Research Article

Prevalence and Virulence Genes of *Vibrio* and *Aeromonas* Species Isolated from Nile Tilapia and Mugil Fish Farms in Egypt

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Abstract | The present study was designed to determine the prevalence of *Vibrio* and *Aeromonas* in Nile tilapia and Mugil fish farms in Egypt and monitoring some virulence genes associated with *V. parahaemolyticus* and *A. hydrophila* isolates. One hundred diseased fishes (50 for each Nile tilapia and Mullet fish) were collected over 10 months from January to October 2019 and submitted to bacteriological and biochemical examination. Randomly selected 5 isolates of *Vibrio* species were submitted for molecular identification using species-specific PCR (*tox*R gene for *V. parahaemolyticus* and *collagenase* for *V. alginolyticus*) and the molecular detection of virulence genes including *rec*A and *trh* in *V. parahaemolyticus* isolates. Other 5 *A. hydrophila* isolates were examined for the molecular detection of virulence genes including *fla*, *aer*A, *hly*A, and *ahcytoen* genes. *Vibrio* species isolated with a prevalence of 65.0% for 100 examined fishes. *Vibrio parahaemolyticus, V. alginolyticus,* and *V. cholerae* were isolated with a prevalence of 55.4, 33.8, and 10.8%, respectively. *Aeromonas* species were isolated with a prevalence of 72.0% for 100 examined fishes where *A. hydrophila* and *A. caviae* were isolated with a prevalence of 99.3 and 9.7%, respectively. PCR results showed *tox*R gene was detected in all five isolated *Vibrio* species, *rec*A virulence gene was detected in three out of the five tested *V. parahaemolyticus* isolates. Meanwhile, the *trh* gene was not detected. On the other hand, *aer*A and *hly*A virulence genes were detected in all five isolated *A. hydrophila*. *Ahcytoen* was detected in 4 out of 5. Meanwhile, the *fla* gene was detected in 1 out of the tested 5 *A. hydrophila* isolates. The high prevalence of *Vibrio* and *Aeromonas* species in this study with an elevated coexistence of their virulence genes threatens the aquaculture industry in Egypt and poses a public health concern.

Keywords | *Aeromonas*, *Vibrio*, Nile tilapia, *Mugil cephalus*, Virulence genes

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INTRODUCTION

Aquaculture industry has expanded rapidly worldwide, which offers better economic income, job opportunities, and high-quality food products (Manage, 2018). Egypt is producing about 73.8% of total cultured fish in Africa and occupies the eighth level all over the world as it produces about 919585 tons of cultured fish that represents 1.54% of total cultured fish all over the world (FAO, 2012). Bacterial diseases seriously affect the fish industry, which may be attributed to the high death rate not only of farmed fish but also wild fish (Khalil and Abd El-Latif, 2013). Several *Vibrio* species are well recognized for their severity to cause fish disease, besides, causing mortality in reared fish is very common during early larval stages and can occur suddenly, leading sometimes to the death of the population (Thompson et al., 2004). The most studied *Vibrio* spp. known to be pathogenic to humans are *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Vibrio vulnificus* (You et al., 2016). Commonly, not all strains of *V.*

parahaemolyticus are considered pathogenic (Dileep et al., 2003), the pathogenic ones that responsible for the onset of disease symptoms and outbreaks are characterized by the production of thermostable toxins and/or TDH-related hemolysin encoded by *tdh* and *trh* genes; respectively (World Health Organization and Food Agriculture Organization, 2011).

