

Molecular characterization of *Vibrio parahaemolyticus* isolated from shellfish and their harvesting water from Suez Canal area, Egypt

¹Youssef, A. I., ²Farag, A. L. and ²Helal, I. M.

¹Department of Zoonoses, Faculty of Veterinary Medicine, Suez Canal University, Round Road, 4.5km, 41522, Ismailia, Egypt

²Food Hygiene Department, Animal Health Research Institute, Nadi El-Seid Street, Dokki, 12618, Giza, Egypt

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Abstract

Vibrio parahaemolyticus is a leading cause of seafood-derived food poisoning throughout the world. The main objectives of this study were to determine the prevalence of contamination of shellfish by *V. parahaemolyticus* in Suez Canal area and to assess its molecular characteristics. The study included 410 samples of shellfish (164 clams, 86 mussels, and 160 shrimps) collected from the three Governorates of the Suez Canal area. Harvesting water samples were collected from the three sites. All samples were collected during the warmest season (June, July, and August) through two years. Samples were processed, and the enriched samples were identified by plating onto TCBS agar. Presumptive *V. parahaemolyticus* colonies were selected, purified, and further identified by API20 and PCR techniques targeting *toxR* gene. The pathogenicity of the isolates was examined by detection of Thermostable Direct Haemolysin (TDH) and related Haemolysin (TRH) genes. Results revealed that the overall prevalence of *V. parahaemolyticus* in shellfish was 38/410 (9.27%), whereas in water was 12/48 (25%). Higher contamination rate was detected in shrimp (15%), and the highest prevalence was recorded in Ismailia governorate (12.2%). The detection rate of TDH and TRH genes among *V. parahaemolyticus* isolates was 21.05% and 5.26% consequently indicating its health hazards to the consumers. This study concluded that the examined shellfish may have the potential human health risk associated with the presence of pathogenic *V. parahaemolyticus*.

Keywords

Vibrio parahaemolyticus
Suez Canal
Shellfish
Water
Zoonoses

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Introduction

Vibrio parahaemolyticus is a Gram-negative halophilic and mesophilic bacterium, commonly found in estuarine environment (McCarter, 1999; Su and Liu, 2007). *V. parahaemolyticus* considered a natural pathogen of the aquatic environment and is also inhabited by fish, shellfish, shrimp, oysters, crayfish, and other aquatic organisms (Cook *et al.*, 2002; Lee *et al.*, 2008). The bivalves accumulate environmental bacteria in their gills and digestive glands becoming potential vectors for many pathogens including *Vibrio* species (Potasman *et al.*, 2002).

Several *Vibrio* bacteria species can cause serious disease in humans or animals. Twelve *Vibrio* species have been recognized as potential foodborne disease agents in humans, of which, *V. parahaemolyticus* is the most common (Adams and Moss, 2008). Although strains of *V. parahaemolyticus* are environmental, many strains are pathogenic to humans. Virulent strains of *V. parahaemolyticus* can cause wound infections, septicemia, or more

commonly acute gastroenteritis which is acquired through the consumption of raw or undercooked seafood, especially shellfish (Letchumanan *et al.*, 2014).

Outbreaks of *V. parahaemolyticus* have been reported in many countries such as the USA, France, and New Zealand because of the increase in seafood consumption and the global warming, which may be resulting in a higher prevalence of *Vibrio* species and increase the risk of *Vibrio*-borne infections (Nair, 2007; Cruz *et al.*, 2015). Recognized infections from *Vibrio* species are increasing. Most researchers predict that climate change will increase cases (Burge *et al.*, 2014; Letchumanan *et al.*, 2014). In Egypt, few studies on *V. parahaemolyticus* in shellfish have been conducted with an incidence ranged from 2.6 to 16.6%, and reached 31% (Eissa *et al.*, 2010, Merwad *et al.*, 2011; Abdel-Elghany and Sallam, 2013).

Not all *V. parahaemolyticus* strains have the same pathogenic potential. Pathogenicity is strongly correlated with two well-characterized hemolysins, the thermostable direct hemolysin (TDH) and the TDH-related hemolysin (TRH) (Ceccarelli

*Corresponding author.

