5 and 31 ppm, respectively. Consequently, after 4 hrs. of the treatment period, the concentrations became 451, 138, 9.2, and 29 ppm, respectively, and then after 6 hrs. of the treatment period, the concentration of factors became 377, 128, 9.2, and 28.7 ppm, respectively. While, the concentrations of factors became 225, 92, 7.4 and 26 ppm after 18 hrs. The reduction percentage was also 50.5, 39, 22 and 16%, respectively.

Table (3) shows the reduction of pollutants concentration from wastewater in terms of factors COD, TOC, TP, and TN using *Rhodotorula mucilaginosa* yeast at a treatment period of 4, 6, and 18 hrs., as the concentrations of factors before treatment were 455, 151, 9.5, and 31 ppm, respectively. The concentration became 455, 140, 9.5, and 31 ppm, respectively, after 4 hrs. of treatment also after 6 hrs. of treatment, the concentrations became 365, 126, 8.7, and 29 ppm, respectively, while the concentrations of COD, TOC, TP and TN became 210, 82, 6.6 and 22.5 after 18 hrs. of treatment with the reduction percentage of 54, 46, 30.5 and 27%, respectively.

Table (4) shows the reduction of pollutant concentrations from wastewater in terms of the factors COD, TOC, TP, and TN using *Kluyveromyces marxianus* yeast for a treatment period of 4, 6, and 18 hrs. The concentrations of the factors before treatment were 455, 151, 9.5 and 31 ppm, respectively. The concentrations became 450, 148, 9, and 30 ppm, respectively, after 4 hrs. of treatment. However, the concentrations of factors became 360, 122, 6.4, and 30 ppm, respectively, after 6 hrs. of treatment. Whilst, the concentrations became 162, 35, 4.6, and 25 ppm after 18 hrs. With the reduction percentage of 64, 77, 32, and 19%, respectively.

The current study showed the high efficiency of the selected yeasts in reducing the concentration of the chemical oxygen requirement of sewage wastewater with a rate ranging from 50-64% compared to the control during a treatment period of 18 hrs. The results illustrated the high ability of the yeast *Kluyveromyces marxianus* to reduce the concentration of pollutants from the wastewater in terms of the factors COD, TOC, TP, and TN compared to the control. The results matched with the study (14) where they showed the ability of the yeast Kluyveromyces marxianus on the production of a group of effective enzymes in biodegradation processes such as β-galactosidase, inulinase, β-polygalacturunases, and a group of peroxidase enzymes, and the current study agreed with (15) as they showed the ability of *Rhodotorula mucilaginosa* to break down the complicated

Factors	Concentration before treatment (ppm)	Concentration after treatment (ppm)			The reduction percentage % after 18 hrs.
		4hrs.	6hrs	8hrs.	
COD	455	454	452	440	3
TOC	151	151	144	128	15
TN	9.5	9.2	8.7	8.5	10
TP	31	29	29.5	26	16

Table 1. Reducing the concentration of pollutants from wastewater for the control in terms of COD, TOC, TP, and TN at a treatment period of 4, 6, and 18 hrs.

Factors	Concentration before treatment (ppm)	Concentration after treatment (ppm)			The reduction percentage % after 18 hrs.
		4hrs.	6hrs	8hrs.	
COD	455	451	377	225	50.5
TOC	151	138	128	92	39
TN	9.5	9.2	9.2	7.4	22
TP	31	29	28.7	26	16

Table 2. Reducing the concentration of pollutants from wastewater in terms of factors COD, TOC, TP, and TN using *Sporobolomyces yunnanensis* yeast for a treatment period of 4, 6, and 18 hours.

Factors	Concentration before treatment (ppm)	Concentration after treatment (ppm)			The reduction percentage % after 18 hrs.
		4hrs.	6hrs	8hrs.	
COD	455	455	365	210	54
TOC	151	140	126	82	46
TN	9.5	9.5	8.7	6.6	30.5
TP	31	31	29	22.5	27

Table 3. Reducing the concentration of pollutants from wastewater in terms of factors COD, TOC, TP and TN using yeast *Rhodotorula mucilaginosa* for a treatment period of 4, 6 and 18 hours.

Factors	Concentration before treatment (ppm)	Concentration after treatment (ppm)			The reduction percentage % after 18 hrs.
		4hrs.	6hrs	8hrs.	
COD	455	455	365	210	54
TOC	151	140	126	82	46
TN	9.5	9.5	8.7	6.6	30.5
TP	31	31	29	22.5	27

Table 4. Reducing the concentration of pollutants from wastewater in terms of factors COD, TOC, TP and TN using *Kluyveromyces marxianus* yeast for a treatment period of 4, 6 and 18 hours.

organic compounds associated with heavy metals in the contaminated water due to its effective enzymatic system. A study (16) proved that the use of yeasts in wastewater treatment processes has an influential role in the biodegradation of organic waste. At the same time, its efficiency is less in the process of reducing the concentrations of nitrogen and phosphorous compounds due to its nutrition system and the effectiveness of its enzymes in fermentation.This was proven by the current study results, as the percentage of reduction in the concentration of nitrogen and phosphorous compounds did not exceed 12-32%.

Efficiency of *Bacillus* and *Pseudomonas* bacteria in reducing pollutants concentration from sewage wastewater

Table (5) shows the reduction of pollutants concentration from wastewater in terms of the factors COD, TOC, TP, and TN using *Bacillus cereus* at a treatment period of 4, 6, and 18 hours, as the concentrations of the factors before treatment, were 455, 151, 9.5 and 31 ppm, respectively. While the concentrations became 450, 147, 8.5, and 29 ppm, respectively after 4 hrs. of treatment, however after 6 hrs. of treatment, the concentration of COD, TOC, TP, and TN factors became 325, 130, 7.8, and 21 ppm, respectively, and after 18 hrs of treatment, the concentrations became 185, 65, 3.5 and 16.5 ppm with a reduction rate of 59, 57, 63 and 46%, respectively.

Table (6) shows the reduction of pollutants concentration from wastewater in terms of factors COD, TOC, TP, and TN using *Pseudomonas aeruginosa* for a treatment period of 4, 6, and 18 hrs., as the concentrations of factors before treatment were 455, 151, 9.5, and 31 ppm, respectively. After 4 hours of treatment, they became 435, 150, 9.5, and 28.7 ppm, respectively; however, the concentrations became 320, 122, 6.6 and 18 ppm after 6 hours of treatment. At the same time, the concentrations after 18 hrs. of treatment became 140, 52, 4.5, and 14 ppm with a reduction rate of 69, 66, 53, and 55%, respectively.

The results of the current study illustrated the high efficiency of the selected bacteria in reducing the concentration of pollutants from the sewage wastewater during the treatment period of 18 hours compared to the control, as the reduction percentage of the concentration of the chemical oxygen requirement for *Bacillus* and *Pseudomonas* was 59-69%, the total organic carbon was 57-66%, as well as for total nitrogen was 53-63%, and total phosphorous was 40-55% compared to the control. So, the results matched with study (18), where they demonstrated the ability of Bacillus to secrete several enzymes that destroy organic and hydrocarbon compounds with high efficiency and use in treating water contaminated with oil derivatives. Also, the results of the current study are in agreement with the study (19); it was showed the efficiency of microorganisms in the biodegradation of organic pollutants, as they demonstrated the ability of *Bacillus* bacteria to produce a group of peroxidase enzymes that decompose and destroy organic materials and aliphatic and aromatic compounds with high efficiency. The results of the current study also coincided with study (20), as they showed in their study the high enzymatic efficiency of *Pseudomonas* bacteria in destroying many organic compounds and hydrocarbon pollutants, including the cytochrome group and the biphenyl dioxygenase group, which have a wide range and high efficiency in the biodegradation of organic pollutants and benzene compounds. The current study's results also agreed with (21) as they showed in their study the ability of bacteria *Pseudomonas* produces a wide range of enzymes that break down hydrocarbons and benzene compounds and their ability to biodegrade these compounds with high efficiency.

Efficiency of *Chlorella vulgaris* in reducing pollutants concentrations from sewage wastewater

Table (7) shows the reduction of pollutants concentration from wastewater in terms of the factors COD, TOC, TP, and TN using *Chlorella vulgaris* for a treatment period of 4, 6, and 18 hrs., as the concentrations of factors before treatment, were 455, 151, 9.5 and 31 ppm, respectively, however, the concentrations became 410, 148, 8, and 29 ppm, after 4 hrs. of treatment. While after 6 hrs. of treatment, the concentrations of the factors became 388, 133, 5.2, and 20

Factors	Concentration before treatment (ppm)	Concentration after treatment (ppm)			The reduction percentage % after 18 hrs.
		4hrs.	6hrs	8hrs.	
COD	455	450	325	185	59
TOC	151	147	130	65	57
TN	9.5	8.5	7.8	3.5	63
TP	31	29	21	16.5	46

Table 5. Reducing the concentration of pollutants from wastewater in terms of COD, TOC, TP, and TN using Bacillus cereus for a treatment period of 4, 6, and 18 hours.

Factors	Concentration before treatment (ppm)	Concentration after treatment (ppm)			The reduction percentage % after 18 hrs.
		4hrs.	6hrs	8hrs.	
COD	455	435	320	140	69
TOC	151	150	122	52	66
TN	9.5	9.5	6.6	4.5	53
TP	31	28.7	18	14	55

Table 6. Reducing the concentration of pollutants from wastewater in terms of factors COD, TOC, TP, and TN using *Pseudomonas aeruginosa* for a treatment period of 4, 6 and 18 hours.

