Prevalence of efflux genes and chromosomal protection protein genes among tetracycline resistant *Aeromonas* spp. isolated from diarrheic patients in Iraq

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(Received 4 September 2015, Accepted 13 October 2015)

Abstract

The study aim to investigate the prevalence of tetracycline resistant *Aeromonas hydrophila* isolated from clinical sources in Hilla city - Iraq, using polymerase chain reaction (PCR) technique. A total of 822 samples were collected from fecal specimens from patients. Samples were collected from rectal swab (routine work) and from those who suffering from diarrhea. Isolates were identified using cultural and biochemical tests to the level of species and then identification was confirmed using viteks 2 system and molecular technique using 16S rRNA specific primer. PCR was used to detect tetracycline resistance isolates. Out of 822 clinical samples collected, 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. Results found that all tetracycline-resistant isolates carried at least one of the tet genes examined. Among efflux genes, tet(A) was the most commonly observed in isolates (No.8), found in 6 isolates (75%), followed by Efflux tet gene (tet B,C) (62.5%) and chromosomal protection protein (tetO) 5 (62.5%). Tet (E) efflux gene was found in two isolates (25%). Efflux pump (tetL) and chromosomal protect gene tet (M) was detected in one isolates (12.5%). However efflux genes tet (D and G) were not detected in any of the isolates. The prevalence of tet O chromosomal protection protein among *A. hydrophila* isolates was first recorded in Iraq and in many other countries.

Key words: *Aeromonas hydrophila*, tetracycline resistance, efflux genes, chromosomal protection protein gene, clinical sources.

سيادة جينات المضخة وجينات الحماية الكروموسومية بين انواع جرثومة الايرومونس المقاومة للتتراسايكلين المعزولة من مرضى الاسهال في العراق

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الخلاصة

هدفت الدراسة الى الكشف عن مدى انتشار بكتريا *Aeromonas hydrophila* المقاومة للتتراسايكلين والمعزولة من عينات الخروج للمرضى والوافدين لمختبر الصحة العامة في مدينة الحلة ، وذلك باستخدام تقنية تفاعل البلمرة المتسلسل ، والتي تمثلت بعض العينات بانها عمل روتيني لفحص العمال في محافظة بابل والوافدين لمختبر الصحة العامة ، وعينات اخرى تمثل اشخاص يعانون من الاسهال ومشكوك بإصابتهم بالكوليرا. تم جمع 822 عينة خروج في مختبر الصحة العامة وتم تشخيصها من خلال اجراء الاختبارات الزرعية والبايوكيميائية وتأكيد النتائج باستخدام نظام Vitek 2، حيث اظهرت النتائج انه من مجموع 822 عينه خروج فان 13 عينه فقط كانت عائدة لبكتريا *Aeromonas spp.* (1.58%) ، على الرغم من انه النسب الاخرى تعود لأنواع بكتيرية اخرى قريبة بصفاتها من بكتريا *Aeromonas spp*. كما تم تأكيد التشخيص باستخدام PCR باستخدام بادئ خاص (S rRNA16) ببكتريا *Aeromonas* ، ان 13 عزله تعود للأنواع التالية ضمن جنس *Aeromonas* : 8عزلات (61.53%) تعود للنوع *Aeromonas hydrophila* ، 4 *Aeromonas salomoncidia* ، وعزلة واحدة تعود الى النوع *Aeromonas sobria* تم في هذه الدراسة الكشف عن وجود جينات

المضخة فضلا عن جينات الحماية البروتينية الكرموسومية ، والتي تمثلت بجينات (*tet A,B,C,D,E,G,L,M,O*). حيث اظهرت النتائج أن الجين (*tet A*) كان ذا سيادة عالية ، يليه الجينات (*tet B,C,O*) بنسبه 62.5%. اما جين المقاومة (*tet E*) فقد وجد في عزلتين (25%) ووجد الجين (*tet L,M*) في عزلة واحدة. في حين لم يتم الكشف عن امتلاك أي من العزلات لجينات (*tet D,G*) ، فضلا عن انه قد تم ولأول مرة الكشف عن جين المقاومة للتتراساكلين (*tet O*) في بكتريا الايروموناس في العراق وبعض الدول الاخرى.

