Prevalence and Molecular Characterization of Vibrio Spp. in Fish and Shellfish From Port Said Coastal Area

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Abstract

This study was carried out during the period from April 2014 to November 2014. A total of 250 seafood samples, 50 samples each namely Mullet, Sardine, Shrimp, Cuttlefish and Mussel, obtained by random sampling from the coast of Port Said Governorate. Samples were subjected to conventional method for the detection of *Vibrio* species. Overall, 32% of the Mullet, 36% of Sardine, 52% of Shrimp, 44% of both Cuttlefish and Mussel samples were found to be positive for *Vibrio spp*. The prevalence of *Vibrio* species was higher in shellfish samples, particularly in shrimp than that of fish samples. Four *Vibrio* species were identified. *V. alginolyticus* was found to be the dominant identified *Vibrio* species with total prevalence of 48.2% (50/104) followed by *V. damsela* 24% (25/104), *V. harveyi* 16.3% (17/104) and *V. parahaemolyticus* 11.5% (12/104). Eight isolates identified phenotypically as *Vibrio* species, were confirmed by PCR.

Introduction

Vibrionaceae are natural pathogens of shellfish, shrimp and other aquatic organisms ***(Raissy et al., 2011)***. *Vibrio spp*. are Gram-negative, comma-shaped, highly motile with one or more polar flagella and halophilic ***(Thompson et al., 2004)***.Within the *Vibrio* genus there are several species that have a high tolerance for different salinity levels ***(Wright et al., 1996)***. In the last several years, ***Thompson et al. (2004)*** introduced a classification strategy for Vibrios that based on 16S rRNA gene sequencing. The 16S rRNA gene considered as the standard for phylogenetic classification ***(Clarridge, 2004)***.Accurate identification at the family and genus levels of Vibrios is obtained by 16S rRNA gene technique ***(Thompson et al., 2004).***

Epizootics of Vibriosis take place in fish in presence of overcrowding, poor hygiene and organically polluted water ***(Noga, 2000)***. Infectious *Vibrio* species can affect a wide range of marine organisms

causing mass mortality ***(Colwell, 2006)***. Diseases caused by *Vibrio* species were reported in aquatic animals such as oysters, fish, shrimp and lobster ***(Chrisolite et al., 2008)***. Twelve *Vibrio* species have been documented as potential food-borne disease agents in humans: *V. cholerae, V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. funissii, V. fluvialis, V. damselae, V. mimicus, V. hollisae, V. cincinatiencis, V. harveyi and V. metchnikovii.* ***(Adams and Moss, 2008)***. Due to increase in seafood consumption and the global warning, which may cause a higher prevalence of *Vibrio* species and increase in the risk of *Vibrio* borne infections, the present study was conducted for investigating the prevalence, biochemical and molecular identification of *Vibrio* species in fish and shellfish samples from Port Said coastal area.

Materials and Methods

1. Fish and shellfish samples:

A total of 250 seafood samples, (100 fish samples): 50 Mullet (*Mugil cephalus*) and 50 Sardine (*Sardinella spp.*), (150 shellfish samples): 50 Shrimp (*Penaeus spp.*), 50 Cuttlefish (*Sepia spp.)* and 50 Mussel (*Donax trunculus*) were collected freshly during the period from April 2014 to November 2014from fishing boats at the time of landing and from dip net near shore region of coastal area of Port Said Governorate. Samples were put in sterile polythene in insulated ice-boxes with ice and conveyed to Port Said laboratory for Food Hygiene, Bacteriology Unit for bacteriological examination. Full descriptions of samples names, number and sampling are showed in Table (1).

2. Bacteriological examination of fish and shellfish samples for detection of *Vibrio spp*.:

2.1. Isolation of *Vibrio* Species from Seafood Samples:

Isolation and identification of *Vibrio* spp. were determined according the methodology outlined in ***FDA's Bacteriological Analytical Manual (2004)***. Briefly, the fish and shellfish samples were homogenized in a Stomacher 400 Circulator at120 rev/for 2 min and 25g of each homogenate was placed in 225ml of alkaline peptone water (APW) then incubated at 35°C for 18-24 h. At the end of incubation period, and without shaking flask, loopful of culture from pellicle (surface growth) was streaked onto Thiosulphate Citrate Bile salt Sucrose (TCBS) agar plates (HiMedia, India) and incubated at 35°C for 18-24 h. Yellow and green colonies from TCBS media suspected to be *Vibrio* species were picked and purified by streaking onto Tryptic Soy Agar (TSA; HiMedia, India) plates supplemented with 2% w/v sodium chloride. The TSA agar plates were incubated at 35C under aerobic conditions for 18–24 h. A loopful of pure isolate was inoculated into semi-solid nutrient agar, incubated at 35C for 18-24 h, tubes were

tightly caped and stored at 20-25ºC to preserve culture and then stored until further analysis.

