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Article

Efflux Pumps, Biofilm Formation, and Susceptibility Testing of Escherichia Coli Isolated from Urinary Tract Infection

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Abstract
Urinary tract infections (UTIs) are infectious diseases of the urinary system caused by several causative agents, including parasites, viruses, fungi and bacteria. The most frequent UTI cause is Escherichia coli (E.coli). Antibiotic resistance in E. coli has been linked to overexpression of the efflux system. This study aimed to isolate various bacteria from UTI and then select E. coli isolates to study the prevalence of the efflux pump genes TetA and MdfA.This study included 150 midstream urine samples from patients suffering from UTI (115 females and 35 males) with ages ranging between(5-70)years. The results showed that only 100 samples exhibited bacterial growth; 72.5% referred to female patients, while 27.5% referred to male patients. Infection with bacteria occurred most frequently in the age group of 21-30 years. Bacterial isolates were identified by macroscopic and microscopic examination, biochemical test and VITEK2 system. The result showed that 40% of these growth were confirmed to be E.coli,19% Klebsiella pneumonia,17% Staph.aureus, 13%Proteus mirabilis,7% Pseudomonas aeruginosa, 2% Staph. saprophyticus,1%Proteus vulgaris and 1%Enterobacter cloaca.The results of sensitivity to antibiotics showed that UPEC isolates were utterly resistant to novobiocin and rifampin 100%, ampicillin 87.5%, cefotaxime 85%, tetracycline82.5%, ciprofloxacin77.5%, trimethoprim-sulfamethoxazole 50%, gentamicin 22.5%, nitrofurantoin 17.5% and meropenem 2.5%. All of the isolates were multidrug resistant. The result of the biofilm-formation ability of E.coli isolates showed that 31/40(77.5%) of isolates producing biofilm were divided into three groups: 1 (2,5%) had strong biofilm formation, 4(10%) were moderate, and 26(65 %) were weak. The phenotypic detection of the efflux pumps was observed in 100% of the bacterial isolates at a concentration of 0.5 mg/l of ethidium bromide(ETBR). The prevalence of the TetA and MdfA efflux pump genes was 72.5% for each. The gel electrophoresis showed that the molecular weight of TetA and MdfA genes were 131bp and 403bp respectively.

Keywords: Efflux pumps, Urinary Tract, Infection, Parasites, E-coli.

Introduction

UTI is one of the most frequent bacterial infections worldwide, impacting millions of people of all ages each year[1]. Following respiratory tract infections, they are the second most prevalent bacterial infections[2]. Furthermore, numerous factors lead to UTIs, including urinary tract abnormalities, age, diabetes, immunocompromised individuals, sexual activity and past UTI therapy(bacterial resistance)[3]. AUPEC is the most common cause of UTI in all age groups because it has various virulence

factors such as adhesions, toxins, polysaccharide capsules, lipopolysaccharides and iron acquisition[4].E.coli is a member of the Enterobacteriaceae, gram-negative bacteria, motile or non-motile, rod-shaped, lactose fermentative and facultative anaerobic[5,6]. Usually, a UTI is treated without an antibiotic sensitivity test to determine the best antimicrobial agent[7]. The use of random therapy for a long time led to the emergence of antimicrobial-resistant bacteria that made the treatment of UTI infection difficult[8]. E.coli uses different mechanisms to resist different groups of antibiotics, including alteration of cell membrane permeability, target modification, alteration of metabolic pathways, production of enzymes and efflux pumps[9]. Efflux pumps are membrane proteins found in all gram-positive and gram-negative bacteria and transport toxic materials such as antibiotics, detergents, toxins, dyes, and waste metabolites out of bacterial cells. Genes of efflux pumps are found in bacterial chromosomes or mobile genetic elements, such as plasmids[10]. There are five major efflux pumps: RND (resistance – nodulation-division), MFS (major facilitator superfamily), MATE(multidrug and toxic efflux), SMR(small multidrug resistance) and ABC(ATP binding cassette)[11]. Depending on the source of energy, efflux pumps are further categorized into primary transporters, such as (ATP-binding cassette (ABC)transporters) that utilize ATP hydrolysis as an energy source and secondary transporters, which utilize the energy stored in the transmembrane electrochemical gradient such as(MFS, RND, MATE and SMR)transporters[12]. Two transporters, MdfA and tetA, which belong to the major facilitator superfamily, are linked to multidrug resistance (MDR) in E. coli when their genes are overexpressed[13].