الكلمات المفتاحية: جرثومة الايروموناس ، المقاومة للتتراسايكلين ، مورثات المقاومة ، مورثات المقاومة الكروموسومية ، اصابات سريرية.

Introduction

Members of the genus *Aeromonas* are facultative anaerobic, rod shaped oxidase positive, gram negative bacteria, mesophilic and facultative anaerobic bacteria. 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 immune compromised persons, serious wound infections in healthy individuals and in patients undergoing medical 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 known to cause severe diarrheal disease of short duration or chronic loose stools in children, the elderly, or the immune compromised individuals, and they have been implicated in travelers' diarrhea. They are commonly isolated from fecal sample of children less than five years old, whereas their isolation from other body sites usually occurred in adult populations (4, 5). Tetracyclines belong to a family of broad spectrum antibiotics that inhibits protein synthesis in gram +ve and gram -ve 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). The aim of this study was to evaluate the incidence and spreading of *A. hydrophila* isolated from diarrheic patients in Hilla city, Iraq, and study the tetracycline resistance at molecular level by detecting efflux pump genes and chromosomal protection protein genes responsible for tetracycline resistance among these isolates.

Materials and methods

A total of eight hundred and twenty two (822) fecal samples were collected from rectal swab (routine work) and from patients suffering from diarrhea who attending public health lab, Hilla city, Iraq. The period of specimen collection and analysis was extended from October 2013 to February 2014. For isolation of and identification of bacterial isolates, all specimens were cultured on alkaline peptone water, then transfer to TCBS and MacConkey ager by swabbing and incubated at 37ºC for 24 hrs. Each primary positive culture was 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). Vitek 2 system (Biomerieux/France) was used to confirm the identification according to the manufacturer's instructions. Finally, identification was confirmed using 16S rRNA specific primer using molecular technique (PCR). For Genomic DNA Extraction, A single colony of cultivated bacteria, which had been incubated overnight, transfer to 2 ml of sterile Louria broth and incubate at 37◦ C for 18-20 hours. The DNA extracted and purified using Genomic DNA kit (EURx/Korea). All Clinical isolates were screened for chromosomal DNA according to manufacture instructions. The total DNA was used to detect 16S rRNA, effluxe pump, and

Table 1: Sequence and concentration of primers used in the study

Target	Primer	Oligo Sequence (5′→3′)	Product (Pb)	Ref.
16sRNA	F	CCA GCA GCC GCG GTA ATA CG	300bp	(Nawaz *et al*, (9)
	R	TAC CAG GGT ATC TAA TCC		
tetA	F	GCT ACA TCC TGC TTG CCT TC	211bp	
	R	GCA TAG ATC GCC GTG AAG AG		
Tet B	F	TCA TTG CCG ATA CCA CCT CAG	391bp	
	R	CCAACCATCATGCTATTCCATCC		
TetC	F	CTGCTCGCTTCGCTACTTG	897 bp	
	R	GCCTACAATCCATGCCAACC		
tetD	R	TGTGCTGTGGATGTTGTATCTC	844 bp	
	F	CAGTGCCGTGCCAATCAG		
tetE	R	ATGAACCGCACTGTGATGATG	744 bp	
	F	ACCGACCATTACGCCATCC		
tetG	R	CGGCCAAGTGCCTGCGAGCCCTATGGGTC	241 bp	Dorsch, (10)
	F	CGGGAACACCATCCATCCCTGCGTGGC		
tetL	R	GGAACACATGAGTGTGTATTAGTTTTCTGG	311 bp	
	F	CCTACAATTGCTAATACCCTGTTCCCTCTG		
TetM	R	CCTAACATGTCATTTATATGGAGAAGACC	305 bp	
	F	CGAAAATCTGCTGGCGAGTACTGAACAGGGC		
tetO	R	CCGCCAAATCCTTTCTGGGCTTCTGTCGG	354 bp	
	F	CGCCCGTGAGAGATATTCCTGCGGTGC		

