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RESEARCH PAPER

Investigation of Nile tilapia Summer Mortality in Kafr El-Sheikh Governorate, Egypt

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Abstract

Aquaculture sector in Egypt has demonstrated a remarkable development; however, it has also faced challenges with respect to disease outbreaks. Nile tilapia is the most widely cultured species in Egypt. Under stress conditions, tilapia is vulnerable to a variety of bacterial diseases that tend to be ubiquitous in the freshwater environment. Semi-intensive Nile tilapia farms in Kafr El-Sheikh Governorate, Egypt are experiencing acute mortality especially during summer months. The samples from these farms were collected and subjected to investigations to identify the etiological agent(s) behind these mortalities. Bacteriological examination revealed that the dominant bacteria were Gram-negative rods and identified as *Aeromonas* spp. through biochemical tests. PCR, restriction fragment length polymorphism (RFLP) assays, and sequencing confirmed the presence of *Aeromonas hydrophila*. In addition, the *aerA* gene, a virulence factor in *A. hydrophila*, was detected by PCR in all identified *A. hydrophila* isolates. In order to confirm that the isolated *A. hydrophila* was the causative agent of tilapia mortality, healthy Nile tilapia were challenged with the isolated strains, which produced the same clinical picture of the collected samples. This study implicates that *A. hydrophila* could be the causative agent of the summer mortality in Nile tilapia farms in Kafr El-Sheikh Governorate, Egypt.

Introduction

Nile tilapia is the main cultured species in Egypt that contributes about 65.15% of Egyptian fish production (GAFRD, 2017). However, tilapia culture has faced higher mortality rates particularly during summer months in recent years (Eissa *et al.*, 2015; Fathi *et al.*, 2017). Several attempts have been made to identify the causative agents of these outbreaks. On one hand, cultured tilapia mass mortalities in El-Sharkia governorate was linked to bacterial infection with the opportunist pathogen *Aeromonas veronii* biovar sobria (Eissa *et al.*, 2015). On the other, Fathi *et al.* (2017) attributed these mortalities to viral infection with Tilapia Lake Virus.

Aeromonas spp. are oxidase-positive, facultatively anaerobic Gram-negative bacteria that belong to the family *Aeromonadaceae* (Deyan Stratev & Odeyemi, 2016). Aeromonads are ubiquitous in the aquatic environment and they have been found in different types of foods, such as fish, shrimps, mussels meat, meat products, milk, and vegetables (Neyts, Huys,

Uyttendaele, Swings, & Debevere, 2000). *Aeromonas hydrophila* has been reported to be the most predominant *Aeromonas* spp. found in tilapia aquaculture (Hassan, Noureldin, Mahmoud, & Fita, 2017).

Identification of *A. hydrophila* was relayed on isolation and biochemical characterization which considered time-consuming and may lead to inaccurate conclusions (Abbott, Cheung, & Janda, 2003). Hence, powerful molecular assays such as polymerase chain reaction (PCR) and DNA sequencing are highly essential (Jagoda *et al.*, 2014).

The development of DNA-based molecular techniques in the identification of bacterial species has highly influenced the identification of prokaryotes based on the small subunit ribosomal RNA (16S rRNA) gene analysis (Ramon & Rudolf, 2001). The 16S rRNA gene provids signature sequences for identification of most *Aeromonas* species (Martinez-Murcia, Benlloch, & Collins, 1992). Consequently, many protocols have been described based on the RFLP patterns of the amplified 16S rRNA gene for identification of most *Aeromonas* spp. utilizing endonucleases (Borrell, Acinas, Figueras, & Martínez-Murcia, 1997; Figueras, Guarro, & Martínez-Murcia, 2000).

