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Original Research Article

Prevalence of Tetracycline Resistant *Aeromonas Hydrophila* Isolated From diarrheic Patients in Hilla City, Iraq

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Abstract

The aim of this study was to investigate the prevalence of tetracycline resistant *Aeromonashydrophila* isolated from clinical sources in Hilla city, Iraq. A total of 822 samples were collected from fecal specimens from patients. Samples were collected from those who suffering from diarrhea. The period of the research was from October 2013 to February 2014 at public health lab, Hilla city. Results of this study revealed that out of 822 fecal samples, 13 isolates (1.58%) were belonged to *Aeromonas spp*. However, other bacterial isolates belonged to other genera similar to *Aeromonas*were also recovered. Out of 13 *Aeromonas spp*., eight *A. hydrophila*isolates (61.53%) were obtained, while the other isolates were distributed as: four isolates of *A. salmoncidia* (30.76%), and one of *A. sobria.* Isolation and detection of *A. salmoncidia* species was first recorded in Iraq. The susceptibly of *A. hydrophila* (NO. 8) to several antibiotics was determined using disk diffusion test (DDT). Results showed that 5 isolates (62.5%) were Multi-drug resistant (MDR) and three isolates (S2, S3, and S5,) were sensitive to most of antibiotic classes tested. The MIC of *A. hydrophila* to tetracycline was also detected using ager dilution mothed according to CLSI guidelines. Results found that only 2 isolates (25 %) were resistant to tetracycline. The MIC of these isolates ranged from 0.25-16 µg/ml. This result confirms tetracycline resistance by these isolates when tested using DDT.

Key words: *Aeromonashydrophila* ,*Aeromonassalomonacidia* , Tetracyclineresistance ,diarrheic patients , Iraq.

الخلاصة

تهدف هذه الدراسة الى الكشف عن مدى انتشار بكتريا *Aeromonashydrophila* المقاومة للتتراسايكلين والمعزولة من عينات الخروج للمرضى لمختبر الصحة العامة في مدينة الحلة. تم جمع ٨٢٢ عينة خروج في مختبر الصحة العامة للفترة من تشرين الاول ٢٠١٣ الى شباط ٢٠١٤ وتم تشخيصها من خلال اجراء الاختبارات الزرعيةوالبايوكيميائية وتأكيد النتائج باستخدام نظام system 2Vitek ، حيث اظهرت النتائج انه من مجموع ٨٢٢ عينه خروج ، ١٣ عينه فقط كانت عائدة لبكتريا *Aeromonasspp.* (١.٥٨%) ،على الرغم من انه النسب الاخرى تعود لأنواع بكتيرية اخرى قريبة بصفاتها من بكتريا *Aeromonasspp*. كما تم تأكيد التشخيص باستخدام تفاعل البلمرة المتعدد PCR باستخدام بادئ خاص ببكتريا الايروموناس (16S rRNA)وبالاعتماد على الصفات المظهرية والزرعيةوالبايوكيمياوية فقد وجد ان العزلات تعود للأنواع ضمن جنس الايروموناس : *8*عزلات (٦١.٥٣%) تعود للنوع *Aeromonashydrophila*، 4 *Aeromonassalomoncidia*، وعزلة واحدة تعود الى النوع *Aeromonassobria.* ان عزل وتشخيص بكتريا *Aeromonassalomonacidia* في هذه الدراسة يسجل لأول مرة في العراق. تم الكشف عن حساسية بكتريا *A. hydrophila* للمضادات الحياتية باستخدام طريقة انتشار الاقراص في الاكار. اظهرت النتائج ان ٥ من ٨ عزلات *A. hydrophila*كانت متعددة المقاومة (٦٢.٥%) لأكثر من ثلاثة أصناف من المضادات الحياتية المدروسة. اما نتائج التركيز المثبط الادنى لمضاد التتراسايكلين والتي تم الكشف عنها باستخدام طريقة التخفيف بالاكار، فقد اظهرت النتائج انه ٢٥% من العزلات كانت مقاومة للتتراسكلين والتي اظهرت انه هناك عزلتين مقاومة لهذا المضاد. ان هذه النتيجة قد تطابقت مع نتائج تجربة انتشار القرص في الاكار لهذا المضاد.