Table 2: PCR conditions for efflux protein genes and ribosomal protection genes

Target	Primer denaturation	Primer annealing	Primer extension
16sRNA	94º C for 30 sec.	52º C for 30 sec.	72º C for 30 sec.
Tet A	94º C for 30 sec.	63º C for 30 sec.	72º C for 30 sec.
Tet B	94º C for 30 sec.	58º C for 30 sec.	72º C for 30 sec.
Tet C	94º C for 45 sec.	65º C for 45 sec.	72º C for 10 sec.
Tet D	94º C for 30 sec.	64º C for 30 sec.	72º C for 40 sec
Tet E	94º C for 45 sec.	65º C for 45 sec.	72º C for 10 sec.
Tet G	94º C for 30 sec.	63º C for 30 sec.	72º C for 30 sec.
Tet L	94º C for 30 sec.	52º C for 30 sec.	72º C for 30 sec.
Tet M	94º C for 30 sec.	52º C for 30 sec.	72º C for 30 sec.
Tet O	94º C for 30 sec.	62º C for 30 sec.	72º C for 30 sec.

ribosomal protection protein genes. For Plasmid DNA Extraction**,** A single colony of cultivated bacteria, which had been incubated overnight, transfer to 2 ml of sterile Louria broth and incubate at 37º C for 18-20 hours. The DNA extracted and purified using High-Speed mini DNA plasmid extraction kit (Geneaid Biotech) according to manufacture instructions. Plasmid DNA was used to detect efflux genes. Chromosomal and plasmid DNAs obtained were used as templates for all PCR experiments. The PCR reactions were carried out in a thermal cycler (Clever, U.K). Before PCR assay, and in order to quantify the DNA concentration (ng µL-1), the quantification of DNA samples was carried out by means of a spectrophotometric reading using 1 µL aliquots of Genomic DNA with a Nano-Drop TM spectrometer (Nano-Drop Technologies), adopting the manufacturer's recommendations. The concentration of DNA was estimated from absorbance at 260 nm. DNA profiles were performed using bacterial DNA and loading buffer according to the manufacturer instructions (Bioneer, Korea). Efflux protein genes (tet *A*, tet *B*, tet *C*, tet *D*, tet *E*), (tet *G* and tet *L*), and Ribosomal protection protein genes tet (*M*) and tet (*O*) were detected using different resistance primers. Also, specific primer (16S rRNA) was used for bacterial identification of *A. hydrophila*. Stock solution of this primer prepared according to the information of manufactured company listed with primers. Primer types and sequences are shown in (Table 1). Five µl of DNA was added to master mix (5 µl), then (10 µl) of primer (3

µl for up and down stream, 4 free distal water) was added to the mix information labeled with master mix. The compassions mixed carefully then placed in thermocycler (after incubation at 94°C for 5 min) and running according to conditions of each primer mentioned above. The following temperature profiles for PCR amplification of tetracycline resistance gene fragments were applied over 40 cycles (Table 2). After PCR, the profiles of amplification products were detected by gel electrophoresis according to the procedure of Sambrook and Russel (11). Five microliters of total reaction mixture was loaded on a 1.2 % agarose gel and electrophoresed at 100 V at 70 mA for 60 min. The amplified DNA fragments were visualized by UV illumination after agarose gel electrophoresis and Ecodye (and ethidium bromide) staining by standard procedures.

