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Phenotypic, molecular detection, and Antibiotic Resistance Profile (MDR and XDR) of *Aeromonas hydrophila* isolated from Farmed *Tilapia zillii* and *Mugil cephalus*

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Abstract

In the present study, *Aeromonas hydrophila* was isolated from *Tilapia zillii* and *Mugil cephalus* samples collected during different seasons from various Suez Canal areas in Egypt. The prevalence of *A. hydrophila*, virulence genes, and antibiotic resistance profile of the isolates to the commonly used antibiotics in aquaculture were investigated to identify multiple drug resistance (MDR) and extensive drug-resistant (XDR) strains. In addition, a pathogenicity test was conducted using *A. hydrophila*, which was isolated and selected based on the prevalence of virulence and resistance genes, and morbidity of natural infected fish. The results revealed that *A. hydrophila* was isolated from 38 of the 120 collected fish samples (31.6%) and confirmed phenotypically and biochemically. Several virulence genes were detected in retrieved *A. hydrophila* isolates, including aerolysin *aer*A (57.9%), *ser* (28.9%), *alt* (26.3%), *ast* (13.1%), *act* (7.9%), *hly*A (7.9%), and *nuc* (18.4%). Detection of antibiotic-resistant genes revealed that all isolates were positive for bla_{pse1} (100%), bla_{SHV} (42.1%), *tet*A (60.5%), and *sul*1 (42.1%). 63.1% of recovered isolates were considered MDR, while 28.9% of recovered isolates were considered XDR. Some isolates harbor both virulence and MDR genes; the highest percentage carried 11, followed by isolates harboring 9 virulence and resistance genes. It could be concluded that the high prevalence of *A. hydrophila* in aquaculture species and their diverse antibiotic resistance and virulence genes suggest the high risk of *Aeromonas* infection and could have important implications for aquaculture and public health.

Keywords Prevalence, *Aeromonas hydrophila*, Virulence genes, Antibiotic resistance, cultured freshwater fish

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Introduction

Aeromonas is a ubiquitous Gram-negative bacterial pathogen, considered the causative agent of septicemic diseases, such as hemorrhagic septicemia, epizootic ulcerative syndrome, and motile *Aeromonas* septicemia [1]. However, several species of the genus *Aeromonas* (*Aeromonas sobria, Aeromonas caviae, Aeromonas veronii*) are known to cause fish diseases. *Aeromonas hydrophila* is considered the main pathogen affecting farmed and wild fishes, leading to mass mortalities in aquaculture and mariculture systems with severe losses of millions of dollars annually [2]. *A. hydrophila* has been linked with general clinical signs of septicemia, including extensive hemorrhages, hemorrhages at the base of fins, tail and fin rot, body ulceration, swelling, and abdominal distention [3–5].

The pathogenicity mechanism of *Aeromonas* species is complicated and multifactorial, it is directly correlated to the presence of single or multiple virulence genes that encode extracellular products and toxins, allowing bacterial invasion, multiplication, and colonization in host tissue, thus disease development occurs [6]. So, the molecular detection of these virulence genes is an essential step in determining the potentiality of pathogenic *Aeromonas* [7]. Heat-stable cytotonic enterotoxin (*ast*), heat-labile cytotonic enterotoxin (*alt*), cytotoxic enterotoxins (*act*), aerolysin (*aer*) [7, 8], hemolysin, adhesins, and cytotoxins [9] are the most common virulence genes detected in the pathogenic strains of *A. hydrophila* isolated from different fish species worldwide.

The use of antibiotics in treatment of bacterial diseases is a common practice in the aquaculture sector, it comes as an effort to control such bacterial infections and disease outbreaks in aquaculture. However, the unlimited and widespread inappropriate use of antibiotics in aquaculture for the treatment of bacterial infections result in antibiotic resistance has been developed in numerous fish pathogens globally [10]. Moreover, there is a risk associated with the transmission of the bacteria containing antimicrobial resistance genes from aquaculture to humans via the accumulation of antibiotic-resistant genes in fish by-products [11]. Thus, in the long run, this will inhibit the beneficial microbiota in the human gastrointestinal tract and reduce the effectiveness of antibiotics in treating human diseases [12–15].

The association/combination between virulence factors and antimicrobial resistance genes within *Aeromonas* bacteria is of great concern, as it reflects bacterial fitness and survivability duration and mechanism within its host Ramadan, et al. [16]. Therefore, the aim of this study was to assess the prevalence of potentially pathogenic *A. hydrophila* isolated from *Tilapia zillii* and *Mugil cephalus* collected from the Suez Canal region of the Ismailia governorate, Egypt; through molecular identification of different virulence genes and assess the multiple drug resistance (MDR) and extensively drug-resistant (XDR) genes present in *A. hydrophila* isolates against commonly used antibiotics and antibiotic agents. Then, a pathogenicity test was conducted using *A. hydrophila* isolates selected based on their prevalence of virulence genes, and the survivability and morbidity of infected fish.

Materials and methods

Sampling and clinical examination

A total of 120 clinically affected fish (60 *Tilapia zillii* and 60 *Mugil cephalus*) were randomly collected freshly dead or moribund from different private fish farms within Suez Canal areas, Ismailia Governorate, Egypt, during different seasons ($n=15$ each season/ each species). Fish with external lesions, such as hemorrhages, fin rot, distended abdomen, and skin darkening were transferred in an ice box (-4 ℃) to the laboratory of the Microbiology Department, Animal Health Institute, for immediate bacteriological analysis. General characteristics and clinical signs of all moribund fishes were observed and recorded following Austin, Austin [17].

Bacterial culturing and isolation

For the detection of *A. hydrophila*, fish external skin surface was first disinfected by spraying with 70% ethyl alcohol before conducting the postmortem examination, as described by Austin, Austin [17]. Kidney, spleen, liver, and gills samples were obtained from each fish and aseptically streaked on Rimler-Shotts (R-S) agar media (HiMedia, India) and *Aeromonas* agar base media (HiMedia, India) supplemented with rehydrated ampicillin (Oxoid®, USA), cultured plates were incubation at 29 °C for 18 to 24 h. *A.hydrophila* colonies were picked and subcultured for purification and bacterial morphology assurance analysis according to Quinn, et al. [18]. Then the purified isolates were kept in Tryptic soy broth containing 20% glycerol (v/v) at -20 °C for further biochemical and molecular investigations.

Bacterial identification

Phenotypic characterization

Conventional phenotypic characterization were performed including the following: characterization of colonial morphology (shape and color), Gram staining, motility testing, cytochrome oxidase, catalase, and oxidation fermentation test (O/F). Different isolates were evaluated for sensitivity to novobiocin antibiotic. The hemolytic activity was detected by streaking the bacterial colonies on TSA supplemented with 5% sheep red blood cells Quinn, et al. [18]. The bacterial proteolytic activity was assessed by plating isolates on brain heart infusion agar with 1% egg yolk and incubated at 37 °C for 48 h

[19]. The API-20 NE kit (Biomerix, France) is used for further confirmation of retrieved *A. hydrophila* isolates.

