



Vibriosis outbreaks in farmed Nile tilapia (*Oreochromis niloticus*) caused by *Vibrio mimicus* and *V. cholerae*

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Abstract

Vibriosis is a common disease in aquaculture. Nile tilapia (*Oreochromis niloticus*) farmed within some commercial fish farms in Kafr El-Sheikh governorate Egypt showed signs of disease and exhibited heavy mortality. In order to get to the root of the problem, ninety moribund tilapia were sampled from the affected fish farms and subjected to bacteriological and molecular examinations. Biochemical characterization of bacterial isolates was performed via the API 20E analytical system. All examined fish samples were infected with *Vibrio* species. Eighteen *Vibrio* isolates, *V. mimicus* ($n = 12$), and *V. cholerae* ($n = 6$) were taken randomly for molecular characterization and further analysis. Isolates were genotyped via sequencing and alignment of the *recA* gene. Isolates possessed numerous virulence traits, including the production of hemolysins, proteases, lipases, and nucleases. The prevalence of *ompU*, *vmh*, *vpsR*, and *fliA* virulence genes in *Vibrio* strains was 61.1%, 66.6%, 27.7%, and 33.3 %, respectively. The *bla*TEM (55.5%), *ap*HAI (50%), and *qnr*VC (27.7%) antibiotic resistance genes were recorded in *Vibrio* strains. All *Vibrio* isolates (100%) were resistant to ampicillin 10 µg and amoxicillin 30 µg, while they showed high sensitivity against florfenicol 30 µg (83.3%) and ciprofloxacin 5 µg (77.7%). Challenge experiments in Nile tilapia confirmed pathogenicity of the isolates. Fish showed symptoms of septicemia and high mortality was observed. Infections induced numerous histopathological alterations in diseased fish. This is the first report of *V. mimicus* outbreaks associated with mass mortality in Egyptian farmed Nile tilapia. The findings form the basis for future development of effective control and preventive measures against one of the most infectious pathogens that affect fish and humans.

Keywords Histopathology · Nile tilapia · *recA* gene sequence analysis · *Vibrio mimicus*, *V. cholerae*, virulence genes

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Introduction

Vibriosis is a common bacterial disease in aquaculture. This disease is caused by bacteria belonging to the genus *Vibrio* (Elgendy et al. 2016). Vibrions (family, Vibrionaceae) are Gram-negative curved or straight rod-shaped bacteria, motile by polar flagella, and typically sensitive to the vibriostatic agent (O/129) (Roberts 2012). *Vibrio* spp. attack numerous aquatic animals in marine and fresh aquatic environments (Elgendy et al. 2015a, b).

Vibrio mimicus is one of the emerging *Vibrio* spp. that can cause disease outbreaks and mortalities in some aquatic animals, including yellow catfish (*Pelteobagrus fulvidraco*) (Zhang et al. 2014); black tiger shrimp (*Penaeus monodon*), red-claw crayfish (*Cherax quadricarinatus*), and yabby (*Cherax albidus*) (Eaves and Ketterer 1994; Wong et al. 1995). *Vibrio mimicus* was biochemically identified previously as atypical *V. cholerae* (Davis et al. 1981). Infections relevant to *V. cholerae* are reported in mortalities affecting numerous wild and farmed fish species (Rehulka et al. 2015). *Vibrio mimicus* and *V. cholerae* can also cause serious human infections (Austin 2010).

Vibrio spp. are ubiquitous in aquaculture production systems in the water and sediment (Moller et al. 2021). Vibrions constitute an integral part of the intestinal flora of fish and shellfish (Elgendy et al. 2016). Vibriosis outbreaks in aquaculture are more common during the summer season and are linked to lack of biosecurity and bad managerial practices (Abdelmoneam et al. 2021). *Vibrio* spp. enter in a dormant viable but not cultivatable stage when the water temperature drops below 10 °C (Baffone et al. 2003). Infections may spread via the water, by direct contact between fish, or by ingesting contaminated materials (Moller et al. 2021). Adhesins, hemolysins, proteases, and DNases are among the virulence components involved in the pathogenicity of *Vibrio* spp. enabling them to invade the host and cause tissue damage (Baffone et al. 2001).

Affected fish commonly exhibit signs of septicemia. Lethargy, anorexia, reddening, and petechial hemorrhages along the external body surfaces are the most common clinical manifestations of vibriosis (Moustafa et al. 2015).

Egypt is one of the world's leading producers of tilapia. The Nile tilapia (*Oreochromis niloticus*) is the main farmed fish species in Egypt, accounting for 65.15% of aquaculture production about (1081202 MT) (GAFRD 2019; Elsheshtawy et al. 2019). Infectious diseases and poor management have caused massive mortality in many Egyptian tilapia farms, particularly during the summer with an economic impact of around 720,000 USD (Ali et al. 2020). Serious vibriosis outbreaks with significant economic losses were reported in farmed tilapia in earlier studies (Elgendy et al. 2015b; Abdelsalam et al. 2021). The present study aimed to identify two closely related *Vibrio* spp. (*V. mimicus* and *V. cholerae*) that initiated mortality episodes in farmed Nile tilapia within some earthen ponds at Kafr El-Sheikh Governorate, Egypt, in autumn 2021 using the recA gene as a genetic marker. Some antibiotic resistance and virulence genes were investigated in the recovered bacterial strains. The pathogenicity of isolates was confirmed via experimental infection trials in Nile tilapia. Furthermore, the study investigated the management practices and inferior water quality parameters that may have predisposed fish to infections.

