Mansoura Veterinary Medical Journal 21:1 (2020) 56-67

https://doi.org/10.35943/mvmj.2020.21.108

Original Article *Fish management and Diseases*

Prevalence, molecular characterization, virulotyping, and antibiotic resistance of motile aeromonads isolated from Nile tilapia farms at northern Egypt

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ARTICLE HISTORY

Received: 27.02.2020

Revised: 18.03.2020

Accepted: 29.03.2020

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ABSTRACT

Objective: This study was aimed to survey Aeromonas spp associated with cultured Nile tilapia *Oreochromis niloticus* (*O. niloticus*) showing signs of motile Aeromonas septicemia (MAS) at different fish farms; molecular characterization and identification of test isolates; and to test the isolates for their antimicrobial and virulence activities that contribute to its pathogenesis.
Design: Observational study.
Animals: 280 Nile tilapia.
Procedures: Clinically diseased 280 Nile tilapia, were collected from different localities at Kafr El-Sheik and Dakahlia governorates. The clinical picture and gross lesions were recorded. Aeromonas spp were isolated and presumptively identified using API20E. The identification was confirmed using PCR. Hemolysin (hylA), lipase, and aerolysin (aerA) virulence genes were detected among isolates obtained from different sampling sites. Besides, antimicrobial activity was reported for the identified *A. hydrophila*.
Results: General septicemic signs were evident on Nile tilapia including, skin hemorrhages and ulcerations, bilateral exophthalmia, congested internal organs with significant mortalities. The prevalence of bacterial infection among naturally diseased Nile tilapia was 79.17, 70, and 58.33 in Kafr El-Sheikh, El- Manzala, and Gamsa fish farms, respectively. The most prevalent bacterial isolates were aeromonads (29.84 %), of all, 65.63 *A. hydrophila*, 18.75 *A. caviae*, and 15.63 *A. sobria*. All isolates were positively amplified using a species-specifying primer to determine *A. hydrophila*. Virulence genes detection revealed that five *A. hydrophila* isolates (83.3 %) harbored the aerA gene. Meanwhile, hylA and lipase genes positive isolates were lower reaching 16.7 % for both genes. *A. hydrophila* was highly sensitive to ciprofloxacin, amikacin, trimethoprim, and chloramphenicol, and MAR index of *A. hydrophila* isolates was ranged from 0.16-0.42
Conclusion and clinical relevance: Our findings demonstrate that Aeromonas spp are among the bacterial pathogens implicated in summer mortalities in tilapia fish farms in Egypt. Besides, determination of the prevalence, virulence genes, and antibiotic resistance pattern associated with the disease outbreaks is critical data that warrant the development of strategies to proper monitoring and farm management practices.

Keywords: Fish, Bacterial pathogens, Molecular diagnosis, Virulence.

1. INTRODUCTION

The fast development of aquaculture and increasing fish demand lead to the intensification of fish culture, magnifying stressors for fish, and thus heighten the risk of diseases **[1]**. Infectious diseases represent the main problem in fish farms, causing massive economic losses due to intensive farming practices **[2]**. *Aeromonas* are opportunistic pathogens for fish, and their prevalence rate is linked to stress conditions such as overcrowding, rough handling, or poor water quality leading to significant epidemic outbreaks **[3, 4].**

Usually, clinical abnormalities of *A. hydrophila* are in the form of skin darkness, scales detachment, extensive irregular hemorrhages on the body surface, ulcers on the skin varied from shallow to deep necrotizing ulcers, exophthalmia, fin erosions, and abdominal distension. Postmortem examination revealed hemorrhage and enlargement in internal organs **[5]**. Extracellular enzymes such as hemolysis, lipases, proteases, β- lactamases, amylases, chitinases and nucleases produced by *Aeromonas* have involved in their ecology, survival pathogenicity **[6]**, and contribute to the ability for their attachment to the host cells and finally, disease development **[7-9]**. Molecular characterization to confirm the biochemically identified aeromonads using the16S rDNA region helps for accurate identification **[10]**. The analysis of the 16S rRNA gene gave a quick and precise

identification of the bacteria **[11]**. 16S rRNA gene is an essential tool when used beside biochemical tests to identify microbes in the diagnostic laboratory **[12]**. Virulence of *A. hydrophila* is multifactorial, resulting from the production or secretion of virulence factors, such as adhesins, cytotoxins, *hylA*, lipases, and proteases as well as the capacity to form biofilms, use specific metabolic pathways and mediate virulence factor expression through quorum sensing **[4]**. Hemolytic toxins as *hylA* and *aerA* released by *aeromonads* contribute to their pathogenicity that has been linked to hemagglutinins, adhesins, and several hydrolytic enzymes **[13]**. Aerolysin gene is recorded to be the putative virulence gene produced by some strains of *A. hydrophila*, which is an extracellular, soluble, and hydrophilic protein exhibiting both hemolytic and cytolytic properties. Further, it binds to proteins of the host red blood cells (RBCs) and forms pores in the cell membrane causing hemolysis. Thus, it can be used for the diagnosis of *A. hydrophila* infection in fishes **[5]**.

Antibiotic sensitivity determination is necessary to select the most effective antibiotic drug to be used. However, resistance due to the vast use of antibiotics has been reported in previous studies in *A. hydrophila* isolates from freshwater fish **[14]**. *Aeromonas spp* were tested for resistance to 12 antibiotics by Odeyemi and Ahmad **[15]** who revealed that all isolates were utterly resistant (100%) to ampicillin, novobiocin, sulphamethoxazole, and trimethoprim, however, isolates were susceptible to tetracycline (100%), kanamycin (5.7%), gentamicin (5.7%) and oxytetracycline (24.5%). Besides, different patterns in antimicrobial resistance have been reported in many previous studies **[16]**.

Therefore, the objectives of this study were to investigate the prevalence of *Aeromonas spp* associated-outbreaks through isolation and characterization of *Aeromonas spp* from infected Nile tilapia at different localities. Besides, evaluation of antimicrobial susceptibility, and characterization of virulence encoding genes leading to potential pathogenicity and MAR patterns of the obtained isolates.

2. MATERIAL AND METHODS

2.1. Sampling sites

Fish samples were collected from five fish farms exhibited mass mortalities in two governorates in Egypt, Kafr El-Sheikh (Baltim, Tolombat, and Elhamol), and Dakahlia governorate (Manzala region and Gamsa area) during January 2017 - 2018. Both sites encountered mass mortalities and apparently were exposed to different types of stressors like high stocking density in different semi-intensive earthen ponds, poor handling, and extremes in some water parameters due to using of agricultural drainage as their water supply.