Bacterial genotyping

For molecular identification, genomic DNA was extracted from purified fresh *A.hydrophila* colonies, using the QIAamp® DNA Mini Kit (Cat. No. D4068, Germany) as directed by the manufacturer. The PCR reaction was conducted in a total volume of 25 µl, which comprises 12.5 µl of PCR master mix (Takara, Japan), 1 µl (20 pmol) of each forward and reverse primers (Invitrogen, Carlsbad, CA, USA), 4.5 µl nuclease-free water, and 6 µl (10 ng/µl) of DNA template. The reaction mixture was done in a T3 Thermal cycler, (Biometra GmbH, Göttingen, Germany). *A. hydrophila* strains were primarily confirmed using the *16SrRNA* gene according to Stackebrandt, et al. [20]. A positive control (*A. hydrophila* ATCC 7966) and a negative control (the reaction mixture without a DNA template) were included with each run. A 100 bp (DNA marker) was utilized to determine the appropriate size of the magnified products. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in Tris borate EDTA buffer and the gel documentation system (Alpha Imager 2200) was used to visualize the gel.

Sequencing and phylogenetic analysis

The amplified bands of *A. hydrophila* were sequenced, and the sequence was analyzed using the MEGA 11 software program and blasted on NCBI [20]. The sequence obtained from NCBI was imported for multiple sequence alignment using the Clustal W program, followed by phylogenetic tree construction using the neighbour-joining with 1000 bootstrap method following Kumar, et al. [20].

Antimicrobial susceptibility testing

Twelve antimicrobial agents belonging to seven antimicrobial classes were used to test *A. hydrophila* isolates susceptibility using the disk diffusion method on Muller-Hinton agar (HiMedia, India) according to the Clinical and Laboratory Standards Institute (CLSI) [21]. Antimicrobials tested (Oxoid, Hampshire, England, UK) were ampicillin (AM, 10 µg), amoxicillin-clavulanic acid (AMC, 30 µg) and oxacillin (OX, 5 µg) belonging to β-lactams class. cefadroxil (CFD, 30 µg) and cefotaxime (CTX, 30 µg) belong to class cephalosporines. amikacin (AK, 30 Mg) and gentamicin (GM, 10 µg) belong to the class Aminoglycosides. ciprofloxacin (CIP, 5 Mg) and levofloxacin (LEV, 5 Mg) belong to class fluoroquinolones. Polymyxin B (PB, 10 µg) belongs to the class polymyxins. Doxycycline (DO, 30 µg) belonging to the class tetracyclines and trimethoprim/sulfamethoxazole (SXT, 25 µg) belonging to the sulfonamides class. The test accuracy was determined using *Escherichia coli* ATCC® 25,922 (Manas sas, VA, USA) as a control group. The diameters of the inhibitory zones were evaluated using standards [21]. The multiple antibiotic resistance (MAR) index was evaluated and elucidated for each isolate based on Krumperman [22], using 0.2 as the modal value.

Virulence and multiple antimicrobial resistance (MAR) genes detection

Molecular identification for detection of virulence was carried out for *A. hydrophila* isolates using specific primers (Invitrogen, Carlsbad, CA, USA) of aerolysin (*aer*A), serine protease (*ser*), *Aeromonas* cytotonic heat-labile enterotoxins (*alt*), *Aeromonas* cytotonic heat-stable enterotoxins (*ast*), cytotoxic enterotoxin (*act*), haemolysin *(hly*A), nuclease (*nuc*) and adhesion (*aha*) virulence genes. The antimicrobial resistance of the retrieved isolated was confirmed by the detection of antimicrobial resistance genes β-lactamase (bla_{pse1}), β-lactamase (bla_{SHV}), sulfonamide (*sul1*), tetracycline (*tet*A). The nucleotide sequence and cycling conditions of the used primers are listed in Table 1.

Pathogenicity test

Two hundred and forty (240) apparently healthy *T. zillii* were obtained from a private fish farm at West Qantara, Suez Governorate, Egypt, with an average body weight of 30.00±3.8 g. The fish were transported to the National Institute of Oceanography and Fisheries, Suez Governorate, Egypt, and acclimated for two weeks in a 1000 L fiberglass tank supplied with de-chlorinated water with continuous oxygen aeration using electric air pumping compressors before the challenge. Fish were fed a commercial pelletized diet twice daily at 3% of their body weight. The water temperature in the aquaria was thermostatically controlled at 26±2 °C [23].After acclimatization, fish were divided into eight groups in duplicate (30 fish/group). The first group received 0.2 mL of sterile normal saline intraperitoneally (IP) as a negative control, whereas the other seven groups received 0.2 mL of an overnight *A. hydrophila* culture at 3×10^8 CFU/mL. The inoculated bacteria was firstly selected for its high virulence and resistance following Kochs postulates, the bacteria was cultivated on tryptic soy broth (Oxoid) at 29 °C for 24 h, then bacterial suspension was prepared and adjusted to the final concentration using a 0.5 McFarland standard and Helber counting chamber. All fish groups were thoroughly inspected daily after the challenge for 2 weeks for any pathological lesions and mortalities [24]. Moribund and freshly dead fish were collected and aseptically examined for bacterial reisolation. At the end of the experiment, the fish were killed by an overdose of anesthesia (200 mg clove oil/L) and hygienically disposed by burning in the incinerator.

Table 1 List of used *A.hydrophila* oligonucleotide primers

Target Gene		Primer sequence (5′-3′)	Amplicon size (bp)	Cycling conditions (35 cycles)			References
				Denaturation	Annealing	extension	
Virulent genes							
Confirmatory gene	*16SrRNA*	F: AGAGTT TGATCCTGGCTCAG R: GGTTACCTTGTTACGACTT	1200	94 ℃ 1 min	55 ℃ 1 min	72 ℃ 2 min	[63]
Aerolysin	*aer*A	F: CACAGCCAATATGTCGGTGAG R: GTCACCTTCTCGCTCAGGC	326	94 ℃ 30 s	52 ℃ 30 s	72 ℃ 30 s	[64]
Serine protease	*ser*	F:ACGGATGCGTTCTTTACTCCA R:CCGTTCATCACACCGTTGTAGTCG	211	94 °C for 1 min	64 °C for 30 s	72 °C for 45 s	[65]
Heat-labile Cytotonic enterotoxin	*alt*	F: TGACCCAGTCCTGGCACGGC R: GGTGATCGATCACCACCAGC	442	94 ℃ 30 s	55 ℃ 40 s	72 ℃ 45 s	[31]
Heat-stable Cytotonic enterotoxin	*ast*	F: TCTCCATGCTTCCCTTCCACT R:GTGTAGGGATTGAAGAGCCG	331				
Cytotoxic enterotoxin	*act*	F:AGAAGGTGACCACCACCAAGAACA R:AACTGACATCGGCCTGAACTC	232				
Haemolysin	*hly*A	F: GGCCGGTGGCCCGAAGATACGGG R:GGCGGCGCCGGACGAGACGGGG	592	95 °C for 2 min	55 °C for 1 min	72 °C for 1 min	[66]
Nuclease	*nuc*	F:CAGGATCTGAACCGCCTCTATCAGG R:GTCCCAAGCTTCGAACAGTTTACGC	504	94 ºC for 1 min	64 ºC for 30 s	72 ºC for 45 s	[65]
Adhesion	*aha*	F:GGTATTGTATCCCGGCTCTGTT R:CGGTCCATCGTCGTCCATCTTG	1082	94 ºC for 30 s	60.4 ℃ for 30 s	72 ℃ for 45 s	[67]
Antimicrobial Resistance genes							
β-lactamase	bla_{pse1}	F: ACC GTATTG AGC CTG ATT R: ATTGAA GCC TGT GTT TGA GCTA	321	96 ℃ 30 s	60 ℃ 30 s	72 ℃ 30 s	[68]
	bla_{SHV}	F: AGGATTGACTGCCTTTTTG R: ATTTGCTGATTTCGCTCG	392	94 ℃ 30 s	54 ℃ 40 s	72 ℃ 40 s	[69]
Sulfonamide	*sul1*	F: CGCACCGGAAACATCGCTGCAC R:TGAAGTTCCGCCCAAGGCTCG	163	95 ℃ 15 s	65 ℃ 30 s	72 ℃ 30 s	[70]
Tetracycline	*tet*A	F: GCTACATCCTGCTTGCCTT R: CATAGATCGCCGTGAAGAGG	210	95 ℃ 15 s	60 ℃ 30 s	72 ℃ 30 s	[71]

