Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

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Original Paper

Bacteriological and molecular assay of Vibrio species affecting some marine and freshwater fishes

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ARTICLE INFO

Keywords

Nile tilapia,
Marine Fishes,
Vibrio species,
toxR gene.

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Received 14/01/025
Accepted 22/03/2025
Available On-Line 01/04/2025

ABSTRACT

Aquatic animals are susceptible to a serious disease called *vibrio*sis that causes high mortality rates, financial losses, and foodborne outbreaks. So, the present study was planned to isolate and identify the prevalence of *Vibrio* species in some freshwater and marine fish in Kafer El-Sheikh governorate, Egypt. A total of 200 fish samples, 100 freshwater fish [Nile tilapia (*Oreochromis niloticus*)], and 100 marine fish [20 red porgies (*Pagrus pagrus*), 30 gilthead seabream (*Sparus aurata*), and 50 European seabass (*Dicentrarchus labrax*)] were subjected to clinical, bacteriological, and molecular diagnosis for *Vibrio* infection. Clinical observations revealed diseased fish included dark skin, redness, detached scales, fin erosion, and irregular hemorrhagic patches. Bacteriological identification showed that the isolation rate of *Vibrio* in Nile tilapia was 23% compared to 34% in marine fish (15%, 17%, and 52% from red porgy, Gilthead seabream, and European seabass, respectively), where the highest percentage was encountered in European seabass. Among Nile tilapia isolates, *Vibrio alginolyticus* (*V. alginolyticus*) was the most detected species (52%), followed by *Vibrio parahaemolyticus* (*V.parahaemolyticus*) (39%) and then *Vibrio cholera* (*V. cholera*) (9%). Meanwhile, isolation from marine fish showed (50%), (41%), and (9%) for *V. parahaemolyticus*, *V. alginolyticus*, and *V. harveyi*, respectively. Moreover, the molecular identification of the isolates by PCR confirmed the identity of the *V. parahaemolyticus*, *V. alginolyticus*, and *V. cholerae* through the presence of the species-specific genes (transmembrane regulatory protein, *tox*R), collagenase, and superoxide dismutase B, *sod*B), respectively. These results indicate the high rate of *Vibrio* infection in marine and freshwater fishes at Kafer El-Sheikh Governorate, Egypt. Moreover, the study results also showed that *V. parahaemolyticus* and *V. alginolyticus* are the most common *Vibrio* species isolated from the examined marine fishes and Nile tilapia, respectively, which are crucial for public health regarding fish consumption.

1. INTRODUCTION

Increased disease outbreaks are linked to contemporary developments in aquaculture farming and production growth, which have a detrimental impact on the industry's sustainability, profitability, and output worldwide. *Vibrio*sis is one of the most prevalent bacterial diseases that causes significant losses in cultured fish and shellfish (Ina-Salwany et al., 2019).

Several strains of *Vibrio* spp., which are Gram-negative, comma-shaped, extremely motile, and contain one or more polar flagella, are very tolerant of varying salinity levels (Sampaio et al., 2022). They are naturally occurring diseases that almost affect marine fishes, shrimp, and other aquatic animals (Yousef et al., 2023). Various factors, particularly the water quality and farm management in addition to *Vibrio* virulence factors, are playing a great role in a *Vibrio*sis occurrence (Sanches-Fernandes et al., 2022). Fish infected with *vibrio*sis display a range of clinical signs, including lethargy, skin and fins ulcers. Additionally, they may experience liquefaction of internal organs, blindness, increased opacity of muscle tissue, and ultimately, impaired growth and mortality (Onohuean et al., 2022).

Due to the fastidious nature of *Vibrio* species, a recent advanced molecular identification of this species was implemented based on the PCR detection of target genes such as *tox*R, *sod*B, and collagenase genes (Federici et al., 2018) since it is quick, sensitive, and dependable for detecting pathogens in various matrices (Park et al., 2013). Therefore, the present study was conducted to investigate the incidence of *Vibrio* infection at Kafer El-Sheikh Governorate among Nile tilapia, red porgy, gilthead seabream, and European seabass through clinical, bacteriological, and molecular diagnosis.

2. MATERIAL AND METHODS

The study was accepted by the Ethical Committee, Faculty of Veterinary Medicine, Benha University, approval BUFVTM04-12-23.