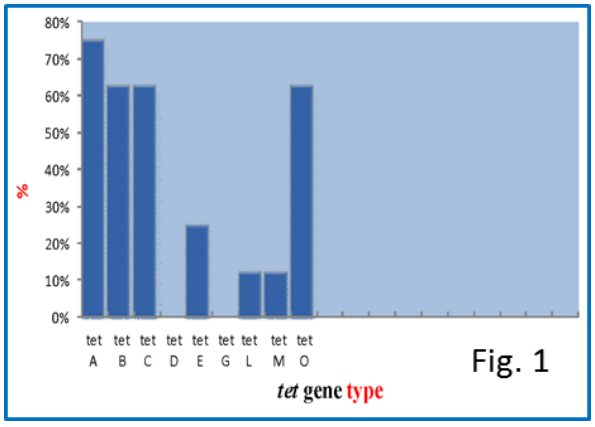
Results

Results were detected the following tet genes: Efflux (*tet A,B,C,D,E ,G and L)* genes, and ribosomal protection protein (*tet M and O)* genes. All tetracycline-resistant isolates carried at least one of the *tet* genes examined (Table 3). Regarding efflux genes, *Tet A* was the most commonly observed in *A. hydrophila* isolates (N= 8), found in 6 (75%) (Fig. 1), followed by *tet B and tet C* (Fig. 2 and 3). *Tet E* efflux gene was found in two isolates (Fig. 6) and *tet L* (Fig. 8) in one isolates. However efflux pump genes, *tet D and G*, were not detected in any of *A. hydrophila* isolates tested (Fig. 5 and 7). Regarding chromosomal protection protein, (*tet O*) was detected in five isolates (62.5%) (Fig. 9) and *tet M* (Fig. 10) was detected in only one isolate (12.5%). Results revealed that *tet O* chromosomal protection protein gene was detected in 5 isolates (62.5%) of *A. hydrophila* (Fig. 1). According to our knowledge, this result is a first record in Iraq and in many other countries.

Table 3: Genetic profile of efflux pump *tet* gene and chromosomal protection protein genes in *A. hydrophila* isolates

Tet gene	*A. hydrophila* isolate No.							
	1	2	3	4	5	6	7	8
Tet A	+	+	+	+	+	-	+	-
Tet B	+	+	-	+	+	+	-	-
Tet C	+	+	+	-	+	-	-	+
Tet D	-	-	-	-	-	-	-	-
Tet E	-	-	+	-	-	-	-	+
Tet G	-	-	-	-	-	-	-	-
Tet L	-	-		-	+	-	-	-
Tet M	-	-	-	-	+	-	-	-
Tet O	+	+	+	-	-	+	+	-

Although very little studies are available concerning tetracycline resistant *A. hydrophila* isolated from human samples, results of this study revealed the prevalence of tetracycline resistant *A. hydrophila* recovered from the clinical sources. The result of this study is a first record in Iraq.



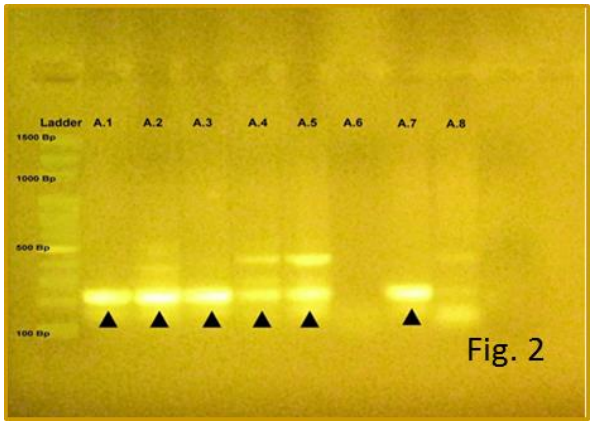


Fig. (1): Percentages of efflux pump genes and chromosomal protection genes of *A. hydrophila*

Fig. (2): Ecodye stained Agarose Gel Electrophoresis (1.2 %) of PCR Amplified products from extracted of DNA of *A.hydrophila* isolates and amplified with primer of (*tet A)* gene (211 bp).

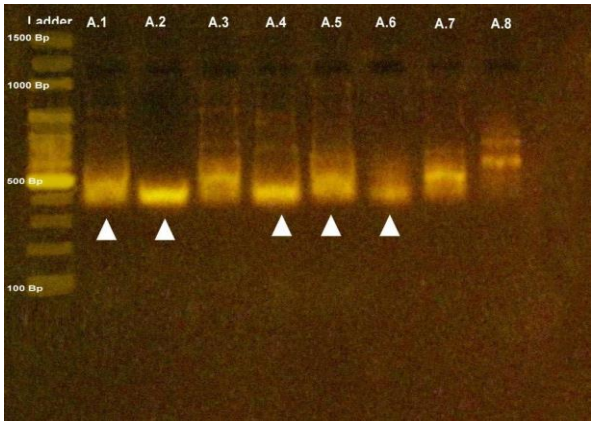


Fig. (3): Ecodye stained Agarose Gel Electrophoresis (1.2 %) of PCR Amplified products from extracted of DNA of *A. hydrophila* isolates and amplified with primer of (*tet B)* gene (391 bp).