Factors	Concentration before treatment (ppm)	Concentration after treatment (ppm)			The reduction percentage % after 18 hrs.
		4hrs.	6hrs	8hrs.	
COD	455	410	388	270	40
TOC	151	148	133	95	37
TN	9.5	8	5.2	1.8	81
TP	31	29	20	5.6	82

Table 7. Reducing the concentration of pollutants from wastewater in terms of factors COD, TOC, TP and TN using *Chlorella vulgaris* for a treatment period of 4, 6 and 18 hours.

ppm, respectively, and after 18 hrs. of treatment, the concentration of the factors COD, TOC, TP, and TN became 270, 95, 1.8 and 5.6 ppm with the reduction rate of 40, 37, 81 and 82%, respectively.

The current study results showed the ability of *Chlorella vulgaris* to reduce the concentration of nitrogenous and phosphorous compounds from sewage wastewater with a reduction rate of 81 and 82% compared to the control.

The current results agreed with (22) in their study to reduce the concentration of nitrogen and phosphorous nutrients from sewage wastewater, as they demonstrated the ability of *Chlorella vulgaris* to reduce total nitrogen concentrations by 87% and total phosphorous by 98% to benefit from them in growth and proliferation, as they are essential elements for algae growth. The current results agree with study (23). They clarified the essential and influential role of using this alga in wastewater treatment processes because of its high ability to reduce nitrogen and phosphorous nutrients concentrations and exploit them in growth and proliferation.

Efficiency of the multiple biofilters in reducing pollutants concentration from sewage wastewater

Table (8) shows the percentage of reducing the concentration of pollutants from wastewater in terms of the factors COD, TOC, TP, and TN for the control treatment by using the multiple biofilters at a treatment period of 18 hrs., as the concentrations of the factors before treatment were 560, 172, 25 and 42 ppm, respectively. As well as, after 18 hours of treatment, distributed in three stages (6 hrs. for each stage). The results showed an increase in COD concentration, which became 820 ppm, while the concentration of TOC, TP, and TN were 168, 13.5, and 39 ppm, with a reduction percentage of 2.10 and 7%, respectively.

Table (9) shows the reduction of pollutants concentration from wastewater in terms of the factors COD, TOC, TP, and TN using the multiple biofilters for a treatment period of 18 hours, as the concentration of the factors before treatment were 560, 172, 25 and 42 ppm, respectively. In addition, it was distributed into three phases (6 hrs. for each phase); after 18 hrs. of treatment, the concentration of the factors became 0, 15, 95, and 0 ppm with the reduction percentage of 83, 91, 100, and 100%, respectively.

The results of the current study showed the high ability of the biofilter used to reduce the concentrations of organic and inorganic pollutants from sewage wastewater and the high efficiency of the microorganisms used within this filter during 18 hrs. of the treatment period, also the nitrogen and phosphorous compounds were removed from the wastewater sample at a rate of 100%, as the results of the current study agreed with (3) in their study demonstrated the ability of biofilters to reduce the concentration of pollutants from wastewater if appropriate microorganisms were used which can biodegrade when the appropriate conditions were provided for the growth of these organisms, and the results of the current study matched with the study (25), as they proved the ability of the biofilter is eliminated of the total nitrogen from sewage waste by 100% and reduced organic pollutants in terms of total organic carbon measurement by

Factors	Concentration before treatment (ppm)	Concentration after 18hrs. treatment ppm)	The reduction percentage % after 18 hrs.
COD	560	820	-----
TOC	172	168	2
TN	15	13.5	10
TP	42	39	7

Table 8. Reducing the concentration of pollutants from wastewater in terms of the factors COD, TOC, TP, and TN for the control treatment using the multiple biofilters for 18 hours.

Factors	Concentration before treatment (ppm)	Concentration after 18hrs. treatment ppm)	The reduction percentage % after 18 hrs.
COD	560	95	83
TOC	172	15	91
TN	25	0	100
TP	42	0	100

Table 9. Reducing the concentration of pollutants from wastewater in terms of factors COD, TOC, TP, and TN using the multiple biofilters for a treatment period of 18 hours.

95%. The results also matched with (26) and (27), as they proved in their study that the efficiency of biofilters depends on the selection of the species of microorganisms used to treat industrial and domestic wastewater used as biological filters and appropriate conditions provide for the continuity of their activity. The reduction of pollutants concentration from wastewater is based on these microorganisms' metabolism and degradation activity.

Conclusions

The current study demonstrated the high efficiency of the multiple biofilters consisting of bacteria, yeasts, and algae in reducing the concentration of pollutants in sewage wastewater in terms of COD, TOC, TP, and TN with reduction rates of up to 100%.

Conflicts of interest

The authors declare no discrepancy of interest related to this article.

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LETTER TO EDITOR / CARTA AL EDITOR

The enemy is the virus, not the vaccine

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If we remember very superficially a little about the complex human immune system, a part of this immune system is made up of leukocytes (especially phagocytes and lymphocytes) that are responsible for detecting invaders and sending them defensive markers that adhere to them (the antibodies) and also to destroy the invaders identified by the system; In addition to the already mentioned leukocytes, our immune system is made up of various other elements such as cells, proteins, tissues and organs, which defend us against germs and microorganisms[1,2].

Speaking specifically about the immune system and COVID-19, the events mainly involved in the immunopathogenesis of COVID-19 and the dysregulation of the immune response include lymphopenia and increased neutrophil-to-lymphocyte ratio, cytokines, lymphocyte depletion and dysfunction, antibody-dependent enhancement, and monocyte and granulocyte abnormalities. It must be emphasized that lymphopenia is an essential finding in most patients with COVID-19, particularly in those with a severe phenotype[3]. It has been shown that multiple viral proteins induce T cells after infection and that T cells that have antiviral signatures related to safety and protection can be achieved by vaccination[4].

There are many questions about immunity-based protection still unresolved due to the wide clinical diversity of symptoms, the association between delayed/weak antibody responses and severe outcomes, and the coexistence between coronavirus and its specific IgG; SARS-CoV-2 infection can either weaken or overactivated the immune system, and patients affected by severe forms of COVID-19 can be distinguished by different "immunotypes" associated with poor clinical pathways[5].

Despite the significant advances that have been evidenced in terms of knowledge about the virus-host interaction in this pandemic, it has been mentioned that the information on the immunopathology of COVID-19 is still limited, and the current evidence may change soon with the accumulation of new knowledge about the biology of SARS-CoV-2 and host immune responses, so the current hypotheses could be incomplete and may even prove wrong in the near future[6].

Every day our understanding of the human body's response to SARS-CoV-2 evolves; however, during this same time, the virus mutates, which leads us to new challenges for our intervention strategies for the patient. Rational drug reuse has primarily focused on drugs with known activity against the cytokine storm (excessive immune response) associated with immune interventions[7,8].

The course of SARS-CoV-2 infection is individual and is influenced by the particularities of immune reactivity. The eradication of the infectious process requires the complete activation of the immune response, innate and adaptive, humoral and cellular, and the synthesis of IFN I, the primary and essential form of antiviral surveillance. The complex interplay between the severity of COVID-19 and the dysregulated host immune response has allowed worldwide evaluation of different immunomodulators currently being investigated for therapeutic effects in COVID-19 patients[9].

IgM and IgG seroconversion rate and timing of SARS-CoV-2, SARS-CoV, and MERS-CoV have been comparatively studied. With inconsistent results across studies, in SARS-CoV-2, the majority seroconverted to IgG within 2 weeks and 100% of patients within 4 weeks; IgG was still detectable at the end of follow-up (49-65 days) in all patients; neutralizing antibodies were detected in 92%-100% of patients (up to 53 days), without clarifying whether reinfection with SARS-CoV-2 is possible, with more suggestive studies of intermittent detection of residual RNA. In SARS-CoV, specific IgG was maintained for 1–2 years after infection and then decreased; although one study detected IgG up to 12 years after infection, neutralizing antibodies were detected up to 17 years in another study. Three studies on MERS-CoV reported that IgG could be detected for up to 2 years[10].

It is worth mentioning that the proposed course of SARS-CoV-2 infection could start in upper respiratory tract infection (mainly as respiratory droplets), continue in lower respiratory tract infection (mainly as pulmonary infection) , followed by viremia and diffusion to other target organs (including the gastrointestinal tract), the latter being very important as it is associated with the theory that the spread of SARS-CoV-2 in hospitals and nursing homes could occur through fecal contamination, involving health personnel in the infection, who are usually able to protect themselves from droplets, wear masks, but less easily during direct care and hygiene of patients, where people are generally bedridden and not autonomous in the handling of their stools, so it should be reiterated in the proper use of personal protection equipment[11].

We wanted to bring this topic up after having treated a 65-year-old female patient, who was referred for consultation to be authorized to be vaccinated against COVID-19 because both her family doctors and several other people had reported her that because she had low defenses, she should not be vaccinated. If a patient has "low defenses," is it not logical to receive medicines that can improve her

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defenses as part of her treatment? It would be the most rational from our point of view. But what happens when this drug is a vaccine?

Reviewing the medical file, the patient had a documented history of almost 20 years with leukopenia, which, due to its mild nature, had not been given much importance; however, in the current scenario of the COVID-19 pandemic, for the patient, it became a very relevant situation from one moment to another since the "low defenses" did not allow her to be vaccinated creating personal conflicts by not being able to go to public or private places where the vaccination card is required and with her family since they were avoiding her for being the only one not to be vaccinated, causing all this feeling of guilt, anxiety and depression.

Decisions about the best method to achieve control of the pandemic should not be left only to doctors and medical authorities. However, they should be shared between vaccine experts, regulators, financial and political leaders, and public representatives to establish the best acceptable safety margins and efficacy thresholds for vaccines, which can increase confidence in the decision process and its results[12].

