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Prevalence of antibiotic-resistant *Aeromonas hydrophila* isolated from the farmed Striped Mullet *Mugil cephalus*

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ABSTRACT

Aeromonas hydrophila is an opportunistic fish pathogen, significantly impacting aquaculture production worldwide. A total number of 150 mullets *(Mugil cephalus)* was collected to detect the presence of virulent *Aer. hydrophila* in fish farms suffering from mass mortality. In addition, the antibiotic-resistant genes were determined for the isolated strains, viz. tetracycline (TetA (A), sulphonamide (Sul1), and quinolones (qnrsA). The isolated strains were identified as *Aer. hydrophila* and were registered at NCBI under accession numbers (OM965641, OM965642 and OM965643). They prevailed at a rate of 96%, 92%, and 86% in the three sites under investigation. The isolated strains harbored virulence genes cytotonic enterotoxins heat-stable (ast) and cytotoxic enterotoxin (act). The isolated *Aer. hydrophila* had resistant genes for TetA(A), Sul1, qnrsA. Therefore, this study was conducted to shed the light on a serious obstacle facing aquaculture. In addition, fish farmers should avoid the excessive and random antibiotic uses resulting in emerging antibiotic-resistant bacteria.

INTRODUCTION

Aeromonas species that belong to the family *Aeromonadaceae* are facultative anaerobic and Gram-negative bacteria, which can be divided into psychrophilic and mesophilic bacteria according to temperature tolerance. These bacteria have a wide range of hosts, with both cold-and warm-blooded fish, and it can infect humans as well **(Nerland, 1996)**. According to Bergey's (systematic manual of bacteriology), the Aeromonads were divided into the psychrophilic species, non-motile *Aer. salmonicida* and three mesophilic species motile (*Aer. hydrophila*, *Aer. caviae* and *Aer. sobria*) **(Popoff, 1984)**. *Aeromonads* have been frequently isolated in aquaculture products such

as fish and shellfish, since they are widely present in the aquatic environment **(Anguita *et al.*, 1993; EI-Jakee *et al.*, 2020)**.

Previous studies stated that the extracellular proteins secreted by *Aeromonas* species are their virulent factors causing fish diseases and human illness. They added that these enzymes, including protease, aerolysin, nuclease, chitinase, elastase, lecithinase, gelatinase, lipase, and amylase were active as cytolytic enterotoxins and hemolysins **(Altwegg *et al.*, 1989; Joseph *et al.*, 1991)**. The gene (act) that is cytotoxic heat-labile enterotoxin of *A. hydrophila* has many toxic impacts, including cytotoxic, hemolytic along with enterotoxin effects **(Chopra & Houston, 1999)**. Other *aeromonads* possess genes, namely *aerA* and *hlyA* with hemolytic activities; the isolated *A. hydrophila* may have more than one gene **(Heuzenroeder *et al.*, 1999)**. Other virulent genes and cytotonic enterotoxins (*ast*, *alt*) genes mainly have diarrhoeic effects **(Sha *et al.*, 2002)**.

Aeromonads are frequently isolated from diseased fish in freshwater farms suffering from high mortality rates without good responses for antibiotic treatments. Pathogenic aeromonads isolated from fish are resistant to some antibiotics, such as ampicillin, amoxicillin, sulbactam and streptomycin **(Abu-Elala *et al.*, 2015)**. **Rosenblatt-Farrell (2009)** claimed that these antibiotic-resistant bacteria get into the aquatic environment through inadequate hygienic systems, agriculture drainages, and treatment plants of wastewater, transferring this genetic information to aeromonads species, which are abundant (ex., transfer of plasmid-mediated quinolone resistance) **(Rhodes *et al.*, 2004)**. Many strains of *Aer. caviae* harbor nalidixic acid, ciprofloxacin, and norfloxacin resistant genes **(Sinha *et al.*, 2004)**. A gradual increase has been observed with regard to the degree of antibiotic-resistant bacterial isolates **(Das *et al.*, 2009; Pal & Bhattacharjee, 2011).**

This study investigated the prevalence of antibiotic-resistant genes in the virulent *A. hydrophila* associated with mass mortalities in fish farms.

