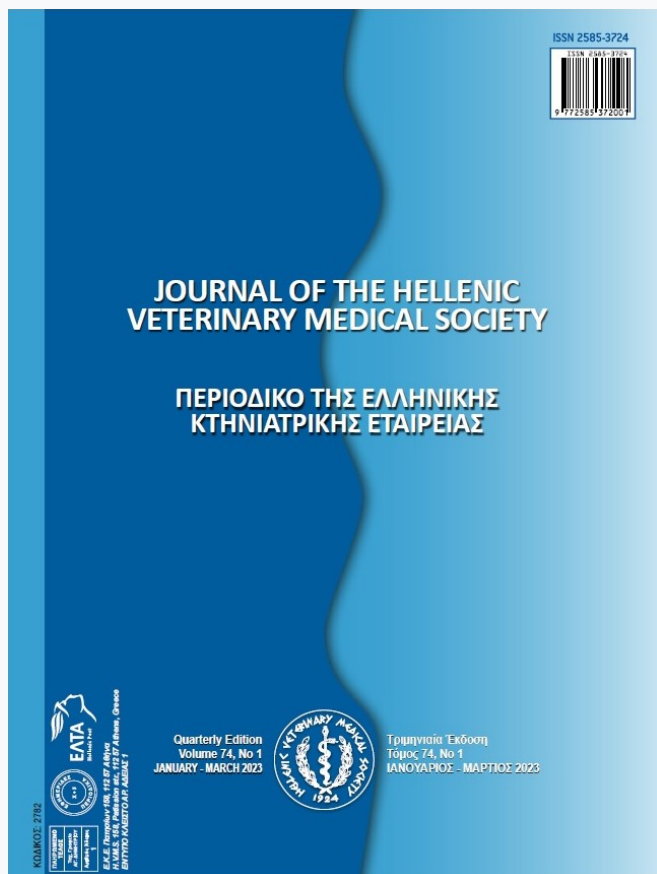


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Molecular Characterization of Antimicrobial Resistance of *Vibrio* Species Isolated from Fish in Egypt

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ABSTRACT: *Vibrio* sp. is the most widely recognized and serious pathogen in fish and shellfish marine aquaculture around the world generated disease, not only to fish but also to human causing gastro-enteritis. *Vibriosis* is a common disease caused by several *Vibrio* spp.; *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus* etc. *Vibrio* sp. is Gram negative, straight or curved short rods, non-sporulating, non-capsulated, arranged singly or in chains, motile, oxidase positive, catalase positive, citrate positive, string test positive, indol positive, urease negative, H₂S negative. The current study was conducted to spot light on identification, molecular characterization and antimicrobial resistance of *Vibrio* sp. isolated from fish in Egypt and to detect the presence of some of β- lactams resistance genes and class 1 integron. About 52 *Vibrio* isolates were isolated from 150 fish (75 *Oreochromis niloticus*, 50 *Mugil cephalus*, 25 *Clarias gariepinus*) in Kafr El-Sheikh Governorate in Egypt. Twenty isolates of *Vibrio* species were tested against 11 antimicrobials for antimicrobial resistance. All the isolates were highly sensitive (100%) to ciprofloxacin and norfloxacin. *V. alginolyticus* showed high resistance to Ampicillin and intermediate resistance to streptomycin, cefotaxime and erythromycin. *V. cholerae* showed high resistance to Ampicillin and intermediate resistance to erythromycin. *V. parahaemolyticus* showed high resistance to gentamycin and intermediate resistance to Ampicillin. *V. fluvialis* showed high and intermediate resistance to Ampicillin. *V. splendidus* showed high resistance to Ampicillin and intermediate resistance to streptomycin and cefotaxime. Five isolates showing multidrug resistance were tested for *bla*_{TEM}, *bla*_{CMY2}, *bla*_{CTX} genes and class 1 integron. The gene *bla*_{TEM} was detected in 100% of the isolates, while *bla*_{CMY2} and *bla*_{CTX} genes were detected in 0% of the isolates. Class 1 integron was detected with a percentage of (100%) in the 5 examined isolates. A class 1 integrons bearing streptomycin/ spectinomycin resistant gene cassette of *aadA2* were discovered on *V. parahaemolyticus* isolate. As there is few reports about antibiotic resistance in *Vibrio*, this study highlight the incidence of some of the β-lactams resistance genes and class 1 integrons in *Vibrio* species.