Aeromonas is the most prominent pathogen that can infect fish; many *Aeromonas* species are responsible for several fish diseases including *A. hydrophila, A. caviae*, and *A. veronii* biotype *sobria*. *Aeromonas hydrophila* infections are characterized by septicemia, ulcerative, hemorrhagic diseases, and significant mortalities in both wild and farmed freshwater and marine fish species. They don't only cause mortality and cytotoxicity but also create uselessness of live fish as they are responsible for spots, lesions, and scale loss on the infected ones (Saikot et al., 2013). The pathogenicity of aeromonads has been linked to exotoxins that were grouped as aerolysin-hemolysins, cytolytic enterotoxins, or cytotonic enterotoxins (Kingombe et al., 1999). *Vibrios* and *Aeromonas* cause a great economic problem for aquaculture and human consumers in Egypt. Therefore, our study aimed for studying the prevalence of these bacteria in fresh Nile tilapia and Mugil cephalus isolated from the different fish farms at Kafr Elsheikh governorate and the molecular detection of some virulence genes.

MATERIALS AND METHODS

Ethical approval

All samples were taken according to the standard sample collection procedure without putting any stress on the fish sample. The current study was approved by the Ethical Committee for Medical Research at the faculty of veterinary sciences, Benha University and Animal Care Guidelines of the General Organization for Veterinary Services, Egypt.

Collection of samples

One hundred (n=100) clinically diseased fish samples,50 Nile tilapia (*Oreochromis niloticus*) and 50 mullet fish (*M. cephalus*), complaining of high mortality rate with signs of septicemia (unilateral or bilateral exophthalmia, skin ulcers, and hemorrhages) were collected from different fish farms at Kafr Elsheikh Governorate, Egypt at the period between January (2019) and October (2019). Each examined sample was taken alone in a strong sterile plastic bag with ½ of its volume water pumped with pressured oxygen, labeled and transferred alive with a minimum delay to the laboratory (Microbiology unit of Animal Health Research, Tanta branch, Egypt) for clinical and bacteriological examination. Three hundred and five samples were collected under strict aseptic conditions from apparently pathognomonic lesions in liver, kidney, spleen, heart, intestine, and gills from the 100 diseased fishes; 157 lesion samples from 50 Nile tilapia (*O. niloticus*) and 148 samples from 50 mullet fish (*M. cephalus*) and submitted to bacteriological and biochemical examination for isolation of *Vibrio* and *Aeromonas* species.

Bacteriological examination

Isolation and identification of *Vibrio* and *Aeromonas* species using the conventional cultural method

According to (Quinn et al., 2002) and (Markey et al., 2013) sterilized loops were introduced through seared lesions using a hot spatula. Then taken from lesions and inoculated into 1% peptone broth (Oxoid, UK) + 3%NaCl for *Vibrio* isolates and 1% peptone broth for *Aeromonas* isolates then incubated aerobically at 37°C for 18-24 hours. Loops from incubated cultured broth were streaked onto selective diagnostic agar media: cholera medium (TCBS, Oxoid) for *Vibrio* spp. and *Aeromonas* selective agar (BSIBG agar, HIMEDIA, M1890-55 G) for *Aeromonas* spp. and incubated for 24 hours at 37°C. After that one separated typical colony from agar medium was picked up and transferred into the nutrient broth (Oxoid, UK) and was aerobically incubated at 37 °C for 18-24 hrs, then 15% glycerol was added, gently mixed, and immediately preserved in a refrigerator at -85 °C. The smears from the suspected pure colonies were stained with Gram's stain and microscopically examined under the oil immersion lens. *Vibrio* spp. (Gram-negative, curved bacilli, comma shape) and *Aeromonas* spp. (Gram-negative, straight rods with a round end, non-capsulated and non-sporulated) were selected for further biochemical identification steps later. The biochemical identification of *Vibrio* and *Aeromonas* isolates was performed according to (Quinn et al., 2002; Nicky, 2004; Markey et al., 2013) by using catalase, oxidase, indole production, citrate utilization, urease test, triple sugar iron, and methyl red tests. *Vibrio* species were positive for oxidase; catalase, indole production, methyl red, citrate utilization except *V. alginolyticus* were citrate utilization negative, and ferment the glucose without gas, negative results for urease test and H2S production except *V. alginolyticus* produced H2S. *Aeromonas hydrophila* isolates were positive for oxidase, catalase, indole, H2S production, and ferment glucose with gas production. While negative for citrate utilization and methyl red tests. *Aeromonas caviae* are similar to *A. hydrophila* except for methyl red +ve, H2S –ve, and not ferment glucose.