MATERIALS AND METHODS

2.1 Sites and fish under investigation

A total of 150 mullet fish *(Mugil cephalus)* were collected from three private freshwater fish farms, located at different sites in Tolompate village, El-Ryad City. *M. cephalus* specimens had an average body weight of 285±25 g and a body length of 32±1.5 cm. Fish were collected randomly from farms suffering from high mortality rates. They were transferred to the bacteriology laboratory of Animal Health Research Institute, Kafrelsheikh in aseptic plastic bags and preserved in ice-tanks.

2.2 Primary bacterial isolation

Bacterial swabs were taken from internal organs, the liver, spleen, and kidney. They were cultivated into tryptic soy broth at 35°C for 48h then streaked on tryptic soy agar with 5% sheep's blood at 28°C for 24h. Afterwards, they were subcultured on aeromonas agar plates to obtain pure cultures of predominant organisms. Phenotypic characterization

of *Aer. hydrophila* isolates were confirmed following the recommendations of **Bergey (1994)** and **Elmer *et al.* (1997)**. *Aeromonas* spp. were biochemically identified using API 20E Strep **(Bio-merieux, 1984)**.

2.3 Molecular bacterial identification

To identify *A. hydrophila*, strains were subjected to PCR assays 16S rRNA.To confirm the pathogenicity of isolates, the virulence genes cytotonic enterotoxins heat-stable (ast), cytotoxic enterotoxin (act), cytotoxic enterotoxin (alt), and haemolysin (hly) genes. While, the antibiotic-resistant genes were detected for tetracycline (TetA(A), sulphonamide (Sul1), and quinolones (qnrsA).

2.3.1 Extraction of DNA was performed according to the instructions of the QIAamp DNA mini kit. PCR Master Mix was prepared according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit. Firstly, the ladder was gently mixed via pipetting up and down, then 6 µl of the formed ladder were directly loaded.

2.3.2 PCR products, purified with a Qiaquick PCR Purification Kit (QIAGEN), and the required primers (Table 1) were added. Agarose gel electrophoreses were performed according to the procedure of **Sambrook *et al.* (1989)**; the obtained gel was photographed by a gel documentation system, and the data was analyzed through a computer software.

2.3.3 Partial sequences of *gyrB* gene. The genomic DNA of *Aer. hydrophila* isolates were extracted as mentioned above. To confirm the identity of *Aeromonas* spp., genus-specific primers pair; (*gyr*B) were used for PCR amplification of the *gyr*B gene as described in the study of **Hu *et al.* (2012)**. Briefly, PCR was carried out in a reaction mixture containing 12.5 µl of Dream Taq Green PCR Mix (2X) (Thermo-Scientific, USA), 2 µl of extracted DNA, 1 µl of each primer and 8.5 µl of distilled water. The thermal cycler program was adjusted as follows: 95°C for 4min (initial denaturation), followed by 30 cycles of (95°C for 30 s (denaturation); 51°C for 30 s (annealing) and 72°C for 90 s (extension)). The reaction was ended at 72°C for 10 min (as a final extension). The amplicons were purified using A GeneJET™ PCR Purification Kit (Thermo Fisher Scientific, USA). PCR products were electrophoresed in 1.5% agarose and visualized under UV light. The amplified *gyr*B gene was sequenced in two directions using an ABI 3730xl DNA sequencer (Applied Biosystems USA). The raw sequences were edited and assembled via BioEdit version 7.0 **(Hall, 1999)**. The assembled gyrB genes were submitted to the database of GenBank. The phylogenetic tree was constructed using MEGA version X **(Kumar *et al.*, 2018)**.

