Occurrence of some Zoonotic Vibrios in Shellfish and Diarrheic Patients with Regard to *tdh* Gene in *Vibrio Parahaemolyticus*

Merwad, A. M. Amin[*1]; El-Ghareeb, W. Rizk [2] and Taisir, S. Mohamed.[3]

[1]Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, Egypt
[2]Department of Food Control, Faculty of Veterinary Medicine, Zagazig University, Egypt
[3]Department of Medical Microbiology and Immunology, Faculty of Medicine, Zagazig University, Egypt
[*]abdallamerwad@yahoo.com

Abstract: Shellfish is a food substrate for some zoonotic vibrios of which these microorganisms, cause food poisoning and diarrhea in human. A total of 245 samples including white shrimps (75), blue crabs (50), oysters (50), water samples from Suez Canal (20) and fecal swabs from diarrheic patients (50) were collected from different localities in Ismailia province and subjected for bacteriological examination. The overall prevalence of *Vibrio* spp. was 57.3% in shrimps, 48% in crabs, 54% in oysters, 25% in water samples and 18% in human stool. Eight *Vibrio* spp. were identified from shrimps with different percentages: *V. parahaemolyticus* (2.6%), *V. vulnificus* (6.6%), *V. fluvialis* (12%), *V. hollisae* (2.6%), *V. furnissii* (6.6%), *V. mimicus* (6.6%), *V. alginolyticus* (10.6%) and *V. damsella* (9.3%). Also, five *Vibrio* spp. isolated from crabs were belonged to *V. vulnificus* (2%), *V. fluvialis* (14%), *V. hollisae* (4%), *V. alginolyticus* (12%) and *V. damsella* (16%). Moreover, oysters showed higher infection rate of *V. fluvialis* (16%) followed by *V. mimicus* (12%), *V. alginolyticus* (10%), each of *V. furnissii* and *V. damsella* (6%) and each of *V. parahaemolyticus* and *V. vulnificus* (2%). From water samples; each *V. vulnificus*, *V. fluvialis*, *V. alginolyticus* showed a similar infection rate of 5%, while for *V. damsella* was 10%. In addition, five *Vibrio* spp. identified from diarrheic patients were belonged to *V. parahaemolyticus* (4%), *V. vulnificus* (2%), *V. fluvialis* (8%), *V. hollisae* (2%) and *V. furnissii* (2%). Thermostable direct hemolysin gene (*tdh*) was positive in 50% and 100% of *V. parahaemolyticus* isolates from human stool and oyster, respectively; where this gene was negative in these isolates from shrimp. Also, *tdh*$^+$ *V. parahaemolyticus* was indicated by presence of 269 bp using PCR. This study throw light on the necessasity of adequate cooking of shellfish, better postharvest handling and monitoring of *tdh*$^+$ *V. parahaemolyticus* to protect human health.

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1. Introduction

Shellfish make an excellent substrate for the microorganisms to live in the aquatic habitats due to loose texture of their flesh. When the aquatic system is contaminated with pathogenic *Vibrio*, these bacteria become part of shellfish microflora **(*Colakoglu et al., 2006*).** Concerning the zoonotic aspect, the hazardous pathogenic *Vibrio* causes life threatening food borne infections **(*Rippey, 1994*)** and poses a considerable public health threat as agents of sporadic and epidemic human infections to be represented an important microbial group in the field of food safety **(*Espineira et al.*, 2010).**

In the last 20 years, many halophilic *Vibrio* species such as *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. hollisae*, *V. fluvialis V. mimicus*, *V. furnissii and V. damsella*, have been implicated in human enteric infections, wound infections and septicemia due to consumption of shellfish and exposure to seawater **(*Geneste et al., 2000 and Thompson et al., 2004*).**

Vibrio parahaemolyticus is often isolated from seawater, sediment and a variety of seafood including shrimp, crab, oyster and clam due to its halophilic characteristics **(*Liston, 1990*)**. This bacterium is one of the leading causes of food borne gastroenteritis associated with ingestion of undercooked shellfish through out the world including the United States, China, Japan and Korea **(*Liu et al., 2004; Jay et al., 2005 ; Su and Liu ,2007*).** Also, this microbial infection is characterized by diarrhea, vomiting, nausea, abdominal cramps and low grade fever **(*Pinto et al., 2008*)**. In addition, *V. vulnificus* is a potentially lethal food borne pathogen and capable of causing primary septicemia and necrotizing wound infections in susceptible individuals **(*Harwood et al., 2004*).**