2.2. Naturally infected Fish

A total number of 280 alive and freshly dead naturally infected Nile tilapia (*Oreochromis niloticus*) exhibited signs of hemorrhagic septicemia were collected from private fish farms in Kafr El-Sheikh, Manzala, and Gamsa. The freshly dead fish were kept on ice in a storage icebox, and alive ones were transported in a separate labeled plastic bag supplied with compressed air as soon as possible to Mansoura Veterinary laboratory of the Animal Health Research Institute of the Agricultural Research Center.

2.3. Clinical and P.M examination

Sampled fish were subjected to the clinical examination of the gross external signs as described elsewhere **[17]**. Autopsy and examination of the internal organs were carried out on freshly dead and moribund fish according to the method described by **Noga [3]**.

2.4. Bacteriological examination

Fish body surface was disinfected before examination by alcohol (70%) (Al-Goumhoria Co, Egypt), then fish body were aseptically opened, samples taken from the kidney, liver, and spleen of the moribund fish were incubated on Tryptic soy broth (TSB) (Oxoid), then loopful of the incubated isolates was streaked on Tryptic soy agar (TSA) (Oxoid). Single colonies were selected and re-streaked on the same type of media. Besides, selective media as *Aeromonas* base media (RYAN; Code M833) with ampicillin selective supplement 5mg/l (code SR 136) was used for selective differential isolation of *Aeromonas* species; all plates were incubated at 28°C for 24-48hr. Pure stock isolates were stored at -80°C in sterile TSA broth supplemented with 50% glycerol for further study.

2.5. Identification of bacterial isolates

Morphological and Biochemical identification bacterial isolates were carried out according to Bergey's Manual **[18].** Briefly, biochemical tests used were as follow, cytochrome-oxidase (Oxoid, USA), catalase test (Al-Goumhoria Co, Egypt) oxidation-fermentation (O-F) medium (BioMérieux Marcy-l'Étoile, France), gas production from glucose, indole test (Elgomehria.co), esculin hydrolysis test (bile esculin agar medium (Difco™, USA), Voges-Proskauer tests, acid production from arabinose, sucrose, lactose and mannose, lysine decarboxylase and arginine dihydrolase and nitrate reduction. Further identification was achieved using an analytical profile index of (API 20 E system (BioMérieux) according to manufacturer instruction.

2.5.1. Molecular identification of Aeromonas species

2.5.1.1. DNA extraction and detection of virulence genes

Genomic DNA was extracted using the DNA extraction kit (DNeasy kit, Qiagen, USA) following the manufacturer's instructions. Primers UB-F (954F) (GCACAAGCGGTGGAG CATGTGG) and UB-R (1369R) (GCCCGGGAACGTATTCACCG) **[19]** were used to amplify 500 bp from general bacterial 16S rRNA. Then all isolates were examined using a species-specifying primer to determine *A. hydrophila* according to **[20]**. Virulence gene was then verified in all isolates confirmed *A. hydrophila*; by PCR using virulence specific primers according to **Yang et al. [16]**. Each reaction mixture was performed in a total volume of 25 µL, containing 12.5 µL

dreamTaq master mix (Green PCR Master Mix (2X), (Thermo scientific), 1 µL of each primer, 2 µL of DNA template, 9.5 mL of H_2O. The PCRs thermal conditions were as follow (Table 1); the first step was denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s at the specified temperature according to each gene (55.5°C for *aerA*; 60.0°C for *hylA* and; 58.2°C for lipase) and an extension step at 72°C for 30 s. After the end of the cycles, one final extension step at 72°C for 10 min was added. The PCR products were subjected to electrophoresis on 1% agarose gels in (1xTris-borate-EDTA (TBE) buffer, stained with GelRed® nucleic acid gel stain (Biotium, USA) visualized with UV transilluminator. A 100-bp DNA ladder (Invitrogen, San Jose, CA) was used as the size standard.

2.5.2. Antimicrobial Susceptibility Testing and MAR index value

All identified *A. hydrophila* strains were tested, by the disc diffusion method **[21]** to determine their sensitivity to the following twelve antimicrobials; chloramphenicol (C, 30µg), ciprofloxacin (CIP,5µg), ampicillin (AM,10µg), neomycin(N,30µg) tetracycline (TE,30µg), clindamycin (DA,2µg), Gentamycin (CN,10µg), amoxicillin (AX,25µg), erythromycin (E,15µg), Nalidixic acid (NA,30µg), trimethoprim/sulfamethoxazole (SXT,25µg), and amikacin (AK, 30µg, Oxoid, Thermo Fisher Scientific Inc., USA).

Pure cultures of identified *A. hydrophila* strains were cultivated in TSB (Oxoid CM0129), incubated at 28°C for 18 hrs, and then streaked, by sterile cotton swabs, on to Mueller Hinton agar (Oxoid CM0337) plates. Results were recorded after incubation for 24 hrs at 28°C. Regarding the diameters of the inhibition zones, tested strains were classified as being sensitive, intermediate, or resistant **[22]**. The degree of sensitivity was determined by measuring the diameter of the inhibitory zone in mm obtained by diffusion of antibiotics, from disc to the surrounding medium.

Multiple Antibiotic Resistance (MAR) index was determined for isolates that showed resistance to more than two antibiotics **[23]**. According to the following equation: MAR index = a/b; where (a) is the number of antibiotics to which the isolate shows resistance. b) is the number of antibiotics to which the isolate was exposed.

When the use of antibiotics is seldom or of low dose use for the animal of treatment, the MAR value is usually equal to or less than 0.2. In contrast, the elevated rate of use or the high risk of exposure of antibiotics for animal treatment will yield a MAR index value, which is more than 0.2.

3. RESULTS

3.1. Clinical examination and postmortem examination of naturally diseased Nile tilapia

Clinical signs in naturally infected fish were evident including, hemorrhagic septicemia in the form of bilateral exophthalmia associated with hemorrhage in gill cover, cloudiness of both eyes, hemorrhage, and severe ulceration on the body surface, abdominal distension and mass mortalities. The postmortem examination showed congested liver with hemorrhage on its surface, distended gall bladder, and hemorrhagic spleen. Kidneys were congested and slightly enlarged (Plate 1, 2).

