Multifactorial causes of mass mortality in *Oreochromis niloticus* in Kafr El-Sheikh, Egypt

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Abstract

Mass mortality episodes have been reported in *Oreochromis niloticus* (Nile tilapia) farms in Kafr El-Sheikh Governorate, Egypt. Physicochemical water analysis revealed a water temperature of 31.33 °C ± 0.77 °C, practical salinity units (PSU) of 45 ± 4.08, and unionised ammonia (UIA) levels of 0.49 ± 0.02 mg/L. In bacteriological examinations, *Aeromonas hydrophila*, *Vibrio alginolyticus*, and *Vibrio cholerae* were isolated from kidney of infected fish (81.3%, 68.8%, and 31.3 % of sampled fish, respectively). Confirmation of the bacterial species isolates was performed using universal primers for the 16S rRNA gene, followed by species-specific gene detection and sequencing analysis for each species isolated. Antibiotic sensitivity testing revealed that the bacterial isolates showed sensitivity to ciprofloxacin (CIP). In summary, the cause of this fish-kill appeared to be multifactorial in nature, due to a variety of environmental, bacteriological causes.

Introduction

Oreochromis niloticus (Nile tilapia) is the most economically important farmed fish in Egypt, as up to 65% of the fish produced annually are tilapia (Shaalan et al., 2018a). However, several mass mortality episodes have been reported, which result in substantial economic losses to the fish farmer (Aboyadak et al., 2015; Elsheshtawy et al., 2019).

Mass fish kills have taken place in Kafr El-Sheikh, Egypt, which are multifactorial and may be attributed to deterioration in water quality, heavy metal contamination, infectious diseases or the interaction between various environmental and biological causes (Abdelsalam et al., 2017; Shaalan et al., 2018a).

Reported bacterial diseases in Egyptian aquaculture are mainly caused by members of family Vibrionaceae, Pseudomonadaceae, Aeromonadaceae and Streptococcaceae (Shaalan et al., 2018a; El-Adawy et al., 2021). *Aeromonas* and *Vibrio* bacteria are associated with natural fish microbiota (Haenen et al., 2013). Several mass mortality episodes due to *Vibrio* and *Aeromonas* bacterial infection were recorded in different geographical regions in Egypt (Aboyadak et al., 2015; Elsheshtawy et al., 2019).

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Within the family Vibrionaceae, *Vibrio alginolyticus* was found to be one of the most widely reported pathogens, severely affecting marine water fishes (Yishan et al., 2011). However, they were also isolated from brackish water fishes (El-Sayed et al., 2019). *Vibrio cholerae* was isolated from gills, kidney and brain tissues of diseased fishes as ayu (*Plecoglossus altivelis*), guppy fish (*Poecilia reticulate*) and Nile tilapia (Halpern and Izhaki, 2017), while *A. hydrophila* has been responsible for high mortality episodes in farmed fish (Aboyadak et al., 2015; Okasha et al., 2016).

Application of good sanitary and biosecurity measures in fish farms, together with antibiotics is known to be effective for the treatment of bacterial disease outbreaks. However, the main adverse effect of randomly administering antimicrobial agents is bacterial antimicrobial resistance development (Shaalan et al., 2018b). This study was performed to investigate the biological and environmental causes behind massive fish kills in cultured Nile tilapia in Kafr El-Sheikh, Egypt during the summer season and to establish the antibiotic of choice based on the antibiotic sensitivity of bacteria isolated from diseased fish.

Materials and Methods

In the late summer of 2019, when air temperature was around 35°C, a sudden outbreak of mass mortalities, reaching up to 75%, was observed in Nile tilapia, weighing 80–90g, in a semi-intensive fish farm in Kafr El-Sheikh Governorate, Egypt (Figure 1). Tilapia fingerlings (5-30 g) were stocked at a density of 24,000 fish per ha. The production cycle was between 5-9 months. Fish size at harvest ranged from 200 to 300 g. The rearing ponds, (varying from 0.5 to 12 ha in size), were earthen and mainly supplied with agricultural drainage from the Kafr El-Sheikh Governorate.

Figure 1. Map of northern part of Egypt to point out the location of Kafr El-Sheikh governorate where the mortality episode occurred.