الكلمات المفتاحية :ايروموناسهايدروفيلا، ايروموناسسالمونسيديا، مقاومة التتراسايكلين، الاسهال، العراق.

Introduction

Members of the genus *Aeromonas*are facultatively anaerobic, non- spore forming, rod shaped oxidase positive, gram negative bacteria motile by polar flagellum, mesophilic and facultative anaerobic bacteria of family Aeromonadaceae whose natural habitat is in the aquatic environment. Some species are pathogenic for animals and humans. *Aeromonas*species are widely distributed in the aquatic environment, including raw and processed drinking water, and have been frequently isolated from various food products such as fish and shellfish, raw meat, vegetables, and raw milk Additionally, in recent years aeromonads have been implicated as causative agents of human disease, ranging from gastroenteritis to wound infections [1-2].

The genus *Aeromonas*comprises important human pathogens causing primary and secondary septicemia in immunocompromised persons, serious wound infections in healthy individuals and in patients undergoing medicinal leech therapy, and a number of less well described illnesses such as peritonitis, meningitis, and infections of the eye, joints, and bones. Gastroenteritis, the most common clinical manifestation, remains controversial [3].

*Aeromonas*species are commonly isolated from fecal sample of children under the age of five years, whereas their isolation from other body sites usually occurred in adult populations. Aeromonads are known to cause severe diarrheal disease of short duration or chronic loose stools in children, the elderly, or the immuno-compromised individuals, and they have been implicated in travelers' diarrhea [4,5].

Tetracyclines belong to a family of broad-spectrum antibiotics that includes tetracycline, chlortetracycline, doxycycline, and minocycline. These antibiotics inhibit protein synthesis in gram-positive and gram-negative bacteria by preventing the binding of aminoacyl-tRNA molecules to the 30S ribosomal subunit and inhibiting protein synthesis [6]. Contributing to higher levels of microbial resistance, especially among the genus *Aeromonas* [5].

This aim of this study was to evaluate the incidence and the spreading of *A. hydrophila* from diarrheic patients in Hilla city, Iraq, and study the antibiotic resistance patterns of the tested organisms to tetracycline and other antibiotics.

Material and Methods

Collection of fecal samples

This cross sectional study was designed to evaluate the incidence and the spreading of *A. hydrophila* from diarrheic patients in Hilla city A total of 822 fecal samples were collected. They were collected from rectal swab (routine work) and from patients suffering from diarrhea who attending public health lab, Hilla city, Iraq.

Specimen collection and analysis was carried out from October 2013 to February 2014.

Isolation and identification of bacterial isolates

All specimens were cultured on alkaline peptone water, then transfer to TCBS and MacConkey agarby swabbing and incubated at 37ºC for 24 hr. Each primary positive culture identified depending on the morphological properties such as (Shape, swarming, odor and lactose or non-lactose fermentation on MacConkey) [7]. Different Biochemical tests were used for identification of bacterial isolates according to standard methods [7, 8] The Vitek 2 system was used to confirm the biochemical test according to the manufacturer's instructions.

DNA Extraction and Purification

A single colony of cultivated bacteria, which had been incubated overnight, transferred to 2 ml of sterile Louria broth

and incubate at 37 ºCfor 18-20 hours. The DNA extracted and purified using Genomic DNA kit (EURx. /Poland Gene MATRIX). All clinical isolates were screened for chromosomal DNA according to manufacture instructions. The total DNA was used to detect16S rRNA. The DNA primers (*16SrRNA F*: CCAGCAGCCGCGGTAATACG, *16SrRNA R*: TACCAGGGTATCTAATCC), 300 bp, were re-suspended by dissolving the lyophilized product after spinning down briefly with TE buffer molecular grad depending on manufacturer instruction as stock suspension. Working primer tube was prepared by diluted with TE buffer molecular grad.