2.1. Samples collection

Two hundred fish samples were collected from Kafr El-Sheikh Governorate between December 2022 and February 2023. Of these, 100 samples (Nile tilapia) with an average body weight of 70 ±5 g and 100 marine fish (20 red porgies with an average body weight of 60 ± 5 g, 30 Gilthead seabream with an average body weight of 70 ± 5 g, and 50 European seabass with an average body weight of 90 ± 5 g). Cultured Nile tilapia samples were obtained alive from a fish farm in the Baltim province in a water tank; meanwhile, the marine fish samples were collected freshly dead from Kafer El-Sheikh markets, whose resource was from Lake Burullus in an icebox.

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Every fish sample was placed in a plastic bag separately and delivered to the lab as rapidly as possible for clinical observations, bacteriological, and molecular diagnosis.

2.2. Clinical examination

Clinical and postmortem examinations were conducted according to Austin and Austin (1987) and Conroy and Herman (1981), where fish samples were examined for any deformities and abnormalities.

2.3. Bacteriological analyses

According to Noga (2010), internal organs (liver, spleen, heart, kidney, a portion of the intestine, and gills) from each fish sample were collected under complete aseptic circumstances to isolate *Vibrio* species bacteriologically. Bacteriological samples were taken from each organ and incubated in alkaline saline peptone water (ASPW) (HiMedia, India) for 24 hours at 37 °C in accordance with Elliot et al. (2001), Then a loopful from ASPW was streaked onto Thiosulfate Citrate Bile Sucrose agar (TCBS) (HiMedia, Mumbai, India) and incubated for 24 hours at 37°C. Colonies were categorized according to their size and color. Gram's stain, motility on semisolid agar, oxidase, catalase, and urease; in addition, indole, methyl red, Voges–Proskauer, and citrate tests (IMVC tests) were used to identify *Vibrio* species according to Quinn et al. (2002). In addition, novobiocin (NV 30 mg) was used to distinguish between yellow colonies of *Vibrio* species and other Gram-negative bacterial isolates following re-streaking on tryptic soy agar (TSA; HiMedia, India) plates supplemented with 2% sodium chloride (NaCl; Vivantis, USA) as recommended by Abd El Tawab et al. (2018). Another confirmatory biochemical test was performed using a commercial diagnostic test (S.R.O. kits®) (GN24: Cat. No. 1001) following the manufacturer's instructions (Diagnostics, 2024).

2.4. Molecular Identification

Ten selected isolates of *Vibrio* were purified: 4 marine fish isolates (two *V. parahaemolyticus* and two *V. alginolyticus*) and 6 isolates from Nile tilapia (two *V. parahaemolyticus*, two *V. alginolyticus*, and two *V. cholera*) were molecularly diagnosed using *tox*R, *collagenase*, and *sod* B genes. With some modifications from the manufacturer's instructions, the QIAamp DNA mini kit (catalog no. 51304, Qiagen, Hilden, Germany) was used to extract DNA from each *Vibrio* isolate. Primers, sequences, and cycle conditions are listed in Tables 1 and 2 for the Emerald Amp GT PCR master mix (Takara, Japan) with Code No. RR310A and 1.5% agarose gel electrophoresis (Sambrook *et al.*, 1989). Positive and/or negative controls were represented by field samples that were previously confirmed to be positive or negative by PCR for the related genes in the reference laboratory for veterinary quality control on poultry production, Animal Health Research Institute for *V. parahaemolyticus* ATCC 17802, *V. alginolyticus* ATCC 17749, and *V. cholerae* ATCC 14035.

Table 1. Cycling conditions of the different primers

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
V. alginolyticus collagenase	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec	72˚C 45 sec	35	72˚C 10 min.
V. parahaemolyticus toxR	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec	72˚C 40 sec	35	72˚C 10 min.
V. cholerae sodB	94˚C 5 min.	94˚C 30 sec.	57˚C 30 sec	72˚C 30 sec	35	72˚C 7 min.

Table 2. Oligonucleotide primers sequence of target genes (bp).

Gene	Primer sequence (5'-3')	Length of amplified product	Genbank accession No.	Reference
V. cholera				
sodB	AAG ACC TCA ACT GGC GGT A GAA GTG TTA GTG ATC GCC AGA GT	248 bp	CP104354	Tarr *et al.*, 2007
V. parahaemolyticus				
toxR	GTCTTCTGACGCAATCGTTG ATACGAGTGGTTGCTGTCATG	368 bp	KT194135	Kim *et al.*, 1999
V. alginolyticus				
Collagenase	CGAGTACAGTCACTTGAAAGCC CACAACAGAACTCGCGTTACC	737 bp	DQ097161	Abu-Elala *et al.*, 2016

3. RESULTS

3.1. Clinical and postmortem examinations

Diseased Nile tilapia revealed dark skin, redness around the mouth, detached scales, fin erosion, and irregular hemorrhagic patches dispersed on the trunk region and at the base of the pectoral and anal fins (Photo 1). Moreover, the clinical examination of marine fish showed fin erosion and hemorrhage at fins bases (Photos 2 and 3).

