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RESEARCH ARTICLE

Phenotypic, genotypic and virulence traits analysis of aeromonads causing massive mortality in farmed *Oreochromis niloticus*

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Motile aeromonads were identified in earthen-pond-farmed *Oreochromis niloticus* that suffered massive mortalities in Egypt during the summer of 2020. The fish showed hemorrhagic septicemic signs. Poor management practices and inadequate water quality measures were observed in the affected earthen ponds. Motile aeromonads (n = 31 isolates) were identified from 70 fish specimens. Based on their phenotypic and molecular characteristics, isolates were identified as: *Aeromonas hydrophila* (n = 12), *A. veronii* (n = 10), *A. caviae* (n = 5), and *A. sobria* (n = 4). Bacteriological examination of farm water samples also revealed aeromonads (n=9) and some fish-nonpathogenic bacteria. The aeromonad isolates recovered from fish exhibited lipase (52.5%) and protease (47.5%) activities and harboured some virulence genes: *Ser* (62.5%), *Aer* (55%), *ela* (37.5%), *gcaT* (32.5%), *Hyl* (25%), *laf-A* (22.5%), and *Act* (20%). They also harboured numerous antibiotic-resistance genes, including *aadA* (37.5%), *tetC* (32.5%), *tetA* (27.5%), *sul 1* (20%), and *blaTEM* (10%). Virulence and antibiotic resistance genes were also noted in some of the *Aeromonas* spp. isolates obtained from farm water. Aeromonads were highly resistant to ampicillin, amoxicillin, and gentamicin but highly susceptible to ciprofloxacin and florfenicol antibiotics. *Aeromonas* spp pathogenicity was confirmed by the experimental infection of *Oreochromis niloticus*. Our results indicate a positive correlation between excessive tilapia mortalities, motile Aeromonas septicemia and adverse water quality parameters measured during the summer. This study provides data on the virulence, pathogenicity, and antibiotic resistance of motile aeromonads affecting fish and humans, which will be useful for developing efficient therapies.

Introduction

Motile *Aeromonas* septicemia (MAS) is one of the most important bacterial diseases in aquaculture caused by various *Aeromonas* species and results in severe economic losses (Elgendy, Moustafa, et al. 2015). Aeromonads are ubiquitous in aquatic environment, but under stressful conditions, they can cause serious infections and high levels mortality in farmed fish (El-Gohary,

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Zahran, and Abd El-Gawad 2020). *Aeromonas hydrophila*, *A. sobria*, *A. caviae*, and *A. veronii* are the most pathogenic *Aeromonas* spp. in aquaculture (Abdelsalam et al. 2021).

The pathogenesis of MAS is multifactorial and requires the interaction of several variables for the disease to develop. *Aeromonas* species secrete various extracellular enzymes and possess numerous virulence genes that enable bacteria to overcome the host's immune system and initiate severe infections (Hu et al. 2012; El-Gohary, Zahran, and Abd El-Gawad 2020).

Nile tilapia (*Oreochromis niloticus*) aquaculture constitutes an integral part of the Egyptian aquaculture industry (Elgendy et al. 2022). Farmed tilapia are susceptible to numerous bacterial diseases causing massive mortalities and colossal economic losses (Abdelsalam et al. 2022). The excessive use of antibiotics by fish farmers to control such infections have detrimental impacts on the aquatic ecosystem and poses a threat to human health due to the emergence of resistant strains (Elgendy, Shaalan, et al. 2021; Ali et al. 2022; Algammal et al. 2022). This study was conducted to analyze *Aeromonas* spp. isolates recovered from farmed *O. niloticus* that suffered massive mortalities during summer. The molecular characteristics, virulence profile, antibiotic resistance and pathogenicity of aeromonads isolates were investigated.

Materials and methods

Case history and sampling

Abnormal mortalities (250 dead fish every day/earthen pond) were recorded in *O. niloticus* (110–200 g) within five earthen ponds in a fish farm in Beheira Governorate, Egypt, in June 2020. Fish were stocked at a density of 30,000 fish/acre. The farm owner used untreated poultry droppings to promote natural food production in the ponds and had rear-Pecking ducks (190 ducks per/acre). Cattle and sheep grazed at the banks between the earthen ponds. There were several dogs straying on the farm. Moribund fish exhibited signs of hemorrhagic septicemia and respiratory distress. The average temperature, dissolved oxygen level, and unionized ammonia level were 27°C, 4 mg/L, and 0.53 mg/L, respectively. A total of 70 moribund fish were collected and transferred in an ice box to the hydrobiology department laboratory NRC. Water samples (n = 15) (3 samples from each affected pond) were collected and analyzed according to Newaj-Fyzul et al. (2008).

