

Article

Isolation and identification of some types of histamine-producing bacteria from shrimp in local markets

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ABSTRACT

Different genera and species of bacteria were isolated from shrimp, which were brought from the Al-Faw area in the Basra governorate, and these isolates were diagnosed after purification through studying their phenotypic characteristics and conducting API confirmation, biochemical tests, motility examination and staining with gram stain, in addition to using different types of diagnostic tools bacteria. Twenty-five bacterial isolates of genera and species of bacteria were isolated and diagnosed from shrimp. *Morganella morganii* sub. *Morganii*, *Klebsiella pneumonia* sub. *Pneumonia*, *Proteus marina*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, *Proteus mirabilis*, *Proteus vulgaris*, *Enterobacter tailored*, *Cronobacter sakazakii*, *Escherichia coli*, *Hafnia alvei*, *Citrobacter amalonaticus*, *Citrobacter freundii*, *Vibrio harveyi*, *Vibrio vulnificus*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Staphylococcus intermedius*, *Staphylococcus saprophyticus*, *Pseudomonas fluorescens*, *Bacillus subtilis*, *Bacillus licheniformis*, *Staphylococcus epidermidis*, *Aeromonas sobria*. The results of the detection of histamine-producing bacteria, which were detected by using broth media such as Histamine decarboxylase broth (HDB), Trypticase Soy broth-histidine (TSBH) and solid media such as Histamine decarboxylase agar (HDA) showed that all bacteria were producing histamine in different proportions except *Aeromonas sobria* and *Hafnia alvei* were non-histamine-producing.

Keywords: histamine, shrimp, bacterial contamination

INTRODUCTION

Thousands of people suffer yearly from food poisoning by foods and drinks contaminated with bacteria, viruses or toxins. They may show symptoms such as high temperature, diarrhea, vomiting and digestive disorders. The foods most often associated with food poisoning are meat and poultry products, unpasteurized milk and dairy products, eggs, uncooked shellfish, fish and rice ¹.

Fish is considered more perishable than red meat due to its high moisture content and its content of protein and carbohydrate compounds, as well as minerals and salts. Its pH represents a favorable condition for the growth of many microorganisms that cause undesirable changes in addition to the softness and

disintegration of fish tissues because fish fat is oxidized faster than other types of meat ².

Crustaceans are invertebrates that lack an internal skeleton, but they are characterized by a rigid external structure called the exoskeleton, which protects the crustacean bodies. The term shrimp is used to refer to some decadal crustaceans. Shrimp is a delicious seafood that is usually a part of every traditional meal, and the demand for its products has increased worldwide. ³. Living organisms, including bacteria, have a significant variation in the production of histamine due to the presence of specific genes responsible for the production of histamine ⁴. Histamine is formed in fish from some microorganisms (HDC) capable of producing the enzyme Histamine decarboxylase, produced by Gram-positive and Gram-negative bacteria. Histidine, accessible to histamine, is naturally present in high levels in the muscles of some fish ⁵.

Biogenic amines such as bio-tricin, cadaverine and tyramine are produced from gram-positive and gram-negative bacteria such as *Staphylococcus* spp and *Photobacterium phosphorus*. Researchers have recorded many bacterial species as histamine products and fermented seafood. They were found to be histamine-producing bacteria. With different species or genera, the ability of histamine synthesis depends on the type of histamine ⁶.

Fish, seafood, and their products are among the foods rich in histamine-producing bacteria ⁷. Incorrect processing or storage of fish is one of the main factors that contribute to the production of histamine in fish, and histamine production can occur during manufacturing processes such as salting, smoking, fermentation, drying, and others until the product is ready for marketing, and when eating these histamine-containing fish by the consumer, many changes will occur within the consumer's body, leading to many risks (fig: 1). ⁸

