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Incidence of *Vibrio* Species in Some Crustaceans and Molluscus .

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Abstract

Crustaceans and Molluscus, as Crab and Oyster are filter-feeding organisms that concentrate microorganisms, especially the human enteric viruses and pathogenic *Vibrio* species. This study aimed to detect and accurate identify *Vibrio* sp. in fresh and marine Crab and Oyster specimens especially that of public health important as *cholerae, Vibrio parahaemolyticus, Vibrio mimicus* and *Vibrio vulnificus*using using biochemical methods and PCR methods (16SrRNA and mPCR). The prevalence of *Vibrio* species was 5%, 50% and 65% in fresh Crab, marine Crab and Oyster samples respectively examined by traditional biochemical method appeared in examined fresh Crab samples as only one *vibrio mimicus* species as (5%). *Vibrio* species in collected marine Crab samples was *V. alginolyticus* (15%), *V. cholera* (5%), *V. parahaemolyticus* (10%) and *V. vulnificus* (20%). *Vibrio* species in oyster was *V. alginolyticus* (5%), *V. cholera* (10%), *V. mimicus* (5%), *V. parahaemolyticus* (20%) and *V.vulnificus* (25%).

Overall, results of the present study indicated that the using of 16SrRNA and m-PCR allowed rapid and accurate identification of the predominant and pathogenic *Vibrio* species which failed to be accurately identified by biochemical method. Furthermore, presence of *Vibrio cholerae and Vibrio mimicus* in examined samples considered a life threatening to human health.

Key words:Clams, *V. vulnificus, V. cholerae, V.parahaemolyticus, V. mimicus*, PCR.

Introduction

Seafood is a nutritious food that constitutes one of the desirable components of a healthy diet due to many nutritional benefits where they contain high-quality protein, low cholesterol level, having omega-3 fatty acids and minerals as well as vitamins. A well-balanced diet includes a variety of fish and shellfish can contribute to heart health and children's growth and development. Nevertheless, there is a health risks associated with the consumption of seafood. One of the major risks involves the consumption of raw or undercooked seafood that may be naturally contaminated by food-borne pathogens present in the marine environment. Such risk

is further increased if the food is mishandled during processing where pathogens could multiply exponentially under favorable conditions.

The majority of these illnesses are associated with the consumption of raw bivalve, including oysters and others, are filter-feeding organisms that pump seawater through their digestive systems to obtain oxygen and feed and in this process, accumulate and concentrate microorganisms. These organisms may be harmless, commensals as well as pathogens; the most prominent types are the human enteric viruses and the pathogenic *Vibrio* species. Since shellfish are frequently consumed whole and raw, they can serve as passive carriers of food borne disease agents **(Drake *et al*., 2007).**

Vibrio species are halophilic, Gram-negative bacteria that occur naturally in aquatic environments and are transmitted to humans primarily through consumption of contaminated water, raw or mishandled seafood. *Vibrio* infections can result in gastroenteritis, causing bloody diarrhea, or even septicemia in individuals with underlying chronic illness **(Centers for Disease Control and Prevention "CDC"2004).**Four species as *Vibrio cholerae, Vibrio parahaemolyticus, Vibrio mimicus and Vibrio vulnificus* are accounted for the majority of *Vibrio* infections in human being **(Tarr et al., 2007).** It was found that between 1998 and 2010, the incidence of *Vibrio*infections increased by more than 115%. The CDC estimates that 80,000 *Vibrio* infections (200 *V. vulnificus*, 45,000 *V. parahaemolyticus* and 35,000 *Vibrio* species) and approximately 100 deaths related to *Vibrio* infections may occur annually in United States**(Scallan et al., 2011).**

Most of these food borne infections are caused by *V.* parahaemolyticus, *V. mimicus, V. cholerae and V.vulnificus* **(WHO, 2005).** *Vibrio parahaemolyticus* is the leading cause of seafood associated illness in the United States and is generally associated with the consumption of raw molluscan shellfish **(Iwamoto et al., 2010**). Also, *Vibrio parahaemolyticus* is continuing to be the top causative agent among all the reported food poisoning outbreaks in recent years **(Adeleye et al., 2010).**

