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Insight into summer mortalities syndrome in farmed Nile tilapia (*Oreochromis niloticus*) associated with bacterial infections

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ABSTRACT

The current investigation was undertaken to determine the potential causes of summer mass mortalities among farmed Nile tilapia (*Oreochromis niloticus*). Pure bacterial colonies were isolated from moribund *O. niloticus* from 13 different fish farms suffered from high mortalities ranged from (50-80%), during the period from April to October 2018. Fish showed external hemorrhagic spots, skin darkening, abdominal distension and exophthalmia. Internally, congestion and enlargement of internal organs with serous or hemorrhagic fluid was the most obvious picture. The phenotypic and biochemical characterization using API20E identified the bacterial isolates as (*A. veronii*, *A. hydrophila*, *A. caviae* and *A. sobria;* Aeromonads), (*Ps. Fluorescence;* Pseudomonas spp), (*E. sakazakii* and *E. cloacae;* Enterobacter spp), (*C. freundii;* Citrobacter spp), (S. *odorifera*, *S. liquefaciens*, and *S. marcescens;* Serratia spp), (*S. lutiensis*, *S. equine;* Streptococcus spp), *Lactococcus lactis* and *Proteus vulgaris*, with the most prevalence to aeromonads. Most isolates were accurately identified by PCR and gene sequencing. Water physicochemical parameters were measured at the farm sites showed an increase in the pH and ammonia levels. In order to confirm the pathogenicity of the bacterial isolates, an experimental infection was conducted using different doses. The results revealed that *A. veronii (HY2)* at dose of 9×10^8 cells/ml was the most pathogenic with mortality rate 100 %. This study concludes that *A. veronii*, *C. freundii*, *P. vulgaris* and *P. fluorescens* are implicated in Nile tilapia summer mortalities, without neglecting the role of water quality in worsening the problem.

1. INTRODUCTION

Globally, estimated aquaculture disease losses reached 3 billion USD (Tan *et al*., 2006). The major constraint of tilapia production in Egypt during the last five years could be summer mass mortalities, precisely the bacterial pathogens that have been incriminated as the most important common cause (Algammal et al., 2020).

Tilapia under stress conditions is susceptible to a wide range of pathogenic bacteria (Shaheen 2013; Eissa *et al.*, 2015). Outbreaks of bacterial diseases cause severe economic losses to fish farms due to high mortalities, especially during the summer period (Elsheshtawy et al., 2019). The capability of a bacterial infection to cause mass mortality in fish is a multi-factorial condition, and variable elements identified with the host, environment, and the microbe may work in show to characterize the course of the disease (Harvell et al., 1999). Also, environmental-related factors e.g. oxygen level, temperature, salinity and pH may be engaged in triggering disease outbreaks, particularly in intensive aquaculture systems (Eissa *et al.*, 2013). Fish suffering from bacterial infection show comparable clinical signs range from stop eating, swim slowly and aimlessly near the surface, in addition to different pathologic conditions e.g. dermal ulceration, tail rot, hemorrhagic fins and skin, red sores, exophthalmia, erythro-dermatitis and erected scale (Austin and Austin, 2007; Baumgartner et al., 2017; Matter et al., 2018). Postmortem lesions associated with bacterial infection were listed by Abdel-Latif and Sedeek (2017) as noticeable focal hemorrhages and areas of necrosis in the liver and congestion in gills and spleen. In contrast to distinguishing phenotypic proof, 16S rDNA sequencing gives specific detailed information even to uncommon isolates (Hossain, 2008).

This work aimed to characterize bacterial pathogens isolated from diseased Nile tilapia, determine their prevalence and molecular identification, concerning the role of water quality in disease severity and pathogenicity of some retrieved bacterial isolates.

2. MATERIAL AND METHODS

2.1. Naturally infected fish samples

A total of 120 clinically diseased Nile tilapia (*Oreochromis niloticus*) with an average weight ranged from 30 ±10 to 200 ±50 g, were collected at the period from April to October 2018 from 13 different fish. These farms suffered from mass mortalities ranged from 50-80%. Freshly dead and moribund fish were packed in an ice box and transported to the laboratory of Aquatic Animal Diseases and Management,

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Faculty of Veterinary Medicine, Moshtohor, Benha University, as quickly as possible, where clinical and bacteriological examinations were carried out as described by Austin and Austin (2007).

2.2. Bacteriological examination

A loopful from gills, liver, kidneys, and spleen of the clinically diseased fish were inoculated into Tryptic soy broth (TSB, Oxoid, UK) then streaked over Tryptic soy agar (TSA, Oxoid, UK), incubated for 24 hrs at 28 °C (Austin and Austin, 2007).

Phenotypic identification (motility, Gram staining and morphology) of the most dominant colonies in the subculture was determined as described by Quinn *et al*. (2002) and Panangala *et al*. (2007).