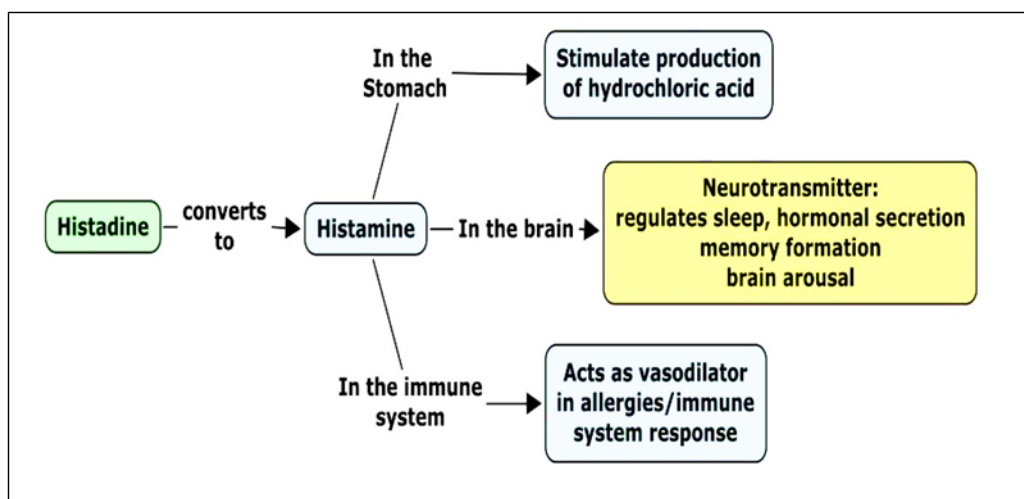


Figure 1. Causes of histamine in humans

Some researchers stated that the bacteria isolated from fish were divided into two groups, a group that did not produce histamine and a group that produced histamines such as histidine, and a moderately thermophilic and cold-resistant group of various marine foods and the bacteria were responsible for the production of histamine isolated from all local and imported fish ⁹.

The study aimed to know the bacterial content of shrimp and its role in the production of histamine and food poisoning events and cases of allergies resulting from eating shrimp. This study was conducted because there are no

studies dealing with the role of histamine-producing bacteria isolated from shrimp.

MATERIALS AND METHODS

Sample sources

One kg of shrimp sample was collected from the Al-Faw area in Basra governorate with 3 replications. The samples were placed in sterile plastic bags, kept in a cork container containing pieces of ice and crushed ice, and then transferred to the Market Research and Consumer Protection Center/ University of Baghdad, Iraq laboratory.

Materials used for isolation and diagnosis

Various cultural media such as Nutrient Agar (NA) medium, Nutrient broth (NB), Eosin Methylene medium (EMB), MacConkey medium and Simmon's citrate agar medium, Brain-Heart infusion broth medium, solid mannitol medium, salmonella detection medium, gelatin decomposition medium, casein decomposition, starch decomposition amid indole production, nitrate reduction medium, carbohydrate fermentation medium, Vibrio medium for sucrose bacteria diagnosis (TCBS)) and urease enzyme test medium, 6%, 7% and 8% NaCl medium, Medium Mueller Hinton broth and medium milk. Ampicillin Dextrin Agar with Medium Additionally (ADA-V) Vancomycin Identification Use many identifiers To diagnose bacteria at the level of type Kit API Hi 25 TM Included: Kit Enterobacteriaceae. Identification Kit API Hi Vibrio. TM Identification Kit API Hi Staph. TM Identification Kit API Hi Carbohydrate TM Identification Kit API Hi Aeromonas TM Kit They are all equipped with Identification Kit Hindi. Himedia.

The culture media is used to detect histamine in bacteria.

Histamine and broth histidine (TSBH) liquid media histamine decarboxylase broth (HDB), and histamine decarboxylase agar media (HDA) were used to identify histamine-producing bacteria, and the pH of the medium was set to 5.3. This media was autoclaved at 121°C For 15 minutes. This method relied on the occurrence of color change in the media.

Isolation and diagnosis of bacteria

Ten gm of the mashed shrimp sample was transferred to sterilized flasks containing 90 ml of sterile physiological dilution solution and mixed well to obtain identical and homogeneous samples using an electric mixer that was previously sterilized with 70% ethanol, Then 1 ml of the dilutions 10^{-7} , 10^{-8} and 10^{-9} were planted in three replications and planted on the different nutrient media in sterile Petri dishes. The media was incubated at 37 ° C for 48-72 hours in aerobic and anaerobic conditions.

Then, the bacterial colonies were purified by taking a colony from a type of bacterial colony of different appearance in shape, color and size. The bacteria were diagnosed by conducting morphological tests, which examine the shape of the colonies and the shape of bacteria through gram staining and bacterial motility examination. Other biochemical tests were performed ¹⁰.

Purification of isolates

Inoculating loops of primary isolates inoculated the different selective culture media at 24 hours of age to conduct sifting by systematically planning the bacterial colonies in the middle of the surface of the medium and the occlusal incubation at 37°C for 48 hours in aerobic and anaerobic conditions. As mentioned, it was repeated 3 times to Obtain pure single colonies to perform diagnostic tests for these isolates.

Diagnostic tests for bacterial isolates

Morphological tests

These examinations included the colonies' shape, color, and size and microscopic examinations, which included endospore-forming, bacterial motility test, and endospore-forming ¹¹.