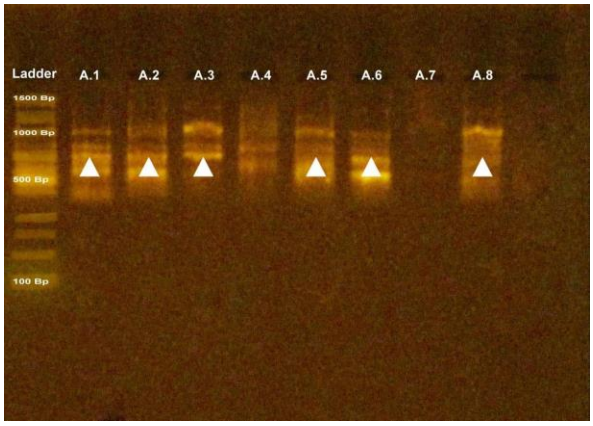


Fig. (4): Ecodye stained Agarose Gel Electrophoresis (1.2 %) of PCR Amplified products from extracted of DNA of *A. hydrophila* isolates and amplified with primer of (*tet C)* gene (897 bp).

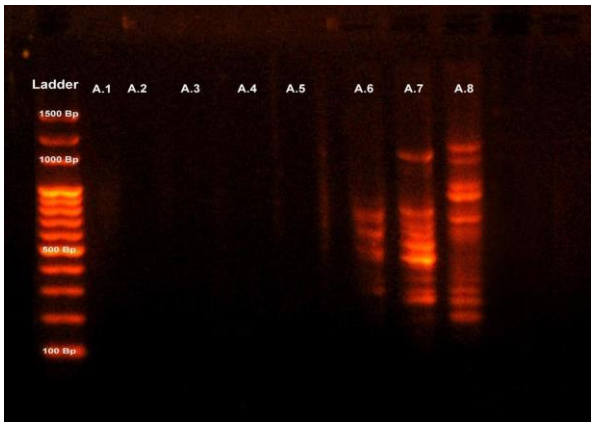


Fig. (5): Ethidium bromide stained Agarose Gel Electrophoresis (1.2 %) of PCR Amplified products from extracted of DNA of *A. hydrophila* isolates and amplified with primer of (*tet D)* gene (844 bp).

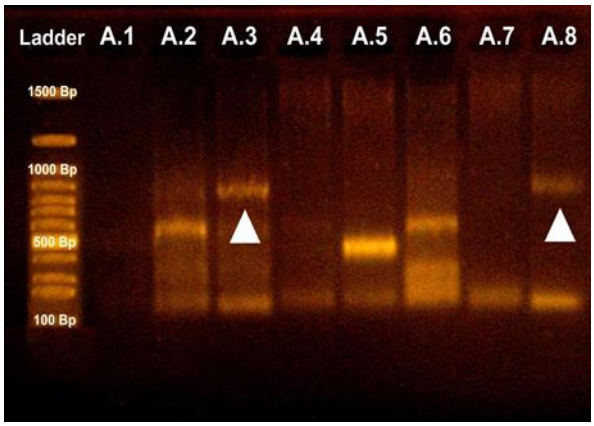


Fig. (6): Ethidium bromide stained Agarose gel electrophoresis (1.2 %) of PCR amplified products from extracted of DNA of *A. hydrophila* isolates and amplified with primer of *tet E* gene (744 bp).