The condition of this patient does not constitute a contraindication for vaccination; moreover, vaccination is currently encouraged in patients with immune compromise since immunosuppressed people are especially vulnerable to COVID-19; however, there is widespread misinformation about the adverse effects and contraindications of the different vaccines, including in health personnel, which often leads us to not offer adequate advice to our patients regarding vaccination[13].

Conclusions

There is a large sector of the world population that, despite all the scientific information available, persists with uncertainty and/or disbelief about the importance of vaccines against COVID-19; however, this is not the first time that vaccines have been rapidly manufactured and tested to deal with a public health crisis and even though accelerated Phase I-II-III clinical programs have been applied, they have followed the traditional development route to ensure the efficacy and safety of vaccines[14]. We hope, for her good, that this patient will decide to get vaccinated. The enemy is the virus, not the vaccine

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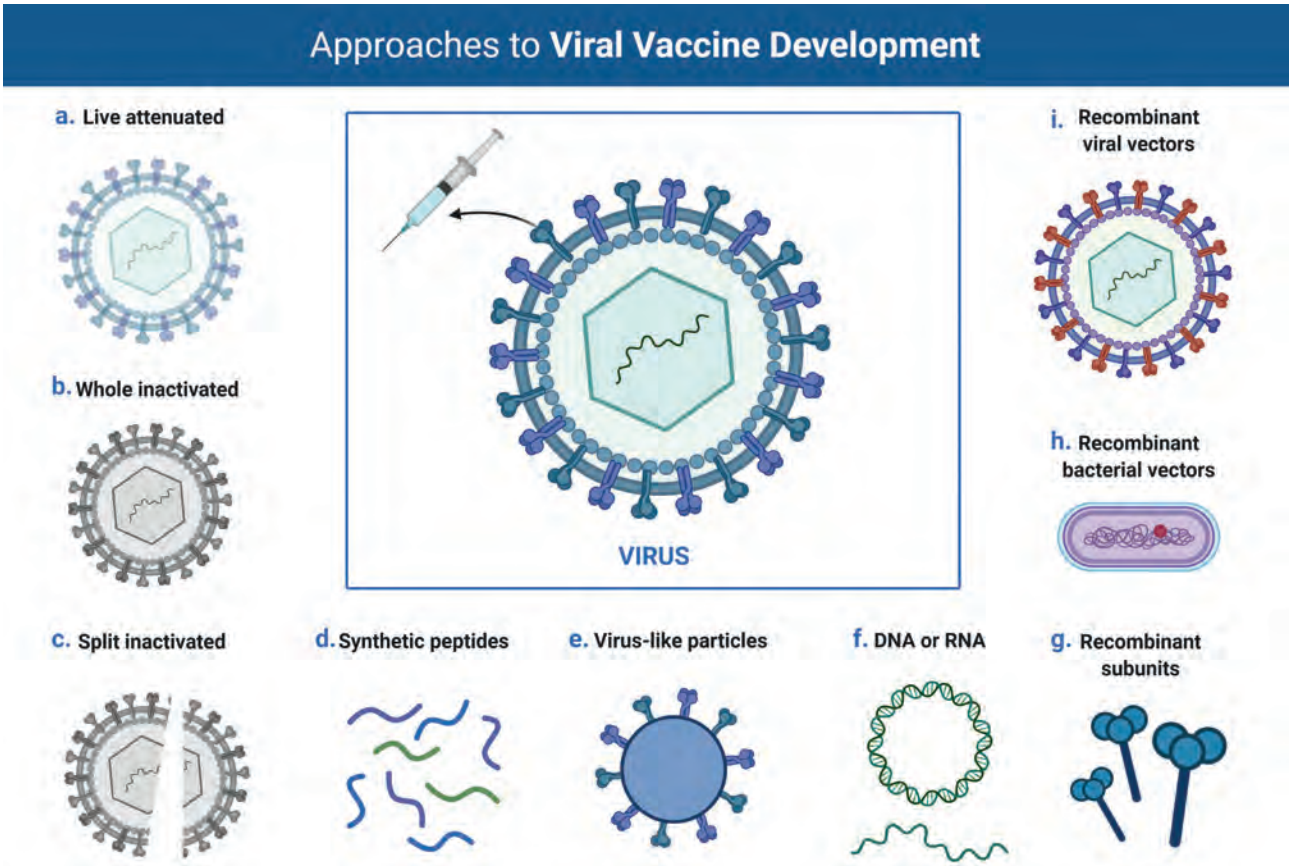


Figure 1. Overview of different platforms for viral vaccine development. Various strategies include live attenuated, whole inactivated, split inactivated, synthetic peptides, and recombinant approaches (Biorender)[15,16].

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NEWS AND VIEWS / NOTICIAS Y OPINIONES

Presentación del libro “Tópicos selectos en ciencias básicas aplicados al enfermo crítico

Presentation of the book "Selected topics in basic sciences applied to the critically ill

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Resumen: El arte de hacer medicina es producto de un intrincado y complejo proceso de razonamiento, conjuga los conocimientos básicos, que mediante la fisiopatología buscan explicar las manifestaciones clínicas que presenta un enfermo y con ello determinar un diagnóstico, tratamiento y pronóstico. El avance vertiginoso en el conocimiento médico, ha revolucionado la práctica sanitaria y ha permitido un crecimiento exponencial de información, cada vez más precisa y molecular; esto, aunque indudablemente es beneficioso, ha abierto importantes brechas entre los hallazgos en ciencias básicas y su aplicación en la práctica clínica. La pandemia actual, por COVID-19, ha sido la vitrina más clara sobre lo escrito en líneas anteriores, en dónde, fue dificultoso amalgamar los descubrimientos y publicaciones que diariamente eran mostrados sobre el impacto del virus a nivel molecular e inmunológico, con el manejo clínico asistencial. En el libro “TÓPICOS SELECTOS EN CIENCIAS BÁSICAS APLICADOS AL ENFERMO CRÍTICO”, planteamos temáticas que a nuestro juicio permitirán anclar conceptos inmunológicos claves, al arsenal de conocimientos de los médicos clínicos, con la finalidad de lograr un manejo holístico, razonado y basado en la evidencia. En siete capítulos, escribimos sobre temas como algunas de las distintas formas de muerte celular: la NETosis, la autofagia y la piroptosis; también, encontraran un espacio para los receptores de reconocimiento de patrón, de ellos, los de tipo TOLL y tipo NOD son explicados a profundidad; la vía JAK/STAT, se analiza con practicidad y se expone los blancos terapéuticos que de ella derivan, muchos ya en aplicación clínica. Finalmente, un tema de interés investigativo extremo, la microbiota y su rol en la enfermedad crítica y en patología humana en general, es abordado.

Palabras clave: Ciencias básicas, enfermedad crítica, muerte celular, microbiota, receptores de reconocimiento de patrón.

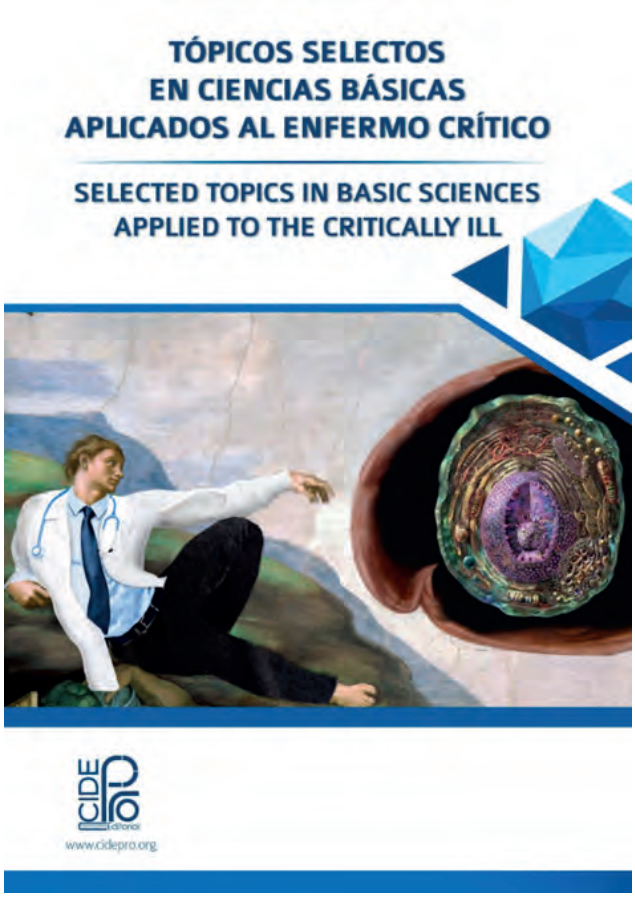
Abstract: The art of doing medicine is the product of an intricate and complex reasoning process, combines basic knowledge, which through pathophysiology seeks to explain the clinical manifestations that a patient presents and thereby determine a diagnosis, treatment and prognosis. The vertiginous advance in medical knowledge has revolutionized health practice and has allowed an exponential growth of information, increasingly precise and molecular; Although this is undoubtedly beneficial, it has opened important gaps between the findings in basic sciences and their application in clinical practice. The current pandemic, due to COVID-19, has been the clearest showcase of what was written in previous lines, where it was difficult to amalgamate the discoveries and publications that were shown daily on the impact of the virus at the molecular and immunological level, with the management healthcare clinician. In the book "SELECTED TOPICS IN BASIC SCIENCES APPLIED TO THE CRITICALLY ILL", we propose topics that, in our opinion, will allow anchoring key immunological concepts to the arsenal of knowledge of clinical physicians, in order to achieve holistic, reasoned management based on evidence. In seven chapters, we write about topics such as some of the different forms of cell death: NETosis, autophagy, and pyroptosis; also, you will find a space for pattern recognition receptors, of which, the TOLL type and NOD type are explained in depth; the JAK/STAT pathway is analyzed with practicality and the therapeutic targets derived from it are exposed, many of which are already in clinical application. Finally, a topic of extreme research interest, the microbiota and its role in critical illness and in human pathology in general, is addressed. As added data, we have the academic endorsement of the Faculty of Medical Sciences of the Central University of Ecuador and the review and prologues of two regional experts in the field of Immunology, who in their letters applaud the intentionality of the translational approach proposed by the book.