Material and methods

Case history and sampling

In September autumn 2021, heavy mortalities were noticed in farmed *O. niloticus* reared in earthen ponds supplied with agricultural drainage water within private fish farms at

Kafr El-Sheikh Governorate, Egypt. Fish were collected from affected farms in the area. Diseased fish showed lethargy, anorexia, and hemorrhages on the external body surfaces and at the base of the fins. Skin erosions and ulcers on the caudle peduncle area were noticed in some fish. The initial fish stocking density in the affected farms was about 26,000–30,000 fish/acre. The mean depth of earthen ponds was about 150–200 m². No paddlewheels were used on the farms. The average values for water quality parameters in the affected earthen ponds were water temperature (28 °C), dissolved oxygen (4 mg/L), and unionized ammonia (0.65 mg/L). A total of 90 moribund *O. niloticus* with a bodyweight of 140–190 g were collected and kept on ice in an isothermal box and transferred within 3 h to the Laboratory of Hydrobiology Department, Veterinary Research Institute, at the National Research Centre Egypt, for further examinations. Skin and gill scrapings were obtained from some moribund *O. niloticus* at the farm and examined microscopically for the presence of ectoparasites.

Bacteriological examination

Loopfuls from the brain, spleen, kidney, and liver of the investigated fish specimens were streaked onto trypticase soy agar (TSA) supplemented with 5% defibrinated sheep blood (Oxoid, USA) and thiosulfate citrate bile saccharose (TCBS) agar plates (Oxoid, USA), and then all the plates were incubated at 28 °C for 48 h. Colonies randomly taken from plates with dense growth were purified via re-streaking on the same media. Bacterial isolates were subcultured in tryptic soy broth (Difco) and kept stored with 20% (v/v) glycerol at – 80 °C (Buller 2004).

Phenotypic and biochemical characterization

Bacterial isolates were characterized by colony morphological characteristics, sensitivity to the vibriostatic agent (O/129), Gram staining, and bacterial cell shape under the microscope. Biochemical characterizations of all the recovered bacterial isolates were performed using the API20 E identification system (BioMerieux™, France), according to Elgendy et al. (2022).

The *recA* gene sequencing

Eighteen *Vibrio* spp. strains ($n = 18$) were taken randomly for molecular characterization and further analysis. Isolates were revived and cultured onto TSA supplemented with 2% NaCl, and incubated at 37 °C for 18 h. After harvesting the pure colonies, the genomic DNA was extracted using the Bacterial DNA Extraction Kit (BioFlux, Tokyo, Japan), following the manufacturer's protocol. The genomic DNA was then preserved at – 20 °C for molecular studies.

This study selected the locus *recA* gene encoding recombination repair protein to identify 18 *Vibrio* spp. strains recovered from diseased tilapia. PCR was conducted using the specific primer pairs of *recA* gene listed in Table 1. PCR amplification was performed in a 25 µl volume reaction containing 5 µl of purified genomic DNA, 5 µl 10X PCR buffer, 1 µl of each primer (with 50 pmol concentrations), 1.25 µl dNTPs (250 µM), 0.25 µl Taq DNA polymerase

(5 units/ μl), and sterilized double-distilled water to a final volume of 25 μl . The samples were placed in a thermal cycler and subjected to 95 °C for 10 min (initial denaturation); then 30 cycles of 1 min at 95 °C (denaturation), 1 min at 50 °C (annealing), and 2 min at 72 °C (extension); and finally, 10 min at 72 °C (final extension). The PCR products were purified using the Biospin PCR Purification Kit (BioFlux, Japan). The amplified fragments of *recA* locus were bidirectionally sequenced in ABI 3730 automated Sanger sequencer (Macrogen, Inc.), using the BigDye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.). The sequenced amplicon was carefully edited, assembled, and checked for any abnormalities using Bio Edit (Hall 1999). The raw sequences were assembled, assessed, and aligned against other sequences placed in the GenBank database using the DNA-associated database (BLASTn). The assembled sequences were used to identify the species and finally deposited in the GenBank database under specific accession numbers.

Based on *recA* gene sequences, the phylogenetic tree was constructed through the maximum likelihood method based on the Kimura 2-parameter model using MEGA X (Kumar et al. 2018). Tree branching support was based on 1000 bootstrap replicates. *Escherichia coli* (CP000247) was selected as the outgroup in this analysis.

Virulence properties

Enzymatic activities

Hemolysis Hemolysin production was studied by culturing bacterial isolates onto blood agar plates with 5% sheep erythrocytes (Buller 2004).

Protease activity It was assessed using casein as the substrate (skim milk at 2% final concentration). Clear zones surrounding the growth indicated proteolytic activity according to Peralta-Figueroa et al. (2021).

