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Aeromonas veronii Causes Hemorrhagic Septicemia in Cultured Nile tilapia in Qena Governorate

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Abstract

In summer 2021, acute mortalities occurred in Nile tilapia farm in Qena governorate. Different hypothesis including the implication of environmental factors, and/or pathogens have been explored. Clinical, parasitological and bacteriological examinations have been performed. In addition, the water quality parameters were assessed. The clinical investigation of the moribund and recently dead fish demonstrated generalized septicaemia. The phenotypic and biochemical characterization of the pure bacterial colonies that isolated from 60 moribund fish revealed Gram-negative rods and identified as *Aeromonas* species. The identity of the isolates was identified as *Aeromonas* species by amplification of *Aeromonas 16S rDNA* gene fragment by PCR and confirmed as *Aeromonas veronii* by amplification and sequencing the *Aeromonas gyrB*-gene. The water quality parameters showed an increase in the pH and ammonia levels over the permissible levels. Experimental infections were performed to confirm the virulence of the isolated bacteria. Results revealed that *A. veronii* isolate produced the same clinical picture of the collected samples with mortality rate 80 %. The present study implicates that *A. veronii* could be the causative agent of the Nile tilapia mortality in Qena, without neglecting the role of water quality in worsening this outbreak.

Keywords: Nile tilapia, Fish mortalities, Gene sequencing, *A. veronii*

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Introduction

Nile tilapia, *Oreochromis niloticus*, is one of the commercially important, fast-growing and well-adapted fish species worldwide (FAO, 2014). Egypt is one of the top three tilapia producers in the world (GAFRD, 2019). However, tilapia culture has faced unusual high mortalities particularly during summer season in recent years (Fathi et al., 2017; Elsheshtawy et al., 2019). Several attempts have been made to identify the causative agents of these mortalities. some studies linked the cultured tilapia mass mortalities to bacterial infection such as *Aeromonas veronii* (Eissa et al., 2015). Other studies suggested that Tilapia Lake Virus could be the causative agent of the Nile tilapia outbreaks (Fathi et al., 2017). In addition, previous studies attributed these mortalities to multidisciplinary microbial and environmental causes (Abu-Elala et al., 2016). Some risk factors were suggested to predispose such high mortality, such as; the type of culture system, inferior source of water, salinity and higher temperature (Ali et al., 2020).

Aeromonads are facultative anaerobic, Gram-negative bacteria that cause serious disease problems in fish (Stratev and Odeyemi, 2016). In Egypt, several aeromonads were found to cause mass mortalities of farmed fish. It was demonstrated that *A. veronii biovar sobria* has been isolated and identified in mass mortalities in cultured Nile tilapia in El-Sharkia governorate (Eissa et al., 2015). Similarly, mass mortalities in farmed Nile tilapia at Port Said province occur due to *A. veronii* (Abd El Latif et al., 2019). *A. veronii*, an etiological organism of hemorrhagic septicemia in fish (Cai et al., 2012), contributed to major losses all-year-round with the cumulative mortality varying from 10 to 100% (Dong et al., 2017). More importantly, these bacteria are shown to infect invertebrates, aquatic vertebrates, and mammals, including humans (Lazado and Zilberg, 2018). In contrast to phenotypic identification, 16S rDNA sequencing gives specific detailed information even to uncommon isolates (Hossain, 2008).

The aim of this study was to identify the causes of Nile tilapia mortalities in Qena through isolation of the causative agent, biochemical characterization of isolated pathogens, molecular identification, assessment the pathogenicity of the isolated bacteria and evaluation the role of water quality in the disease severity

Materials and Methods

Sampling and clinical examination of fish

A about 10 moribund and freshly dead naturally infected Nile tilapia from a private fish farm were sampled daily with total number of 60 (100 ± 15 g) and transported to the Aquatic Diagnostic Laboratory, Fish diseases Department, Faculty of Veterinary Medicine, South Valley University for investigating the cause(s) of mortalities. No ethical approval has been requested for the fish sampling because experimental research has been conducted to determine the possible causes of mortalities. Clinical examination and necropsy were performed on the collected fish to determine the presence of any clinical signs and to demonstrate the post-mortem lesions according to Noga, (2010).