Fish sample collection

Eighty moribund and freshly dead fish were randomly collected from the affected ponds. The fish samples were visually inspected and transported on ice to our laboratory within 2 h. Sampling and handling of fish were approved by the Ethical Committee for Animal Experimentation, Faculty of Veterinary Medicine, Cairo University, Egypt, Approval number VetCU23012020106

Physicochemical and heavy metal analysis of water

During the mortality episode, dissolved oxygen (mg/L), temperature, pH, salinity (PSU), and total dissolved solids (TDS) (mg/L) were measured three times from three different points of the pond, including the inlet and outlet water, using a portable multiparameter waterproof meter HI98194 (Hanna Instruments Inc., RI, USA). The water samples

were collected 30 cm below the water surface, stored in sterilised glass bottles (500 mL capacity), and transferred to the laboratory for analysis of unionised ammonia (UIA) (mg/L), nitrate (mg/L), nitrite (mg/L), and total hardness (mg/L) as $CaCO_3$. Heavy metal analysis for cadmium (Cd), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), and zinc (Zn) were also determined according to APHA (2012).

Bacteriological Examination

Kidney tissues were aseptically cultured on thiosulphate-citrate-bile salts-sucrose agar (TCBS) and *Aeromonas* isolation medium base supplemented with rehydrated ampicillin and Trypticase Soya agar (TSA). Bacterial plates were incubated for 24–48 h at 28 °C. Bacterial colonies from the plates were identified using Gram staining and biochemical tests, such as oxidase and catalase, together with API 20E & NE (Biomerieux, France) profiles. The purified isolates were cultured in brain heart infusion (BHI) broth supplemented with 15% glycerol for further confirmatory molecular identification.

Molecular identification and sequencing analysis

The extraction of genomic DNA was performed using QIAamp® genomic DNA kits. *Aeromonas* universal 16S rRNA gene and *Vibrio* universal 16S rRNA gene were used to identify *Aeromonas* and *Vibrio* bacteria from sampled fish, respectively. The *GyrB* gene was used to confirm isolation of *A. hydrophila*, and *Aerolysin* gene was used to detect virulent *A. hydrophila* strains. Species-specific *collagenase* gene and *Ctx* gene were used for confirming the identity of *V. alginolyticus* and *V. cholerae* isolates (Table 1).

The amplified regions of target genes were purified using a PCR Purification Kit (Qiagen), and sequenced using a Genetic Analyzer 3500 sequencer,

Table 1. Oligonucleotide sequence of primer sets used in the study

Bacteria	Gene (amplified region)	Primer sets	Amplicon size	Reference
Aeromonas sp.	Aeromonas Universal (*16S rRNA* gene)	F:CGACGATCCCTAGCTGGTCT R:GCCTTCGCCACCGGTAT	461bp	Persson et al., (2015)
A. hydrophila	*GyrB* gene	F: TCCGGCGGTCTGCACGGCGT R: TTGTCCGGGTTGTACTCGTC	1100bp	Abu-Elala et al., (2015)
A. hydrophila	*Aerolysin* (*aer.*)	F:CCTATGGCCTGAGCGAGAAG R:CCAGTTCCAGTCCCACCACT	431bp	Oliveira et al., (2012)
Vibrio	Vibrio_ universal *16S rRNA*	F: CAGGCCTAACACATGCAAGTC R:GCATCTGAGTGTCAGTATCTGTCC	700 bp	Montieri et al., (2010)
V. alginolyticus	*Collagenase*	F:CGAGTACAGTCACTTGAAAGCC R: CACAACAGAACTCGCGTTACC	737 bp	Abdelaziz et al., (2017)
V. cholera	*Ctx* gene	F-CAGTCAGGTGGTCTTATGCCAAGAGG R-CCCACTAAGTGGGCACTTCTCAAACT	167 bp	Wong et al., (2012)

and the sequence analysed using the NCBI BLAST program. The resulting sequences were registered in the NBCI gene bank using the BankIt program.

Antibiogram

The bacterial isolates were tested for antibiotic sensitivity by disk diffusion method (CLSI, 2018). The Mueller–Hinton plates were incubated at 30 °C for 24 h. Finally, zones of inhibition were measured and compared with the reference data of the antibiogram.