Lipase activity Bacterial isolates were streaked onto a nutrient basal agar plate containing 10% (v/v) egg yolk emulsion. Clear zones indicate lipase production (West and Colwell 1984).

Production of nucleases Production of DNase was assessed by streaking bacterial culture onto DNase agar (West and Colwell 1984).

Detection of virulence genes

Vibrio spp. ($n=18$ isolates) were PCR-screened against 4 different virulence genes: the outer membrane protein (*ompU*), the hemolysin (*vmh*), (*vpsR*) regulatory gene involved in biofilm formation, and the (*fliA*) gene-regulating flagella synthesis in response to environmental changes. The specific primers and annealing temperatures are demonstrated in Table 1. The PCR reactions were performed in a standard 25 μL volume containing 1 μl of template DNA, 12.5 μl of 2X DreamTaq Green Master Mix (Fermentas, USA), and 0.25 μl of each primers using a C1000 Touch™ Thermal Cycler (Bio-Rad, UK). The thermocycling program utilized in detection of these genes was adjusted following the protocols described in previous studies: *ompU* (Singh et al. 2002) and *Vmh* (Shi et al. 2000), while *vpsR* and *fliA* genes followed Gennari et al. (2012) as shown in in Table 1.

Table 1 Primer sequences for the detection of virulence and antibiotic resistance genes in *Vibrio* spp

Gene	Oligonucleotide sequence 5'-3'	Amplicon size (bp)	Annealing temperatures	References
Recombination repair protein (<i>recA</i>)	F-TGARAARCAARTTYGGTAAAGG R-TCRCCNTTTRTAGCTRTACC	837	50 °C	Zhang et al. (2014)
Outer membrane protein (<i>ompU</i>)	F-ACGCTGACGGAAATCAACCAAAG R-GCGGAAAGTTGGCTTGAAGTAG	869	62 °C	Singh et al. (2002)
Hemolysin Vmh	F-GGTAGCCATCAGTCTTATCAG R-ATCGTGTCCCAATACITTCACCG	390	54 °C	Shi et al. (2000)
vpsR	F-GGTGAGTAGCCATAAGCAAG R-CATCCAGCACCAAGATATCT	911	59 °C	Gennari et al. (2012)
fliA	F-GAGGCAACAGCACCATCAAA R-CGCATCTATATCAGGGACAA	503	55 °C	Gennari et al. (2012)
Beta-lactams resistant gene <i>bla</i> TEM	F-TTTCGTGTGCGCCCTTATTC R-CCGGCTCCAGATTTATCAGC	690	60 °C	Bailey et al. (2011)
Aminoglycosides resistant gene <i>aph</i> HA1	F-ATGGGCTCGCGATAATGTC R-CTCACCGGAGGCAGTTCCAT	600	50 °C	Maynard et al. (2004)
Fluoroquinolones resistant gene <i>qnr</i> VC	F:CCCTCGAGCATGGATAAAACAGACCAG TTATA R:CGGGATCCCTTAGTCAGGAACACTACTATT AAACCT	521	62 °C	Xia et al. (2010)

Briefly, the PCR reaction started with an initial denaturation for 4 min at 94 °C. The reaction was continued for 35 cycles, with each cycle consisting of denaturation for 40 s at 94 °C, annealing at the optimal temperature for each specific primer (Table 1), extension at 72 °C for 90 s, and a final extension phase at 72 °C for 6 min. The amplified PCR products were electrophoresed (1.5% agarose), and the amplicons were ethidium bromide-stained and visualized under UV light.

PCR detection of antibiotic resistance genes

The antibiotic resistance genes, *bla*TEM (β -lactams), *ap*HAI (aminoglycosides), and *qnr*VC (fluoroquinolones), were investigated in the recovered vibrio isolates. The PCR reactions were performed as formerly described by Gxalo et al. (2021) using specific primers as shown in Table 1. The cycling conditions used comprised initial denaturation for 5 min at 94 °C, 35 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at optimum temperatures as described in Table 1, extension at 72 °C for 91 s, and final elongation at 72 °C for 5 min.

Antibiotic susceptibility testing

Antibiotic susceptibility of *Vibrio* isolates ($N=18$) was assayed using Muller-Hinton agar (Oxoid, USA) via the disc diffusion assay (Bauer et al. 1966). The turbidity of the bacterial culture was adjusted to match 0.5 McFarland standards. Bacterial isolates were tested against the following antimicrobial agents: tetracycline (30 μ g), ciprofloxacin (5 μ g), florfenicol (30 μ g), trimethoprim/sulfamethoxazole (1.25/23.75 μ g), amoxicillin (30 μ g), ampicillin (10 μ g), and gentamycin (10 μ g). Each antibiotic sensitivity testing was performed in duplicate. Results were interpreted as susceptible (S), intermediate sensitive (IM), or resistant (CLSI 2010a, b).