Email: ayoussef22@gmail.com

et al., 2013; Raghunath, 2015). TDH gene has been recognized as a primary virulence factor in pathogenic *V. parahaemolyticus* (Pinto et al., 2008). TDH gene is currently used as pathogenicity marker since most clinical isolates of *V. parahaemolyticus* possess TDH gene (Bej et al., 1999; Nordstrom et al., 2007). Generally, 0.2 to 3% of environmental *V. parahaemolyticus* isolates are potentially pathogenic based on the presence of TDH gene (Nordstrom et al., 2007). The TDH and TRH genes are known to occur in 99% of clinical strains whereas; their presence in environmental strains is relatively rare (2–3%) (Nishibuchi and Kaper, 1995).

The present study aimed to investigate the prevalence of *V. parahaemolyticus* in shellfish, mollusks and its harvesting water at Suez Canal area, and to assess its pathogenicity.

Material and Methods

Sample collection and preparation

This study included the analysis of a total of 410 samples of wild shellfish consisted of 164 clams, 86 mussels and 160 white shrimps (*Penaeus latisulcatus*). Samples were collected from the three Governorates of the Suez Canal area, Egypt, which are Port Said, Ismailia and Suez Governorates. Shellfish samples were collected from the fish market from the Suez Canal Gulf and Suez Canal and the Mediterranean Sea. In addition, a total of 48 harvesting water samples were collected from the same collection areas. All samples were collected during the warmest seasons (June, July, and August) each sampling year through two years. Samples were transported with minimal delay under chilling conditions to the laboratory. One sample represented by 25 grams of the shellfish after shelling under a sterile condition which was collected from an average from 5-10 shell fish according to the species and the size.

Isolation and identification of *V. parahaemolyticus*

Isolation and biochemical identification of *V. parahaemolyticus* were carried out according to Kaysner and DePaola (2004). Samples were externally sterilized before being shelled on a clean sterile surface using sterile scissors and forceps. Twenty-five grams of shelled samples were added to 225 ml sterile phosphate buffer saline (PBS) (Oxoid) in a stomacher bag and mixed in a stomacher for 60s. This constituted the 1:10 dilution, from which, 1:100, 1:1000, 1: 10,000 dilutions were prepared. The homogenized samples were enriched by adding 1ml of the homogenized sample to 9 ml of in Alkaline peptone water (APW) followed by incubation at

35°C for 24 hr. A loopful from the top 1 cm of APW tubes containing the three highest dilutions of the enriched sample was plated onto Thiosulfate Citrate Bile Sucrose (TCBS) agar (Oxoid) and incubated at 35°C for 24 hr. Presumptive *Vibrio* spp. that appeared round, opaque, green or bluish colonies, 2 to 3mm in diameter on TCBS agar were selected and purified on TCBS agar. Each single colony was screened for Gram's staining, motility, cytochrome oxidase, urease activity, NaCl requirement (0%,1%, 3%, 6%, 8%, or 10%), citrate utilization test, triple sugar iron agar test, arginine dehydrolase test, lysine and ornithine decarboxylase tests, O/129 sensitivity test, Vogues Proskauer test, indole test, Onitrophenyl-b-d-galactopyranoside (ONPG) hydrolysis and acid production from sucrose, lactose, arabinose, cellobiose, mannitol and mannose. The suspected colonies were further confirmed by API20E test (BioMerieux, France). The reference of *V. parahaemolyticus* strains (ATCC 17802) was used as positive control.

Molecular identification of *V. parahaemolyticus* and detection of TDH and TRH genes.

All the identified colonies as *V. parahaemolyticus* were subjected to PCR targeting species-specific gene toxR gene to be confirmed to *V. parahaemolyticus*. The molecular experiments were performed in the Laboratory of Zoonoses, Department of Animal Hygiene and Zoonoses, Faculty of Veterinary Medicine, Suez Canal University,

DNA extraction from the enriched samples was carried out by the boiling method as described by Tunung et al. (2011).

PCR amplification reactions were performed using primer sequences Forward: 5'-GTCTTCTGACGCAATCGTTG-3' and Reverse: 5'-ATACGAGTGGTTGCTGTCATG-3', which amplify an amplicon of 367 bp of TOXR gene as described by Kim et al. (1999). TDH and TRH genes were examined using primer sequences as described by Tada et al. (1992) as following: Forward 5'-CCACTACCACTCTCATATGC-3', reverse 5'-GGTACTAAATGGCTGACATC-3', amplifying an amplicon size of 251 bp of TDH gene and sequences of forward: 5'-GGCTCAAATGGTTAAGCG-3', and reverse 5'-CATTTCCGCTCTCATATGC-3' amplifying an amplicon size of 250 bp of TRH gene. The primers were ordered from Operon Company, (Operon, Japan) as nucleotide sequence. All primers were diluted according to the company instructions using sterile TE buffer.