Pure colonies were sub-cultured on Rimler-Shotts (RS, Oxoid, UK) medium, Thiosulphate citrate bile salt sucrose agar (TCBS, Oxoid, UK), Pseudomonas Cetrimide agar base medium supplemented with CFC (Cetrimide, Fucidin, and Cephaloridine supplement and 1.5% (w/v) NaCl), MacConkey agar (Oxoid, UK) (for detection the fermentation activity) and Bile esculin Azide agar (Techno Pharmchem. Company) and incubated at 25 ℃ for 18-48 hrs. Pure colonies were stored at -80 °C in TSB containing 20% glycerol for further studies following Hollander (1954). All isolates were identified biochemically by streaking bacterial colonies over TSA and incubated at 28 °C for 24 hrs then identified at the genus level using API20E strips, according to Buller (2004).

2.3. Molecular identification of bacterial isolates

Bacterial colonies' DNA was extracted using a bacterial genomic DNA extraction kit (QIAamp DNA mini kit, Catalogue no. 51304, USA) following the manufacturer's instructions.

PCR assay targeting the 16S rRNA gene was carried out to confirm the bacterial isolates using universal primers (Lagacé *et al.*, 2004)

16S-F (5′- AGAGTTTGATCTGGCTCAG-3′)
16S-R (5′ TACGGTACCTTGTTACGACTT -3′)

in a DNA thermal cycler (T3 Thermal cycler, Biometra). Reactions were performed in a final volume of 25 µl, and conditions were as follows: 94 °C for 15 min, 35cycles at 94 °C for 30 sec, 56 °C for 1 min, 72 °C for 1 min, followed by a final elongation at 72 °C for 10 min. The PCR products (20 µl) were analyzed by electrophoresis on 1.5% agarose gels, stained with ethidium bromide and photographed by gel documentation system (Alpha Innotech).

PCR products were purified using QIA quick PCR Purification kit (QIAGEN, USA) following the manufacture's protocol. Then the purified product was sequenced in the forward and/or reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI 3130, USA*)* using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Cat. No. 4336817, Perkin-Elmer/Applied Biosystems, Foster City, CA). The resultant sequenced *Aeromonas* strains were analyzed using the National Center for Biotechnology Information (NCBI), Basic Local Alignment Search Tool (BLAST) program, and the neighbor-joining blast tree against the database of strain types (the most prevalent and pathogenic *Aeromonas* isolates) and published valid prokaryotic nomenclature.

2.4. Water physicochemical parameters

Water samples were collected from the same fish farms, where fish were collected. Pre-cleaned polyethylene sampling bottles (1.5-liter capacity) were immersed about 50 cm below the water surface, and 500 mL of water were taken at each sampling site. Measuring of water parameters including pH (pH meter, HANNA), dissolved oxygen and water temperature (DO/Temp Meter (PDO-408) and ammonia (ammonia checker, HANNA) were carried out immediately in all water samples. The remaining of the samples was transported to the laboratory in an icebox and prepared for analysis of heavy metals.

2.5. Determination of heavy metals

The collected water samples were analyzed for the determination of cadmium, lead, copper, iron, and zinc levels according to APHA (1995). All measurements were carried out according to the international standards.

2.6. Pathogenicity of the retrieved bacterial isolates

2.6.1. The experimental condition

A total number of 250 apparently healthy Nile tilapia with an average body weight of 20 ± 5 g were transported alive from a fish farm at Kafr El-Sheik to the wet laboratory of Aquatic Animal Diseases and Management, Faculty of Veterinary Medicine, Benha University. Fish were kept in well-prepared fiberglass tanks (750 L capacity) for two weeks to be acclimated for laboratory conditions. Each tank was supplied with an air pump and dechlorinated tap water. The water parameters were adjusted as following; water temperature 26±2 ℃, dissolved oxygen 6.0±0.5 mg/L, ammonia concentration 0.53±0.07 mg/L, and pH 7.0±0.2. Fish were fed with a 4% body weight basal diet (30% protein) twice daily. Random samples of fish were sacrificed and examined for parasites, mycotic, and bacterial pathogens as described by Austin and Austin (2007) to ensure their normal healthy status. Fish were then distributed in well-prepared glass aquaria (100 × 100 × 50 cm) for experimental laboratory studies. Each aquarium was supplied with air pumps for continuous aeration (Sicalls, Pietes, Italy) and dechlorinated tap water.

2.6.2. Preparation of the isolates

The identified isolates, including five strains of *Aeromonas veronii (A. veronii) (A/HY1, A/HY2, A/HY3), A/HY4)* and *A/HY6), Citrobacter freundii*, *Pseudomonas fluorescence*, and *Proteus vulgaris* were chosen, since they were the most prevalent isolated species. The preserved isolates at -80 °C were inoculated into TSB media and incubated at 28 °C for 24 hrs. From each strain, 3 doses were prepared for 3 subgroups (10 fish each) using McFarland opacity tube (D1, D2 and, D3) at a concentration of 3, 6 and 9×10^8 cells/ml using a spectrophotometer at 620 nm.