Bacteriological examination

Bacteriological smears were obtained from fish hematopoietic organs (kidneys and liver) and subcultured onto tryptic soy agar. Water samples were collected and examined according to Newaj-Fyzul et al. (2008). Bacterial strains were purified and identified using the Vitek 2 compact device (bio-Merieux).

Molecular identification of Aeromonas spp.

DNA was extracted from purified bacterial strains according to the PrepMan® Ultra Sample Preparation Reagent protocol. Polymerase chain reaction was conducted using *gyrB*-specific primers as described by Hu et al. (2012). The amplified *gyrB* was directly sequenced in two directions using the Sanger DNA sequencer ABI 3730xl DNA sequencer (Applied Biosystems™, USA) at Sigma (Cairo, Egypt), assembled using Bio Edit version 7.0 (Hall 1999) and aligned to other interrelated sequences in the GenBank database using BLASTN search (National Center for Biotechnology Information). The neighbor-joining phylogenetic tree was constructed using MEGA version X (Kumar et al. 2018).

Virulence characteristics of Aeromonas spp.

EXTRACELLULAR ENZYMES

Lipase and protease activities were analyzed according to Carrazco-Palafox et al. (2018) and Sokol, Ohman, and Iglewski (1979).

DETECTION OF VIRULENCE AND ANTIBIOTIC RESISTANCE GENES

The presence of aerolysin (*Aer*), hemolysin (*Hyl*), lateral flagella A (*laf-A*), elastase (*ela*), serine protease (*Ser*), cytotoxic enterotoxin (*Act*), and glycerophospholipid-cholesterol acyltransferase (*gcaT*) genes in the recovered aeromonads strains was investigated according to Sun et al. (2016) using specific primers (Table 1). The strains were also analyzed for the presence of the following antibiotics resistance genes: aminoglycosides (*aad*A), β-lactams (*bla*TEM), tetracycline (*tet*C & *tet*A), and sulphonamide (*sul* 1) according to Ndi & Barton (2011) using specific primers (Table 1).

ANTIBIOTIC SUSCEPTIBILITY TESTING

The susceptibility of the *Aeromonas* spp. isolates to ampicillin 10 µg, amoxicillin 30 µg, gentamicin 10 µg, trimethoprim/sulfamethoxazole 1.25/23.75 µg, florfenicol 30 µg, ciprofloxacin 5 µg, and tetracycline 30 µg was evaluated using the disk diffusion method according to CLSI (2010).

Pathogenicity

A total of 720 healthy Nile tilapia with an average weight of 55 g were collected and acclimatized in aerated aquaria (50 L each) at 25°C ± 1°C. The fish were divided into groups of 10 fish/aquarium in duplicates. They were anesthetized using MS-222 (Sigma) (150 μgL^{-1}). One isolate representative for each *Aeromonas* spp. was chosen at random and used to determine the LD_{50} value. Bacterial cultures were serially diluted, and each fish group was injected intraperitoneally (I/P) with 0.1 ml of the relevant bacterial culture suspension at concentrations ranging from 10^2 to 10^9 CFU/ml/fish (one isolate and one challenge dose per tank). Control fish were injected with 0.1