3.2. Bacteriological identification and biochemical characterization of A. hydrophila

Presumptive *A. hydrophila* colonies on *Aeromonas* media base appeared rounded smooth colonies 2-3 mm in diameter and dark green with a dark center. These colonies were gram-negative short rods, and positive for oxidase, esculin hydrolysis, Voges Proskauer, and gas from glucose (Table 2). Further identification of these purified colonies was carried out using API 20E kits and was confirmed as *A. hydrophila*.

3.3. Molecular identification of presumptive Aeromonas isolates and virulence genes detection

Molecular identification was carried out for six representatives selected *A. hydrophila* strains. All isolates were positively amplified at 500 bp using universal bacterial UB-F (954F) and UB-R (1369R). Then all isolates were positively amplified at 100 bp using a species-specifying primer to determine *A. hydrophila*. Virulence genes detection revealed that out of the six selected isolates, five *A. hydrophila* (83.3 %) harbored the *aerA* gene. Meanwhile, *hylA* and lipase genes' positive isolates were lower, reaching 16.7 % for both genes.

3.4. Prevalence of bacterial pathogens in different fish farms

A total of 200 diseased fish out of 280 examined fish with a prevalence of infection of 71.43%. The prevalence of bacterial infection among naturally infected Nile tilapia was 79.17, 70, and 58.33 in Kafr El-Sheikh, El- Manzala, and Gamsa fish farms, respectively. The total percentage of infection was 33.93, 25, and 12.5 in Kafr El-Sheikh, El- Manzala, and Gamsa fish farms, respectively (Table 3).

Bacteriological examination and biochemical confirmation showed that the most prevalent bacterial isolates were aeromonads 128 isolates (29.84%), among them; *A. hydrophila* was the predominant species 84 isolates (65.63%) and followed by *A. caviae* 24 isolates (18.75%) and *A. sobria* 20 isolates (15.63%) (Table 4). The prevalence of *A. hydrophila* among naturally diseased Nile tilapia was 65.75, 61.53, and 75 in Kafr El-Sheikh, El- Manzala, and Gamasa fish farm; respectively. Prevalence of *A. hydrophila* to total No. of *Aeromonas* isolates was 37.5%, 18.75, and 9.37 in Kafr El-Sheikh, El- Manzala and Gamasa fish farm; respectively (Table 5).

3.4.1. Prevalence of isolated bacterial pathogen among various organs of naturally diseased Nile tilapia

The prevalence of *A. hydrophila* infection in the liver, kidney, and spleen was 54.76, 69.69, and 75%, respectively. The prevalence of *A. caviae* in the liver, kidney, and spleen was 16.66, 22.72, and 10%, respectively. Additionally, the

Table 1. Primers used for PCR detection of virulence genes

Gene	Primer	Identification (5–3)	Reference
Aerolysin	*aer*-F *aer*-R	CCTATGGCCTGAGCGAGAAG CCAGTTCCAGTCCCACCACT	**[16]**
Hemolysin	*hly*-F *hly*-R	CACAGCCAATATGTCGGTGAAG GTCACCTTCTCGCTCAGGC	**[28]**
Lipase	lip-F lip-R	ATCTTCTCCGACTGGTTCGG CCGTGCCAGGACTGGGTCTT	**[16]**

Table 2. Biochemical characteristics of obtained Aeromonas isolates from naturally infected Nile tilapia

Characteristic	Results
Gram stain	-
Morphology and motility	Motile, short bacilli
Growth on TSA media	Creamy, round, raised , entire colony
Growth on *Aeromonas* base media	Dark greenish colony with dark center
Oxidase & catalase	+
Aero-Key	
Acid from mannitol	+
Aesculin hydrolysis	+
Gas from Glucose	+
Acid from Arabinose	+

Table 3. Prevalence of bacterial infection from examined naturally infected *Nile* tilapia in different sampling sites.

Sampling site	No. of fish sampled	No. of infected fish	Prevalence %	Total percentage %
Kafr El-Sheikh farms	120	95	79.17	33.93
El-Manzala farms	100	70	70	25
Gamasa farms	60	35	58.33	12.5
Total	280	200	71.43	71.43

Table 4. The prevalence of *Aeromonas* infection.

Examined fish	Bacterial isolates	*Aeromonas* isolates	*Aeromonas* infection	*A. hydrophila*		*A. caviae*		*A. sobria*	
No.	**No.**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
280	429	128	29.84	84	65.63	24	18.75	20	15.63

Table 5. Prevalence of *Aeromonas* infection among the examined Nile tilapia in different farms.

Sampling sites	No. of fish	Bacterial isolates	*Aeromonas* isolates	*Aeromonas* infection / area	*Aeromonas* infection	*A. hydrophila* isolates	*A. hydrophila* in area	*A. hydrophila*
	No.	**No.**	**No.**	**%[a]**	**%[b]**	**No.**	**%[a]**	**%[c]**
Kafr El-Sheikh	120	210	73	34.76	17.01	48	65.75	37.5
El-manzala	100	150	39	26	9.09	24	61.53	18.75
Gamasa	60	69	16	23.18	3.72	12	75	9.37
Total	280	429	128	29.83	29.83	84	65.62	65.62

[a]prevalence of Aeromonas in relation to the No. of bacterial isolates in area.
[b]prevalence of *Aeromonas* infection in relation to the total number of isolated bacteria (429).
[c]prevalence of *A. hydrophila* infection in relation to the total number of aeromonads bacteria (128).

Table 6. Intensity of bacterial isolates in examined tissues and organs in naturally infected Nile tilapia.

Organ	Total No. of sample	Total No. of bacterial isolates		*A. hydrophila*		*A. caviae*		*A. Sobria*	
	No	No	%[a]	No	%[a]	No	%[a]	No	%[a]
Liver	95	42	44.21	23	24.21	7	7.37	12	12.63
Kidney	110	66	60	46	41.83	15	13.63	5	4.54
Spleen	75	20	26.67	15	20	2	2.67	3	4
Total	280	128	45.71	84	30	24	8.57	20	7.14

[a]The percentage in relation to the No. of sample.

Table 7. Seasonal prevalence of *Aeromonas* spp in naturally infected Nile tilapia.