Keywords: β-lactam resistance genes, Class 1 integron, fish, PCR, *Vibrio* species

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INTRODUCTION

Vibrio species are the most predominant heterotrophic microscopic bacteria in the marine environment and are widely distributed in the coastal seawaters and/or brackish waters and have been isolated from seawater, fish, and shellfish (Alonzo *et al.*, 2017 and Drais *et al.*, 2018). They are likewise found on the surface and/or in the gastrointestinal tract of marine animals or other organisms (Colweli and Grimes, 1984; Austin and Austin, 1993 and Jun and Woo, 2003). Because of the rapid expansion of intensive mariculture and the consequent deterioration of culture conditions, the disease; vibriosis; caused by the genus *Vibrio*, occurs frequently worldwide, which influences a large number of fish species (Austin and Austin, 1993). Members of this family *Vibrionaceae* are Gram-negative, facultative, non-spore forming mostly characterized with comma-shaped rod.

Some of *Vibrio* sp. are referred to as significant human pathogens such as *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* (Moriarty, 1997), and others cause major financial losses in the aquaculture sector, such as *V. harveyi* and *V. anguillarum* that cause vibriosis with high mortality rates (Rippey, 1994).

Antimicrobial resistance is one of the most challenging public medical issues (Llor *et al.*, 2014) that are directly associated with the management and control of diseases (Ansari and Raissy 2010) and it has been seriously studied in traditional fish farming systems in temperate waters (Alderman *et al.*, 1998). Intensive fish farming in the world has resulted in growing issues of bacterial diseases, which prompted subsequent heavy antimicrobial use (Cabello, 2006). Resistance to the antimicrobials is viewed as another significant contributor to the virulence of the fish pathogen. Recently, antimicrobial resistance has been risen and evolved in *Vibrio* spp. resulting from the excessive utilization of antibiotics in agriculture, aquaculture systems and human medicine (Cabello *et al.*, 2013). Most of the common antibiotics which are frequently used are no longer effective. The wide use and misuse of antibiotics in agriculture, aquaculture, and livestock production are considered one of the key factors that impact the spread and development of antimicrobial resistance. Multidrug-resistant bacteria are another emerging challenge when a bacterial cell gets resistant to multiple antibiotics (Faja *et al.*, 2019).

The majority of hereditary determinants which give resistance to antibiotics are found in the plasmid.

Plasmids and portable hereditary elements such as integrons are considered contributors in the dissemination of antibiotic resistance genes and regarded as promoters of multi-drug resistance (Gonzalez-Plazaa *et al.*, 2019). Antibiotic contamination from facilities of manufacturing advanced the horizontal mobility of plasmids carrying antibiotic resistance genes among aquatic bacteria (Flach *et al.*, 2015). Integrons are portable hereditary elements that have a significant job in the spread and acquisition of antimicrobial resistance genes. Class 1 integron is composed of two conserved regions (CS): 5'CS contains the integrase gene, and 3'CS that normally contain *qacEΔ1* and *sul1* resistance genes to quaternary ammonium compounds and sulfonamide, respectively. Between these CSs, there is a variable region where gene cassettes can be embedded (Li and Zhao 2018). These cassettes present protection from most classes of antibiotics (Deng *et al.*, 2015).

Class 1 integrons are the most frequently found in clinical isolates of gram-negative bacteria (Mala *et al.*, 2016). Class 1 integrons are found in environmental and clinical isolates of *V. cholerae*, while very few resistance integrons have been found in *Vibrio* species other than the *V. cholerae* (Taviani *et al.*, 2008).

Just little reports have revealed antibiotic resistance determinants in the *Vibrio* spp. that are a reservoir of resistance genes.

The current study was carried out to spotlight on the incidence of some of the β -lactams resistance genes and class 1 integrons in *Vibrio* species isolated from fishes in Egypt.

MATERIAL AND METHODS

A- Materials

1. Fish Samples

A total of 150 clinically diseased fish samples (75 *Oreochromis niloticus*, 50 *Mugil cephalus* and 25 *Clarias gariepinus*) were collected alive from private fish farms at KafrelSheikh governorate (Gobarah *et al.*, 2021). The collected fish were transported immediately to the Department of Microbiology, Animal Health Research Institute in KafrelSheikh governorate and held in well-prepared glass aquaria supplied with sufficient amounts of dechlorinated tap water with continuous aeration using an electric air pump (Eissa, 2016) till investigation.