Table 1. Primers used in the study

Genes	Primers	size/ bp	References
The *gyrB* gene	F: TCCGGCGGTCTGCACGGCGT R TTGTCCGGGTTGTACTCGTC	1100	Hu et al. (2012)
Cytotoxic enterotoxin (*Act*)	F: GAGAAGGTGACCACCAAGAACA R: AACTGACATCGGCCTTGAACTC	232	
Aerolysin (*Aero*)	F: GAGCGAGAAGGTGACCACCAAGC R:TTCCAGTCCCACCACTTCACTTCC	417	Nam and Joh (2007)
Serine protease (*Ser*)	F: ACGGAGTGCGTTCTTCCTACTCCAG R: CCGTTCATCACACCGTTGTAGTCG	211	
Cholesterol acyltransferase (*gcaT*)	F: CATGTCTCCGCCTATCACAACAAGC R: CCAGAACATCTTGCCCTCACAGTTG	339	
Elastase gene (*ela*)	F: ACACGGTCAAGGAGATCAAC F: CGCTGGTGTTGGCCAGCAGG	513	Sen and Rodgers (2004)
Lateral flagella A (*Laf-A*)	F: GGTCTGCGCATCCAACTC R: 5GCTCCAGACGGTTGATG	550	Merino et al. (2003)
Hemolysin (*hly*)	F: GGCCGGTGGCCCGAAGATACGGG R: GGCGGCGCCGGACGAGACGGG	579	Heuzenroeder, Wong, and Flower 1999)
Aminoglycoside resistance gene (*aad*A)	F:GAGAACATAGCGTTGCCTTGG R:TCGGCGCGATTTTGCCGGTTAC	198	Sunde and Norstrom (2005)
β-lactamase resistance gene (*bla*TEM)	F: ATG AGT ATT CAA CAT TTC CG R: CTG ACA GTTACC AATGCT TA	867	Rasheed et al. (1997)
Tetracycline resistance gene *Tet*A	F: GTA ATT CTG AGC ACT GTC GC R: CTG CCT GGA CAA CAT TGC TT	956	Schmidt et al. (2001)
Tetracycline resistance gene (*tet*C)	F: TCT AAC AAT GCG CTC ATC GT R: GGT TGA AGG CTC TCA AGG GC	588	
Sulphonamides resistance gene (*sul*1)	F: CTT CGA TGA GAC CCG GCG GC R: GCA AGG CGG AAA CCC GCG CC	436	Sundstrom et al. (1988)

ml of sterile phosphate buffered saline (PBS). All fish were monitored for 13 days. The LD_{50} value was calculated according to Reed J. and Muencha H. (1938).

Histopathology

Haematoxylin and eosin (H&E) stained histopathological sections were prepared from tissue specimens collected from experimentally infected fish following the methods described by Bancroft and Gamble (2008).

Results

Clinical examination

Moribund tilapia demonstrated signs of hemorrhagic septicemia. Petechial hemorrhages, fin rot, and skin erosions were widely distributed on the external body surface. Internally, the liver, spleen, and kidney were congested and enlarged. Ascites and exophthalmia were noted in some fish (Fig. 1).

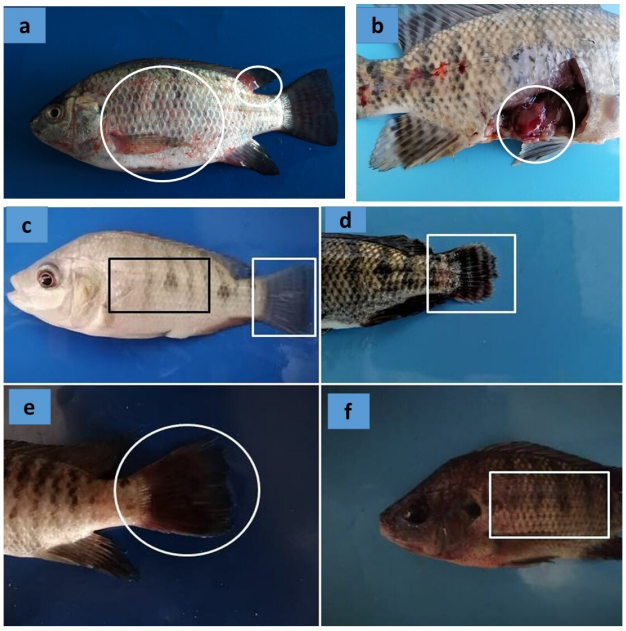

Fig. 1. (a) naturally infected tilapia showing hemorrhages on the external body surface and at the base of fins (circle); (b) naturally infected tilapia showing congestion and enlargement of the liver (circle). (c, d, e, f): Fish used in the pathogenicity testing (c) Nile tilapia injected with saline showing normal appearance of external body surface (square) and caudle fin (square); (d, e, & f) tilapia injected with aeromonads showing: (d) erosions and tail rot (circle); (e) hemorrhages on the caudal fin (circle); and (f) skin darkening (square).

Bacteriological examination

In total, 31 *Aeromonas* spp. isolates were recovered from the investigated fish specimens and identified as *A. hydrophila* (n = 12), *A. veronii* (n = 10), *A. caviae* (n = 5), and *A. sobria* (n = 4). Nine *Aeromonas* spp. isolates were discovered in the farm water samples, including *A. hydrophila* (n = 4), *A. sobria* (n = 2), and *A. caviae* (n = 3). *A. veronii* was not detected in the water samples. Some nonpathogenic bacteria (*Proteus* spp., *Citrobacter freundii,* and *Shigella* spp.) were also detected in some water and fish samples.