Season	No. of fish	Total isolates		*A. hydrophila*		*A. caviae*		*A. sobria*	
	No	No	%[a]	No	%[a]	No	%[a]	No	%[a]
Winter	24	5	20.84	4	16.67	1	4.17	0	0
Spring	52	28	53.85	18	34.62	6	11.54	4	7.69
Summer	110	73	66.36	49	44.55	14	12.72	10	9.09
Autumn	94	22	23.40	13	13.83	3	3.19	6	6.38
Total	280	128	45.71	84	30	24	8.57	20	7.14

[a]The percentage in relation to the No. of fish in a season

Table 8. Antibiotic resistant of identified *A. hydrophila* isolates.

Antibiotic	Concentration	Resistant (%)	Intermediate (%)	Sensitive (%)
Chloramphenicol	30µg	0	16.66 (1)	83.33 (5)
Ciprofloxacin	5 µg	0	0	100 (6)
Ampicillin	10µg	100 (6)	0	0
Neomycin	30µg	50 (3)	50 (3)	0
Tetracycline	30µg	0	33.33 (2)	66.66 (4)
Clindamycin	2µg	83.33 (5)	16.66 (1)	0
Gentamycin	10µg	66.66 (4)	33.33(2)	0
Amoxicillin	25µg	100 (6)	0	0
Erythromycin	15µg	83.33 (5)	16.66 (1)	0
Nalidixic acid	30µg	50 (3)	50 (3)	0
Trimethoprim/sulfamethoxazole	25µg	0	0	100
amikacin	30µg	0	33.33 (2)	66.66 (4)

Table 9. Antimicrobial resistance phenotypes and MAR index value of *Aeromonas* spp. isolated from infected Nile tilapia.

A.hydrophila strain	*Resistant antibiotic	MAR
Ah1	AM, AX, DA,CN, E	0.42
Ah2	AM , AX, DA, CN, E	0.42
Ah3	AM , AX	0.16
Ah4	AM , AX, DA, E	0.33
Ah5	AM , AX, DA, CN, E	0.42
Ah6	AM, AX, DA, CN, E	0.42

**A. hydrophila* strain (Ah) ampicillin (AM , 10µg), clindamycin (DA 2 µg), amoxicillin (AX25 µg), erythromycin (E15 µg), Gentamycin (CN10 µg).

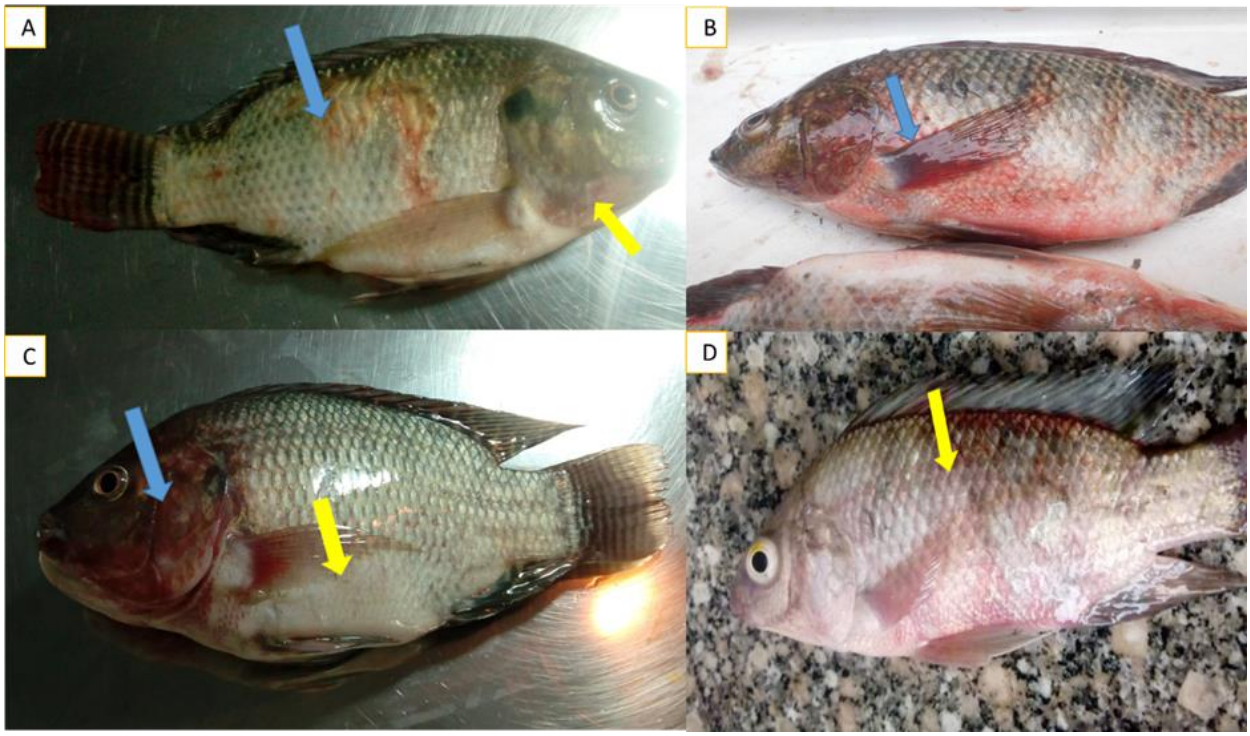

Plate.1. Naturally infected Nile tilapia showing (A) Sever ulceration of skin (blue arrow) and hemorrhage of operculum (yellow arrow), (B) Sever petechial hemorrhage all over the body surface and pectoral fin (arrow), (C) Abdominal distention (yellow arrow), associated with hemorrhage in gill cover (blue arrow), (D) Sever hemorrhage and ulceration of skin (yellow arrow).

