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Research Article

Antibiotic resistance profile and virulence factor genes of *Aeromonas sobria* isolated from AL-Hillah River in Babel (Iraq)

Zeena Hadi Obaid Alwan*
Department of Biology, College of Science, University of Babylon, Iraq
Rafal Ahmed Lilo
Department of Biology, College of Science, University of Babylon, Iraq
Liqaa Y. Mohsen
Department of Biology, College of Science, University of Babylon, Iraq

* Corresponding author email: sci.zeena.hadi@uobabylon.edu.iq

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Abstract
Although Aeromonas are common in aquatic habitats and have been marked as an arising risk to human health, some information dealing with antibiotic resistance profiles and virulence factor genes involved in pathogenicity are understood. The objective of this study was to evaluate the resistance profile for aquatic *A. sobria* and to identify the virulence factor genes. *Aeromonas sobria* isolates were collected from AL-Hillah River in Babel near the hospital swage water from January until May 2021. VITEK 2 system was used to diagnose isolates of the anaerobic G-ve *A. Sobria* bacteria, which were then confirmed by PCR for 16S RNA. Eight different groups of antibiotics were examined in *A. sobria* isolates using the disk diffusion method on a Mueller-Hinton agar. Genes encoding for virulence factor genes (act, ast, ela, alt, lip, asa, hly, and aer) were detected using conventional PCR. The isolates showed resistance to β-lactam drugs, while they were susceptible to ciprofloxacin, erythromycin, tetracycline, clindamycin and presented susceptibility to the gentamycin at rate 57%. Gel electrophoresis results of PCR products variably displayed clear bands for virulence factor genes (act, ast, ela, alt, lip, and asa), previously reported to be associated with some diseases. This is the first study in provinces of middle Iraqi dealing with aquatic *A. sobria* that evaluated the antibiotic sensitivity and investigated the virulence factor genes, including cytotoxic enterotoxins and enzymes. Virulence factor genes detection and 16S RNA gene for species identification were achieved by designing specific primers in the present study.

Keywords: *Aeromonas sobria,* AL-Hillah River, Antibiotics, Virulence factors genes

INTRODUCTION

Aeromonas is a genus of oxidase-positive, facultative, anaerobic G-ve bacteria that live in the aquatic environment. They are classified into two large groups based on their physiological bioactivities and host specificity. The first type consists of flagellated aeromonads, with *Aeromonas hydrophila* being a perfect representative that primarily causing human infection. The other one comprises non-motile species, such as *A. salmonicida*, which are related to fish disease (Igbinosa *et al*., 2012). Typically, biochemical tests are the prime criteria used in identifying species. However, 16S rDNA gene sequencing has proven to be useful in identifying *Aeromonas* spp. (Demarta *et al*., 1999). Recently, Aeromonas spp. have been identified as a relevant causative pathogen in gastrointestinal infections (Janda and Abbott, 1998; Pemberton *et al*., 1997; Vila *et al*., 2003), as well as extraintestinal infections such as wound infections, cellulitis, septicemia, and urinary tract infections (Abrami *et al*., 1998). Three Aeromonas species are considered clinically significant: *A. hydrophila, A. veronii* biotype sobria and *A. caviae*. (Abrami *et al*., 1998; Pemberton *et al*., 1997). Hemolysins, enterotoxins, cytotoxins, lipases, DNases, proteases, and adhesins have all been discovered as possible virulence agents in aeromonads (Cascón *et al*., 2000; Rabaan *et al*., 2001). In order to determine the pathogenicity of Aeromonas isolates, virulence genes must be identified (Yogananth *et al.,* 2009).Genes encoding for these virulence factors have been extracted and sequenced, enabling signature regions identification of these genes and the assessment of their presence in clinical and environmental isolates *(*Cascón *et al.,* 2000*;* Chacón *et al*., 2003). Furthermore, the pathogenicity of Aeromonas species is influenced by the regulation and

