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High mortalities caused by *Aeromonas veronii*: identification, pathogenicity, and histopathological studies in *Oreochromis niloticus*

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Abstract
The current study planned to identify suspected causes of mass mortalities in *Oreochromis niloticus* at private fish farms in Port Said Governorate, Egypt, during the 2017 summer season. One hundred twenty diseased moribund *O. niloticus* fish were randomly collected from different locations. The examined fish showed lethargy, dark skin, bilateral exophthalmia with corneal opacity, and varying degrees of ulcers. Internal examination revealed generalized inflammation, congestion, hemorrhaging, and enlargement of most internal organs. Identification was accomplished through analytical profile index (API) 20E and polymerase chain reaction (PCR) and confirmed by sequencing and phylogenetic analysis. An antibiogram was constructed based on results from a disc diffusion test. Experimental infection was also performed and showed 80% mortality. Virulence genes were also assayed. Histopathological examination of experimentally challenged fish revealed prominent pathological lesions (mainly necrosis), degenerative changes, and cellular vacuolation. In conclusion, *Aeromonas veronii* can be considered one of the main causes of summer mass mortalities in *O. niloticus* in the study area.

Keywords Identification · *Aeromonas veronii* · Antibiogram · Histopathology · Virulence

Introduction

Aeromonas spp. infections are well known in aquaculture and usually cause severe losses among cultured fishes (Tukmechi et al. 2010; Cai et al. 2012; Eissa et al. 2015; Dong et al. 2017; Peatman et al. 2018). Mortality caused by *Aeromonas* in fish exposed to temperature stress under certain environmental conditions can reach up to 80% (Noga 1996). Also, in intensive aquaculture, mortality caused by *A. hydrophila* infections showed an increase in levels from the end of spring season to the beginning of summer (Faisal et al. 1989).

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Aeromonads are characterized by great diversity, and with the current developments in diagnostic techniques, some new members have been recorded (Nováková et al. 2009; Lazado and Zilberg 2018), suggesting that more members have yet to be recognized. *Aeromonas veronii* is one of those overlooked members that has been shown to be a cause of epizootic ulcerative syndrome and hemorrhagic septicemia in fish (Cai et al. 2012). In addition, *A. veronii* is shown to infect invertebrates, aquatic vertebrates, and mammals, including humans (Lazado and Zilberg 2018). *A. veronii* was also reported to cause severe mortalities in cultured Nile tilapia in Egypt (Eissa et al. 2015) with a mortality rate of 100% in the Nile tilapia (Dong et al. 2017).

A few studies have studied and reported the detection of *A. veronii* biovar *veronii* infection as a cause of mass mortality in *Oreochromis niloticus* in Egypt. Thus, in the current study, we aimed to investigate molecular profiles, pathogenicity, and histopathological alterations produced by isolated *A. veronii* from *O. niloticus*.

Materials and methods

Naturally infected fish

A total of 120 diseased *O. niloticus* were randomly collected from different private fish farms in Port Said Governorate, Egypt. The average fish body weight was 200 ± 15 g, and total length ranged from 20 to 30 cm. Fish exhibiting clinical signs were collected alive and transported in double polyethylene bags (one-third filled with pond water and the other two-thirds filled with oxygen) at 25 °C and within 3 h of the collection period. Fish were transported to the Faculty of Veterinary Medicine, Moshtohor, Egypt. Fish, moribund and/or freshly dead, were subjected to postmortem examination according to the method described by Noga (2010).

Clinical and postmortem examinations

Fish were carefully examined for any abnormal behavior, and any abnormal signs were recorded. Postmortem examinations were performed according to Noga (2010).

Isolation and identification

Loopfuls of tissue samples from kidney, spleen, liver, and skin lesions of infected fish were tapped into tryptic soy broth (Difco, USA) and incubated at 30 °C for 24 h and then subcultured onto Rimler-Shotts (RS) agar medium (HiMedia, India) followed by incubation for 18 to 24 h on *Aeromonas* base agar media (Merck, Germany) and incubation at 30 °C for 24 to 48 h as previously described by Cai et al. (2012), Dong et al. (2017), and Matter et al. (2018).