Vibrio vulnificus is another organism that can become life threatening if the bacterium enters the blood stream causing septicemia, septic shock and blistering skin lesions **(Levin, 2005).** Moreover, *V. cholerae* is the etiological agent of cholera, a severe diarrheal disease that occurs most frequently in epidemic form **(Wachsmuth et al., 1994).***Vibrio cholerae* infections if untreated, can result in fluid loss and osmotic shock, death may occur within few hours **(Cockburn and Cassanos, 1960).**

This study aimed to detect and accurate identify Vibrio sp. in clam's specimens especially that of puplic health important as *cholerae, Vibrio parahaemolyticus*,

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Vibrio mimicus and *Vibrio vulnificus* using biochemical methods and PCR methods (16S rRNA and mPCR).

Material and Methods

Sample Preparation and bacteriological examination:

A grand total of (60) fresh crab, marine crab and oyster specimens were collected from Olobour city, Benha city and Elmonofea governorate markets (20 of each) during summer of 2015 and 2016. All samples were transported into the laboratory without any delay in an ice box then subjected to bacteriological examination for detection of *Vibrio* species according to **FDA (2004).**

Biochemical identification:

according to **ISO/TS 21872-1(2007)** and **ISO/TS 21872-2 (2007).**

Polymerase Chain Reaction (PCR):

1. Primer sequences used in the current study:

1.1. Primer sequences of *Vibrio* species used for PCR identification system:

Application of PCR for identification and characterization of *Vibrio* species using different primers (Pharmacia Biotech) as shown in the following table:

Table (1): Primer sequences of *Vibrio* species:

Vibrio Spp.	Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
All	16S rRNA (F)	5′ CGGTGAAATGCGTAGAGAT ′3	663	**Tarr *et al.* (2007)**
	16S rRNA (R)	5′ TTACTAGCGATTCCGAGTTC ′3		
V.mimicus	sodB (F)	5′ CATTCGGTTCTTTCGCTGAT ′3	121	
	sodB (R)	5′GAAGTGTTAGTGATTGCTAGAGAT′3		
V.cholerae	sodB (F)	5′ AAGACCTCAACTGGCGGTA ′3	248	**Rao and Surendran (2013)**
	sodB (R)	5′ GAAGTGTTAGTGATCGCCAGAGT′3		
V.alginolyticus	gyrB (F)	5′ATTGAGAACCCGACAGAAGCGAAG′3	340	
	gyrB (F)	5′CCTAATGCGGTGATCAGTGTTACT′3		
V.vulnificus	Hsp (F)	5′ GTCTTAAAGCGGTTGCTGC ′3	410	
	Hsp (R)	5′ GTCTTAAAGCGGTTGCTGC ′3		
V. parahaemolyticus	flaE (F)	5′ GCAGCTGATCAAAACGTTGAGT′3	897	
	flaE (R)	5′ ATTATCGATCGTGCCACTCAC ′3		

1.2. Primer sequences of *V. parahaemolyticus*for virulence factors:

Characterization of virulence factors including thermostable direct hemolysin (tdh), thermolabilehemolysin (tlh) and tdh-related hemolysin (trh) genes of *V. parahaemolyticus* was adopted.

Table (2): Primer sequences of *V. parahaemolyticus*

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
tdh (F)	5′ TTGGAAACGGTTAAAACGAA′3	269	**Bej *et al.* (1999)**
tdh (R)	5′ GAACCTTCCCATCAAAAACA ′3		
tlh (F)	5′ TCGCATCAAACTGACAAACG ′3	450	
tlh (R)	5′ GCGGTACTCTATAAGTGCC ′3		
trh(F)	5′ CTAGTTTGGTAATATCTCCT ′3	500	
trh(R)	5′ TAATGCTATATCTTATAGGG ′3		

2. DNA extraction from the bacterial culture (Tarr et al., 2007):

After overnight culture on tryptic soy agar plates with 3% Nacl incubated at 35°C, a single colony was scraped from the plate's surface and suspended in 200µl of 1× Tris-EDTA of pH 8.0. The suspension was then heated at 95°C for 10 minutes and centrifuged for 2 minutes to pellet cellular debris. The pellet was then re-suspended in 500 µl of TE buffer (pH 7.8) and vortexed. All the samples were finally lysed for 10 minutes in a boiling water bath. Further, the lysate was cooled in an ice bath. From this suspension, 2 µl aliquot was directly used as a template for PCR amplification.