secretion of virulence factor mechanisms along with the host response. (Galindo *et al*., 2006; Sha *et al*., 2004; Sha *et al*., 2005). Resistant pathogens potentially result in raises of inadequate treatment (Kapil, 2005; Ventola, 2015), leading to the delay and impeding treatment (Fair and Tor, 2014; Prestinaci *et al*., 2015), as well as negative effects associated with these drugs or death (Al-Khikani and Al-Janabi, 2019; AL-Khikani and Ayit, 2020). The aim of this work was to investigate the antibiotic resistance profile and virulence factors genes encoding for cytotoxic enterotoxin (act), enterotoxin (ast), elastase (ela), cytotonic enterotoxin (alt), lipase-like protein (lip), aerolysine (asa), hemolysin (hly), and aerolysin-like toxin gene (aer) among *A. sobria* isolates collected from AL-Hillah River close to the medical wastewater.

MATERIALS AND METHODS

Sample's collection and cultural characteristics

Freshwater samples were collected from January until May 2021 from different areas of AL-Hillah River in Babel city near the hospital wastewater. The samples were collected from different regions in the same place and saved in plastic bottles before culturing in MacConkey and Blood agar for 24 hours at 37 ºC on the same day of collection. The water temperature was 18 ºC. After incubation, the growing colonies were purified by growing in the same media along with Chromogenic Agar (UTIC) selective medium and colonies were identified depending on the morphological properties (Al-Fatlawy and Al-Hadrawy, 2014).

VITEK 2 system diagnosis

To confirm the diagnosis of bacterial isolates, Standard methodologies were carried out for the rapid and accurate identification of Aeromonas at the species level. VITEK2 System was used, and the assay was carried out in accordance with the manufacturer's instructions. Plane tubes with three millilitres of normal saline were inoculated with each isolated colony (aged 24hr). The inoculum of the standardized colony was put in the cassette. The specimen identification number was added to the software system using a barcode, so the VITEK2 card was linked to the specimen ID number. The cassette was then situated in the filler module, and once the cards were filled, it was switched into the reader/ incubator module.

Primer design

Primers have been designed based on the sequence data available for *A. sobria* in the database of NCBI for 16S RNA and for each virulence gene Reference Sequences. Several accession data were aligned together to find the consensus sequence and avoid the possibility of the mutation that may affect primer binding specificity. The nucleotide sequences of the primers are shown in Table 1.

PCR protocol

Genomic DNA extraction was achieved using a bacterial genomic extraction kit (Supplied from Solarbio). Molecular identification and subtyping of *Aeromonas* isolates were completed by using of 16S rDNA gene conserved region amplified by PCR technique and eight genes (Act, Aer, Alt, Asa, Ast, Ela, Hly, and Lip) encoding for virulence factor were also determined. PCR protocol was applied based on the instruction of the Supplier company (Solarbio), 2X hot start master mix was used. The protocol of PCR was as follows- the first step is denaturation at 95°C for 5 minutes followed by 30 cycles of 95°C (30 sec.), annealing temperature was according to what is mentioned in Table 1 for 25 sec., and the extension was done at 72°C 1 min/kb, the final extension was 72°C for 10 min.

Antimicrobial susceptibility test

The identified isolates by VITEK 2 system and PCR assay were tested against 8 antibiotics (Erythromycin E 15µg, Tetracycline TE 10 µg, Gentamycin CN 10 µg, Penicillin P 10 µg, Ampicillin Am 25µg, Clindamycin DA 15µg, Ciprofloxacin CIP 10 µg, Amoxicillin Ax 25 µg) as described by (Humphries *et al*., 2018; Borty *et al*., 2016), using the disk diffusion method. To prepare the inoculum, freshly bacterial colonies grown on nutrient agar medium were inoculated in 5 ml sterile normal saline and the turbidity was compared with (1.5X 108 cl/ml) McFarland standard tube (0.5). From this suspension, 100 µl were streaked evenly on Mueller-Hinton agar (Lab M, UK) and left to dry, then incubated overnight at 37C. After incubation, the inhibition zone was measured and the results were interpreted into sensitive, resistance, and intermediate according to the CLSI (2016) (Borty *et al.,* 2016).