2.2. Morphological and biochemical characterization of isolates:

The isolates were identified at the species level on the basis of the scheme and the methodology outlined in ***FDA's Bacteriological Analytical Manual (2004)*** and that proposed by ***Alsina and Blanch (1994a and 1994b)***.

Characterization of isolates included Gram staining, motility test, Oxidase test, catalase test, reactions on KIA, sucrose utilization, carbohydrate fermentation test, Halophilisms test (growth on media containing 0, 3, 6, 8, 10% Nacl), Amino acid decarboxylase test (Arginine dihydrolase, Lysine decarboxylase, Ornithine decarboxylase), MR-VP test, urease test, citrate utilization, growth at 42 ºC, beta-galactosidase (ONPG) and sensitivity to Vibriostatic agent O/129 (150 and 10 µg).

2.3. Molecular Identification of Isolates:

Eight *Vibrio* isolates from the above assay results (1 isolates from Mullet, 1 from Sardine, 2 from Shrimp, 2 from Cuttlefish and 2 from Mussel) were further confirmed using PCR. The 16S rRNA gene were used as target sequences to confirm the identities of the presumptive *Vibrio* isolates to the genus level using specific primers in the polymerase chain reaction assay.

2.3.1. DNA extraction:

The presumptively identified *V.* species were grown overnight in Tryptone soy broth (TSB) supplemented with 3% NaCl, DNA extraction was carried out according to **QIAamp DNA mini kit instructions**.

2.3.2. DNA Molecular weight marker: Gel Pilot 100 bp plus ladder (cat. no. 239045) supplied from QIAGEN (USA).

Size range: 100-1500 bp.

2.3.3. Oligonucleotide Primers: Oligonucleotide Primers used to amplify *Vibrio* species are listed in Table (2).

2.3.3. PCR assays: PCR amplification of the target DNA was carried out in a thermal cycler .The reaction conditions according to ***Sambrook et al. (1989)*** were as follows: 94°C for 10 minutes (to make Primary denaturation), then 94°C for 45 sec. (to make Secondary denaturation), then to make cycle 50˚C for 45 sec. (Annealing) and 72°C for 45 sec (initial Extension). All are 35 cycles followed by 1 cycle of 72°C for 10 minutes (to make Final extension).Then the ladder was mixed gently by pipetting up and down. 6 µl of the required ladder were directly loaded. Twenty µl of each PCR product samples, negative control and positive control were loaded to the gel. The power supply was 1-5 volts/cm of the tank length. The run was

stopped after about 30 min and the gel was transferred to UV cabinet. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

Table (1): *Species and total numbers of fish and shellfish examined samples:*

Family name	English name	Local name	Latin name	No. of sample	Sampling
Mugillidae	Mullet	Bori	*Mugil cephalus*	50	Composite samples comprising whole body parts
Clupeidae	Sardine	Sardina	*Sardinella spp.*	50	
Penaeidae	Shrimp	Gambary	*Penaeus spp.*	50	Pooling of 13-15 shrimp
Sepiidae	Cuttlefish	Sepia	*Sepia spp.*	50	Pooling of muscles
Donacidae	Mussel	Um elkholol	*Donax trunculus*	50	Pooling of 20 Mussel include meat and liquor
Total Samples analyzed				**250**	

Table (2): *Primer used in PCR identification of Vibrio species:*

Gene	Primer	Sequence 5′–3′	Amplified product	Reference
16S rRNA	*V.16S-700F*	CGGTGAAATGCGTAGAGAT	663 bp	**Tarr *et al.*, (2007)**
	V.16S-1325R	TTACTAGCGATTCCGAGTTC		

Results

Table (3): *Morphological and biochemical characteristics of Vibrio spp. isolated from fish and shellfish samples:*