Biochemical tests

The following tests included the catalase test, glucose production test, carbohydrate fermentation test, focus procure test, casein hydrolysis test, starch hydrolysis test, oxidase test, ammonia production test from arginine, nitrate reduction test, gelatin hydrolysis test, indole production test, sulfur dioxide production test Urease, methyl red test, esculin decomposition test and temperature effect incubation in bacterial growth and growth in anaerobic conditions ¹².

Identification by using of Analytic Profile Index E

From the liquid or solid medium specific to the type of bacteria that had previously been inoculated with the bacterial isolate to be studied, 48 hours at a temperature - and after incubating the tape for 24 37 m, the results were recorded and interpreted based on and followed the same method as Analytical Profile Index with different diagnostic systems according to the API type diagnosis systems Bacterial system. This test was carried out by inoculating 5 ml of physiological solution with pure colonies of bacteria to be tested, then mixing the bacterial suspension well. In light of the appearance of the results, the type of bacteria underdiagnosis was determined by referring to the Himedia kit, which the Indian company supplied. The numbers of bacteria and the percentage frequency of isolates isolated from shrimp samples were calculated ¹³.

RESULTS

The results showed the presence of different genera and types of Bacteria distributed as follows: four species of the *Bacillus* genus (*B. pumilus*, *B. alvei*, *B. subtilis* and *B. licheniformis*), and two species of *Pseudomonas* (*P. fluorescens* and *P. aeruginosa*) and three type of *Staphylococcus* genus (*S. saprophyticus*, *S. epidermidis* and *S. intermedius*) and four species of *Aeromonas* (*A. veronii* *A. schubertii*, *A. sobria* and *A. encheleia*) and five different species of *Vibrio* (*V. fischeri*, *V. vulnificus*, *V. alginolyticus*, *V. fluvialis*, *V. parahaemolyticus*) and ten species belonging to the family Enterobacteriaceae, which included (*C. amalonaticus* and *C. freundii*) for the genus *Citrobacter* and three species belonging to the genus *Enterobacter* (*E. aerogenes*, *E. cloacae* and *E. taylorae*) and three species belonging to the genus *Proteus* (*P. mirabilis*, *P. vulgaris*, and *P. marina*) And two species belong to genus *Serratia* (*S. fonticola* and *S. marcescens*) And two species of the *Klebsiella* genus (*K. oxytoca* and *K. pneumonia* sub. *Pneumonia*), In addition to *Escherichia coli* bacteria and *Morganella morganii* sub. *morganii* in addition to *Hafnia alvei* bacteria.

All biochemical diagnostic tests for bacteria in shrimp showed that there are species of the genus *Bacillus* spp. The isolates were rod-shaped, gram-positive and spore-forming, and the results agreed with some studies ¹⁴. The results agreed with ^{15, 7}. By isolating them with *Bacillus subtilis* from shrimp Blacktiger, *Bacillus amyloliquefaciens* was also isolated from shrimp by ¹⁶.

Also, The results showed that the colonies growing on the *Pseudomonas* selective media it was distinguished as large, and its colonies are sticky and shiny and showed a green color where the color was clear green bluish when growing on

nutrient agar medium because it is characterized by the production of types of pigments like pyocyanin dye. The bacterial isolates of *Pseudomonas* were Gram-negative and mobile, where negative for the production of gas from glucose sugar and negative for sucrose sugar lactose, galactose, and for the starch and methyl red tests and indole and focus pressure and positive for tests Catalyzes, citrates, fermentation of glucose and xylose and grow at a temperature of 4 ° C ¹⁷.

The medium Cetrimide Agar was used to detect *Pseudomonas aeruginosa*. Three replicates were made for each sample after dilution. It was grown on dishes containing the medium as mentioned above and incubated at 37 °C.

The results agreed with ¹⁸. by the diagnosis of bacteria *Pseudomonas* spp from sea shrimp. Also, the results agreed with ¹⁹ by diagnosing *P.aeruginosa* from tissues and muscles of *Penaeus monodon* Shrimp.

As for the results of *Staphylococcus* spp isolating bacteria, the results showed that all isolates had it gave a positive result on the (selective Mannitol medium) and mannitol sugar, as well as on nutritious agar, circular colonies have appeared Smooth, slightly convex. The microscopy showed Colonies have aggregated gram-positive spherical cells in clusters, pairs, short chains, not mobile, and the results of the biochemical tests were they were all positive for catalase and negative for oxidase.

The bacterial isolates were subjected to the API HiStaph. TM Identification Kit. It has shown the results of the diagnosis are in complete agreement with the results of ²⁰. The tests the aforementioned biochemical for staphylococcal bacteria, which included the examination of carbohydrate fermentation and other enzymatic assays.