RESULTS

Isolation and identification of *A. sorbia*

For identification of *A.sorbia, the colonies on MacConkey agar* were pale shaped, whereas, on Blood agar, they were smooth, convex, and spherical. Moreover, they produce dark blue color in UTI Chromogenic agar (Fig. 1). From the total isolates, 22% were distinguished as *A. sobria* by VITEK 2 system using the analytical profile index of this system (the identification percentage is id% = 93.8). The biochemical tests of the identification profile of VITEK 2 system are listed in Table 2. The PCR amplification confirmed the identification for the targeting sequence in the 16S rDNA, including the general fragment for all *Aeromonas spp.* at

Table 1. Primer designing for 16SrRNA and virulence factor genes of A. sobria

Gene name	primer	Sequence	Tm (°C)	PCR product length (bp)	NCBI accession number used for primer design
16S rRNA gene	F-rRNA16	CAGTTGGAAACGACTGCTAATAC	58	656	NR_043638.1, NR_037012.2
	R-rRNA16	GTGGACTACCAGGGTATCTAATC			
	R_rRNA16_Sobria	GCTGACAGATATTAGCTGCCAAC	58	325	
Cytotoxic enter-otoxin (act)	Act-F	CTACAAGGCTGACATCTCCTATC	57	193	KP942445.1, KP942444.1, KP942443.1, KP942442.1, and KP942441.1
	Act-R	CTTGTCCCACTGGTAACGAATG			
Aerolysin-like toxin gene (aer)	Aer-F	CGGTAATCACAGCCAATATGTCG	57	694	AF443394.1, AF443393.1, and AF443392.1
	Aer-R	TTGTCCCACTGGTAGCGAATGC			
Cytotonic enter-otoxin (alt)	Alt-F	CATCGCCAATGCCCTGAAAGC	59	367	KP942461.1 and KP942457.1
	Alt-R	CTGCATGCCGGCATAGATCTG			
Aerolysine (asa)	Asa_F	CTGGAACCCGACTCCTTCAGC	60	143	JX293341.1, JX293340.1, and JX293339.1
	Asa_R	CAGTTGGTGGCCTTGTCGTAC			
Enterotoxin (ast)	Ast_F	CCGCTGGTCAATACCCGGG	59	160	HG977092.1, HG977091.1, and MN748903.1
	Ast-R	GGGCCTCGTTGAGGAAGCG			
Elastase (ela)	Ela-F	CACTACTTCGGCAACGTGGTG	58	357	MN210396.1, KY753923.1, KY753921.1
	Ela-R	GCCGTTGCCCTTGAAGATCTG			
Hemolysin (hly)	Hly-F	CAACCTTGTCAGCTCTGGATATTC	57	260	KP942374.1 and KP942375.1
	Hly-R	GACCTTGATGGCCGTCTTCTC			
Lipase-like protein (lip)	Lip_F	CAGGAGCAGACCATCAACGG	57	212	AB206038.1 and AB206037.1
	Lip_R	GTGTTGCCCTTGGCATCCTG			

656bp and specific target sequence in *A. sobria* species at 325bp as shown in Fig. 2.

Antimicrobial susceptibility test

The results of *A. sobria* isolates tested for antibiotic susceptibility according to (Borty et al., 2016, Cockerill *et al*., 2012) showed that all *A. sobria* isolates were resistant to the Ampicillin, Amoxicillin and Penicillin. Conversely, all the isolates were susceptible to the Ciprofloxacin and Erythromycin antibiotics and 85% and 71% were also susceptible to the Tetracycline and Clindamycin, respectively. However, isolates presented susceptibility to Gentamycin at the rate of 57% (Fig.3).

Detection of virulence genes

The presence of multiple virulence genes was common among isolates of *Aeromonas* sps. virulence factors genes encoding for cytotoxic enterotoxin (act), enterotoxin (ast), elastase (ela), cytotonic enterotoxin (alt), lipase-like protein (lip), aerolysine (asa), hemolysin (hly), and aerolysin-like toxin gene (aer) among *Aeromonas sobria* have been identified. Gel electrophoresis results of PCR products (Fig. 4) presented bands with sizes 193bp, 160bp, 357bp, 367bp, 212bp, and 143bp for the virulence factor genes act, ast, ela, alt, lip, and asa, respectively. Genes of ast and alt were presented only in 80% of isolates. However, no bands were detected for the aer and hyl genes.