PCR thermocycling conditions and agarose gel electrophoresis:

The PCR tubes were placed on the PCR machine and the right PCR cycling program parameters conditions were as follows: 94º C 3min 1x, 94ºC 30sec, 52º C 30sec 30x, 72º C, 30 sec, and 72º C 10 min. 1x.

The amplified PCR products were detected by agarose gel electrophoresis and visualized by staining with Ecodyes. The electrophoresis result was detected by using Biometra gel documentation system.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility patterns of isolates to different antibiotics were determined using disk diffusion test (DDT) and interpreted according to CLSI guidelines [10]. The following antibiotics were obtained (from Oxoid, UK, and Himedia, India) as standard reference disks as known potency for laboratory use: penicillin (P, 10 units), Piperacillin (PRL,100 µg), amoxicillin-clavulanate (AMC, 20/10 µg), Imipenem (IMP,10 µg), Meropenem (MEM,10 µg), Gentamycin (CN,10 µg), Tobramycin (TM,5 µg), Amikacin (AK10 µg), Ceftazidime (CAZ,30µg), Cefotaxime (CTX,30 µg), Ciprofloxacin (CIP,10 µg), Tetracycline (TE,10µg), and Chloramphenicol (C, 30 µg)

Determination of MICs of tetracycline

The ager dilution susceptibility method was used for determination of MICs of tetracycline according to CLSI documentations [10]. The ranges of appropriate dilutions of tetracycline MIC determination were 0.25-256 (µg/ml). To determine ager dilution break points, the plates were placed on dark surface , and the MIC was recorded as the lowest concentration of the antimicrobial agent that completely inhibits growth. MIC values were compared with the break points recommended by [10].

Results and Discussion

Isolation of *Aeromonashydrophila*

Results of this study revealed that out of 822 clinical sample 13 isolates (1.58%) were belonged to *Aeromonas*spp., however other bacterial isolates belong to other genera similar to *Aeromonas*were also recovered (Table 1).

Table 1: Occurrence of *Aeromonas* spp. recovered from fecal specimens

Sample	Bacteria type	NO. of isolates	%
Positive	*Aeromonas* spp.	13	1.58%
Negative	*Pseudomonas* Spp., *Pantoea spp.* and *Proteus* Spp. and E*nterobacter cloacae*	809	98.4%
Total		822	100 %

The low isolation rate of this bacteria 1.58% may be attributed to the fact that most cases of diseases caused by this bacteria are occurred usually in warm months, while the collection of samples in this study was in cold months. Agge*et al.* reported that *A. hydrophila* similar to other enteric pathogens was seen more often in hot months. the frequency of *A. hydrophila* cases during warm months was 1.25 case per month, whereas during cold months it was 0.83 case per month [11]. The other reason of this result may be due to fact that *A.hydrophila* infects mainly children, elderly, and immunocompromised persons, while this study was focused on subjects of youth age group. Most studies are focusing isolating bacteria from feces of children only, and the fact that bacteria *Aeromonas* occur in children under two years at high rates, because of lack immune system completely, and abase infant formula of milk plays role in promoting the growth and reproduction of bacteria [12].

In a local study, Obaid [13] reported that 2.7% of *A. hydrophila* isolates were recovered from 479 patients from different ages and sexes. Naji [14] isolated this bacteria from children, the isolation rate of *A. hydrophila* was 4.08%. However, several authors found higher isolation rate of *Aeromonas* from clinical cases. AL-Fathlawy [15]obtained 20.17% of*A. hydrophila* from clinical and environmental sample. On contrast, Borchardt*et al* [16] showed low isolation rate (0.66%) of *A.hydrophila* among 2565 diarrheic stool specimens submitted to a Wisconcin clinical reference laboratory.

Results showed that (8) isolates were diagnosed as *A. hydrophila* (61.53%), while the other isolates were distributed as (4, 30.7%) *A. salmoncidia* and (1, 7.6%) *A. sobria*.