Previous studies determined the occurrence of *V. parahaemolyticus* in shellfish in different geographic areas over the world: in shrimp, ***Jaksic et al. (2002)*** in Croatia; ***Gopal et al. (2005)*** in India; ***Hassanin (2007)*** in Egypt, and in oyster, ***Kirs et al. (2011)*** in New Zealand, whereas in mussels, ***Baffone et al. (2000)*** in Italy; ***Colakoglu et al. (2006)*** in Turkey;

Blanco- Abad et al. (2009) in Spain. Many literatures reported other pathogenic *Vibrio* in different seafood: ***Ripabelli et al. (1999)*** isolated *V. vulnificus* from mussels in Italy; ***Sung et al. (2001)*** cited *V. furnissii*; *V. hollisae* from shrimp in Taiwan and ***Hidalgo et al. (2008)*** found *V. alginolyticus*, *V. fluvialis* in mollscan shellfish in Spain.

Regarding the public health hazard, vibrios have been implicated in food poisoning and gastroenteritis; *V. parahaemolyticus*, ***Fuenzalida et al. (2007)***; *V. fluvialis*, ***Ballal et al. (2010)*** **and** *V. vulnificus*, ***Horseman and Surani (2011)***. Also, in Egypt, ***Mohamed et al. (2000)*** recovered *V. parahaemolyticus*, *V. hollisae* and *V. fluvialis* from diarrheic patients.

The presence of *V. parahaemolyticus* with the thermostable direct hemolysin gene (*tdh*), encoding for heat stable hemolysin, raises important health issues ***(Richards, 1988).*** AS, *tdh* is currently used as pathogenicity marker since most clinical isolates of *V. parahaemolyticus* possess this gene **(*Bej et al., 1999; Davis et al., 2004 and Nordstrom et al., 2007*).** Thereby, monitoring of pathogenic *V. parahaemolyticus* in shellfish and diarrheic patents is crucial. Due to limited information available on vibriosis associated with shellfish in Egypt, the objectives of this study are to investigate the occurrence of zoonotic vibrios in shellfish, water samples and diarrheic patients in Ismailia province belonging to Egypt, and to detect pathogenic *V. parahaemolyticus* carrying *tdh* gene.

2. Material and Methods

2.1. Sample collection and preparation

A total of 245 samples including shellfish, seawater and fecal swabs from diarrheic patients were aseptically collected from different localities in Ismailia Province during the period extending from July to November, 2010.

One hundred and seventy five shellfish samples including white shrimps, *Penaeus setiferus* (75); blue crabs, *Callinectes sapidus* (50) and oysters, *Crassostrea gigas* (50) were collected from Talateen Fish Market in Ismailia Province, then packed in sterile polyethylene bags placed in an ice box. White shrimps, blue crabs and oyster were classified taxonomically according to ***Boudry et al., 2003 Calo-Mata et al., 2009*** **and** ***Havens et al., 2011,*** respectively.

From water, twenty samples were taken from various depths of Suez Canal, where the shellfish samples were caught. Water samples were collected in sterile bottles. Also, 50 fecal swabs were collected from diarrheic patients admitted the University Hospital belonging to Faculty of Medicine, Suez Canal University. All samples were transferred to Zoonoses and Food Control Laboratories, Faculty of Veterinary Medicine, Zagazig University.

2.2. Enrichment procedures

Five grams of individual shellfish flesh were incised using a sterile scalpel after removal of the carpace. These 5 gm flesh samples were homogenized in 45 ml of 3% NaCl containing 1% alkaline peptone water (APW, pH: 8.6) using a sterile blender. The shellfish homogenates were incubated at 37°C for 18 hr **(*Jaksic et al., 2002*** **and** ***Pinto et al., 2008*).** While, water samples were enriched by adding 100 ml of each sample aseptically to equal volume of 1% alkaline peptone water containing 3% NaCl then were incubated at 37°C for 18 hr **(*Bockemuhl et al., 1986*).** Also, each fecal swab of diarrheic patients was directly transferred to 1% alkaline peptone water containing 3% NaCL, and incubated at 37°C for 18 hr **(*Elliot et al., 1995*).**

2.3. Isolation procedures

Following incubation, the shellfish homogenate and enriched samples of water and fecal swabs were inoculated on Thiosulphate Citrate bile salts sucrose agar media (TCBS, Hi Media, India) using an inoculating loop and kept at 37°C for 18 hrs **(*Donovan and Netten, 1995*** **and** ***Colakoglu et al., 2006*).**

