



# Isolation and Molecular Detection of Pathogenic *Vibrio* Species among Economic Fish from Red Sea in Egypt

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## Authors' contributions

This work was carried out in collaboration between all authors. Authors SS, AA and MWAA designed the study, performed the statistical analysis and wrote the protocol. Author AA wrote the first draft of the manuscript and managed literature searches under supervision of author SS. Authors MWAA, AA, MIEB and SS managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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## ABSTRACT

A total of 105 samples were collected from *Siganus rivulatus*, *Mulloidichthys vanicolensis*, and *Lethrinus lentjan*, freshly captured from the Red Sea along Hurghada City coastline zone, Egypt. Clinical and post mortem findings revealed the presence of characteristic clinical signs and lesions similar to those reported in vibriosis. Out of 43 putative *Vibrio* species isolates obtained by culturing; 30 isolates were presumptively discriminated into *Vibrio cholera* (n=11), *Vibrio anguillarum* (n=8), *Vibrio fluvialis/ Vibrio furnissii* (n=4), *Vibrio harveyi* (*Vibrio carchariae*) (n=4) and *Vibrio alginolyticus* (n=3), but it was not initially possible to approve or repudiate that the remaining 13 isolates were *Vibrio* species through phenotypic characterization. By using PCR, targeting

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*Vibrio*-specific 16S rRNA gene, the presumptive 30 *Vibrio* isolates and 9 out of the remaining 13 isolates were confirmed as *Vibrio* species. The prevalence of *Vibrio* species was 37.1% among the examined fish species; 47.1%, 34.3% and 30.6% in *Mulloidichthys vanicolensis*, *Lethrinus lentjan* and *Siganus rivulatus*, respectively. The occurrence of *Vibrio* species pathogenic for aquatic animals and humans was confirmed which possess public health concerns. Also, the utility of molecular technique to improve the identification of phenotypic *Vibrio* like species is recommended.

**Keywords:** *Vibrio* species; fish; phenotypic characterization; PCR; 16S rRNA gene; Egypt.

## 1. INTRODUCTION

Bacterial diseases outbreaks are a critical obstacle challenging aquaculture sector improvement worldwide [1,2]. The aquatic environment is a habitat for many bacterial pathogens, obligatory and opportunistic pathogens. Stresses exerted on fish due to the poor environmental conditions make them immunocompromised and, subsequently, more susceptible to pathogens infection [2,3].

*Vibrio* species are a normal part of the bacterial flora in aquatic environments and some species are commonplace in/on aquatic animals; considered to be mostly opportunistic pathogens [4,5]. Several *Vibrio* species; *Vibrio anguillarum* and *Vibrio tapetis*, associated with diseases of aquatic animals, whereas other species including *Vibrio cholerae*, comprise serious pathogens of human. A comparatively small number of *Vibrio parahaemolyticus* and *Vibrio vulnificus*, causes disease in both aquatic animals and human [5,6].

*Vibrio* species are responsible for vibriosis, one of the most devastating bacterial diseases in cultured fish and other aquatic animals globally [6,7]. The main pathogenic *Vibrio* species for marine fish are *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio carchariae*, *Vibrio cholerae*, *Vibrio ordalii*, *Vibrio vulnificus* and *Vibrio parahaemolyticus*, whereas, *Vibrio mimicus* and *Vibrio cholerae* are the main pathogenic *Vibrio* species for fresh water fish [8,9].

*Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio alginolyticus*, *Vibrio mimicus*, *Vibrio fluvialis*, *Vibrio furnissii*, *Vibrio metschnikovii*, *Vibrio hollisae* and *Vibrio damsela* are also pathogenic for humans, causing infections such as gastroenteritis, septicemia, wound and ear infections [10,11].

The 0/129 vibriostatic test is a useful as a primary screen for identification and differentiation of members of *Vibrio* species [12,13].