Key words: Basic sciences, critical illness, cell death, microbiota, pattern recognition receptors.

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Presentación

Ante todo, este libro representa una amalgama de conocimientos de ciencia básica muy profundos y detallistas, ahondando en los mecanismos moleculares y proteómicos, pero en perfecta concordancia con su aplicación en la ciencia clínica, y la perspectiva de su aplicación a corto o mediano plazo.

Cada capítulo inicia con una revisión bastante profunda y técnica de los elementos constituyentes de un determinado mecanismo inmunológico, pasando por las variantes de la expresión de estos componentes, y proyectándose al rol fisiológico y patológico que cumplen cada una de estas moléculas en el complejo funcionamiento del sistema inmune.

Aparte de esto, muestran la utilidad de estas moléculas, sea intra o extracelulares, en las patologías comunes, y, sobre todo hacen especial rigor al rol desempeñado en situaciones críticas como es la situación de sepsis y la reciente crítica situación de la infección por COVID-19.

En la primera sección se discute el rol crucial que cumplen dos elementos un poco olvidados de la respuesta inmune como las plaquetas y las trampas extracelulares de neutrófilos, que en los últimos años ha dado mucha información y posibilidades de intervenciones terapéuticas, que muy bien han sido resaltada por los autores.

En la segunda sección otras moléculas ya conocidas, pero aun incompletamente aprovechadas, como son los receptores Toll-Like Receptors (TLRs), sus variantes y sus posibilidades de uso terapéutico.

El tercer capítulo revisa en detalle otro tipo de receptores, en este caso, intracelulares, los NODs y su importancia en sepsis y sus potenciales aplicaciones terapéuticas.

En el cuarto capítulo se revisa el mecanismo poco conocido de la Autofagia, en sus roles fisiológicos de defensa y reguladores, como en sus disfunciones en patologías graves como la sepsis.

El quinto capítulo dirigido al entendimiento del mecanismo recientemente reconocido de la Piroptosis y sus implicancias en condiciones de salud y sepsis.

El sexto capítulo se concentra en mecanismos de trasmisión de señales intracelulares a través de tirosin kinasas y factores de transcripción, de crítica importancia en mecanismos de activación normal, disfunciones del sistema inmune por enfermedades autoinmunes, o por sobreactivación por infecciones severas, y que ya, de hecho, se están utilizando y se avecinan muchos nuevos productos que actúan a estos niveles.

El séptimo y último capítulo dedicado a una de las líneas de investigación de mayor importancia y con mayor potencialidad de uso clínico en salud y enfermedad, el tema de la microbiota y sus aplicaciones.

Una de las ventajas de los autores es que se proyectan en los usos potenciales tanto del desarrollo de nuevos productos farmacéuticos, como de producto terapéuticos no farmacológicos, que, desde el punto de vista integrativo, es uno de los enfoques más prospectivos y esperanzadores.

Es para mí un gran orgullo, que dentro de los autores se encuentren alumnos de la Maestría de Inmunología, que ojalá haya servido para darles esa visión holística del conocimiento en salud y enfermedad.No me queda más que desearles a los autores mis mejores augurios de una obra de trascendental importancia para profesionales de la salud médicos y no médicos, y a los lectores, les garantizo que la lectura y el entendimiento de estos temas les aperturará una visión de las medicina y ciencias biológicas, que antes quizá la sospechaban, pero nunca como con este libro, lo verán tan claro y entendible.

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ARTICLE / INVESTIGACIÓN

Comparative anatomical study to skeleton for same species of Turtles in Iraq

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Abstract: Bones were recorded in the skeleton of some species of Iraqi turtle *Mauremys rivulata*; the objectives of this study came in light of current conditions, environmental developments, talents and techniques of biological studies taking place in the country, need for an anatomy guide in river turtles of Iraqi species, to identify all kinds of similarities and differences with their preaching, this work or study has become written in response to those modern needs. It is designed to be one of the resources for those interested in biological studies, beginners or professionals, and veterinarians, distinguishing them from marine and global species. Turtles were dissected in the laboratories of the Research Center and Museum of Natural History / the University of Baghdad. The specimen was dissected by removing the abdominal cortex, muscles, and internal viscera and imaging the bone starting from the skull to the hind leg bones. This first study was in Iraq.

Key words: Turtles (*Mauremys rivulata*), anatomy, skeleton, bone, Iraq.

Introduction

The history of turtles(*Mauremys rivulata*) goes back to the Jurassic period when they were found about 210 million years ago. They are considered the oldest primitive armored animal with some standard features with the others to this day, and the most prominent thing in common is a bony shell that consists of a group of ribs that fuse to form that apparent solid plate[1]. Turtles are classified into two main types: 1- pleurodires that bend to one side for each head and neck. 2- Cryptodires that pull the head back in a vertical and straight line between their shoulders is known as the movement of the letter S[2]. These river turtles produced many sea turtles that trace their origins to the Cretaceous period, about 65 million years ago. Based on fossils found in different regions of the world[3].

Mauremys rivulata is a turtle that descends from the coasts of Croatia, Albania, and Macedonia with Greece and part of southwestern Turkey, through the Middle East, Iraq, Jordan, and Syria, Palestine, and Lebanon[4]. Its presence in these places is due to the weather and the moderate environment that suits it[5]. *M. rivulata* in static and slow waters such as lakes, irrigation streams, swamps, estuaries, ponds, and even dams are the habitats you prefer to be in rather than fast-flowing water[6,7].

The turtle's body outline is classically represented as a result of morphology and development, as it has a dorsal shield and also there is ossified abdominal support with four legs, two front and two hinds; the nature and shapes of the turtle shields are still under the interest of scientists, the shield is a part of the axial cartilaginous skeleton of the thoracic vertebrae and the ribs with a group. The dermis that overlaps with it, and there are many dermis forms, magnifies its component in this great mosaic[8,9].

The ossification of the skeleton in the skull is typical of vertebrates. However, it is rare after the cranium that the ribs change according to the limbs and the girdles between them, the abdominal or thoracic brace interferes with the thoracic or pelvic girdles in the front and backside of the ribs due to its close association with the dermis. To maintain the position Dorsal, tetrapods are related to the axis of the dorsal girdle ventral deep; in this study, the bony parts will be clarified[10].

Some studies author study the evolutionary inclusion of the turtle's body and how it evolved from the primitive tetrapods to the more typical shape as a result of a developmental conflict between similarity to the structural elements in turtles[11,12].

The skeleton of sea turtles is characterized by heavyweights that reach about a (1ton) and a length of about 2 meters13. Also consists of the dorsal and abdominal supports, axial skeleton, and four legs. The number of bones was found up to (280) bones of different shapes and sizes[14-16].

Turtles have been exploited over the years as they are considered a nutritional value for some coastal areas; the turtle shell has been used in various cultures[17-19]. In comparison, the bones do not attract scientists much attention to their relatively large number with the diversity of their shapes and sizes for the prominent types of turtles with the recording of numbers of bones In some records of archaeological areas[18,19]. The movement of the spine is an essential requirement for every animal. The reason for movement is not to obtain food but because it needs movement to escape predators[20]. The turtle shell is an excellent shield for it because it contributes to protecting the head and neck and the internal viscera, and the head retracts vertically or horizontally laterally inside the shell[21]. A turtle's neck consists

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of eight cervical vertebrae articulated with the skull with a group of complex joints that facilitate movement[22].

Cost Components

- Kingdom: Animalia
- Phylum: Chordata
- Class: Reptilia
- Order: Testudines
- Suborder: Cryptodira
- Superfamily: Testudinoidea
- Family: Geoemydidae
- Genus: Mauremys
- Species: M. rivulata
- Binomial name[23,24].

Materials and methods

Samples were taken from irrigation creek water from Babel and Diyala provinces during the summer season (June- July 2021). Four species of adult turtles *Mauremys-rivulata* (2 ♂, 2 ♀), male weight 660 ± 3.30 g and length of 25 ± 1.39 cm and female weight 560 ± 2.30g and length of 23 ± 1.24 cm, The animal was anesthetized, and then the abdominal shell was removed with anatomical tools. The internal viscera was removed, all parts of the shell and bones were cleaned from the remains of muscles, the bones were extracted for the front and back legs with the head and tail and studied in terms of shape and location and compared to each other[25].

Results

The shell

At the beginning of the study, the female had shells darker in color than the males. The dorsal shells have 40 in number and the ventral ones have 12 in number, while the dorsal shells of the males have 37 and the ventral ones have 11, according to (Fig.1) ., and from here, it was found that the females are more comprehensive to they carry eggs inside them.

The shells were fused to the turtle's spine with a trace of the ribs that may have been fused with the shells for additional protection and support for the animal from dangers as a defense mechanism (Fig.2).

