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The Occurrence, Hemolytic, Cytotoxic Activity and Antibiotic Susceptibility of *Aeromonas hydrophila* Isolated from Fish Samples in Baghdad

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Summary

In this study a total of 60 samples of lived fishes (common carp) and frozen fishes were collected from 15 local markets in Baghdad city ,for isolation of *Aeromonas hydrophila* to determine the hemolytic ,cytotoxic activity of the isolates and their antibiotic susceptibility , 65% of our samples were found to be positive for *Aeromonas hydrophila* isolation 76.6% were in life fish samples and 53.3% in frozen fish 94.87% exhibited α and β hemolysis, 100% of life fish isolates show β hemolysis while frozen fish isolates show 85.7% β hemolysis and 14.3% α hemolysis , 97.43% of isolates show cytotoxic effect on Vero cells the highest frequency occur in the isolates of life fish group 60.50% , all isolates were 100% resistant to pencillin , ampicillin , Cloxacillin and Bacitracin in sensitivity test , and the resistance to other antibiotics appear to oxytetracycline 56.5% ,tetracycline 33.4% ,cephoxetin 30.8% , chloramphinicol and kanamycin 28.2% , at last the isolates show resistant to streptomycin and rifampicin in 23.1% and 15.4% respectively. These results demonstrated the presence of virulent food borne *Aeromonas hydrophila* in fish with multiple antibiotic resistance in Baghdad markets.

Keywords: hemolytic, cytotoxic, *Aeromonas hydrophila*

عزل بكتريا *Aeromonas hydrophila* المنتجة لعوامل تحلل الدم وعوامل تسمم الخلايا وحساسيتها للمضادات الحياتية من الاسماك في مدينة بغداد

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الخلاصة

في هذه الدراسة تم جمع 60 عينة من اسماك الكارب الحي والسمك المجمد من 15 سوق محلي في مدينة بغداد لغرض عزل وتشخيص بكتريا الايرومونص هايدروفيلا وتحديد قابليتها على تحليل الدم واظهار التأثير السام لخلايا الفيرو مع تحديد حساسيتها ومقاومتها للمضادات الحيوية , وكانت 65% من العينات موجبة للعزل و 76.6% في مجموعة عينات السمك الحي و 53.3% في مجموعة السمك المجمد و 94.87% من العزلات اظهرت تحلل للدم نوع الفا وبيتا وكانت نسبة 100% من نوع بيتا في مجموعة السمك الحي في حين مجموعة السمك المجمد اظهرت تحلل نوع بيتا 85.7% ونسبة 14.3% نوع الفا و 97.43% من العزلات اظهرت تأثير سام لخلايا الفيرو وكان اعلى تردد في عزلات السمك الحي بنسبة

60.5% وكانت جميع العزلات 100% مقاومة للمضاد الحيوي البنسلين والامبسلين والكلوكساسلين والباستراسين وقد اظهرت العزلات مقاومة ضد الاوكسيتيتراسايكلين بنسبة 56.5% و 33.4% للتيتراسايكلين و30.8 % للسيفوكستين و 28.2% لكل من الكلورامفنيكول والكانامايسين واخيرا اظهرت مقاومة للستريبتومايسين والريفامبسين بنسب 23.1% و 15.4% على التوالي وهذه النتائج اظهرت وجود لبكتريا الايرومونص هايدروفيلا الضارية والمقاومة للمضادات الحيوية في الاغذية المتمثلة بالاسماك المباعة في اسواق مدينة بغداد .