Moreover, the pathogenicity of *A. hydrophila* has been linked to the expression of a number of exotoxins such as aerolysin, hemolysin, lipases, cytotonic enterotoxin, and proteases (Castro-Escarpulli *et al.*, 2003; Howard, Mac Intyre, & Buckley, 1996; J.M. Janda & Abbot, 1996). Aerolysin, with the structural gene named *aerA*, a pore-forming toxin, is considered the most pivotal virulence factor. Moreover, the presence of *aerA* strongly indicates the virulence in *A. hydrophilia* pathogenic isolates (Biscardi, Castaldo, Gualillo, & De Fusco, 2002; Ormen, Regue, Tomas, & Granum, 2003). Furthermore, several researchers have detected the *aerA* gene only in enterotoxic, cytotoxic, and hemolytic *A. hydrophila* strains (Lior & Johnson, 1991; Oliveira, Veneroni-Gouveia, & Costa, 2012). In addition, Singh, Rathore, Kapoor, Mishra, and Lakra (2008) found *aerA* in all isolated *A. hydrophila* strains from fish demonstrated hemorrhagic septicemia in their study. Hence, there is a strong positive correlation between the pathogenicity of *A. hydrophila* and the presence of *aerA* gene.

The aim of this study was to identify the causes of summer mortalities in tilapia semi-intensive farms in Kafr El-Sheikh Governorate, Egypt.

Materials and Methods

Fish Samples

A total of 150 live Nile tilapia fish, exhibiting septicemia signs, with the average weight ranging between 150 and 300 g were randomly collected as from five different semi-intensive polyculture fish farms with a history of mortalities during the last few years in Kafr El-Sheikh governorate, Egypt, during summer (June-September). The collected samples were packed in sterile polyethylene bags, sealed, and transferred immediately in a cooled insulated box under aseptic conditions to the food hygiene laboratory, Faculty of Veterinary Medicine, Kafr El-Sheikh University, where clinical and postmortem examinations were carried out according to Noga (2010). Fish tissues (kidney, spleen, and liver) were sampled for molecular investigations. Water samples were also taken from the same ponds to the laboratory for the determination of water-quality parameters (dissolved oxygen, nitrite, ammonia, ammonium, phosphate, and pH) using the commercially available testing kit (Sera®, GmbH, Germany).

Bacterial isolation and Biochemical Characterization

For bacterial isolation, the samples from the ulcers, kidney, liver, and spleen of the diseased Nile tilapia were inoculated onto blood agar plates (CM0271, Oxoid) and tryptone soya agar (TSA) plates (CM0131, Oxoid) and incubated for 48 h at 28°C. Dominant colonies from the plates were selected and sub-cultured on the TSA. Bacterial isolates were characterized by morphology of colony on agar, Gram-stain, and cell motility in semisolid media. Biochemical characterizations of the bacterial isolates were performed using the VITEK® 2 Compact microbial identification system (BioMerieux TM, France). Suspected *Aeromonas* isolates were subcultured onto Aeromonas medium base (RYAN, CM0833, Oxoid) for confirmation. The isolates were stored in Brain Heart Infusion (BHI) broth medium with 20% (v/v) glycerol at −80°C.

Molecular Analysis

Genomic DNA Extraction

Genomic DNA was extracted from the Nile tilapia tissues and bacterial isolates by using the Thermo Scientific GeneJET Genomic DNA purification Kit according to the manufacturer's protocol. Briefly, fish tissues (kidney, spleen, and liver) were ground in liquid nitrogen, and then 20 mg of tissue powder was transferred into 1.5-mL microcentrifuge tubes that previously cooled in liquid nitrogen. After addition of digestion solution (180 µL) and proteinase K (20 µL), the samples were incubated for 3 h at 56°C until complete lyses of the tissues. DNA extraction was then completed as per the manufacturer's instructions. The extracted DNA was kept at –20°C until use.

Furthermore, 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 digestion solution (180 µL), and then the genomic DNA was extracted according to the manufacturer's instructions and the bacterial DNA was kept at −20°C until use.