The skull and vertebrae

Is unpretentious in composition, consisting of the temporal bone fused with the orbit of the eye socket and the nasal bone opening. Additionally, the two ear holes and jaw bone discriminate between the upper and lower mandible containing the teeth (Fig.3). The 6 cervical vertebrae are fused with the skull; the straightforward form is fused. The last vertebra is fused with the sternum, collarbone, and dorsal shell. (Fig.4). thoracic vertebrae 8 in number is, fused with the shells. The remains of the ribs and shells are shown in (Fig.5).

The lumbar vertebrae are 4 in a number smaller than the thoracic vertebrae. They also fuse with the dorsal shell. Furthermore, the sacral or coccygeal vertebrae show 3vertebrae fused to form one bone fused with the dorsal shell (Fig.6).

The caudal vertebrae in females were smaller in size than in males. They contain the shape of the vertebrae in the advanced vertebral organism. Their number ranges from 20-22 vertebrae that are not fused with the shells. (Fig.7).

Appendicular skeleton

Forelimbs, the scapula is a flat bone showed that does not contain the protrusion of the scapula and articulation with the humerus bone, which was more prominent in males than females, and the presence of the radius and ulna; these two bones were relative in shape to each other, but the radius bone was thicker than the ulna (Figs 6,8, and 9).

It articulates with the carpal bone, which is 10 tiny bones in each of the anterior vertebrae, fused and articulating with the phalanges (Fig.9).

The fingers in both sexes were 5 in the front limbs and 4 in the hind limbs, which were longer than the front fingers and contained claws, hind limbs, The femur bone, which articulates with the pelvic bone on the proximal side, and articulates with the tibia and fibula on the distal side, the tibia, and fibula, in two sexes tibia thicker than fibula but male thicker than female and those two bones. Articulation with 5 small metacarpal bones fused together and connected with phalanges and fingers (Fig.10 and 11).

The pelvic bone in females is more comprehensive than in males due to the presence of the ovary and the female reproductive organs. It contains three fused bones that make up the pelvis: ischium, ilium, and pubis. (Fig. 7).

Discussion

Auther used almost all sea turtles - including marine ones belonging to the family Cheloniidae –to have a shell consisting of a dorsal and ventral shield, and the material that does not consist of it is carnelian brown in color[26]. Like river turtles with different colors, they tend to the dorsal shell to dark brown color in males and females, while the shell The abdomen is dark green. The name of the tortoiseshell in the literature came (ar chae o log i cal and eth no Graph ic). It is an unclear term referring to the shell in the dorsal and ventral parts, considered a carotenoid shell or bony horn. In many cases, this is clear from context[27]. The osteology section of this atlas is The bones of the skull (prefrontal, frontal, parietal, postorbital, supraoccipital, squamosal, quadratojugal, jugal, and maxilla) and mandible (dentary, angular, surangular, prearticular, splenial, and articular bones) while.

The results observed that the skull had undifferentiated fused bones consisting of the temporal bone fused with the orbit of the eye socket and the nasal bone opening, the two ear holes, and the discrimination of the upper and lower mandible containing the teeth[28]. The observed author explained that the neck is long for evasion and capture of prey, consisting of cervical vertebrae that help it to move freely, crouching inside the body when sleeping and feeling fear, comparable to what was explained[29,30]. The neck is long in all species, whether aquatic or semi-aquatic. Con-

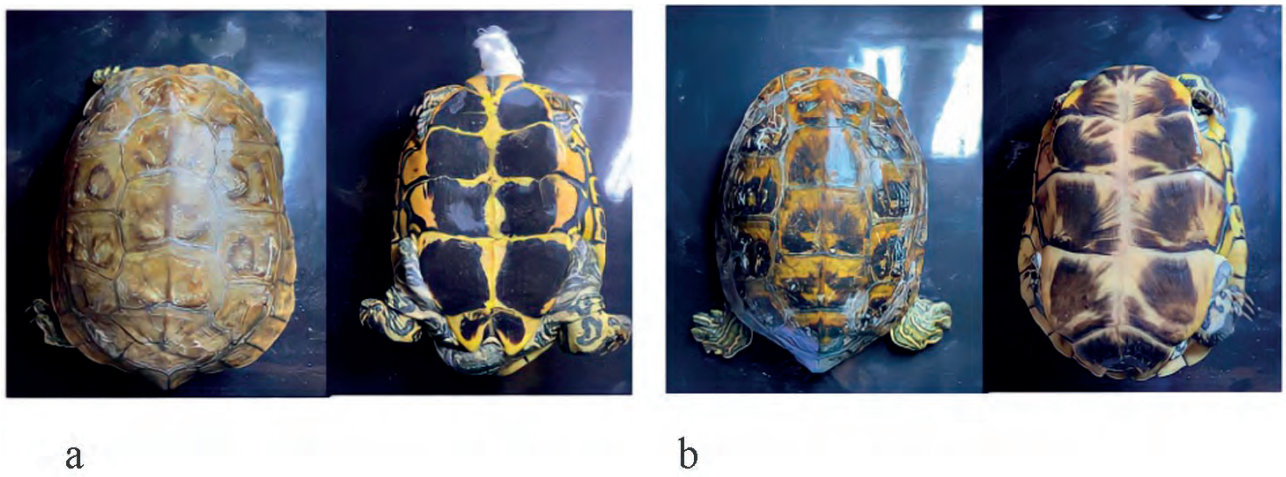


Figure 1. Show dorsal and ventral (a) male and (b) female shall.

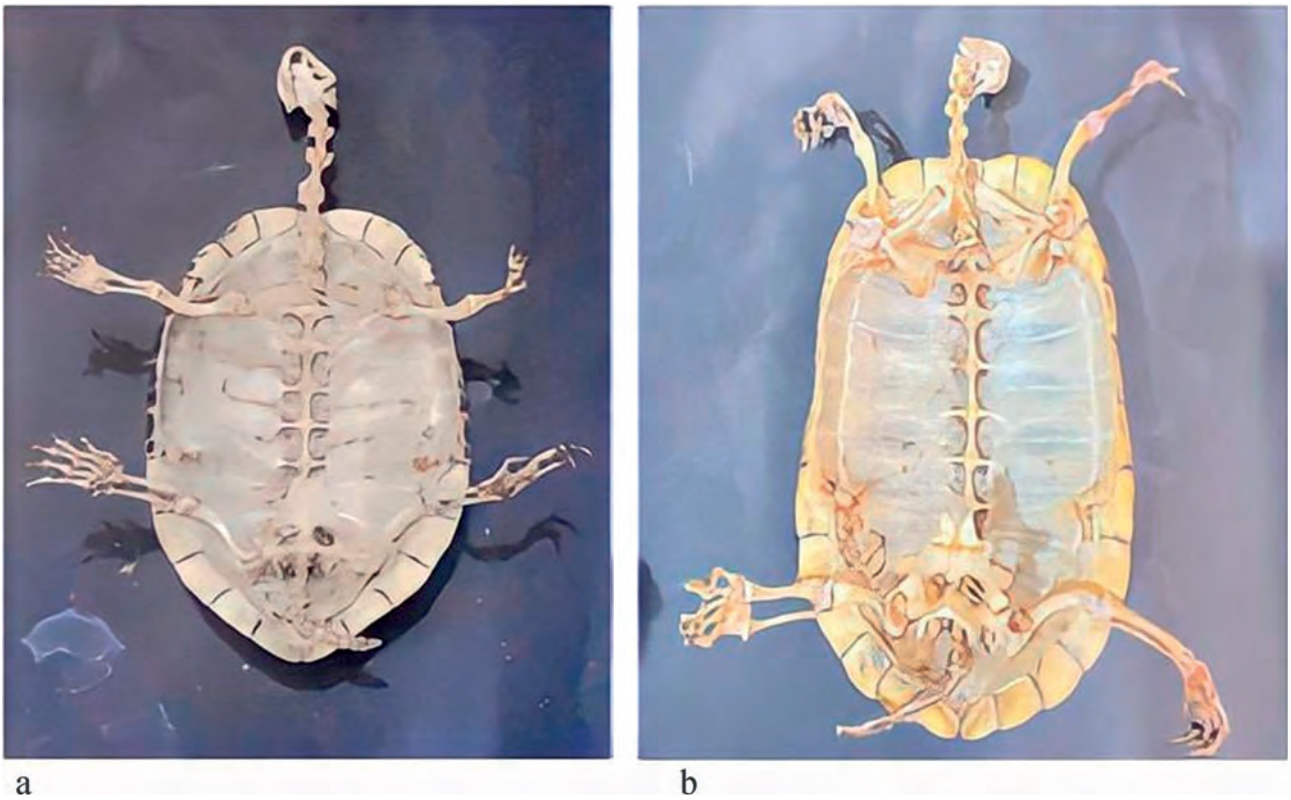


Figure 2. Show ribs fused with shall in (a) male and (b) female shall.

trol of the neck is crucial for turtles as it is multi-joint with a complex structure consisting of developed vertebrae to the neck, body, and head of the vertebra are articulated to make them more accurately and efficiently and control their freedom during movement during rapid movements in proportion to Its nature, and this is indicated by (22) where this development of the cervical vertebrae is proportional to the accompanying muscles that allow them to move vertically or horizontally river turtles in Iraq are little studied. However, cervical vertebrae were found in all types of turtles that supply eight rectangular vertebrae (C1 to C8) and nine joints. observed[31].