Molecular identification of Aeromonas spp.

A comparison of *gyrB* sequences revealed that all isolates (n = 40) belonged to the genus *Aeromonas,* the identity of which was confirmed as *A. hydrophila* (n = 16), *A. caviae* (n = 8), *A. veronii* (n = 10), and *A. sobria* (n = 6).

A. hydrophila isolated from water samples (OL321924, OL321926, OL321928, and OL321930) and that isolated from fish specimens (OL321922, OL321923, OL321925, OL321927, OL321929, OL321931–OL321933, and ON086954–ON086957) exhibited 99.72%–98.05% similarity to the following *A. hydrophila* strains: JN711793^T, JN711794^T, JN711795^T, AB436660^T, and JN711793^T (typing strains) and HQ701864.1, GQ471011.1, JQ805070.1, AB473095.1, KF873661.1,

MK484180.1, JN711794.1, and AB473068.1. The intraspecies similarity was 97.74%–100% for 12 isolates of *A. hydrophila* recovered from tilapia and water, with nucleotide differences ranging from 10 to 24 bp.

A. caviae recovered from fish (OL321939, OL321941, and ON086965–ON086967) and that recovered from water (OL321940, OL321942, and ON086968) showed 98.92%–98.22% similarity to the following *A. caviae* strains: AJ868400^T, JN829530^T, and KC924126^T (typing strains) and MT371974.1, LC003106.1, MN855498.1, KJ747132.1, MN855516.1, KR140073.1, KC924174.1, and MN855511.1. The intraspecies similarity was 99.19%–100% for four *A. caviae* isolates recovered from fish and water, with eight nucleotide differences.

The sequence alignment of *A. sobria* isolated from fish (OL321918, OL321920, ON086963, and ON086964) and that recovered from water (OL321919 and OL321921) exhibited 98.91–98.15% similarity to the following *A. sobria* strains: AB473084^T, HQ442698^T, AF417631^T (typing strains) and MG263589.1, AB473086.1, JN829516.1, MG263541.1, KP115770.1, KJ743530.1, AY101781.1, and HQ442698.1. The intraspecies similarity was 99.20%–100% for four *A. sobria* isolates recovered from Nile tilapia and water, with eight nucleotide differences.

The sequence alignment of *A. veronii* isolated from fish (OL321934–OL321938 and ON086958–ON086962) showed 99.34%–98.84% similarity to the following *A. veronii* strains: FN796748^T, HM584508^T, and AF417626^T (typing strains) and MN659233.1, LC644255.1, AB829112.1, JF938686.1, KR140071.1, MN025464.1, LC003119.1, and JX025899.1. The intraspecies similarity was 98.56%–100% for five *A. veronii* isolates recovered from tilapia, with nucleotide differences ranging from 6 to 16 bp. The phylogenetic analysis confirmed the identity of the isolates (Fig. 2).

Virulence characteristics of Aeromonas spp.

PROTEOLYTIC AND LIPOLYTIC ACTIVITIES

Proteolytic and lipolytic activities were noted in (52.5%) and (47.5%) of isolates recovered from fish, respectively, and in (20%) and (17.5%) of isolates from water, respectively.

DETECTION OF VIRULENCE AND ANTIBIOTIC RESISTANCE GENES

Analysis of *Aeromonas* spp. isolates recovered from fish showed that *Ser* was the most commonly detected gene (62.5%), followed by *Aer* (55%), *ela* (37.5%), *gcaT* (32.5%), *Hyl* (25%), *laf-A* (22.5%), and *Act* (20%) gene. *laf-A* was missing in *A. veronii* and *A. caviae* isolates. *Act* and *Hyl* were absent