Tetracyclines are antibiotics with a broad spectrum of action commonly used to treat the most common gram-positive and gram-negative bacterial infections. Tetracycline efflux pumps, such as TetA and TetB, are responsible for most resistance to tetracycline in E.coli[11].

MdfA is a member of the major facilitator superfamily, which confers resistance to a wide range of harmful chemicals such as fluoroquinolones, erythromycin, chloramphenicol, aminoglycosides tetraphenylphosphonium, ethidium and rhodamine. MdfA has a second physiological function in pH regulation because of its activity as an H+/Na+, K+ antiporter[14].

Materials and Methods

Sample collection

A total of (150) midstream urine samples were collected from UTI patients attending the different hospitals in Kirkuk/Iraq, in the age groups (5 to 70) years from November 2021 to March 2022.

Bacterial Identification

The midstream urine samples were inoculated on Blood agar, Eosine Methylene Blue agar and MacConkey agar and incubated at 37℃ for 24 hours. Then, the isolates were identified using macroscopic examination according to their color, size, shape and type of hemolysis. Further identification was made by microscopic examination (Gram stain), biochemical tests including catalase test, oxidase test, urease test, and IMVIC test including (indol, methyl red, Voges-Proskauer and citrate).In addition, E. coli isolates were confirmed by the vitek-2 system (bio-Mérieux, France)[15].

Antimicrobial Susceptibility Testing

40 E. coli isolates were tested for susceptibility to 10 antibiotics by using the Kerby-Bauer method. These antibiotics include Novobiocin, Rifampin, Ampicillin, Cefotaxime, Tetracycline, Ciprofloxacin, Trimethoprim-Sulfamethoxazole,

Gentamycin, Nitrofurantoin, and Meropenem and the results were compared with the national committee for clinical laboratory standards (CLSI 2019) and reported only as resistant, intermediate, or sensitive[16].

Detection of Biofilm using microtiter plate method

The microtiter plate method was used to detect the formation of E. coli biofilm. The biofilm formation was identified by crystal violet staining, whose color intensity is directly related to biofilm concentration. An Enzyme-Linked Immunosorbent Assay (ELISA)was used to measure the absorbance at 630nm[17].

Phenotypic detection of Efflux pumps using Ethidium Bromide (EtBr) agar cartwheel method

The EtBr cartwheel method[18] tested the efflux of EtBr.Bacterial isolates were cultured overnight at 37℃, and the next day, the cell concentration was adjusted to 0.5 of a McFarland standard. The tryptone soya agar containing different concentrations of EtBr (0.5,1,1.5,2,2.5) mg/L, was divided by radial lines, making a cartwheel pattern. The bacterial isolates were then swabbed with a sterile cotton swab and incubated for 16h at 37℃. The colonies on tryptone soya agar plates were examined under an ultraviolet(UV) transilluminator.

DNA extraction

E. coli isolates were grown overnight at 37℃, and DNA was extracted using a genomic DNA purification kit supplemented by the manufacturing company (Geneaid, Taiwan).DNA was purified and its purity was measured using nanodrop (AcTGen, Taiwan).

Molecular detection of efflux pumps

Efflux pump genes (TetA and MdfA) in UPEC isolates were detected using a polymerase chain reaction (PCR) assay. The extracted DNA was used as a template for PCR amplification of Uropathogenic E.coli TetA and MdfA genes with two separate primer pairs (Bioneer, Korea). TetA: F:5-ATCATGGTCCTGCTTGCTTC-3 and reverse primer R:5-ATCGAGGTCAGGCTGGTG-3, MdfA: F-5-CCTATTTTCGGGGCGTTAAT-3 and reverse primer R:5-TAACCATCAGCGACAGCAAC-3, PCR reaction took place in the total volume of 25µL, the reaction mixture was contained 5 µL of AccuPower®PCR premix (Bioneer, Korea),5 µL template DNA,1 µL F-primer,1 µL R-primer and 13 µL of deionized nuclease-free water (Bioneer, Korea). The conditions used for PCR assay are as follows: 5 minutes of initial denaturation at 95℃ (1 cycle) followed by denaturation of DNA at 95℃ for 30 seconds, annealing for TetA and MdfA gene 58℃ and 57℃ respectively, extension at 72℃ for 40 seconds(35 cycles) and final extension at 72℃ for 5 minutes (1 cycle).