3. DNA amplification:

3.1. Amplification of *Vibrio* species (Espeneira *et al*., 2010):

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). Multiplex PCR assays were adopted by amplification of targeted DNA in 20 µl reaction volumes. A typical 20 µl reaction contained 1.2 µl of crude lysate, 0.5 µM each of *V. cholerae* species specific primers, 0.5 µM each of *V. mimicus* primers, 0.25 µM each of *V. vulnificus* primers, 0.16 µM each of *V. alginolyticus* primers and 1 µM each of *V. parahaemolyticus* primers and 18 µl of master reaction mix containing 10 mMTris-HCl, pH 8.3; 50 mMKCl, 1.5 mM MgCl2, 200 µM each dATP, dCTP, dGTP and dTTP and 1U of Taq polymerase. The thermal cycling profile was as follows: an initial denaturation of template DNA at 93°C for 15 min; 35 amplification cycles wherein each cycle

consisted of denaturation at 92°C for 40 sec, primer annealing at 57°C for 1 min, extension at 72°C for 1.5 min and final extension at 72°C for 7 min. Accurately, 10 µl of the amplified products were run in 2% agarose gel electrophoresis (Applichem, Germany, GmbH) in 1x TBE buffer stained with ethidium bromide and captured as well as visualized on UV transilluminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

4.1. Amplification of virulence genes of *V.parahaemolyticus* (Khalil *et al.*, 2014):

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). The final volume of the reaction mixture was adjusted to 50 µL with sterile deionised distilled water. Amplification of DNA segment was performed) with the following temperature cycling parameters; initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 sec, primer annealing at 57°C for 30 sec, primer extension at 72 °C for 1 min and a final extension at 72 °C for 2 min. Ten µL of each amplified product was loaded in 1.5% agarose gel in 1X Tris-boric acid-EDTA buffer [TBE: 0.089M Trisbase, 0.089M boric acid and 0.002 M EDTA (pH 8.0)] at 100 volts for 40 minutes. After electrophoresis, amplification products were captured and visualized on UV transilluminator.

Results and Discussion

Biochemical identification of *Vibrio* spp. in examined fresh crab samples collected in table (3) revealed that the incidence of only one *Vibrio mimicus* species as (5%) the overall incidence of the examined samples.

But the incidence of *Vibrio* species in collected marine crab samples was *V. alginolyticus* 3 as (15%), *V. cholera* 1 as (5%), *V. parahaemolyticus* 2 as (10%)*and V. vulnificus* 4 as (20%).

Table (4) revealed that the incidence of *Vibrio* species in collected oyster samples was *V. alginolyticus* 1 as (5%), V. cholera 2 as (10%), V. *mimicus* 1 as (5%), *V. parahaemolyticus* 4 as (20%) and *V.vulnificus* 5 as (25%).

These results were lower than that recorded by **Matté et al. (1994a)**who analyzed oyster samples (Crassostreagigas) originating from the southern coast of the State of São Paulo-Brazil for *Vibrio* species, and observed the highest incidence for *V. alginolyticus* (81%), followed by *V. parahaemolyticus* (77%), *V. cholerae* non O1 (31%), *V. fluvialis*(27%), *V. furnissii* (19%), *V. mimicus* (12%) and *V. vulnificus* (12%). Also, lower than that recorded by **Elsabagh –Rasha (2013)** who recorded the prevalence of *Vibrio* species in examined oyster samples collected from Suez gulf as

18 (45%) for the all examined samples, from which 7 (17.5%) for *V. mimicus*, 3 (7.5%) for *V. alginolyticus*, 2 (5%) for V. damsela, 1 (2.5%) for each *V. fluvialis*, V. furnissii, *V. parahaemolyticus* and *V. vulnificus* , 2 (5%)for mixed isolates. *While, V. cholerae* failed to be detected biochemically.