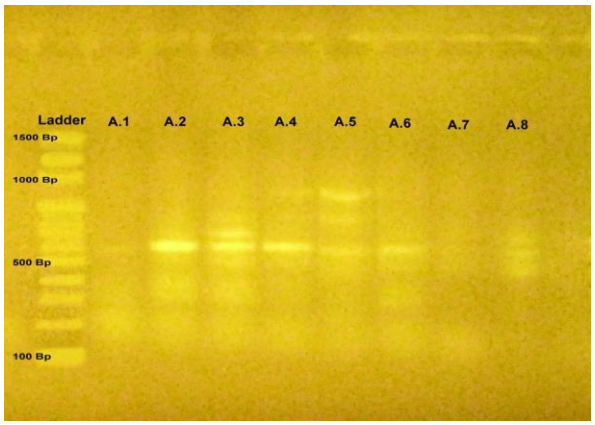


Fig. (7): Ecodye stained Agarose Gel Electrophoresis (1.2 %) of PCR Amplified products from extracted of DNA of *A. hydrophila* isolates and amplified with primer of *tet G* gene (241 bp).

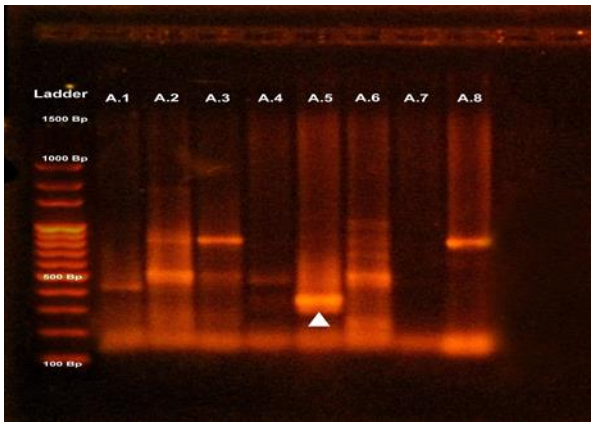


Fig. (8): Ethidium bromide stained Agarose Gel Electrophoresis (1.2%) of PCR Amplified products from extracted of DNA of *A. hydrophila* isolates and amplified with primer of *tet L* gene (311 bp).

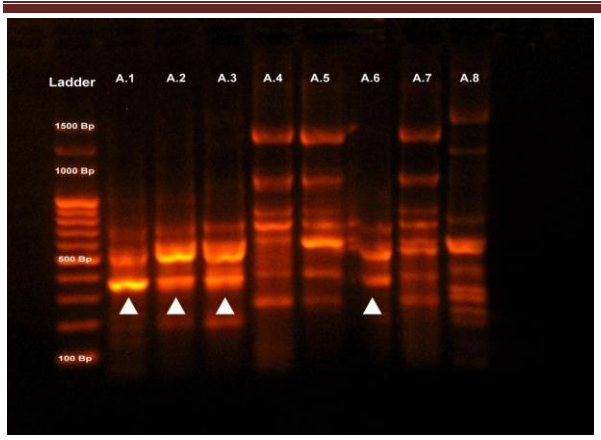


Fig. (9): Ethidium bromide stain Agarose Gel Electrophoresis (1.2%) of PCR Amplified products from extracted of DNA of *A. hydrophila* isolates and amplified with primer of (*tet O)* gene (354 bp).

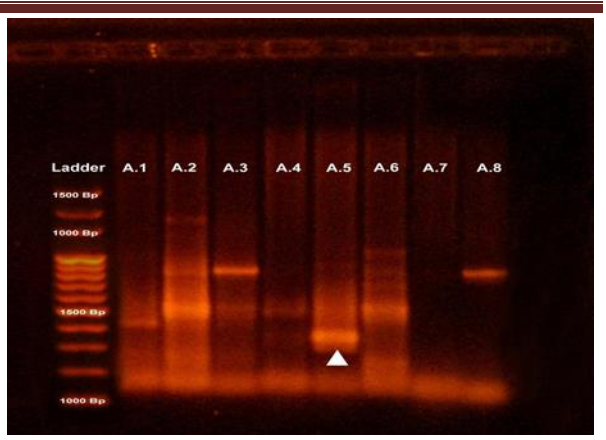


Fig. (10): Ethidium bromide stain Agarose Gel Electrophoresis (1.2%) of PCR Amplified products from extracted of DNA of *A. hydrophila* isolates and amplified with primer of (*tet M*) gene (305 bp).