2.4. Identification of bacterial colonies

The isolated colonies were subjected to Gram staining and growing at various salt concentrations by transferring colonies into tubes containing peptone water and 0%, 3%, 6% and 10% NaCl, and these tubes were incubated at 37°C for 24 hrs **(*Lhafi and Kuhne, 2007*).** Also, all bacterial colonies from different samples; growing on TCBS plates were selected to be streaked onto the surface of Trypticase Soya agar slants (TSA; Oxoid, UK) supplemented with 2% NaCL, then incubated at 37°C for 24 hrs **(*Musa et al., 2008*).**

The further identification of *Vibrio* spp. were done using morphological, physiological and different biochemical tests which are listed in Table 1 **(*Poda, 1997*** **and** ***Farmer et al., 2005*).**

Table 1: Cultural, morphological and biochemical characters of *Vibrio* spp. isolated from shellfish, water samples and diarrheic patients.

Characters	*V. parahaemolyticus*	*V. vulnificus*	*V. fluvialis*	*V. hollisae*	*V. furnissii*	*V. mimicus*	*V. alginolyticus*	*V. damsella*
Growth on TCBS media	Blue green	Blue green	Yellow	Yellow	Yellow	Green	Yellow	Green
Gram stain	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S
Oxidase production	+	+	+	+	+	+	+	+
Voges-proskauer reaction	-	-	-	-	-	-	+	+
Indole production	+	+	+	+	-	+	+	-
Growth on:								
0% NaCl	-	-	+	-	+	+	-	-
3% NaCl	+	+	+	+	+	+	+	+
6% NaCl	+	+	+	+	+	-	+	+
10% NaCl	-	-	-	-	-	-	+	-
Utilization of :								
Citrate	+	+	+	+	+	+	+	+
Urea	-	-	-	-	-	-	-	+
Sucrose	-	+	+	-	+	-	+	-
Arabinose	+	-	+	+	+	-	-	-
lactose	-	+	-	-	-	-	-	-

-/S = negative stain + = positive - = negative

2.5. Molecular detection of pathogenic *V. parahaemolyticus* carrying *tdh* gene from stool of diarrheic patients, oyster and shrimp

Vibrio parahaemolyticus isolated from diseased marine fish (Sea bream) was supported by Central Lab for Aquaculture Research, Abbassa, Sharkia Province, and considered as a positive control.

All *V. parahaemolyticus* isolates streaked on TSA slants were subcultured on TCBS plates, and then incubated at 35°C for 24 hr. Afterwards, *V. parahaemolyticus* colonies were cultured in T1N1 broth medium (10% tryptone, 1% NaCL) at 35°C overnight **(*Atlas, 1993*).**

2.5.1. DNA extraction

One milliliter pure culture of *V. parahaemolyticus* identified by biochemical tests was centrifuged at 13000 g for 5 min at room temperature. The DNA was then extracted using the QIA amp DNA Mini Kit (QIAGEN, Germany) and eluted from the QIA amp spin column in 80 UL of elution buffer. The DNA concentration and purity were measured by absorbance at 260 nm to absorbance at 280 nm using a spectrophotometer (U.V- VIS), U.V. 2500 (Labomed, Inc) (***Pinto et al., 2008)***.

2.5.2. Polymerase chain reaction (PCR)

Amplification and detection of *tdh* gene was done according to the method previously described by **Bej *et al.* (1999**) with some modifications. All oligonucleotides were synthesized in Bio Basic Inc. (Canada). The sequences of primers are TDH-L, 5′GTA AAG GTC TCT GAC TTT TGG AC 3′ and TDH-R, 5′TGG AAT AGA ACC TTC ATC TTC ACC 3′.

The PCR was performed in a total volume of 25µl reaction mixtures contained 5µl of DNA as template, 20 pmol of each primer and 1X of PCR master mix (Taq Master/ High yield, Jena Bioscience) which provide 2.5 units per reaction of DNA polymerase, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 1 X PCR buffer (with 1.5 mM – $MgCl_2$).

The amplification cycles were carried out in a PT-100 thermocycler (MJ Research, USA). Reaction conditions were optimized to be 95°C for 15 min as initial denaturation, followed by 30 cycles of 94°C for 30 seconds as denaturation, 60°C for 30 seconds as annealing and 72°C for 45 seconds as extension. The final extension step was followed at 72°C for 10 min. Negative control (no template DNA) was included.