2.6.3. Evaluation of bacterial isolates pathogenicity

A total of 250 fish (20 ±5 g average body weight) were divided into nine groups. Each of the first eight groups were divided into three subgroups (10 fish/ subgroup). Fish in each subgroup were injected (IP) at dose 0.2 ml containing bacterial cell concentration 0.6×10^8 cells/fish (D3), 1.2×10^8cells/ fish (D2), 1.8×10^8 cells/fish (D3). The ninth group (n=10 fish) was kept as control and injected with sterile saline. Food was offered after challenge and throughout the remainder of the study twice daily at a rate of 4% of body weight. The clinical signs and mortality rates among the infected and control groups were recorded daily for 10 days post-challenge. The re-isolation of bacterial strains from different organs (liver, kidneys, and spleen)

were performed and completely identified as previously described.

3. RESULTS

3.1 Clinical picture

Clinical examination of Nile tilapia collected from fish farms suffered from mass mortality revealed congestion, redness of the skin, operculum, and at the base of fins. Abdominal distention and protruded anal opening were observed. Besides, there was fin rot, detached scales, skin ulceration**,** and unilateral exophthalmia (Fig. 1). Internally, the diseased fish collected from different localities showed enlargement and congestion in the liver, kidneys, spleen, and distended gall bladder. Moreover, in some cases, the abdominal cavity contained serous fluid (Fig. 2).

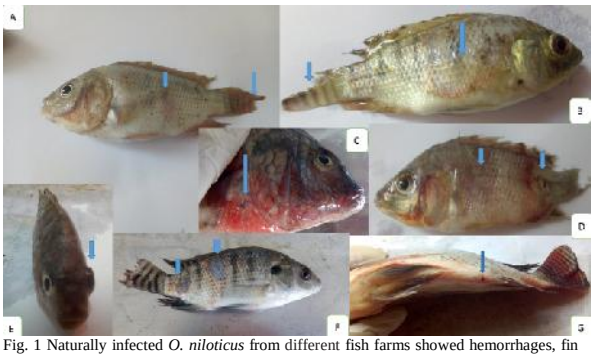

Fig. 1 Naturally infected *O. niloticus* from different fish farms showed hemorrhages, fin rot, detached scales and skin ulceration (arrows) (A, B, C and D), unilateral exophthalmia (E), Skin ulceration and eroded tail (F) and congested anal opening (arrow) (G).

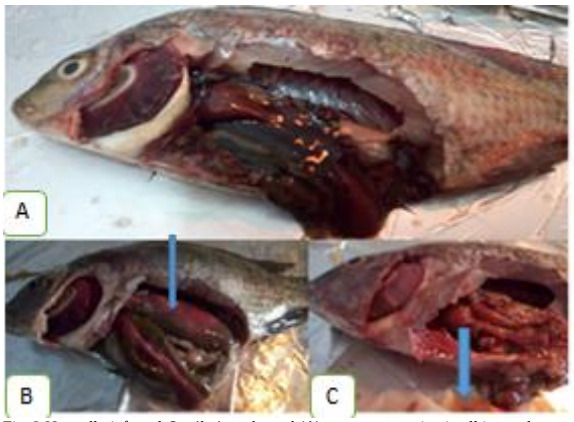

Fig. 2 Naturally infected *O. niloticus* showed (A) severe congestion in all internal organs and gills, (B) severe distended and congested stomach (arrow) and (C) accumulation of ascetic fluid in abdominal cavity, which flow out after dissection (arrow).

3.2. Bacteriological identification

The majority of bacterial isolates (Aeromonas spp., *Pseudomonas fluorescence, Vibrio cholera, Citrobacter freundii, Proteus vulgaris*, Serratia spp, and Enterobacter spp.) were Gram-negative bacteria. Interestingly, Streptococcus spp. and Kurthia spp. were the only Gram-positive bacterial pathogens incriminated in such outbreaks. Streptococcus strains were identified directly by gene sequencing after morphological and phenotypic identification.

Aeromonas spp. suspected colonies appeared round, convex, shiny, and creamy on TSA media. On RS medium, they appeared as yellow colonies after 24 hrs of incubation, non-lactose fermentable on MacConkey agar. Pseudomonas spp. suspected colonies appeared spindle shape on TSA media, and after 24 hrs incubation produced a diffusible yellow-green fluorescence pigment. They were non-lactose fermenter on MacConkey agar, and on RS media appeared greenish after 24 hrs incubation. Both Aeromonas and Pseudomonas spp. colonies were gram-negative coccobacilli to rod-shaped bacteria. *Citrobacter freundii* was lactose fermenter on MacConkey agar and produced yellow colonies on RS media and yellow colonies with black center on TCBS media after 24 hrs of incubation. *Proteus vulgaris* was non-lactose fermentative bacteria on MacConkey agar and produced yellowish colonies on TCBS media. *Citrobacter freundii* and *Proteus vulgaris* spp. were gram-negative rod-shaped bacteria.