Water quality parameters

Simultaneously with fish sampling, water was sampled for determination of water-quality parameters (water temperature, dissolved oxygen, ammonia and pH). Dissolved oxygen-meter was

used for measuring the level of dissolved oxygen (DO) in the water and pH- meter for measuring the pH values. Aquarium ammonia testing strips were utilized for measuring the levels of un-ionized ammonia. All analyzed parameters of the water samples were recorded in comparison with the standard permissible limits of WHO (2004) and Hespanhol and Prost (1994).

Parasitological examination

Fish were clinically examined externally to record any apparent clinical signs or any abnormalities according to Noga, (2010). Subsequently, skin and gill scraping were examined microscopically for detection any parasitic infestation. Incision was made in the skin between the lateral line and dorsal fin this part was peeled off with forceps and fillets of muscle tissues were cut and examined grossly for any cysts. Impression smears were made from the muscles by pressing the cut surface of the muscles several times against the surface of the slide. Afterwards, each fish was dissected, and the internal organs were examined macroscopically for any visible cysts. Stomach, intestine, liver, spleen and kidneys were removed and examined for detection of any abnormalities or cysts. Impression smears were made from each organ by pressing the cut surface of these tissues several times against a glass slide and examined microscopically. Diagnosis was done according to Lucky, (1977). The samples were examined using a compound microscope (Olympus) at low magnification (X10). The entire wet mount was scanned from top to bottom and from left to right to count the numbers of parasite according to the parasitological identification keys (Hoffman, 2019).

Bacteriological examination

Following surface sterilization of fish skin and swapping with 70% ethyl alcohol, kidney, spleen, and liver were sampled, under complete aseptic condition, for bacteriological investigations. Samples were cultured directly on Tryptic Soy Agar for 48 h at 28°C, TSA (Oxoid, England). Dominant colonies were selected and sub-cultured on the TSA. The conventional phenotypic, and biochemical identification were used to identify the bacterial isolate according to Austin and Austin, (2012). The conventional tests including colony characteristics, Gram- staining, cytochrome oxidase (oxidase strips), catalase (hydrogen peroxide 3% solution), motility on semisolid agar, sugar utilization (triple sugar iron, TSI), citrate utilization (with Simmons's citrate), esculin hydrolysis (bile esculin agar), indole (Kovac´s method), voges-proskauer, methyl red, and H2S production, growth on 6- and 10% sodium chloride, and resistance to 150 g/ml vibriostatic agent 0/129 (Oxoid) were performed according to Buller, (2004).

Suspected isolates were sub-cultured on Aeromonas selective agar-base, ASA (Biolife, Italy), at 28°C for 24-48 h. After incubation, a single colony from the suspected isolate was picked up and re-streaked on a new plate of its perused selective culture media and re-incubated at the same conditions. When pure colonies have been grown, a loopful of each pure culture was streaked onto slope of semisolid nutrient agar medium to be used as a stock culture for further biochemical identification. Bacterial isolates were stored in Brain Heart Infusion, BHI (Oxoid, England) broth medium with 20% glycerol and kept at −80°C for further use.

Molecular identification of the suspected isolates

DNA extraction

Bacterial isolates were grown overnight in tryptic soy broth (TSB) at 28°C, and then pelleted by centrifugation at 5,000 ×g for 10 min. The pellets were resuspended in lysis buffer, then extraction of bacterial DNA was completed using Gene JET genomic DNA purification kit (Thermo Scientific, EU) according to the manufacturer's recommendations. The DNA was eluted with 100 µl and kept at -20°C until used.