Molecular identification of virulence genes in *Vibrio* and *Aeromonas* isolates

PCR was used for the confirmation of *Vibrio* species (5 random isolates) using species-specific genes (*tox*R gene for *V. parahaemolyticus* and *collagenase* gene for *V. alginolyticus*), as well as the detection of virulence genes

of *V. parahaemolyticus* and *A. hydrophila* species by primers targeting different virulence genes including *rec*A and *trh* genes for *V. parahaemolyticus* as well as *aer*A, *hly*A, *ahcytoen* and *fla* genes for *A. hydrophila* (Table 1).

Extraction of DNA from *Vibrio* and *Aeromonas* isolates

It was performed by QIAamp® DNA Mini Kit (Catalogue no. 51304) according to the manufacturer's instructions.

Amplification and cycling protocol of PCR for *Vibrio* and *Aeromonas* isolates

The cycling condition for each gene was prepared according to Emerald Amp GT PCR master mix (Takara, Cat PR310A) kit (Table 1). The amplification was performed on Eppendorf MasterCycler® (Eppendorf AG, Hamburg, Germany) in a total reaction volume of 25 µl containing 12.5 µl EmeraldAmp GT PCR Master Mix, 1 µl of each forward and reverse primers, 4.5 µl molecular biology grade water, and 6 µl test DNA.

Detection of PCR products

The PCR amplicons were analyzed by electrophoresis using a 1.5 % agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH = 8.3). A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

RESULTS AND DISCUSSION

The prevalence of *Vibrio* and *Aeromonas* species in diseased fishes

Out of 100 diseased fish samples (50 from each Nile Tilapia and Mullet fish), 65 isolates of *Vibrio* species were isolated and biochemically identified. A total of 36 *V. parahaemolyticus* strains (55.4%) were isolated and identified, 20 (30.76%) from *O. niloticus*, and 16 (24.6%) from *M. cephalus*. Meanwhile, 22 *V. alginolyticus* strains (33.8%) were isolated, 13 (20.0%) from *O. niloticus*, and 9 (13.8%) from *M. cephalus* fish. Besides 7 *V. cholera* strains (10.8%) were isolated, 5 (7.7%) from *O. niloticus*, and 2 (3.1%) from *M. cephalus* (Table 2).

Table 1: Oligo-nucleotide primers and cycling conditions of the primers used in this study.

Bacterial spp.	Target genes	Oligonucleotide sequence (5′ → 3′)	Product size (base pairs)	Primary Denaturation	Amplification (35 cycles)			Final extension	References
					Secondary denaturation	Annealing	Extension		
Vibrio alginolyticus	*collagenase*	F CGAGTACAGTCACTTGAAAGCC R CACAACAGAACTCGCGTTACC	737	94°C 5 min.	94°C 1 min.	50°C 1 min.	72°C 1 min.	72°C 10 min.	Abu-Elala et al., 2016
Vibrio parahaemolyticus	*Tox*R	F GTCTTCTGACGCAATCGTTG R ATACGAGTGGTTGCTGTCATG	368	96°C 5 min.	94°C 1 min.	63°C 1.5min.	72°C 1.5min.	72°C 10 min.	Kim et al., 1999
	*rec*A	F TGARAARCARTTYGGTAAAGG R TCRCCNTTRTAGCTRTACC	793	95°C 5 min.	95°C 35 sec.	55°C 1 min.	72°C 1 min.	72°C 7min.	Casandra et al., 2013
	trh	F ACCTTTTCCTTCTCCWGGKTCSG R RCCGCTCTCATATGCYTCGACAKT	484	94°C 5 min.	95°C 1 min.	62°C 1 min.	72°C 1 min.	72°C 2 min.	
Aeromonas hydrophila	*fla*	F TCCAACCGTYTGACCTC R GMYTGGTTGCGRATGGT	608	94°C 5 min	94°C 30 sec.	55°C 40 sec.	72°C 45 sec.	72°C 10 min.	Nawaz et al., 2010
	*aer*A	F CACAGCCAATATGTCGGTGAAG R GTCACCTTCTCGCTCAGGC	326	94°C 3 min.	94°C 30 sec.	52°C 30 sec.	72°C 30 sec.	72°C 10 min.	Singh et al., 2008
	*hly*A	F GGCCGGTGGCCCGAAGATACGGG R GGCGGCGCCGGACGAGACGGGG	592	95°C 5 min.	95°C 2 min.	55°C 1 min.	72°C 1 min.	72°C 7min.	Rozi et al., 2017
	Ahcytoen	F GAGAAGGTGACCACCAAGAACAA R AACTGACATCGGCCTTGAACTC	232	94°C 5 min.	94°C 30 sec.	56°C 30 sec.	72°C 1 min.	72°C 10 min.	Cagatay and Şen, 2014