3.3. Biochemical characterization

Phenotypic and biochemical characterizations using API20E, identified the bacterial isolates as Aeromonas spp. (*A. hydrophila, A. veronii, A. caviae*, and *A. sobria*). *Pseudomonas fluorescence*, *Citrobacter freundii*, Serratia spp (*S. liquefaciens*, and *S. plymuthica*), Enterobacter spp (*E. sakazakii* and *E. cloacae*), *Lactococcus lactis*, *Vibrio cholera* and Kurthia spp (Table 1).

3.4. Molecular identification of most prevalent bacterial isolates.

Six isolates of *Aeromonas veronii*, one isolate of Pseudomonas spp, one isolate of Citrobacter spp, one isolate of Proteus spp, one isolate of Kurthia spp, three isolates of *Lactococcus spp*, two isolates of Vibrio spp and two isolates of Streptococcus spp) using 16S r DNA region which retrieved band at 1485bp. Blast result for the raw data showed that samples showed identity with the following strains: one isolate *proteus vulgaris*, three isolates *Lactococcus lactis*, six isolates *Aeromonas veronii*, one isolate *Strep. equinus*, one isolate *Citrobacter freundii*, one isolate *Strep. lutetiensis*, *one* isolate *Pseudomonas fluorescens*, one isolate *Kurthia populi* and two isolates *Vibrio cholera.*

Description of aeromonads isolates analyzed in this study, including the ID, organ, geographic origin, and NCBI accession number were shown in table 2. Based on the sequence analysis of the selected six *Aeromonas veronii*, a phylogenetic relationship was confirmed. *A. veronii* HY1 and *A. veronii* HY5 strains showed high phylogenetic relatedness (100%)**.** In comparison, *A. veronii* HY2, *A. veronii* HY4 and *A. veronii* HY6 showed moderate phylogenetic relatedness (50%). *A. veronii* HY2, *A. veronii* HY3, *A. veronii* HY4 and *A. veronii* HY6 were assembled in a separate tree with a 50% similarity level with other isolates. On the other hand, *A. veronii* HY4 and *A. veronii* HY6 revealed a 100% similarity (Fig. 3).

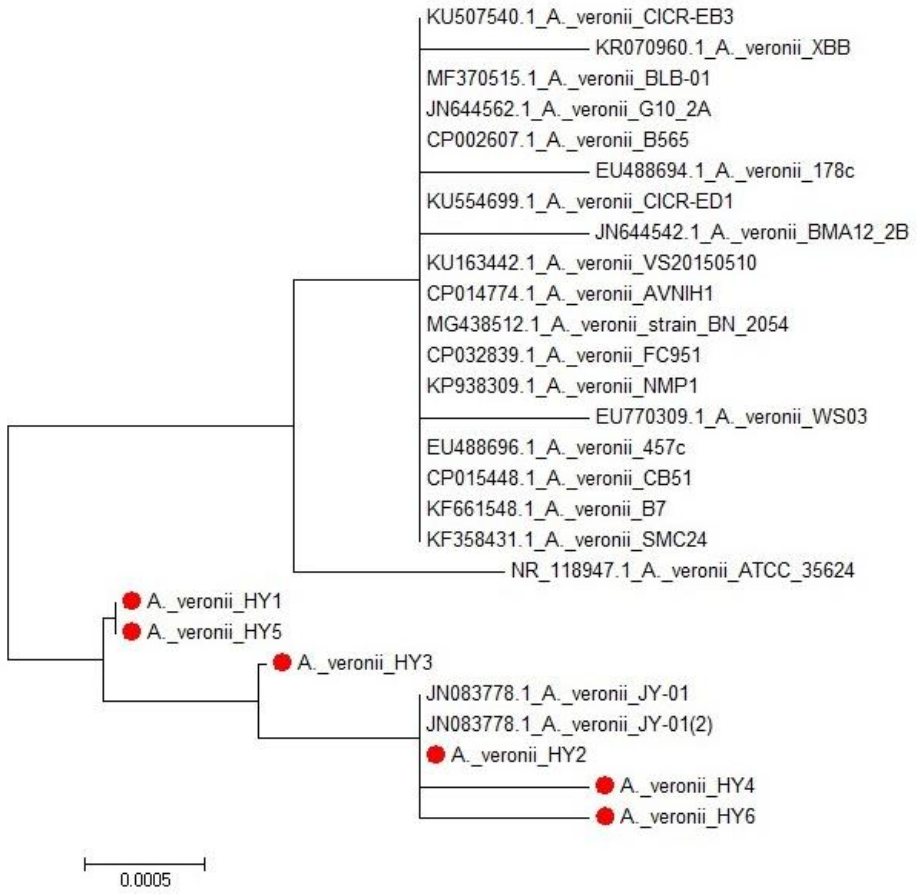

Figure 3 The phylogenetic tree showing relationships of the isolated six *A. veronii* strains (*HY1, HY2, HY3, HY4, HY5* and *HY6*) with other *A. veronii* strains based on 16S rRNA gene sequences were constructed by the neighbor-joining method.