Hurghada coastline is exposed to a variety of stresses as a result of anthropogenic activities, sewage pollution, hypersaline water rejection of desalination plants, shipping operations, and landfilling [14]. Stressed aquatic animals are more susceptible to microbial infections [1-3]. Vibriosis in fish is accompanied with stress but some strains, especially of *Vibrio anguillarum*, *Vibrio ordalii* and *Vibrio salmonicida* appear to be highly infectious primary pathogens. Several pathogenic *Vibrio* species outbreaks are known to be commonly associated with water contaminated with human feces or sewage [15,16]. Vibrios are typically more common in geographic regions having temperate or tropical climates [17]. Salinity, specifically sodium salt ( $\text{Na}^+$ ), is the most important factor governing the environmental distribution of vibrios [18].

The prevalence of bacterial pathogens has been confirmed in several cultured and wild freshwater fish species, however; only a few bacteriological surveys have involved the marine species disease outbreaks [19]. The present study is aimed to assess the prevalence *Vibrio* species, using molecular and conventional methods, among three economic marine fish species captured from the Red Sea along Hurghada City coastline, Egypt.

## 2. MATERIALS AND METHODS

### 2.1 Specimens and Clinical Findings of Captured Fish Species

Over a 4 months period (May through August, 2012), a total of 105 samples from three different fish species (Fig. 1) were freshly captured in Hurghada city coastline zone, the Red Sea, Egypt by using small fishing vessels and gill nets. Whole fish were transported in ice-cooled insulated box to the laboratory within few hours, where they were investigated, Table 1 and Fig. 1. The clinical and post mortem (PM) examination were carried out according to the methods described by [13].

**Table 1. Data of fish species captured and sampled in this study**

Fish species	Common name	Fish no.	Average body weight (g)
<i>Siganus rivulatus</i>	Marbled spinefoot fish	36	70±20
<i>Mulloidichthys vanicolensis</i>	Yellow fin goatfish	34	65±15
<i>Lethrinus lentjan</i>	Pinkear emperor fish	35	80±25

No = Number; g = Gram

**Fig. 1. Showing the morphological features of captured fish species in Hurghada City coastline zone, the Red Sea, Egypt**(A) Marbled spinefoot fish (*Siganus rivulatus*), (B) Yellowfin goatfish (*Mulloidichthys vanicolensis*) and (C) Pinkear emperor fish (*Lethrinus lentjan*)

## 2.2 Isolation of *Vibrio* Species

Fish liver, spleen and kidneys were sampled and cultured on general and selective media; tryptone soya agar (TSA, Oxoid) supplemented with 2% (w/v) NaCl and thiosulfate-citrate-bile salts-sucrose agar (TCBS, Oxoid), the inoculated media were incubated in aerobic conditions at 30°C for 2-5 days.

## 2.3 Initial Phenotypic Screening for Putative *Vibrio* Species

Pure cultures of putative *Vibrio* isolates were initially identified via analyzing phenotypic properties following the criteria described by [12,13].

## 2.4 Molecular Identification of *Vibrio* Species Using Polymerase Chain Reaction (PCR)

Presumptive *Vibrio* species isolates were grown in tryptone soya broth (TSB, Oxoid) supplemented with 2% (w/v) NaCl and incubated at 30°C for 18-24 h. The broth cultures were then transferred into 1.5 ml Eppendorf tubes and centrifuged at 5000 xg at room temperature. The resulting pellet was used for nucleic acid extraction.

### 2.4.1 DNA extraction from pure culture

The DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen, CA, USA) according to manufacturer's instructions. Briefly, the culture pellet was re-suspended in ATL buffer. Then, cell lysis was performed by proteinase K and incubation in a shaking water bath at 56°C/60-

90 min. Then, AL buffer was added and incubated for a further 10 min at 70°C. The DNA precipitation was performed by ethanol (96%). The purified DNA was eluted by AE buffer. DNA concentration and purity were measured by spectrophotometer.