The study reached the isolation and identification of *Aeromonas* spp. Its colonies were yellow, slightly above the surface of the medium (ADA-V). The biochemical tests showed its fermentative ability and its ability to withdraw the carboxyl radical from the amino acid lysine. It grew in the presence of 3% and 6% sodium chloride, respectively, as the results showed that all obtained isolates were gram-negative and all isolates were motile.

And it was found through the results of tests and based on the diagnostic kit API HiAermonas. TM identification kit, the presence of types of these bacteria in the shrimp samples, and these types were *Aeromonas veronii* *Aeromonas schubertii* *Aeromonas Aeromonas sobria* and *Aeromonas encheleia*.

The results agreed with ²¹ By isolating three types of bacteria identical to what was found in the current study, *Aeromonas schubertii*, *Aeromonas sobria* and *Aeromonas veronii* in the shrimp. The study concluded that six vibrio species were isolated by growing dilutions of samples on TCBS, a differential selection medium on which only vibrio spp grows. The colonies were Yellow or green, after which the bacteria were stained by gram stain. All isolates were negative for gram stain, and it showed that the shape of bacteria cells was in the form of small villi, and all of them were motile, and the results showed that all isolates were positive for the oxidase assay ²².

And the results of the study showed there are eight genera of Enterobacteriaceae. The different genera and types of bacteria were diagnosed using the API Hi TM Enterobacteriaceae Identification kit, and two species of the genus *Citrobacter* were identified. They are *C. freundii* and *C.amalonaticus*, while three species belonging to the genus *Enterobacter* have been identified they are *E.taylorae*, *E.aerogenes*, and *E.cloacae*. There are another species belonging to *Cronobacter sakazakii* and isolates belonging to the species *Hafnia avail*, and the genus *Proteus* have been identified .Two species, *P.mirabilis* and *P.vulgaris*, were diagnosed. It was found through the results that *Proteus* bacteria was negative for

the lipoprotein test and negative for oxidase testing, pectin degradation and ONPG production And negative for fermentation of mannose sugar, Onositol, mannitol, adonitol, and D-arabitol are positive for the tests of the enzyme ornithine deaminase and the deaminase enzyme from phenylalanine, growth on potassium cyanide, production of gas from glucose and nitrate reduction, positive for the production of H₂S gas, liquefaction of gelatin and the production of lipases, and weak in the consumption of citrate, decomposition of urea while bacteria *Morganella morganii* sub. *morganii* was negative for the tests mentioned above in addition to citrate consumption and positive for fermentation of mannose sugar and urease test as in ^{23, 24}.

The presence of the intestinal family in fish is an indicator of sewage contamination, and there are strains of Enterobacteriaceae that may cause diarrhea and can cause spoilage in a wide range of foods in addition to giving undesirable flavors and odors due to the action of bacterial enzymes on proteins and fats in foods ⁴. As for the frequency of emergence of bacteria isolated from shrimp, the results indicated that *Pseudomonas fluorescens* was more frequent than the rest of the species.

Primary detection of histamine-producing bacteria in liquid and solid media:

The local bacterial isolates were subjected to a sifting process to determine their ability to produce histamine depending on the color change of the medium to violet color around the colonies growing on the medium HCM. The growth results of bacteria isolated from shrimp in liquid and solid media (HCB, TSBHA and TSBH) gave a color change in the media, indicating histamine production due to the bacteria producing the histidine decarboxylase enzyme and converting it to histamine, which in turn is caused by the histamine pigment, which is the dye. The pH value of the medium (from acidic to basic) and the color changed from green to violet due to bromocresol purple.

After the incubation period, in suitable conditions, such as temperature, etc. In solid media, a violet aura appeared around the growing bacterial colonies. The results showed the presence of strong bacteria in the production of histamine in the solid histamine production medium, and other bacteria that were weak in production in the same medium, as well as other bacteria that were negative in production, meaning that they do not possess the enzyme Histamine decarboxylase (HDC) and have no role in the production of histamine.

This test used the liquid media HCB and TSBH containing bromocresol purple dye. After the incubation period, the color of the liquid media noticeably changed to violet color for the bacteria producing the enzyme Histamine decarboxylase (HDC). It did not change for the bacteria that did not produce histamine. The results showed the isolates also In its enzyme productivity through the variation of the color density of the liquid media during the incubation period.