Table 1 Biochemical and morphological characteristics of the bacterial isolates isolated from diseased Nile tilapia using API 2 E kits.

Bacteria	Aeromonas spp	*Pseudomonas fluorescens*	*Vibrio cholera*	*Citrobacter freundii*	Enterobacter spp	Serratia spp	*Lactococcus Lactis*	*Proteus vulgaris*	*Kurthia Spp*
Gram stain	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve
Motility test	Motile	Motile	Motile	Motile	Motile	Motile	Non-motile	Motile	Motile
			API20E						
Oxidase	+	+	+	+	-	-	-	-	-
ONPG	+	-	+	+	+	+	-	+	+
ADH	+	+	+	+	_	+	+	+	+
LDC	+	-	-	-	+	+	-	+	+
ODC	V	-	+	-	+	+	-	-	-
CIT	V	+	+	+	-	+	-	+	-
H_2S	-	-	-	+	-	-	-	+	-
URE	-	-	-	-	-	V	-	+	-
TDA	+	+	+	-	+	+	-	+	+
IND	+	-	V	-	-	-	-	+	+
VP	+	-	+	-	-	V	+	-	+
GEL	+	-	V	-	+	+	-	+	+
GLU	+	-	+	+	+	+	+	+	+
MAN	+	-	+	+	V	V	+	+	+
INO	V	-	-	+	V	V	-	-	-
SOR	-	-	-	+	V	V	-	-	-
RHA	-	-	V	+	V	V	-	-	-
SAC	+	-	+	+	V	V	+	+	+
MEL	-	+	V	+	V	V	-	-	-
AMY	V	-	V	+	V	V	+	+	+
ARA	-	-	V	+	V	V	+	-	-

Oxidase (Cytochrome oxidase), ONPG (β-galactosidase), ADH (Arginine dihydrolase), LDC (Lysine decarboxylase), ODC (Ornithine decarboxylase), CIT (Citrate utilisation), H_2S (H_2S production), URE (Urea hydrolysis), TDA (Tryptophan deamination), IND (Indol production), VP (Acetoin production), GEL (Gelatin hydrolysis), GLU (Glucose fermentation), MAN (Mannitol), INO (Inositol), SOR (Sorbitol), RHA (Rhamnose), SAC (Sucrose), MEL (Melibiose), AMY (Amygdalin) and ARA (Arabinose).

Table 2 Description of Aeromonas spp isolates which analyzed in this study, including the ID, organ, geographic origin, and NCBI accession number.

NO	Strain	ID	Organ	NCBI accession number
1	*aeromonas veronii*	HY1	Kidney	MK584925 A. veronii HY1
2	*aeromonas veronii*	HY2	Liver	MK584926 A. veronii HY2
3	*aeromonas veronii*	HY3	Kidney	MK584927 A. veronii HY3
4	*aeromonas veronii*	HY4	Liver	MK584928 A. veronii HY4
5	*aeromonas veronii*	HY5	Liver	MK584929 A. veronii HY5
6	*aeromonas veronii*	HY6	Liver	MK584930 A. veronii HY6

3.5. Prevalence of bacterial isolates among the examined Nile tilapia:

The most prevalent bacterial isolates were belonging to Aeromonas species (14 isolates), which represent 48.3%, including *A. veronii* (6 isolates), which are considered the most predominant species. The percent of the obtained *Pseudomonas fluorescence*, *Citrobacter freundii*, Serratia spp (*S. liquefaciens*, and *S. plymuthica*), Enterobacter spp (*E. sakazakii* and *E. cloacae*), Streptococcus spp (*Strep. Lutetiensis* and *Strept. equines*) and Kurthia spp were 3.4% for each one. While the prevalence of *Lactococcus lactis* and *Vibrio cholera* were 10.3 and 6.9% respectively (Table, 3).

Table 3 Types of the isolated bacteria, number and percentage of bacterial isolates and distribution of the isolated bacteria among organs of naturally infected Nile tilapia

Type of recovered bacteria	Number of bacterial isolates from examined cases	% *	Organs of isolation
Aeromonas spp	14	48.3	Kidneys, Liver, Brain, Gills, Spleen
Ps. Fluorescence	1	3.4	Muscle
Proteus vulgaris	1	3.4	Kidneys
S. liquefaciens	1	3.4	Kidneys
S. plymuthica	1	3.4	Liver
C. freundii	1	3.4	Brain
E. sakazakii	1	3.4	Kidneys
E. cloacae	1	3.4	Liver
Lactococcus lactis	3	10.3	Kidneys, Liver
Strep. Lutetiensis	1	3.4	Kidneys
Strept. Equines	1	3.4	Kidneys
V. cholera	2	6.9	Kidneys, Liver
Kurthia spp	1	3.4	Liver
Total	29	99.5	

%* percentage of isolation in relation to the total number of isolates

3.6. Water quality

The mean values of water temperature, dissolved oxygen, pH and NH3 were 34.33±2.73 ℃, 6.68±1.35 mg/l, 10.17±0.44 and 1.87±0.63 mg/l, respectively. Heavy metals (Cadmium, lead, Copper, Ferrous and Zinc) levels were 0.01±0.00, 0.05±0.01, 0.03±0.01, 0.68±0.35 and 0.04±0.01, respectively.