Table 1. Sequence of primers used in this study

Gene	Primer sequence (5'-3')	Length of amplified product	Annealing	Reference
16S rRNA	F: GAAAGGTTGATGCCTAATACGTA R:CGTGCTGGCAACAAAGGACAG	625bp	55˚C 40 sec.	**Gordon *et al.* (2007)**
gyrB	F: TCCGGCGGTCTGCACGGCGT R:TTGTCCGGGTTGTACTCGTC	1100 bp	51˚C 30 sec.	**Hu *et al.* (2012)**
Hly	F: GGCCGGTGGCCCGAAGATACGGG R: GGCGGCGCCGGACGAGACGGGG	592 bp	55˚C 40 sec.	**Rozi *et al.* (2017)**
Act	F: AGAAGGTGACCACCACCAAGAACA R: AACTGACATCGGCCTTGAACTC	232 bp	55˚C 30 sec	**Nawaz *et al.* (2010)**
Ast	F: TCTCCATGCTTCCCTTCCACT R: GTGTAGGGATTGAAGAAGCCG	331 bp	55˚C 30 sec.	
Alt	F: TGACCCAGTCCTGGCACGGC R: GGTGATCGATCACCACCAGC	442 bp	72˚C 45 sec	
qnrA	F: ATTTCTCACGCCAGGATTTG R: GATCGGCAAAGGTTAGGTCA	516 bp	72˚C 45 sec.	**Robicsek *et al*. (2006)**
Sul1	F: CGGCGTGGGCTACCTGAACG R: GCCGATCGCGTGAAGTTCCG	433 bp	72˚C 45 sec	**Ibekwe *et al*. (2011)**
tetA(A)	F: GGTTCACTCGAACGACGTCA R: CTGTCCGACAAGTTGCATGA	576 bp	72˚C 45 sec	**Randall *et al.* (2004)**

2.4 Pathogenicity test for isolated strains

LD_{50} of *A. hydrophila* was estimated following the procedure of **Reed and Muench (1938)**. Briefly, *M. cephalus* (40 ± 3 g b.w.) was acclimated in the wet laboratory of Animal Health Research Institute. After anesthetizing the fish using tricaine methanesulfonate (MS222; Sigma, St. Louis, MO, USA), groups of 10 fish were intraperitoneally injected with serial 10-fold dilutions of the *Aer. hydrophila* cultured in brain heart infusion broth at 30°C for 24h. Firstly, 100 µl of *Aer. hydrophila* suspension was adjusted to 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8, 1×10^9 or 1×10^{10} (CFU/mL) in normal saline (0.65%), and the suspension was injected into duplicate groups of 5 fish. Mortality rates were recorded for fourteen days, and *Aer. hydrophila* was re-isolated from the dead moribund fish and confirmed by PCR.

2.5 Biosafety procedure

This study followed the biosafety measures concerning the pathogen safety data sheets: Infectious substances- *Aer. hydrophila*, Pathogen Regulation Directorate, **Public Health Agency of Canada (2010)**.

RESULTS

3.1 Prevalence of *Aeromonas hydrophila* in examined sites

In Table (2), *M. cephalus* were collected from fish farms at the end of the production season (October 2021); fish farms had exaggerated mortality during the production cycle. The prevalence of *A. hydrophila* was 96%, 92%, and 86% in sites 1-3, respectively.

Table 2. Prevalence rate of *A. hydrophila* in the examined fish farm

Item	**Site 1**	**Site 2**	**Site 3**
Fish no.	50	50	50
Moribund	45	40	41
Fish Carrier	48	46	43
PR%	96	92	86

Note: no. number, PR; prevalence rate

3.2 Bacterial identification

By using traditional bacterial methods (isolation on selective media and API20E), *A. hydrophila* was isolated from fish liver, spleen, and kidney samples revealing the

presence of *A. hydrophila*. The number of isolates obtained by API 20 was (7456754, 7467754, and 7576755). In Fig. (1), the PCR examination of 16S rRNA revealed the presence of *A. hydrophila*.