Fig. 1. Nile tilapia infected with *vibrio* species showing scattered irregular hemorrhagic spots at the base of pectoral fins

Fig. 2. Gilthead seabream infected with *vibrio* species showing erosion of caudal fin.

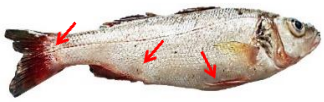

Fig. 3. European seabass infected with *vibrio* species showing hemorrhage at all fins bases

Fig. 4. The PM finding in European seabass infected with vibrio species showing congested spleen, liver, gills.

Fig. 5. Red porgy infected with vibrio species showing multiple skin ulcer and erosion of fins.

3.2. Bacteriological examination

3.2.1. Morphological and cultural characteristics

Gram-negative, motile, straight or curved short rods, and grouped singly or in chains were all characteristics of the *vibrio* spp. isolates. The colonies on TCBS medium were either green or yellow (Fig. 6) and (Table 3). On TSA media, the colonies had a round, creamy yellow appearance.

Fig. 6. Yellow colonies (A) and green colonies (B) of *vibrio* spp. isolates on TCBS medium.

All *vibrio* spp. isolates were oxidase positive, catalase positive, indole positive, as was shown in Table 3.

The result of sugar fermentation (glucose, sucrose, lactose, and mannose) and amino acids detection (arginine, lysine, and ornithine) were shown in S.R.O. kites results which appear in Fig 7.

Table 3. Morphological and Biochemical characters of isolated *vibrio* spp.

Parameters	*V. parahaemolyticus*	*V. alginolyticus*	*V. cholerae*	*V. harvyei*
Catalase	+	+	+	+
Citrate	+	+	+	-
Gram stain	-	-	-	-
H_2S	-	-	-	-
Indole	+	+	+	+
Methyl red	+	+	+	+
Motility	+	+	+	+
On TCBS	Green	Yellow	Yellow	Yellow
Oxidase	+	+	+	+
Urease	-	-	-	+
Vogus-proskaure	-	+	-	-

3.4. Prevalence of *vibrio* species:

Vibrio species were detected in 57 of the 200 fish samples, yielding a 28.5% overall prevalence rate. Out of these isolates of *Vibrio* species, 34 (34/100) were from marine fishes and 23 (23/100) from Nile tilapia. The total prevalence rates of *Vibrio* species in different marine fish types were 15% (3/20), 16.67% (5/30), and 52% (26/50) from red porgy, gilthead seabream, and European seabass, respectively. The highest *Vibrio* prevalence rate in marine fishes was from European seabass (Table 4).

Fig. 7. Results of S.R.O. identification of the detected *Vibrio* isolates

Across all fish types analyzed in this study, the overall prevalence rates of *V. parahaemolyticus*, *V. alginolyticus*, *V. cholera*, and *V. harveyi* were 13%, 13%, 1%, and 1.5%, respectively. *V. parahaemolyticus*, *V. alginolyticus*, and *V. cholera* were recovered at the following percentages: 39%, 52%, and 9% from Nile tilapia, whereas *V. parahaemolyticus, V. alginolyticus, and V. harveyi* were detected at a prevalence rate of 50%, 41%, and 9%, respectively. The greatest isolation rates for *V. parahaemolyticus* and *V. alginolyticus* were exhibited in Nile tilapia and European seabass (39% and 52% & 29% and 38%, respectively) (Table 5).

Table 4. The prevalence of *Vibrio* species in the examined fish samples

	Marine water fish				Freshwater fish	Total prevalence
	Red porgy	Gilthead Seabream	European Seabass	Total	Nile tilapia	
Number of samples	20	30	50	100	100	200
No. of Positive samples	3	5	26	34	23	57
% *	15	17	52	34	23	28.5

* The percent is calculated in relation to the total number of each fish type.