3.7. The experimental infection trial

The highest mortality rate (100 %) were recorded for *A. veronii* (*HY2*) at a dose of 9×10^8 cells/ml, followed by 90 % for *A. veronii (HY4)*, 80% for *C. freundii* and *P. vulgaris*, 60% for *A. veronii* (*HY3*, *HY6*) and 40% for *P. fluorescence* (Fig. 4). The mortality rate was seen with *A. veronii* (*HY2*) strain (70%) at dose 6×10^8 cells/ml followed by *A. veronii* (*HY4*) and *Ps. vulgaris* (60 %), (*C. freundii* and *Ps. Fluorescence*) (40%) and *A. veronii* (*HY3, HY6*) (30%). *A. veronii* (*HY2*, *HY4*) strains showed (40 %) mortality at dose 3×10^8 cells/ml followed by (*A. veronii* (*HY3*,*HY6*) and *C. freundii*) (30 %), *Ps. vulgaris* (20%), and *Ps. Fluorescence* (10%). No mortalities were recorded in the control group and *A. veronii HY1* group throughout the experimental period. Clinically, groups infected with *A. veronii (HY2*, *HY3*, *HY4*, *HY6)*, especially at dose 9×10^8 cells/ml and 6×10^8 cells/ml, exhibited similar clinical signs to that observed in the naturally infected fish (Fig. 5). Based on the mortality rates, *HY2* and *HY4* were found to be virulent, *HY3* and *HY6* showed moderate virulence and *HY1* was avirulent (recorded no mortalities).

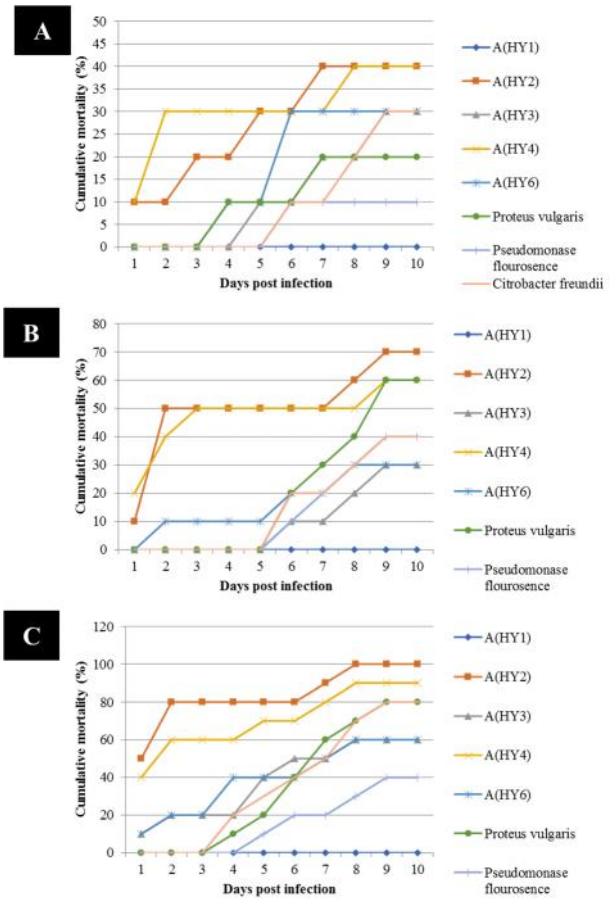

Fig. 4 Cumulative mortality rate of Nile tilapia challenged (I.P) with (A) 3×10^8, (B) 6×10^8 and (C) 9×10^8 of *A. veronii (A(HY1), A(HY2), A(HY3), A(HY4)* and *A(HY6)), Citrobacter freundii*, *Ps. Fluorescence* and *Proteus vulgaris*.

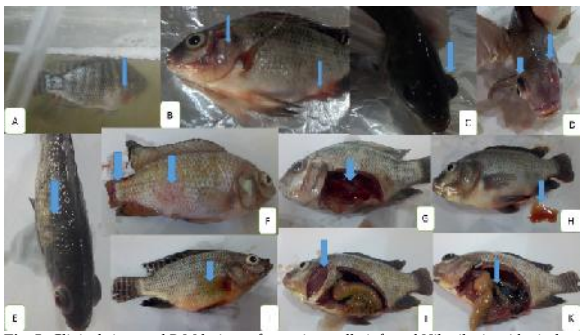

Fig. 5: Clinical signs and P.M lesions of experimentally infected Nile tilapia with virulent strains of *A. veronii* at dose 9×10^8 cells/ml *(HY2,HY4)* showed (A and B), abdominal distension and congestion of the body surface at base of fins and anal opening(arrows), Exophthalmia (unilateral and bilateral (C-E)(arrows), severe congestion in body surface and skin ulceration, eroded caudal fin (F)(arrows), congestion of the internal organs, bloody ascetic fluid in the abdominal cavity (G-K) (arrows).