Smears of suspected pure bacterial colonies of cultured samples for the three isolates were prepared, Gram-stained, and microscopically examined. Finally, motility was examined. Isolates were then preserved as glycerol stocks at − 80 °C as reported in *Bergey's Manual of Determinative Bacteriology* (7th ed.; 1964).

Biochemical characterizations were performed using the API 20E (BioMerieux, USA) rapid identification profiling test following the enclosed company's protocol.

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PCR amplification, DNA sequencing, and phylogenetic analysis

A QIAamp DNA Mini Kit (Qiagen, Germany) was used for DNA extraction from the three isolated bacteria with a 94% probability previously identified using the API 20E. Briefly, 200 μl of each of the three bacterial isolate suspensions was added to 20 μl of proteinase K and 200 μl of lysis buffer and incubated at 56 °C for 10 min. Two hundred microliters of absolute ethanol was added to the lysate followed by washing and centrifugation according to the company's procedure (Qiagen, Germany). Nucleic acids were eluted with 100 μl of elution buffer provided in the kit. Primers were supplied by Metabion (Germany) as shown in Table 1. Amplification was done with the Applied Biosystem 2720 thermal cycler, and sequences were recovered using the Applied Biosystems 3130 genetic analyzer (Hitachi, Japan) (Altschul et al. 1990).

MegAlign module of Lasergene DNAStar was chosen for the phylogenetic analyses and building-related tree (Thompson et al. 1994; Tamura et al. 2013).

Antibiogram

Antibiogram profile was constructed according to the National Committee for Clinical Laboratory Standards against eight antibiotics (Oxoid, UK), including ampicillin (Amp, 10 μg), gentamycin (GN, 10 μg), lincomycin (MY, 10 μg), nalidixic acid (NA, 30 μg), tetracycline (TE, 30 μg), ofloxacin (OFX, 10 μg), oxytetracycline (OT, 30 μg), and sulfamethoxazole-trimethoprim (SXT, 25 μg). Isolates were inoculated in Muller-Hinton broth and incubated at 28 °C followed by streaking on Muller-Hinton agar using sterile swabs followed by incubation at 28 °C for 24 h.

Challenge test

Twenty-five healthy *O. niloticus* fish weighing 35 g were obtained from a private fish farm. Health status was evaluated according to the Environmental Protection Agency (EPA) guidelines (Klemm et al. 1993). Fish were allocated into five groups with five fish in each group and allowed to acclimate for 10 days.

Three isolated samples of *A. veronii* from glycerol tubes were spread on trypticase soy agar (TSA) and incubated at 30 °C for 18 h. Separate colonies from each isolate were selected, and 1% of the bacterial suspension was inoculated into tryptic soy broth (Difco, USA) according to Dong et al. (2017) and incubated with shaking for 2 h. Optical density was adjusted to 1.5, 3.0, 6.0, and 9.0×10^8 colony-forming units (CFU) using a set of McFarland standard tubes. Four treatment groups were injected intraperitoneally (IP) with 0.2 ml each of *A. veronii* following the protocol described by El-Asely et al. (2014), except for the control (consisting of a 0.1 ml of sterile thiosulfate citrate bile salts (TCBS) agar injection). After injection, fish were returned to glass tanks and supplied with basal diet (3% body weight). Water temperature was adjusted to 25.0 ± 1.0 °C. Signs and fatalities were recorded every 6 h for 7 days. Bacterial isolation and histopathological examinations were performed for confirming bacterial identification of the three isolates following the protocol by Austin and Austin (1989).