### 2.4.2 PCR amplification of 162 bp of the *Vibrio*-specific 16S rRNA gene

The eluted DNA was used as a template for PCR detection of *Vibrio*-specific 16S rRNA gene using two primers targeting 162 bp; forward primer 5'-GTCAAAGCGATGCAGGTG-3' and reverse primer 5'-CTTCGCCACCGGTATTCCTT-3' [20].

PCR amplification was performed according to manufacturer's instructions. A total 25 µl volume contains 12.5 µl of HotStarTaq Master Mix (Qiagen, CA, USA), 1.5 µl of 10 µM of each primer and 3 µl of DNA template. The mixture was processed in a gradient thermal cycler (LongGene, A200, Japan) with the following condition; 95°C/15 min as an initial activation step for HotStarTaq DNA polymerase. To minimize PCR products derived from mis-priming events, the actual amplification was initiated with a "touchdown" PCR step consisting of 19 cycles at 94°C/30 sec, 60-51°C/30 sec (decreasing 0.5°C/ cycle) and 72°C/1 min followed by 11 cycles of 94°C/30 sec, 51°C/30 sec and 72°C/1 min. The process was finished with a single cycle at 72°C/5 min. The template-free reactions were included in the PCR setup as negative controls.

### 2.4.3 PCR product visualization

The amplified products were analyzed by electrophoresis on a 1.5% agarose gel in 1x TAE

buffer at 100 V for 45 min and visualized by ethidium bromide staining and a UV transilluminator (ECX-F20. M, France) and photographed by MicroDoc (Cleaver Scientific, UK). A 100-bp DNA Ladder (Promega, G210A, USA), consisting of DNA fragments ranging in size from 1500 to 100 bp, was used as a molecular weight marker.

### 3. RESULTS AND DISCUSSION

Fish were captured during May through August, 2012. Fish diseases including vibriosis outbreaks are more prevalent in spring and summer, high temperature is a major predisposing risk factor for most types of vibriosis, making it a summer disease in most, but not all cases [16].

Fish liver, spleen and kidneys are iron-rich filtering organs that make them predilection sites for *Vibrio* species localization [16,21,22].

The clinical examination of fish samples, revealed the presence of some clinical signs similar to those reported by [19] for vibriosis including, skin darkness and scales detachment, ulcers, small and large areas of hemorrhages distributed over many parts of the body, particularly at fins bases, mouth region, and abdomen area which varied in its severity from fish to another. The clinical signs were well marked in *Mulloidichthys vanicolensis*, mild in *Siganus rivulatus* and no clinical signs were observed in *Lethrinus lentjan*. Haemorrhages were most common in *Mulloidichthys vanicolensis* while only few *Siganus rivulatus* samples showed small areas of haemorrhages distributed over some parts of the bodies.

The PM examination of fish species, revealed the presence of lesions characteristic to vibriosis; varied degrees of congestion, enlargement of internal organs especially liver, spleen and kidneys, distention of gall bladder, congestion of intestines, and accumulation of body fluids in the abdominal cavity similar to those reported by [23,24]. The severity of PM lesions was in accordance with the clinical signs.

A total of 43 putative *Vibrio* isolates were obtained from TCBS agar [25]. The phenotypic properties analyzing of 30 putative *Vibrio* species isolates came in accordance with [12,13]. The 30 isolates (*Siganus rivulatus* (10), *Mulloidichthys vanicolensis* (12) and *Lethrinus lentjan* (8) were discriminated phenotypically into five presumptive *Vibrio* species including, *Vibrio cholera* (n=11), *Vibrio anguillarum* (n=8), *Vibrio*

*fluvialis/ Vibrio furnissii* (n=4), *Vibrio harveyi* (*V. carchariae*) (n=4) and *Vibrio alginolyticus* (n=3), as outlined in Table 2. The remaining 13 isolates (*Siganus rivulatus* (3), *Lethrinus lentjan* (6) and *Mulloidichthys vanicolensis* (4) were proved to be Gram-negative rods, motile, oxidase positive and have requirements for sodium chloride but were resistant to vibriostatic 0/129 agent at both concentrations (10 and 150 µg). Hence, it wasn't initially possible to repudiate that the 13 isolates are *Vibrio* species through phenotypic properties analyzing (Table 3).