Introduction

In developing countries fish and fishery products contribute a major food item of common man, these products are contaminated by various food borne pathogens (1) They act as a vehicle for pathogenic bacteria naturally occurring in the aquatic environment referred as indigenous or derived from post harvest contamination (2) may lead to cause human morbidities and mortalities worldwide (3). It is well established in the fish industry that bacterial infections are responsible for heavy losses in fish farms ,among the etiological agents of bacterial fish disease, the motile Aeromonas group , especially *Aeromonas hydrophila* is considered an important pathogen causing primary infection in wounds or the secondary problem following stress from temperature change, handling or poor water quality (4and5) and it emerged as an important food borne pathogen worldwide (6). It's an aerobic ,non sporulating gram negative bacilli that are ubiquitous inhabitants of fresh and brackish water (7) These organisms have been readily isolated from a wide variety of foods like fish ,eggs, meat, meat products, milk and milk products (8and9). Furthere more ,they have been found in a variety of aquatic environments including lakes, rivers, streams ,springs, rain water, swimming pools and sea water and have also been isolated from tap water and soil (10). These species have been recognized as pathogens of fish ,reptiles and amphibians for many decades ,but it is only recently that they have been recognized as significant human pathogens (7). *Aeromonas hydrophila* , a ubiquitous aquatic microorganism is an opportunistic pathogen that has been associated to wound infections, gastroenteritis, septicemia and travelers' diarrhea in humans (1). It associated with tail and fin rot , hemorrhagic septicemia and epizootic ulcerative syndrome in many fish species (11and12) Several biochemical properties and virulance factors ((enzymes such as (proteases, lipases, Dnases , elastase and gelatinase) ,hemolysins, aerolysin, enterotoxins, cytotoxin, endotoxin lipopolysaccharide , outer membrane proteins , dermonecrotic factor)) have been reported as potential indicators of pathogenicity in *Aeromonas hydrophila* (1and13) .

The antimicrobial agents are great value for devising curative measures against bacterial infections (14). Mounting concerns for emergence of drug

resistance among *Aeromonas spp*. are reflected in a number of reports (15,16and17). The present study was performed to determine the occurance , hemolytic , cytotoxic activity and antibiotic resistance of *Aeromonas hydrophila* isolated from fish samples in Baghdad .

Materials and Methods

In this study, a total of (60) samples of life fish (common carp) and frozen fish were collected from 15 different local markets in Baghdad city and taken for analysis.

Bacterial isolation: The bacterium was isolated in Al-Kindy company for veterinary vaccines and drugs by washing the surfaces of collected fish with 70% ethanol . Liver, kidney and 25 g of fish meat were obtained a sceptically and washed three times with sterile saline . the samples were then homogenized with a tissues terror and the homogenate was inoculated onto brain heart infusion agar (BHIA) and incubated at 28 °C for 2 days every colony was reisolated and subcultured on a new (BHIA) , the motile ,novobiocin-resistant, oxidase positive and glucose fermenting colonies were all considered as *Aeromonas spp.* and further identified by API 20 NE system following the procedure as described in the instruction manual(18) .

Hemolytic activity: The Hemolytic activity of the isolates were determined by blood agar plate assay (19) and recorded after 24 hr. incubation at 37 °C (3).

Preparation of cell free supernatant: *Aeromonas hydrophila* isolates were cultured in 10 ml of brain heart infusion broth (BHIB) and incubated at 37 °C for 18 hr. , supernatant was carefully collected after centrifugation at 8000 rpm for 5 min. at 4 °C and filtered using 0.45 millipore filter.

Cytotoxicity: Vero cells obtained from Al-Kindy company for veterinary vaccines and drugs . Were used for the cytotoxicity analysis of *Aeromonas hydrophila* isolates ,Vero cells were grown in 96 well flat bottom microtitre plate (falcon) in Eagles minimum essential medium supplemented with 10% fetal bovine serum and antibiotics ,the cell suspension (10 4 CFU.ml-1) was seeded in every well and incubated at 37 °C for 48 hr. in 5% CO2 for the formation of confluent monolayer , these monolayer of cells were exposed to the cell free filtrate and its dilutions , the cytotoxic titer was expressed as the highest dilution which showed a positive response.