Table 2: Prevalence of *Vibrio* species isolated from examined fishes.

Type and number of fish samples	No. of examined fish samples	No. of examined lesion samples	Positive samples for *Vibrio* species						Total	
			V. parahaemolyticus		*V. alginolyticus*		*V. cholerae*			
			No.	%*	No.	%*	No.	%*	No.	%*
Nile tilapia (*O. niloticus*)	50	157	20	30.7	13	20.0	5	7.7	38	58.5
Mullet (*M. cephalus*)	50	148	16	24.6	9	13.8	2	3.1	27	41.5
Total	100	305	36	55.4	22	33.8	7	10.8	65	100

*Percentage in relation to the total number of *Vibrio* species isolated (65).

Table 3: Prevalence of *Aeromonas* species isolated from examined fishes.

Type and number of fish samples	No. of the examined fish samples	No. of examined lesion samples	Positive samples for *Aeromonas* species					
			A. hydrophila		*A. caviae*		Total	
			No.	%*	No.	%*	No.	%*
Nile tilapia (*O. niloticus*)	50	157	26	36.1	1	1.4	27	37.5
Mullet (*M. cephalus*)	50	148	39	54.2	6	8.3	45	62.5
Total	100	305	65	90.3	7	9.7	72	100.0

*Percentage in relation to the total number of *Aeromonas* species isolated (72).

Table 4: Types and prevalence of virulence genes in *V. parahaemolyticus* and *A. hydrophila* strains.

V. parahaemolyticus				*Aeromonas hydrophila*							
*rec*A		*trh*		*fla*		*aer*A		*hly*A		*ahcytoen*	
No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
3	60.0	0	0.0	1	20.0	5	100.0	5	100.0	4	80.0

A total of 72 *Aeromonas* species were isolated and identified. Sixty-five *A. hydrophila* strains (90.3 %) were isolated, 26 (36.1%) from *O. niloticus* and 39(54.2%) from *M. cephalus*. Meanwhile, 7 *A. caviae* strains (9.7 %) were isolated, 1 (1.4%) from *O. niloticus* and 6 (8.3%) from *M. cephalus* (Table 3).

Molecular identification of *Vibrio* species using PCR

As a result of the molecular screening of 5 *Vibrio* species isolates using species-specific PCR (for *V. parahaemolyticus* (*tox*R gene) which generated at 368 bp and for *V. alginolyticus* (collagenase gene) which generated at 737bp), all five isolates were identified as *V. parahaemolyticus*. On the other hand, no *V. alginolyticus* isolates were identified (Figure 1).