Figure (1) Agarose gel showing results of the 16S rRNA analysis for samples collected and examined that ten isolates **for** *V.mimicus* **(2)** at **(121 bp),** *V. alginolyticus* (2) were positive at **(340 bp)**, *V.cholerae* (2) at **(248 bp)** & **for** *V.parahaemolyticus* **(2)** at **(897 bp)**and also (2)*V.vulnificus* **at (410 bp) .**

Figure (2) Agarose gel electrophoresis of multiplex PCR of tdh (269bp), tlh (450 bp) and trh (500 bp) virulence genes for characterization of *V. parahaemolyticus* showed that positive results *V. parahaemolyticus* strain for tdh for tdh, tlh and trh genes.

Table (5) revealed the comparative results between m-PCR method and cultural methods for detection of Vibrio species,the prevalence of *V. alginolyticus, V. cholera, V. mimicus, V. parahaemolyticus and V. vulnificus* in all examined samples collected were found to be 4, 3, 2, 6 and 9 respectively, by culture method, and 2 only by m-PCR method were positive for each vibrio species.

A clear variation in results was observed between traditional and multiplex PCR methods; this may be attributed to that accurate phenotypic identification of *Vibrio* species is problematic, largely because of the great variability in biochemical characteristics **(Thompson et al., 2004).** The main reason is the great variability of diagnostic phenotypic features, e.g., arginine dihydrolase and lysine and ornithine decarboxylase, susceptibility to the vibriostatic agent 0/139, flagellation, indole production, growth at different salinities and temperatures as well as carbon utilization. Traditionally used as clear-cut tests for identification of species, the latter should thus be interpreted with greatest care **(Austin et al., 1997).**

(Rippey, 1994) recorded that *V. mimicus* has been associated with diarrhea following consumption of raw or undercooked seafood

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Table (3): Prevalence of Vibrio species isolated from Fresh and marine Crab samples using biochemical methods. (n= 20 of each).

Isolates	Fresh Crab		Marine Crab N = 20*	
	Positive samples		Positive samples	
	NO	%	NO	%
V. alginolyticus	--	--	3	15
V. cholera	--	--	1	5
V. mimicus	1	5	--	--
V. parahaemolyticus	--	--	2	10
V. vulnificus	--	--	4	20
Total	1	5	10	50

Table (4): Prevalence of *Vibrio* species isolated from Oyster samples using biochemical methods. (n= 20)

Isolates	Oyster	
	Positive samples	
	NO	%
V. alginolyticus	1	5
V. cholera	2	10
V. mimicus	1	5
V. parahaemolyticus	4	20
V. vulnificus	5	25
Total	13	65

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Table (5): Comparison of m-PCR method and biochemical methods for identification of *Vibrio* species. (n= 60)

***Vibrio* species**	**ALL EXAMINED SAMPLES (N = 60)***	
	biochemical	**m-PCR**
	NO. of positive samples	NO. of positive samples
V. alginolyticus	**4**	**2**
V. cholera	**3**	**2**
V. mimicus	**2**	**2**
V. parahaemolyticus	**6**	**2**
V. vulnificus	**9**	**2**
Total	**24**	**10**

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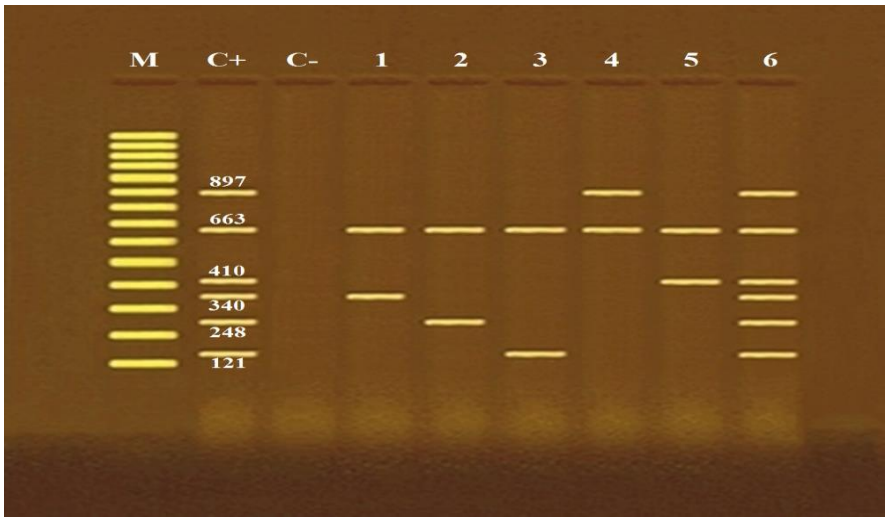
Figure (1): Agarose gel electrophoresis of multiplex PCR for characterization of vm-sodB (121 bp) for *V.mimicus*, Vc-sodB (248 bp) for *V.cholerae*, gyrB (340 bp) for *V.alginolyticus*, hsp (410 bp) for *V.vulnificus*, flaE (897 bp) for *V.parahaemolyticus* and 16S rRNA (663bp) for all *Vibrio* Spp.