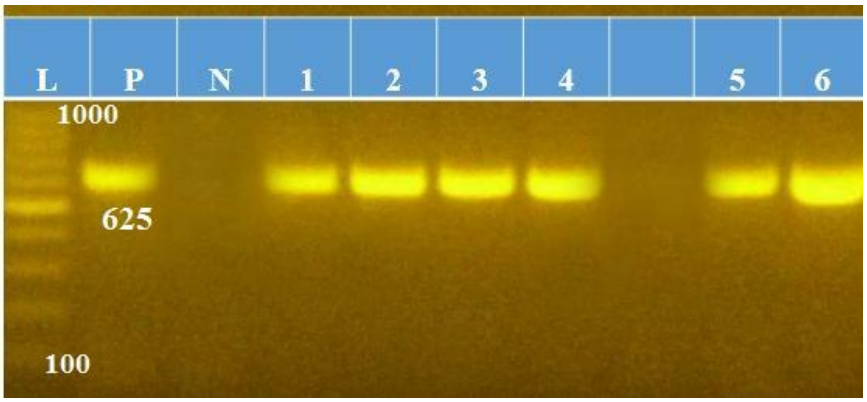

Fig. 1. *Aeromonas hydrophila* 16S rRNA. Notes: L= Lader; P= positive; N=negative; 1,2= Site1; 3,4 = Site2; 5,6= site 3.

Fig. (2) shows the genetic identification of *Aeromonas* spp. Approximately, 1100 bp of the *gyrB* gene was obtained from three bacterial isolates. Comparison of the multiple sequences alignment showed that all three isolates were deeply rooted in the *Aeromonas* spp. group. The multiple alignments of these sequences showed 99.49-98.32% similarity to the subsequent accession numbers of *A. hydrophila* (FR681749.1, FR681606.1, MH697731.1, KP781955.1, FR681599.1, CP083944.1, AB473091.1, JX275837.1, AP023398.1, and CP010947.1). Therefore, these *Aeromonas* spp. isolates were genetically confirmed as *A. hydrophila*. The accession numbers of *A. hydrophila* were OM965641, OM965642, and OM965643 at sites 1-3, respectively. The intraspecies similarity of these isolates was 99.53–100% for the three strains of *A. hydrophila*, isolated from grey mullet with nucleotide differences ranging from 4 to 5 bp.

The phylogenetic analysis exhibited two large lineages. The first clade was subdivided into two subclades. The first subclade was composed of three branches. The first branch clustered the three isolates of *A. hydrophila* in this study, with other *A. hydrophila* strains retrieved from the GenBank database to form one branch. The second branch clustered *A. caviae* isolates that was separated from the third branch that clustered *A. veronii* isolates. The second subclade grouped *A. sobria* isolates with a bootstrap value of 97%. The second clade is formed isolates of *A. salmonicida* to form a monophyletic clade, with a high bootstrap value of 100%. (Fig. 2). *Shewanella aquimarina* (FJ589042) was used as an outgroup isolate.