Amplification products were electrophorezed in 1.5% agarose gel containing 0.5 X TBE at 70 volts for 60 min., and visualized under ultraviolet light **(*Sambrook et al., 1989*).** To assure that the amplification products were of the expected size, a 100 bp DNA ladder (Promega, Cat. No. G_{2101}) was run simultaneously as a marker. The presence of 269 bp DNA fragment indicated a positive sample.

2.6. Statistical analysis

The data was analyzed using Chi square according to ***SAS, 2002***.

3. Results and Discussion

The food poisoning associated with consumption of shellfish either raw or slightly cooked, contaminated with *Vibrio* spp. causes intestinal infection characterized by diarrhea, abdominal cramps, sicknes, vomiting, fever and severe headache **(*Espineira et al., 2010*)**. Human infections with *Vibrio* spp. are common; and shrimps, oysters, crabs, lobsters, clams and mussels have all been implicated in transmission **(*Altekruse et al., 2000*)**.

In Table 2, the overall prevalence of zoonotic vibrios was 57.3% (43 out of 75) in white shrimps, 48% (24 out of 50) in blue crabs and 54% (27 out of 50) in oysters. *Vibrio parahaemolyticus* are regularly linked to human food borne infections caused by consumption of undercooked or recontaminated shellfish, but there are also occasional reports of food borne or waterborne infections caused by environmental *Vibrio* e.g. *V. mimicus* **(*Shah and Deokule, 2006*)**, *V. alginolyticus* **(*Yoder et al., 2008*)** and *V. hollisae* **(*Edouard et al., 2008*)**. There was no significant difference (P> 0.05) regarding the infection rate for each *Vibrio* microorganism isolated from shrimp, crab, oyster and water as illustrated in Table 2. In the current study, the infection rate of shrimps with *V. parahaemolyticus* was 2.6% (2 out of 75). Nearly similar result of 2.8% in tropical shrimp culture in East coast farm in India was reported **(*Gopal et al., 2005*)**. Otherwise, the percentage of *V. parahaemolyticus* in shrimps harvested from Dardanelles Market in Turkey was zero **(*Colakoglu et al., 2006*)**. Some studies reported higher infection rates of *V. parahaemolyticus* in shrimps: ***Hassanin, 2007*** (27.6%) in Abu-Kir fishing ground, Egypt; ***Gopal et al., 2005*** (12.2%) in West coast farm, India and ***Jaksic et al., 2002*** (4%) in Croatia. The differences between studies concerned percentages of *V. parahaemolyticus* in shrimps may be attributed to the differences in the range of variation of salinity level and the sample sizes of studies as was supported by ***Parveen et al., 2008***. *Vibrio parahaemolyticus* were not detected from crabs as noted in Table 2. From zoonotic point of view, detection of *V. parahaemolyticus* in shrimps suggests a probable risk for health of people consuming raw seafood. Therefore, it is recommended to pay attention to postharvest handling and adequate cooking to safeguard public health.

Oysters pose high risk of *V. parahaemolyicus* food borne illness (***Zhuang et al., 2007*** *and* ***Shen et al., 2009*****)** due to their ability to concentrate pathogenic vibrios and toxins during the filter feeding process **(*Rippey, 1994*)**. This study clarified an infection rate of 2% (1 out of 50) for *V. parahaemolyticus* in oysters (Table 2). Nearly similar findings of 2.04% in clams and 2.7% in mussels were recorded in Italy ***(Baffone et al., 2000)***. Slightly lower infections of *V. parahaemolyticus* were cited in mussels by **Ripabelli *et al.*, 1999** and ***Colakoglu et al., 2006***, where they reported 1.6% in Italy and 1.2% in Turkey, respectively. On the other hand, higher percentages were recorded in previous studies: ***Cavallo and Stabili, 2002*** (5% for mussels) in Italy; ***Jaksic et al., 2002*** (12% for bivalve mollusks) in Croatia; ***Pinto et al., 2008*** (32.6% for mussels) in Italy; ***Blanco – Abad et al., 2009*** (11.2% for mussels) in Spain and ***Kirs et al., 2011*** (94.8% for oysters) in New Zealand. The higher reports of *V. parahaemolyticus* in the former studies may be associated with the growing of bivalve mollusks in uncontrolled waters subjected to contamination and their peculiar characteristic of filtering large amounts of water **(*Baffone et al., 2000*)**.