PCR amplification

The extracted DNA from the bacterial isolates was subjected to polymerase chain reactions (PCR) assay for amplification both of the1502 bp hypervariable segment of the *Aeromonas 16S-rDNA* gene using genus-specific primers according to Borrell et al., (1997) and a 1100 bp fragment of *Aeromonas gyrB*-gene (Yanez et al., 2003). PCR amplification was performed in the Verti-thermal cycler (Applied Biosystems, USA). Oligonucleotide primers are listed in (Table 1).

Table 1: Primers used in the present study.

Target genes	Primer sequence	Product sizes/bp	Reference
gyrB	F 5' TCCGGCGGTCTGCACGGCGT 3'	1100	(Yanez et al., 2003)
	R 5' TTGTCCGGGTTGTACTCGTC 3'		
16S-rDNA	F 5' AGAGTTTGATCATGGCTCA 3'	1502	Borrell et al., (1997)
	R 5' GGTTACCTTGTTACGACTT 3'		

Analysis of the PCR products

The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis in Tris-acetate EDTA (TAE) buffer, stained with ethidium bromide (50 µl/L). 100 bp DNA plus ladder (Qiagen, GmbH) was used to determine the fragment sizes. The gel was photographed and visualized on UV transilluminator system (MultiDoc- It, UVP, UK).

Gene sequencing of *gyrB* gene

PCR products were purified from the gel utilizing Zymoclean Gel DNA Recovery Kit (Zymo Research, USA) as per manufacturer`s instructions. The purified PCR products were sequenced in a commercial sequencing laboratory (Macrogen Humanizing Genomics, biotechnology company, Seoul, South Korea). The *gyrB* gene sequences were compared against other sequences deposited in GenBank using a BLAST® analysis (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/BLAST/) to establish sequence identity (Altschul et al., 1997).

Pathogenicity of the *A. veronii* isolates

Experimental design

For challenge experiment, apparently healthy Nile tilapia (n=60) were obtained from fish hatchery with no history of bacterial septicemia and transported a live

to the aquatic laboratory at Faculty of Veterinary Medicine, South Valley University, Qena, Egypt, and acclimated to laboratory conditions for 2 weeks in fiber glass aquaria supplied with dechlorinated tap water and aeration according to the recommendations for the maintenance of bioassay fish described by (Ellsaesser and Clem, 1987). Fish were admitted in the Department of Fish Diseases, Faculty of Veterinary Medicine, South Valley University following the ethical consideration of experimental animals of South Valley University (No 9a/13.12.2020).

Immersion challenge test

An *A. veronii* isolate was grown on trypticase soya broth (TSB, Biolife) at 28 °C until log phase (Stevenson et al., 2016). Colony Forming Unit (CFU) counts were done following the method previously described by Goldman and Green, (2015) using a ten-fold serial dilution of the bacterial culture in sterile saline (0.85%). Acclimated fish with an average body weight of 20 ± 5 g were divided into 2 groups. The first group (10 fish) was challenged by immersion in *A. veronii* culture (1×106 CFU/ml) for 1 h. The second group (10 fish) was set as a control. The entire study was done in three replicates. Clinical signs, PM lesions, and mortalities were recorded daily for up to two weeks. Moribund fish were bacteriologically examined to re-isolate the causative *Aeromonas* strain from the internal organs. Identification of re-isolated bacteria was conducted by the molecular approaches as described previously in this study.

Results

Clinical signs and post-mortem findings in naturally infected Nile Tilapia

The diseased fish was lethargic with sluggish movements, loss of reflexes, dark in colour and loss of appetite. Clinical investigation showed haemorrhagic areas with scale loss and redness especially below the opercula and at the base of all fins. Deep haemorrhagic ulcers were found in the body musculature below the dorsal fin, and ascitic belly, and swollen haemorrhagic protruded anal opening. Exophthalmia, and corneal opacity. Furthermore, the postmortem examination demonstrated congestion of gills and internal organs, enlarged liver and gall bladder and body cavity filled with bloody ascitic fluids (Figure 1).