Virulence gene assays

Primers and cycling conditions are described in Table 1. Polymerase chain reaction (PCR) products were separated via 1.5% agarose gel electrophoresis (Applichem, Germany) and 100 bp GelPilot,

Table 1 Primer sequences, genes of interest, and cycling conditions for reverse transcription polymerase chain reaction (RT-PCR)

Target gene	Primer sequences	Amplified segment (bp)	1st denaturation	Amplification (35 cycles)			Final extension	Reference
				2nd denaturation	Annealing	Extension		
16S rRNA	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	1485	94 °C 5 min	94 °C 30 s	56 °C 1 min	72 °C 1.2 min	72 °C 12 min	Lagacé et al. (2004)
qacED1	TAA GCC CTA CAC AAA TTG GGA GAT AT GCC TCC GCA GCG ACT TCC ACG	362	94 °C 5 min	94 °C 30 s	58 °C 40 s	72 °C 40 s	72 °C 10 min	Chuanchuen et al. (2007)
kan	GTGTTTATGGCTCTCTTGGTC CCGTGTCGTTCTGTCCACTCC	621	94 °C 5 min	94 °C 30 s	54 °C 40 s	72 °C 45 s	72 °C 10 min	Frana et al. (2001)
qnrS	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	417	94 °C 5 min	94 °C 30 s	55 °C 40 s	72 °C 45 s	72 °C 10 min	Randall et al. (2004)
blaNDM	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	621	94 °C 5 min	94 °C 30 s	52 °C 40 s	72 °C 45 s	72 °C 10 min	Nordmann et al. (2011)
blaTEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	94 °C 5 min	94 °C 30 s	54 °C 40 s	72 °C 45 s	72 °C 10 min	Colom et al. (2003)
tetA(A)	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576	94 °C 5 min	94 °C 30 s	50 °C 40 s	72 °C 45 s	72 °C 10 min	Randall et al. (2004)
sul1	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433	94 °C 5 min	94 °C 30 s	60 °C 40 s	72 °C 45 s	72 °C 10 min	Ibekwe et al. (2011)
dfrA	TGGTAGCTATATCGAAGAATGGAGT TATGTTAGAGGCGAAGTCTTGGGTA	425	94 °C 5 min	94 °C 30 s	60 °C 40 s	72 °C 45 s	72 °C 10 min	Grape et al. (2007)
aadB	GAGCGAAATCTGCCGCTCTGG CTGTTACAACGGACTGGCCGC	319	94 °C 5 min	94 °C 30 s	58 °C 40 s	72 °C 40 s	72 °C 10 min	Frana et al. (2001)
floR	TTTGGWCCGCTMTCRGAC SGAGAARAAGACGAAGAAG	494	94 °C 5 min	94 °C 30 s	50 °C 40 s	72 °C 45 s	72 °C 10 min	Doublet et al. (2003)
mphA	GTGAGGAGGAGCTTCGCGAG TGCCGCAGGACTCGGAGGTC	403	94 °C 5 min	94 °C 30 s	58 °C 40 s	72 °C 40 s	72 °C 10 min	Nguyen et al. (2009)
ermB	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	425	94 °C 5 min	94 °C 30 s	51 °C 40 s	72 °C 45 s	72 °C 10 min	Nguyen et al. (2009)
aada1	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCATT	484	94 °C 5 min	94 °C 30 s	54 °C 40 s	72 °C 45 s	72 °C 10 min	Randall et al. (2004)

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Table 1 (continued)

Target gene	Primer sequences	Amplified segment (bp)	1st denaturation	Amplification (35 cycles)			Final extension	Reference
				2nd denaturation	Annealing	Extension		
aerolysin (Aero)	CACAGCCAATATGTCGGTGAAG GTCACCTTCTCGCTCAGGC	326	94 °C 5 min	94 °C 30 s	52 °C 40 s	72 °C 40 s	72 °C 10 min	Singh et al. (2008)
act	AGAAGGTGACCACCACCAAGAACA AACTGACATCGGCCTTGAACTC	232	94 °C 5 min	94 °C 30 s	55 °C 30 s	72 °C 30 s	72 °C 7 min	Nawaz et al. (2010)
alt	TGACCCAGTCCTGGCACGGC GGTGATCGATCACCACCAGC	442	94 °C 5 min	94 °C 30 s	55 °C 40 s	72 °C 45 s	72 °C 10 min	
lipase	ATCTTCTCCGACTGGTTCGG CCGTGCCAGGACTGGGTCTT	382	94 °C 5 min	94 °C 30 s	55 °C 40 s	72 °C 45 s	72 °C 10 min	Sen and Rodgers (2004)