Vibrio vulnificus is one of the emerging food and waterborne zoonotic bacteria that represents a human health hazard **(*Canigral et al., 2010*)**. This pathogen causes gastroenteritis and primary septicemia due to consumption of contaminated oysters, while skin and soft tissue infection results from handling contaminated shellfish or from exposure of open wounds to sea water **(*Horseman and Surani, 2011*)**. In Table 2, shrimps showed an infection rate of 6.6% (5 out of 75) for *V. vulnificus*. This result contrasts the findings of ***Jaksic et al., 2002*** and ***Colakoglu et al., 2006***; who reported higher percentages of 12 and 16.6, respectively. Also, lower infection rate of 2.2% in East coast shrimp was cited **(*Gopal et al., 2005*)**. Moreover, crabs and oysters showed a similar infection rate of 2% (1 out of 50) for *V. vulnificus*. However, previous studies cited irrelevant higher reports: ***Cavallo and Stabili,*** **2002** (4.4%) in mussels; ***Canigral et al. 2010*** (10%) in oysters and ***Kirs et al., 2011*** (17.2%) in oysters. Regarding the zoonotic significance, *V. vulnificus* are usually acquired through ingestion of shellfish or through contaminating open wounds during swimming, crabbing, shellfish cleaning and other marine activities as was previously sustained

by ***Heelan (2001)***,and are implicated in epidemic human gastroenteritis **(*Ballal et al., 2010*)**.

With respects to *V. fluvialis*, oysters was higher infected, 16% (8 out of 50) followed by crabs, 14% (7 out of 50) and shrimps, 12% (9 out of 75). Otherwise, lower infection rates were detected in other studies. ***Ripabelli et al., 1999*** cited 1.6% in mussels; ***Cavallo and Stabili, 2002*** reported 1.5% in mussels; ***Gopal et al., 2005*** recorded 4.6% in shrimps and ***Hidalgo et al., 2008*** obtained 3.7% in clams. The variations in the incidence of *V. fluvialis* in shellfish may be accounted for the differences in water contamination levels in many geographic areas. In addition, higher reports of *V. fluvialis* in this study may be associated with presence of planktons which are a tool for survival and distribution of these bacteria in aquatic environments as was advocated by ***Gugliandolo et al., 2005***. From Table 2, *V. hollisae* showed higher infection rate (4%) in crabs followed by shrimps (2.6%), while not detected in oysters. ***Sung et al., 2001*** reported irrelevant higher finding (5%) in shrimps in Taiwan. However, lower infection rate (1.5%) was recorded in mussels in Italy **(*Cavallo and Stabili, 2002*)**. Public health concerns were coupled with the finding of ***Shorr et al., 1997*** who reported the first case of gastroenteritis, diarrhea and bactermia from *V. hollisae* in an immunocompetant host after ingesting raw shellfish in Baltimore, Maryland.

Moreover in Table 2, the prevalence of *V. furnissii* was 6.6% in shrimps and 6% in oysters, while not recorded in crabs. ***Esteve et al. (1995)*** isolated V. *furnissii* for the first time as a potential pathogen from European eel. On the other hand, higher infection rate of *V. furnissii* (15%) in shrimp were cited in Taiwan **(*Sung et al., 2001*)**. Concerning the role of shellfish in transmitting other zoonotic virbios, *V. mimicus* represented an infection rate of 12% in oysters followed by 6.6% in shrimps, while crabs were free from infection. This finding disagreed with previous studies of ***Cavallo and Stabili, 2002*** and ***Gopal et al., 2005***; who recorded 4.4% in mussels and 1.7% in shrimps, respectively. In this study, the infection rate of *V. alginolyticus* in crabs was 12% followed by 10.6% in shrimps and 10% oysters. This result was nearly close to the finding of ***Baffone et al., 2000*** (8.16% in clams in Italy). Lower incidences of 4% and 6.3% from shrimp in Croatia and clams in Spain were recorded by ***Jaksic et al., 2002*** and ***Hidalgo et al., 2008***, respectively. *Vibrio alginolyticus* is associated with white spot in shrimp in India and Taiwan **(*Lee et al., 1996*)**, while the zoonotic hazard of this pathogen has been implicated in ear, soft tissue and wound infections in human **(*Horrii et al., 2005*)**. Otherwise, previous studies cited higher infection rates for *V. alginolyticus*: ***Vandenberghe et al., 1998*** (17.2%, shrimp, China); ***Ripabelli et al., 1999*** (32.2%, mussels, Italy); ***Gopal et al., 2005*** (17.8%, shrimp, India); ***Colakoglu et al., 2006*** (50%, shrimp, Turkey) and ***Hassanin, 2007*** (40%, shrimp, Egypt). Also, crabs were highly infected by *V. damsella* (16%) followed by shrimp (9.3%) and then oysters (6%). This study revealed eight *Vibrio* spp. from shellfish, and all of them have a zoonotic importance. Therefore, the surveillance of contaminant *Vibrio* in shellfish is crucial for sustenance of public health.