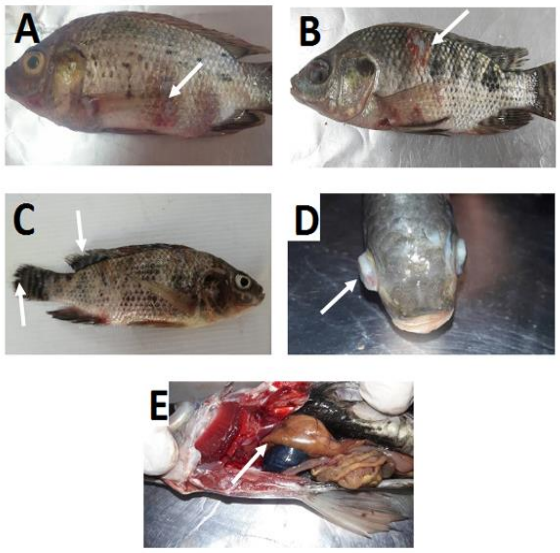

Figure 1: Naturally infected *O. niloticus* showed hemorrhages (A), skin ulceration (B), fin and tail rot (C), bilateral exophthalmia with corneal opacity (D) and severe distended and congested internal organs (E) (arrows).

Water quality parameters

All analyzed parameters of the water samples were recorded in (Table 2). In our study, the water quality parameters revealed high water temperature, relatively low DO level and elevated ammonia and pH value.

Table 2: Chemical properties of pond water in comparison with the standard permissible limits.

Water parameters	Results of this study	Permissible limits
Temperature (°C)	29 – 29.5	–
Dissolved oxygen (DO) (mg/L)	4.5 – 4.8	5 – 6
Un-ionized ammonia (NH3) (mg/L)	0.5	0.01
pH	8.2	8.0 – 8.5

Parasitological examination

External and internal parasitological examinations do not reveal any potential causes of mortalities.

Morphological and biochemical identification of isolated bacteria

The bacterial isolates from the diseased fish formed round, convex, shiny, and creamy colonies on TSA agar. Light microscopy examination of the bacterial smears revealed Gram-negative bacilli. The traditional microbial identification revealed that, the isolates have the same biochemical profiles, which were positive for oxidase, motility, catalase activities, indole reaction, gas from glucose fermentation, esculin hydrolysis, production of acetyl methyl carbinol (Voges–Proskauer) and hydrogen sulfide formation; resistant to vibriostatic agent 0/129; negative for methyl red and citrate utilization and have the ability to grow in media containing 6% NaCl. The bacterial colonies were also confirmed by growth on Aeromonas selective agar-base, and the colonies appeared smooth with yellow colour. All the isolates were identified to be belong to the genus *Aeromonas* according to Bergey's Manual of Systematic Bacteriology (Martin-Carnahan et al., 2005). The study revealed that, out of the 60 examined Nile tilapia samples, Aeromonas spp. was isolated and identified from 25 samples (42%) according to biochemical identification.

Molecular Analysis

PCR successfully amplified the expected fragment (1502 bp) of the *Aeromonas 16S rDNA* gene from 25 (42%) of isolates (Figure 2) which were confirmed as *Aeromonas* spp. In order to Know the *Aeromonas* species sequencing of *gyrB*-gene purified amplicon was done and the alignment results of the 25 isolates