Results

External and Necropsy findings

The surviving fish exhibited sluggish swimming and loss of equilibrium with no reflexes. Moribund and freshly dead fish displayed extensive skin hemorrhages, especially at the fins and base of fins, congested hemorrhagic gills, and detached scales (Figure 2A–B). Congestion, enlargement, and hemorrhage in the spleen, liver, and kidneys were evident in the necropsy (Figure 2C–D).

Water Quality parameters

The physicochemical analysis of water samples (n=9) (mean ± SD) was as follows: water temperature 31.33 °C ± 0.77 °C; pH (7.65 ± 0.01); dissolved oxygen (6.43 ± 1.52 mg/L); salinity (*PSU)* 45 ± 4.08; TDS (mg/L) 422.6 ± 18.78; total hardness (mg/L) 263.30 ± 16.45 as CaCO nitrate (mg/L) 0.26 ± 0.01; nitrite (mg/L) 0.22 ± 0.02 and a significant increase in unionised ammonia (mg/L) 0.49 ± 0.02 was recorded. Heavy metal content was below the permissible limits according to APHA (2012). The summary of the standard water quality requirements for farmed tilapia is presented in Table 2 (Bhatnagar and Devi, 2013).

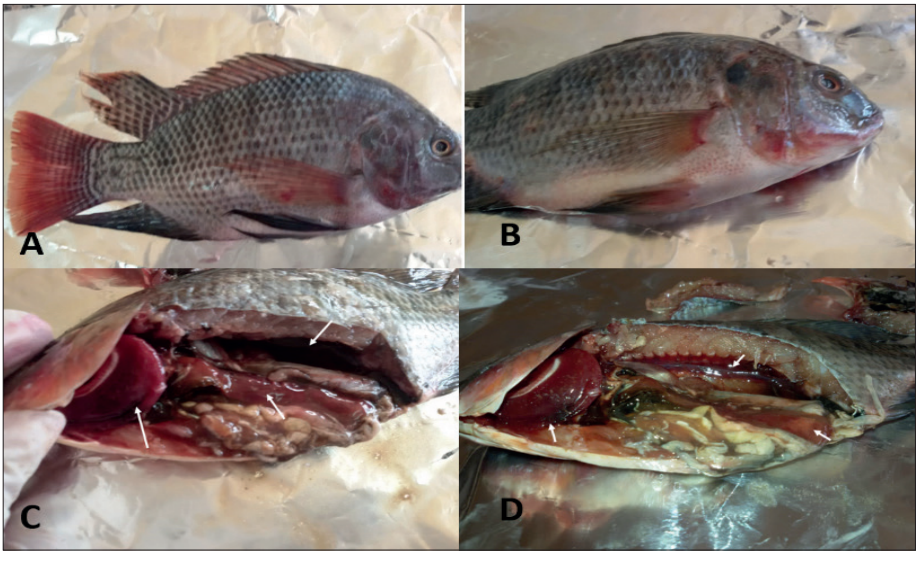

Figure 2. External lesions and necropsy findings of naturally infected *Oreochromis niloticus*: (A, B) Extensive skin hemorrhages especially at fins and the base of fins. (C, D) Congestion in gills, liver and kidney

Bacteriological identification

Aeromonas hydrophila colonies were yellow, shiny, and rounded on *Aeromonas* isolation base medium, and appeared creamy on the TSA agar plates. On the TCBS media, *V. alginolyticus* colonies were yellow, swarming, large and sticky, whereas *V. cholerae* colonies were yellow and rounded. *A. hydrophila* appeared as short rods, whereas *V. alginolyticus* and *V. cholerae* appeared as comma-shaped, slightly curved rods. All the isolates were gram negative, motile and oxidase and catalase positive. The API20NE-derived codes of *A. hydrophila* and *V. alginolyticus* were 5576755, 5575755 and 7452244, 7454744, respectively, whereas the API20E identification code of *V. cholerae* was 5306124.