Antibacterial sensetivity test: Sixteen antibacterial agents were used for in-vitro sensetivity test of *Aeromonas hydrophila* isolates from fish using the disc diffusion assay (20).

Results

Bacterial isolation: From 60 collected samples, 30 were from the life fish and the other 30 samples from frozen fish, all these samples send for analysis. Fig. 1- shows 35% (n=21/60) were negative while the other samples show positive results for the presence of *Aeromonas hydrophila* infection 65% (n=39/60) the bacterial isolation show higher frequency in life fish 76.6% (n=23/39) while in frozen fish the frequency was 53.3% (n=16/39).

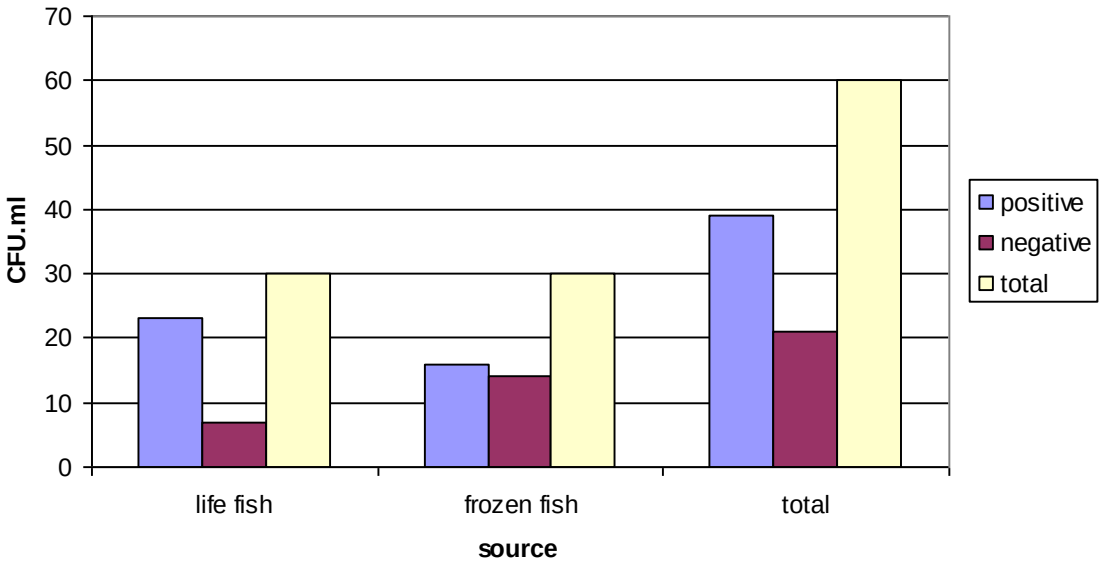

Figure – 1: The frequency percentage of *Aeromonas hydrophila* isolated from fish samples

Hemolytic activity: Hemolytic activity of the isolates were determined for its importance as a virulent factor , Table 1- represent these activities of *Aeromonas hydrophila* isolates we found that 100% (n=23/23) isolates from the life fish group show β hemolysis while isolates from the frozen fish group show 85.7% (n=12/14) β hemolysis and 14.3% (n=2/14) α hemolysis . From all isolates we found that only 5.4% (n=2/37) show α hemolysis and 94.6% (n=35/37) show β hemolysis , at the same time 5.12% (n=2/39) isolates did not show any hemolytic activity these were from the frozen fish group and the remain 94.87% (n=37/39) isolates show α and β hemolysis.