Result of isolation rate in the present study was similar to many studies conducted worldwide, Kannan *et al.* [17] found that the isolation rates of *A. hydrophila* were 60%, and 58.8% respectively, also they found that several species of *Aeromonas* were detected from acute diarrhea which were *A. caviae* (20%), *A. veronii* (10%), *A. schubertii* (4%), *A. jandaei* (3%), and *A. trota* (3%).

A. hydrophila and *A. sobria* tended to cause acute infection in human [18], while *A. salmonicida* cannot grow at 37°C, it is not pathogenic to humans [19]. Authors also referred to isolate *Aeromonas* spp. from different clinical specimens like blood (63%), wounds (11%), ascites (9%), feces (8%), and bile (3%). In addition to different unknown body sites [20].

Identification of *A. hydrophila*

Members of the genus *Aeromonas* are not difficult to isolate from clinical specimens in the diagnostic laboratory, but are often misidentified as belonging to the genus *Vibrio* or *Plesiomonas*[20]. Results of the phenotypic characteristics of the colonies *Aeromonas* had shown conformity with that reported by several authors [21, 22,23]. Bacterial isolation showed a good growth of *A. hydrophila* on TCBS medium and isolates produced yellow colonies /green color due to sucrose fermentation, with diameter of colonies ranged from 2-3 mm, while on blood agar, colonies

appeared dark grey color beta- hemolytic (Figure 1).

On the MacConkey agar formed relatively small pale colonies is non- lactose fermenter (Table 2). also *A. hydrophila* showed good growth in anaerobic condition because, *Aeromonashydrophila* facultative anaerobic, that is known to be pathogenic in humans [24].

Table 2: Characters of *A. hydrophila*isolates on different culture media

Medium	Characters of colonies
TCBS agar	yellow shin with diameter ranged from (2-3)mm.
MacConkey agar	As pale like shaped indicated that *A.hydrophila*is unable to ferment lactose sugar
Blood agar	smooth, convex, rounded and β-hemolytic colonies and pale white to grey color

A B

Figure 1: Characters of *Aeromonashydrophila* on (A) TCBS agar, (B) Blood agar

The microscopic examination of the bacteria stained by gram stain showed that the cells were gram negative, rod shaped, and the cells appeared singly to pairs, or as short chains [25].

Results ofbiochemical tests carried out for identification of isolates were compared with standard methods [10,1]. All isolates were positive for oxidase, and catalase .Oxidase test is used for differential of *A. hydrophila* from other enteric bacteria. Results also found that *A. hydrophila* isolates had the ability to ferment glucose on Kligler iron ager (Alk/acid). They appeared positive to heamolysis test, motility test, and utilization of citrate, but they were negative to string test and urease test. String test is used to differentiate between *A.hydrophila*and *V. cholera* isolates [26].

Identification of *A. hydrophila* was confirmed using Vitek 2 system. Out of 13 *A.hydrophila*isolates (identified using biochemical tests), only 8 isolates was identified as *A.hydrophila.* The other 5 isolates were identified as *A. soberi* (1 isolate) and *A. salamoncidia* (4 isolates) had showed 85- 99% identification percentage probability.

Molecular identification of *A. hydrophila*

16S rRNA fragment was used for molecular identification of *A.hydrophila* isolates. Results found that 16S rRNA gene showed 100% similarity with *A. hydrophila* (Figure 3)that were identified previously as *A. hydrophila* using Vitek 2 system.

Identification of *Aeromonas* to the species level is difficult and complex due to their phenotypic and genotypic heterogeneity. The 16S rRNA ribosomal PCR amplifiedproduct size was 300 bp. that

selected specific primer to this gene according to Jun *et al*., [27]. The difficulty in identifying *Aeromonas* to the level of species was solved through diagnoses by viteks 2 system especially between *A. hydrophila*and *A. cavia*. However, the molecular identification of isolates confirmed that isolate 8 of *Aeromonas* spp. isolates were belonged to *A.hydrophila.*

The first attempts to identify *Aeromonas*genotypically relied upon differences in16S ribosomal DNA sequences was by Martinez-Murcia *et al.* [28], and several investigators developed probes for detection of various *Aeromonas*spp. [28, 29].Several authors referred that 16rRNA gene was a specific and a good marker in identification of all strains of *A.hydrophila* [20,30, 14].