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100 bp Plus Ladder (Qiagen, Germany), and GeneRuler 100 bp ladder (Fermentas, Thermo) were used to determine fragment sizes. The reaction was conducted in an Applied Biosystem 2720 thermal cycler following the manufacturer's recommendations as previously described.

Histopathological examination

Tissue samples were collected from gills, livers, kidneys, spleens, muscles, brains, and eyes of moribund fish after 15 days of experimental challenge and preserved in 10% buffered formalin solution. Fixed tissue was stained with hematoxylin and eosin (H&E), examined, and evaluated according to Bancroft et al. (1996).

Results

Clinical examination

The clinical signs of naturally diseased *O. niloticus* included loss of appetite, detached scales, ascites, skin darkness, and hemorrhagic patches covering the body. Also, congestion in all internal organs especially in kidneys, livers, and spleens was recorded.

Isolation of *A. veronii* from naturally diseased *O. niloticus*

Suspected colonies of the three isolates of *Aeromonas* spp. appeared as round, convex, shiny, and/or creamy colonies on TSA media. Colonies were Gram-negative, motile coccobacilli to rod-shaped bacteria. On the RS medium, colonies appeared yellow after 18 to 24 h of incubation and were nonlactose fermentable on MacConkey agar, while on *Aeromonas* base agar media, small, dark green colonies with dark centers were observed.

API 20E showed positive results for β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, and citrate utilization (Table 2). According to Abbott et al. (2003), the three isolates had biochemical characteristics resembling *A. veronii bv. veronii*.

Molecular confirmation of isolated *A. veronii*

Sequencing of the PCR (900 bp) and BLASTing 100% confirmed the identity of the three isolates as *A. veronii*. Results of the derived neighbor-joining phylogenetic tree showed clear clustering of the three isolated strains (*A. veronii*_Egy_strains 1, 2, and 3) with different *A. veronii* strains uploaded from the gene bank (Fig. 1).

Antibiotic susceptibility

Antibiotic susceptibility of the three isolated *A. veronii* strains revealed that they were resistant to all tested antibiotics with the exception of ofloxacin and sulfamethoxazole-trimethoprim.

Challenge test

O. niloticus challenged with the three strains of *A. veronii* showed signs of darkness, unbalanced swimming, and anorexia. Internally, diseased fish showed massive hemorrhaging

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Table 2 Biochemical characterization of *Aeromonas veronii* isolated from naturally infected *Oreochromis niloticus* using the API 20E system

	The 3 isolated Egyptian strains (*A. veronii*_Egy_strains 1, 2, and 3)	*A. veronii bv. veronii*[a]
Character		
Gram stain	Negative	Negative
Motility test	Motile	Motile
API 20E reactions		
β-Galactosidase (ONPG)	+	+
Arginine dihydrolase (ADH)	+	−
Lysine decarboxylase (LDC)	+	+
Ornithine decarboxylase (ODC)	+	+
Citrate utilization (CIT)	+	V
H_2S production	−	ND
Urea hydrolysis (URE)	−	−
Tryptophan deamination (TDA)	+	ND
Indol production (IND)	+	+
Voges-Proskauer (VP)	V	+
Gelatin (GEL)	+	+
D-Glucose (GLU)	+	+
D-Mannitol (MAN)	+	+
Inositol (INO)	−	−
D-Sorbitol (SOR)	−	−
L-Rhamnose (RHA)	−	−
D-Sucrose (SAC)	+	+
D-Melibiose (MEL)	−	−
Amygdalin (AMY)	V	+
L-Arabinose (ARA)	−	−
NO_2 production	+	+