*Initial denaturation of 5 min at 94 ℃. * Final extension at 72 ℃ extended by 10 min

Statistical methods

The distribution data assessments were carried out using the Chi-square test in R-software (version 4.0.2; https://www.r-project.org/), with a significance level of $P<0.05$.

Results

Clinical and postmortem observation

Clinical examination revealed that all 120 fish (*T. zillii* and *M. cephalus*) exhibited extensive hemorrhages, hemorrhagic fin erosions, skin darkening, hemorrhages in the eyes and around the gill cover, and some fishes showed abdominal distention. (Fig. 1A). Internally, enlargement and congestion of the internal organs and congested gills were observed (Fig. 1B).

Aeromonas hydrophila isolation and phenotypic characterization

A total of 38 purified *A.hydrophila* isolates were retrieved, on Rimler-Shotts media *Aeromonas* colonies were small, smooth and yellow. On *Aeromonas* agar base media, the colonies were dark green with dark centers. On tryptic soy agar, the colonies were creamy circular, convex, and glistening. On non-lactose fermented MacConkey's agar, the colonies were pale in color. Moreover, on blood agar, *Aeromonas* colonies showed a beta-hemolytic zone, and are round, grayish and proceeded to dark green color after a long-time incubation. Additionally, all isolates exhibited high resistance patterns against novobiocin.

Microscopically, *A.hydrophila* were gram-negative, short rod-shaped bacilli, and motile with single polar flagella. Conventional biochemical tests revealed that *Aeromonas* is a facultative anaerobic bacteria (O/F +/+) and cytochrome oxidase and catalase tests positive. Moreover, the bacterial Proteolytic activity was evaluated by observing the appearance of a visible proteolytic zone surrounding the bacterial cells cultured on Brain Heart Infusion Agar (HiMedia) with 1% fresh egg yolk and incubated at 30 °C for 48 h.

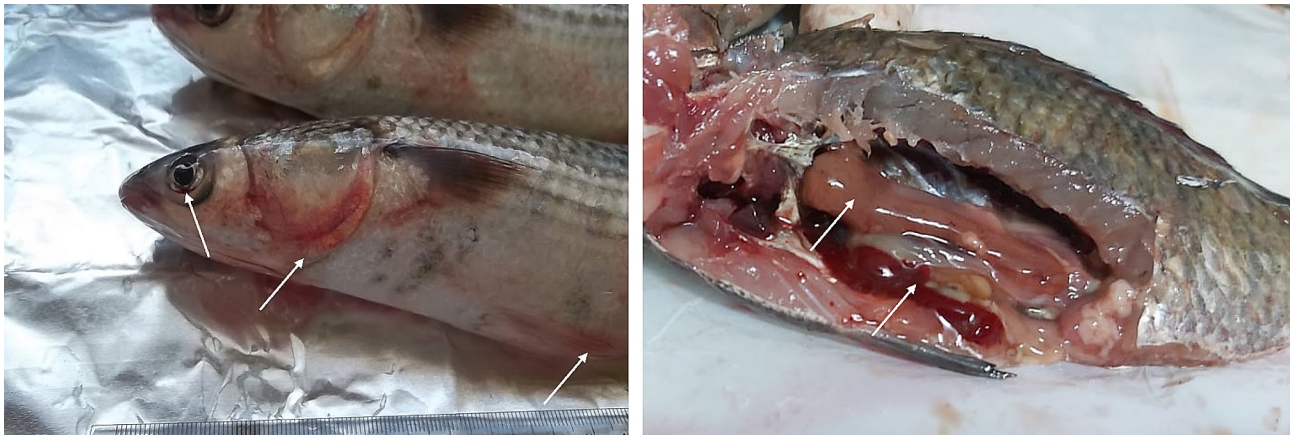

Fig. 1 (**A**) Naturally infected *M. Cephalus* showing external hemorrhages on the eye, gill cover and fins, (**B**) Naturally infected *Tilapia zillii* showing congestion, hemorrhages and enlargement of internal organs

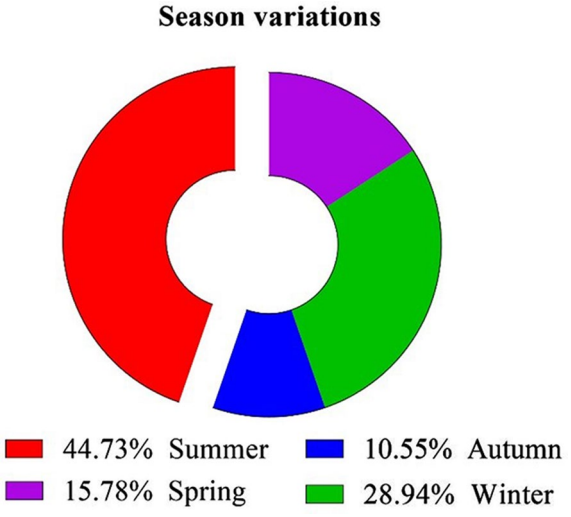

Fig. 2 Seasonal variation of *A. hydrophila* prevalence

Based on API-20 NE, the retrieved isolates were confirmed as *A. hydrophila* that react positively to nitrate reduction, glucose assimilation, gelatine liquefaction and negatively to citrate utilization and urease production.

Bacterial prevalence

A. hydrophila was detected in 38 of the 120 examined fish samples with a prevalence percentage (31.6%), 29 isolates were detected in *T. zillii* 48.3% (29/60) and the remaining nine isolates were isolated from *M. cephalus* 15% (9/60). The bacterial isolation from internal organs revealed that *A.hydrophila* were highly prevalent in the liver (16, 42.1%) followed by the kidney (14, 36.8%) and the spleen (5, 13.1%) and prevalence in the gills was the least (3, 7.9%). There was a statistically significant difference in the *A. hydrophila* prevalence among different internal organs of the examined fish ($X^2=13.16$, $P<0.05$).

Seasonally, the prevalence of *A. hydrophila* in naturally infected *T. zillii* and *M. cephalus* varies significantly throughout the year; the summer season recorded the highest percentage of infection (44.73%), followed by winter (28.94%), spring (15.78%) and autumn (10.52%) (Fig. 2). There was a significant difference in *A. hydrophila* prevalence among different seasons ($X^2=10.632$, $P<0.05$). Based on the Molecular identification, all the recovered isolates ($n=38$) were positive for *16SrRNA*.