4. DISCUSSION

Bacterial fish diseases are involved in genuine warns to aquaculture (Bentzon-Tilia et al., 2016), and it causes outbreaks with high mortalities (Sekar et al., 2008). The isolated bacterial pathogens from Nile tilapia add more

evidence of the wide geographical distribution of bacterial diseases. In the current study, the isolated bacteria were identified as Aeromonas spp. (*A. hydrophila*, *A. veronii*, *A. caviae*, and *A. sobria*), *Pseudomonas fluorescence*, *Citrobacter freundii*, Serratia spp (*S. liquefaciens*, and *S. plymuthica*), Enterobacter spp (*E. sakazakii* and *E. cloacae*), *Proteus vulgaris*, Streptococcus spp (*S. Lutetiensis*, *S. equines*), and *Vibrio cholera.* The examined fish showed generalized septicemic signs that are comparable to those recorded by Austin and Austin (2007) and Matter *et al*. (2018). These signs may be due to the virulence factors of the pathogenic enteric bacteria like somatic antigens, adhesins, lipopolysaccharides (Lipid-A), colicins, and siderophores (Seker *et al.,* 2010). These factors might penetrate the epithelial layers of the intestinal mucous cells facilitating pathogenicity (Tan *et al.,* 2002). Besides, extracellular proteins, hemolysin and leukotoxins were also involved in bacterial pathogenicity (Paraje *et al.,* 2005).

Conventional morphological methods for the diagnosis of bacterial infections in fish are complicated and time-consuming with a delay in the implementation of control measures, which causes massive economic losses. Moreover, many pathogens share common morphological characteristics and cause similar clinical signs in diseased fish. Hence, molecular identification techniques are adopted (Bader *et al.,* 2003, Altinok, 2011; Tsai *et al.,* 2012). In the current study, isolation of some highly pathogenic bacteria, like *Kurthia sp., Citrobacter sp,* and *Proteus sp,* from diseased fish, indicates fecal pollution of the fish farm water (Gonzalez et al., 2001; Wogu and Maduakol, 2010).

API 20 E results identified numerous strains that were suspected to be Aeromonas spp., these results nearly similar with that recorded by Lund *et al.* (2002) and Cantas *et al*. (2012), who reported that some of standardized API20E test, showed a variation in results. Beside Aeromonas spp.; there were other isolated species of *Proteus vulgaris*, Pseudomonas spp., *Serratia* spp., *Citrobacter freundii*., *Vibrio cholera*, Kurthia spp and *Enterobacter* spp. Several authors observed nearly similar results, like Sato et al. (1982), Baya et al. (1992), Mohamed (2000), Lund *et al*. (2002) and Austin and Austin (2007).

Molecular characterization using conventional PCR using 16S r DNA region retrieved a specific band at 1485 bp. These results agreed with that of Panigrahy *et al*. (2011), who described PCR amplification of the 16S rDNA coding region of aeromonads isolates at the 1485 bp. Genetic variability and relatedness among 6 strains of *Aeromonas veronii* from *Oreochromis niloticus* at different localities were assessed by unweighted pair group method with arithmetic mean (UPGMA) analysis showed heterogeneity and distance within the phylogenic tree. In the same manner, Sarkar et al., (2012) recorded that the six isolates of aeromonads isolated from different sources were all *A. veronii*; where genetic heterogeneity and distance within the phylogenic tree was evident. The total prevalence of bacterial infection was (99.5 %), these findings were higher than that recorded by Aly et al. (2012), who found the total prevalence of *Enterobacteriaceae* in diseased Nile tilapia was 44.1% and nearly similar to Hassan et al. (2012) who found the total prevalence of bacterial infection in Nile tilapia was 92.5% and the identified bacteria were; *E. coli, Salmonella arizonae, C. braakii, Enterobacter sakazakii, C. freundii, Raoultella ornithinolytica, Enterobacter cloacae, Klebsiella ozaenae* and *Proteus vulgaris* were 27%, 21.6%, 19%, 10.8%, 8.1%, 5.4%, 2.7%, 2.7%, and 2.7%, respectively. This difference may be attributed to the site of sample collection, the number of examined fish, the size of fish and environmental conditions. Also, the bacterial isolation in this work 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 bacterial infection. The most prevalent bacterial isolates were *aeromonads* (14 isolates, 48.3 %). These findings nearly like that recorded by Hanafy (2005), Dahdouh et al. (2016) and Abd El-Kader and Mousa-Balabel (2017). Almost all the obtained bacteria were isolated from kidneys and liver samples. These findings were similar with those obtained by Thompson *et al.* (2004) Toranzo *et al*. (2005), and Kusdarwati *et al.* (2017). These findings were attributed to the nature of these organs (hematopoietic organs contain high blood supply, so highly susceptible to infection**.**