Each PCR reaction mixture consisted of a final volume of 25 µl divided to 5 µl of the extracted DNA,

12.5 µl of 2X PCR Master Mix (Biotek corporation), 0.5 µl of each primer (5 pmol concentration) and 6.5 µl sterile distilled water. The PCR assays were performed using a Thermal Cycler (Eppendorf). The amplification procedure consisted of an initial denaturation step at 94°C for 2 mins, followed by 30 cycles with denaturation at 94°C for 30s, annealing at 57°C for 45 s and extension at 72°C for 30 s. A final extension step was carried out at 72°C for 5 mins. Aliquots from amplification reactions were analyzed by 1% agarose gel electrophoresis and viewed and photographed under UV light using gel documentation system (Biospectrum 310 imaging system).

Statistical analysis

Chi-square was used for calculation of significance between the prevalences at $P < 0.01$ using GraphPad QuickCalcs program.

Results

Total prevalence of *Vibrio* spp. isolated from shellfish examined.

As tabulated in Table 1, results revealed that the total prevalence of *Vibrio* spp. isolated from the shellfish examined was 17.07% (70/410). The total isolation rates of *Vibrio* spp. were 14.63% from clams, 11.63% from mussel, and 22.5% from shrimp. The isolation rate of *vibrio* spp. was highest in Port Said (20.51%), followed by Ismailia (19.12%) and Suez (6.67%).

Prevalence of *V. parahaemolyticus* among governorates of Suez Canal area.

As tabulated in table 2, results revealed that the total prevalence of *V. parahaemolyticus* isolated from the examined shellfish was 9.27% (38/410). The total isolation rates of *V. parahaemolyticus* were 6.01% from clams, 4.65% from mussels, and 15% of shrimp. The isolation rate of *V. parahaemolyticus* was highest in Ismailia (12.2%), followed by Port Said (8.94%) and Suez (4.44%). Results revealed that there were no significant differences between the two years of sampling using Chi-square.

The prevalence of *V. parahaemolyticus* in relation to *Vibrio* spp.

As illustrated in table 3, out of 70 isolates of *vibrio* spp., 38 (54.29%) was confirmed to *V. parahaemolyticus* by microbiological and molecular techniques. The percentage of isolation of *V. parahaemolyticus* to *vibrio* spp. was 66.67% (24 out of 36), whereas it was nearly similar in

Table 1. Total prevalence of *Vibrio* species from shellfish examined

Shellfish	Sampling sites			Total +ve/Examined (%)
	Port Said +ve/Examined (%)	Ismailia +ve/Examined (%)	Suez +ve/Examined (%)	
Clams	14/56 (25%)	10/80 (12.5%)	0/28 (0%)	24/164 (14.63%)
Mussel	6/24 (25%)	4/28 (14.29%)	0/34 (0%)	10/86 (11.63%)
Shrimp	12/76 (7.89%)	18/56 (32.14%)	6/28 (21.43%)	36/160 (22.50%)
Total	32/156 (20.51%)	32/164 (19.12%)	6/90 (6.67%)	70/410 (17.07%)

Table 2. Total isolation rate of *V. parahaemolyticus* from Shellfish.

Shellfish	The sampling sites			Total +ve/Examined (%)
	Port Said +ve/Examined (%)	Ismailia +ve/Examined (%)	Suez +ve/Examined (%)	
Clams	4/56 (7.14%)	6/80 (7.5%)	0/28 (0)	10/164 (6.01%)
Mussel	2/24 (8.33%)	2/28 (7.14%)	0/34 (0)	4/86 (4.65%)
Shrimp	8/76 (10.53%)	12/56 (21.43%)	4/28 (14.29%)	24/160 (15%)
Total	14/156 (8.94)	20/164 (12.20%)	4/90 (4.44%)	38/210 (9.27%)

clams 10/24 (41.67%), and 4/10 (40%) in mussel. Regarding the location, the highest percentage of *V. parahaemolyticus* was in Suez 4/6 (66.67%), followed by Ismailia 20/32 (62.5%), and Port Said 14/34 (41.18%).

Detection of the virulence genes TDH and/or TRH genes among the *V. parahaemolyticus* isolates.