Gel Electrophoresis

The PCR products were migrated by 1% agarose gel electrophoresis with 2 µL of ethidium bromide in 1X (TBE) buffer using a DNA ladder (100-1000)bp (Bioneer, Korea) at 90 volts for 80 minutes. The DNA was examined and photographed using a UV Transilluminator[19].

Results

The study revealed that the infection distribution was 72.5% in female and 27.5% in male patients. The higher incidence of infection in females compared to males may be attributed to anatomical reasons, as female urinary tracts are shorter and broader compared to males, which allows bacteria to enter and remain in the urethra and bladder. The distribution of bacterial agents associated with UTIs in the study is visualized in Table 1 as follows: E.coli 40%, K. pneumonia 19%, S. aureus 17%, P.mirabilis 13%, P. aeroginosa 7%, S.saprophyticus 2%, P.vulgaris 1% and E.cloaca 1%.

Bacterial isolates	No.of samples	percentage
E. coli	40	40%
K.pneumonia	19	19%
S.aureus	17	17%
P.mirabilis	13	13%
P.aeroginosa	7	7%
S.saprophyticus	2	2%
P.vulgaris	1	1%
E.cloaca	1	1%
Total	100	100%

Table 1. Prevalence of bacterial isolates in the study.

E. coli is the most common isolate. The results of these studies agree with previous studies of [6, 20], which revealed that E. coli formed 35% and 37.82% of the organisms that were isolated in the urine culture. On microscopic examination, all E.coli isolates appeared gram-negative rod and non-spores forming bacteria, pink color(lactose fermenter) on MacConkey agar, dark-blue with green metallic sheen on (EMB), and off-white or beige color on blood agar, while biochemical tests revealed that all E.coli isolates were positive to catalase, indol, methyl-red while negative to citrate utilization, Voges- Proskauer and urease tests, and E.coli isolates were acid/acid no H2S with gas production on Kliglller iron agar[21].

Antibiotic susceptibility of E.coli

In the antibiotic sensitivity test, E.coli isolates showed 100% resistance to novobiocin and rifampin, ampicillin 87.5%, cefotaxime 85%, tetracycline 82.5%, ciprofloxacin 77.5%, trimethoprim-sulfamethoxazole 50%, Gentamycin, nitrofurantoin and meropenem showed the lowest resistance as 22.5%, 17.5% and 2.5% respectively as shown in (Table 2).

Gandra et al. [22] were consistent with our results; they found that isolates of E.coli resisted meropenem at a rate of 8.11%, while the current study differs from the study of Gandra et al. [22], which found the resistance to this antibiotic was 76.7%. The study of Odongo et al. 23 showed that E. coli is resistant to nitrofurantoin at a rate of 30%. In contrast, the present study for resistance to this antibiotic differed from the study of Tajbakhsh et al. 24, which found that the resistance percentage to this antibiotic was 2%. The results of our study for the E.coli resistance of Gentamycin were in agreement with the results of Al-Saadi et al. 25. They showed the resistance percentage of Gentamycin was 30%; our results were in disagreement with the result of Al-Najar et al. 6, which showed the resistance percentage to this antibiotic was 80%. Our study showed high resistance to novobiocin and rifampin and agreed with the result of Avşar and Berber [26], which showed that E. coli resistance to novobiocin was 100%.