PCR Using the Universal Bacterial Primer

The extracted DNA from the bacterial isolates and tissue samples was subjected to PCR using universal bacterial primers according to Niannian, Peng, Wang, Wang, and Peng (2004) with some modifications. Briefly, the reaction mixture in 25-µL volume contained 12.5 µL of Thermo Prime 2x ReddyMix PCR Master Mix, 1 µL each of forward and reverse primers (10 pmol each) (Table 1), 3 µL of extracted DNA, and 7.5 µL of sterile deionized distilled water. The cycling profile was 94°C for 5 min, then 35 PCR cycles of 94°C, 58°C, and 72°C for 1 min each, with a final extension step of 72°C for 10 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis in Tris acetate−EDTA buffer (TAE), stained with 0.5 µg mL^{-1} ethidium bromide, and examined using Gel Documentation System. A 100-bp DNA ladder, H3 RTU (Nippon Genetics, Europe), was used to determine the size of the PCR products. The amplified products were purified using a MinElute Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The purified PCR products were sequenced in a commercial sequencing laboratory, then subjected to BLASTn search for sequence similarity against GenBank database according to Altschul *et al.* (1997).

Amplification and Endonuclease Digestion of the *Aeromonas* spp. 16S rRNA Gene

Aeromonas spp. 16S rRNA gene (1502 bp) was amplified using the DNA extracted from the bacterial isolates and tissue samples, according to Borrell *et al.* (1997), with some modifications. Briefly, the 25 µL end-volume reaction mixture contained: 12.5 µL of Thermo Prime 2x ReddyMix PCR Master Mix, 1 µL forward primer (20 pmol), 1 µL reverse primer (20 pmol) (Table 1), 3 µL of extracted DNA, and 7.5 µL sterile deionized distilled water. The cycling profile was 94°C for 5 min, then 35 PCR cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, with a final extension step of 72°C for 10 min. PCR products were analyzed and purified as mentioned earlier. A fraction of the purified PCR products was used for sequencing. The other part was subjected to enzymatic digestions, according to Ghatak, Agarwal, and Bhilegaonkar (2007), using FastDigest Eco105I following the manufacturer's instructions. Restriction digestion products were analyzed by agarose gel electrophoresis using 1.5% gel stained with ethidium bromide and examined using the Gel Documentation System.

Detection of *A. hydrophila aerA* Gene

The amplification of the *aerA* gene segment (431 bp) was performed using the DNA extracted from the bacterial isolates and tissue samples according to Oliveira *et al.* (2012) with some modifications. Briefly, a reaction mixture in 25 µL contained 12.5 µL ThermoPrime 2x ReddyMix PCR Master Mix, 1 µL forward primer (15 pmol), 1 µL reverse primer (15 pmol) (Table 1), 0.5µL $MgCl_2$, 3 µL of extracted DNA, and 7 µL sterile deionized distilled water. The cycling profile was 94°C for 3 min, then 35 PCR cycles of 94°C for 1 min, 55.5°C for 45 s, and 72°C for 30 s, with a final extension step of 72°C for 10 min. The PCR products were analyzed by gel electrophoresis using 1.5% agarose in TAE buffer, stained with ethidium bromide, and visualized using the Gel Documentation System. A 100-bp DNA ladder (Fisher Bioreagents) was used to determine the size of the PCR products. The amplified products were purified and subjected to sequencing as mentioned above.

Establishing the Pathogenicity of *A. hydrophila* Isolate in Nile Tilapia

Apparently healthy Nile tilapia (n=80, average weight 150±10 g) were purchased from a local farm and transferred alive to the laboratory for the challenge experiment. After acclimatization to laboratory conditions for three weeks, fish were randomly divided into four groups (20 fish/group). Three groups were challenged with the isolated *A. hydrophila*