+ positive, − negative, *V* variable

[a] Data from Abbott et al. (2003)

and liver congestion. The percentage mortality showed the highest value (80%) from 9×10^8 CFU dose for the three isolated *A. veronii*_Egy strains followed by 60% of the 6×10^8 CFU dose for *A. veronii*_Egy strains 1 and 2 and 40% of 6×10^8 CFU dose for the *A. veronii*_Egy strain 3. The lowest percentage (20%) was found with the 3×10^8 CFU dose for *A. veronii*_Egy strains 1 and 3 and in 1.5×10^8 CFU dose for *A. veronii*_Egy strain 2 compared with the control group that revealed no mortalities.

Virulence genes

Results of virulence and antibacterial sensitivity gene amplifications demonstrate that the virulence (*lipase* and the enterotoxins, *alt*, *act*, and *aero*) and antibiotic resistance genes (*floR*, *sul1*, *qacED1*, *qnrS*, and *aada1*) were all present in the three *A. veronii*_Egy isolates, indicating that the isolates were multidrug resistant.

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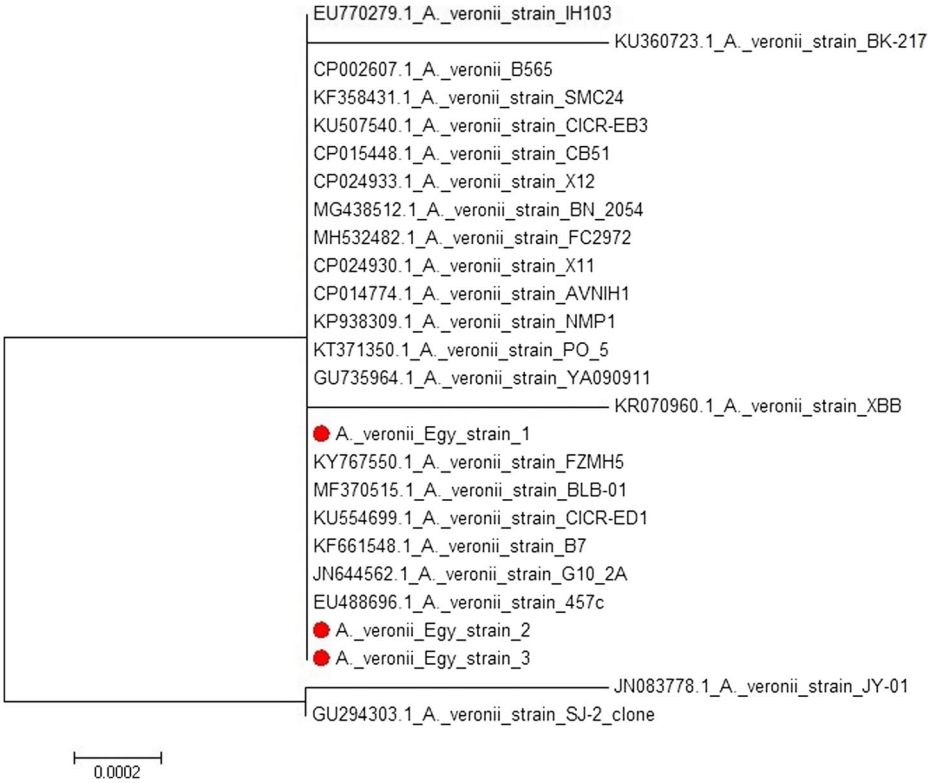

Fig. 1 Phylogenetic tree for *16S rRNA* partial sequences that were generated using maximum likelihood, neighbor joining, and maximum parsimony in MEGA6. Phylogenetic tree showed clear clustering of the three isolated Egyptian strains (*A. veronii*_Egy_strains 1, 2, and 3) with different *A. veronii* strains uploaded from the gene bank