Observed appendicular skeleton in their study, there were some similarities in the skeleton of the thoracic and pelvic girdle and the fore and hind limbs in the reptiles studied; *P. guttatus* and *A. boskianus*[32]. With the presence of incommodious differences in the length of the angled ends of the bones in the thoracic and pelvic, which is similar at the same time to river turtles with fewer bones in size and thickness, a similarity was found in terms of the presence of double wishbones on the non-double-bone river turtles are more developed than them[33]. The skeleton of the forelimbs of the same studied, which consists of the humerus, radius, ulna, and metacarpal bones, was observed, and it is similar to the river turtles studied in this research, and it is connected with the thoracic girdle, and in general, it is identical to mammals and birds The bone radius was a long bone articulated with the carpal uncle and ulna as in turtles, while the ulna was more prominent and thicker in sources, unlike river turtles, it was a slight difference in thickness. Studies of reptiles assured *P. guttatus* and *A. boskianus* having a pelvic girdle consisting of the pubic, iliac, and ischium; It fused and met the acetabulum, and they left a wide foramen (fenestra ischio-pubic fenestra). There was a clear difference in the

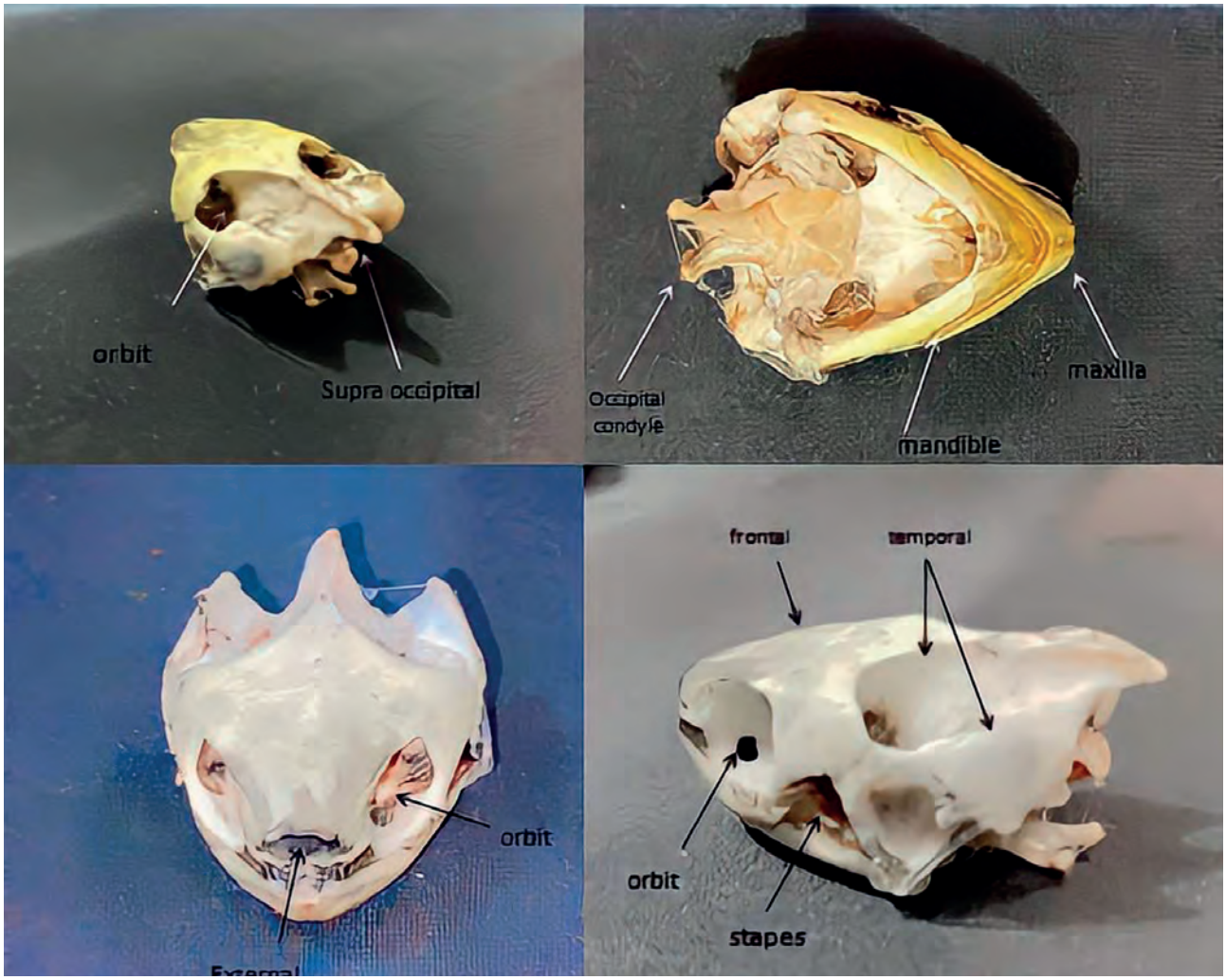


Figure 3. Show skull in the two species the same.



Figure 4. Show the occipital condyle articulation with 1st cervical vertebrae

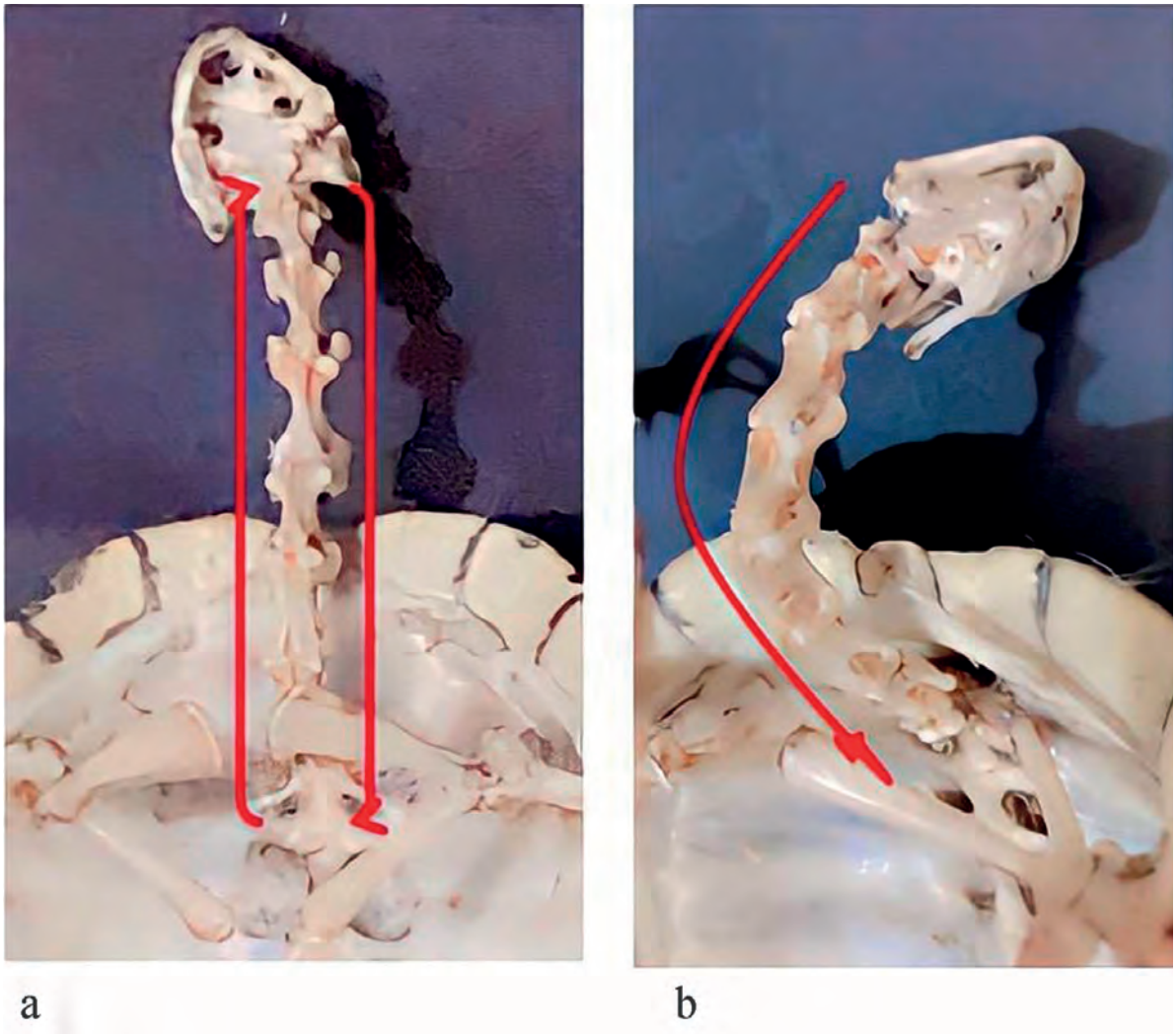


Figure 5. Show cervical vertebrae (a) male (b) female.



Figure 6. The black arch show thoracic vertebrae fused with ribs and shall ,red circle the pelvic bone (ileum +ischium ,pubis , red arrow its scapula ,black arrow the clavicle bone blur arrow the coracoids bone.