No product was detected when genomic DNA from organisms other than *A. hydrophila* was used. 16S rRNA is a significant target to the molecular level identification. The upstream region of 16S rRNA is known to be highly conserved in species to species so this region could also be used for the verification of the thermodynamic stability on the basis of conserved secondary structures of RNA.

Different sources (other than 16S rRNA) of *A. hydrophila*have also been detected by the amplification of aerolysin gene [31], which targets 209 bp fragment of *aero* gene coding for the aerolysin toxin.

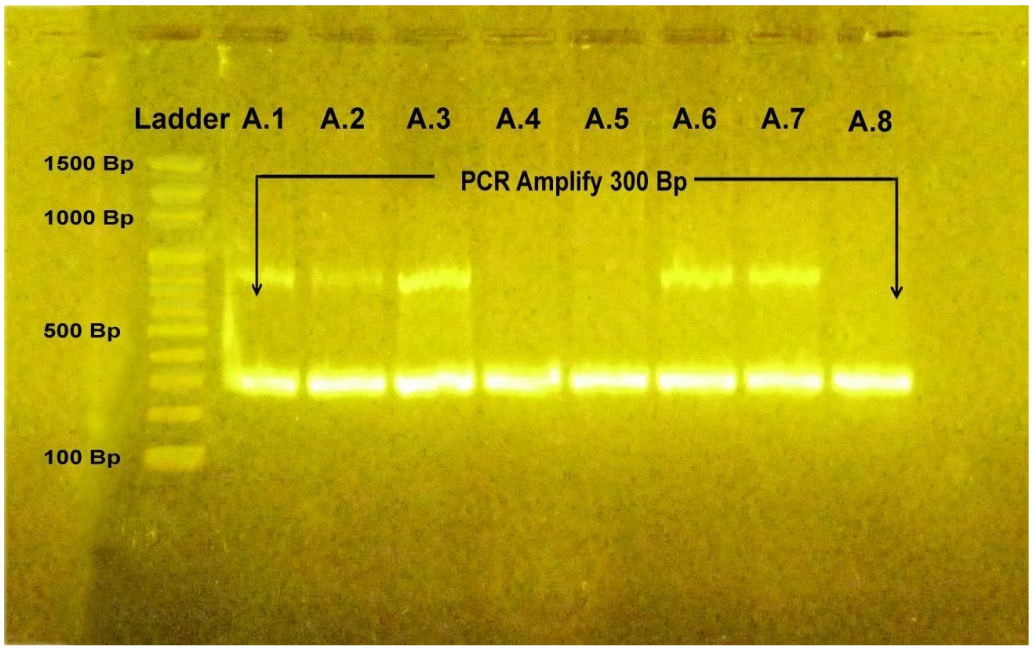

Figure 2: Ecodye stained agarose gel electrophoresis (1.2 %) of PCR amplified of *16 SRNA* gene (300) bp of *A. hydrophila* isolates

Antimicrobial susceptibility testing

Results of susceptibility testing using DDT of *A. hydrophila* isolates (No.= 8) showed that 5 isolates (62.5%) were MDR. The definition of antibiotic resistance patterns was determined according to [36] who defined the MDR phenotype as resistance to representative antimicrobial agents of at least 3 different classes of drugs. Only three isolates (S2, S3, and S5,) were sensitive to most of classes of antibiotics (Table 3). However, no isolate showed XDR or PDR pattern of resistance. Results also showed that most isolates (87.5%) were resistant to amoxicillin-clavulanic acid and gentamycin. Six of *A. hydrophila*isolates (75%)were resistant to ceftazidime, whereas more than half of

them were susceptible to amikacin, cefotaxime, and tetracycline. However 3 isolates (37.5%) were highly susceptible (100%) to imipenem, meropenem and chloramphenicol (Figure 3).