Table 1. Primers used in this study

Primer	Primer sequences (5'–3')	Target genes	Specificity	Size of amplified product (bp)	References
Univer.Bac.For	GCACAAGCGGTGGAGCATGTGG	16S- rRNA	Bacterial	variable	(Niannian *et al.,*
Univer.Bac.Rev	GCCCGGGAACGTATTCACCG		pathogens		2004)
A.hyd_16S_For	AGAGTTTGATCATGGCTC AG	16S- rRNA	*Aeromonas* spp.	1502 bp	(Borrell *et al.,*
A.hyd_16S_Rev	GGTTACCTTGTTACG ACT T				1997)
A.hyd_*aer*_For	CCTATGGCCTGAGCGAGAAG	aerolysin	*Aeromonas*	431 bp	(Oliveira *et al.,*
A.hyd_*aer*_Rev	CCAGTTCCAGTCCCACCACT		*hydrophilia*		2012)

intraperitoneally (I/P) using 0.5 mL of 5 × 10^6 CFU/mL, and one group (sham-control) was injected with 0.5 mL sterile saline I/P. The aquaria were supplied with enough water; aeration was carried out by an electric aerator, and the temperature was maintained at 27±2°C.

Results

Clinical Signs in the Diseased Nile Tilapia

The diseased Nile tilapia in the affected farms showed sluggish movements, dullness, and swimming near the surface water with the absence of reflexes. Clinical investigation of the sampled fish demonstrated exophthalmia, hemorrhages on the body surface, detached scales with pale skin patches, ulcerations, and abdominal dropsy. Furthermore, the postmortem examination of the sampled fish exposed congestion of gill and kidney, hepatosplenomegaly, distended gallbladder, hemorrhagic spots in the liver, and accumulation of bloody fluids in the abdominal cavity. The cumulative mortality of the infected fish was 30–60% during summer months.

Biochemical Characteristics of Bacterial Isolates

The bacterial isolates from the, kidney, liver, spleen and ulcers, of the diseased fish formed smooth, convex, rounded, and β-hemolytic colonies on blood agar and produced round, convex, shiny, and creamy colonies on TSA agar. Light microscopy examination of the bacterial smears revealed Gram-negative, rod-shaped bacteria. The microbial identification system (VITEK® 2 Compact) showed that, all the isolates have the same biochemical profile, which was positive for motility, oxidase, and catalase activities, resistant to vibriostatic agent 2,4-diamino–6,7-diisopropylpteridine (O129), methyl red, and citrate utilization; growth in media containing 6.5% NaCl, hydrolysis of urea; o-nitrophenyl-b-D galactopyranoside (ONPG) production; indole reaction, ornithine and lysine decarboxylase and arginine dihydrolase activities; gas from glucose fermentation; esculin hydrolysis; production of acetyl methyl carbinol (Voges–Proskauer) and elastase; hydrogen sulfide formation in the gelatin-cysteine-thiosulfate medium; oxidation of potassium gluconate; fermentation of L-arabinose, D-mannitol, salicin, mannose, cellobiose, sucrose, m-inositol, glycerol, L-rhamnose, and D-sorbitol; and utilization of sodium citrate, DL-lactate, urocanic acid, and DNAse. The bacterial isolates were also confirmed using Aeromonas medium base, and the colonies appeared dark green, opaque with a black center, with the diameter range of 0.5–1.5mm. All the isolates were identified as *A. hydrophila* according to Bergey's Manual of Systematic Bacteriology (Martin-Carnahan & Joseph, 2005). The study revealed that of the 150 Nile tilapia samples examined, *A. hydrophila* was isolated and identified from 120 samples (80%) according to biochemical identification.

Molecular Analysis

The universal bacterial primers amplified the expected (~500 bp) conserved region of the bacterial 16S rRNA gene from the isolates. BLASTn results of these sequences reveled 98% identity with the different *Aeromonas* species. In addition, the expected fragment (1052 bp) of the *Aeromonas* spp. 16S rRNA gene was amplified from all samples and isolates using the specific primers (Figure 1). In order to discriminate between the different *Aeromonas* species, restriction digestion of the purified *Aeromonas* spp. 16S rRNA PCR amplicons was performed, which exhibited a single cut with two