Pathogenicity testing and determination of the LD50

Experimental fish

Apparently, healthy *O. niloticus* ($n=305$) with a mean weight of 45 g were brought from a private fish farm in Giza governorate. Fish were acclimatized in tanks (50 L) containing de-chlorinated, continuously aerated water at 25 ± 1 for 2 weeks. Randomly taken fish ($n=5$) were bacteriologically examined to confirm that the fish were not infected with bacteria using the same procedures performed in the bacteriological examination of naturally infected fish. After the acclimatization period, the experimental fish were randomly divided into groups of (10 fish/aquaria) in duplicates. Two bacterial isolates showing the most studied virulence traits (*V. mimicus* OM994477 and *V. cholerae* OM994488) were used in the pathogenicity test. Bacterial culture was prepared via culturing into LB broth with 1% NaCl, incubated at 28 °C with shaking for 18 h, centrifuged at 5 000 rpm min^{-1} for 10 min at 4 °C, and re-suspended in sterile phosphate-buffered saline (PBS). Experimental fish were intraperitoneally injected with 0.1 ml of serially diluted (tenfold) bacterial culture suspension in normal saline from 10^2 to 10^8 CFU/ml for each isolate (one isolate and one challenge dose per tank). Control fish were injected with 0.1 ml of sterile PBS. Fish were anesthetized using 150 $\mu\text{g l}^{-1}$ MS-222 (Sigma) before injection. All fish were monitored for

10 days, and mortalities were recorded. The LD50 was calculated using the Probit analysis statistical tool. The cause of death was confirmed by the re-isolation and identification of injected vibrios from dead fish. All the fish sampled in this study were handled following the guidelines of welfare and ethics of Laboratory Animals approved by the Animal Ethics Committee National Research Centre, Egypt.

Histopathology

Skin, liver, spleen, kidney and gill tissue samples were collected from freshly dead fish, fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin wax. Sections were examined microscopically using a light microscope after being stained with hematoxylin and eosin (H&E) (Suvarna et al. 2012).

Statistical analysis

The challenge experiments and the LD50% were analyzed using the Probit analysis statistical tool.

Results

Clinical examination

Affected fish showed lethargy, dark coloration, and hemorrhages on the external body surfaces, around the vent, mouth, and at the base of fins. Some fish displayed fin rot, inflamed vent, exophthalmia with hemorrhages in the eye, skin erosions, and ulcers. Distention of gall bladder, hepatomegaly, and splenomegaly were the common post-mortem lesions. The liver was pale-colored in some fish. In most investigated specimens, abdominal distension and reddish fluid in the peritoneal cavity were noticed (see Fig. 1).

Bacteriological examination

All examined fish samples ($n=90$) were infected with *Vibrio* spp. The majority of fish samples ($n=67$) were infected with a mixed infection with *V. mimicus* and *V. cholerae*. The remaining fish samples ($n=23$) was infected with a single infection either with *V. mimicus* ($n=14$) or *V. cholerae* ($n=9$). Some nonpathogenic bacterial species were discovered in the investigated fish samples. A total of $n=18$ *Vibrio* isolates were taken randomly for complete phenotypic and molecular examination. Isolates were Gram-negative, slightly curved rod-shaped, oxidase-positive, and sensitive to the vibriostatic agent (O/129) (2,4-diamino-6,7-di-iso-propylpteridine phosphate). The biochemical characterization identified isolates as *V. mimicus* ($n=12$) and *V. cholerae* ($n=6$), using the API 20 E identification system. All *V. mimicus* strains ($n=12$) produced green-colored colonies onto thiosulfate-citrate-bile-salt-sucrose agar media, indicating failure to ferment sucrose. The majority of *V. cholerae* strains ($n=5$) produced yellow-colored colonies pointing to fermentation of sucrose, while one isolate ($n=1$) produced green-colored colonies onto the TCBS (see supplementary Table 1). There were no parasite infestations discovered in the examined scrapings of the skin and gills.