As tabulated in table 4, the total detection rate of the TDH gene among *V. parahaemolyticus* isolates was 8/38 (21.05%), whereas the detection rate of TRH gene was 2/38 (5.26%). The positive TRH gene isolate was also TDH gene positive.

The Isolation rate of *V. parahaemolyticus* and distribution of TDH and TRH genes in the water samples.

Among 48 water samples, 20 (40%) was *vibrio* spp. positive. Of them, 12 (25%) was identified as *V. parahaemolyticus*. TDH gene was detected in 2/12 (16.67%) of the *V. parahaemolyticus* in the water samples whereas TRH gene was not detected.

Table 3. Percentage of *V. parahaemolyticus* in relation to *Vibrio* spp.

Shellfish	The sampling sites			Total
	Port Said	Ismailia	Suez	
	+ve/Examined (%)	+ve/Examined (%)	+ve/Examined (%)	
Clams	4/14 (28.57%)	6/10 (60%)	0/0 (0)	10/164 (6.10%)
Mussel	2/6 (33.33%)	2/4 (50%)	0/0 (0)	4/86 (4.65%)
Shrimp	8/12 (66.67%)	12/18 (66.67%)	4/6 (66.67%)	24/160 (15%)
Total	14/32 (41.18%)	20/32 (62.5%)	4/6 (66.67%)	38/410 (9.27%)

Discussion

V. parahaemolyticus is a zoonotic pathogen and is one of the most significant foodborne pathogens causing gastroenteritis, wound infections, and septicemia (Pinto *et al.*, 2008; Letchumanan *et al.*, 2014). Although *V. parahaemolyticus* is disseminated throughout the world (Yeung and Boor, 2004; Su and Liu, 2007), rare pathogenic variants of *V. parahaemolyticus* can cause human gastric infections most often from the consumption of raw or improperly handled seafood and wound infections from recreational aquatic activities (McCarter, 1999; Scallan *et al.*, 2011).

Seafood dishes are preferred in the Suez Canal area for both residents and visitors. The widespread practice of eating seafood could be associated with a potential risk of food poisoning in that area. The Egyptian dietary habits not including the eating of raw fish and seafood, but there is a potential risk of food borne infections in case of eating improperly cooked seafoods.

In the present study, the overall prevalence of *vibrio* spp. was 22.5% which was lower than that reported by Eissa *et al.* (2011) in shrimp in Egypt (31.1%). Another study revealed that the total prevalence of *Vibrio* spp. in the Northeast of Egypt was 57.3% in shrimp, 54% in oysters (Merwad *et al.*, 2011). The Incidence of *Vibrio* spp. was 43.4% in shrimp in China (Xu *et al.*, 2016). The high prevalence of *Vibrio* spp. isolation from shellfish that detected in the study area could be attributed to the collection of samples during the hot season. It has been reported that *V. parahaemolyticus* may be detected year-round in locations where water temperatures do not drop below 15°C with the number of organisms detected in water, sediment and oysters increasing as water temperatures rise and the risk of exposure to an infectious dose of pathogens increases (Su and Lu, 2007; Eissa *et al.*, 2011). On the other hand, oysters may have a concentration of *V. parahaemolyticus*

Table 4. Prevalence of the virulence genes TDH and/or TRH among *V. parahaemolyticus* isolates from shellfish.

Shellfish	Pathogenicity genes		
	TDH	TRH	TDH/TRH
	+ve/Examined (%)	+ve/Examined (%)	+ve/Examined (%)
Clams	2/10 (20%)	0/10 (0%)	0 (0%)
Mussel	0/4 (0%)	0/4 (0%)	0 (0%)
Shrimp	6/24 (25%)	2/24 (8.33%)	2/24 (8.33%)
Total	8/38 (21.05%)	2/38 (5.26%)	2/24 (8.33%)

up to 100-fold higher than surrounding waters due to filter feeding particularly in the summer season which increases the chances of infection (Morris, 2003). Controversy, it has been reported that the temperature and total abundance do not exclusively explain contamination rates with *V. parahaemolyticus* as some infections occur when water temperatures and abundance of total *V. parahaemolyticus* are low (Zimmerman *et al.*, 2007).

Results of the current study revealed that the prevalence of *V. parahaemolyticus* in shellfish in Suez Canal area was lower matched with another study conduct in the same study area which was 22.5% in shrimps and 16.7% in shellfish samples (Abd-Elghany and Sallam, 2013). The incidence of *V. parahaemolyticus* in shellfish was 36.2% in China (Xu *et al.*, 2016).