Sequencing of the isolated *A. Hydrophila*

One selected strain for high virulence and resistance was sequenced, and the sequence was submitted to the Gene Bank with accession number (MW582865, https://www.ncbi.nlm.nih.gov/nuccore/MW582865.1/). The final alignments showed that isolate MW582865 had a high similarity with strains CP053859, CP028418.1, and CP018201 with a percentage of 96%, 78%, and 77%, respectively. It had a low similarity with strains CP046954, AP024234, CP050012, CP016989.1, and AP019193.1 each with 29%. The derived neighbor-joining phylogenetic tree revealed an apparent clustering of the isolated strain of *A. hydrophila* with various strains of *A. hydrophila* uploaded from the gene bank (Fig. 3). The nucleotide percentage of adenine (A), thymine (T), cytosine (C), and guanine (G) were 17% (39), 16% (33), 34% (79), and 33% (77), respectively (Fig. 3).

Antimicrobial susceptibility testing

Results of Antibiotic sensitivity of isolated *A.hydrophila* showed that all isolates from *T. zillii* and *M. cephalus* samples displayed a different degree of resistance to all the tested antibiotic agents. The isolates showed exceptional sensitivity to fluoroquinolones; levofloxacin (100%) and ciprofloxacin (89.5%), aminoglycosides; gentamycin (94.7%) and amikacin 86.6% but were highly resistant to *β-lactamase;* oxacillin, ampicillin (100%) and amoxicillin-clavulanic acid (89.5%). cephalosporins;both cefotaxime and cefadroxil (89.5%) (Table 2; Fig. 4). Susceptibility to

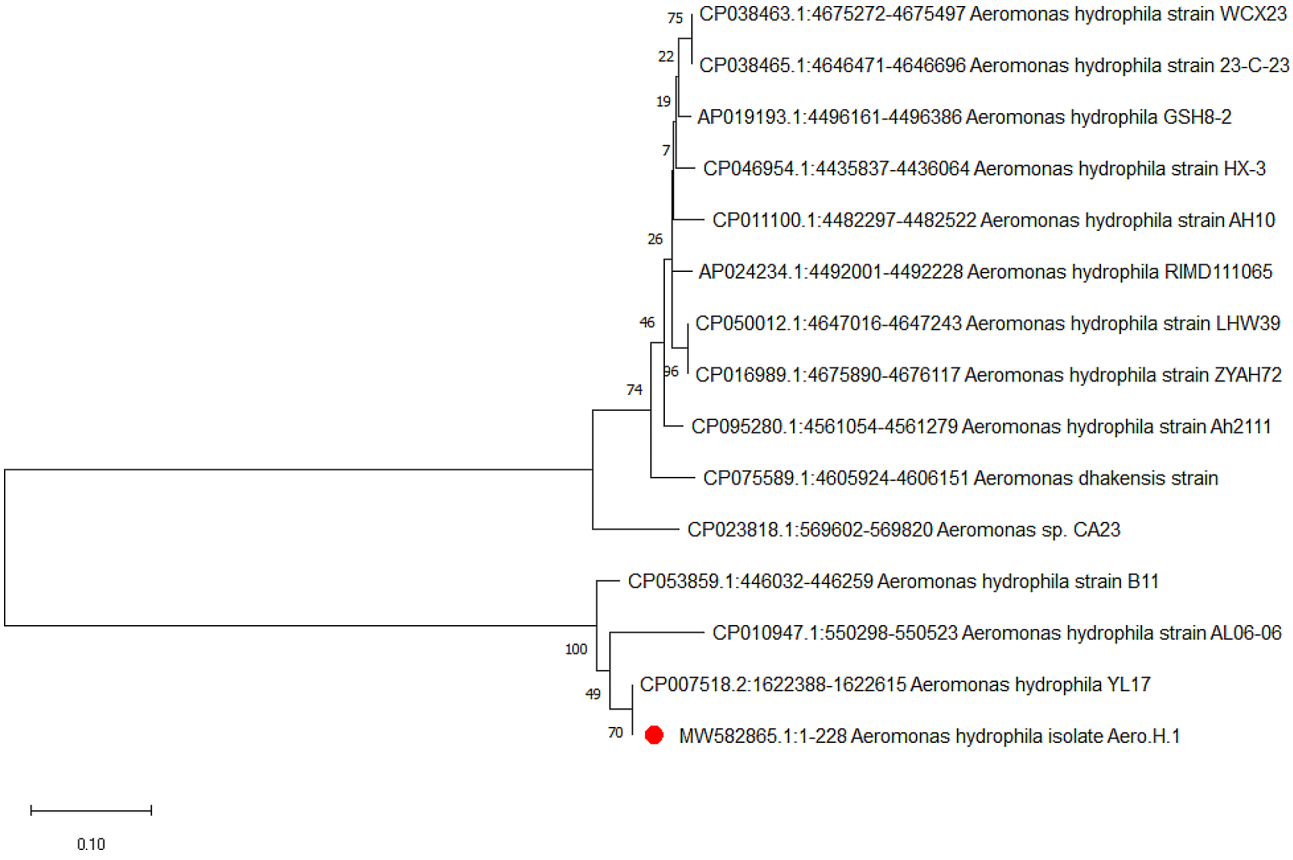

Fig. 3 Phylogenetic tree of *Aeromonas hydrophila*

Table 2 Antimicrobial susceptibility pattern of *A. hydrophila* isolates ($n = 38$)

Antimicrobial class	Antimicrobial agent	Interpretation					
		Sensitive		Intermediate		Resistance	
		N	%	N	%	N	%
β-lactamase	Oxacillin	-	-	-	-	38	100
	Ampicillin	-	-	-	-	38	100
	Amoxacillin + clavulanic acid	-	-	4	10.5	34	89.5
Cephalosporins	Cefotaxime	-	-	4	10.5	34	89.5
	Cefadroxil	3	7.9	1	2.6	34	89.5
Aminoglycosides	Amikacin	33	86.6	4	10.5	1	2.6
	Gentamycin	36	94.7	1	10.5	1	10.5
Fluoroquinolones	Levofloxacin	38	100	-	-	-	-
	Ciprofloxacin	34	89.5	4	10.5	-	-
Polymyxins	Polymyxin B	11	28.9	8	21.1	19	50
Tetracyclines	Doxycycline	12	31.6	2	5.2	23	60.5
Sulfonamides	Trimethoprim-Sulfamethoxazole	20	52.6	2	5.2	16	42.1
Chi-square		176.14 $P < 0.0001$		25.2		138.15 $P < 0.0001$	
P value				0.008521			