Test /species	*V. alginolyticus*	*V. damsel*	*V. harveyi*	*V. parahaemolyticus*
TCBS agar	Y	G	Y	G
Gram stain	-	-	-	-
Shape	Rods	Rods	Rods	Rods
Motility	+	+	+	+
Catalase test	+	+	+	+
Growth in 0% NaCl	-	-	-	-
Growth in 3% NaCl	+	+	+	+
Growth in 6% NaCl	+	V	+	+
Growth in 8% NaCl	+	-	V	+
Growth in 10% NaCl	+	-	V	+
Growth at 42ºC	+	-	V	+
Sucrose	+	-	V	-
D-Cellobiose	-	+	V	V

Lactose	-	-	V	-
Arabinose	-	-	-	+
D-Mannose	+	+	+	+
D-Mannitol	+	-	+	+
Voges-Proskauer test	+	+	-	-
Methyle red test	V	+	+	+
Citrate utilization	-	-	+	-
Kligler Iron Agar reaction	K/A	K-A/A	K/A	K/A
Arginine dihydrolase	-	+	-	-
Lysine decarboxylase	+	V	+	+
Ornithine decarboxylase	+	-	+	+
ONPG	-	-	-	-
Sensitivity to 10 µg 0/129	R	S	R	R
Sensitivity to 150 µg 0/129	S	S	S	S
Urease test	-	-	V	V

Abbreviations: TCBS, thiosulfate-citrate-bile salts-sucrose Y = yellow, G = green, V = variable, + = positive, - = negative, K/A =Slant alkaline /Butt acidic, S = susceptible, R = resistant.

Table (4): *Total identified Vibrio spp. and Percentage of occurrence in the examined fish and shellfish samples:*

Identified isolates	**No.**	**%**
V. alginolyticus	50	48.2
V. damsela	25	24
V. harveyi	17	16.3
V. parahaemolyticus	12	11.5
Total	104	100

NB: Percentage was calculated according to the total number of the isolates (104).

Table (5): *Prevalence and frequency of occurrence of Vibrio spp. in fish and shellfish samples from costal area of Port Said (n =50):*

Samples	Positive samples No. (%)	Identified *Vibrio* species No. (%)	
Mullet	**16 (32%)**	*V. alginolyticus*	9(56.25)
		V. damsela	3(18.75)
		V. parahaemolyticus	4 (25)
Sardine	**18 (36%)**	*V. alginolyticus*	9 (50)
		V. damsela	4 (22.2)
		V. harveyi	5 (27.8)
Shrimp	**26(52%)**	*V. alginolyticus*	10 (38.5)
		V. damsela	6 (23)
		V. harveyi	8 (30.8)
		V. parahaemolyticus	2(7.7)
Cuttlefish	**22 (44%)**	*V. alginolyticus*	14 (63.6)
		V. damsela	6 (27.3)
		V. parahaemolyticus	2 (9.1)
Mussel	**22(44%)**	*V. alginolyticus*	8 (36.3)
		V. damsela	6 (27.3)
		V. harveyi	4 (18. 2)
		V. parahaemolyticus	4 (18. 2)

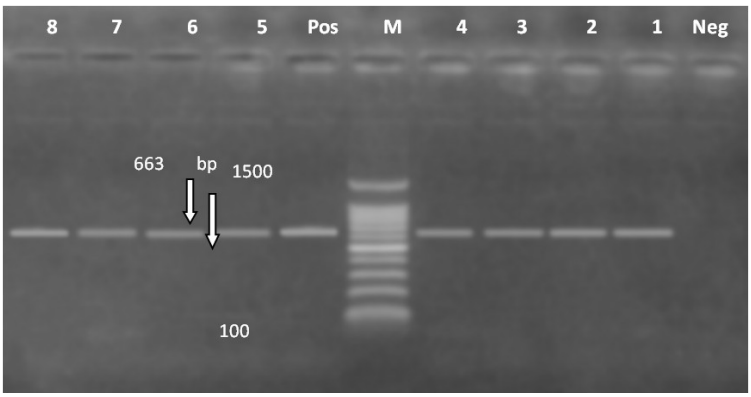

Fig. (1): *Gel electrophoresis of the PCR products of some of the confirmed Vibrio species:*

Lane (M): Lane 1 to 8 is positive to *Vibrio* spp. with a 663 bp.
Eight *Vibrio* isolates: no (1) isolate from Mullet, no (2) from Sardine, no (3, 4) from Shrimp, no (5, 6) from Cuttlefish and no (7, 8) from Mussel.