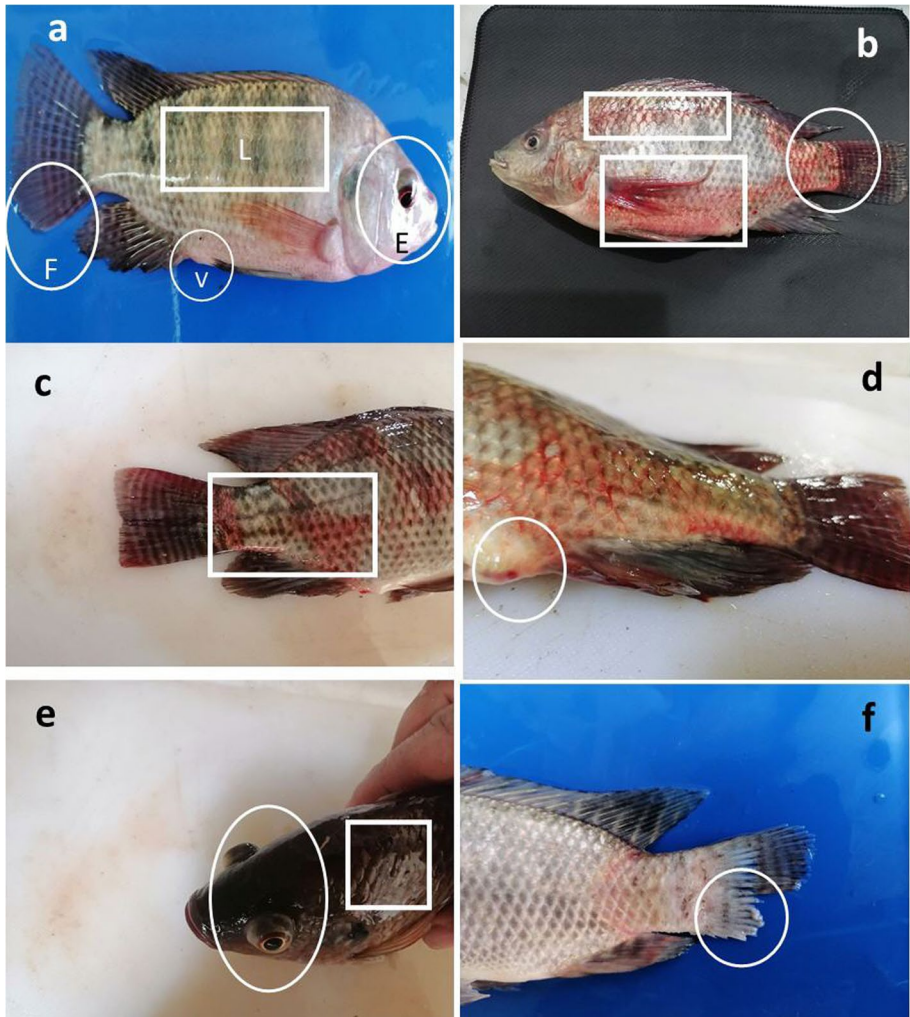


Fig. 1 Clinical examination of naturally infected fish. **(a)** Healthy Nile tilapia showing normal clinical picture; **(L)** lateral surface; **(f)** intact fins; **(v)** normal vent; **(e)** normal eye. **(c, d, e, f)** Nile tilapia naturally infected with vibrios showing; **(b, c)** severe hemorrhages on the external body surfaces (square) and ulceration on the skin (circle). **(d)** Inflammation of the vent (circle). **(e)** Bilateral exophthalmia and darkening of the fish coloration (square). **(f)** Severe fin rot

Molecular identification

The alignment analysis of *recA* gene sequences revealed that all 18 *Vibrio* spp. isolates belonged to the *Vibrio* species. The identities of 12 isolates of *Vibrio* species were confirmed to be *V. mimicus* and were deposited in the GenBank database under the following accession numbers: OM994472, OM994473, OM994474, OM994475, OM994476, OM994477, OM994478, OM994479, OM994480, OM994481, OM994482, and OM994483. The intraspecies similarity in the partial

recA gene sequences was 98.43–100% for *V. mimicus* (OM994472) against the other 12 isolates recovered from diseased Nile tilapia, and the nucleotide differences ranged from 4 to 12 bp. The alignment analysis of the recA gene sequences of *V. mimicus* isolates showed 99.74–98.81% similarity to the accession numbers of *V. mimicus* (CP016383.1, KJ604710.1, EF990319.1, CP046823.1, EF990316.1, AF301121.1, CP046846.1, and EF990317.1).

Alternatively, the identities of six isolates of *Vibrio* species were confirmed to be *V. cholerae*, and their sequences were deposited in the GenBank database under the following accession numbers: OM994484, OM994485, OM994486, OM994487, OM994488, and OM994489. The intraspecies diversity for six strains of *V. cholerae* was 97.90–100%, and the nucleotide differences ranged from 7 to 16 bp. The alignment analysis of recA gene sequences of *V. cholerae* isolates exhibited 99.57–95.22% similarity to the following accession numbers of *V. cholerae* (KF421475.1, KP833904.1, KC493027.1, CP013014.1, FJ479704.1, CP000627.1, AF301074.1, and KP833871.1).

The constructed phylogenetic tree demonstrated two main lineages and different *Vibrio* species arranged as polyphyletic. The first lineage was additionally alienated into two subclades, and the first subclades are further divided into two branches. The phylogenetic tree of the recA genes sequences of *V. mimicus* and *V. cholerae* are grouped with other *V. mimicus* and *V. cholerae* sequences and formed two separated branches with a bootstrap value of 98% as exhibited in Fig. 2. The first branch contained *V. mimicus* isolates separated from *V. cholerae* and other *Vibrio* species. The current isolates of *V. mimicus* are grouped with other *V. mimicus* isolates with a high bootstrap value of 99%. The second branch encompassed *V. cholerae* in this study and gathered with other sequences of *V. cholerae* with a high bootstrap value of 100%. Other *Vibrio* species are arranged in different clades and branches.

Virulence properties

Enzymatic activities

Hemolysis, protease, lipase, and nuclease production All the analyzed *Vibrio* strains (100%) were hemolytic on sheep blood agar plates. Production of protease, lipase, and nuclease were noticed in 61.1%, 72.2%, and 38.8% of isolates, respectively, as shown in supplementary Table 1.