In contrast, this disagreement with the study of Al-Saadi et al. 25, which found the percentage resistance of E.coli to this antibiotic was 74% and agreement with her study about Rifampin, which showed that the resistance of E.coli to this antibiotic

was 94%. Our study also showed a high resistance to *β*-lactam antibiotics such as Ampicillin and the third generation of cephalosporin (Cefotaxime). These results were in agreement with the result of Jamil et al. 27, which showed that the percentage resistance of E.coli isolates to this antibiotic was 100%; Sah et al. [28] described that the E.coli resistance *β*-lactam antibiotics by the production of β-lactamase enzymes like CTX-M and TEM, while the present study for the resistance rate of these two antibiotics was different from the study of Avşar and Berber [26] which found that the resistance percentage to Ampicillin was 12.5% and the study of Hamada et al[29] which showed that the resistance percentage to Cefotaxime was 33.3%. Furthermore, the current study revealed that 82.5% of E. coli isolates resisted tetracycline. These results were in agreement with the results of [6], which showed that the percentage resistance of E.coli to this antibiotic was 85.7%, a higher resistance rate due to the bacteria's having antibiotic resistance genes TetA and TetB, which are carried on plasmids and are responsible for these resistance rates.

The results of our study for E.coli resistance to Ciprofloxacin agreed with the results of Odongo et al. 23, which found that the resistance percentage to Ciprofloxacin was 90%. In contrast, the present study for resistance to this antibiotic differed from the previous studies [6, 25], which found the percentage resistance of E.coli to this antibiotic was 57.2% and 52%, respectively. The resistance of E.coli to this antibiotic is due to the bacteria's efflux pumps (MdfA, AcrAB and YhiV), change in the cell membrane permeability, and alteration of target location[30]. Finally, resistance to Trimethoprim-Sulfamethoxazole showed that 50% of the isolates were resistant. This result was in agreement with the result of Ferdosi-Shahandashti et al. 31, which showed that the percentage resistance of E.coli to this antibiotic was 63%, while our results were in disagreement with the results of Tajbakhsh et al. [24], which found the resistance rate of E.coli to this antibiotic was higher 71.25%.

Antibiotic	Sensitive	Intermediate	Resistance
Novobiocin	0	0	100%
Rifampin	0	0	100%
Ampicillin	12.5%	0	87.5%
Cefotaxime	10%	5%	85%
Tetracycline	12.5%	5%	82.5%
Ciprofloxacin	20%	2.5%	77.5%
Trimethoprim-Sulfamethoxazole	42.5%	7.5%	50%
Gentamycin	67.5%	10%	22.5%
Nitrofurantoin	72.5%	10%	17.5%
Meropenem	92.5%	5%	2.5%

Table 2. Uropathogenic E.coli susceptibility pattern.

Biofilm-forming ability of E.coli isolates

A total of 40 E.coli isolates from UTI,31/40(77.5%) of isolates producing biofilm were divided into three groups based upon the 〖OD〗_630 of bacterial biofilm formation: 1(2.5%) was strong biofilm formation, 4(10%) were moderate biofilm formation, and 26 (65%) were weak biofilm formation as in Figure 1.

The previous studies of [32] [33] for the biofilm formation ability of E.coli showed that the biofilm production percentage (84.6%) and (93.7%), respectively, were compatible with the current study. In contrast, the result of González et al. 34 showed that the biofilm production rate was 41.4%. This study found that UPEC isolates have a high propensity to form a biofilm(more than 75%). Also, most studies in the present review showed a significant correlation between biofilm formation with virulence factors and antibiotic resistance.

The Materials and Methods should be described with sufficient details to allow others to replicate and build on the published results. Please note that the publication of your manuscript implies that you must make all materials, data, computer code, and protocols associated with the publication available to readers. Please disclose any restrictions on the availability of materials or information at the submission stage. New methods and protocols should be described in detail, while well-established methods can be briefly described and appropriately cited.

Research manuscripts reporting large datasets deposited in a publicly available database should specify where the data have been deposited and provide the relevant accession numbers. If the accession numbers still need to be obtained at submission, please state that they will be provided during review. They must be provided prior to publication.

Interventional studies involving animals or humans and other studies that require ethical approval must list the authority that provided approval and the corresponding ethical approval code.

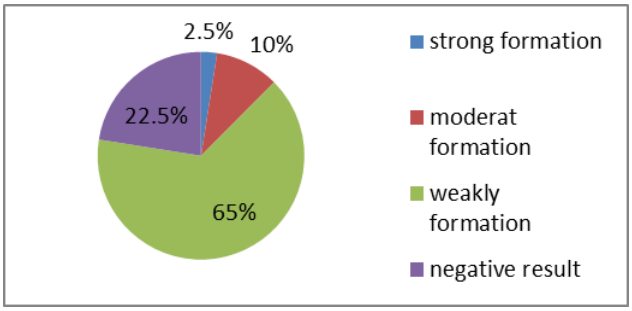

Figure 1. Biofilm formation rate by E.coli isolates.