Discussion

In the present study a total of 250 samples, 50 of each Mullet, Sardine, Shrimp, Cuttlefish and Mussel were examined. A full bacteriological investigation of isolated *Vibrio* spp. was done by Morphological, colonial and biochemical characters.

The results obtained from morphological and biochemical tests have been shown in Table (3).

On the basis of all biochemical and morphological characteristics, isolates were found closely related to 4 species namely *V. alginolyticus, V. damsela, V. harveyi* and *V. parahaemolyticus*. The morpho-chemical characteristics of 50 identified *V. alginolyticus* isolates in the present study coincided with the *V. alginolyticus* profiles reported by ***Costinar et al., (2010)***. *V. alginolyticus* is one of the most dangerous pathogens causing damage in finfish, crustaceans and shellfish ***(Hormansdorfer et al., 2000)***. *V. alginolyticus* has been associated with human infection such as cellulitis, wound infection and seawater-related otitis media ***(Matsiota-Bernard and Nauciel, 1993 and Mukherji et al., 2000)***.

The morpho-chemical characteristics of 25 identified *V. damsela* isolates were in accordance with biochemical profile of *V. damsela* reported by ***Labella et al., (2010)***.*V. damsela* was isolated from outbreaks affecting several fish species in southern Spain. Moreover, *V. damsela* has been reported to cause diseases in human and was considered as zoonotic pathogen ***(Labella et al., 2011)***.

In the current study, the biochemical profile of 17 isolates assigned as *V. harveyi* coincided with the *V. harveyi* profiles that reported by ***Robertson et al., (1998)***. *V. harveyi* is a well-known pathogen of marine finfish and shellfish and is the causative agent of luminous disease, which resulted in 80 to 100% mortality in *Penaeus Monodon* hatcheries ***(Austin and Austin, 1999)***. Insight analysis of the morpho-chemical characteristics for the 12 of isolates in the present study coincided with the *V. parahaemolyticus* profiles which was in accordance with that reported by ***Alsina and Blanch (1994a)***. *V. parahaemolyticus* is responsible for mass mortalities among fish stocks in many marine fish farms throughout the Mediterranean area and severe economic losses in aquaculture worldwide ***(Actis et al., 1999)***. *V. parahaemolyticus* is one of the twelve *Vibrio* species occurring in human and represents one main cause for foodborne gastroenteritis, especially in Asia and the United States ***(Sua and Liu, 2007)***.

The present results tabulated in Table (4) revealed that *V. alginolyticus* was found to be the dominant identified *Vibrio* species with total prevalence of 48.2% (50/104) followed by *V. damsela* 24% (25/104), *V. harveyi* 16.3%(17/104) and *V. parahaemolyticus* 11.5% (12/104), in that order, high preponderance of *V. alginolyticus* (57% incidence) was also reported by ***Bhasker and Setty (1994)***. Also ***Thararat et al., (2009)*** found that contamination of raw seafood by *V. alginolyticus* was most frequent (61.5 %).

The present data in Table (5) revealed that the samples of sea foods analyzed microbiologically in this study showed varying degree of *Vibrio* contamination. Overall, 32% of the Mullet, 36% of Sardine, 52% of Shrimp, 44% of both Cuttlefish and Mussel samples were positive for *Vibrio spp*. These results were nearly agreed with the results obtained by ***Abd-El-Latif et al. (2008)*** who isolated *Vibrio* spp. with a percentage of 33.75% from healthy *Mugil cephalus* fish. Also the present results go with findings obtained by ***(Kriem et al., 2015)*** who found that the overall prevalence of *Vibrio* spp. in shrimps was 55.8%. In India, shrimp samples (41%) and clam samples (42%) harboured heavy load of Vibrios ***(Bhasker et al., 1998)***. In a study by ***Merwad et al. (2011)***, the overall prevalence of Vibrios was 57.3% in white shrimps, 48% in blue crabs and 54% in oysters. However, ***Pinto et al. (2008)*** reported 32.6% for mussels in Italy. Since *Vibrio spp*. can occur naturally in an aquatic environment, the presence of these organisms in raw seafood may be expected ***(El-Hadi et al. 2004)***. The difference in prevalence may be attributed to water quality and temperature. In this concern, ***Sung et al. (1999)*** reported that the prevalence of *Vibrio* species varied according to the season.