Table 2: Occurrence of some zoonotic vibrios in shellfish and water samples from Suez Canal in Ismailia Province.

Samples / *Vibrio* spp.	White shrimps No= 75	Blue crabs No=50	Oysters No=50	Water No=20	Chi value	P value
V. parahaemolyticus no (%)	2 (2.6)	- (0)	1 (2)	- (0)	1.33 NS	0.72
V. vulnificus no (%)	5 (6.6)	1 (2)	1 (2)	1 (5)	2.87 NS	0.41
V. fluvialis no. (%)	9 (12)	7 (14)	8 (16)	1 (5)	0.96 NS	0.80
V. hollisae no (%)	2 (2.6)	2 (4)	- (0)	- (0)	2.5 NS	0.47
V. furnissii no (%)	5 (6.6)	- (0)	3 (6)	- (0)	3.6 NS	0.30
V. mimicus no (%)	5 (6.6)	- (0)	6 (12)	- (0)	7.01 NS	0.07
V. alginolyticus no (%)	8 (10.6)	6 (12)	5 (10)	1(5)	0.458 NS	0.9
V. damsella no (%)	7 (9.3)	8 (16)	3 (6)	2 (10)	5.57 NS	0.13
Overall *Vibrio* no (%)	43 (57.3)	24 (48)	27 (54)	5 (25)		

No= Number of examined shrimps, crabs, oysters and water samples.
no= Number of infected samples with *Vibrio* spp.
(%) = Percentage of infection of samples with *Vibrio* spp.
NS = Non significant difference at (P> 0.05).

The family Vibrionaceae is autochthonous to aquatic environments including estuarine, coastal waters and sediments worldwide, and some species are well- known pathogens of marine organisms including fish and shellfish **(*Gomez-Leon et al., 2005*)**. The overall prevalence of *Vibrio* spp. in water samples from Suez Canal was 25% (5 out of 20) in this study. In Table 2, it was clearly that *V. parahaemolyticus*, *V. mimicus*, *V. furnissii* and *V. hollisae* were not detected in water samples collected from Suez Canal. On the contrary, higher reports of *V.*

parahaemolyticus in water were noticed in other studies: ***Mohamed et al., 2000*** (6.25%, from River Nile tributaries at Damietta Province, Egypt); ***Mahmoud et al., 2006*** (20.8%, Japan); ***Masini et al., 2007*** **(**6.5%, Italy); ***Hassanin, 2007*** (40%, Abu-Kir fishing farm, Egypt) and ***Blanco- Abad et al., 2009*** (5.6%, Spain). In the presents study, *V. parahaemolyticus* was not detected in Suez Canal water may be associated with sample size and water clearness .This finding was supported by ***Watkins and Cabelli (1985)*** and ***Zimmerman et al. (2007)***, who observed that *V. parahaemolyticus* levels in water are strongly correlated with turbidity during summer.

On the other side, some zoonotic vibrios were detected in water samples with a similar infection rate (5%) for *V. vulnificus*, *V. alginolyticus* and *V. fluvialis*, while that of *V. damsella* was 10% (Table 2). Nearly similar result of *V. fluvialis* (4.1%) was recorded **(*Cavallo and Stabili, 2002*)**. Otherwise, low incidence of *V. fluvialis* (0.6%) was reported **(*Gopal et al., 2005*)**. Also, the infection rate of *V. alginolyticus* in water in this study contrasts other studies cited by ***Mohamed et al., 2000*** (12.5%); ***Masini et al., 2007*** (28.5%) and ***Hassanin, 2007*** (60%). Compared with prevalence of *V. vulnificus* in water in the present work, lower percentages of 2 and 3.7 were detected by ***Masini et al. (2007)*** and ***Gugliandolo et al. (2005)***, respectively. However, higher infection rates were found in previous reports; ***Mohamed et al., 2000*** (12.5%) and ***Canigral et al., 2010*** (32%). The different results of *Vibrio* infections in sea water may be attributed to differences in level of contamination of investigated geographic areas **(*Maugeri et al., 2006*)**.

