Detection, Identification, and Prevalence of Pathogenic *Vibrio parahaemolyticus* in Fish and Coastal Environment in Jordan

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Abstract: *Vibrio parahaemolyticus* is widely distributed in the marine environments and considered the leading cause of human gastroenteritis in Asian countries. A total of 150 marketed fish and 50 water and sediment samples from the Gulf of Aqaba were examined for the prevalence of pathogenic strains of *V. parahaemolyticus*. A total of 132 typical isolates obtained from the primary selective medium (thiosulfate-citrate bile salt sucrose agar) and showed positive biochemical properties were subjected to confirmation by polymerase chain reaction targeting the *gyr*B and *tox*R genes. These genes were confirmed at rates of 82% (108 isolates) and 72% (95 isolates), respectively. The *tox*R positive isolates were tested for the presence of thermolabile hemolysin (*tlh*), thermostable direct hemolysin (*tdh*), and *tdh*-related hemolysin (*trh*) virulence genes. Accordingly, the prevalence rates of pathogenic *V. parahaemolyticus* were 4%, 8%, and 12% in sediment, water, and fish samples, respectively. The 16S rRNA amplification and sequences were conducted for confirmation of the isolates and showing the relatedness among these isolates. The results showed that both 16S rRNA and *tox*R assays had same sensitivity and tested isolates had high nucleotide similarity irrespective of their sources.

Keywords: coastal environment, fish, *Vibrio parahaemolyticus*

Practical Application: The prevalence rate of pathogenic *V. parahaemolyticus* in seafood and coastal water in Jordan is close to the rates reported in other studied coastal regions. The *tox*R gene alone cannot be used as confirmatory test for pathogenic *V. parahaemolyticus.*

Introduction

Vibrio parahaemolyticus is a foodborne pathogen that is characterized as Gram-negative, halophilic, mesophilic, rod-shaped bacterium. It is widely distributed in the marine environments and frequently isolated from a variety of seafood (Liston 1990; Su and Liu 2007). Consumption of raw or undercooked seafood that is contaminated with *V. parahaemolyticus* may cause acute gastroenteritis. Worldwide, *V. parahaemolyticus* is identified as the leading cause of human gastroenteritis associated with consumption of seafood (Xu and others 2014). *V. parahaemolyticus* has been implicated in several seafood-borne infections in different regions of the globe including the United States (McLaughlin and others 2005), Japan (Kubota and others 2008), Spain (Lozano-León and others 2003), Italy (Paydar and others 2013), and Brazil (Leal and others 2008). The prevalence of *V. parahaemolyticus* in seafood samples from different parts of the world has been reported in the range of 8% to 51% (Abd-Elghany and others 2013; Gjerde and Boe 1981; Robert-Pillot and others 2004; Tilburg and others 2000; Wang and others 1996; Xu and others 2014); however, most isolates of this species are nonpathogenic to humans (Nishibuchi and Kaper 1995). The prevalence rate of pathogenic strains of *V. parahaemolyticus* in seafood has not exceeded 13% in different global locations (Abd-Elghany and others 2013; Paydar and others 2013; Suffredini and others 2014; Xu and others 2014). In environmental samples (sediments and water), the prevalence of pathogenic strains of *V. parahaemolyticus* was reported 17% in water samples in Malaysia (Mohammad and others 2005), and 8.5 % in sediment samples in China (Chao and others 2009b), 4 % in Norway (Gjerde and Boe 1981), 5% in France (Urdaci and others 1988) and 6.3% in Spain (Rodriguez-Castro and others 2010).

Presence of the regulatory gene of the toxin operon *toxR* gene or *gyrB* gene that encodes the B subunit protein of DNA gyrase (topoisomerase type II) is generally used for the detection of virulence factors. Taniguchi and others (1986) described the presence of a thermolabile hemolysin (*tlh*) in all virulent *V. parahaemolyticus* strains but not in other spp. Moreover, genes encoding the thermostable direct hemolysin (*tdh*) and the thermostable direct hemolysin-related hemolysin (*trh*) are strongly correlated with the virulence of *V. parahaemolyticus*. The presence of these 2 virulent factors (*tdh* and *trh* genes) is a useful tool for rapid investigation of suspected pathogenic *V. parahaemolyticus* strains in food samples (Gutierrez West and others 2013). The 16S rRNA gene is highly conserved among different species of bacteria. In certain cases, the 16S rRNA gene contains hyper variable region that can provide species-specific useful signature sequence for bacteria identification (Gerlach 2012). For the *V. parahaemolyticus*, the amplification of 16S rRNA has been conducted in many studies to confirm the detection and the relatedness among different *V. parahaemolyticus* (Gauger and Gomez-Chiarri 2002; González-Escalona and others 2005).