As shown in Fig. 2 and Table 3 when the 43 isolates were submitted for molecular identification using PCR via targeting *Vibrio*-specific 16S rRNA gene, the 30 vibriostatic 0/129 agent sensitive isolates were found positive for 16S rRNA gene. As well, 9 out of the 13 vibriostatic 0/129 agent resistant isolates were found positive (*Siganus rivulatus* (1/3), *Lethrinus lentjan* (4/6) and *Mulloidichthys vanicolensis* (4/4) while the remaining 4 isolates were negative,. Subsequently, TCBS medium is associated with limitations and is not 100% selective for the isolation of *Vibrio* species where bacteria other than *Vibrio* such as *Enterobacteriaceae* could be grow on it [26,27].

Vibriostatic 0/129 agent sensitivity test is of a great value in differentiating some *Vibrio* species from other Gram-negative rods and particularly from aeromonads, which are characteristically resistant to vibriostatic 0/129 agent. The degree of sensitivity of vibrios to 0/129 agent can also be used as a diagnostic feature in differentiation of *Vibrio* species; *Vibrio anguillarum* is sensitive to 0/129 agent at both concentrations while *Vibrio fluvialis* is sensitive to 0/129 agent at 150 µg and resistant at 10 µg [12,13]. It is important to emphasize that negative 0/129 agent test must be interpreted with caution; 9 vibriostatic 0/129 agent resistant isolates were identified as *Vibrio* species using PCR.

The identity of putative *Vibrio* species isolates obtained from TCBS medium should therefore be confirmed employing a number of phenotypic and genetic techniques to accurately identify the atypical isolates [16,28,29]. PCR technique was definitely able to confirm 30 phenotypically presumptive *Vibrio* species and to assert the suspicions related to 13 vibriostatic 0/129 agent resistant isolates.

No *Vibrio* species were isolated from some *Mullicthys vanicolensis* and *Siganus rivulatus*

samples in spite of observing clinical signs, and vice versa. Consequently, the clinical signs might be due to *Vibrio* species infection and/or other etiological agents.

**Table 2. Phenotypic profiles of *Vibrio* species isolated from examined fish**

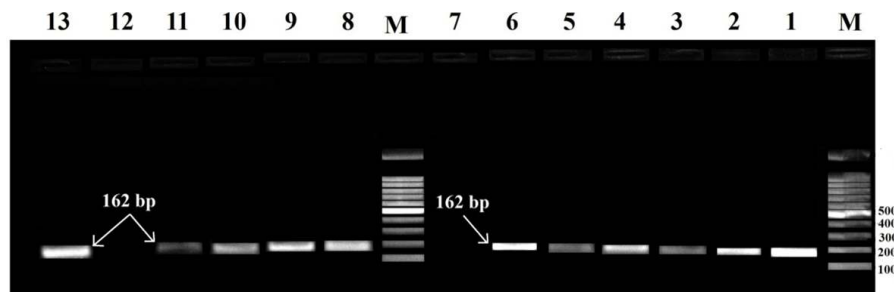
Test	Percentage of isolates positive in the corresponding test (%)				
	<i>V. cholerae</i> (n=11)	<i>V. fluvialis</i> / <i>V. furnissii</i> (n=4)	<i>V. harveyi</i> ( <i>V. carchariae</i> ) (n=4)	<i>V. anguillarum</i> (n=8)	<i>V. alginolyticus</i> (n=3)
TCBS	100	100	100	100	100
Gm	0	0	0	0	0
Mot	100	100	100	100	100
Ox	100	100	100	100	100
Cat	100	100	100	100	100
Ind	0	50	100	0	100
MR	0	75	75	0	100
VP	81.8	0	0	100	100
Cit	72.2	75	100	100	100
ONPG	100	100	100	100	0
LDC	100	0	100	0	100
<b>NaCl requirement/ tolerance test:</b>					
0% NaCl	100	0	0	12.5	0
3% NaCl	100	100	100	100	100
6% NaCl	18.2	100	100	100	100
10% NaCl	0	0	25	0	100
<b>Vibriostatic agent (0/129) sensitivity test:</b>					
At 10 µg	100	0	0	100	0
At 150µg	100	100	100	100	100