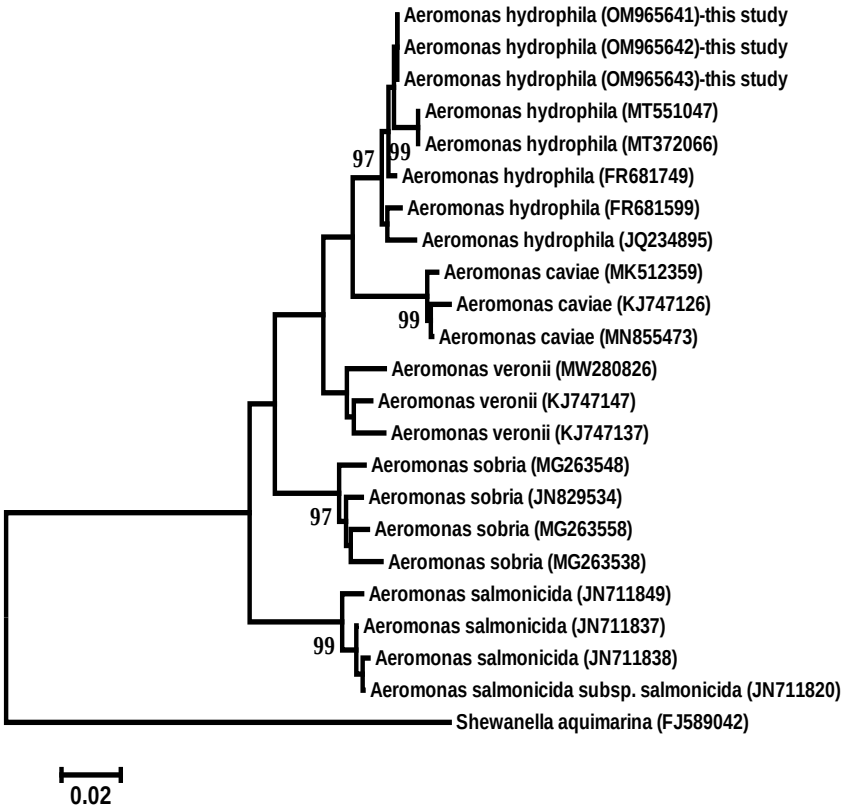

Fig. 2. Phylogenetic tree of *Aer. hydrophila* isolates

3.3 Multi-drug resistant genes and virulence genes

The three bacterial isolates *(Aer. hydrophila)* were examined for the presence of antibiotic-resistant genes, all isolates were harbor resistant genes for tetracycline (TetA(A), sulphonamide (Sul1) and quinolones (qnrsA) (Fig. 3).

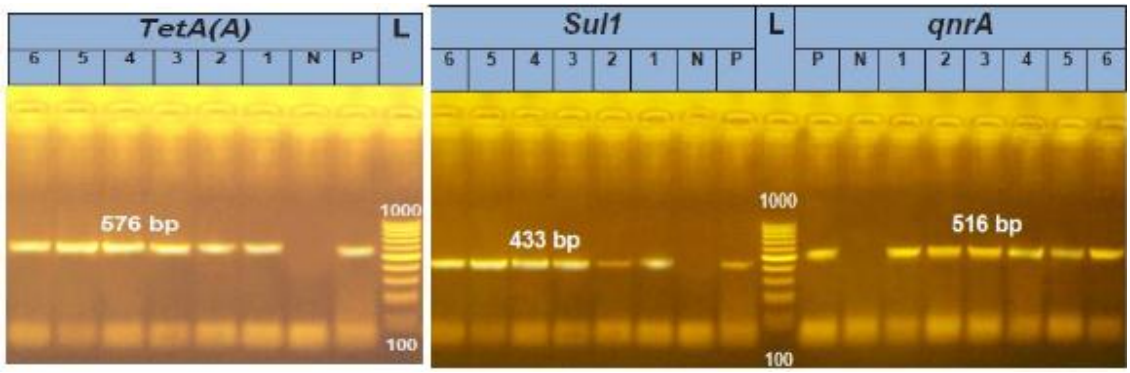

Fig. 3. Electrophoresis gel showing bands of multi-drug resistant bacterial isolates. Tet A; tetracycline, Sul1; sulphonamide, qnrA; quinolones, L; ladder, P; positive, and N; negative.

Fig. (4) exhibits the isolated *Aer. hydrophila* harboring a number of virulence genes, with cytotonic enterotoxins heat-stable (ast) and cytotoxic enterotoxin (act), while they lack the cytotoxic enterotoxin (alt) and haemolysin (hly) genes.