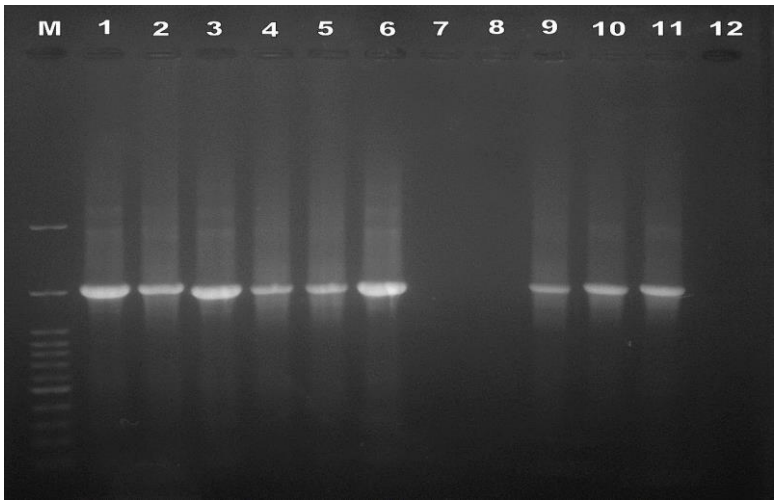

Figure 1. 1.5% agarose gel electrophoresis of 10-samples, representative for the 150 tested samples, showing DNA bands of 16S rRNA PCR amplification of *Aeromonas* spp. (1502 bp). Lane M= 100 bp DNA ladder, Lanes 1–6 & 9–10 = Positive sample that demonstrated the expected 1502 bp fragment of *Aeromonas* 16s rRNA gene. Lanes 7–8= Negative samples that did not demonstrate the expected 1502 bp fragment of *Aeromonas* 16s rRNA gene; Lane 11= Positive control, Lane 12= no-template control.

fragments 1039 and 462 bp (Figure 2), implicating that the isolated species is *A. hydrophila*. Our study revealed that 80% of the examined samples and all the isolates were identified as *Aeromonas* spp. by 16S rRNA gene amplification and confirmed as *A. hydrophila* by RFLP pattern. The 16S rRNA purified PCR amplicons were sequenced, and the BLAST search analysis of the sequences obtained revealed 100% similarity between the isolates and 98% identity with 16S rRNA gene of different *A. hydrophila* strains; for example, the following GenBank accession numbers: AY987741, AY966887, LC420129, AB680307, and KJ522794. The *A. hydrophila* 16s rRNA sequence obtained from this study was submitted to the GenBank under the accession number KX012004.

The 431 bp fragment of *A. hydrophila aerA* gene was detected in all the examined samples and isolates as shown in Figure 3. The *aerA* gene was detected in all the isolates and 80% of the examined fish samples during this study. For the validation of *A. hydrophila* identification, purified PCR products were subjected to sequencing. BLAST search analysis of the sequences obtained from *A. hydrophila aerA* gene PCR amplicon revealed 98% identity with *A. hydrophila aerA* gene, for example, the following GenBank accession numbers HM853019, KU527551, and AF410644. The *A. hydrophila aerA* gene sequence obtained from this study was submitted to the GenBank under the accession number KX138395.

Results of Experimental Infections

In experimental infections, the bacterial isolates exhibited virulence to Nile tilapia and led to severe symptoms as observed in postmortem pictures with a mortality rate of 14 fish per group (70%) in the challenged groups and the mortalities peak was on the fourth day post-infection. However, no mortalities were recorded in the sham-controlled groups. The inoculated bacteria were re-isolated from the lesions of the challenged fish and confirmed to be *A. hydrophila* based on the biochemical profiles and molecular sequence analysis.

Results of Water Samples Analysis

All analyzed water-quality parameters of the water samples were within the normal range.

Discussion

Despite the fact that the aquaculture sector in Egypt has witnessed a remarkable development, it has also been facing numerous challenges with respect to disease outbreaks (Soliman & Yacout, 2016). Nile tilapia is the most widely cultured species in Egypt. Tilapia, similar to other cultured fish species, under stress conditions, is susceptible to various bacterial diseases that tend to be ubiquitous in the freshwater environment (Dong *et al.*, 2017; Eissa *et al.*, 2015). The present study was carried out to identify the causative agent of fish mortalities in tilapia semi-intensive farms in Kafr El-Sheikh, Egypt.