TCBS= Growth on thiosulfate-citrate-bile salts-sucrose agar; Gm= Gram stain test; Mot= Motility test; Ox= Oxidase test; Cat= Catalase test; Ind= Indole production test; MR= Methyl red test; VP= Voges-Proskauer test; Cit= Citrate utilization test; ONPG= O-nitrophenyl-β-galactopyranoside (β-galactosidase test); LDC= Lysine decarboxylase test

**Table 3. PCR-based identification of presumptive *Vibrio* species isolates and screening of vibriostatic 0/129 agent resistant isolates for *Vibrio* species**

Fish species	Presumptive <i>Vibrio</i> species isolates				0/129 agent resistant isolates			
	No. of isolates	Positive isolates		No. of isolates	Positive isolates			
		N	%		N	%		
<i>S. rivulatus</i>	10	10	100	3	1	33.3		
<i>M. vanicolensis</i>	12	12	100	4	4	100		
<i>L. lentjan</i>	8	8	100	6	4	66.7		
Total	30	30	100	13	9	69.2		

N= number of isolates; % =Percentage of isolates positive for *Vibrio*-specific 16S rRNA



**Fig. 2. The PCR product obtained using specific primers targeting 162 bp of *Vibrio* spp 16S rRNA gene after agarose gel electrophoresis and staining by ethidium bromide (0.5 ug/ml) and photographed by MicroDoc system using Canon digital camera**

M= 100 bp DNA ladder; Lane 7: negative control (master mix without DNA); Lane 13: Positive control (*V. anguillarum*); Lanes: 1-6 and 8-11 *Vibrio* species positive samples; Lane 12: *Vibrio* species negative sample

As outlined in Table 4, *Vibrio* species were detected in 37.1% of the total examined fish samples including, *Vibrio cholerae* (10.5%), atypical *Vibrio* species (8.6%), *Vibrio anguillarum* (7.6%), 3.8% for each of *Vibrio fluvialis*/ *Vibrio furnissii* and *Vibrio harveyi* (*Vibrio carchariae*), and *Vibrio alginolyticus* (2.9%). The prevalence of different *Vibrio* species varied among the examined fish species, *Mulloidichthys vanicolensis* showed the most prevalent *Vibrio* species (47.1%) followed by *Lethrinus lentjan* (34.3%) and *Siganus rivulatus* (30.6%). *Vibrio cholerae* was the most predominant (28.2%) among isolated *Vibrio* species. It was found that *Vibrio cholera* showed the highest isolation rate from *Mulloidichthys vanicolensis* (14.7%) followed by *Siganus rivulatus* (13.9%) and *Lethrinus lentjan* (2.9%). The *Vibrio anguillarum* was detected in 14.7%, 8.6% and 0% of *Mulloidichthys vanicolensis*, *Lethrinus lentjan* and *Siganus rivulatus*, respectively. *Vibrio alginolyticus* was detected only in *Lethrinus lentjan* with a level of 8.6%. Atypical *Vibrio* species were detected in 11.8% and 11.4% and 2.8% of *Mulloidichthys vanicolensis*, *Lethrinus lentjan* and *Siganus rivulatus*, respectively. The variations of *Vibrio* species occurrences and infection rates in the different fish species may be associated to various factors, including *Vibrio* species virulence, fish immunity, fish genetic factors, and physiological status, as well as the environmental stress conditions [1,2,30].