Table 5. The prevalence rate of each detected Vibrio species in different fish isolates

Source	Vibrio spp. / Fish spp.	V. parahaemolyticus		V. alginolyticus		V. cholera		V. harveyi		Total vibrio spp.	
		NO.	%	NO.	%	NO.	%	NO.	%	NO.	%
MWF (n=34)	Red porgy	3	9	0	0	0	0	0	0	3	9
	Gilthead Sea bream	4	12	1	3	0	0	0	0	5	15
	European Seabass	10	29	13	38	0	0	3	9	26	76
Total		17	50	14	41	0	0	3	9	34	100
FWF (n = 23)	Nile tilapia	9	39	12	52	2	9	0	0	23	100
Total of all fish	types (n = 200)	26	13	26	13	2	1	3	1.5	57	28.5

MWF: marine water fish FWF: freshwater fish

3.5. Molecular identification

From the ten studied *Vibrio* isolates, the 4 *V. alginolyticus* isolates had collagenase gene at 737bp (Fig. 8), and the 4 *V. parahaemolyticus* isolates harboured *tox*R gene at 368bp (Fig. 9). While the *sod*B gene were found in 2 *V. cholera* isolates at 248bp (Fig. 10).

In four marine fish isolates, the two *V. parahaemolyticus* isolates, from Gilthead Seabream and European Seabass, had *tox*R (species specific) gene while the collagenase gene was found in the two *V. alginolyticus* isolates.

In freshwater fish isolates, the *tox*R, collagenase, and *sod*B genes were detected in the two *V. parahaemolyticus*, the two *V. alginolyticus* and the two *V. cholera* isolates respectively.

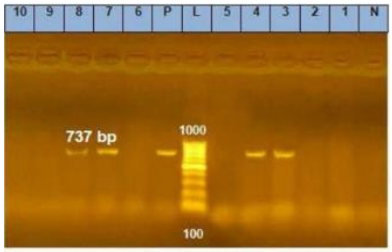

Fig. 8. Amplification of *collagenase* gene at 737pb. MWM-molecular weight marker (100-1000bp DNA ladder),+control(P:positive,*Vibrio alginolyticus* ATCC 17749 , N: negative)
Samples 3-4 and 7-8 were positive
Samples 1-2, 5-6 and 9-10 were negative

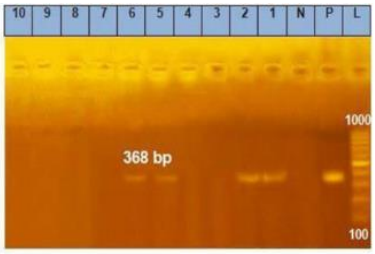

Fig. 9. Amplification of *toxR* gene at 368bp. MWM-molecular weight marker (100-1000 bp DNA ladder), + control (P: positive, *Vibrio parahaemolyticus* ATCC 17802, N:negative) Samples 1-2 and 5-6 were positive Samples 3-4 and 7-10 were negative

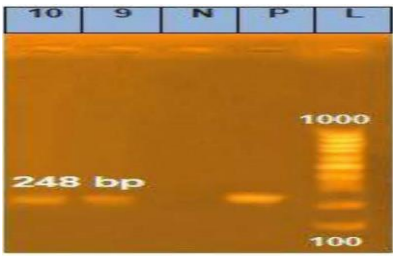

Fig. 10. Amplification of *sodB* gene at 248bp. MWM-molecular weight marker (100-1000 bp DNA ladder), and controls (P: positive, *Vibrio.cholerae* ATCC 14035 N: negative). Samples 9-10 were positive.

4. DISCUSSION

*Vibrio*sis, a common bacterial disease in aquatic animals, is a major cause of death in fish worldwide, particularly those of marine origin. It causes a significant economic loss due to the nearly 50% mortality rate of affected fish (El-Galil and Mohamed 2012). Fish *vibriosis* also can impose a serious risk to human health due to the ability of the causative bacteria to cause some significant zoonotic diseases (Sanches-Fernandes *et al.*, 2022).

In the present results, the detection of *vibrio* infection at a rate of 28.5% is parallel to the reported detection rate of Abdelaziz et al. (2017), who examined a total of 100 samples of wild *Solea aegyptiaca*, *Epinephelus marginatus*, and *Mugil cephalus* that were collected from the Gulf of Suez and Qarun Lake, where vibrio spp. was detected in 29% and 64% of the examined samples from the Gulf of Suez and Qarun Lake, respectively. The discrepancy between different records may be attributed to the differences in the climate temperature and the levels of salinity or the nutrition quality. Indeed, of the factors affecting *vibrio*sis, the nutritional and environmental status was supposed to play a major role in the severity of *vibrio*sis spread (Jun and Woo, 2003). Moreover, water quality, water salinity, water temperature, water pollution, and the presence of macro- or micro-organisms play critical roles in *vibriosis* development and outbreaks (Huicab-Pech *et al.*, 2016).