Table 3: Antibiotic susceptibility patterns of *Aeromonashydrophila* isolates

Isolate	AMC	CAZ	CTX	IPM	MEM	CN	AK	C	CIP	TE	Resistance Pattern
S1	R	R	R	R	S	R	S	S	S	S	MDR
S2	R	S	S	S	S	R	R	S	S	S	Sensitive
S3	R	S	S	S	S	R	R	S	S	S	Sensitive
S4	R	R	S	S	S	R	S	S	S	S	MDR
S5	S	R	S	S	S	S	S	S	S	S	Sensitive
S6	R	R	R	S	S	R	S	S	S	S	MDR
S7	R	R	R	S	S	R	R	S	S	R	MDR
S8	R	R	R	S	S	R	R	S	S	R	MDR
% of resistance	87.5	75	12.5	37.5	0	87.5	37.5	0	0	25	

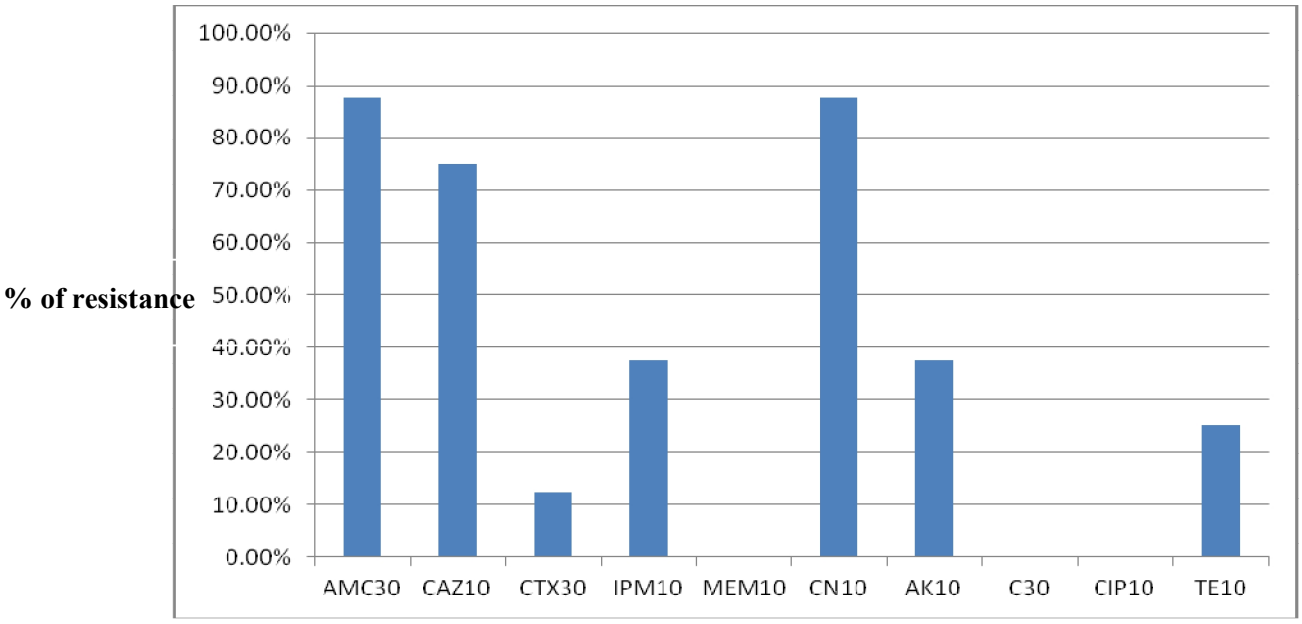

Figure 3: Antibiotic susceptibility testing of *A. hydrophila* isolates using DDT

Absolute resistance of *Aeromonas*to ampicillin and oxacillin [33]. However, in this study susceptibility of *A. hydrophila* isolated were not tested for these antibiotic due to that they are not included in CLSI documentations released from 2005 through 2014 [10, 34].

Several authors [37,38] found a similar findings regarding susceptibility to ceftazidime, cefotaxime, ciprofloxacin, and

chloramphenicol. They revealed that *A. hydrophilia*isolated from Alice showed susceptibility to these antibiotics at 99%, 100%, 83.3%, and 83.3% respectively.