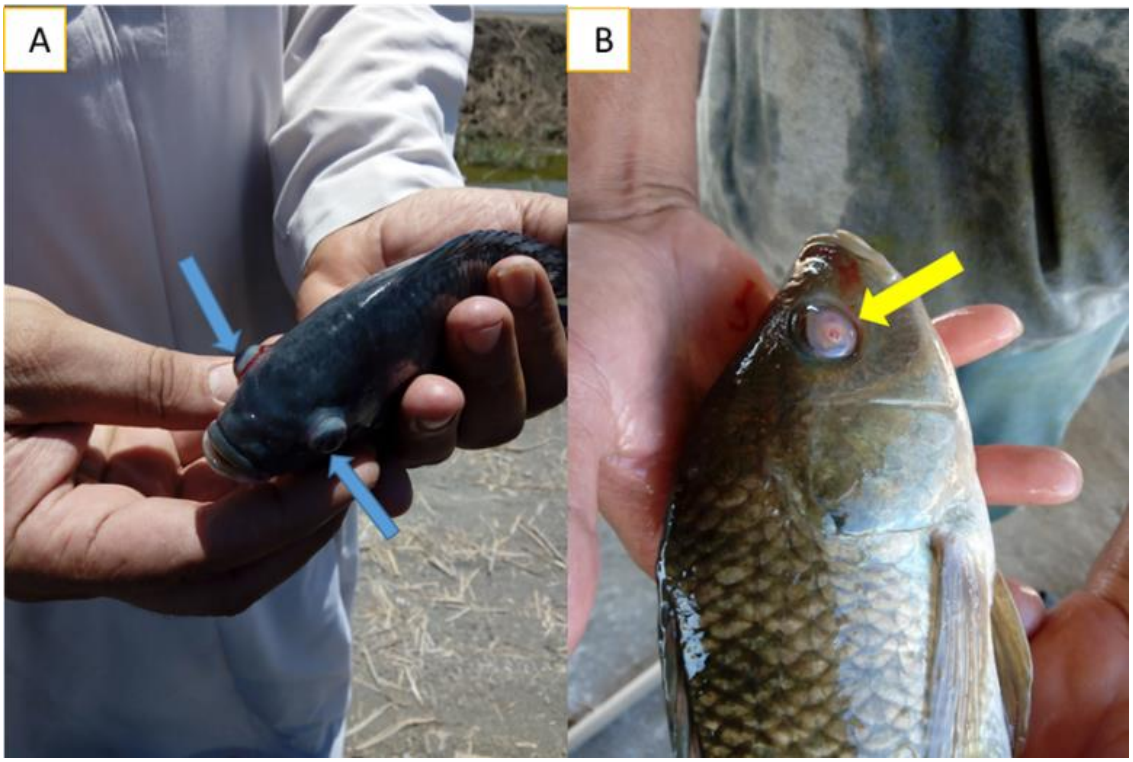

Plate.2. Naturally infected Nile tilapia showing (A) Bilateral exophthalmia (arrow). (B) Eye cloudiness (arrow).

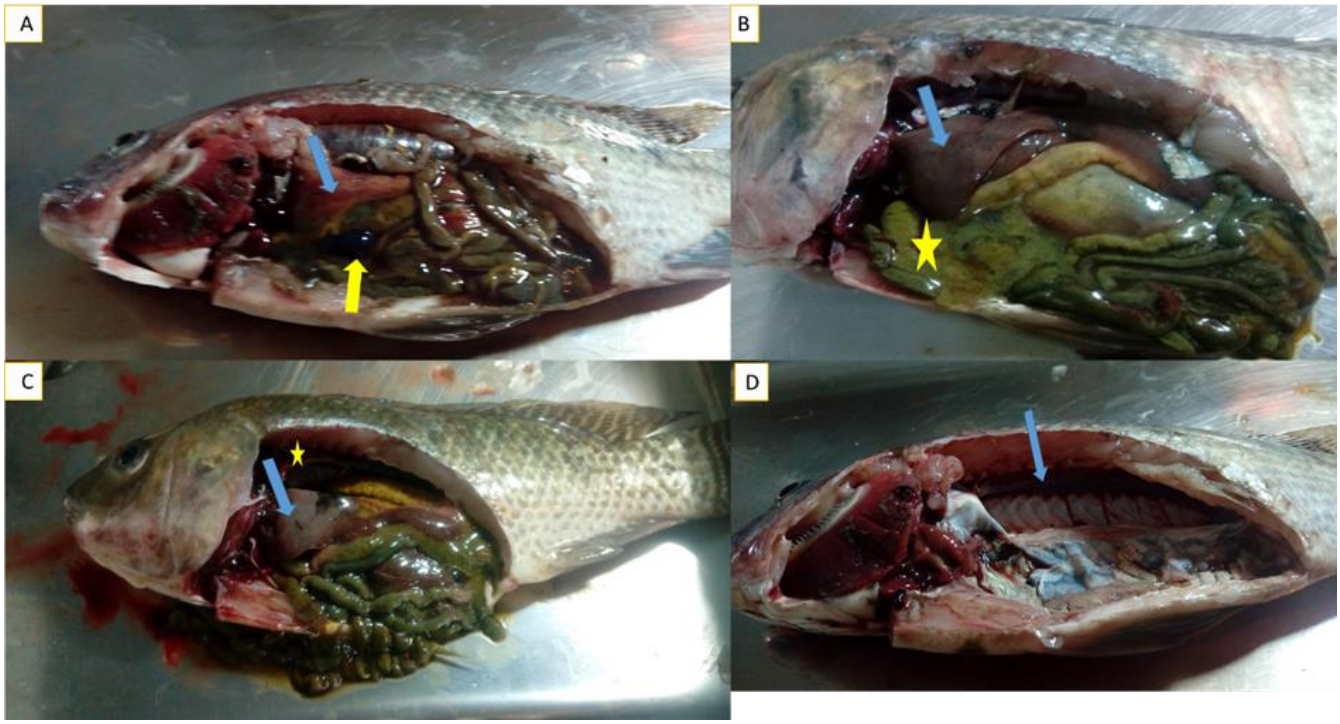

Plate.3. Naturally infected Nile tilapia showing (A) Congested and hemorrhagic liver (blue arrow) with congested spleen (yellow arrow), (B) Congested liver (arrow) with bladder was distended with bile and ascetic fluid in abdominal cavity (star), (C) Congested kidney (star) and congested liver (arrow), (D) Congested and hemorrhagic kidney.