One of these pathogenic bacteria is *A. hydrophila*, which is considered to be of growing interest due to its pathogenicity in aquatic organisms, its potentially pathogenic effects in humans, and its spoilage action in foods (Janda & Abbott, 2010). It is well known that *A. hydrophila* is a ubiquitous, opportunistic pathogen to warm and cold-blooded animals, and its occurrence has

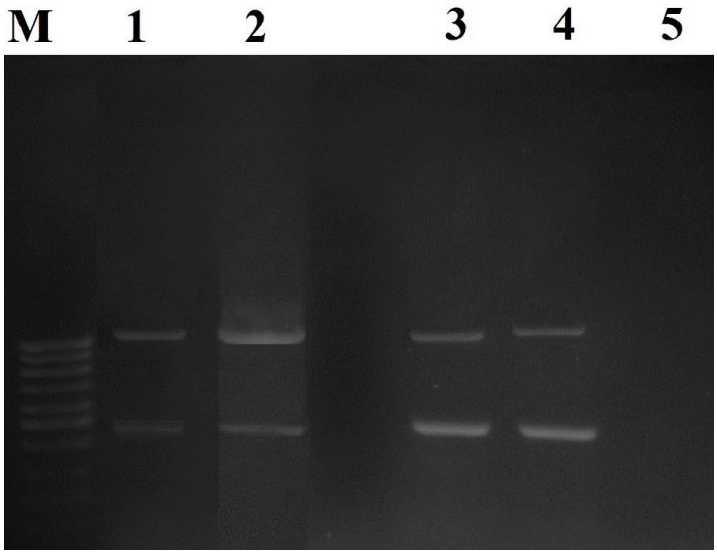

Figure 2. 1.5% agarose gel electrophoresis showing restriction digestions of *Aeromonas* spp. 16S rRNA PCR amplicons (RFLP patterns) at (462–1039 bp). Lane M= 100 bp DNA ladder, lanes 1–3 = Demonstrating the characteristic RFLP pattern of *Aeromonas hydrophila* (462–1039 bp) where Lane 4 is the positive control. Lane 5= No-template control.