B- Methods:

1. Clinical and postmortem examinations:

The gathered fish were clinically examined to identify any external changes or clinical abnormalities (McVicar, 1982). Postmortem examination of the internal organs was carried out on sacrificed and freshly dead fish according to Austin and Austin (2012) and Eissa (2016).

2. Bacterial isolation

Tissue samples were aseptically collected from the skin, liver, kidney, heart and gills of the examined fish, pre-enriched on Tryptic Soy Broth (TSA) with 3% NaCl and incubated for at 30°C for 18-24 hrs. After that, they were streaked on tryptic soy agar and incubated at 30°C for 48 hrs. Pure single colonies on TSA were further streaked on selective media; Thio-sulfate Citrate Bile Salts Sucrose Agar (TCBS -Himedia) media and incubated for 18-24 hrs at 30 °C.

3. Phenotypic identification of bacterial isolates

Phenotypic identification of the bacterial isolates was identified according to Bergey's, (1994) and Madigan and Martinko (2005). Bacterial colonies that were suspected to be *Vibrio* has shown yellow or green color on TCBS agar. Pure cultures were subjected to Gram staining and motility test and then viewed microscopically (Cruickshank *et al.*, 1975). Further conventional biochemical tests following the rules proposed by Kreig and Holt (1984), MacFaddin (2000), and the FDA manual (Jayasinghe *et al.*, 2010) were performed.

4. Antimicrobial susceptibility test:

Antimicrobial susceptibility testing was performed by the Kirby-Bauer disc diffusion method (Stratev *et al.*, 2015) utilizing Mueller-Hinton agar (Difco), as indicated by the National Committee for Clinical Laboratory Standards "NCCLS" (2001) guidelines.

A total of 11 different antimicrobial sensitivity discs were utilized to investigate the resistance of the isolates. Sensitivity discs with variable concentrations were used to determine the susceptibility of the isolated bacterial strains to different antimicrobial agents (Oxoid Limited, Basingstoke, Hampshire, UK). The tested antimicrobial agents and their corresponding concentrations were as follows: gentamycin (10 µg/disk), ampicillin (10 µg/disk), streptomycin (10 µg/disk), nitrofurantoin (300 µg/disk), ciprofloxacin (5 µg/disk), sulpha-trimethoprim (25 µg/disk), nalidixic acid (30 µg/disk), cefotaxime (30 µg/disk), erythromycin (15µg/disk), amikacin (30µg/disk) and norfloxacin (10µg/disk). After incubating the inoculated plates at 30 °C for 18-24 hrs, the interpretations of the zones of inhibition were estimated as the maximal inhibition zone for the growth of microbe is said to that antibiotic had greatest impact on the microbe growth according to the guidelines stipulated by National Committee for Clinical Laboratory Standards "(CLSI, 2010)".

5. Molecular identification by Polymerase Chain Reaction (PCR) :

DNA extraction

The Genomic DNA was extracted from a pure culture of bacterial isolates using the DNA extraction kit (QIAamp DNA Mini Kit - Catalogue no. 51304 - Hilden, Germany) following the manufacturer's instructions.

Detection of β - lactams resistance genes and class 1 integron.

Five isolates of *Vibrio* sp., showing multidrug resistance, were screened for the presence of some of β - lactams resistance genes and class 1 integron. PCR assays were applied to target *bla*_{TEM}, *bla*_{CMY2}, *bla*_{CTX} genes and class 1 integron. Details of primers used are listed in (Table 1). Initial optimization experiments for

Table 1. Primers used to detect β - lactams resistance genes, class 1 integron and virulence genes

Target gene	Sequence	Amplified product	Reference
<i>bla</i> _{TEM}	5'-ATCAGCAATAAACACAGC 3'-CCCCGAAGAACGTTTTTC	516 bp	Colom <i>et al.</i> , 2003
<i>bla</i> _{CMY2}	5'-TGG CCA GAA CTG ACA GGC AAA 3'-TTT CTC CTG AAC GTG GCT GGC	462 bp	Pérez-Pérez and Hanson, 2002
<i>bla</i> _{CTX}	5'-ATG TGC AGY ACC AGT AAR GTK ATG GC 3'-TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593 bp	Archambault <i>et al.</i> , 2006
class 1 integron cassettes	5'-CSGGC ATC CAA GCA GCA AG 3'-CSAAG CAG ACT TGA CCT GA	Variable	Sow <i>et al.</i> , 2007