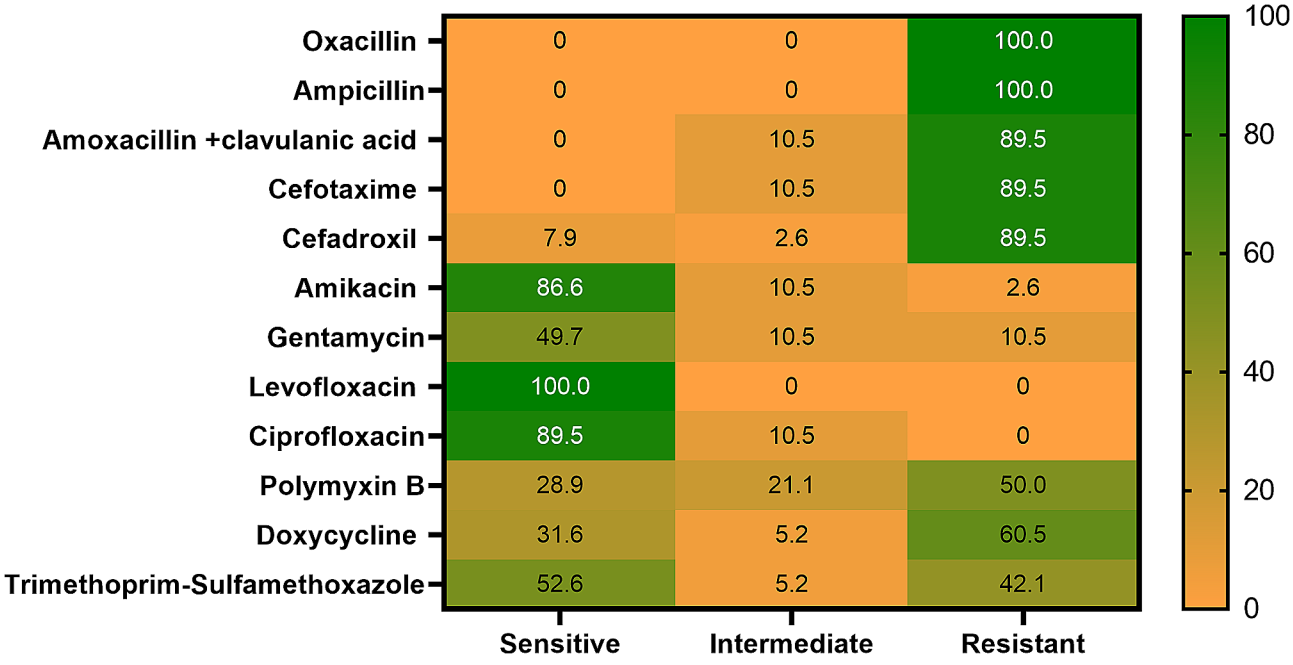

Fig. 4 Antibiotic resistance for the recovered *A. hydrophila* isolates

Table 3 Virulence genes distribution and antimicrobial resistance genes among *A. hydrophila* isolates ($n=38$)

Gene function	Target gene	Prevalence		Statistical analyses
		No	%	
Confirmatory gene	*16 S rRNA*	38	100	104.73 $P<0.0001$
Virulence genes	*aerA*	22	57.9	
	ser	11	28.9	
	alt	10	26.3	
	ast	5	13.1	
	act	3	7.9	
	hlyA	3	7.9	
	nuc	7	18.4	
	aha	0	0	
Antimicrobial-resistance genes	*β-lactamase pse1*	38	100	15.505 0.001432
	β-lactamase SHV	16	42.1	
	*sul*1	16	42.1	
	*tet*A	23	60.5	

the different tested antibiotics was statistically significant ($P<0.05$).

Molecular identification of virulence genes and multiple antibiotic resistance (MAR) genes among *A. hydrophila* isolates

Molecular identification was carried out for all *A. hydrophila* isolates retrieved from diseased fish samples using specific virulence genes primers (*aer*, *act ast*, *alt*, *hly*A, *ser*, *nuc* and *aha*), that produce positive amplicons with a percentage of detected virulence genes in *A. hydrophila* are, *aer*A (22/38, 57.9%), *act* (3/38; 7.9%), *ast* (5/38, 13.1%), *alt* (10/38, 26.3%), *hly*A (3/38, 7.9%), *ser* (11/38, 28.9%) and *nuc* (7/38, 18.4%) respectively. The (*aha*) gene was not detected in any sample as presented in Table 3. and Fig. 5.

Regarding antibiotic resistance, All isolates were positive for *pse*1 (38 /38, 100%), *tet*A (23/38, 60.5%), *sul*1 (16/38, 42.1%) and bla_{SHV} (16/38, 42.1%). The tested isolates of *A. hydrophila* revealed a significant difference between antimicrobial resistance genes ($P<0.05$) and a nonsignificant difference among virulence genes ($P>0.05$) (Table 3).

Association between antimicrobial agents, virulence and antimicrobial resistance genes

The relation between the phenotypic multi-drug resistance and the antimicrobial resistance genes for *A. hydrophila* isolates is represented in (Table 4; Fig. 6) and showed a strong correlation between DO and *tet*A ($r=1$); SXT and *sul*1 ($r=1$); GM and AK ($r=1$); CFD and CTX ($r=1$). The results of the MAR index varied between (0.16–0.83) are shown in Table 4. Heatmap and hierarchical clustering grouped isolates into five clusters (L1, L2, L3, L4, and L5) based on AMR phenotypes, virulence genes, and antimicrobial resistance genes (Fig. 6). L1, L2, and L3 had related isolates, whereas L4 and L5 had other related isolates. Despite no grouping, isolates 25,30 had identical AMR phenotypes, virulence genes, and antimicrobial resistance genes. 28.9% (11/38) of recovered isolates were considered XDR, while 63.1% (24/38) of recovered isolates were considered MDR (Table 4). It was found that some isolates harbor both virulence and MAR genes; where the highest percentage of isolates

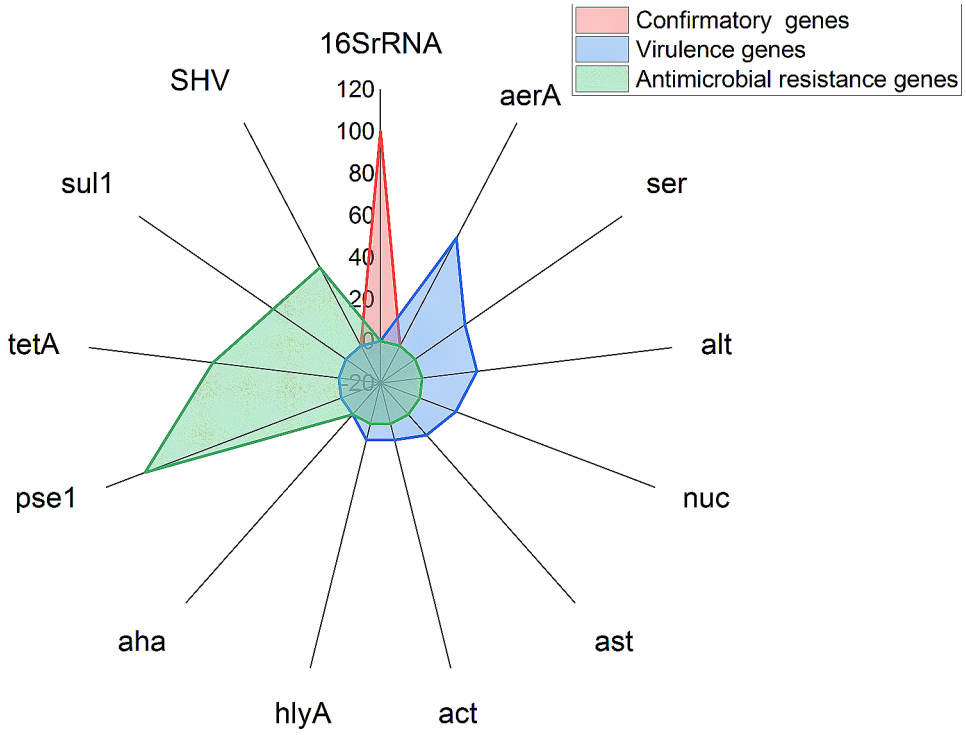

Fig. 5 Distribution of different *A.hydrophila* confirmatory, virulence and Antimicrobial resistance genes among recovered isolates