In Jordan, the real situation of food poisonings caused by *Vibrio* spp. is unknown, and no data exists on the prevalence of *V. parahaemolyticus* in fish and costal environment. Thus, the objectives of

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this study were (i) detection and identification of the pathogenic *V. parahaemolyticus* in fresh fish and in costal environment in Jordan, and (ii) identification of the virulence properties and relatedness among *V. parahaemolyticus* isolates from different sources.

Materials and Methods

Sample collection

Local marketed fish samples and water and sediment samples from The Gulf of Aqaba (northern tip of the Red Sea) were collected throughout an 8-month period extended from March to October, 2011, at biweekly intervals. A total of 150 fresh, refrigerated and organoleptically sound fish samples representing different species—*Greasy grouper* (35 samples), *Thunnus* spp. (15 samples), *Lethrinus nebulosus* (20 samples), *Aprion* spp. (10 samples), *Cyprinus carpio* (10 samples), *Argenteus pampus* (20 samples), *Commerson scomberomorous* (10 samples), *Sciaenops ocellatus* (10 samples), *Threadfin bream* (10 samples), and *Pagellua* spp. (10 samples)—were collected from the local market (each sample represented by 3 fish). Each sample was stored in clean bag and immediately placed in cleaned disinfected ice box. Twenty-five surface water samples (1 L each) from different points of the Gulf of Aqaba coast were collected in sterile plastic bottles. Twenty-five sediment samples (250 g each) from the upper 2 cm surface of different locations on the coast were also collected using sterile bags. All samples were transported to the laboratory under aseptic and cooling conditions in cleaned disinfected ice boxes. The samples were kept refrigerated at 0 °C and were analyzed within 24 h.

Isolation and identification of *V. parahaemolyticus* using conventional methods

V. parahaemolyticus (ATCC 17802) bacterial strain was used in this study as positive control. This strain is positive for *gyr*B, *toxR*, *tlh*, *trh*, and *tdh* genes. Standard culture methodology as described in the International Organization for Standardization (ISO 2007) with slight modification according to Sujeewa and others (2009) was followed. Twenty-five gram samples from whole fish skins and gills or from sediment samples were homogenized in 225 mL alkaline peptone saline water (APSW, Hi Media, Bombay, India), whereas 50 mL of water samples were inoculated in 450 mL APSW. The homogenate was mixed thoroughly for 1 min at 260 rpm using Stomacher Circular Unit 400 (Seward Ltd., London, UK), and incubated at 41.5 °C for 8 h.

A 10th milliliter of the incubated homogenate was streaked in duplicate on thiosulfate citrate bile salts sucrose agar (TCBS, Hi Media) and tryptone soy agar (Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with 3% NaCl (TSA + 3% NaCl). The inoculated plates were incubated at 37 °C for 18 to 24 h. Suspected colonies were streaked again on TSA + 3% NaCl to obtain a pure isolate. Typical colonies were tested for oxidase, motility, and urease tests and biochemically identified using Microgen™ Gn A+B -ID System (Microgen Bioproducts Ltd. Surrey, UK).