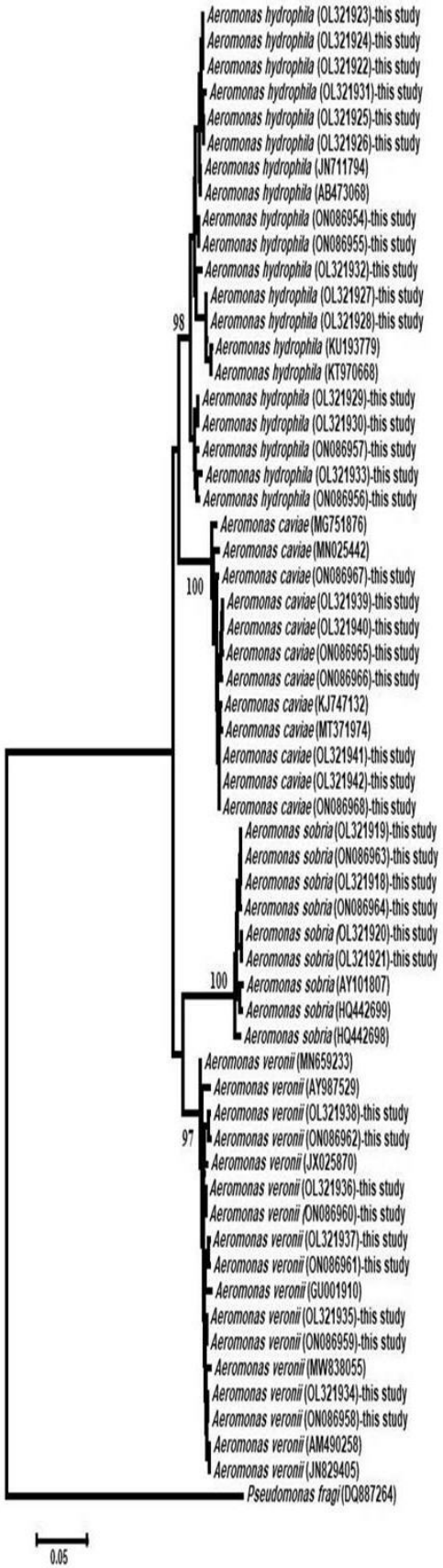

Fig. 2. Phylogenetic tree based on the *gyrB* gene of *Aeromonas* spp.

in *A. sobria* isolates. The *Aeromonas* spp. isolates recovered from water also harboured *Aer* (17.5%), *Ser* (12.5%), *laf-A* (12.5%), *ela* (5%), *Hyl* (2.5%), and *gcaT* (2.5%) genes, while *Act* gene was missing in these isolates.

The *aad*A resistance gene was the most detected among *Aeromonas* spp. isolates obtained from fish (37.5%), followed by *tet*C (32.5%%), *tet*A (27.5%), *sul* 1 (20%), and *bla*TEM (10%). *Aeromonas* spp. isolates recovered from water harbored *aad*A (20%), *tet*C (15%), *tet*A (7.5%), and *sul* 1 (10%) genes, while *bla*TEM was not detected in any of these isolates.

ANTIBIOTIC SUSCEPTIBILITY TESTING

All *Aeromonas* spp. isolates (100%) recovered from both fish and water were resistant to ampicillin 10 µg, amoxicillin 30 µg, and gentamicin 10 µg. Isolates were highly susceptible to ciprofloxacin 5 µg (47.5%), florfenicol (40%) and trimethoprim 1.25 µg/sulfamethoxazole 23.75 µg (32.5%).

PATHOGENICITY OF *AEROMONAS* SPP.

Tilapia injected with *Aeromonas* spp. exhibited skin darkening, and hemorrhages on the external body surfaces, while some fish showed erosions and fin rot (Fig. 1). Control fish showed normal clinical signs. The LD_{50} value determined for the four tested aeromonad isolates was as follows: *A. veronii* (5.5×10^5), *A. hydrophila* (4.21×10^6), *A. sobria* (5.6×10^6), and *A. caviae* (8.48×10^6). Congestion and enlargement of the liver, spleen, and kidneys were the most common post-mortem lesions. Bacterial strains were reisolated from dead fish, and no mortalities were recorded in the control fish.

Histopathological observation

Degenerative, necrotic changes and mononuclear inflammatory cell infiltrations were the most commonly seen histopathological alterations (Fig. 3)

Discussion

Bacteriological and molecular examinations confirmed the identity of motile aeromonads involved in Nile tilapia mortality. The phenotypic and molecular characteristics of the isolates were consistent with previous findings (Abu-Elala et al. 2015). Outbreaks of MAS in the investigated farm was due to inadequate management and lack of biosecurity practices that negatively impacted the physiological and immunological status of fish, rendering them more susceptible to infections (Elgendy, Moustafa, et al. 2015; Elgendy, Soliman, et al. 2015). Untreated poultry manure acts as a source of infections (Abu-Elala et al. 2015).