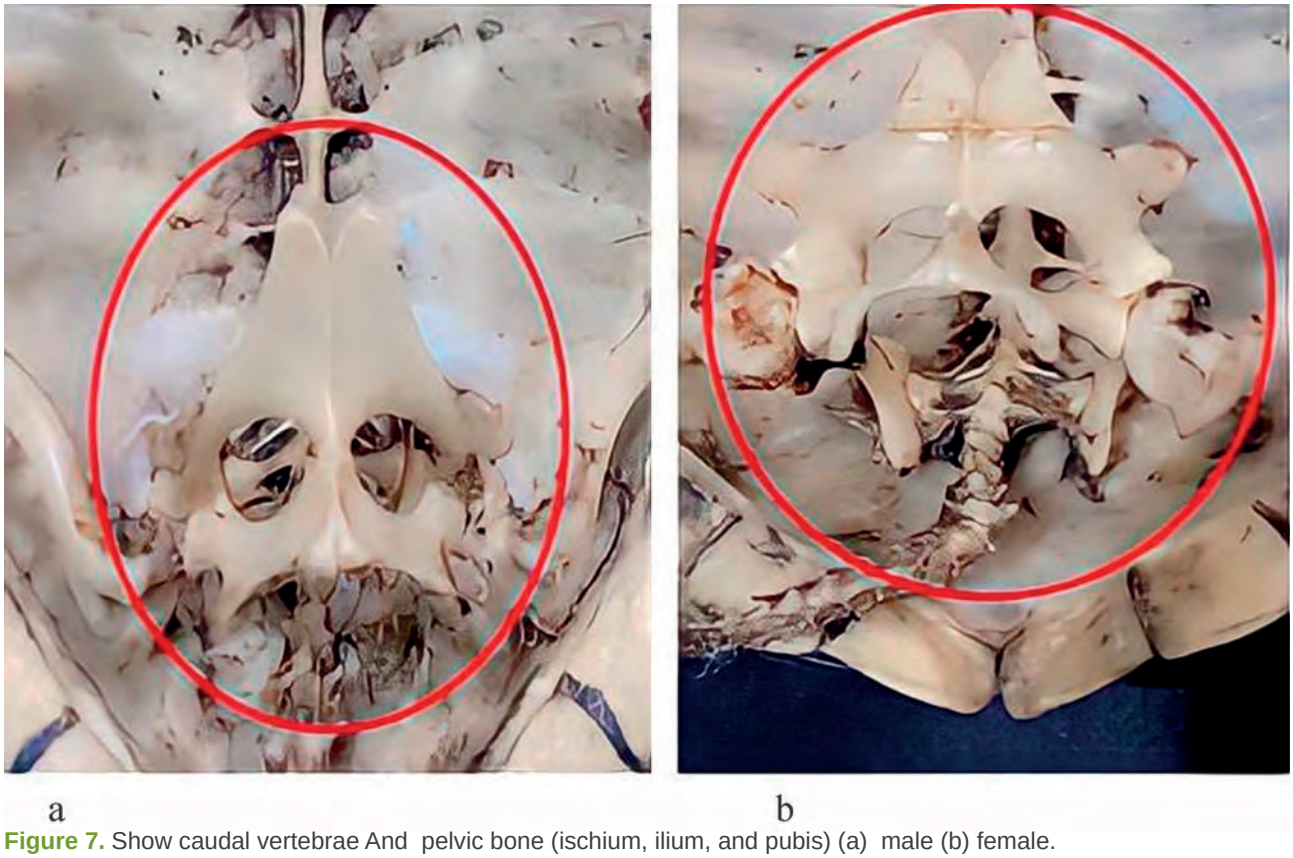


Figure 7. Show caudal vertebrae And pelvic bone (ischium, ilium, and pubis) (a) male (b) female.

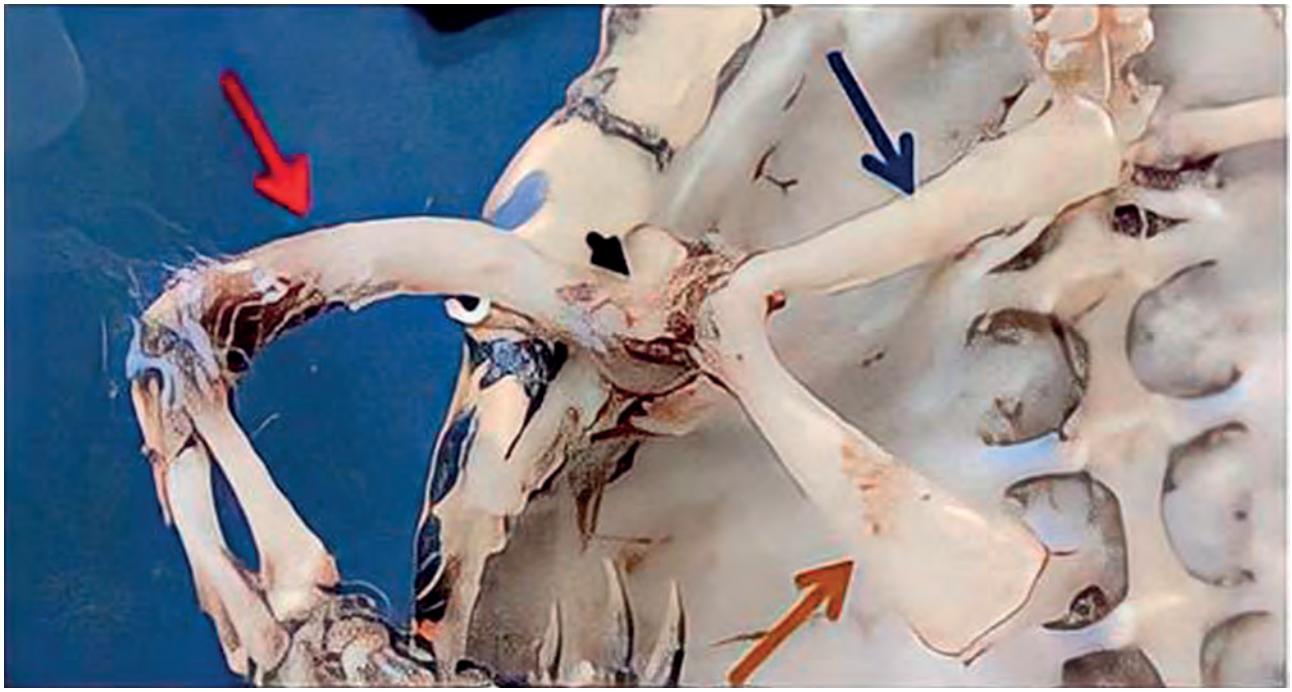


Figure 8. Show forelamb red arrow humerus bone ,black head arrow head of humerus ,brown arrow scapula and blue arrow the clavicle bone.

shape of the pubis, ischium, and iliac of the two species studied with river turtles in terms of size and thickness with pelvic bones[32,34].

The study contained the tibia and fibula of approximately equal length but shorter and thinner than the femur and also noted that the tibia is more straight than the fibula and articulated with the femur by the tibial condyle of the femur. The distal part is articulated with the heel bone and is analogous to the study of a developed vertebrate[35].

The phalanges and their total number in the anterior and posterior extremities, respectively (manus: 2-3-3-3-2, pes: 2-3-3-3-1). It is similar to river turtles except that the back end contains five digits, only in *C. flavomarginata*, it is also found with a missing phalanx in the 5 digit of manus and pes; They also appear in species in the pes of *C. mouhotii*. In *C. flavomarginata*, the fifth digit of pes is also missing.

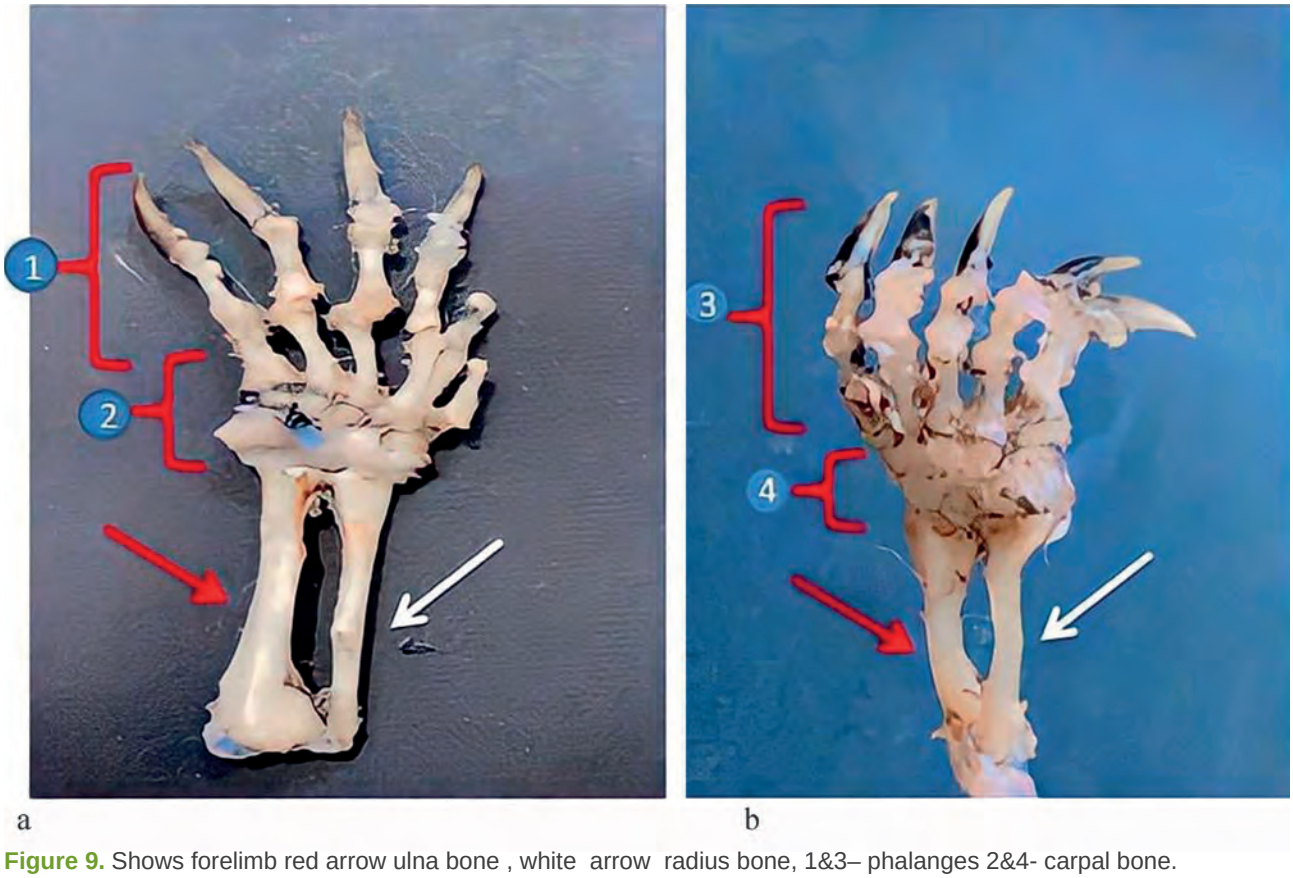


Figure 9. Shows forelimb red arrow ulna bone , white arrow radius bone, 1&3– phalanges 2&4- carpal bone.