Phenotypic detection of efflux pumps

The EtBr-agar cartwheel method is used for phenotypic detection of efflux pumps in 40 E.coli as in(figure 2.). The fluorescent growth refers to no efflux, while no fluorescent growth refers to the isolates having active efflux pumps[18]. Our study showed that all isolates (100%) do not show fluorescence at a concentration of 0.5 mg/l of EtBr (positive for phenotypic detection of efflux pumps). In contrast, they showed fluorescence at a concentration of (1,1.5,2,2.5)mg/l of EtBr.Our study's results agreed with the result of [35] while differing from the result of [25], which found that only 70 % of E. coli isolates were positive for phenotypic detection of efflux pumps. The presence of efflux pumps in bacteria is closely associated with antibiotic resistance. Efflux pumps can expel many antibiotics and other toxic substances from cells.

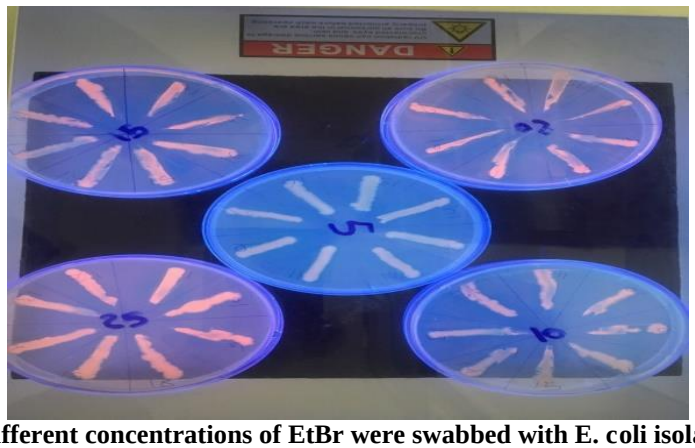

Figure 2. Tryptic soy agar plates containing different concentrations of EtBr were swabbed with E. coli isolates.

Detection of Antimicrobial Resistance Genes: TetA gene

TetA gene detection was performed by using the PCR technique. A total of 40 extracted DNA samples were used for screening the TetA gene. The results showed that only 29(72.5%) isolates had the TetA gene (figure 3). These results agreed with the study carried out by Kadhum et al[37] which showed that 77.4% of E.coli isolates had this gene and described how the bacteria resist tetracycline by three mechanisms, including modifying the ribosome to prevent adequate tetracycline binding, limiting tetracycline from reaching the ribosomes and releasing tetracycline-inactivating enzymes. Limiting the reach of tetracycline into ribosomes may reduce the intracellular concentration of tetracycline by pumping antibiotics out of the cell. A study by Boroumand et al.[38] showed that 59.7% of E. coli isolates carried the TetA gene.

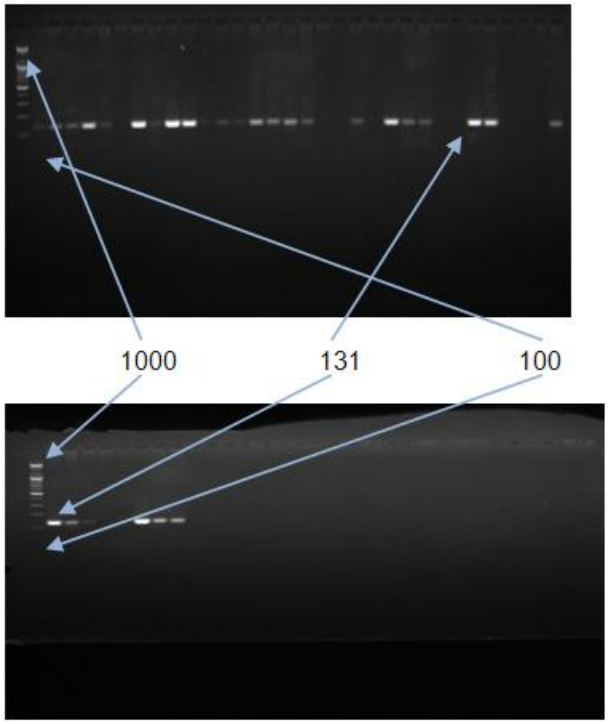

Figure 3. Agarose gel electrophoresis of TetA genes. The product size is 131bp.