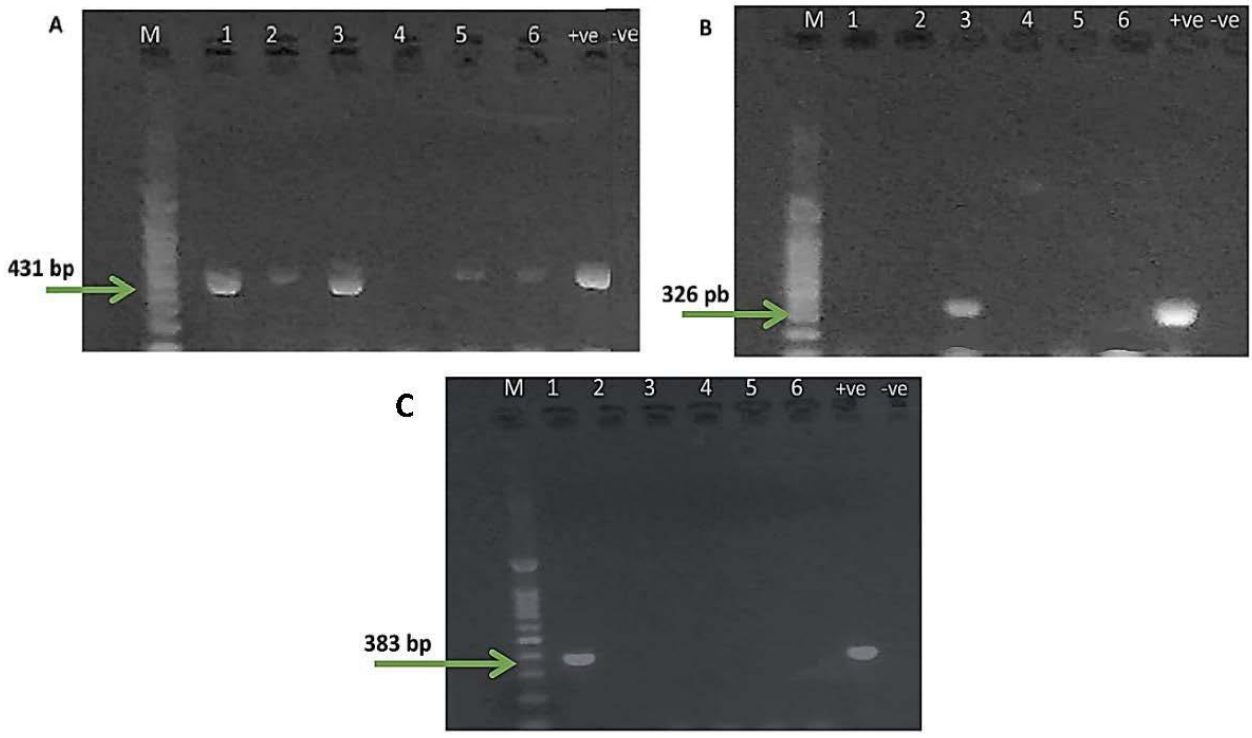

Plate.4. Agarose gel electrophoresis of amplicons of positive *Aeromonas* isolates for (A) aerolysin gene 431bp, Lane M DNA ladder 100 bp, Lane 1,2,3,5,6 positive *Aeromonas* isolates. (B) hemolysin gene 326bp, lane M DNA ladder 100 bp, lane 1 positive *Aeromonas* isolates. (C) lipase gene 382 bp, lane M DNA ladder 100 bp, lane positive 3 *Aeromonas* isolates. Lane7 in A, B, and C is the control positive *A. hydrophila* strain.

prevalence of *A. sobria* in the liver, kidney, and spleen was 28.57, 7.57, and 15%, respectively (Table 6).

3.4.2. Seasonal prevalence of isolated bacterial species in naturally diseased Nile tilapia

The prevalence of bacterial infection was recorded during winter, spring, summer, and autumn seasons as 3.91, 21.87, 57.03, and 17.18%, respectively. The prevalence of *A. hydrophila* in winter, spring, summer, and autumn was 80, 64.28, 67.12, and 59.09%, respectively. The prevalence of *A. caviae* in winter, spring, summer, and autumn was 20, 21.43, 19.18, and 13.63%, respectively. The prevalence of *A. sobria* in spring, summer, and autumn was 14.28, 13.69, and 27.27, respectively (Table 7).

3.5. Antimicrobial susceptibility and MAR index value of identified A. hydrophila strains

An antibiogram sensitivity test was performed on the six *A. hydrophila* strains and revealed that *A. hydrophila* was highly sensitive to ciprofloxacin, trimethoprim, chloramphenicol, amikacin, and tetracyclin. Intermediate resistance was exhibited against neomycin and nalidixic acid. However, a higher resistance pattern varied among the other tested drugs; the highest resistance (100%) was recorded against ampicillin, amoxicillin followed by clindamycin and erythromycin (83.33%); and gentamycin (66.66 %) (Table 8). The MAR index values revealed that the MAR index of *A. hydrophila* was ranged from 0.16-0.42 (Table 9). The identified isolates of *A. hydrophila* showed multiple resistant patterns, where four strains were commonly resistant to five antibiotics with 66.6% multi-resistance patterns, while one strain was resistant to two antibiotics with 16.6% multiple resistance patterns. Also, one strain showed resistance to four antibiotics, with 16.6% multiple resistance patterns.

4. DISCUSSION

Aquaculture is considered a good source of animal protein suitable for human consumption in developing coast countries **[24]**. In Egypt, Nile tilapia is one of the most cultured freshwater fish and considered an important species in commercial fisheries **[5]**. Naturally infected Nile tilapia are usually prone to one or more stress factors, including rough handling, overcrowding, malnutrition, and high free ammonia (NH_3) **[25, 26]**. Our results revealed that Nile tilapia, exposed to such stressors, were susceptible to disease outbreaks, mainly bacterial ones. Prevalent bacterial pathogens contributed to mass mortalities in different fish farms in Kafr El-Sheikh and Dakahlia governorate were surveyed during the period of January 2017 – 2018.

Clinical and postmortem findings of the surveyed naturally infected Nile tilapia showed hemorrhages on the external surface, the base of pectoral fin, ulcer on the skin, abdominal distention, unilateral or bilateral exophthalmia, prolapsed anus, and fin rot. Postmortem examination revealed the accumulation of yellowish watery fluid in the abdominal cavity, pale anemic, and friable liver with some hemorrhagic patches with the distended gall bladder. The kidney and spleen were congested and slightly enlarged. The observed clinical and postmortem findings were similar to those described elsewhere **[5, 27, 28]**.

All-over external and P.M findings can be attributed to bacterial invasion, multiplication, colonization, and toxins produced by invading microorganisms **[29]**. Additionally, The action of toxic extracellular metabolites of *A. hydrophila,* including *hylA*, *aerA*, and cytotoxic toxins, which possess hemolytic, cytolytic, and enterotoxic activities altogether induce liver necrosis, renal tubules degeneration, and render the tissue hemorrhagic, with exudates of serum and fibrin **[29]**.