Table 2. Cycling conditions of PCR

Target gene	Primary denaturation	Amplification			No. of cycles	Final extension	Reference
		Secondary denaturation	Annealing	Extension			
bla _{TEM}	94°C/ 5 min.	94°C/ 30 sec	54°C/ 40 sec	72°C/ 45 sec	35	72°C / 10 min.	Colom <i>et al.</i> (2003)
bla _{CMY2}	94°C/ 5 min.	94°C/ 30 sec	55°C/ 40 sec	72°C/ 45 sec	35	72°C/ 10 min.	Pérez-Pérez and Hanson, (2002)
bla _{CTX}	94°C/ 5 min.	94°C/ 30 sec	54°C/ 40 sec	72°C/ 45 sec	35	72°C/ 10 min.	Archambault <i>et al.</i> (2006)
class 1 integron cassettes	94°C/ 5 min.	94°C/ 1 min.	50°C/ 1 min.	72°C/ 2 min.	35	72°C/ 10 min.	Sow <i>et al.</i> (2007)

Table 3. Prevalence of *Vibrio* species isolated from the examined fishes (Gobarah *et al.*, 2021)

Recovered isolates	<i>Oreochromis niloticus</i> (75)		<i>Mugil cephalus</i> (50)		<i>Clarias gariepinus</i> (25)		Total (150)	
	NO	%	NO	%	NO	%	NO	%
<i>V. alginolyticus</i>	9	12	7	14	8	32	24	16
<i>V. cholerae</i>	3	4	6	12	2	8	11	7.33
<i>V. parahaemolyticus</i>	4	5.33	2	4	2	8	8	5.33
<i>V. fluvialis</i>	0	0	2	4	3	12	5	3.33
<i>V. splendidus</i>	1	1.33	1	2	1	4	3	2
<i>V. anguillarum</i>	1	1.33	0	0	0	0	1	0.66
Total	18	24	18	36	16	64	52	34.6

each primer were conducted to ascertain optimal PCR conditions for MgCl₂ and annealing temperatures and the reaction mixture of the total volume of 25 µL was prepared. The PCR conditions used are presented in (Table 2).

RESULTS

1. Clinical and postmortem examination

The external gross lesions of the examined fish revealed scale detachment, fins erosion, corneal opacity, pale gills, skin darkening, ulcers, redness at base of anal fin and hemorrhagic areas around the mouth. Meanwhile, postmortem examination revealed congestion, swelling of the spleen, liver and kidney and hemorrhage in abdominal cavity.

2. Bacterial isolation and Incidence of *Vibrio* species

Bacterial isolation in the current data revealed that *Vibrio* sp. could be isolated from 18 out of 75 *Oreochromis niloticus* samples; with an incidence of 24% and the isolated *Vibrio* species were identified as *V. alginolyticus* (9), *V. cholerae* (3), *V. parahaemolyticus* (4), *V. fluvialis* (0), *V. splendidus* (1) and *V. anguillarum* (1). Moreover, *Vibrio* sp. could be isolated from 18 out of 50 *Mugil cephalus* with an incidence of 36%.

The isolated *Vibrio* species were identified as *V. alginolyticus* (7), *V. cholerae* (6), *V. parahaemolyticus* (2), *V. fluvialis* (2), *V. splendidus* (1) and *V. anguillarum* (0). However, in the case *Clarias gariepinus*, it could be isolated from 16 out of 25 fish; with an incidence of 64%. The isolated *Vibrio* species were identified as *V. alginolyticus* (8), *V. cholerae* (2), *V. parahaemolyticus* (2), *V. fluvialis* (3), *V. splendidus* (1) and *V. anguillarum* (0) (Table 3) (Gobarah *et al.*, 2021).