MdfA gene detection was performed by using the PCR technique. From 40 E. coli isolates, only 29(72.5%) of isolates carried the MdfA gene, as in (figure 4). In our study,5% of the ciprofloxacin-resistant isolates did not have mdfA overexpression. Thus, fluoroquinolone resistance could be attributed to alternative mechanisms such as topoisomerase IV or gyrase mutation. This result agreed with another study

by [13], which showed 82.1% of E.coli isolates carried the MdfA gene, While the current study disagreed with the result of [39], which showed 34.4% of E.coli isolates carried the mdfA gene. They suggested that many factors can lead to overexpression of efflux pumps, such as gender, indwelling urinary catheter, hospitalization and previous use of fluoroquinolones or other antibiotics.

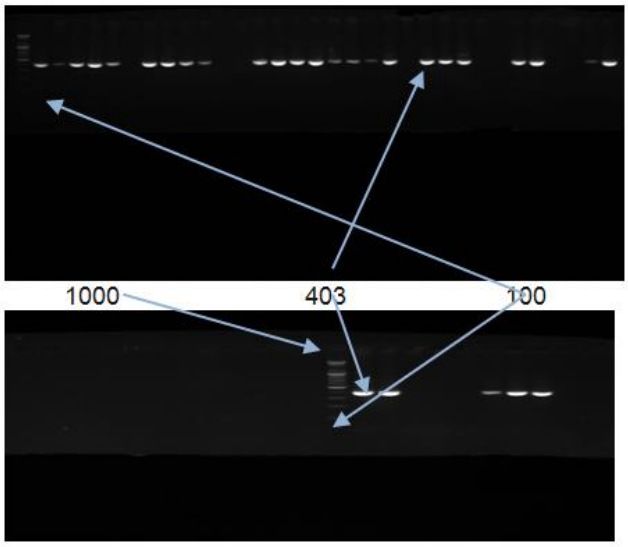

Fig 4. Agarose gel electrophoresis of MdfA gene. The product size is 403bp.

Discussion

UTI is more common in females than males. The most common bacterial infection in UTI patients was E. coli. Meropenem, Nitrofurantion and Gentamycin showed the highest E.coli sensitivity. This study reveals the prevalence of MDR E. coli mediated by efflux pumps among UPEC. 100% of E. coli isolates were positive for phenotypic detection of efflux pumps. The percentage of E. coli isolates having efflux pumps and biofilm formation was 77.5%. This is owing to efflux pumps critical function in biofilm formation. They may contribute to biofilm formation by importing or exporting essential chemicals to biofilm formation[40]—the significant correlation between biofilm formation and E.coli MDR, which results in chronicity of urinary tract infection[41]. MDR E. coli is on the rise due to incorrect antibiotic use, so choosing the right antibiotics is critical to avoiding the emergence of drug-resistant bacteria.

Conclusions

The results of sensitivity to antibiotics showed that UPEC isolates were utterly resistant to novobiocin and rifampin 100%, ampicillin 87.5%, cefotaxime 85%, tetracycline82.5%, ciprofloxacin77.5%, trimethoprim-sulfamethoxazole 50%, gentamicin 22.5%, nitrofurantoin 17.5% and meropenem 2.5%. All of the isolates were multidrug resistant. The result of the biofilm-formation ability of E.coli isolates showed that 31/40(77.5%) of isolates producing biofilm were divided into three groups: 1 (2,5%) had strong biofilm formation,4(10%) were moderate, and 26(65 %) were weak. The phenotypic detection of the efflux pumps was observed in 100% of the bacterial isolates at a concentration of 0.5 mg/l of ethidium bromide(ETBR). The prevalence of the TetA and MdfA efflux pump genes was 72.5% for each. The gel electrophoresis showed that the molecular weight of TetA and MdfA genes were 131bp and 403bp, respectively.

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