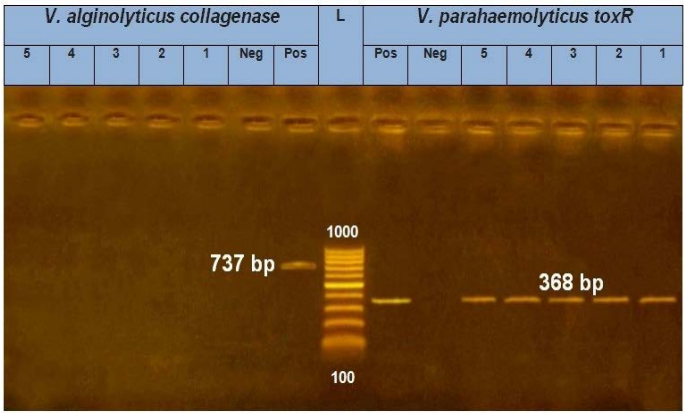

Figure 1: PCR amplification of the *tox*R gene (*V. parahaemolyticus*) and *collagenase* gene (*V. alginolyticus*) on agarose gel 1.5%. Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control (*Enterobacteriaceae* strain). Pos.: Positive control (local strain obtained from Central Lab. for quality control of poultry production, El-Giza, Egypt (CLQP)) (at 368 bp for *tox*R gene and 737bp for *collagenase* gene). Lanes 1-5: *V. parahaemolyticus* (*tox*R gene) positive and Lanes 1-5: *V. alginolyticus* (*collagenase* gene) negative.

Prevalence of some virulence genes among some isolated *V. parahaemolyticus* and *A. hydrophila* using PCR

PCR results showed the *rec*A virulence gene was detected in three out of five random isolated *V. parahaemolyticus*. Meanwhile, the *trh* virulence gene was not detected in any of the tested 5 *V. parahaemolyticus* isolates (Figure 2). Also, five randomly selected *A. hydrophila* isolates were submitted for the screening of virulence genes by PCR. Results revealed that *aer*A and *hly*A virulence genes were detected in all five random isolated *A. hydrophila* (Figure 3), Meanwhile, *Ahcytoen* was detected in 4 out of 5 *A .hydrophila* studied strains and *fla* virulence gene was detected in 1 out of 5 *A. hydrophila* isolates (Figure 4) (Table 4).

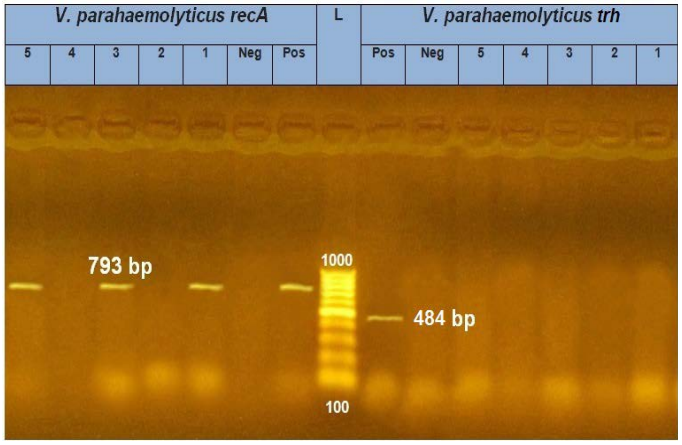

Figure 2: PCR amplification of *rec*A and *trh* genes of *V. parahaemolyticus* on agarose gel 1.5%. Lane L: 100-1000 bp. DNA Ladder, Neg.: Negative control (*Enterobacteriaceae* strain), Pos.: Positive control (local strain obtained from CLQP) (at 793 bp for *rec*A gene and 449 bp for *trh* gene), Lanes 1, 3 and 5: *V. parahaemolyticus* (*rec*A gene) positive, Lane 2 and 4: *V. parahaemolyticus* (*rec*A gene) negative, and Lanes 1-5: *V. parahaemolyticus* (*trh* gene) negative