The total prevalence of *Vibrio* species (37.1%) in naturally infected marine fish species which appears to be higher than that reported by [31] which could be due to that most of the collected fish samples were freshwater fish while most *Vibrio* species are halophilic what decreases the

viability and variability of *Vibrio* species in freshwater fish; this fact is very clear with comparison between *Vibrio* species incidences in *Siganus rivulatus* (50%) and *Oreochromis niloticus* (12.8%) in the same study. Also, they only sampled fish fleshes but in the present study liver, kidneys and spleen of examined fish were sampled [16,21,22]. The different fish species under both studies could give rise to variations in their susceptibility to *Vibrio* species infections. The high prevalence of *Vibrio* species may be attributed to the stresses induced by abundant anthropogenic activities in Hurghada City coastline zone in contrary to cultured fish farms which could be under good sanitary and management conditions. One of the most strong possible causes is that [31] depended in their study only on bacteriological analysis of examined fish and did not apply any molecular technique for screening their bacterial isolates for additional *Vibrio* species isolates while during the present study, applying of PCR technique added 9 *Vibrio* species isolates plus 30 initially phenotypically identified and PCR confirmed *Vibrio* species isolates.

The total prevalence of *Vibrio* species (37.1%) appears to be lower than that reported by [32] who analyzed 150 samples from two types of freshwater fish (included, catfish "*Pangasius hypophthalmus*" and red tilapia "*Oreochromis spp*") commonly sold at hypermarkets in state of Selangor, Malaysia, for the presence of *Vibri*species applying PCR via targeting *Vibrio*-specific 16S rRNA gene. Different fish species under both studies could give rise to variations in their susceptibility to *Vibrio* species also, they sampled the flesh and gills of fish which are in direct contact with aquatic environments bacteria

**Table 4. Prevalence and distribution of *Vibrio* species among the examined fish**

Fish name	<i>Vibrio</i> spp.													
	<i>V. cholerae</i>		<i>V. fluvialis</i> / <i>V. furnissii</i>		<i>V. carchariae</i> / <i>V. harveyi</i>		<i>V. anguillarum</i>		<i>V. alginolyticus</i>		Atypical <i>Vibrio</i> species		Total <i>Vibrio</i> species	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%
<i>S. rivulatus</i> (n=36)	5	13.9	1	2.8	4	11.1	-	-	-	-	1	2.8	11	30.6
<i>M. vanicolensis</i> (n=34)	5	14.7	2	5.9	-	-	5	14.7	-	-	4	11.8	16	47.1
<i>L. lentjan</i> (n=35)	1	2.9	1	2.9	-	-	3	8.6	3	8.6	4	11.4	12	34.3
Total = 105	11	10.5	4	3.8	4	3.8	8	7.6	3	2.9	9	8.6	39	37.1

N= number of isolates; %= Percentage of isolates calculated to the number of examined fish

including, *Vibrio* species and sampled fish intestinal tracts where genus *Vibrio* dominates microbiota [33,34].

#### 4. CONCLUSION

The pathogenic *Vibrio* species are prevalent among the examined economic fish in Red Sea, along Hurghada City coastline, Egypt which possess public health concerns, necessity need for applying hygienic measurements to avoid water contamination and spread of infection among aquatic animals and human. The usage of combination of molecular and conventional methods for *Vibrio* species identification is necessary.

#### SIGNIFICANCE AND IMPACT OF THE STUDY

*Vibrio* species are common inhabitants in/on the aquatic animals, some species are pathogenic for aquatic animals and others are pathogenic for humans, meanwhile other species are pathogenic for both aquatic animals and humans. So, this study identifies *Vibrio* species isolated from three economic fish species in the Red Sea, Egypt, and assesses their prevalence and distribution using the conventional and molecular methods. The pathogenic *Vibrio* species for aquatic animals and humans are prevalent among economic fish in Egypt which possess public health concerns. The usage of molecular and conventional methods, together, for *Vibrio* species identification is necessary.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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