3. Phenotypic identification of bacterial isolates

Phenotypic analysis of *Vibrio* sp. colonies on TCBS agar media revealed that the bacterial colonies of *V. alginolyticus* were large (2-4 mm) and mucoid yellow; colonies of *V. cholerae* and *V. fluvialis* were large (2-3mm) and smooth yellow, while the colonies of *V. parahaemolyticus* were large (2-5mm) and green. By Gram staining, all colonies revealed Gram-negative, straight or curved short rods, non-sporulating, non-capsulated, arranged singly or in chains, motile, oxidase positive, catalase positive, citrate positive, string test positive, indole positive, urease negative, H₂S negative while the other biochemical tests differed from species to another (Gobarah *et al.*, 2021).

4. Antimicrobial susceptibility test:

Antimicrobial susceptibility test was done for 20

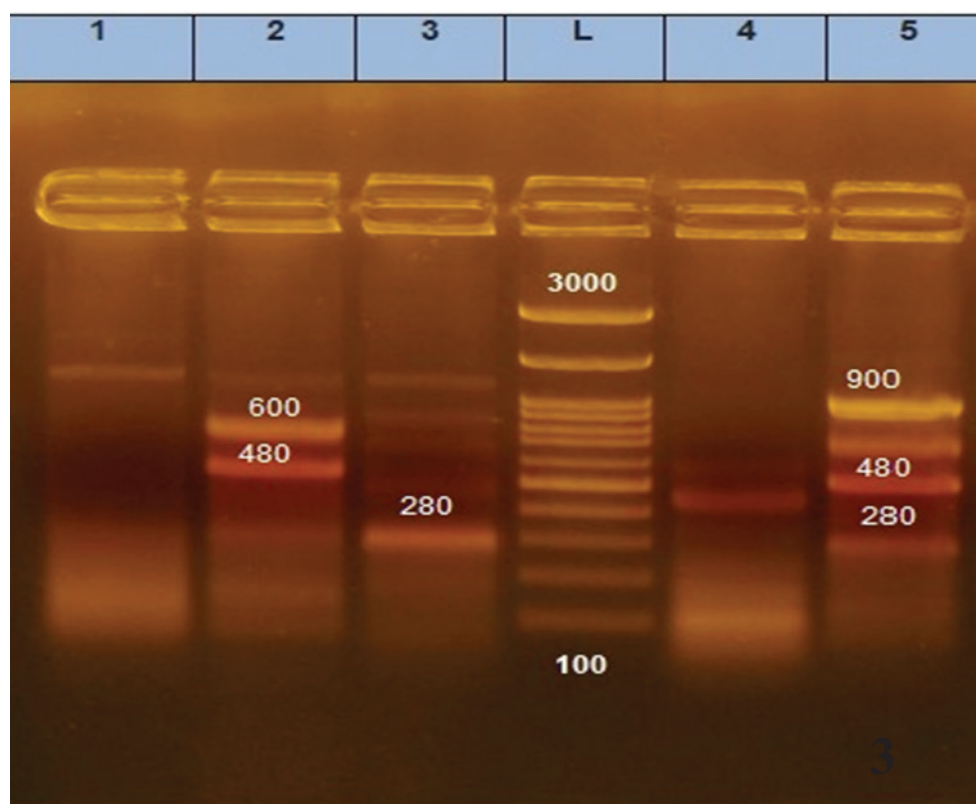


Figure 3. Agarose gel electrophoresis of PCR products of class 1 integrons in *Vibrio* isolates. lane (1,2,3,4,5) different strains of *vibrio* (*V. alginolyticus*, *V. parahaemolyticus*, *V. fluvialis*, *V. cholerae* and *V. cholerae* respectively).

Isolates 1, 2, 3, 4 and 5 are positive for class 1 integron..

Isolate no. 2: an empty integron (480 bp, 600 bp).

Isolate no. 3: an empty integron (280 bp).