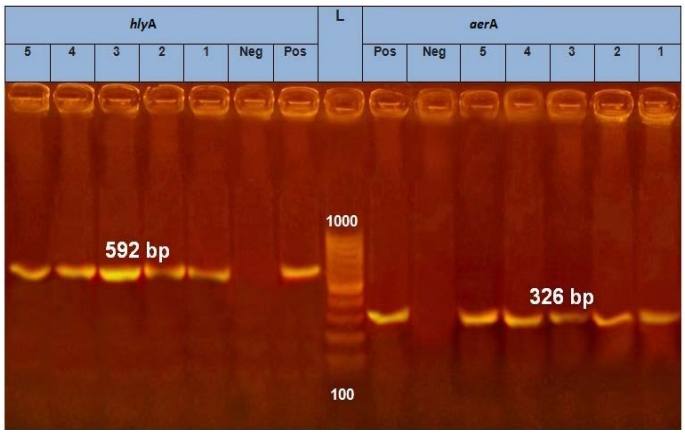

Figure 3: PCR amplification of Aerolysin (*aer*A) gene and hemolysin (*hly*A) on agarose gel 1.5%. Lane L: 100-1000 bp. DNA Ladder, Neg.: Negative control (*Enterobacteriaceae* strain), Pos.: Positive control (local strain obtained from CLQP) (at 326 bp for *aer*A gene and 592 for *hly*A gene), Lanes 1-5: *A. hydrophila* (*aer*A gene) positive and Lanes 1-5: *A. hydrophila* (*hly*A gene) positive.

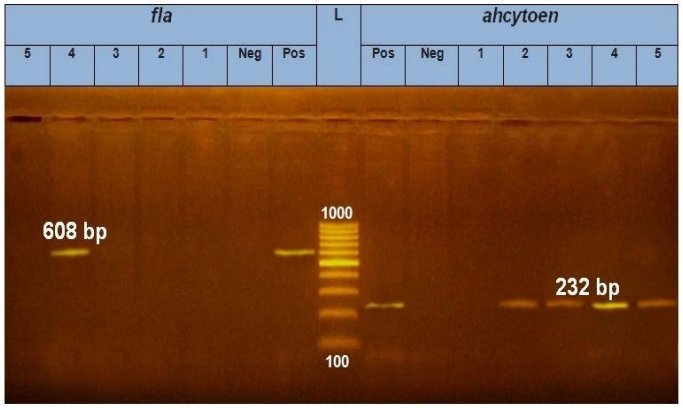

Figure 4: PCR amplification of *fla* and *ahcytoen* genes on agarose gel 1 %. Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control (*Enterobacteriaceae* strain), Pos.: Positive control (local strain obtained from CLQP) (at 608bp for *fla* gene and 232bp for *ahcytoen* gene, Lane 4: *A. hydrophila* (*fla* gene) (Positive), Lanes 1, 2, 3and 5: *A .hydrophila* (*fla* gene) (Negative), Lanes 2, 3, 4 and5: *A. hydrophila* (*ahcytoen* gene) (Positive) and Lane 1: *A. hydrophila* (*ahcytoen* gene) (Negative).