Table 4 The relation between the phenotypic multi-drug resistance and the antimicrobial resistance genes for *A. hydrophila* isolates

NO. of Isolates	Phenotypic antibiotic resistance	Antimicrobial resistance genes	MAR	
1	OX, AM, AMC, CTX, CFD, PB, DO, SXT, AK, GM	*pse*1, *sul*1, *tet*A, *SHV*	0.83	XDR
1	OX, AM, CTX, CFD, PB, DO, SXT	*pse1*, *sul1*, *tetA*, *SHV*	0.75	XDR
9	OX, AM, AMC, CTX, CFD, PB, DO, SXT	*pse*1, *tet*A, *sul*1, *SHV*	0.66	XDR
6	OX, AM, AMC, CTX, CFD, PB, DO	*pse*1, *tet*A	0.58	MDR
3	OX, AM, CTX, CFD, DO, SXT	*pse*1, *sul*1, *tet*A, *SHV*	0.5	MDR
1	OX, AM, AMC, CTX, CFD, PB	*pse*1, *SHV*	0.5	MDR
2	OX, AM, CTX, CFD, SXT	*pse*1, *sul*1	0.41	MDR
3	OX, AM, CTX, CFD, DO	*pse*1, *tet*A	0.41	MDR
8	OX, AM, CTX, CFD	*pse*1	0.33	MDR
1	OX, AM, AMC	*pse*1, *SHV*	0.25	MDR
3	OX, AM	*pse*1	0.16	DR

(5.3%) was carrying 11/12 virulence and resistance genes, followed by (5.3%) isolates harboring 9/12 virulence and resistance genes (Table 5).

Pathogenicity test

Seven isolates of *A. hydrophila* were selected for pathogenicity test depending on the prevalence of their virulence genes. The first three experimentally infected groups showed 100% mortality, revealing the high pathogenic capability of the injected strains, Furthermore, the other experimentally infected groups showed mortalities (96.67%, 93.33%, 86.67%, and 80%). The mortality (%) and survivability (%) were calculated as shown in Table (6). The pathogenic strains were re-isolated from freshly dead fish that exhibited high mortalities and the experimentally infected *T. zillii* fish displayed sluggish activity, skin darkening, and dispersed hemorrhagic patches, especially at the base of fins, fin rot, and detached scales. Statistically, there is a significant difference ($P<0.5$) in the survival rate between different groups.

Discussion

Aeromonas species are characterized by their widely ubiquitous distribution in fresh, eustarian, and marine ecosystems, *A. hydrophila* is one of the members of this genus that is most commonly isolated from diseased and apparently healthy fishes [3]. In the present study, moribund fishes that found infected with *A.hydrophila* displayed similar clinical signs and gross lesions as those reported in several previous studies [24, 25]. Also, these results were parallel with the results obtained by Ayoub, et al. [26]; Al-Mokaddem, et al. [1], who found that a clinical examination of the obtained naturally infected Nile tilapia (*Oreochromis niloticus*) with *Aeromonas* species demonstrated abundant hemorrhages, fin and tail fraying, corneal opacity, and body depigmentation.

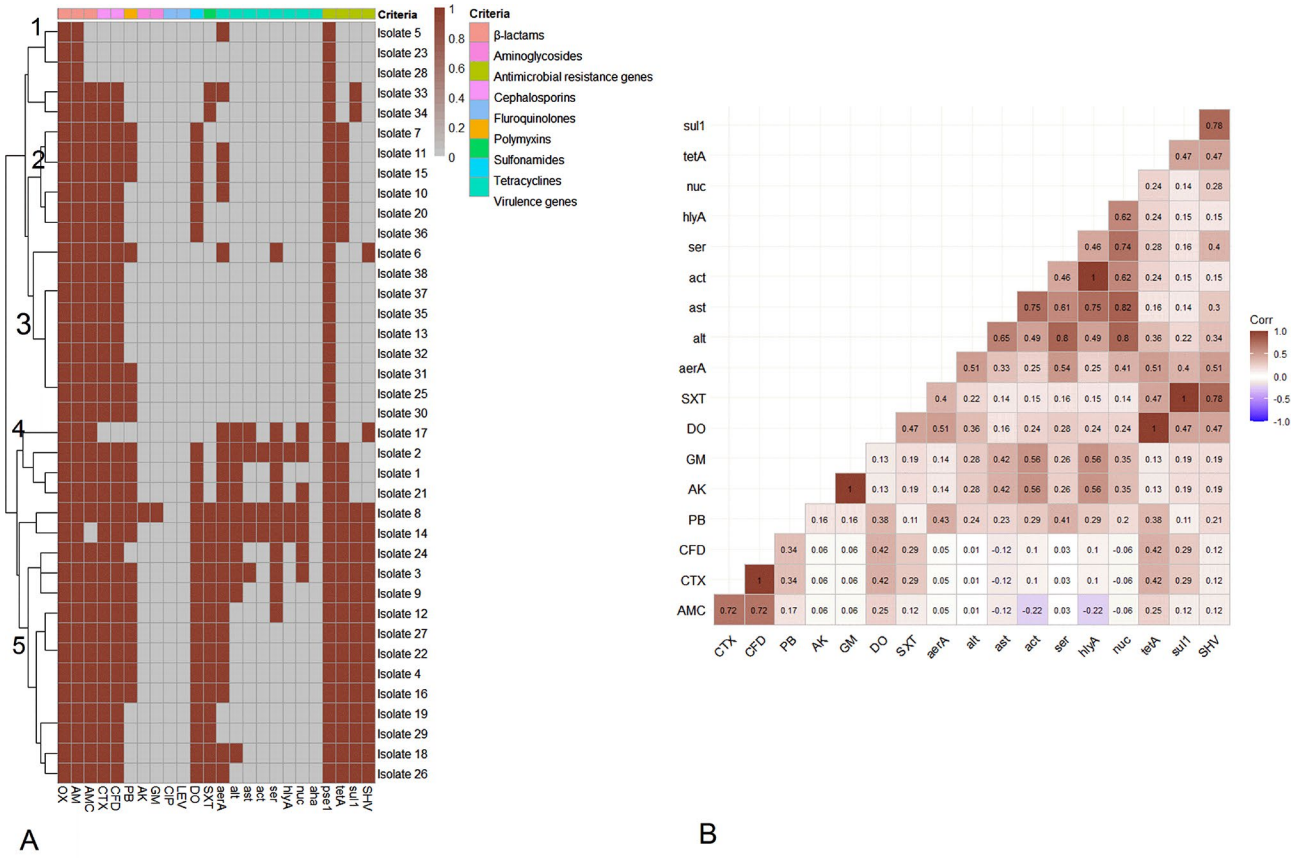

Fig. 6 **A** A heatmap of antimicrobial resistance phenotypes, virulence genes, and antimicrobial resistance genes in examined isolates. Dark red squares indicate presence; grey squares indicate absence. The figure shows five clusters (L1–L5). **B** The correlation coefficient (r) between various tested antimicrobial resistance phenotypes, virulence genes, and antimicrobial resistance genes

These clinical signs may be attributed to the burst of tiny blood vessels because of the *A. hydrophila* invasion and releasing of the extracellular materials that cause symptoms such as anemia, lethargy, anorexia, ulceration, and hemorrhage. *A. hydrophila* is one of the main pathogens causing Motile *Aeromonas* septicemia in fish and leads to substantial losses in aquaculture [27, 28].