In the current study, the lower rate of detection in freshwater fish (23%) compared to 34% in marine fish is mostly attributed to the lower level of salinity in freshwater, as *vibrio*sis was reported to be increased with increasing salinity levels (Baker-Austin *et al.*, 2018). Moreover, the incidence of *vibrio*sis in the freshwater fish and the marine-water fish in the current study is in accordance with a previously reported incidence of *vibrio*sis (26.0% and 48.0% in freshwater fish and marine fish, respectively) by Saad et al. (2015).

The results of the present study revealed a higher prevalence of *vibrio*sis in European seabass (52%), followed by gilthead seabream (17%) and red porgy (15%), which is different from the previously reported results by Abdel-Aziz et al. (2013) (44% and 28% in gilthead seabream and European seabass, respectively). This incidence discrepancy can be attributed to the locality's differences. In respect to the *Vibrio* species, *V. parahaemolyticus* came in at rates of 29%, 12%, and 9% in European seabass, Gilthead seabream, and red porgy, respectively, which is slightly different from the previously reported rates by Abdel-Aziz et al. (2013) and Yiagnisis et al. (2011). This difference could be related to the season of sampling, geographical differences, feed habits, and size of the sampled fish. While *V. alginolyticus* was detected at rates of 52%, 38%, and 3% in Nile tilapia, European seabass, and Gilthead seabream, respectively, it is lower than the previously reported incidence of 82.2% and 87.3% in European seabass and Gilthead seabream, respectively (Abdel-Aziz *et al.*, 2013), and 57.72% in seabream (Ismail *et al.*, 2024). The lower rate of *V. alginolyticus* isolation in the current study may reflect the lower bacterial load, especially *V. alginolyticus*, in the Kafr El-Shiekh coastal seawater compared to the other reported localities in Egypt. Noteworthy, *V. alginolyticus* is a well-known enteropathogen to humans, causing seafood-borne illness and mortality (Mizan *et al.*, 2017). The high rate of *V. alginolyticus* detection in freshwater fish and marine fish highlights the potential of these fish types being vectors for *V. alginolyticus* infection to humans. Nine percent (9%) detection rate of *V. cholerae* and the fact that it was not found in the seawater fish support the earlier theory that *V. cholerae* is an opportunistic fish pathogen and a normal flora of tilapia (Halpern and Izhaki, 2017). This is further supported by the fact that it was not found in the internal organs of Nile tilapia kept in floating cages in Thailand (Thaotumpitak *et al.*, 2023). Since the current study was unable to isolate from marine fish, despite the fact that *V. cholerae* has been isolated from a variety of freshwater and marine fish, freshwater fish are believed to be the main reservoir of *V. cholerae* strains, which could pose a risk of infection to humans (Halpern and Izhaki, 2017). Moreover, *V. cholerae* caused a severe and fatal diarrhea in humans mediated by cholera toxin and represents a major public health concern (Thaotumpitak *et al.*, 2023). Therefore, its detection in tilapia at this rate should be taken into consideration when dealing with tilapia or its products, especially the raw tilapia fish.

Vibrio spp. identification showed high variability in their genotypic traits, which necessitates the importance of implementing the molecular identification technique as a valid diagnostic alternative (Ismail *et al.*, 2024). In the current study, the molecular detection of different *Vibrio* spp. has depended on the identification of the major characteristic genes in each *Vibrio* spp. by PCR. The primers used were the Collagenase gene for *V. alginolyticus*, *the toxR* gene for *V. parahaemolyticus*, and the *sod*B gene for *V. cholera.* In the current study, the use

of these respective genes for *Vibrio* spp. identification was in accordance with the morphological, cultural, and biochemical identification. This is consistent with validation of the molecular identification of *Vibrio* spp. (Abdallah *et al.*, 2011; Mustapha *et al.*, 2013; Aly *et al.*, 2020). Taken together, these results indicate the validity of the molecular technique for V*ibrio* spp. identification.

5. CONCLUSIONS

The results of this study showed higher prevalence of *Vibrio* spp. in marine water fish compared to freshwater fish with *V. parahaemolyticus* and *V. alginolyticus*, respectively. *Vibrio cholerae* was detected only in Nile tilapia, indicating the importance of considering this fish type as a potential risk for human health concern. Finally, the study showed the validity of the molecular technique as *a Vibrio* spp. identification tool.

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