DISCUSSION

In the present study, 22% of isolates were identified as bacteremia-related to *A. sobria*, previously reported as naturally occurring aquatic organisms (Ashbolt *et al*., 1993). Important types of aeromonads may be a useful key to water quality to some extent because they were the initial bacteria to re-grow (from biofilms) in drinking water after the disappearance of disinfectant residuals (Araujo *et al*., 1991). VITEK 2 (an automated bacterial identification system) was dependent as it provides rapid, reliable and highly reproducible results for identifying bacterium as *A. sobria*. (Ling *et al*., 2001; Ling *et al*., 2003; Kabroot AL-Fatlawy *et al*., 2021). It is characterized by rapid detection of bacterial species without wasting numerous culture media and, simultaneously, reduces the contamination of bacterial cultures. For the respective gene, PCR of the 16S rRNA was dependent on confirming the identification since

Table 2. Details of biochemical tests for VITK 2 system of A. sobria

Biochemical details	Results	Biochemical details	Results
Ala-Phe-Pro-arylamidase (APPA)	+	L- Arabitol (IARL)	-
H2S production (H2S)	-	D- Glucose (dGLU)	+
Beta-glucosidase (BGLU)	-	D-Mannose (d MNE)	+
L-Proline arylamidase (ProA)	+	Tyrosine arylamidase (TyrA)	+
Saccharose /Sucrose (SAC)	+	Citrate (Sodium) (CIT)	+
L- Lactate alkalinisation (ILATk)	-	Beta-N-acetyl-galactosaminidase (NAGA)	-
Glycine arylamidase (GlyA)	-	L- Histidine assimilation (IHISa)	-
O/129 resistance [comp.vibrio.] (O129R)	+	Ellman (ELLM)	+
Adonitol (ADO) -	-	D-cellobiose (dCEL)	-
Beta –N- acetyl-glucosaminidase (BNAG)	+	Gamma-glutamyl transferase (GGT)	-
D-Maltose (dMAL)	+	Beta-xylosidase (BXYL)	-
Lipase (LIP)	-	Urease (URE)	-
D- Tagatose (dTAG)	-	Malonate (MNT)	-
Alpha-glucosidase (AGLU)	-	Alpha- galactosidase (AGAL)	-
ORNITHINE DECARBOXYLASE (ODC)	-	Coumarate (CMT)	+
Glu-Gly-Arg-arylamidase (GGAA)	+	L- Lactate assimilation (ILATa)	-
L-Pyrrolydonyl- arylamidase (PyrA)	-	Beta-galactosidase (BGAL)	+
Glutamyl arylamidase pNA (AGLTp)	-	Fermentation/glucose (OFF)	+
D-Mannitol (dMAN)	+	Beta-alanine arylamidase pNA (BAlap)	-
Palatinose (PLE)	-	D-Sorbitol (dSOR)	-
D- Trehalose (dTRE)	+	5-Keto -D- Gluconate (5KG)	-
Succinate alkalinisation (SUCT)	+	Phosphatase (PHOS)	-
Lysine decarboxylase (LDC)	-	Beta- glucoronidase (BGUR)	-
L-Malate assimilation (IMLTa)	+		