Discussion

Regarding prevalence of tetracycline resistant *A. hydrophila* recovered from the clinical sources. Very little studies are available concerning tetracycline resistant *A. hydrophila* isolated from human samples. Results found that all tetracycline-resistant *A. hydrophila* isolates carried at least one of the *tet* genes examined. The result of this study is a first record in Iraq. Most of the studies focused on tet genes of *A. hydrophila* in fish because of the wide spreading of aeromonades is a result of their adaptation towards a different aqua-medium including lakes, rivers and drinking water (12,13). Furushita *et al*. (14) suggested that *tet* genes from fish farm bacteria have the same origins as those from clinical strains, they also found that sequence analysis indicated the identity in *tet* genes between the fish farm bacteria and clinical bacteria: 99.3 to 99.9% for *tet B*, 98.2 to 100% for *tet C*, 99.7 to 100% for *tet D*, 92.0 to 96.2% for *tet G*, and 97.1 to 100% for *tet Y*. Igbinosa and Okoh (15) assessed the prevalence of antibiotic-resistant *Aeromonas* species isolated from Alice and Fort Beaufort wastewater treatment plant in the Eastern Cape Province of South Africa, and they found *Tet C* was not detected in any of the *Aeromonas* isolates. Balassiano (16) analyzed the involvement of *tet A and tet E* genes in the tetracycline resistance of 16 strains of genus *Aeromonas*, isolated from clinical and food sources. Polymerase chain reactions revealed that 37.5% of the samples were positive for *tetA*, and also 37.5% were *tet E* positive. One isolate was positive for both genes. The prevalence of efflux gene (*tet A)* (75%) of *A. hydrophila* was also reported by several authors. Jacobs and Chenia (17) isolated *Aeromonas* spp. from South African aquaculture systems, and they reported the prevalence of *tet A and tet E* genes. They reported that single and multiple class A family tet determinants were observed in 27% and 48.7% of isolates, respectively, with tet A being the most prevalent tet determinant type. Hedayatianfard (18) showed that the most widely distributed resistance gene was gene *tet* A and at the least known resistance genes was *tet* (M) among the studied bacteria of the genus *Aeromonas*. On the other hand, Andersen and Sandaa (19) reported that tet A determinant was detected in only 5% of the isolates, and tet D was detected in 4% of the isolates. While they found that *tet E* found in 63% of isolates and was the most wide spread determinant in bacterial isolates obtained from polluted and unpolluted marine sediments in Norway and Denmark. Results of this study found that efflux pump gene (*tet D* and *tet G*) not detected in any of *A. hydrophila* isolates. In contrast, several authors reported prevalence efflux tet gene

(*tet D),* in *A. hydrophila* isolated from fish pathogens (20). Data from another study showed that bacterial isolates from aquaculture sources in Australia harbor a variety of tetracycline resistance genes. The tet *A*, tet *D*, tet *E and* tet *M genes* were found in Aeromonas spp. No *tet B, tet C* or *tet Y* determinants were detected in this study. Interestingly, one of our isolates also contained both *tet* A and *tet* E genes. Therefore, it is evident that tetracycline resistance determinants *tet* E, *tet* A and *tet* D (in that order) are also present in aquaculture in Australia, just as in other geographical regions. However the widespread occurrence of *tet* M in this isolates is not surprising because this class of tetracycline resistance determinant has been recently described in aquaculture (21). Furushita *et al*. (14) in a study of 66 *tet* resistant strains isolated from fishes collected at three different fish farms located in the southern part of Japan, reported prevalence of tet B in 31 of *tet* resistant bacteria, then *tet* Y in six, and *tet* C, *tet* D in four strains. Jacobs and Chenia (17) reported the occurrence of more than one *tet* resistance genes with the majority of isolates possessing two *tet* determinants with the *tet* A and *tet* E as well as *tet* B and *tet* D/*tet* H combination being observed. Three *tet* genes (*tet* D, *tet* E, *tet* M) were found in two strains of *Aeromonas* spp.

In conclusion, prevalence of tetracycline resistant *A. hydrophila* recovered from the clinical sources in this study is first record in Iraq. Very little studies are available concerning tetracycline resist in *A. hydrophila* isolated from human samples. Also the prevalence of *tet O* chromosomal protection protein among *A. hydrophila* isolates in the present study is first recorded in Iraq and in many other countries.

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