Isolate no. 5: *aadA2* (900 bp) an empty integron (280 bp, 480 bp)

Table 4. Resistance phenotypes and incidence of resistance genes in some *Vibrio* sp. isolated from fishes:

NO	Isolate	Resistance pattern	MDR	Resistance Genes	Class 1 integron
1	<i>V. alginolyticus</i>	AMP, SXT, STR	+	<i>bla</i> _{TEM}	
2	<i>V. parahaemolyticus</i>	AMP, STR, ERY, NIT	+	<i>bla</i> _{TEM}	
3	<i>V. fluvialis</i>	AMP, GEN, ERY	+	<i>bla</i> _{TEM}	
4	<i>V. cholerae</i>	AMP, NIT, ERY	+	<i>bla</i> _{TEM}	
4	<i>V. cholerae</i>	AMP, GEN, ERY	+	<i>bla</i> _{TEM}	<i>aadA2</i>

DISCUSSION

Bacterial diseases are the major serious problems in aquaculture (Ibrahim *et al.*, 2013). Bacterial fish pathogens are normally present in the fish surrounding environment; nonetheless, under certain stress conditions, they may cause extraordinary financial mortalities with 80% mortalities in fish farms. That is why protecting the aquaculture industry from bacterial diseases is a priority (Ljubojevic Pelic, 2018; Hamouda *et al.*, 2019; Hamouda and Moustafa 2020).

The infected fish showed scale detachment, fins erosion, this may be due to the lytic activity of the bacterial infection. The hemorrhages all over the body

may be due to the hemolysin secreted by the isolated bacteria which make hemolysis of the RBCs as well as, elastase enzyme which contribute significantly to vascular damage because the blood vessels are mainly composed of elastic and collagenous fibers (Hamouda *et al.*, 2019 and Hamouda and Moustafa 2020). The post mortem changes may be attributed to the isolated septicemic bacteria and their virulence genes. The clinical signs displayed in the current study nearly corresponded with that recorded by previous authors (Eissa, 2016; El-Gamal *et al.*, 2018 and Hardi *et al.*, 2018)

Regarding the incidence of *Vibrio* sp.; 18 out of

75 *Oreochromis niloticus* samples; (24%) ; and the isolated *Vibrio* sp. were identified as *V. alginolyticus* (9), *V. cholerae* (3), *V. parahaemolyticus* (4), *V. fluvialis* (0), *V. splendidus* (1) and *V. anguillarum* (1). Moreover, *Vibrio* sp. was 18 out of 50 *Mugil cephalus* with an incidence of 36% and the isolated *Vibrio* sp. were identified as *V. alginolyticus* (7), *V. cholerae* (6), *V. parahaemolyticus* (2), *V. fluvialis* (2), *V. splendidus* (1) and *V. anguillarum* (0). However, in case *Clarias gariepinus*, 16 out of 25 fish could be isolated; with an incidence of 64%; and the isolated *Vibriosp.* were identified as *V. alginolyticus* (8), *V. cholerae* (2), *V. parahaemolyticus* (2), *V. fluvialis* (3), *V. splendidus* (1) and *V. anguillarum* (0). These findings may differ completely or partially with that recorded by many authors and this could be due to abiotic and biotic conditions of the environments where the studies were performed (El-Gamal *et al.*, 2018; Hardi *et al.*, 2018; Hamouda *et al.*, 2019; Hamouda and Moustafa 2020).

The colonies morphology, Gram staining and the biochemical profile of the detected bacteria are nearly identical to those reported by many other previous authors (Ahmed and Shoreit, 2001; Noga, 2010; Abd El-Kader and Mousa-Balabel, 2017; El-Gamal *et al.*, 2018 and Hardi *et al.*, 2018)

The antimicrobial susceptibility testing method is of major time-wise; was applied to distinguish antibiotic resistance and to decide the best treatment for a particular bacteria. Clinical microbiology relies on these techniques to select the agent of choice for a bacterial infection, and to know the local and the global epidemiology of antimicrobial sensitivity. The information on measure of antimicrobials that utilized in aquaculture are not available in many nations, there is evidence recommends that the amount of antimicrobials utilized in aquaculture in developing countries is limited in certain countries including Egypt, huge amounts of antimicrobials are normally utilized in aquaculture without consultation or supervision (WHO, 2006). The spread and development of antimicrobial resistance as a result of exposure to antimicrobial agents is recorded widely in both human and veterinary medicine. It is also well known that aquatic bacteria and pathogens of fish can develop resistance due to exposure to antimicrobials (Sørum, 2006).

One of the main findings in the current study was the wide spread of resistance to antimicrobials in *Vibrio* spp. isolated from fishes. The resistance to ampicillin and erythromycin was prevalent, the results

were in concurrence with the previous studies (Kitiyodom *et al.*, 2010). This is not surprising as both antibiotics are naturally created and scattered in the environment (Rosser and Young, 1999; Bani *et al.*, 2007). Resistance to cefotaxime and gentamycin, although detected in only a few strains, it could be of noteworthiness, because the presence of such resistance is an indication of the potential to spread.