The present results revealed that the prevalence of *Vibrio* species were higher in shellfish samples, particularly in shrimp than that of fish samples. Shrimp is one of the most important fishery products of the coastal area of Port Said provinces. Numerous studies have been done on Vibriosis in shrimp the incidence of *Vibrio* in shrimp is of significant importance ***(Ansari and Raissy 2010)***.

Regarding the molecular characterization, the representative gel photo of the PCR has been shown in Figure (1). The PCR amplifications of 16S rRNA from the 8 selected isolates were successfully carried out using PCR primers designed in the study. The molecular analysis carried out on 8 selected isolates, identified as *Vibrio* spp. gave positive results for selected 8 strains. As it is observable, the 663bp. bands that have appeared on the gel for the *Vibrio* spp. corresponding to the 16S rRNA gene. Molecular approaches to the identification and characterization of *Vibrio* spp. has been developed and highly utilized due to their higher sensitivity and specificity rather than the conventional methods ***(Di Pinto et al., 2005)***. The 16S rRNA gene is used for both phylogenetic studies and as a taxonomic marker ***(Thompson et al., 2005)***. The relationship studies based on the 16S rRNA comparison have been extensively used in *Vibrio* classification due to most of *Vibrio* species have more than 90% 16S rDNA similarities ***(Aznar et al., 1994)***.

Conclusion and recommendation

It can be concluded that local fish and shellfish in Port Said coast were contaminated with *Vibrio* species. The prevalence of *Vibrio* species was higher in shellfish samples, particularly in shrimp than that of fish samples.

Monitoring the prevalence of *Vibrio* is particularly important especially with the increasing utilization of sea, brackish and island waters near coast to cultivated and fatten fish of various species. The presences of these organisms in the fresh seafood samples showed that seafood is predisposed to contamination by *Vibrio*. Thus, it is becomes advisable that sea foods be adequately subjected to proper boiling and cooking before consumption.

Referances

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الملخص العربى

انتشار و التوصيف الجزيئي لانوع الفيبريو في الاسماك والمحاريات في المنطقة الساحلية ببورسعيد

حمزه محمد عيد **زينب إبراهيم سليمانالشيماء توفيق حنفي**

**قسم البكتريولوجي و المناعة و الفطريات - كلية الطب البيطري- جامعة قناة السويس*

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تهدف هذه الدراسة الى عزل وتصنيف انواع الفيبريو المختلفة من الاسماك والمحاريات من الشريط الساحلى لمدينة بورسعيد. تم جمع ٢٥٠ عينة عشوائية بواقع ٥٠ عينة من كل من اسماك البورى والسردين وام الخلول والجمبرى وكذلك السيبيا فى الفترة من ابريل ٢٠١٤ الى نوفمبر ٢٠١٤ والتى تم نقلها مبردة و تم فحصها بمعمل فحوص صحة الاغذية ببورسعيد بمجرد وصولها للكشف عن ميكروب الفيبريو بالطريقة البكتريولوجية والبيوكيميائية. اسفرت الدراسة عن وجود ميكروب الفيبريو فى ٣٢% و ٣٦ % و ٥٢% و ٤٤% و٤٤% من عينات البورى والسردين والجمبرى وام الخلول والسيبيا على التوالى. وقد تبين ان العترات المعزولة تنتمى الى اربعة انواع من جنس الفيبريو وقد سجل الفيبريو الجيلنوليتكس اعلى نسبة من المعزولات وكانت ٤٨,٢ % (٥٠/ ١٠٤) يليه الفيبريو دامسيلا ٢٤% (١٠٤/٢٥) ثم الفيبريو هارفى ١٦,٣ % (١٧/ ١٠٤) واخيرا الفيبريو باراهيمولتيكس بنسبة ١١,٥ % (١٢/ ١٠٤) من العترات التى تم تصنيفها بالطريقة البيوكيميائية. هذا وقد تم اختيار ٨ معزولات من التى تم عزلها للتاكد من انها تنتمى لجنس الفيبريو بالفحص الجزئى واختبار البلمرة الجزئى وقد تبين ان العترات تنتمى الى جنس الفيبريو.