The prevalence of *A. hydrophila* in *T. zillii* and *M. cephalus* was 48.3% and 15% respectively similarly to Jimoh, Jatau [29], Balaji, et al. [30] who reported 47% and 41.7% prevalence percentage of *A. hydrophila* in *Oreochromis niloticus*. The prevalence in internal organs was the highest in liver (42.1%) followed by the kidney (36.8%) and the spleen (13.1%). In contrast, the prevalence in the gills was (7.89%). In the current study, the difference in prevalence percentage could be assigned to fish species, geographical allocation differences, and sampling time [26, 31].

The highest prevalence of *A. hydrophila* among infected *T. zillii* and *M. cephalus* was recorded in the summer season (44.73%), while the lowest was in the autumn (10.52%). This variation may be attributed to the alterations in other water quality parameters in relation to increase in the water temperature, which is considered a stress factor for fish, increasing their susceptibility to infection and aids in bacterial proliferation [32, 33].

Genotypic identification of retrieved *A. hydrophila* isolates using the *16SrRNA* gene is considered an accurate and rapid tool for preliminary bacterial confirmation. In this study, all the isolates carried at least one of the virulent genes. This confirms the high virulence and pathogenicity of *A. hydrophila* isolated from *T. zillii* and *M.cephalus* and their high affinity to cause disease, which matches with the results of the previous researches [34, 35]. In regards to the detected virulent genes, Aerolysin *(aer*A*)* gene was the most frequently detected virulence gene in isolated *A. hydrophila* strains, this comes in agreement with other studies [16, 24, 34]. Aerolysin plays an important role in the pathogenesis of *A. hydrophila* as a pore-forming toxin that destroys membrane permeability, causing osmotic lysis that ends with cell death [36]. Motile Aeromonads, potential foodborne pathogens, require aerolysin (*aer*) and cytotoxic enterotoxins as *act*, *alt* and *ast* genes as *Aeromonas* heat-labile and heat-stable cytotonic enterotoxins. Type II secreted pore-forming cytotoxic enterotoxin gene (*act*) encodes cytotoxic and cytolytic proteins [37]. Five isolates of *A. hydrophila*

Table 5 The percentage of isolates harbor both virulence and multiple antibiotic resistance genes

A. hydrophila isolates		Virulence genes (*n* = 8)	Antibiotic-resistant genes Detected (*n* = 4)	Total number of detected genes (*n* = 12)
NO.	%			no
2	5.3	*hly*A, *aer*A, *ser*, *alt*, *nuc*, *act*, *ast*,	*pse*1, *tet*A, *sul*1, bla_{SHV}	11
1	2.6	*hly*A, *aer*A, *ser*, *alt*, *nuc*, *act*, *ast*	*pse*1, *tet*A	9
1	2.6	*aer*A, *ser*,*alt*,*nuc*,*ast*	*pse*1, *tet*A, *sul*1, bla_{SHV}	9
1	2.6	*aer*A, *ser*,*alt*,*nuc*	*pse*1, *tet*A, *sul*1, bla_{SHV}	8
2	5.3	*aer*A, *ser*,*alt*	*pse*1, *tet*A, *sul*1, bla_{SHV}	7
1	2.6	*aer*A, *ser*,*alt*,*nuc*	*pse*1, bla_{SHV}	6
1	2.6	*aer*A, *ser*,*alt*,*nuc*	*pse*1, *tet*A	6
1	2.6	*aer*A, *alt*	*pse*1, *tet*A, *sul*1, bla_{SHV}	6
1	2.6	*aer*A, *ser*	*pse*1, *tet*A, *sul*1, bla_{SHV}	6
5	13.2	*aer*A	*pse*1, *tet*A, *sul*1, bla_{SHV}	5
1	2.6	*aer*A, *ser*,*alt*	*pse*1, *tet*A	5
1	2.6	*aer*A, *ser*	*pse*1, bla_{SHV}	4
2	5.3	-	*pse*1, *tet*A, *sul*1, bla_{SHV}	4
3	7.9	*aer*A	*pse*1, *tet*A	3
1	2.6	*aer*A	*pse*1, *sul*1	3
2	5.3	-	*pse*1, *tet*A	2
1	2.6	-	*pse*1, *sul*1	2
1	2.6	*aerA*	*pse1*	2
10	26.3	-	*pse1*	1

with a prevalence of 13.1% harbor the *ast* gene, which increases intestinal vascular permeability and intestinal mucosal detachment, in contrast to Ramadan, et al. [16], who identified the *ast* gene in 46% of *Mugil cephalus* isolates, and El-Bahar, et al. [24], who couldn't detect the *ast* gene in any sample of the Aeromonas isolates from Nile tilapia.

The *alt* gene was detected in ten out of thirty eight *A.hydrophila* isolates, which was lower than those reported by Rather, et al. [38]. Heat-labile enterotoxin (*alt*) induces intestinal fluid retention in animals [39]. Our study revealed that the *hly*A gene was detected in only three strains of *A. hydrophila* with a prevalence of 7.9%, this result is lower than those reported by Hayati, et al. [40]; Simon, et al. [41] who confirmed the presence of *hyl*A in 95% and 39% of *A. hydrophila* isolates, respectively. Protease activity is crucial to *Aeromonas* spp. pathogenesis as it causes tissue damages, or activates toxins and overcomes host defenses [42]. Regarding to serine protease (*ser*) gene, nine isolates were detected with a total prevalence of 28.9% and this result is lower than those found by Yu, Chu [43] and Abu-Elala, et al. [44] who reported a higher percent of the *ser* gene (89%) and (55%) respectively detected in *Aeromonas* isolates. More frequently than in environmental samples, the nuclease (*nuc*) gene has been determined to be a virulence factor in clinical samples [45]. . The total prevalence of *nuc* gene among isolated *Aeromonas* spp was 18.4% lower than those described by Onuk, et al. [46] who detected the *nuc* gene in 54.54% of *Aeromonas* isolates.