DISCUSSION

The bacterial infection is one of the main obstacles to aquaculture, which leads to wide economic losses (Gophen, 2017). This study was planned for the determination of the prevalence of *Vibrio* and *Aeromonas* infection in Nile tilapia and Mullet fish farms and the molecular detection of some virulence genes. The obtained results of naturally infected fishes with *Vibrio* species indicating disease problem and the clinical signs varied from the dark coloration of skin with detached scales, and hemorrhage at the base of the fins with some erosion which support the findings of El-Bouhy et al. (2016), and Al-Taee et al. (2017) in Mugil and Tilapia. *Vibrio parahaemolyticus* was the most highly detected with a prevalence of 55.4 % and detected in Nile tilapia higher than mullet fish (Table 2) which may be due to the flesh texture of tilapia fish or their living habits. These results were in agreement with that obtained by El-Hady et al. (2015). The reverse result obtained by Abdelaziz et al. (2017) where *V. alginolyticus* recorded the highest prevalence of infection followed by *V. parahaemolyticus* is probably due to differences in fish species (*Solea senegalensis* and *Mugil capito*). Lower prevalence of *V. parahaemolyticus* was recorded by Abdelaziz et al. (2017); Ahmed et al. (2018), and Deng et al. (2020) which may be due to differences in the geographical origin or season. Five random *Vibrio* species isolates were further genetically verified by PCR for the detection of *V. parahaemolyticus tox*R gene. Kim et al. (1999) clarified that the *V. parahaemolyticus tox*R sequence is perfectly conserved among *V. parahaemolyticus* strains and always be an appropriate diagnostic test. In our study, all five isolates were identified as positive for the *tox*R gene as *V. parahaemolyticus* (Figure 1). These results agreed with that recorded by Al- Othrubi *et al.* (2014) and Ashrafudoulla et al. (2019) where the *tox*R gene was detected in all *V. parahaemolyticus* isolates. PCR results showed that the *trh* virulence gene was not detected in any of the tested 5 *V. parahaemolyticus* isolates indicating that the majority of isolates were found to be non-pathogenic to humans due to the lack of the pathogenic *trh* gene (Figure 2). These results agreed with that recorded by Mohamad et al. (2019) and Tan et al. (2020). Meanwhile, the *rec*A virulence gene was detected in three out of five tested *V. parahaemolyticus* isolates (Figure 2). Theethakaew et al. (2013) identified frequent interspecies horizontal gene transfer and intra genetic recombination at the *rec*A locus, which plays a great role in the apparent evolutionary classification of *V. parahaemolyticus* (Han et al., 2014). *Aeromonas* species cause severe diseases among fish and humans, as Motile *Aeromonas* Septicemia (MAS) in fish which is caused by *A. hydrophila* leading to high mortalities and high economic losses (Shayo et al., 2012). The results of clinical and postmortem examinations of studied fish were similar to those reported by Sayed (2017) and Algammal et al. (2020). The results of bacteriological examination (Table 3) revealed that *A. hydrophila* was isolated in mullet fish higher than Nile tilapia. These results agreed with these of Abd El Tawab et al. (2017), and Enany et al. (2019). Lower results were recorded by Gufe et al. (2019), and Algammal et al. (2020) which was probably due to differences in the geographical distribution. The *aer*A gene was amplified in all five random isolated *A. hydrophila* studied strains (Figure 3). These results were similar to Algammal et al. (2020), and Sonkol et al. (2020) who reported that the aerolysin (*aer*A) gene was detected in 100% of the isolates. Meanwhile, they have disagreed with that recorded by

Nagar et al. (2011) and Ibrahim (2015) who failed to detect *aer*A virulent gene in these strains. The *hly*A gene was amplified in all five random isolated *A. hydrophila* studied strains (Figure 3). Similar results were decided by Ruhil-Hayati et al. (2015) and Abd El Tawab et al. (2017). Lower results were reported by Sonkol et al. (2020). The *Ahcytoen* gene was amplified in 4 out of 5 *A. hydrophila* studied strains (Figure 4). Similar results were recorded by Abd El Tawab et al. (2017). The *fla* gene was amplified in 1 out of 5 *A. hydrophila* studied strains (Figure 4). Similar results were reported by Blaszk (2013) and Aravena et al. (2014). While higher results were reported by Nawaz et al. (2010) and Dahanayake et al. (2020).

CONCLUSIONS AND RECOMMENDATIONS

This study revealed that *Vibrio* and *Aeromonas* species contribute to the occurrence of severe economic losses in the aquaculture industry in Egypt that requires a constant detection of these isolates to reduce the spread of infection. The high frequency of virulence genes in the isolates obtained in this work revealed the important role of these virulence genes in the emergence of the clinical signs.

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AUTHOR'S CONTRIBUTION

All authors contributed equally.

Conflict of interest

The authors have declared no conflict of interest.

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