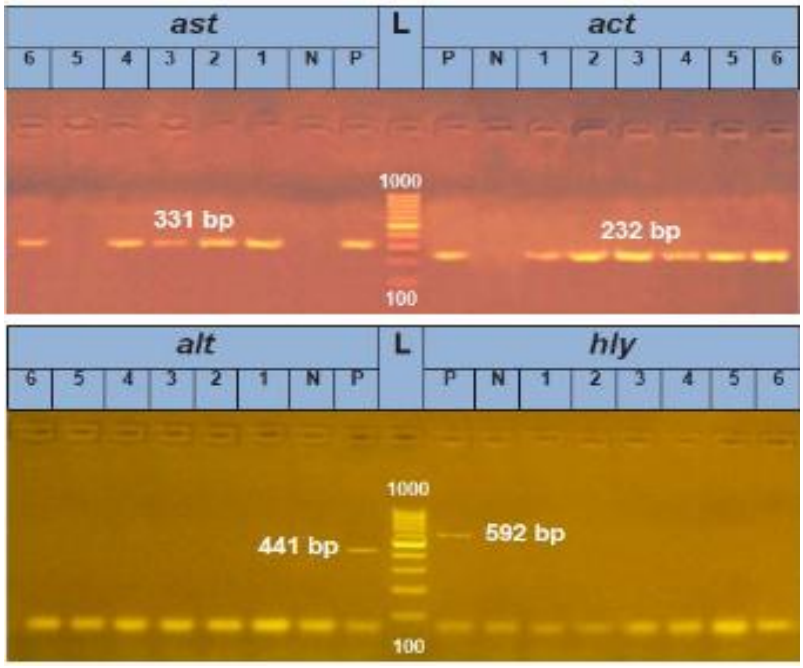

Fig. 4. Virulence genes in present in *Aeromonas hydrophila* isolates

3.4 Median lethal dose of the bacterial isolates

After traditional and molecular identification of *Aer. hydrophila* isolates the LD_{50} at the water temperature of 27 °C. LD_{50} for the three isolates (OM965641, OM965642, and OM965643) was 2.2×10^5, 2.2 × 105, and1.18×10^6 CFU/mL, respectively, within fourteen successive days.

DISCUSSION

In this work, the *Aer. hydrophila* was isolated from the farmed *M. cephalus* and the phenotypic characterization, antibiotic-resistant genes, and virulence genes of the isolates were determined. In both freshwater and marine fish species, *Aeromonas*-infection is associated with disease conditions known as Epizootic Ulcerative Syndrome (EUS) and Motile Aeromonas Septicaemia (MAS) **(Yogananth *et al.*, 2009; Viji *et al.*, 2011)**.

Motile *Aeromonas* prevailed with a rate of 66% and 34% of finfish and shellfish, respectively; the source of infection could be via the pond waters and the reared fish for the gut fish species contain *Aeromonas* spp. **(Aberoum & Jooyandeh 2010)**. In this study, *A. hydrophila* were isolated at a rate of 96%, 92%, and 86% from the farmed *M. cephalus* at the sites (1-3) under investigation, respectively. These high results are similar

to those obtained by **Ibrahim-Lamis (2015)**, while lower incidences were obtained in the studies of **Abu-Leila (2005)**, **Yucel *et al.* (2005)** and **Nawaz *et al.* (2006)**. *Aeromonas* spp., isolated from the gut content of the farmed fish is considered a typical member of the microbiota in the United States of America and Japan **(Sreedharan *et al.*, 2012)**; it is isolated from the intestinal tract of *O. niloticus* in Egypt **(Sherif *et al.*, 2020, 2021a)**.

The API20E system was used to identify the bacterial isolates with numbers of (7456754, 7467754, and 7576755). Similarly, the morphological characteristics of *Aeromonas* spp. were previously reported **(Jayavignesh *et al.*, 2011; Niamah 2012)**.

16S rRNA was used to identify the isolates after the sequence of the gyrB gene of *Aer. hydrophila* to define the obtained *Aer. hydrophila* in this study. They were deposited in NCBI (OM965641, OM965642, and OM965643). In addition, the phylogenetic tree was illustrated to elucidate the intraspecies phylogenetic relationships within *Aer. hydrophila*. In accordance, **Yanez *et al.* (2003)** used the gyrB gene (1100 bp) in the phylogenetic analyses. Whereas, the current study used the gyrB gene rather than the 16S rRNA gene. Accordingly, phylogenetic analyses of *Aeromonas* spp., based on 16S rRNA genes indicate low phylogenetic marker **(Yanez *et al.*, 2003; Küpfer *et al.*, 2006)**. On the other hand, it was recommended that gyrB (which encodes the B-subunit of DNA gyrase, a type-II DNA topoisomerase) could be more appropriate for *Aeromonas* spp. sequencing **(Yi *et al.*, 2013)**.