The Bacteria frequently possess virulence and antimicrobial-resistance genes on the chromosome or on mobile genetic elements such as plasmids, transposons, and integrons [47]. This association is significant because these genes' successive acquisition and expression may affect bacterial fitness and host survival [48, 49]. Janda, Abbott [50] noted that bacteria can express up to three β-lactamases through a coordinated process. β-lactam antibiotics cure bacterial illnesses best. However, resistant bacterial strains produce β-lactamases, reducing their efficacy. *Aeromonas* has a β-lactamase gene, which hydrolyzes the β-lactam ring to inactivate the antibiotic [51]. As shown in Table (2), 100% and 42.1% of *A.*

Table 6 Mortality and survivability % in pathogenicity test

Fish group	Virulence genes	Total No. of fish	No of dead fish	Final No of fish	Survival %	Mortality %
Group 1 Control negative	-	30	0	30	100%	0
Group 2 (Isolate8)	*hyl*A, *aer*A, *ser*, *alt*, *nuc*, *act*, *ast*, *pse*1, *tet*A, *sul*1, *SHV*	30	30	0	0	100%
Group 3 (Isolate 14)	*hyl*A, *aer*A, *ser*, *alt*, *nuc*, *act*, *ast*, *pse*1, *tet*A, *sul*1, *SHV*	30	30	0	0	100%
Group 4 (Isolate 2)	*hyl*A, *aer*A, *ser*,*alt*,*nuc*, *act*,*ast*,*pse*1,*tet*A	30	30	0	0	100%
Group 5 (Isolate 3)	*aer*A, *ser*,*alt*, *nuc*, *ast*, *pse*1, *tet*A, *sul*1, *SHV*	30	29	1	3.33%	96.67%
Group 6 (Isolate 24)	*aer*A, *ser*, *alt*, *nuc*, *pse*1, *tet*A, *sul*1, *SHV*	30	28	2	6.67%	93.33%
Group 7 (Isolate 17)	*aer*A, *ser*, *alt*, *nuc*, *ast*, *pse*1, *SHV*	30	26	4	13.33%	86.67%
Group 8 (Isolate 9)	*aer*A, *ser*, *alt*, *pse*1, *tet*A, *sul*1, *SHV*	30	24	6	20%	80%

hydrophila isolates possessed bla_{pse1} and bla_{SHV} genes, respectively, which confirmed that genes that code for β-lactamase increase resistance to β-lactam antimicrobials (penicillins and derivatives, cephalosporins, carbapenems, and monobactams), SHV enzymes can hydrolyze monobactams and carbapenems due to modifications in amino acids that alter the active site structure of β-lactamases [50, 52].

The present study also revealed the presence of genes encoding resistance to tetracyclines (*tetA*) and sulfonamides (*sul 1*) in 60.5% and 42.1% of *A. hydrophila* isolates, respectively. The resistance gene of *sul1* had been detected at a high rate of 87.1% and 75% respectively in *Aeromonas* spp. isolated from rainbow trout, *Oreochromis niloticus* and *Clarias gariepinus* [53, 54]. In another study, although *sul1* was present in *A. hydrophila* (41%, 7/17), it could not be detected in *A. sobria* and *A. caviae* [55]. While variable occurrences of the *tet*A (A) resistance gene had been reported in *Aeromonas* from several studies 50%, 87.5% [53], and 67.44% [56].

Antibiotic susceptibility assessments are critical to monitoring the severity of antibiotic resistance and choosing the appropriate drugs for disease treatments in aquaculture to minimize risks to human health. In intensive aquaculture systems, antimicrobial agents are extensively used to control infectious diseases and are often unregulated [57]. All the tested isolates in this study were sensitive (100%) to levofloxacin and ciprofloxacin. In contrast, lower resistance (11.2%) to amikacin and gentamicin agrees with the results obtained by Ramadan, et al. [16] who demonstrated lower resistance to gentamicin for the bacterial isolates from fish samples. *Aeromonas* species are susceptible to Fluoroquinolones [50]. Ciprofloxacin is known to be the most effective treatment for most diseases. The high levels of resistance to ampicillin and oxacillin were identical to those reported by [58]. They observed that all the isolates tested were highly resistant to amoxicillin and ampicillin. Similar results have been reported in isolates borne on zebrafish and Nile tilapia [59].

Our results revealed a high prevalence of MAR in *A. hydrophila* isolates from freshwater and saltwater fish in Egypt. The higher frequencies of antibiotic resistance of the isolates may imply that antimicrobial agents are used more frequently in aquaculture. The multiple antibiotic resistance index (MAR) has been used to specify the degree of antibiotic use. The value of the MAR index is higher than 0.2 reflecting the bacterial isolates from high-risk sources of antibiotic contagion where antibiotics are frequently used. Higher values of the MAR index (>0.2 to 0.93) were expressed by Krumperman [22], Tartor, et al. [35], Vivekanandhan, et al. [60], who noted that MAR indices were displayed in 87.2% of *A. hydrophila* isolates. These results nearly agree with those captured by Kusdarwati, et al. [61]. Depending on the antibiotic resistance phenotype, 63.16% (24\38) of tested *A. hydrophila* isolates exhibited multidrug resistance (MDR) to five or more antimicrobial classes and 28.95% (11/38) of tested isolates exhibited extensive drug resistance (XDR) to eleven or more antimicrobial classes, these results agreed with those found by Algammal, et al. [62]; Algammal, et al. [12]. Isolates that demonstrated resist at least one agent in all antibiotics from multiple classes (except for 1 or 2) are categorized as XDR. Isolates that demonstrated resistance to three or more drugs were categorized as MDR, as previously documented. [33, 75].

Conclusion

Our findings showed that most recovered *A. hydrophila* isolates from the Suez Canal area, Egypt carried both virulence and antibiotic-resistant genes. It showed that the prevalence and distribution of various virulence and antibiotic-resistant genes in *A. hydrophila* is crucial in the occurrence of the septicemic disease, furthermore, the presence of such antibiotic-resistant strains in aquaculture will be a constrain in treatment or even control of infected fishes. In addition, these findings raise a public health concern regarding the illegal use of antibiotics in fish farms and the expected human health implications.

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Author contributions

Conceptualization; Hala Ayoub, Ahmed Khafagy, Aboelkheir Esawy, Noura El moaty Reham M. ELTarabili, and Reham A. Ibrahim. Data curation; Hala Ayoub, Ahmed Khafagy, Noura El moaty, Reham M. ELTarabili and Reham A. Ibrahim. Formal analysis; Hala Ayoub, Aboelkheir Esawy and Reham M. ELTarabili. Funding acquisition; Khairiah Mubarak Alwutayd and Abdallah Tageldein Mansour. Investigation; Hala Ayoub, Noura El moaty and Reham M. ELTarabili.; Methodology; Hala Ayoub and Dalia A. Ab-del-moneam Project administration; Aboelkheir Esawy. Resources; Aboelkheir Esawy, Khairiah Mubarak Alwutayd and Abdallah Tageldein Mansour. Software; Khairiah Mubarak Alwutayd, Abdallah Tageldein Mansour and Reham M. ELTarabili. Supervi-sion; Ahmed Khafagy and Reham M. ELTarabili. Validation; Ahmed Khafagy, Noura El moaty, Abdallah Tageldein Mansour and Reham M. ELTarabili. Writing – original draft; Hala Ayoub. Writing – review & editing; Hala Ayoub, Reham M. ELTarabili, Abdallah Tageldein Mansour, Reham A. Ibrahim and Dalia A. Abdel-moneam.

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Data availability

Data and materials are available upon request.

Declarations

Ethics approval and consent to participate
The handling of fish was carried out by well-trained scientists. The fish farm owners provided their oral consent prior to the collection of fish samples, informed about the objectives of the study, and their contact details were also recorded to obtain the results of the survey.
All protocols including live animals were approved by the Animal Ethics Review Committee of Suez Canal University guidelines (AERC-SCU 2023026).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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