Lane M: 100 bp ladder as molecular size DNA marker, Lane C+: Control positive for all tested genes, Lane C-: Control negative, Lane 1: Positive *V.alginolyticus* for 16S rRNA and gyrB genes, Lane 2: Positive *V.cholerae* for 16S rRNA and Vc-sodB genes, Lane 3: Positive *V.mimicus* for 16S rRNA and Vc-sodB genes, Lane 4: Positive *V.vulnificus*for 16S rRNA and hsp genes, Lane 5: Positive *V.parahaemolyticus* for 16S rRNA and flaE genes, Lane 6: Positive Mixture of all tested *Vibrio* species.

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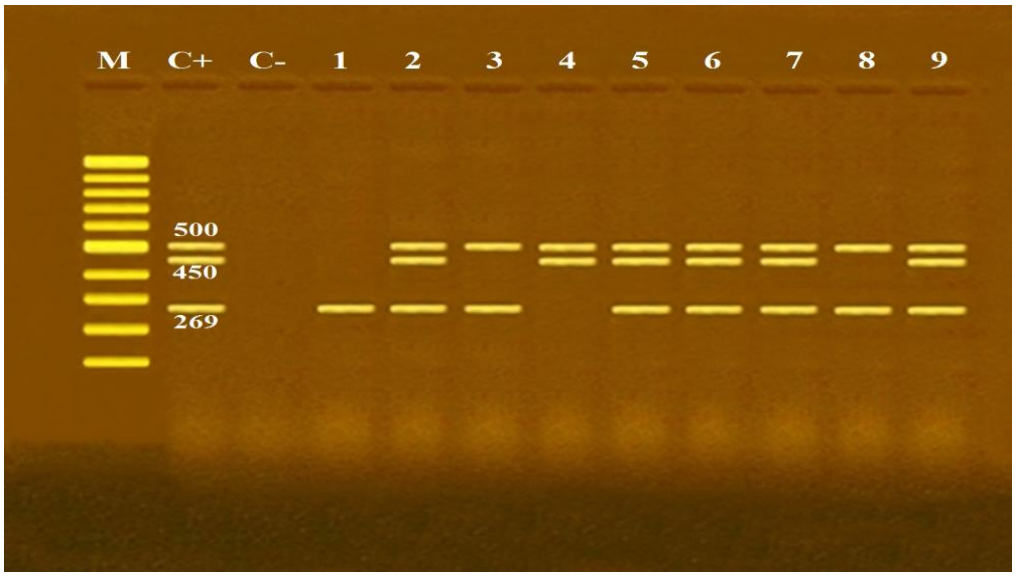
Figure (2): Agarose gel electrophoresis of multiplex PCR of tdh (269bp), tlh (450 bp) and trh (500 bp) virulence genes forcharacterization of *V. parahaemolyticus.*



Lane M: 100 bp ladder as molecular size DNA marker, Lane C+: Control positive *V.parahaemolyticus* for tdh, tlh and trh genes, Lane C-: Control negative, Lane 1: positive *V. parahaemolyticus* strain for tdh gene, Lanes 2, 5, 6, 7 & 9: positive *V. parahaemolyticus* strains for tdh, tlh and trh genes, Lanes 3 & 8: positive *V. parahaemolyticus* strains for tdh and trh genes, Lane 4: positive *V. parahaemolyticus* strain for tlh and trh genes.

Conclusion:

Based on the above mentioned study, it was concluded that the novel multiplex PCR assay developed in this study was more specific, sensitive, rapid and accurate method for the detection of *Vibrio* species than the traditional culture method. Furthermore, presence of *Vibrio cholera* and *Vibrio mimicus* in examined samples considered a life threatening to human health.

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