Frequency of bacterial infection

A. hydrophila was the most predominant bacteria present; representing 81.3 % of fish sampled, followed by *V. alginolyticus* with 68.8% and *V. cholerae* with the lowest frequency of 31.3%

Molecular identification and sequencing analysis

On the basis of phenotypical identification, the 16S universal *Aeromonas* primers gave positive amplicons for *Aeromonas* bacteria at 461bp. (Figure 3A), while the presence of *A. hydrophila* species was confirmed by detection of the *GyrB* gene at 1100 bp. The *Aerolysin* gene was detected in most *A. hydrophila* isolates amplifying a 431bp amplicon (Figure 3B). The 16S universal *Vibrio* primers produce positive 700 bp amplicons in all isolated *vibrio* species (Figure 3C). Presence of *V. alginolyticus* was confirmed by the production of *collagenase* gene amplicons at 737bp (Figure 3D) while the PCR of the *Ctx* virulent gene of *V. cholerae* was negative. Sequencing results of *A. hydrophila, V. cholerae* and *V. alginolyticus* amplicons, when blasted, showed high similarity with sequences registered in GenBank and showed a high percentage identity and query coverage with reference sequences reported in GenBank. The accession number of submitted nucleotide sequences were MN123791 for *A. hydrophila*, MK880216 for *V. cholerae* and MK880217 for *V. alginolyticus*.

Table 2. Summary of standard water quality requirements for fish farming (according to Bhatnagar and Devi, 2013)

Parameter	Recorded value	International standards
Dissolved oxygen(mg/L)	6.43 ± 1.52	> 5
Salinity (PSU)	45 ± 4.08	Varies according to geographical region
PH	7.65 ± 0.01,	6.5-9
Total dissolved solid (mg/L)	422.6 ± 18.78	0.13
Total hardness (mg/L)	263.30 ± 16.45	50-150
Un ionised ammonia (mg/L)	0.49 ± 0.02	< 0.02
Nitrate (mg/L)	0.26 ± 0.01	0.1 to 4.5
Nitrite (mg/L)	0.22 ± 0.02	< 0.02

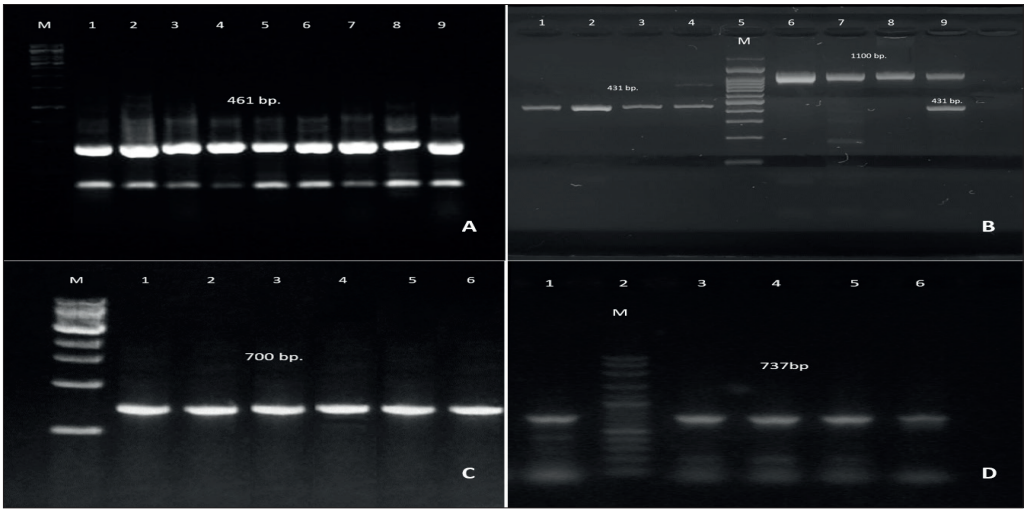

Figure 3. Agarose Gel electrophoresis of PCR products representing (A) amplification of universal *16S rRNA* gene of *Aeromonas* species at 461 bp, (B) Amplification of *GyrB* gene at 1100bp and *Aerolysin* gene at 431bp. (C) Amplification of universal *16S rRNA* gene of *Vibrio* species at 700 bp, (D) Amplification of *collagenase* gene of *V. alginolyticus* at 737 bp.