In the present study, isolates obtained were gram-negative, rod-shapedd, motile bacilli. Further, biochemical identification revealed that isolates were positive for catalase, indole test, Voges Proskauer, oxidase test, citrate utilization, carbohydrate utilization, and triple sugar iron agar medium; therefore, these isolates were presumptively identified as *A. hydrophila*. These results indicate the diversity of motile aeromonads in the freshwater aquatic environment of this region, *A. hydrophila* is the predominant species that represent a potential threat to the fish population under stress condition. Most of the phenotypic characteristics of the obtained isolates were similar to those reported in Bergey's manual of determinative bacteriology **[18]**, and Our findings were in-line with those reported elsewhere **[30, 31]**.

Furthermore, identification using API20E confirmed presumptive *A. hydrophila* isolates, and these isolates were amplified at 500bp using 16S rDNA and species-specific *A. hydrophila* primer. Polymerase chain reaction (PCR) used in the diagnosis of bacterial fish diseases offered a very rapid and accurate method **[32]**, and 16S rDNA is considered a useful marker for species identification and the determination of the phylogenetic relationships of *Aeromonas* spp **[33, 34]**. Such consistent identification to the species level of *Aeromonas* isolates is necessary to establish outbreak management, source tracing, and threat analyses. Consequently, there is an essential need for intensive epidemiological studies **[35]**.

PCR-based assays have been used in characterizing the virulence genes such as *aerA* and *hlyA* of particular strains to determine their virulence **[36]**. Aerolysin gene is a strong indication of virulence in pathogenic isolates of *A. hydrophila* **[37]**. Lipase is the most frequent and important virulence gene which has the potential to change the histochemical identity of the cell membrane of the infected cells so allowing *A. hydrophila* colonization and inducing cell necrosis **[32]**. Lipase can alter the cytoplasmic membrane structure of the host cells and facilitate pathogenicity **[38]**.

The presence of virulence genes positive *A. hydrophila* strains represents a major public health risk, as virulence factors related to extracellular products play a critical role in the translocation in the epithelium **[39]**. Some studies have reported the correlation between the higher numbers of virulence genes and their potential for determining diseases, *Aeromonas* had *aerA*, cytotonic heat-labile enterotoxin (*alt*) and a cytotonic heat-stablee enterotoxin (*ast*) that are considered important virulence factors **[40]**. Besides, exotoxins are major virulence factors of aeromonads that include aerolysin/hemolysin; (*alt*), lipase or phospholipase, and (*ast*) **[41]**. The previous study revealed that *Aeromonas* strains isolated from diseased, healthy fish, and water samples had three or more virulence genes in different combinations. Most *A. hydrophila* strains from infected fish were classified as a virulent, even though some strains lack one or two virulence genes. These indicate the importance of

performing biological assays for assessing the virulence of the strains and determine the potential pathogenicity of *A. hydrophila* owing to its possible public health risk **[35]**.

In the present study, *aerA*, *hylA,* and lipase virulence genes were detected and thus, related to the pathogenicity of *Aeromonas spp*. Out of the six selected isolates, five *A. hydrophila* (83.3 %) harbored the *aerA* gene where lipase and *hylA* gene-positive isolates were lower, reaching 16.7%. Our finding is consistent with **Attia et al. [42]**, who found that a high number of *A. hydrophila* were positive for the *aerA* gene (81.8%) and **Abd-El-Malek [43]** who stated that the percent of lipase in *Aeromonas* isolates was 17.14%. Contrastingly, a previous study reported that *A. hydrophila* possesses a high percentage of the *hylA* gene **(**100%) **[44]**. Similarly, *A. hydrophila* was reported for the presence of *hlyA* genes (50%) in fish farms in East Delta **[45]**. The *aerA* recorded (100%) in *A. hydrophila* that isolated from fish farms in East Delta **[45]**. Additionally, The lipase activity recorded (100%) of the *A. hydrophila* isolates **[46]**. Lipase gene was highly detected (81.8%) of *A. hydrophila* strains recovered from diseased Nile tilapia **[47]**.

As observed, the prevalence of *A. hydrophila* isolates was (65.63%), *A. caviae* (18.75%) and *A. sobria (*15.63%). Our findings might be attributed to the ubiquitous and opportunistic nature of the microorganism in the aquatic environment, and its presence as normal flora in the fish intestine **[48]**. Similarly, Motile *Aeromonas* (*A. sobria*, *A. vernoii*, *A. jandae*, *A. hydrophila*, and *A. cavae*) were the most prevalent bacterial pathogens affecting the Nile tilapia and common carp **[49]**. Similar findings have been reported by **Wamala et al. [50]** who identify the *A. hydrophila* (43.8%), *A. sobria* (20.8%), and *Edwardsiella tarda* (8.3%) as the most prevalent bacterial pathogens in Nile tilapia in Uganda. A similar previous study revealed that summer mortalities were due to *A. hydrophila,* with a prevalence of 78% **[51]**. On the contrary, **Dahdouh et al. [28]** found that the prevalence of *A. hydrophila* was 47% in 170 fishes (100 freshwater, 40 brackish water, and 30 marine water fishes) from different farms in Alexandria, Kafr Elsheikh, and El-Behera governorates. Similar results were seen where *A. hydrophila* and *A. veronii* were the most prevalent species found in samples from fish and water **[35]**.

However, previous studies reported a comparable prevalence to our study, and this might be attributed to species, sampling time, geographical range, sensitivity, and specificity of the techniques used to identify the bacteria **[52]**. For example, **Ebeed et al. [53]** reported that the prevalence of *Aeromonas* spp. in Nile tilapia was 34 (68%) and the most frequently identified *Aeromonas* species were *A. caviae* 18 (36%), *A. sobria* 14 (28%) and *A. hydrophila* 7 (14%). Similarly, the prevalence of *A. hydrophila* strains isolated from cultured Nile tilapia was 25% **[5]**. Same to **El-Gamal et al. [54]** who revealed that the isolated bacteria; were *Aeromonas* sp. (25.9%) as *A. hydrophila* (23.3%) and *A. caviae* (2.6%). Additionally, *A. caviae* was predominant spp in tilapia **[55]**. *A. sobria* was the most prevalent strain of aeromonad in relation to other strains in tilapia and common carp 46.8% and 76.4%, respectively **[49]**.