Histopathological findings

Various pathological lesions in gills, livers, kidneys, spleens, muscles, brains, and eyes were observed in infected fish. Gills showed congestion of the central venous sinus and severe necrosis in gill lamellae with leukocytic cellular infiltrations. The livers revealed diffuse vacuolation and coagulative necrosis of hepatocytes and pancreatic acinar cells. The kidney displayed tubular degeneration and hemorrhage in-between renal tubules. The spleens showed marked degenerative changes and diffuse splenic hemorrhages. Congestion of cerebral blood vessels with mild perivascular lymphocytic infiltrations was the most prominent pathological change detected in brain tissues (Fig. 2A). The eyes revealed retinal ulcerations characterized by degenerative changes in the photoreceptor layer of the retina, including cones and rods (Fig. 2B).

Discussion

Aeromonad infections are currently considered the most important bacterial disease affecting freshwater fish (Noga 2010). However, *A. hydrophila* is not the only member reported to cause

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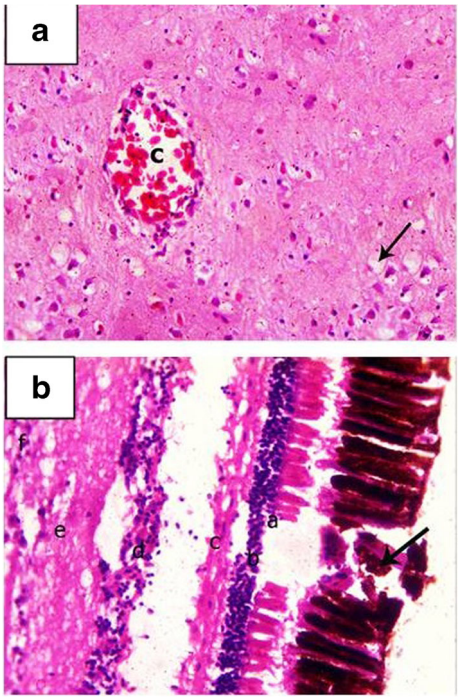

Fig. 2 H&E stained sections of the brain (A) and eye (B) of *O. niloticus* infected with *A. veronii*. (A) Congestion of cerebral blood vessels (c) with mild perivascular lymphocytic infiltrations with vacuolation of the neurons (arrow, × 200). (B) Focal areas of entire desquamation of the pigmented epithelium layer (arrow) with extension of the degenerative changes to cones and rods (a) and degeneration of outer nuclear layer (b), outer plexiform layer (c), inner nuclear layer (d), inner plexiform layer (e), and ganglion cell layer (f) (× 200)

disease conditions in freshwater fish (Peepim et al. 2016; Zhu et al. 2016). The high interspecies similarity of the 16S rRNA gene sequence among aeromonads, which ranges from 96.7 to 100% (Martínez-Murcia et al. 2007), in addition to overlapping biochemical profiles and minimal information about the correlation between phenotypic and genotypic identification makes its taxonomy complex (Ormen et al. 2005).

With the significant development in molecular tools, a great number of other aeromonads have been discovered, but only a minimal amount of information about their pathogenicity is available (Janda and Abbott 2010; Soto-Rodriguez et al. 2013).

A. veronii is known as a cause of hemorrhagic septicemia (Cai et al. 2012) among different fish species worldwide (Eissa et al. 2015; Cai et al. 2012). Additionally, *A. veronii* has previously been reported as an important threat for tilapia (Dong et al. 2017; Peepim et al. 2016).