Polymerase chain reaction (PCR) procedures

Pure *V. parahaemolyticus* colonies from TSA + 3% NaCl were used to prepare the DNA templates. The DNA was extracted using the method described by Tada and others (1992) and Kim and others (1999). PCR assay was performed separately for *gyrB*, *toxR*, *tlh*, *tdh*, *trh,* and 16S rRNA genes of the suspected *V. parahaemolyticus* isolates. The amplification of a 285 pb DNA fragment of *gyrB* gene (VP1: 5′ CGG CGT GGG TGT TTC GGT AGT3′ VP2 R:5′ TCC GCT TCG CGC TCA TCAATA 3′) was conducted as described by Venkateswaran and others (1988). The isolates that possess *gyrB* gene were further tested for *toxR* gene. The amplification of a 368 bp DNA fragment of *toxR* gene (R: 5′GTC TTC TGA CGC AAT CGT TG 3′ F: 5′ATA CGA GTG GTT GCT GTC ATG 3′) was conducted as described by Kim and others (1999). The isolates that possess *toxR* gene were further tested for the presence of virulence genes (*tlh*, *trh,* and *tdh*). The amplification of a 450 bp DNA fragment of *tlh* gene (R-5′ GCT ACT TTC TAG CAT TTT CTC TGC 3′ F-5′ AAA GCG GAT TAT GCAGAA GCA CTG 3′) was performed as described by Kaysner and DePaola (2004). The amplification of a 251 bp DNA fragment of *tdh* (R-5′ CCA CTA CCA CTC TCA TAT GC 3′ F- 5′GGT CTAA ATG GCT GAC ATC 3′) and 250 bp DNA fragment of *trh* (R- 5′GGC TCA AAA TGG TTA AGCG 3′ F-5′ CAT TTC CGC TCT CAT ATGC 3′) genes were performed as outlined by Tada and others (1992).

The PCR reaction mixtures for 16S rRNA gene were prepared as for *tox*R gene. The amplification conditions of a 1125 bp fragment of the 16S rRNA gene (R- 5′ GCA AAC AAG GAT AAG GGT TGC GCT3′ F- 5′ TGA TCC TGG CTC AGA TTG AAC GCT 3′) were 35 cycles of amplification: denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 7 min.

The reaction mixtures were resolved by electrophoresis in 2% agarose gel and visualized under UV light. The presence of the goal bands compared with the DNA molecular weight standard (100 bp marker) was recorded. PCR products of the 16S rRNA were gel purified and sent for sequencing by dideoxynucleotide sequencing chemistry to Macrogen sequencing facility in Korea.

Table 1–Prevalence of *Vibrio parahaemolyticus* identified by conventional methods in fish and costal samples.

Sample source	Nr of samples	Nr (%) of positive samples on TCBS Agar	Nr (%) of positive samples by biochemical tests
Fish	150	101 (67)	101 (67)
Sediment	25	13 (52)	13 (52)
Water	25	18 (72)	18 (72)

Phylogenetic analysis of the isolates based on 16S rRNA sequencing

Sequences of 16S rRNA were viewed and edited using the BioEdit software. After editing, the sequences were aligned by MegAlign software of the DNA STAR software. Aligned 16S rRNA sequences were used for preparing the phylogenetic tree by the neighbor-joining method (Saitou and Nei 1987) using the MegAlign software.

Results

A total of 132 different samples showed typical *Vibrio* colonies on TCBS agar. Two typical colonies from each sample (264 colonies) were tested for biochemical identity. All of the isolates were proved biochemically as *V. parahaemolyticus* (Table 1). Out of these positive suspected samples, only 8 samples carried urease positive isolates.

Isolates that were identified as *V. parahaemolyticus* by the biochemical tests, represented by one isolate from each positive sample, were examined for *gyrB* and *toxR* genes targeting chromosomal locus at 285 and 368 bp, respectively. Accordingly, 108 samples harbored the *gyrB* gene, and 95 samples harbored the *toxR* gene (Table 2).

Table 2–Prevalence of *Vibrio parahaemolyticus* isolates containing *gyrB, toxR, tlh, tdh*, and *trh,* and genes.

Sample source	Nr of samples	*gyrB* gene Nr (%) of samples	*toxR* gene Nr (%) of samples	*tlh* gene Nr (%) of samples	*tdh* gene Nr (%) of samples	*trh* gene Nr (%) of samples
Fish	150	82 (55)	73 (49)	18 (12)	12 (8)	6 (4)
Sediment	25	12 (48)	10 (40)	1 (4)	1 (4)	0 (0)
Water	25	14 (56)	12 (48)	2 (8)	2 (8)	0 (0)
Total	**200**	**108 (54)**	**95 (48)**	**21 (11)**	**15 (7)**	**6 (3)**

The isolates that were positive to *toxR* gene (95 samples) were tested for the presence virulence genes (*tlh*, *tdh,* and *trh)* targeting chromosomal locus at 450, 251, and 250 bp, respectively. Twenty-one samples were positive for *tlh* gene. The number of samples that were positive for the presence of *tdh* or *trh* genes was 1, 2, and 12 samples for *tdh* gene and 0, 0, and 6 samples for *trh* gene for sediment, water, and fish samples, respectively. Only 2 samples were positive for the presence of both *tdh* and *trh* genes (Table 2).