Table 1: Hemolytic activity of *Aeromonas hydrophila* isolated from fish samples

Source	α	%	B	%	total	%
life fish	0	0	23	100	23	100
frozen fish	2	14.3	12	85.7	14	100
Total	2	5.4	35	94.6	37 #	100

 # (n=2/39)5.12% isolates did not show any hemolytic activity

Cytotoxic activity: All the positive isolates from life fish and frozen fish samples were subjected to cytotoxicity analysis on Vero cells. Table 2- and Fig. 2- show the cytotoxic effects on Vero cells started with in the first 6 hours of bacterial supernatant addition , complete cell death was observed with in 24 hours 2.56% (n=1/39) isolates did not show cytotoxic effects on Vero cells while the remaining isolates (n=38/39) all have a cytotoxic effect on Vero cell (97.43%). The life fish group isolates show their cytotoxic effect on Vero cells with percentage 60.5% (23/38) and in frozen fish group 39.5% (15/38) , the highest frequency occur at the titer (1:16) 63.2% (n=24/38). In life fish group the highest percentage was 70.83% (n=17/23) while in frozen fish the percentage was 29.17% (n=7/15), and the most powerful effect was at titer(1:64)2.6% (n=1/38) isolate from life fish group.

Table 2: The results of the cytotoxic effects of *Aeromonas hydrophila* on Vero cells

Titer	Total no.	%	Life fish samples	%	Frozen fish samples	%
1:64	1	2.6	1	100	0	0
1:32	3	7.89	2	66.66	1	33.33
1:16	24	63.2	17	70.83	7	29.17
1:8	8	21.1	3	37.5	5	62.5
1:4	2	5.3	0	0	2	100
total	38 #	100	23	60.5	15#	39.5