Vibrio parahaemolyticus may spread into humans orally via contaminated mollscan shellfish particularly oysters **(*Depaola et al., 2003*** and ***Drake et al., 2007*)** leading to development of gastroenteritis with diarrhea **(*Cho et al., 2008*)** accounting for 60-80% of cases, wound infections in 34% and 5% have septicemia **(*Butt et al., 2004*)**. Although few data exist on *V. parahaemolyticus* infections in human, a notable increase in its worldwide incidence has been reported **(*Croci and Suffredini, 2003*)**. In Table 3, there were significant differences between the infection rates of each of five *Vibrio* spp. isolated from diarrheic patient (P≤ 0.05). The overall infection of *V. parahaemolyticus* in stool of diarrheic patients was 4% (2 out of 50). Lower percentage of 2.4 in Damietta province, Egypt was cited **(*Mohamed et al. 2000*)**; although higher finding of 22.5% in Taiwan was reported **(Wu *et al.*, 1996)**. Other studies detected *V. parahaemolyticus* in stool of diarrheic patients: ***Nolan et al. (1984)*** reported six cases in Washington after eating raw oysters, and ***Fuenzalida et al. (2007)*** recorded 19 patients in Chile as a result of mussel consumption. In addition, ***Lee et al. (2003)*** isolated two *V. parahaemolyticus* strain from patients stool with acute gastroenteritis and edible mollusk abalone and their implication as the first evidence of laboratory acquired zoonosis.

Table 3: Occurrence of *Vibrio* spp. of public health importance in diarrheic patients attending the University Hospital in Ismailia Province.

***Vibrio* spp.** \ **Diarrheic patients**			**Total** No=50
V. parahaemolyticus	no	(%)	2 (4)
V. vulnificus	no	(%)	1 (2)
V. fluvialis	no	(%)	4 (8)
V. hollisae	no	(%)	1 (2)
V. furnissii	no	(%)	1 (2)
Overall *Vibrio*	no	(%)	9 (18)
Non-infected patients			41 (82)%
Chi value			17.73*
P value			0.003

No= Number of examined patients suffering from diarrhea.
no = Number of infected patients with *Vibrio* spp.
(%) = Percentage of infected patients with *Vibrio* spp.
* = Significant differences at (P≤0.05).

Table (3) clarified the overall infection rate of *V. vulnificus* in stool samples was 2% (1 out of 50). For the zoonotic hazard, 252 cases of *V. vulnificus* infection were recorded of which 116 cases followed consumption of crabs **(*Barton and Ratard, 2006*)**. Also, consumption of raw shellfish among immunocompromised patients is a risk factor for severe *V. vulnificus* infection **(*Gholami et al., 1998*)**. Infection due to *V. fluvialis* most commonly present as gastroenteritis and diarrhea **(*Lesmana et al., 2002*)**. In Table 3, the total infection rate of *V. fluvialis* in stools from diarrheic patients was 8% (4 out of 50). This result was nearly close to the finding of ***Altekruse et al. (2000)***, who recorded 10% in Mexico region, U.S.A. On the contrary, lower result of 1.2% was reported in Egypt **(*Mohamed et al., 2000*)**. Regarding the public health hazard, *V. fluvialis* was reported in a human case of severe watery diarrhea and bacteremia in Taiwan **(*Lai et al., 2006*)**. Also, *V. fluvialis* was implicated in an outbreak of food poisoning and gastroenteritis in India during 1981 with an infection rate of 64.28% **(*Thekdi et al., 1999*)**.

This study showed an infection rate of 2% for *V. hollisae* (Table 3). Otherwise, higher infection rate (18.8%) was cited in adult patients **(*Mohamed et al.,***

***2000*)**. Thirty three cases of human infection with V. hollisae have been described after eating raw oysters **(*Carnahan et al., 1994*)**. In Table 3, the overall infection rate of *V. furnissii* in diarrheic patients was 2% (1 out of 50). Concerning the public risk, *V. furnissii* has been linked with infantile diarrhea and diarrheal disease from 16 patients in Brazil **(*Magalhaes et al., 1993*)**. From zoonotic point of view, five *Vibrio* spp. namely: *V. parahaemolyticus*, *V. vulnificus*, *V. fluvialis*, *V. hollisae* and *V. furnissii* were isolated from stool of diarrheic patients, and associated with consumption of undercooked shellfish. The differences in infection rates of patients with *Vibrio* spp. may obey to variation in cultural food habits and geographic areas.