Detection of virulence genes

The *ompU* gene was noticed in 61.1% of isolates: *V. cholerae* (4/6 isolates) and *V. mimicus* (7/12 isolates). The *vmh* gene was noticed in 66.6% of isolates: *V. cholerae* (0/6 isolates) and *V. mimicus* (12/12 isolates). The *vpsR* gene was noticed in 27.7% of isolates: *V. cholerae* (5/6 isolates) and *V. mimicus* (0/12 isolates). The *fliA* gene was detected in 33.3% of isolates: *V. cholerae* (6/6 isolates) and *V. mimicus* (0/12 isolates) (see supplementary Table 1).

Antibiotic resistance genes

Vibrio spp. isolates possessed some antibiotic resistance genes: 55.5%, 50%, and 27.7% for *bla*TEM, *ap*HAI, and *qnr*VC, respectively, as shown in supplementary Table 1.

Fig. 2 Phylogenetic tree constructed based on the comparative sequences of the partial *recA* gene sequences of different 18 isolates *Vibrio* spp. retrieved from naturally infected Nile tilapia in this study and related isolates of *V. mimicus* and *V. cholera*

Antibiotic susceptibility testing

All *Vibrio* isolates (100%) were resistant to ampicillin 10 µg and amoxicillin 30 µg. The highest sensitivity was noticed against florfenicol 30 µg (83.3%), ciprofloxacin 5 µg (77.7%), and trimethoprim 1.25 µg/sulfamethoxazole 23.75 µg (66.6%), respectively (see supplementary Table 2).

Pathogenicity

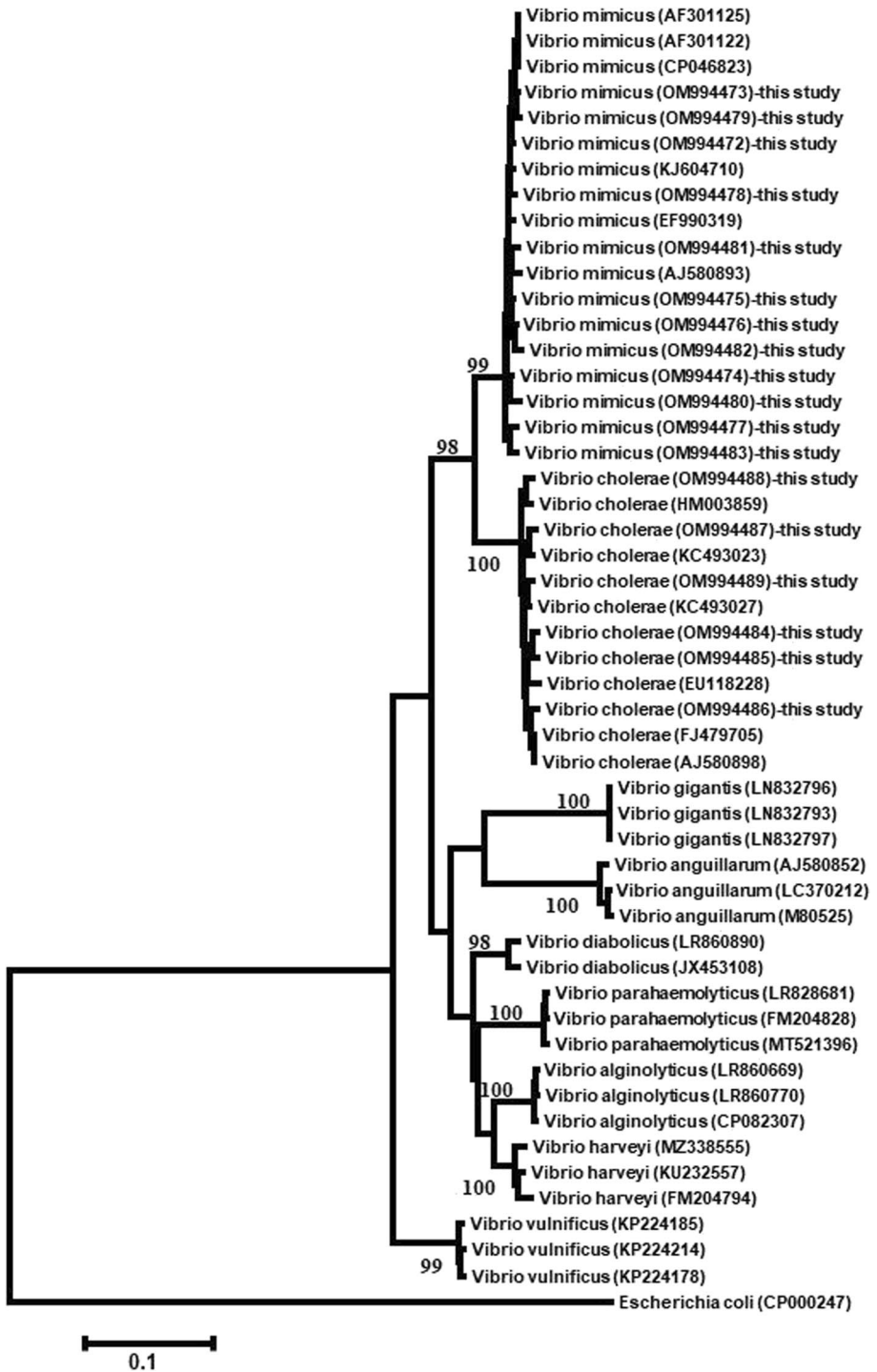
The tested bacterial isolates were pathogenic to Nile tilapia. Experimentally infected fish showed signs of septicemia. Darkening and petechial hemorrhages on the external body surfaces and at the base of the fins were frequently observed. Severe skin erosions were commonly noticed. Internally, severe congestion and enlargement were noticed in the liver, spleen, and the kidneys. The LD₅₀ value of *V. mimicus* and *V. cholerae* isolates was determined at 8.2×10^5 and 1.62×10^6 CFU/ml, respectively. Bacterial isolates were reisolated in pure culture from all dead fish. Control fish remained healthy and showed no mortalities.