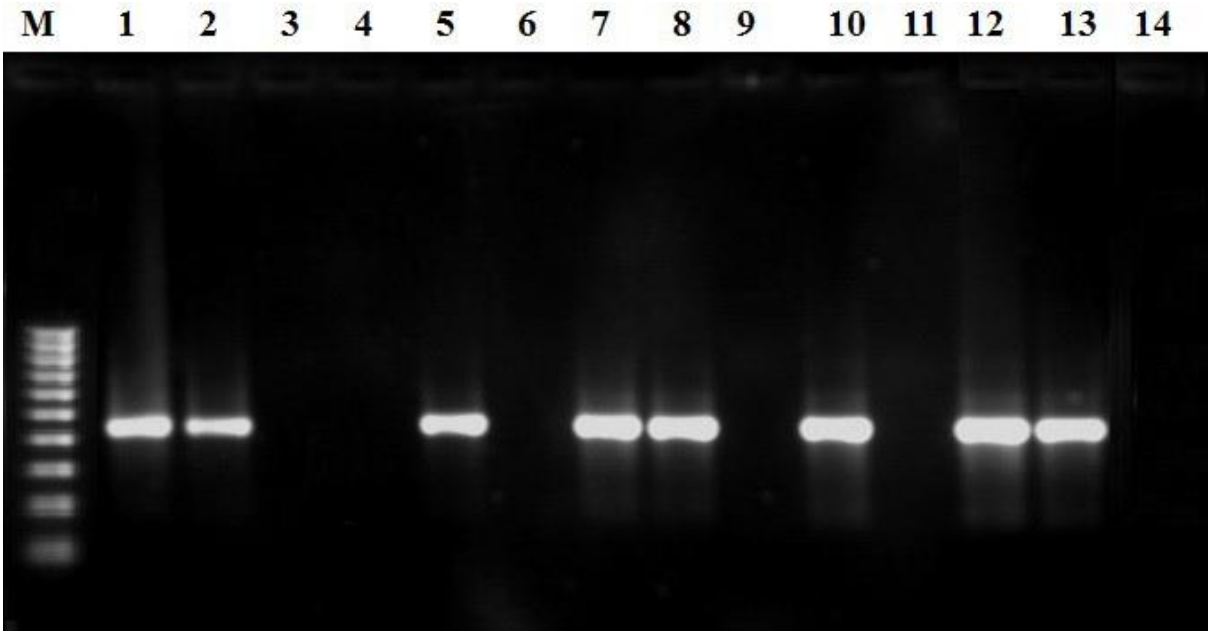

Figure 3. 1.5% agarose gel electrophoresis showing DNA bands of aerolysin gene fragment amplification of *Aeromonas hydrophila* Lane M= 100 bp DNA ladder, lanes 1, 2, 5, 7, 8, 10 & 12 = Positive samples that demonstrated the expected 431 bp amplicon of *Aeromonas hydrophila* aerolysin gene fragment. Lanes 3, 4, 6, 9 & 11= Negative samples that did not demonstrate the expected 431 bp amplicon of *Aeromonas hydrophila* aerolysin gene fragment. Lane 13= Positive control, Lane 14= No-template control.

been reported in numerous sources so far by many researchers.

The observed clinical signs of infected Nile tilapia in the current study are similar to those reported by Dahdouh, Basha, Khalil, and Tanekhy (2016); Harikrishnan and Balasundaram (2005); Sarkar and Rashid (2012). These gross lesions, including hemorrhagic patches, can be attributed both to bacterial invasion, multiplication, and colonization as well as to toxins produced by invading microorganism(s) (Miyazaki & Kaige, 1986). Moreover, the postmortem examination revealed that the affected Nile tilapia showed septicemic lesions, which is in agreement with the findings of Afifi, Al-Thobiati, and Hazaa (2000); Noor El Deen, Dorgham-Sohad, Hassan-Azza, and Hakim (2014); Omar, Moustafa, and Zayed (2016). The current postmortem findings could be due to the action of toxic extracellular metabolites of *A. hydrophila* including hemolysin, aerolysin, and cytotoxic toxins, which possess hemolytic, cytolytic, and enterotoxic activities that induce liver necrosis, renal tubules degeneration, and render the tissue hemorrhagic, with exudates of serum and fibrin (Miyazaki & Kaige, 1986).

Basic isolation and preliminary identification methods were followed for the identification of the causative agent of the mortalities. The microbiological examination revealed that the causative agent is *A. hydrophila*, and it was detected in 80% of the investigated fish samples. These results were at par with those reported by Aboyadak, Ali, Goda, Aboelgalagel, and Alnokrashy (2015) and Ullmann, Krause, Knabner, Weber, and Beutin (2005). However, lower prevalences were detected by Neyts *et al.* (2000); Hassan (2006); Yogananth, Bhakyaraj, Chanthuru, Anbalagan, and Nila (2009); Omeje and Chukwu (2013); Alhazmi (2015); and Stratev, Daskalov, and Vashin (2015). However, a higher prevalence of *A. hydrophila* (95.06%) was reported by Gobat and Jemmi (1993). Variations in the incidence level of *A. hydrophila* in the fish worldwide can be attributed to species, sampling time, and geographical range (Vivekanandhan, Hatha, & Lakshmanaperumalsamy, 2005). The higher incidence of *A. hydrophila* 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 occurred during summers when the water temperature was high, which act as a stress factor for the fish and increase their susceptibility to infection.