Thermostable direct hemolysin gene (*tdh*) has been recognized as primary virulence factor in pathogenic *V. parahaemolyticus* **(*Okuda et al.. 1997*** and ***Pinto et al., 2008*)**. Based on studies conducted in different regions of the world, generally 0.2 to 3% of environmental *V. parahaemolyticus* isolates are potentially pathogenic based on presence of *tdh* gene **(*Nordstrom et al., 2007*)**. Table (4) illustrated the occurrence and distribution of *tdh* gene among *V. parahaemolyticus* from stool of diarrheic patients, oysters and shrimps using PCR. The positive sample (*tdh*$^+$ *V. parahaemolyticus* isolate) was indicated by presence of 269 bp DNA fragment, as listed in Figure 1. In the current study, *tdh* gene was detected in 1 out of 2 *V. parahaemolyticus* isolates (50%) from stool of diarrheic patients. This finding was nearly in accordance to ***Robert – Pillot et al. (2004)***, who cited that 46% of *V. parahaemolyticus* isolates from patient stool was *tdh*$^+$ in France. However, each microbial isolate from human stool was *tdh*$^+$ (100%) in Chile.

Table 4: Occurrence and distribution of *tdh* gene among *V. parahaemolyticus* isolates from stool of diarrheic patients, oysters and shrimps using PCR.

Source	Number of isolates (%)		
	Total isolates	*tdh*$^+$ (%)	*tdh*$^-$(%)
Stool of diarrheic patients	2	1(50)	1(50)
Oysters	1	1 (100)	- (0)
Shrimps	2	- (0)	2 (100)

From Table 4, the percentage of pathogenic *V. parahaemolyticus* isolates from shrimps (*tdh*$^+$) was zero. Otherwise, previous studies recorded variant distributions of *tdh* positive *V. parahaemolyticus* of shellfish origin: ***Robert- Pillot et al., 2004*** (0.8%, seafood product, France); ***Pinto et al., 2008*** (33.3%, mussels, Italy) and ***Messelhauser et al., 2010*** (18.18%, shrimps, Germany). Also, *tdh* was detected in the single *V. parahaemolyticus* isolate from oysters (100%), Table 4. Compared with other studies targeting tdh gene in *V. parahaemolyticus* isolates from oysters where 44% of pacific oysters from Alaska **(*Nordstrom et al., 2007*)**, 44-56% of Eastern oysters from Mexico **(*Zimmerman et al., 2007*)**, 20% of Eastern oysters from Chesapeake bay **(*Parveen et al., 2008*)**, 3.4% of oysters from India **(*Raghunath et al., 2009*)** and 3.4% of oysters from New Zealand **(*Kirs et al., 2011*)** have been found *tdh*$^+$ *V. parahaemolyticus*. Thereby, detection of *V. parahaemolyticus* isolates bearing *tdh* gene from patients stool and oyster constitutes a public health hazard, where this microbial infection may cause food poisoning and gastroenteritis. This finding was well in line with that of ***Messelhausser et al. (2010)***, who pointed that PCR assay is a time saving and a reliable solution for detection of pathogenic *V. parahaemolyticus*.

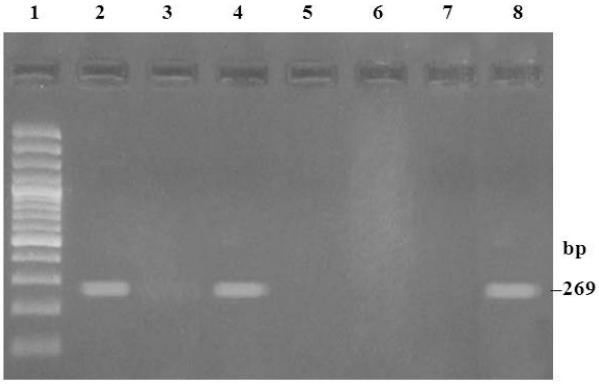

Figure 1: Electrophoretic profile of *tdh* gene among *V. parahaemolyticus* isolates from stool of diarrheic patients, oyster and shrimp using PCR.

Lane 1: 100 bp DNA ladder; Lane2: Positive stool sample; Lane 3: negative stool sample; Lane 4: positive oyster sample; Lanes (5 & 6): negative shrimp samples; Lane 7: negative control (no DNA); Lane 8: Positive control (*V. parahaemolyticus* isolate from diseased Sea bream).

In conclusion, shellfish acts as an important food vehicle for some zoonotic vibrios, of which these microorganisms are implicated in outbreaks of food poisoning and diarrhea in humans. *V. parahaemolyticus* carrying *tdh* gene in oyster and diarrheic stool using PCR could be useful as basis for a preventive consumer protection policy. This study recommended further investigation for other virulent genes in pathogenic *V. parahaemolyticus*.

Corresponding author
Merwad, A. M. Amin
Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, Egypt
abdallamerwad@yahoo.com

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