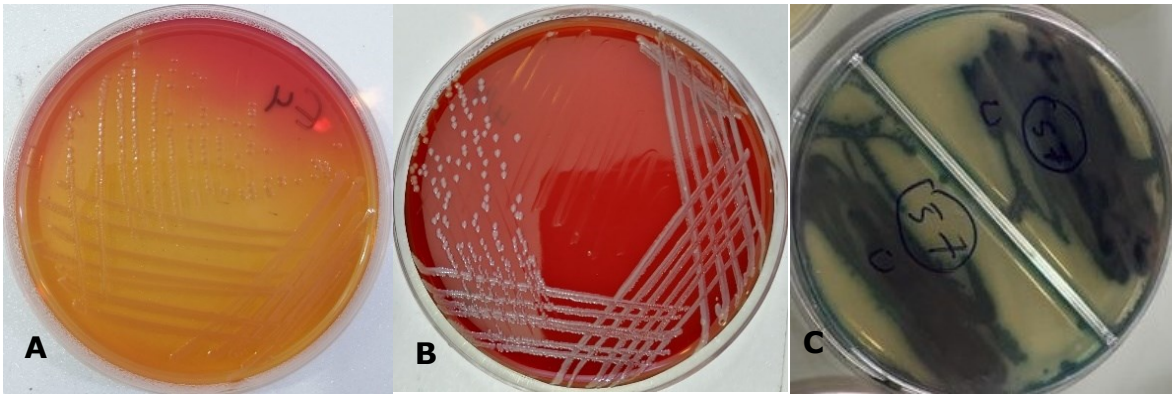


Fig. 1. *Aeromonas sobria colonies in A- MacConkey, B- Blood, and C- UTI Chromogenic Agar media*

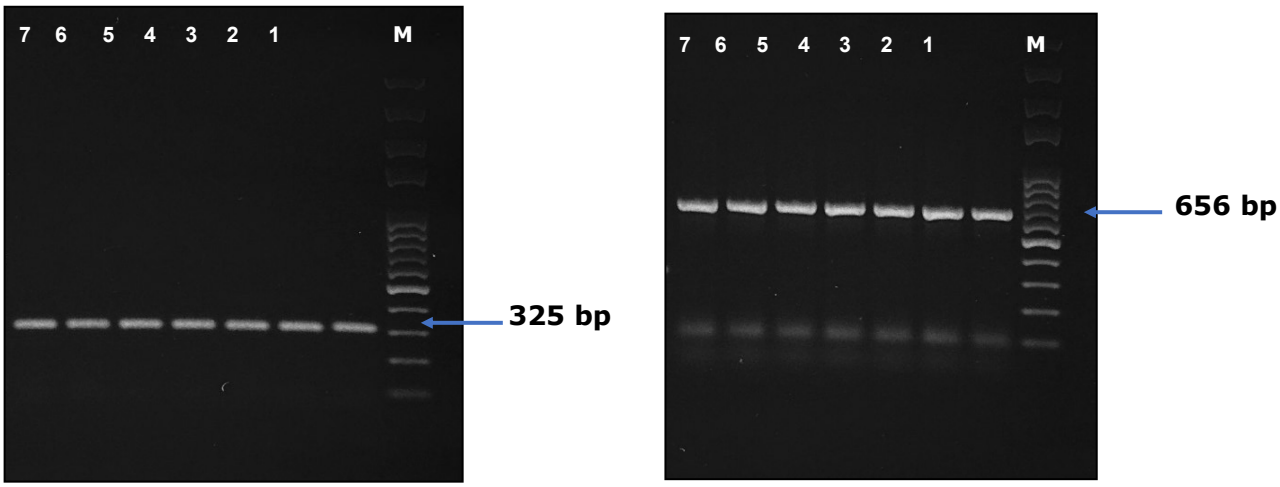
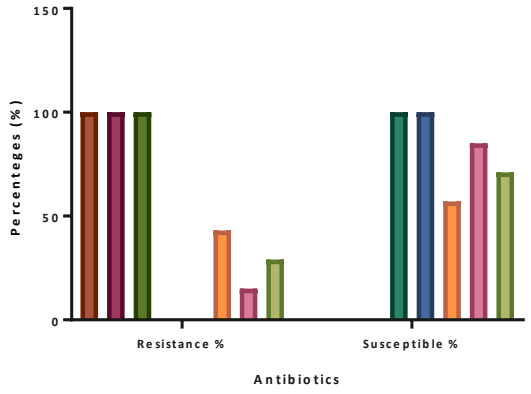


Fig. 2. *Agarose gel electrophoresis photo shows PCR product of 16S RNA: A- PCR product with bands size 325 bp using primers specific for A. sobria, B- PCR product with bands size 656 bp by using primers aligned with Aeromonas sps. M: 100 bp DNA ladder and 1-7 represents isolates. Samples run in 1% Agarose gel at 100 volts for 1 hour*

this protocol was performed by designing specific primers targeting the 16S RNA conservative sequences, including region-specific for *Aeromonas* spp. as well as the particular sequence for *A. sobria* sp. The sizes of the PCR products were compared with the results of NCBI accession number used for primer designing (NR_043638.1, NR_037012.2) for this species; therefore, all isolates were mainly confirmed and related to the above species.