Aeromonas species are slightly susceptible to gentamycin. The aminoglycosides (amikacin, gentamicin, and tobramycin) showed excellent activity against almost all the isolates of the aeromonads except a few isolates of *A. cavie*[37].

Ashiru *et al*, [38] showed that all the species of *Aeromonas* (*A. caviae*, *A. sobria* and *A. hydrophila*) isolated from were resistant to nitrofurantoin and augmentin and randomly sensitive to ceftriazone, gentamycin, cotrimozazole and amoxicillin.

Abulhamd [39] reported that a total of 10 motile *Aeromonas* strains were detected in water samples, Antimicrobial sensitivity patterns of the *Aeromonas* isolates revealed that 100% were sensitive to gentamicin, 80% to sulpha-methoxazole–trimethoprim, 70% to chloramphenicol, 50% to ciprofloxacin, 40% to neomycin, (30% to tetracycline, 20% to streptomycin and 10% to erythromycin.

Regarding to tet resistance, Ashiru*et al*. [40] revealed that *A. caviae*, *A. sobria*, and *A. hydrophila* isolated from water treatment were all resistant to tetracycline. The resistance to tetracycline has been reported to be acquired and encoded by plasmids or transposons. Tetracycline inhibition have been reported to give excellent activity against the Aeromonads [41].

Other studies showed Antimicrobial susceptibility testing of the bacterial isolates using tetracycline discs demonstrated strong resistance to tetracycline in several isolates, i.e., *Aeromonas* spp., *Citrobacterfreundi*, *Yersinia ruckeri*, *Pseudomonas putida* [42].

Antibiotic resistance frequencies and profile varied according to the source of the strains. In this sense, one isolate exhibited resistance to seven antibiotics including three aminoglycosides, tetracycline, chloramphenicol, and trimethoprim/sulfa methoxazole), two were resistant to streptomycin, two were resistant to four aminoglycosides, and three were resistant to tetracycline and trimethoprim/ sulfa methoxazole[43].

MIC of *A.hydrophila*isolates

Results of MIC of tetracycline for *A. hydrophila* isolates (NO= 8) found that only 2 isolates (25 %) were resistant to tetracycline (Table 4). The MIC of these isolates ranged from 0.125-16 µg/ml. This result confirms tetracycline resistance by these isolates when tested using DDT (Table 3).

Table 4: Determination of MIC of *A.hydrophila*isolates

Isolates NO.	MIC of tetracycline (≥ 16 µg / ml)
S1	0.125
S2	0.125
S3	0.125
S4	0.125
S5	0.125
S6	0.125
S7	≥16
S8	≥16

Changhesh *et al*. (2013) [44] recorded that the percentage of *A. hydrophila* isolated from diarrheic children (n=22) to tetracycline was 18.2%. Ko*et al*. [20] reported that fifty-one of all isolates of *A. hydrophila* from blood were susceptible to tetracycline.

Fass and Barnishan [46]carried out the MIC of 32 antimicrobial agents for 20 strains of *A. hydrophila* using by microdilution method and they found that the MIC values of tetracycline ranged from 0.5 -2 µg/ml. They also showed among the other antimicrobial agents studied, only tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole were consistently active.

Change and Bolton [47] proved differences in resistance patterns were observed between strains isolated from different geographic locations and between *A. sobria* and *A. hydrophila* isolates. They also found that susceptibility to tetracycline was high (94.36%), consistent with previous reports from Australia and the United States.

Two local studies conducted in Iraq, found that all *A. hydrophila* isolated from clinical and environmental sources had 100% sensitivity to tetracycline when tested by DDT [14,15].

Conclusion

The important conclusions in the present work can be summarized in the following points: *Aeromonashydrophila* was predominant among other species of *Aeromonas*and the isolation of *A. salmoncidia* from human specimens in the present study represented as a first record in Iraq. the results of susceptibility testing using DDT of *A. hydrophila* isolates showed that five isolates were MDR, Only three isolates were sensitive to most of classes of antibiotics. Results of MIC found that only 2 isolates were resistant to tetracycline.

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