Some virulence genes were found in the isolated *Aer. hydrophila*, such as cytotonic enterotoxins heat-stable (ast) and cytotoxic enterotoxin (act), while cytotoxic enterotoxin (alt) and haemolysin (hly) genes were absent. In contrast, the hly gene was present in *Aer. hydrophila* and *Aer. caviae* strains, and by amplification, the PCR products of 1500 bp was given **(Wang *et al.*, 2003; Nam & Joh, 2007; Yousr *et al.*, 2007; Yogananth *et al.*, 2009; Cagatay & Şen, 2014; Stratev *et al.*, 2016; Sherif *et al.*, 2022)**. Similarly, act gene was present in the isolated *Aer. hydrophila* and *Aer. caviae* strains and was amplified giving PCR products of 332 bp **(Abdullah *et al.*, 2003; Ashok *et al.*, 2009; Bin Kingombe *et al.*, 2010; Nawaz *et al.*, 2010; Yi *et al.*, 2013; Furmanek 2014; Sayed 2017; Sherif *et al.*, 2022),** the ast gene was present in *Aer. hydrophila* and *Aer. caviae* giving PCR products of 331 bp **(Sha *et al.*, 2002; Ashok *et al.*, 2009; Bin Kingombe *et al.*, 2010; Yi *et al.*, 2013; Aravena *et al.*, 2014)**. It was noticed that the cytotonic enterotoxins (ast, alt) genes were not degraded in the intestine tract, while alt enterotoxin is a heat-labile gene, the ast is a heat stable gene at 56°C **(Chopra & Houston, 1999)**, and both genes exhibited hemolytic and cytolytic properties **(Niamah, 2012)**.

In this study, the isolated *A. hydrophila* harbored resistant genes of TetA(A), Sul1, and qnrsA. Similarly, The ARGs for sulphonamide and tetracycline were the most prevalent ones in the isolated *Aer. hydrophila*, *Aer. veronii*, *Pseudomonas fluorescens* and *P. aeruginosa* in *O. niloticus* and *M. cephalus* **(Sherif *et al.*, 2021b)**. *Aeromonas* spp.

act recently as a reservoir of antibiotic-resistant genes for quinolones (qnr) **(Young, 1993)**. In accordance, fish pathologists usually apply tetracycline to fish ponds and fish feed to control diseases in aquaculture worldwide and in the Mediterranean region. Additionally it was noticed that, about 26.3% of the bacterial pathogens have at least one of the tetracycline RGs **(Rigos & Troisi, 2005)**. Similarly, *Aer. hydrophila*, *Aer. veronii*, *P. fluorescens*, and *P. aeruginosa* had more than one ARG for tetracycline and sulphonamide **(Sherif *et al.*, 2021b)**. Accordingly, *Aeromonas* strains that were isolated from the European, were harbored quinolone RG, which is a serious alert concerning fish treatment since quinolone was highly effective in the treatment of *Aeromonas* spp. **(Goni-Urizza *et al.*, 2000; Cattoir *et al.* (2007)**.

The LD_{50} of *Aer. hydrophila* isolates (OM965641, OM965642, and OM965643) were 2.2×10^5, 2.2×10^5 and1.18×10^6 CFU/mL, respectively. Different LD_{50} of *Aer. hydrophila* were obtained by several aquatic pathologists due to differences of fish kind, experimental condition and bacterial strain. Therefore, the LD_{50} of *Aer. hydrophila* was 0.3×10^8 CFU/Ml in *Cyprinus carpio* **(Alsapher *et al.*, 2012)**, 1.5×10^7 CFU/mL in *Clarias gariepinus* **(Hussein *et al.*, 2017)**; whereas, in *C. carpio*, it was 0.3×10^8 CFU/mL **(Sherif *et al.*, 2021c)**. The pathogenicity of *Aer. hydrophila* is attributed to the production of extracellular enzymes and lethal toxins **(Angka *et al.*, 1995; Nieto *et al.*, 1991).**

CONCLUSION

It is concluded that, mass mortality of *M. cephalus* was associated with *Aer. hydrophila* infection. The isolated strains of *Aer. hydrophila* were deposited at NCBI (OM965641, OM965642, and OM965643), and strains were isolated at a rate of 96%, 92%, and 86%. Moreover, it was noted that the isolated strains have cytotonic enterotoxins heat-stable (ast) and cytotoxic enterotoxin (act) virulence genes in addition to some resistant genes, viz. TetA(A), Sul1 and qnrsA.

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