 # 2.56% (n=1/39) isolates did not show cytotoxic effect from the frozen fish group

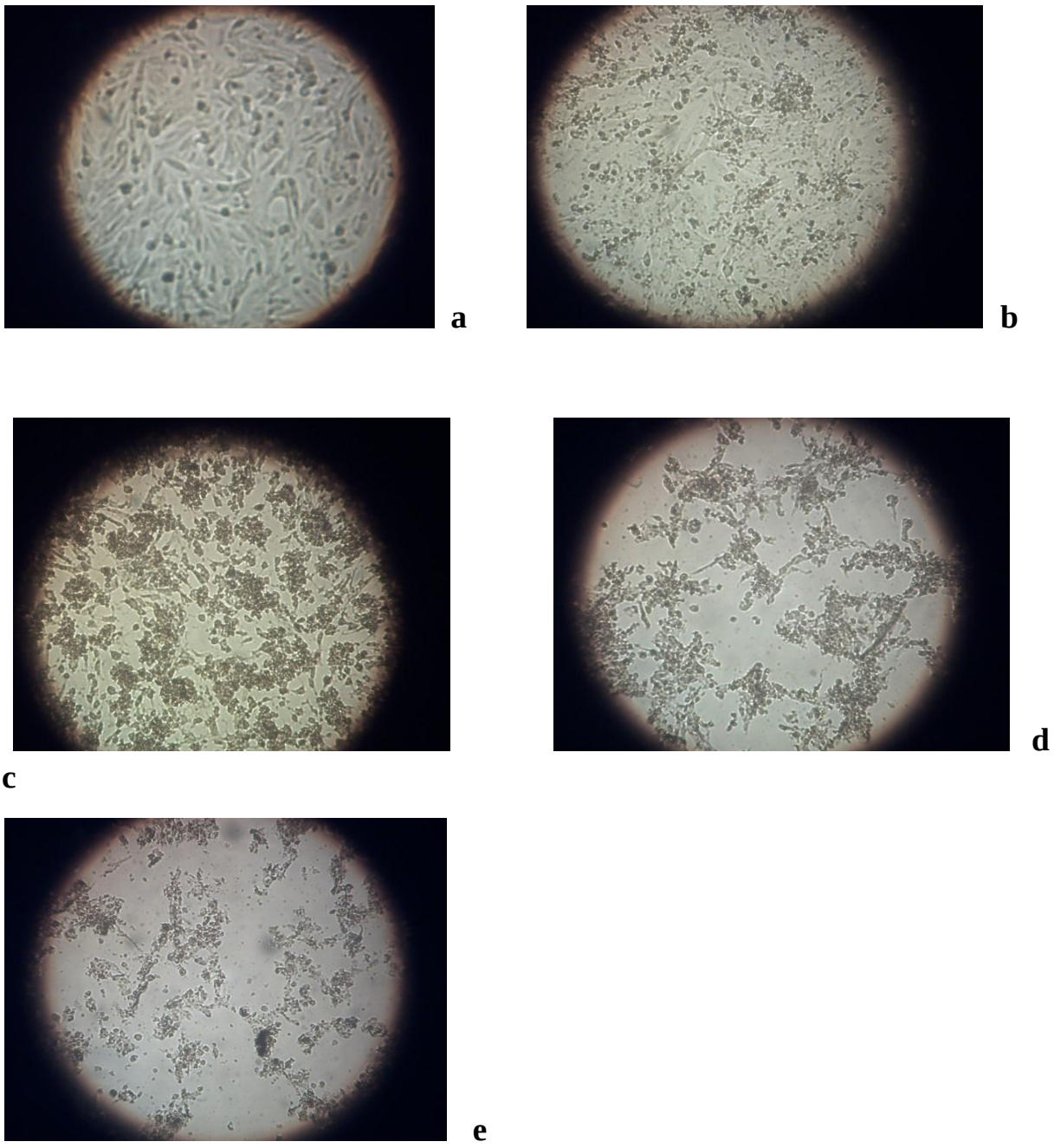

Fig 2- cytotoxic effects of *Aeromonas hydrophila* isolates on Vero cell line. (a = Control Vero cell b, c, d = represent the progression of cytotoxic effects on Vero cell manifested by the clusters of rounding cells and cell shrinkage e) complete cell detachment that appears with in 12 hours for the isolates that give the titer of 1:16 .

Antibacterial sensitivity test: Table 3- shows the results of invetro sensitivity testing of (39) isolates of *Aeromonas hydrophila* , we found that the isolates were sensitive (100 %) to Doxycyclin, furaltadone, ciprofloxcin, gentamicine, and nalidixic acid . but sensitive to streptomycin in (76.9 %),rifampicin(84.6 %) , chloramphinicol and kanamicin (71.8 %) , cephoxetin (69.2 %) , tetracyclin

(66.6 %) , oxytetracyclin (43.5 %). In other hand the isolates were resistant (100 %) to cloxacillin , ampicillin , bacitracin , and penicillin . while to oxytetracycline (56.5 %) , tetracycline (33.4 %) , cephoxetin (30.8 %), chloramphinicol and kanamicin (28.2 %), streptomycin (23.1 %),and to rifampicin (15.4 %)

Table 3: shows the results of antibacterial sensetivity test

Drugs	No. of sensetive	%	No. of resistant	%
Streptomycin 10 Mg	30	76.9	9	23.1
Cloxacillin 5Mg	0	0	39	100
Rifampicin 5Mg	33	84.6	6	15.4
Ampicillin 25Mg	0	0	39	100
Bacitracin 10 unit	0	0	39	100
Oxytetracyclin 30Mg	17	43.5	22	56.5
Doxycyclin 30Mg	39	100	0	0
Furaltadone 300 Mg	39	100	0	0
Ciprofloxacin 5Mg	39	100	0	0
Gentamicin 10Mg	39	100	0	0
Chloramphinicol 30Mg	28	71.8	11	28.2
Tetracycline 30Mg	26	66.6	13	33.4
Penicillin 10Mg	0	0	39	100
Cephoxetin 30Mg	27	69.2	12	30.8
Kanamicin 30Mg	28	71.8	11	28.2
Nalidixic acid 30Mg	39	100	0	0

Discussion

In our study we found that infection occur in 65% (n=39/60) samples collected from life and frozen fish the isolation was higher in frequency in life fish 76.6% (n=23/39) from the frozen fish samples 53.3% (n=16/39) and these result was more than the result of (Seethalakshmi(3) when he found that 71.23% (n=52/73) *Aeromonas hydrophila* isolates from fish samples collected from 5 major fish markets in Chennai, TamilNadu, India .