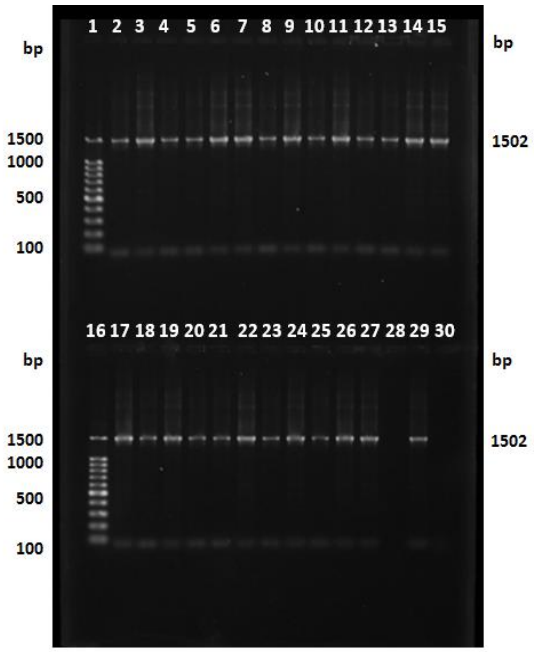

Figure 2: Amplifying a 1502 bp fragment of *16S rDNA* gene of clinical isolates of *Aeromonas* using *Aeromonas*-specific primers. Lane 1 and 16: 100 bp ladder, Lane 2-15 and 17- 27: Clinical isolates of the present study, Lane 29: Positive control, Lane 30: No-template control

revealed 100% similarity and the BLASTn results of these sequences revealed various percentages of similarity with different *A. verronii* species sequences available in GenBank. The greatest similarity was with *A. verronii* (accession number LC003059.1), which revealed 99.12% sequence identity, and *A. verronii* (accession number HM584517.1), which revealed 99.11% sequence identity.

Challenge experiment

In experimental infection, the bacterial isolates exhibited virulence to the challenged Nile tilapia and led to severe symptoms as observed in postmortem pictures with a mortality rate of 80% in the challenged group and the mortalities peak was at the 4th day post-infection. However, no mortalities were recorded in the control group. The inoculated bacteria were re-isolated from the lesions of the challenged fish and confirmed to be *A. veronii* based on the biochemical profiles and molecular identification.

Discussion

Bacterial fish diseases are one of the critical obstacles affecting the expansion of tilapia aquaculture worldwide (Bentzon-Tilia et al., 2016), and it causes outbreaks with high mortalities (Sekar et al., 2008). The isolated bacterial pathogens from Nile tilapia added more evidence of the wide geographical distribution of bacterial diseases. Combination of factors commonly works together to initiate fish infections (Eissa et al., 2021).

In the present study, the isolated bacteria were identified as *A. veronii*. which is globally recognized as one of the dominant and virulent pathogenic agents in freshwater fishes (Sun et al., 2016; Zhang et al., 2018) that causes hemorrhagic septicemia, ulcerative lesions, paleness of the body surface, dropsy, and fin and tail rot in various fish species (Hassan et al., 2017; Hoai et al., 2019).

The examined fish showed generalized signs of septicemia like those previously recorded (El Asely et al., 2020; Matter et al., 2018). These signs may be due to the virulence factors of the pathogenic enteric bacteria such as adhesins, somatic antigens, lipopolysaccharides (Lipid-A), siderophores, and colicins (Seker et al., 2010). These factors can help the pathogen penetrate the epithelial layer of the intestinal mucosa (Tan et al., 2002). In addition, extracellular proteins, hemolysins and leukotoxins have also been implicated in the pathogenicity of bacteria (Paraje et al., 2005).

The phenotypic and biochemical characterization identified numerous strains that were suspected to be *Aeromonas* spp., these results nearly similar with that recorded in previous studies (Eissa et al., 2015; Emeish et al., 2018).

Conventional morphological methods for the diagnosis of bacterial infections in fish are time-consuming and complicated with a delay in the implementation of control measures, that causes massive economic losses. Moreover, many pathogens share common morphological features and cause similar clinical manifestations. Therefore, molecular identification techniques have been adopted (Bader et al., 2003; Tsai et al., 2012). Accordingly, all the isolates in this study were confirmed on the basis of amplification and sequencing of both *Aeromonas- 16S rDNA* and *gyrB* genes. The study revealed that 42% of the examined samples were identified as *Aeromonas* spp. by the *16S rDNA* gene amplification and identified as *A. veronii* by sequencing, which nearly agreed with the findings of Hussain et al., (2014) who confirmed that 56% of the fish samples contained *Aeromonas* spp. based on the presence of the *16S rRNA* gene. As well as, a higher prevalence was observed by Lee et al., (2002) who identified by the *16S rRNA* gene-based PCR that 78% of the examined fish samples were infected with *Aeromonas*. These differences may be attributed to the sampling time, different

fish species., and geographical range. A previous study found that the distribution of *Aeromonas* spp. showed a relationship between the time of sampling and dominant species especially pathogenic species, such as *A. hydrophila, A. veronii* and *A. caviae*, which were dominant at all sites during warm months (Lee et al., 2002).