Histopathology

Degenerative and necrotic changes were commonly observed in tissue sections with chronic inflammatory cells infiltration as shown in supplementary Fig. 1.

Discussion

Vibriosis in the present study was exacerbated by some inferior water quality measures and bad managemental practices in the affected tilapia earthen ponds. Vibrios constitute an integral part of the microbiota of aquatic environments. Aquatic animals become susceptible to *Vibrio* spp. infections when they suffer a stress condition similar to what was observed in the present study (Elgendy et al. 2016). Overstocking renders farmed fish stressed and more vulnerable to infections (Attia et al. 2021). Fish competition for food can induce abrasions on their external body surfaces, which may serve as effectual entry routes for numerous microorganisms. The water quality in the aquaculture production system deteriorates when overstocking is not combined with proper management (Odhiambo et al. 2020). The dissolved oxygen and unionized ammonia values in the present study were unfavorable for the health of the earthen-pond reared tilapia (4 mg/L and 0.65 mg/L, respectively). The negative consequences of these qualities exacerbate when combined with a high temperature (28 °C in the affected earthen ponds); these records are not comparable to the international standards recommended for fish farming. The optimum oxygen level in aquaculture ponds is about 6 mg/l (Bhatnagar and Devi 2013), while the acceptable unionized ammonia level is (0.05–0.1 mg/l) (Boyd 1990; Francis-Floyd et al. 2009), and it should not exceed 0.02 mg/l according to Bhatnagar and Devi (2013). Unionized ammonia damages the gills and other fish tissues (Francis-Floyd et al. 2009), facilitating invasions with infectious agents.



The bacteriological examination of moribund fish specimens indicated involvement of the detected *Vibrio* spp. in tilapia mortalities. The findings were consistent with earlier reports that described significant mortality rate in catfish in China (80–100%) relevant to *V. mimicus* pandemic (Geng et al. 2014). Affected catfish in this epizootic exhibited lethargy, anorexia, skin discoloration, and skin ulcers. Authors attributed these clinical manifestations to the pathogenic factors of *V. mimicus*, such as hemolysins and enterotoxins. Outbreaks relevant to *V. mimicus* were recorded also in numerous crustaceans such as *Cherax albidus* and *Cherax quadricarinatus* (Eaves and Ketterer 1994; Wong et al. 1995). Similarly, Abdel-moneam et al. (2021) isolated *V. cholerae* (31.3%) from mass mortality affecting Nile tilapia farms in Kafr El-Sheikh Governorate, Egypt. Authors concluded that tilapia fish kill was due to combination of environmental stressors and bacterial infections. Enany et al. (2019) identified *V. cholerae* with a prevalence of (12.5%) in the summer mortality affecting farmed Nile tilapia. Hassan et al. (2020) discovered *Vibrio* spp. in Nile tilapia mass mortality but at a lower prevalence (16.48%) than in the present study. Results agreed with Elgendy et al. (2015b) who reported infections with *Vibrio* spp. in Nile tilapia mass kill (98%) in an Egyptian fish farm. The same authors attributed the enhanced bacterial infections to the impaired immune mechanisms triggered by the unfavorable aquatic conditions.

The phenotypic characteristics of the recovered *Vibrio* spp. strains were similar to the published descriptions for *V. mimicus* and *V. cholerae* strains (Davis et al. 1981; Buller 2004; Farmer et al. 2005; Geng et al. 2014). The recovered *Vibrio* isolates showed typical and atypical phenotypic traits in line with previous studies (Davis et al. 1981; Vieira et al. 2001). The findings corroborated those of Lupiani et al. (1993) and Davis et al. (1981), who detected *V. mimicus* and *V. cholerae* in wild and hatchery-reared fish. The same authors reported obvious resemblance in the biochemical profiles of the recovered *Vibrio* isolates, with the exception of sucrose fermentation. The phenotypic similarities between *V. mimicus* and *V. cholerae* may lead to misdiagnosis highlighting the need of molecular characterization for the accurate identification of *Vibrio* isolates. Results of the current study confirmed the usefulness of the *recA* gene as a genetic marker to characterize the causative agents of vibriosis affecting tilapia fish. The alignment analysis of the *recA* gene sequences confirmed the results of phenotypic characterization and grouped isolates into *Vibrio* spp. The identity of *Vibrio* strains was confirmed as *V. mimicus* ($n = 12$) and *V. cholerae* ($n = 6$), including one atypical isolate ($n = 1$). The phylogenetic analysis demonstrated two main lineages and different *Vibrio* spp. arranged as polyphyletic. The phylogenetic tree of the *recA* genes sequences formed two separated branches with a bootstrap value of 98%. The first branch contained *V. mimicus* isolates separated from *V. cholerae* and other *Vibrio* spp. with a high bootstrap value of 99%. The second branch encompassed *V. cholerae* strains gathered with other sequences of *V. cholerae* with a high bootstrap value of 100%. Other *Vibrio* species are arranged in different clades and branches. Results confirmed that genetic information derived from the *recA* gene analysis could be used to differentiate *V. mimicus* and *V. cholerae*. Both *Vibrio* spp. have a high level genetic relatedness, including similar 16S rDNA sequences and virulence-associated genes (Desmarchelier and Reichelt 1984; Boyd et al. 2000). The findings support previous reports demonstrating that sequencing the (*recA*) gene has a greater discriminating value than 16S rDNA and can be used as a genetic marker for phylogeny estimation (Thompson et al. 2004).