A number of virulence factors derived from *Aeromonas hydrophila* have been explained in an effort to explain the pathogensis of infections due to this organism, toxins with haemolytic, cytotoxic and enterotoxic activities have been described in many *Aeromonas.* Spp. As a virulent factors (21) .the genetic detection of these virulent factors will be very useful to give a reliable results , like using the PCR assay (22and23).

Most of *Aeromonas hydrophila* isolates show haemolytic activity 94.87% (n=37/39), β hemolysis appear in 94.59% (n=35/37)from this percentage 100% (n=23/23) was in life fish ,and 85.7% (n=12/14) in frozen fish , while α hemolysis appear only in samples collected from frozen fish 14.3% (n=2/14). So our results was in contrary to the results of Seethalakshmi (3) who found in his study that equal distribution of α & β hemolysis activity was observed in 52 isolates of fish samples that show 46.15% (n=24/52) α hemolysis and 40.38% (n=21/52) show β hemolysis and 13.46% (n=7/52) γ hemolysis , and to the results of Palumbo (24) who reported that all strains isolated from retail foods of animal origin were β hemolysis. In cytotoxic effect we found that 97.43% (n=38/39) from the isolates , 60.5%(n=23/38) , 39.5% (n=15/38) was in life fish and frozen fish respectively , only 2.63% (n=1/38) isolates from life fish group show its effect on Vero cell at titter 1:64 and 7.89% (n=3/38) isolates 2 of them was in life fish group at the titter 1:32 while the higher percentage 63.15% (n=24/38) appear its cytotoxic effect at titter 1:16 , from this 70.83% (n=17/24) in life fish and 29.16% (n=7/24) in frozen fish , these results reflect that the most cytotoxic releasing isolates were in life fish group . These results resemble to Seethalakshmi(3) results who found that 98.61% isolates exhibited cytotoxic effect on Vero cells 98.07% was in samples from fish , while Martin(25) reported only 73% of the food isolates show cytotoxic effect , except Sridhara (26) who write that 100% of clinical isolates produce cytotoxicity on Vero cells. When we done the sensitivity test for different antibiotics we saw that our isolates have multiple drug resistant (100 %) to cloxacillin , ampicillin , bacitracin , and penicillin and were resistant to oxytetracycline 56.5% (n=22/39) , tetracycline 33.4% (n=13/39), cephoxetin 30.8% (n=12/39), chloramphinicol and kanamycin 28.2% (n=11/39), streptomycin 23.1% (n=9/39), rifampicin 15.4% (n=6/39) which agree with the results of Vivekanandhan(15) and Zheng (27) , while Hassan(28) found the isolates from fish were resistant to (amoxicillin, meropenem , oral cephalosporin , cefaclor, cephalexin , cephalothin , colistin) , Gold and Salit(29) found the resistance was to pencillin , ampicillin , flucloxacillin, carbencillin , cefuzolin , Abdel-Gwad and Abdel-Rahman(30) found in vitro susceptibility of the *Aeromonas hydrophila* isolates to a variety of antibiotics revealed to 100% of isolates were resistant to penicillin and ampicillin , Soliman(31) all isolates were resistant to (ampicillin, and novobiocin)the similar results recorded by Sohair and Eman(32) who reported that isolates were resistant to (penicillin ,ampicillin) , Kaskhedikar and Chhabra (14) show 100% of isolates were resistant to (ampicillin and colistin) antibiotics . also Yucel &Ctak (16), Emekdas(17) Soliman(33) , Chardrakanthi(34) , reported that all isolates were 100% resistant

to (penicillin and colistin). All these resurges found the same resistant that it is may be due to the β-lactamase enzyme production of these isolates, so they agree with the results of our study which show 100% resistance to Cloxacillin , penicillin and ampicillin antibiotics , and from this results we find that these isolates have multiple drug resistance and we agree in this with Vivekanandhan(15) and Zheng(27) .