Antibiogram results

Antibiotics sensitivity patterns of *A. hydrophila, V. alginolyticus* and *V. cholerae* are shown in Table 3. Briefly, *A. hydrophila* was sensitive to Ciprofloxacin (CIP), Streptomycin (ST) and Trimethoprim (TMP), Trimethoprim sulfamethoxazole (SXT), Imipenem (IPM) and Gentamicin (CN) in contrast with *V. cholerae* that was resistant to six antimicrobials but sensitive to CIP only. *V. alginolyticus* was recorded to be sensitive to Chloramphenicol (C), Oxalinic acid (OA) and also CIP (Table 3). Multiple antimicrobial resistance indices (MAR) for *A. hydrophila, V. alginolyticus. V. cholerae* were 0.1, 0.3 and 0.7, respectively.

Discussion

The disease dynamics in Egyptian aquatic environments is highly correlated with a shift in water quality parameters, such as temperature, pH, and nitrogen compounds (UIA and NO_3), which negatively influences fish immunity, enhances pathogen replication, and converts normal non-pathogenic bacteria to a pathogenic form (Karvonen et al., 2010).

With regard to the physicochemical analysis of water, the water temperature was 31.33 °C ± 0.77 °C. High water temperature can lead to an increase in the multiplication of fish pathogens and the prevalence of both parasitic infestation and bacterial invasion (Karvonen et al., 2010; Shayo et al., 2012). Both pH (7.65 ± 0.01) and dissolved oxygen (6.43 ± 1.52 mg/L) were within normal values (Bhatnagar and Devi, 2013), salinity (*PSU)* 45 ± 4.08 and TDS (mg/L) 422.6 ± 18.78 were higher than normal. High water salinity has been strongly correlated with *Vibrio* infections in fish (Abdelaziz et al., 2017), and a significant increase in UIA (0.49 ± 0.02 mg/L)was also recorded. Fish, especially Nile tilapia, are very sensitive to high levels of UIA, specifically above 0.02–0.05 mg/L (ICAR, 2006), which affect growth and productivity,

and contribute to gill pathologies as observed in our study.

GyrB and *Aerolysin* genes are commonly detected virulence factors among pathogenic strains of *A. hydrophila* (Abu-Elala et al., 2015; Oliveira et al., 2012). Molecular detection of these genes in our study, reflects the external signs of septicemia that were present on Nile tilapia naturally infected with the bacterium, seen as extensive hemorrhaging over the body surface, fins, and the base of fins. Gross lesions similar to these this have been reported by Okasha et al. (2016). Moreover, the toxic metabolites of *Aeromonas* spp. induce congestion in the gills, hemorrhaging and degenerative cellular changes in the spleen, liver, and kidneys, as reported by Miyazaki and Kaige (1985) and Elsheshtawy et al. (2019).

Phenotypically, the swarming and sticky characteristics of *V. alginolyticus* colonies on TCBS agar may indicate its adhesive and virulence properties (Rameshkumar et al. 2017).

Biochemical identification of the suspected *V. alginolyticus* and *V. cholerae* isolates using API20NE was confirmed by the amplification of universal *16S rRNA Vibrio* gene, which acts as a preliminary rapid tool for *Vibrio* identification. However, this gene has a low discriminatory power among closely related *Vibrio* spp.; hence, species-specific *Vibrio* primers were used. For *V. alginolyticus*, *collagenase* was used as a biomarker gene for virulence (Yishan et al., 2011), with *V. alginolyticus* reported in several mass mortality outbreaks in different fish species (Rameshkumar et al. 2017; El-Sayed et al., 2019). The virulence of *V. alginolyticus* is related to its ability to form a siderophore-mediated iron-sequestering system, and its production of extracellular products containing many proteases with hemolytic activities, collagenase, cytotoxins, and enterotoxins that cause signs of extensive hemorrhage, ulceration, and tail and fin rotting (Yishan et al., 2011). The negative PCR result for the *Ctx* virulent gene suggests that the *V. cholerae* isolates recovered were non-pathogenic (Hounmanou, 2015). Non-virulent strains of *V. cholerae* are found in the aquatic ecosystems that contain low quality sewage water, and may pose a threat to fishermen and consumers, who consume raw fish (Singh et al., 2002).