The high detection rate of *V. parahaemolyticus* in shrimp detected in the present study 9.27% was higher compared to 2.6% another study (Merwad *et al.*, 2011). However, Eissa *et al.* (2011) detected high incidences of *V. parahemolyticus* in shrimp in Suez-bay (31.1%).

In this study, *V. parahaemolyticus* was isolated from water samples collected from the study area. Merwad *et al.* (2011) identified a prevalence of 25% *Vibrio* spp. in water samples from Suez Canal however, they did not detect *V. parahaemolyticus* in the same study area. It has been reported that *V. parahaemolyticus* levels in water are strongly correlated with turbidity during summer (Zimmerman *et al.*, 2007). Water contamination by *V. parahaemolyticus* indicated contamination potentials of the seafoods in that area and also direct contamination during entering these waterways.

Concerning the zoonotic aspect, the hazardous pathogenic *Vibrio* causes life-threatening foodborne infections and poses a considerable public health threat as agents of sporadic and epidemic human infections (Rippey, 1994). It is worth mentioned that these results are not accepted concerning the

Egyptian standard (EOS, 2005), as it should be free from *V. parahaemolyticus*. *V. parahaemolyticus* was identified in 4% in diarrheic patients in Egypt (Merwad et al., 2011; Abdel-Ghany and Sallam, 2013). The difference in the incidence of *V. parahaemolyticus* among samples from the sampling sites could possibly be contributed by the original source from which the shellfish were collected, post-harvest practices and hygiene standards applied during handling, transportation, and difference in storage temperature of seafood products (Yang et al., 2008).

To assess the actual risk to human health posed by the presence of *V. parahaemolyticus* in seafood, the incidence of pathogenic strains need to be identified by detection of the toxigenic genes responsible for causing diseases in humans. Strains of *V. parahaemolyticus* carrying TDH and/or TRH are considered pathogenic (Turner et al., 2013). Previous investigations revealed that 1–2% of the environmental strains harbored the TDH and TRH genes under natural conditions (Hervio-Heath et al., 2002; Abd Elghany and Sallam, 2013).

In the present study, TDH and TRH genes were detected by a higher percentage than that detected by Abd-Elghany and Sallam (2013) who found that 3/120 (2.5%) seafood samples were positive for one or both TDH and TRH genes. In a previous study conducted in Italy, out of 35 *V. parahaemolyticus* isolates from mussels, one (2.86%) and three strains (8.57%) were positive for TDH and TRH genes, respectively (Ottaviani et al., 2005). TDH-positive *V. parahaemolyticus* was detected in 3.4% of oysters in New Zealand (Kirs et al., 2011). Lower prevalence of TDH and TRH positive isolates were recorded. None of 145 *V. parahaemolyticus* isolates possessed TDH or TRH genes (Xu et al., 2016). Of the 38/71 (53.5%) *V. parahaemolyticus* isolates were positive for the TRH gene and 71 (100%) were negative for the TDH gene (Kang et al., 2016). Conversely, higher incidences of virulent *V. parahaemolyticus* isolates from seafood were identified in the USA by Bej et al. (1999) who could detect TDH and TRH genes in 32.56% (14/43) and 23.3% (10/43), respectively in the examined seafood isolates. Much higher incidences of 85% (17/20) of shellfish samples tested in Chile were positive for TDH (Fuenzalida et al., 2007). Additionally, both TDH and TRH genes were detected in 44% (12/27) and 52% (14/27), respectively in oyster samples tested in Alaska (Nordstrom et al., 2007). The percent 44-56% of Eastern oysters from Mexico (Zimmerman et al., 2007). The differences in the frequency of the TDH and/or TRH pathogenicity genes may depend on the location, sample source,

and detection methods (Cook et al., 2002; Hervio-Heath et al., 2002). Therefore, continued monitoring of both the prevalence of *V. parahaemolyticus*, with surveys expanded to the national level, is important to ensure shellfish safety.

Conclusions

In conclusion, the occurrence of pathogenic *V. parahaemolyticus* in shellfish and water could pose a serious threat and hazard to susceptible people through consumption of raw or undercooked shellfish. Thus, it is recommended that monitoring of harvesting areas for the prevalence of *V. parahaemolyticus* is important to ensure shellfish safety. In addition, post-harvest practices and hygiene standards should be applied during handling, transportation, and storage of seafood products and ensuring adequate cooking before consumption.

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