The most important fact, one should remember about *Aeromonas hydrophila* infection of fish is a zoonotic disease which can be spread from animal to man and vice versa. Accident exposure to this bacteria may lead to get the disease by cutting ourselves while butchering affected fish or impaling a sharp fin into our hand is a sure way to infect ourselves, also ,people who may be immuno-deficient or immune-incompetent such as (the very young ,the elderly or those with other diseaseproblems) are at the highest risk (35).

Generally , the main routes of exposure in humans are ingestion of contaminated foods and drinking water, or direct contact with recreational waters or contaminated mud , human exposure to *Aeromonas hydrophila* has risen due to the increased usage of aquatic recreational sites especially with its warm climate (1,28and36) because these infections occur sporadically and infrequently and they are more common in warmer climates (37), and the Aeromonas wound infections are most commonly caused by *Aeromonas hydrophila* and have been reported after accidental puncture of the skin followed by exposure to contaminated water or soils (10, 29 ,37 and 38) .

Despite the number of surveys on the incidence and occurrence of Aeromonas spp. In food products, there have been few studies on frozen fish (39) especially in Iraq .

For this reason we conducted this study to determine the hemolytic and cytotoxic activity also to determine the antibiotic resistance of *Aeromonas hydrophila* which isolated from life fish and frozen fish in Baghdad city , because the fish and fishery products are of great importance world wise due to their nutritional value, clear health benefits and wholesome properties (40) , fish as a seafood are highly prone to contamination , they act as a vehicle for pathogenic bacteria naturally occurring in the aquatic environment referred as indigenous or derived from post harvest contamination (2) .and this may lead to human to be infected. Furthermore the Aeromonads associated with economic loss in fish culture worldwide ,these bacteria are opportunistic pathogens though they are part of the normal intestinal micro flora of the healthy fish (41) .

These bacteria associated in human with 2 type of gastroenteritis 1) similar to cholera cause rice water diarrhea. 2) dysenteric gastroenteritis which cause loose stools filled with blood and mucus last for multiple weeks , also its associated with wound infections ,cellulitis , myonecrosis and eczema in people with promised immune systems, but in fish this bacteria cause fish ulcers , tail rot, fin rot ,and hemorrhagic septicemia (13,42 and43) .because it is capable of expressing a number of virulence factors (3). The increasing antibiotic resistance causes health problems in human beings (3). These problems are more intricate in developing nations (14) . The prevalence and multiple antibiotic resistance of *Aeromonas hydrophila* in seafood samples have been reported by Seethalakshmi(44). At present ,the chief means of controlling the diseases caused by *Aeromonas hydrophila* is by antibiotic treatment and improvement of management (45) but the extensive use of antibiotics lead to an increase in antibiotic resistance among them which causes health problems in human being (3and46). The proper medical attention to any cut or laceration (no matter how small) and bandaging of open wounds (35) because the wound infections caused by *Aeromonas hydrophila* often progress rapidly and may require surgical debridement or the amputation of limbs or digits (47). fatal *Aeromonas* wound infections in healthy adults have also been reported (48and49) and because the fact that members of this genus of bacteria are universally resistant to some antibiotics which used in wound treatment (50).

Conclusion: This study demonstrated the presence of virulent food borne *Aeromonas hydrophila* in fish with multiple antibiotic resistance in Baghdad city. There are other virulence factors like (enzymes , aerolysin, enterotoxins, cytotoxin, endotoxin lipopolysaccharide , outer membrane proteins , dermonecrotic factor) have been reported as potential indicators of pathogenicity in *Aeromonas* that must be studied in relation to genetic control , molecular mechanisms, and net work of these factors.

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