The pathogenesis of *Vibrio* spp. is mediated by various processes, including the generation of extracellular enzymes. All of the *Vibrio* strains studied were hemolytic (100%), and isolates produced protease, lipase, and nuclease (61.1%, 72.2%, and 38.8%, respectively). These findings are comparable to results from a study by Alam et al. (2006) which revealed

protease activity in 95% of *V. mimicus* strains. Additionally, Beshiru (2018) reported that protease and DNases production were noticed in 90% and 100% of tested *V. mimicus* strains. Davis et al. (1981) reported lipase activity in 10% of the tested atypical *Vibrio cholerae* strains. These enzymes enhance the invasiveness and the spread of vibrios through tissues. The investigated vibrio strains harbored some virulence genes. *V. mimicus* isolates possessed the *ompU* and *vmh* genes, while *V. cholerae* encoded the *ompU*, *vpsR*, and *firA* genes. Similar results were reported by Gennari et al. (2012), Gxalo et al. (2021), and Hernandez-Robles et al. (2021). These genes regulate many virulence functions in *V. mimicus* and *V. cholerae* strains as their presence potentiates pathogenicity. The outer membrane protein (*ompU*) gene mediates adherence to host cells. In contrast, the *vpsR* gene is involved in biofilm formation and environmental persistence, whereas the *firA* gene controls flagella production in response to environmental changes (Liu et al. 2015; Gennari et al. 2012).

The existence of antibiotic resistance genes (*bla*_{TEM}, *ap*_{HAI}, and *qnr*_{VC}) in the analyzed *Vibrio* spp. highlighted the dilemma of antibiotic resistance in aquaculture, one of the imminent challenges to aquatic animals and human health worldwide. Similarly, Gxalo et al. (2021) reported the detection of these resistance genes in *Vibrio* strains collected from the aquatic environment. All *Vibrio* isolates (100%) were resistant to ampicillin 10 µg and amoxicillin 30 µg, while they showed a higher sensitivity against florfenicol 30 µg (83.3%) and ciprofloxacin 5 µg (77.7%). The use of drainage water in aquaculture might speed up the propagation of resistant strains in the aquatic environment. The high resistance against the tested antibiotics may be linked to their improper use to treat fish infections. Immunostimulation using medicinal plants and vaccination are new therapies in aquaculture. However, there are some factors related to vaccination that should be considered including cost, mode of administration, safety for fish, and environment. Long-term disease prevention and control require the adoption of biosecurity measures and good management practices. Ali et al. (2020) discovered a link between disease occurrence in farmed tilapia and some farming practices in Egyptian tilapia farms.

The experimental infection trials confirmed the pathogenicity of *V. mimicus* and *V. cholerae* strains. Challenged fish displayed septicemic signs similar to naturally infected fish. Necrotic and degenerative histopathological alterations were observed in tissue sections concordance with previous reports (Zhang et al. 2014). These changes may be relevant to the toxic products produced by *Vibrio* spp. including extracellular enzymes and toxins. The recovery of bacterial isolates from all succumbed fish confirmed the pathogenicity of *Vibrio* spp. and specificity of the cause of fish mortality.

Conclusion

V. mimicus and *V. cholerae* are important *Vibrio* spp. in aquaculture causing vibriosis disease in Nile tilapia and can result in significant mortalities. The occurrence of vibriosis increases with poor aquatic conditions. The *recA* gene can be used as a genetic marker for the molecular characterization of *V. mimicus* and *V. cholerae* infections affecting fish. *Vibrio* spp. produce several extracellular enzymes including proteases, lipases, and nucleases potentiating their virulence. Numerous virulence genes are encoded by *V. mimicus* and *V. cholerae* strains which enhance their pathogenicity. Vibriosis infections cause severe histopathological alterations in diseased fish. The antibiotic susceptibility patterns varied among the obtained *Vibrio* isolates. Immunostimulation using medicinal plants and vaccination are new therapies in aquaculture.

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Data availability All data generated or analyzed during this study are included in this published article.

Code availability Not applicable.

Declarations

Ethics approval The study followed the guidelines and instructions of the Institutional Animal Care and Use Committee, National Research Centre, Egypt.

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