The most prevalent bacterial isolates were *A. veronii*. These results are nearly similar to that recorded previously (El Asely et al., 2020; Hu et al., 2012). The obtained bacteria were isolated from the kidney, spleen and liver. These findings were similar to those obtained previously (Kusdarwati et al., 2017; Toranzo et al., 2005). These findings were attributed to the nature of this organ (hematopoietic organs contain a high blood supply), so highly susceptible to infection.

The higher incidence of *A. veronii* in our study may be attributed to the ubiquitous, opportunistic, and psychrotrophic nature of the microorganism in the aquatic environment, and its presence as normal flora in fish intestine. In addition, this outbreak was during the summer season, as high temperature, low dissolved oxygen, and the subsequent other alteration in water parameters that could induce stress on fish and compromise the immune response rendering the fish more susceptible to infection.

From an environmental prospect, the results obtained have confirmed that water quality is an integral part of aquaculture system. In most instances, the diseases result from a complex interplay between fish, pathogen and environmental stress, that affect the host susceptibility to disease (Song et al., 2008). Environmental stressors can affect the homeostatic mechanisms of fish, thereby reducing their resistance to the pathogens (Small and Bilodeau, 2005).

With regard to water temperature, the higher temperature measured in fish farm may make fish more susceptible to bacterial diseases and facilitate the transmission of infectious diseases, which could lead to disease spread and wider epidemics (Karvonen et al., 2010). In addition, bacteria may exhibit stronger virulence, for instance, *A. hydrophila* showed a greater virulence in largemouth bass (*Micropterus salmoides*) at warmer temperatures due to either reduced host resistance or to an increased expressions of virulence factors (Marcogliese, 2008).

The dissolved oxygen levels were below the optimal recommended values. These relatively low DO levels interacted with other viable environmental aquatic components to produce the notable cases of mortalities (Haley et al., 1967). The higher pH and ammonia levels than the recommended values seem to be conducive to the higher prevalence of bacterial infections recorded in this study. High levels of ammonia also promote microbial infections by suppressing fish immunity (Cheng et al., 2004).

In the present study, the experimental pathogenicity trial showed a high mortality rate. These results were in agreement with Eissa et al., (2015), who found that Nile tilapia challenged with 1.2x108 cells/mL of *A. veronii biovar sobria*, caused mortalities of 70-35% of (IP) and (IM) groups, respectively. Also, similar results were found by (El Asely et al., 2020) where a high mortality rate was recorded in *A. veronii* (HY2-100 %) at dose 9×108 cells/mL followed by *A. veronii* (HY4-90%) then *A. veronii* (HY3, HY6-60%). The reported mortalities varied based on

the pathogenesis and virulence as well as the amount and severity of the toxins among each bacterial isolate.

Conclusions

Mortalities affecting the Nile tilapia are multifactorial. water quality, impaired fish immune mechanisms and bacterial pathogens are collectively posing an impact on initiating summer mortality. Tilapias, under stress conditions are susceptible to various facultative pathogenic bacteria. These secondary invaders tend to be ubiquitous in freshwater environments. Therefore, maintaining a good fish culture, environment, and good husbandry practice is crucial for growing healthy fish. *A. veronii* was the most predominant bacterial pathogen that was isolated from the diseased Nile tilapia in the present study. Therefore, it is strongly involved in the mortality events affecting the Nile tilapia in Egypt, and therefore, should be included in the future strategies of prophylaxis and treatment of farmed Nile tilapia.

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