The polymerase chain reaction has been proven to be a more accurate and rapid method for the identification of bacterial pathogens. The 16S ribosomal RNA gene is a highly conserved region present in bacteria which plays a major role in gene coding. It is considered as a standard marker for bacterial phylogenetic analysis to differentiate the species (Nagpal *et al.*, 1998).

In the present work, the β -lactam resistant genes were identified by the use of the specific primers of these genes (*bla*_{TEM} gene had molecular weight 516 bp, *bla*_{CMY2} gene had molecular weight 462 bp and *bla*_{CTX} gene had molecular weight 593 bp). It was observed that (100%) of the tested isolates had *bla*_{TEM} gene, while *bla*_{CTX} and *bla*_{CMY2} genes were not detected in the tested isolates of *Vibrio* sp. (0%). These results are in accordance to many authors; Rojas *et al.* (2011) found that all isolates of *V. parahaemolyticus* isolated from oysters and mussels possessed the *bla*_{TEM-IIIb} gene; Silvester *et al.* (2019) investigated that all the *Vibriosis* isolated from Cochin Estuary, seafood and shrimp farms harbored the *bla*_{TEM} gene and detected the *bla*_{CTX-M} gene in only 1.1% of the *Vibrio* sp. And Li *et al.*, (2015) who found *bla*_{TEM} gene in 1 of 2 strain of *V. parahaemolyticus* that screened for β -Lactamase genes and first detected *bla*_{CMY2} gene in 1 isolate of 2 isolates of *V. parahaemolyticus*. On other hand, Ceccarelli *et al.* (2016) screened the *V. cholerae* isolates showing reduced susceptibility in Bangladesh to detect the ESBL (*bla*_{CTX}, *bla*_{TEM} and *bla*_{SHV}) and found that all the isolates were negative.

The PCR result showed the presence of a class 1 integron in all (100%) of the five isolates of *Vibrio* species. DNA sequencing results of the purified PCR fragments showed the presence of one antibiotic resistance gene cassettes inside the class 1 integron. The *aadA2* gene cassettes were found in the *V. cholerae* isolate at 900 bp while the other isolates of *Vibrio* were devoid of resistance genes. The *aadA2* allele is widespread among bacteria species around the world. This is similar to the result obtained by Jiang *et al.* (2014) who found that all isolates harbored class1 in-

tegrons but he found that only one carried gene cassette without any resistance genes while Canto de Sá *et al.* (2010) found *aadA2* gene cassettes at 918bp in class 1 integrons in 1 genotypes of environmental *V. cholerae* non-O1/non-O139 and Dalsgaard *et al.* (2000) also detect *aadA2* gene cassettes at 1009 bp in *V. cholerae*.

CONCLUSION

The results of the current study concluded that *Vibrio sp.* is Gram-negative, straight or curved short rods, non-sporulating, non-capsulated, arranged singly or in chains, motile pathogen, not only to fish but also to human causing gastroenteritis. Moreover, the presence of multidrug-resistant *Vibrio sp.* harboring mobile genetic elements in fishes. The presence of antimicrobial resistance determinants with transferable genetic elements can promote the rapid dissemination of antimicrobial resistance among *Vibrio sp.* All the

isolates were highly sensitive (100%) to ciprofloxacin and norfloxacin. Five isolates showing multidrug resistance were tested for *bla*_{TEM}, *bla*_{CMY2}, *bla*_{CTX} genes and class 1 integron. The genes *bla*_{TEM} was detected in 100% of the isolates, while and *bla*_{CTX} genes were detected in 0% of the isolates. Class 1 integron was detected with a percentage of (100%) in the 5 examined isolates. A class 1 integrons bearing streptomycin/spectinomycin resistant gene cassette of *aadA2* were discovered on *V. cholerae* isolate. Further studies in Egypt and elsewhere are required to investigate the usage level of antibiotics in aquatic farms, to determine antimicrobial resistance, the dissemination and acquisition of resistance determinants and to evaluate the danger of the transfer of resistant bacteria or genes to humans through the food chain.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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