Regarding the clinical findings from naturally infected fishes in the present study, signs appeared similar to those obtained by Cagatay et al. (2015), who reported signs of septicemia in some freshwater fish. Investigated fish came with previous histories of exposure to environmental stress conditions. At the same time, the same signs were reported in other studies in which a correlation between stress conditions and occurrence of disease was revealed. This correlation indicated that opportunistic pathogens, such as *Aeromonas* spp., had the capability of causing infection only when the fish immune response has been compromised under different environmental stressful conditions, such as high temperature and overcrowding (Noga 2010; Eissa et al. 2015).

Isolated bacterial colonies appeared as round, convex, shiny, and creamy, Gram-negative, motile coccobacilli to rod-shaped bacterial colonies on TSA media. On RS medium, bacteria

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appeared as yellow colonies. The bacteria were nonlactose fermentable on MacConkey agar, while on *Aeromonas* base agar media, small, dark green colonies with dark centers were observed. The same results were previously recorded by Dong et al. (2017), who had isolated *A. veronii* and reported the same morphological and biochemical characteristics as aeromonads.

The API 20E results revealed that the isolated bacteria had biochemical characteristics resembling *A. veronii*. Similarly, Dong et al. (2017) reported the same reactions to isolated *A. veronii* as the isolated bacteria were indole producers, Voges-Proskauer-, gelatin-, and amygdalin-positive.

Molecular characterization of biochemically identified species targeting the 16S rDNA retrieved a specific band at 900 bp. This finding is in agreement with Matter et al. (2018) and Panigrahy et al. (2011), who described aeromonad isolates and reported that they all had bands at the 900-bp DNA fragment level.

Drug resistance results revealed that the three isolated *A. veronii* were resistant to all of the tested antibiotics except for sulfamethoxazole-trimethoprim and ofloxacin, which are the most commonly used antibiotics in aquaculture. Similarly, Lazado and Zilberg (2018) recorded that their *A. veronii* isolate was highly sensitive to trimethoprim/sulfamethoxazole and florfenicol; however, it was resistant to oxytetracycline, neomycin, and norfloxacin. Also, Jagoda et al. (2014) reported their isolate was resistant to oxytetracycline. This raises the impact of using those antibiotics in aquaculture that might affect antimicrobial resistance levels; therefore, attention should be paid toward the use of those drugs in aquaculture (Guardabassi et al. 2000).

The challenge experiment revealed that the three isolates caused 80% mortality in Nile tilapia, suggesting that *A. veronii* could represent a threat to farmed tilapia and should be considered a potential risk to fish health.

Previous studies have revealed the occurrence of genes encoding hemolysin (*hlyA*), aerolysin (*aerA*), serine protease (*ahpA*), and cytotonic enterotoxins (*alt* and *ast*), which have been shown to be responsible for *Aeromonas* spp. virulence (Sha et al. 2002). In the present study, the *A. veronii*_Egy isolate was highly virulent to healthy *O. niloticus* and had four virulence genes (*lipase*, *alt*, *act*, and *aero*) and antibiotic resistance genes (*qacED1*, *qnrS*, *Sul1*, *floR*, and *aada1*). This was in agreement with previous studies in which it was shown that the presence of *hlyA*, *aerA*, and *ahpA* genes in *Aeromonas* isolates confirmed their strong virulence (Nawaz et al. 2010; Zhu et al. 2007, 2016).

Furthermore, various pathological lesions, mainly necrosis and degenerative changes, were recorded in mostly all internal organs of infected fish. So far, few studies have reported that *A. veronii* is pathogenic to *O. niloticus*. Dong et al. (2017) prepared an artificial infection using individual *A. veronii* bacterial species and demonstrated that the isolate killed Nile tilapia and produced severe disease symptoms without the same kind of previously described stress inducers even at varied dose levels of infection.

In conclusion, this study reported that the three *A. veronii*_Egy isolates were associated with Nile tilapia disease and mortality, suggesting its risk to fish heath. Hence, fish producers should pay attention to other members of aeromonads that are capable of causing disease and mortality in tilapia and were previously misdiagnosed as *A. hydrophila.*

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The authors followed all institutional guidelines for the care and use of animals.

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