From an environmental prospect, the achieved results have confirmed that water quality is an integral part of any aquaculture system. In most instances, the disease occurs as the result of complex interactions between pathogen, fish and environmental stress, which affect the susceptibility of the host to disease. Song et al. (2008) reviewed the role of the stress in the susceptibility of fish to diseases. Environmental stressors can affect the homeostatic mechanism of fish, thus reducing their resistance to the pathogenic organisms (Small and Bilodeau, 2005)**.**

In respect to water temperature, the higher warm temperature detected in some fish farms could be strongly incriminated in the increasing susceptibility of fish to bacterial diseases. Environmental stressors such as high temperature, poor water quality and high organic content, primarily contributed to the onset and severity of fish infections caused by bacterial pathogens belongs to Enterobacteriaceae (Zheng et al., 2004). Increased temperature may also enhance the transmission of infection, leading to a higher prevalence of the disease and to more widespread epidemics (Karvonen et al., 2010). Furthermore, bacteria might show a greater virulence, for instance, *A. hydrophila* showed a greater virulence in largemouth bass (*Micropterus salmoides*) at warmer temperatures because of either reduced resistance of the host or to an increased expression of virulence factors (Marcogliese, 2008).

In respect to the dissolved oxygen levels (DO), lower levels than the optimal recommended values were recorded (5.25±1.00 mg/l). These relatively low DO levels synergized with other viable environmental aquatic components in both aquaculture facilities to produce the notable cases of mortalities **(**Haley *et al.,* 1967**).**

Results demonstrated that the pH and ammonia levels were higher than the recommended values in different fish farms; the recorded values seem to be conducive to the higher prevalence of bacterial infections recorded in the study. High ammonia levels also enhance the microbial infections through suppressing the fish immunity (Cheng et al., 2004). Regarding the heavy metals, our findings also confirmed the synergistic effect of some heavy metals, particularly iron and copper in disturbing the health status of cultured tilapia speeding up the course of outbreaks. The mean of some detected metal values was higher than the levels recommended by WHO (1993). It was documented that fish reared in copper and iron contaminated habitats are more susceptible to a wide range of bacterial pathogens infection (Avendano *et al.,* 2005). This may be attributed to the properties of heavy metals like iron which act as an excellent

oxygen transporter; stimulating the growth of bacteria (Kutsky, 1982).

In the current study, the experimental pathogenicity trial showed varying mortality rates depending on the species of the bacterial isolate, where a high mortality rate was recorded in *A. veronii* (*HY2*-100 %) at dose 9×10^8 cells/mL followed by *A. veronii (HY4*-90%), (*C. freundii*, *Ps. vulgaris*) (80%), *A. veronii (HY3*, *HY6*-60%) then *Ps. fluorescence* (40%), respectively. The reported mortalities varied based on the pathogenesis and virulence as well as the amount and severity of the toxins among each bacterial isolate. These results were in agreement with Eissa *et al.* (2015), who found that Nile tilapia challenged with $1.2x10^8$ cells/mL of *A. veronii biovar sobria*, caused mortalities of 70-35% of (IP) and (IM) groups, respectively. Also, similar results were reported by Eissa *et al.* (2015). The mortality rate of *C. freundii* was higher than that reported by Aly *et al.* (2012), who revealed that (IP) experimental infection of Nile tilapia with *C. freundii* with 0.5 mL of bacterial suspension at concentration of 1.5×10^8/fish gave no distinctive clinical signs of infection with mortality rate (9.99%). Also, mortality rate of *Proteus vulgaris* were higher than that reported by Saad EL-Deen (2013), who found that Nile tilapia challenged with *P. vulgaris* showed mortality rate of 33.33% with similar clinical signs as petechial hemorrhage on the body surface, congested gills. *P. fluorescence* results were nearly similar to Mahmoud *et al.* (2014), who challenged Nile tilapia (IP) with 0.3 ml of sterile saline containing 1.5×10^8 CFU/ml of Pseudomonas fluorescens showed 33.33% mortalities.

In summary *A. veronii*, *C. freundii*, *Ps. vulgaris*, and *P. fluorescence* are considered to be the most threatening bacterial pathogens involved in the septicemic bacterial infections of Nile tilapia, and therefore, should be included in the future strategies of prophylaxis and treatment of farmed Nile tilapia. Our results also showed that the adverse environmental conditions have a great role in the spread of infection and the occurrence of high mortality. Thus, maintaining a favorable fish culture, environment, and good husbandry practice are crucial for growing healthy fish.

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