Aeromonas hydrophila was the most prevalent bacteria found in fish (i.e. 81.3% of fish sampled), similarly to levels detected (i.e. 75%) by Aboyadak et al. (2015), reflecting the ubiquitous nature of *Aeromonas* spp. present in the gut flora of fish. In addition, the increased water temperature may have increased the fish's susceptibility to *A. hydrophila* infection as reported by Shayo et al. (2012). *Vibrio alginolyticus* was isolated from 68.8% of fish sampled, which is similar to that reported by Sabir et al. (2012) who found 70.2% of fish to be infected with. *V. alginolyticus,* which they believe to be the result of high-salinity and warm seawater in Tamouda bay, Morocco, increasing the stress of the fish and increasing their susceptibility to infection. Non-pathogenic *V. cholerae* were recorded at a frequency rate of 31.3%. *Vibrio cholerae* was detected in fish in summer season in Czech Republic, where sewage water utilisation, together with high water temperature enhance its presence (Rehulka et al., 2015).

Antibiotic sensitivity testing was performed to determine which antibiotics are suitable to control these mass kills. Several studies mentioned CIP and florfenicol to be among the most

Table 3. Antibiotic sensitivity of *Aeromonas hydrophila*, *Vibrio alginolyticus* and *Vibrio cholerae*

Antibiotic (µg/disk)	Diameter of inhibition zone in mm*			Bacterial isolates inhibition zone in mm		
	S	I	R	*A. hydrophila*	*V. alginolyticus*	*V. cholerae*
Chloramphenicol (C) 30 µg	≥ 18	13–17	≤ 12	10	27	13
Ciprofloxacin (CIP) 5 µg	≥ 21	16–20	≤ 15	33	32	22
Gentamicin (CN) 10 µg	≥ 15	13–14	≤ 12	16	15	11
Imipenem (IPM) 10 µg	≥ 23	20–22	≤ 19	25	18	8
Kanamycin (K) 30 µg	≥ 18	14–17	≤ 13	14	12	11
Oxalinic acid (OA) 2 µg	≥ 15	≤ 15	NO ZONE	10	16	9
Streptomycin (ST)10 µg	≥ 15	12–14	≤ 11	22	17	Not detected
Trimethoprim (TMP) 5 µg	≥ 16	11–15	≤ 10	18	18	Not detected
Trimethoprim sulfamethoxazole (SXT) 25 µg	≥ 16	11–15	≤ 10	26	9	Not detected

*** S,** I, and R stand for susceptible, intermediate, and resistant, respectively.

applied antibiotics in Egypt and other major aquaculture producing countries between 2008 and 2018 (Lulijwa et al., 2020).

The antibiogram of isolated bacteria showed that *A. hydrophila* was sensitive to CIP, TMP, CN and SXT but was resistant to C. This result is similar to that recorded by John and Hatha (2014), but in contrast with Čížek et al. (2010), who isolated *Aeromonas* spp. resistant to CIP and TMP, but sensitive to C. In the case of *V. alginolyticus*, we recorded its sensitivity to CIP, C, and ST, and similarly, El-Sayed et al. (2019) had isolated CIP-sensitive *V. alginolyticus* from cultured *O. niloticus*. Regarding *V. cholerae,* it showed high susceptibility to CIP, which is similar to the results of Hounmanou (2015). However, other *V. cholerae* isolates were reported to be CIP-resistant (Noorlis et al., 2011). Overall, the observed antibiotic resistance reflects the misuse of antibiotics and agricultural/municipal drainage contributing to the high levels of antibiotics found in aquaculture systems (EL-Sayed et al., 2019). In brief, CIP appears to be the most suitable antibiotic to treat the tilapia under study here to prevent the mass kills that were observed in this study.

In conclusion, the mass mortality that occurred in farmed tilapia in Kafr El-Sheikh in the late summer is multifactorial, including deteriorated water quality as evidenced by high levels of UTA, which can suppress fish immunity and make them more susceptible to bacterial invasion. The choice of suitable antibiotics to control these bacterial outbreaks should be based on sensitivity tests. In this study, bacteria were sensitive to ciprofloxacin.

Declaration of competing interests

None

Author contribution

MS designed the study, performed the pathological examination and wrote the final version of the manuscript. DAA performed the molecular biology and drafted the manuscript. RAI performed microbiology. MN performed the sampling. All authors revised the manuscript and approved it.

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