Although aeromonads are common in water ecosystems and have been classified as a certain risk for human health (Shope and Oaks, 1992; Zhang *et al*., 2021), little knowledge is obviously investigated about the resistant profile of aeromonads from freshwater systems for the antibiotic and metal, including shallow rural and urban playa lakes. According to (Alcaide *et al*., 2010), the resistance of Aeromonas spp. to antimicrobial drugs has risen because emerging resistance was determined not only in clinical types but also in bacteria found in food and water (Saavedra *et al*., 2004). In this study, *A. sobria* isolates showed resistance to the β-lactam antibiotics, including Ampicillin, Amoxicillin and Penicillin. However, they all were susceptible to the Ciprofloxacin (Quinolones) and Erythromycin (Macrolides) antibiotics. Resistance to all β-lactam antibiotics was also detected in *A. sobria* isolates from patients who had symptoms of diarrheal infections in Najaf governorate (Kabroot AL-Fatlawy *et al*., 2021) . Moreover, 85% and 71% of isolates were also susceptible to Tetracycline (Tetracyclines) and Clindamycin (lincosamides). Some isolates presented susceptibility to Gentamycin (Aminoglycosides) at a rate of 57%. Aeromonas spp. produce a variety of β-lactamase enzymes, so it can display resistance to a wide range of β-lactam antibiotics, as that agrees with our findings (Chen *et al*., 2012). This genus produces these enzymes and expresses them coordinated, indicating that a single mechanism controls the expression. Aeromonas isolates are sensitive to quinolones, although rarely, resistance to these drugs is reported and that is compatible with the results adopted by (Mudryk *et al*., 2015) for Aeromonas spp. isolated from the Ustka beach, which showed resistance to β-lactam and lincosamide antibiotics, whereas the higher percentage of isolates display sensitivity to ciprofloxacin, gentamycin, erythromycin, oxytetracycline and tetracycline. The



Antibiotic	Susceptible (%)	Resistant (%)
Ampicillin	0	100
Penicillin	0	100
Amoxicillin	0	100
Erythromycin	100	0
Ciprofloxacin	100	0
Gentamycin	57	43
Tetracycline	85	15
Clindamycin	71	29

Fig. 3. *Antibiotic resistance pattern in Aeromonas sobria*

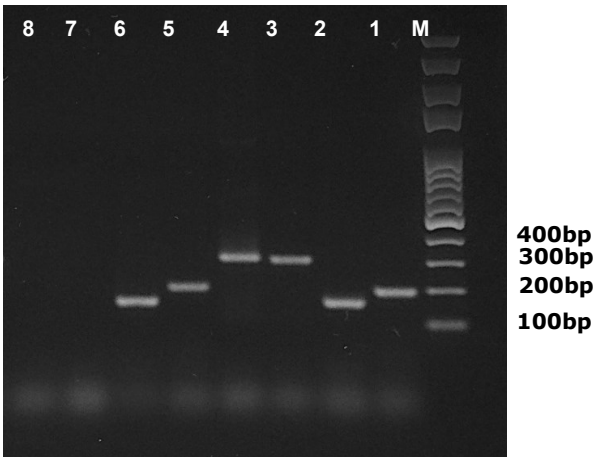


Fig. 4. *Agarose gel electrophoresis photo shows PCR product of several virulence factor genes (act, ast, ela, alt, lip, and asa) in A. sobria with bands sizes 193, 160, 357, 367, 212, and 143 bp respectively. No PCR product was achieved for hyl (260bp) and for aer (649bp) in lanes 8 and 9 respectively. M: 100 bp DNA ladder and 1 -7 represents virulence factor genes. Samples run in 1% agarose gel at 100 volts for 1 hour*

key point for therapeutic option participation, the use of antibiotics against the infection with Aeromonas sp. is the development of resistance and rapid adaptation to new generations of antibiotics, posing a risk to public health (Belém-Costa and Cyrino, 2006). However, the health risk of Aeromonas in recreational waters is frequently overlooked because their presence in water is unrelated to the coliform concentration (the traditional indicator of faecal pollution) (Dumontet *et al*., 2000).