The routine microbiological procedures for isolation and identification of *Aeromonas* spp. may be time-consuming and laborious, whereas regular biochemical identification may lead to misidentification of *Aeromonas* isolates due to the lack of uniformity of some biochemical characteristics (Abbott *et al.*, 2003; ICMSF, 1996). Accordingly, all the fish samples used in this study were confirmed on the basis of amplification of bacterial 16S rRNA, followed by restriction digestion of *Aeromonas* spp. 16S rRNA PCR amplicons to be able to discriminate most *Aeromonas* species (Borrell *et al.*, 1997). The *Aeromonas* strain that exhibited single cut with two fragments (1039 and 462 bp) was identified as *A. hydrophila* strain according to Borrell *et al.*, (1997).

The study revealed that 80% of the examined samples were identified as *Aeromonas* spp. by the 16S rRNA gene amplification and identified *as A. hydrophila*

by the RFLP pattern, which nearly agreed with the findings of Lee, Cho, Lee, Lee, and Kim (2002) who identified by the 16S rRNA gene-based PCR that 78% of the examined fish samples were infected with *Aeromonas* spp., whereas only 14% were identified by RFLP as *A. hydrophilia*. A Lower prevalence was observed by Onuk *et al.* (2013), who identified 23.33% as *A. hydrophila* using PCR-RFLP pattern analysis as well as by Hussain, Jeyasekaran, Shakila, Raj, and Jeevithan (2014) who confirmed that 56% of the fish samples contained *Aeromonas* spp. based on the presence of the 16S rRNA gene. These differences may be attributed to the different species, sampling time, and geographical range. Lee *et al.* (2002) found that the distribution of *Aeromonas* spp. showed a close relationship between the sampling time and dominant species especially pathogenic species, such as *A. hydrop*hila, *A. caviae*, and *A. veronii*, which were dominant at all sites during warmer months.

Variety of virulence factors, that are important for their pathogenicity, were released from the strains of *Aeromonas* spp. (Janda & Abbott, 2010). The majority of *A. hydrophila* virulent strains secrete at least two types of hemolytic toxins: aerolysin (aerA) and hemolysin (AHH1), both toxins contributing to the virulence in *A. hydrophila* (Wang *et al.*, 2003). In *Aeromonas* food poisoning, aerolysin is considered the most vital virulence factor (Ormen *et al.*, 2003). The presence of aerolysin is a strong indication of virulence in pathogenic isolates of *A. hydrophilia* and the *aerA* gene has mostly been detected in enterotoxic, cytotoxic, and hemolytic strains of *A. hydrophila* (Lior & Johnson, 1991). PCR was used for the amplification of *aerA* gene in all the examined samples, and the *aerA* gene was detected in 80% of the examined fish samples in this study. This result nearly agreed with the result obtained by Singh *et al.* (2008), who found the *aerA* gene in 85% of the isolates recovered from fish and pond water. Moreover, Ramachandran , Hemamalini , and Srimathy (2014) detected *aerA* gene in 80% of *A. hydrophila* strains recovered from fish samples. Moreover, Oliveira *et al.* (2012) recorded the *aerA* gene in 78.95% of *A. hydrophila* isolates obtained from fish samples. Abdel-Latef (2015) found the *aerA* gene in fish samples at a lower rate of 3.3%. Furthermore, Herrera, Santos, Otero, and Garcia-Lopez (2006) detected the *aerA* gene in 16% of the examined fish samples. The differences in the prevalence of *aerA* gene detection may be attributed to the primer design divergence and the limited number of strains. It may also be attributed to the species, different water salinities, number of examined fish, and environmental conditions.

The challenge experiment was conducted to demonstrate whether the identified bacterial isolates could induce disease in healthy Nile tilapia. Experimental infection using individual bacterial isolates revealed that these isolates killed 70% of the challenged fish and produced severe disease symptoms.

Conclusions

It can be concluded that *A. hydrophila* is one of the causes of summer mortality outbreaks in Nile tilapia in Egypt. Nevertheless, further studies are needed to determine the other causes of these outbreaks because they could be attributed to multifactorial causes.

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