The previous results showed that all isolates varied in their ability to produce the enzyme to varying degrees. The results also showed the presence of some bacterial species that gave a negative result for the examination in liquid and solid media. However, they can produce histamine from the decomposition of histidine, which may be because the medium's pH does not suit bacteria, which causes them to not grow in the medium, such as *Hafnia alvei*. *Klebsiella pneumoniae* and *Klebsiella oxytoca* bacteria produced histamine in TSBH liquid media, and the results agreed with what was found ²⁵. There are genera of the family Enterobacteriaceae that produce high a level of histamine, and this was also mentioned ²⁶.

Where ²⁷ confirmed that Staphylococcus bacteria were histamine-producing, while ²⁸ Stated that 76% of Staphylococcus isolates, especially Staphylococcus xylosus, were histamine-producing.

It also showed ²⁹ that Staphylococcus bacteria were histamine-producing for Staphylococcus carnosus, and Staphylococcus piscifermentans bacteria were also found by ³⁰.

Staphylococcus bacteria were also histamine-producing for Staphylococcus plymuthica and Staphylococcus fonticola.

It was found that most of the histamine-forming bacteria are living at moderate temperatures, so the biogenic amines, especially histamine, increase at temperatures ranging between 15-20° C ³¹ and the results also agreed with ³², who found that Enterobacter aerogenes And Morganella morganii has given a positive result for the production of histamine.

shrimp Metapenaeus affinis					
name of bacteria	fre- quencie s	percent- age	Type of bac- teria	fre- quencies	per- centage
Cronobacter sakazakii	4	5.63	A. veronii	1	1.40
E.cloacae	3	4.22	A. schubertii	1	1.40
E.aerogenes	2	2.81	A. encheleia	2	2.81
E.taylorae	2	2.81	A. sobria	2	2.81
P. vulgaris	1	1.40	V. vulnificus	2	2.81
P. marina	4	5.63	V. fischeri	1	1.40
P. mirabilis	2	2.81	V. alginolyticus	1	1.40
S. aprophyticus	1	1.40	V. parahaer-nolyticus	2	2.81
S. epidermidis	1	1.40	V. fluvialis	2	2.81
S. intermedius	2	2.81	S. fonticola	3	4.22
C.amalonaticus	2	2.81	S. marcescens	2	2.81
C. freundii	2	2.81	B. alvei	2	2.81
E. coli	2	2.81	B. licheni-formis	2	2.81
H. alvei	2	2.81	B.pumilus	2	2.81
M. morganii sub. morganii	2	2.81	B.subtilis	1	1.40
P .aeruginosa	3	4.22	K. oxytoca	2	2.81
P . fluorescens	5	7.04	K.pneumonia	2	2.81
V. harveyi	1	1.40			

Table 1. Types of bacteria isolated from shrimp Metapenaeus affinis, their frequencies and percentage.

Name of the bacteria	Positive result	Name of the bacteria	Weak result	Name of the bacteria	Negative result
K. pneumonia sub. pneumonia	++	S. intermedius	+	A. schubertii	-
K. oxytoca	++	E. coli	+	A. veronii	-
E. cloacae	++	Serratia marcescens	+	A. encheleia	-
P. marina	++	Serratia fonticola	+	A. sobria	-
V. alginolyticus	++	P. fluorescens	+	H. alvei	-
C. sakazakii	++	P. aeruginosa	+		
E.taylorae	++	B. subtilis	+		
P. vulgaris	++	B. licheniformis	+		
P. mirabilis	++	B. alvei	+		
C. freundii	++	B. Pumilus	+		
C. amalonaticus	++	S. aprophyticus	+		
V. vulnificus	++	S. epidermidis	+		
V. harveyi	++				
V. fischeri	++				
V. parahaemolyticus	++				
V. fluvialis	++				
E.aerogenes	++				

Table 2. Test of isolated bacteria for histamine production on solid and liquid histamine production media.

The results agreed with ⁹. that *Aeromonas* spp does not produce histamine, while *Morganella morganii*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Proteus vulgaris* were histamine-producing. ³³ It was found that samples containing different types and numbers of bacteria belonging to the family Enterobacteriaceae that produce histamine had high histamine levels.

The results agreed with ³⁴, who found that the genus *Bacillus*, especially *Bacillus coagulans*, weakly produced histamine in fish products in Taiwan.

The results also agreed with ³⁵, which found that *Serratia marcescens*, *Klebsiella variicola* and *Enterobacter* spp. isolated from tuna fish products produced histamine weakly in the liquid media containing histidine TSBH.

The use of culture media methods to detect histamine-producing bacteria that depend on the change in the pH number, its decrease in the culture media affects the growth of some histamine-producing microorganisms such as *Photobacterium damsela*, which do not grow well in that number, and raising it can affect the failure of The test. Several researchers confirmed that the change in the medium's acidity could be due to the metabolism of some microorganisms that do not produce biological amines.

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