Regarding the isolation of *Aeromonas* from the internal organs, it was noticed that the highest prevalence of *Aeromonas* isolation was reported from the liver, kidney, and spleen with 32.81, 51.56, and 15.62%; respectively. Similar to our findings, a study revealed that the liver and kidneys were target organs of acute septicemia. These organs were apparently attacked by bacterial toxins and lose their structural integrity **[56, 57]**. The highest number of isolates were isolated from the liver, followed by kidney, spleen, while the lowest number of isolates were isolated from the skin (Nile tilapia, catfish, and mullets) infected by (*Aeromonas hydrophila*, *Pseudomonas fluorescens*, *Streptococcus faecalis*, *Citrobacter spp.* and *Edwardsiella tarda* **[58]**, suggesting that most-bacterial infections affect mainly hemopoietic systems such as liver, kidney, and spleen.

Concerning seasonal prevalence, our results revealed that the highest prevalence of *Aeromonas* was recorded in summer 57.03%, followed by spring season 21.87%, then autumn 17.18%, while the lowest recorded in winter 3.91%. This might be due to the higher water temperature in summer accompaniedby low dissolved oxygen that stresses the fish and renders their immune response weak and more susceptible to bacterial infections. Several researchers investigated that MAS outbreaks were highly encountered during the summer season **[45, 58, 59]**, these observations were in-line with our findings.

With the steady expansion of the fishery industry, the vast use of antibiotics is very common. To increase production, farmers always used different antibiotics to prevent and treat pathogenic bacterial infections in fish **[60]**. The continuous and extensive use of antibiotics in humans also led to the emergence of antimicrobial-resistant strains worldwide **[14]**.

In our study**,** the susceptible, intermediate, and resistance rates of the examined *A. hydrophila* isolates concerning 12 antibiotics revealed that *A. hydrophila* was highly sensitive to ciprofloxacin, amikacin, and chloramphenicol. Meanwhile, isolates were intermediate to tetracycline, trimethoprim, gentamicin, neomycin, and oxytetracycline. Interestingly, isolates were resistant to ampicillin, amoxicillin, erythromycin, and clindamycin. These results suggest that *A. hydrophila* isolates have been originated from a high-risk source of contamination. Thus, it may be important to evaluate variations in antimicrobial resistance profiles of *A. hydrophila* strains.

Previous studies concerned about antimicrobial resistance of different bacterial isolates reports that genus *Aeromonas* is an indicator bacterium for antimicrobial resistance in the aquatic ecosystem **[61, 62]**. Our findings were in-line with other previous works concerning antimicrobial resistance of *A. hydrophila* **[63, 64]**. The resistance observed against ampicillin and other related drugs could be explained by the role of various β-lactamases produced by *aeromonads* that confer resistance to a broad

spectrum of β-lactam antibiotics**.** The microbial resistance in aeromonads is chromosomally mediated; however, β-lactamases may be coded by plasmids or integrons **[65]**. *Aeromonas* spp. produce different β-lactamases, which confer resistance to a broad spectrum of β-lactam antibiotics **[14]**.

Our findings are consistent with Ahmed et al. [66], who reported that the *A. hydrophila* isolates showed high resistance rates to both nalidixic acid and tetracycline (76% and 72%, respectively). Similarly, *A. hydrophila* isolates from fresh, brackish, and marine fishes were resistant to ampicillin, erythromycin, nalidixic acid, and spectinomycin but, they showed high sensitivity to enrofloxacin, ofloxacin, and gentamicin **[28]**. Also, *Aeromonas* strain was resistant to amoxicillin and nalidixic acid **[67]**.

Similar to our results, the sensitivity of *Aeromonas* isolates to ciprofloxacin was 100% reported by other studies **[68]**. However, Other investigations **[66, 69, 70]** reported a lower sensitivity (43%, 6.3%, and 48%) against ciprofloxacin.

Multiple resistance patterns have been reported in fish **[71]**. Due to the misuse of antimicrobials, *Aeromonas spp*. can obtain resistance to antibiotics, resulting in multidrug antimicrobial-resistant (MAR) bacteria **[72]**. Our result revealed that the MAR index values of the six identified *A. hydrophila* strains were ranged from 0.16-0.42, which is consistent with **Laith and Najiah [63],** who reported that the MAR index of *A. hydrophila* isolates was ranged from 0.10 - 0.50. The same was seen in the study of **Jacobs and Chenia [73],** where the MAR index ranged from 0.20-0.30. Previously, the aeromonads isolated from ornamental fish culture systems displayed high MAR index values (0.24 - 0.46) **[74]**. MAR index greater than 0.2 is considered to be originated from high-risk sources of bacterial contamination and where the antibiotics are frequently used **[75]**. Besides, a very high MAR index, suggesting that the origin of the isolates is from an area highly contaminated with antibiotics; consequently, this may impose a much higher risk of spreading MAR to the aquatic environment, which will affect aquaculture production **[76]**.

Our findings could be explained in the view that the resistant bacteria transferred from humans and livestock to fish **[77]**, the use of animal wastes to fertilize fish ponds **[78]** and the naturally occurring resistant bacteria in the aquatic environment and soils **[79], altogether** could contribute directly to this effect bypassing over antibiotic resistance genes to fish bacteria. There is a high risk of infection and the spread of bacteria in high densities environment like the case in intensive aquaculture **[80]**.

Conclusion

Aeromonas spp. are serious fish pathogens, frequently isolated from Nile tilapia during an outbreak of disease mass mortalities from different fish farms at the different sampling sites. Our findings demonstrated that *A. hydrophila* is one of the main causes of summer mortalities outbreaks in Nile tilapia farms in Egypt. Determination of the prevalence and virulence genes associated with the disease outbreaks is critical data for actions that need to be taken for fish disease management. With the reported antibiotic resistance pattern herein, alternative approaches should be knocked to avoid hazards due to this phenomenon. Further, these data could represent a baseline for future references.

Acknowledgment

This research was supported by Laboratory of Fish Diseases, Department of Internal Medicine, Infections and Fish Disease, Faculty of Veterinary Medicine; and Animal Health Research Institute

Conflict of interest

Authors declare that they have no conflict of interest

Research Ethics committee permission

All experimental procedures were in compliance with the Animal Care and Use Guidelines at Mansoura University.

Authors´ contributions

M.S. performed the experiment and drafted the MS; E.Z. reviewed and edited MS; E.Z. R.S. and V. Z. supervised the whole research work and revised the MS.

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