The motile aeromonads isolated from fish and water displayed numerous virulence factors, including the production of extracellular enzymes that enable aeromonads to adapt to unfavourable environmental conditions and

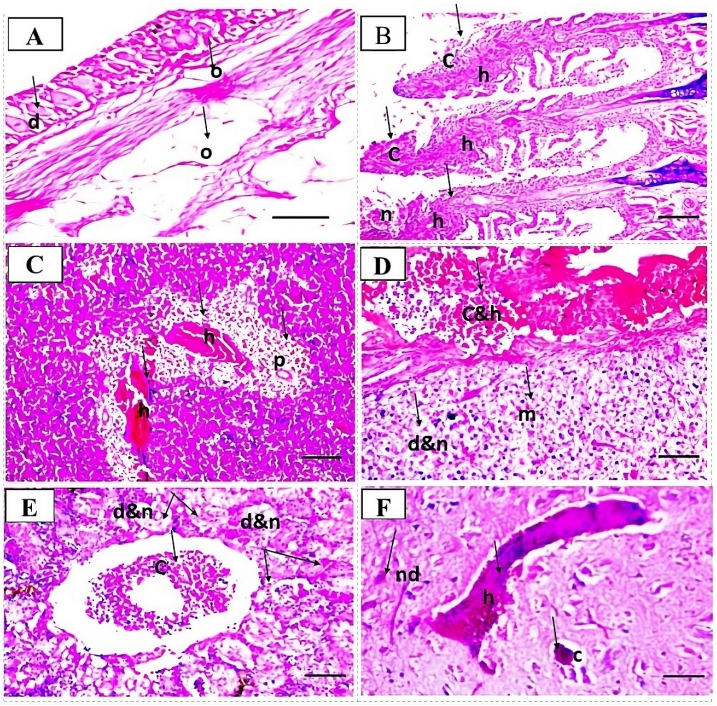

Fig. 3. Histopathological lesions in Nile tilapia experimentally infected with aeromonads, haematoxylin and eosin (H&E) stained sections. **(A)** Skin showing degenerative changes in epidermal cells (d) edema in dermis and underlying muscular layer (o), Bar = 50 µm. **(B)** Gills showing (h) severe hyperplasia, (c) congestion and (n) mononuclear inflammatory cells infiltration, Bar = 50 µm. **(C)** Spleen showing severe hemorrhages (h) and lymphocytic depletion (p), Bar = 20 µm. **(D)**: Liver showing severe congestion (c), hemorrhages (h), diffuse vacuolar degeneration of hepatocytes (d), necrosis (n) and mononuclear inflammatory cells infiltration in between the hepatic parenchyma (m), Bar = 50 µm. **(E)** kidneys showing congestion (c), degenerative and necrotic changes in the tubular epithelium and in the endothelial lining the glomerular tufts (d &n), Bar = 50 µm. **(F):** Brain showing marked neuronal degeneration (nd), congestion (c) and hemorrhages (h), Bar = 50 µm.

facilitated their invasion into fish (Chuang et al. 1997). The virulence genes of the aeromonads have an important role in their pathogenesis. A relatively similar virulence gene profile to the one seen here was reported by El-Gohary, Zahran, and Abd El-Gawad (2020).

The recovered isolates were resistant to most of the antibiotics tested and harbored numerous resistance genes similar to those reported by Ndi and Barton (2011). This dilemma is exacerbated by the contamination of fish farms with agricultural discharges and the use of untreated poultry manure. *Aeromonas* spp. are intrinsically resistant to ampicillin, amoxicillin/clavulanic acid, and cefazoline antibiotics (CLSI 2010). Application of good biosecurity measures, use of medicinal plants as alternative to chemotherapeutics, prudent usage of antimicrobials and performing antibiotic sensitivity testing are critical issues to reduce antibiotic resistance in aquaculture (Elgendy, Awad, et al. 2021; Elgendy et al. 2022).

The challenge experiment confirmed the pathogenicity of *Aeromonas* spp. isolates. The extracellular components and virulence genes of *Aeromonas* spp. are among the primary determinants of their pathogenicity (El-Gohary, Zahran, and Abd El-Gawad 2020).

Conclusion

Stressed fish lose their physiological condition and become vulnerable to motile aeromonad infections. Motile aeromonads harbour numerous virulence and antibiotic-resistance genes. Appropriate management and biosecurity practices are essential in aquaculture for protecting the health of farmed tilapia.

Conflicts of interest/Competing interests

All authors declare that they have no conflict of interest.

Authors' contributions

This study was conducted in cooperation between all authors.

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Ethics approval

The study followed the guidelines of the Institutional Animal Care and Use Committee, National Research Centre, Egypt under the number (231192022).

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