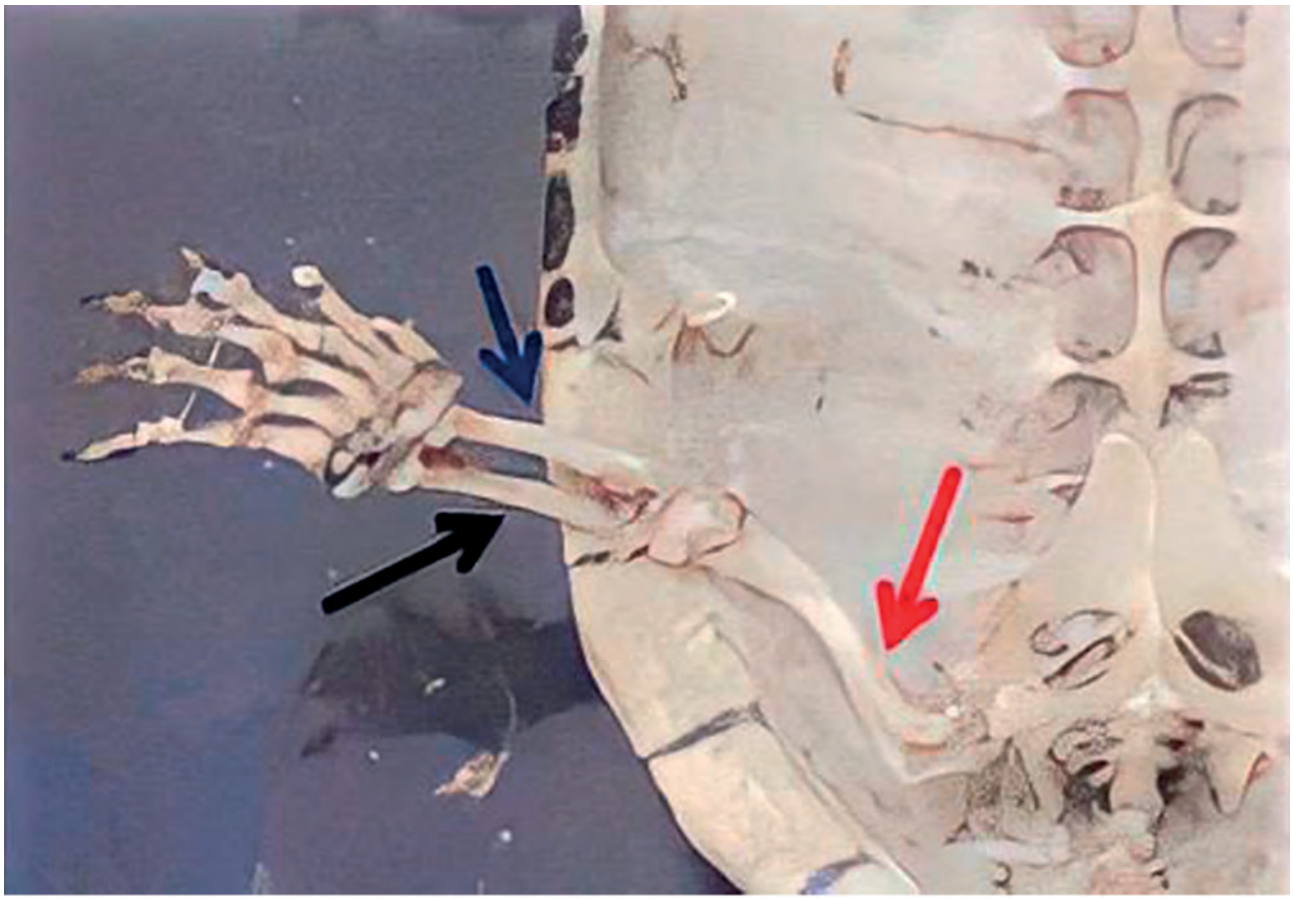


Figure 10. Show hind lamb red arrow femur bone, black arrow tibia bone, blue arrow fibula bone.

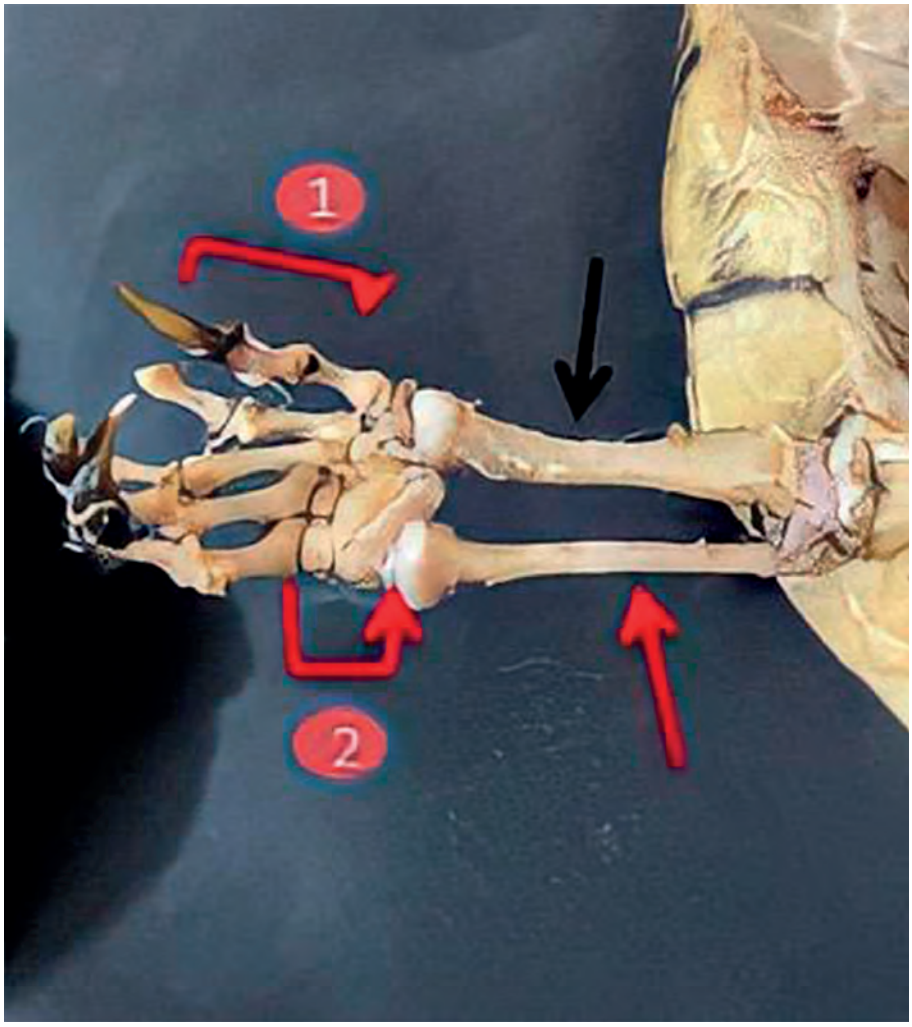


Figure 10. Red arrow tibia bone, black arrow fibula bone.
1- Phalanges
2- Metacarpal (tarsal) bone

Due to changes in some other terrestrial and semi-terrestrial geological materials (*Cuora mccordi*, *Heosemys spinosa*, part *C. mouhotii* and *Leucocephalon yuwonoi*) they also took a similar pattern which led to the disappearance of the fifth finger of pes. It is also found in turtles (Testudinidae), and in another group of terrestrial Chelonians, and land turtles of the genus Terrapene (Emydidae); missing phalanxes or whole digits are known to be found in the species *Malayemys subtrijuga*, *Morenia petersi*, *Pangshura smithii*, and *Siebenrocki* that have been studied where The finger found the fifth digit of the manus(2-3-3-3-3); on one side of the body there are phalanxes of P. 3-3) These are aquatic turtles came similar to the study of river turtles[36].

Since there are few studies of river turtles, the writer discussed the jaw with reptiles, and as it is known, their lower jaw consists of several bones. Cuvier gave them a descriptive name in line with the jaws of crocodiles, turtles, lizards, and snakes in the order named with slight differences in turtles

In (37) the wishbone was not distinct or was small in the thoracic girdle, while in river turtles, the wishbone is found clearly and distinctly. It has thick and convex borders.

Conclusions

This study observed the skeleton of Iraqi river turtles in general and is considered the first study in Iraq. The simplicity of the composition concerning the skull is obvious, with the development of the limbs and their differentiation and difference from sea turtles in the presence of claws, and it required a separate study for each part of the skeleton.

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