Aeromonas is the major pathogens in fish (Austin and Adams, 1996) and they have been linked to diarrhoea disease in humans and wound infections (Janda and Abbott, 1998; Huddleston *et al*., 2006; Kühn *et al*., 1997; Kabroot AL-Fatlawy *et al*., 2021). Exposure to contaminated water causes many opportunistic wound infections. Moreover, accidents involving swimming and boating, alligator bites, and fishing hooks have all been considered reasons for infected people with Aeromonas (Janda and Abbott, 1998). The isolated Aeromonas species from Nile tilapia and Mugil cephalus was not only related to the fish pathogens but also concerned with the consumers' health (Kishk *et al*., 2020).

Out of eight virulence factor genes screened, six genes, encoding for cytotoxic enterotoxin (act), enterotoxin (ast), elastase (ela), cytotonic enterotoxin (alt), lipase-like protein (lip), aerolysine (asa), were detected in the *A.sobria*. Bacterial isolates having a variety of combinations of these genes, ast and alt were presented only in 80% of isolates. Genes encoding for hemolysin (hly), and aerolysin-like toxin (aer) have not been presented in PCR products. (Sen and Rodgers, 2004) revealed the presence of 6 virulence factor genes in *Aeromonas spp*., elastase (ahyB), lipase (pla/lip/lipH3/alp-1) flagella A and B (flaA and flaB), the enterotoxins, act, alt and ast, using PCR and out of 205 isolates, only one isolate including all these virulence genes. However, results by (Roges *et al*., 2020) indicated that among different sources of *A. hydrophila* isolates, at least 92.7% presented one of the virulence factor genes and the most variation was identified in human isolates than in food sources and animals. Some extracellular virulence factor genes associated with Aeromonas have been well described including exotoxins, cytotoxins, cytotonic toxins, dermonecrotic toxins, leucocidins, cytolytic proteins, hemolysins which confer the ability to bind to and destroy epithelial cells, to explain the process of pathogenically of these organisms (Hatha *et al*., 2005; Matyar *et al*., 2010). The aerA and altA virulence factor genes were identified with percentages 64.2% and 38.5%, respectively, in *A. hydrophila* isolates from Nile Tilapia-affected fishes and 30.8% in Mugil cephalus fishes (Kishk *et al*., 2020).

Exotoxins, cytotoxins, cytotonic toxins, dermonecrotic toxins, leucocidins, cytolytic proteins, and hemolysins are exogenous virulence factors that can bind to and enter epithelial cells, which helps to explain how these organisms become pathogens.

Conclusion

The antibiotic resistance profile of *A. sobria* isolated from AL-Hillah River near the hospital sewage water has been well identified and all isolates were resistant

only to β-lactam antibiotics and displayed variable susceptibility to other groups of antibiotics. These isolates variably displayed clear bands of virulence factor genes, previously reported to be associated with some human diseases. Numerous opportunistic infections of wounds could result from exposure to contaminated water with Aeromonas sps. due to their extracellular virulence factors that support the capacity to attach and invade epithelial cells. This is the first study in provinces of middle Iraqi dealing with aquatic *A. sobria* that evaluated the antibiotic sensitivity and investigated the virulence factor genes. 16S RNA and Virulence factor genes detection was achieved by designing our own specific primers for the conventional PCR. Further studies are required for different sources of isolates of this bacterium to evaluate their emerging sensitivity to different antibiotics and to monitor the virulence and adhesion factors.

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Ethical approval

The plane and sampling protocol were reviewed and approved by the University of Babylon/ College of Science ethical committee.

Conflict of interest

The authors declare that they have no conflict of interest.

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