The *gyrB* gene positive samples (108 samples) were tested for 16S rRNA. Primers used were targeting chromosomal locus 1125 bp. Ninety-five samples were positive for 16S rRNA. In another way, all *toxR* positive isolates showed the presence of the target gene in 16S rRNA (Table 2).

Phylogenetic analysis based on the 16S rRNA sequences was conducted to show the nucleotide similarity among the isolates from sediment, water, and fish samples (Figure 1). Generally, high similarities were recorded among fish samples. One sediment isolate (isolate No. 1) had similarity of 98.8% and 98.3% toward 2 water isolates (isolates No. 5 and 7). Three water isolates (5, 7, and 8) had high similarity toward one fish isolate (isolate No. 9). One fish isolate (isolate No. 40) had high similarity to water isolates (isolate No.5). In comparison, three fish isolates (37, 38, and 40) also were with high similarity to each other (Table 3).

Discussion

The most pathogenic *Vibrio* spp. like *V. parahaemolyticus*, *V. fulnicus*, and *V. cholera* are closely related to each other (Oliver and Kaper 1997). Therefore, conventional bacteriology methods, selective agar and biochemical tests, are inadequate techniques for the identification of *V. parahaemolyticus*. Based on the results obtained from the conventional method (TCBS agar and biochemical tests), the percentages of presumptive positive understudy samples for fish, sediment, and water samples were 67% (n = 150), 72% (n = 25), and 52% (n = 25), respectively. Out of the 108 positive samples for the *gyrB* gene, only 95 samples were carrier for *toxR* gene. Therefore, *toxR* gene is considered more specific than *gyrB* gene for identification of *V. parahaemolyticus* (Kim and others 1999). Therefore, this might be related to the 86.8% sequences homology in *gyrB* gene between *V. parahaemolyticus* and *V. aglinolyticus* (Venkateswaran and others 1988), whereas the degree of homology of the *toxR* gene between *V. parahaemolyticus* and *V. cholera* is only 52% (Kim and others 1999). The 16S rRNA has high similarity among *Vibrio* spp. It has been reported that the degree of homology between *V. parahaemolyticus* and *V. cholera* is 91% to 92% (Kita-Tsukamoto and others 1993; Lin and others 1993), whereas 99.7% homology does exist between *V. alginolyticus* and *V. parahaemolyticus* (Venkateswaran and others 1988). In this study, the result of PCR amplification of 16S rRNA was in complete agreement with that of *toxR* gene. This similarity could be related to the designed primers used in the current study. Accordingly, *toxR* gene can be used as a confirmatory step for *V. parahaemolyticus*. The prevalence rates for *V. parahaemolyticus* were 49% in fish, 40% in sediment, and 48% in water samples. These rates are similar to those reported in other studies from different developing global locations. A prevalence rate of 44% was observed in fish and costal water samples in Kenya (Kagiko and others 2001) or 43% for seawater samples and 37% for fish in Mexico (Cabrera-García and others 2004). A rate of 51% for seafood, water, and sediment samples reported in Malaysia (Sujeewa and others 2009), and 35% to 55% in fresh marine and brackish water fish in India (Sanjeev 2002). All of the 95 PCR (*toxR)* confirmed *V. parahaemolyticus* isolates were examined for virulence genes. Twenty isolates were positive for *tlh* gene, 14 isolates were positive for *tdh* gene, 6 for *trh* gene, and 2 of these isolates harbored all 3 virulence genes. Eight (8.4%) of 95 confirmed *toxR* positive isolates were positive for urease production and of these 8 only 6 isolates were positive for *trh* gene. It has been suggested that the production of urease can be used as screening test for *trh* gene (Suthienkul and others 1995), but as pointed out above not all urease positive necessarily carried *trh* gene (Sujeewa and others 2009). The prevalence of pathogenic *V. parahaemolyticus* in fish (12%) and water (2%) comes close to 6.3% reported for coastal water in Spain (Rodriguez-Castro and others 2010), 10% for fish caught off Chennai, Indian Ocean (Rajapandiyan and others 2009), and 8.5% and 1.5% of *tdh* and *trh* positive strains, respectively, for aquatic products in china (Chao and others 2009a). By the same meaning, a prevalence of 4%

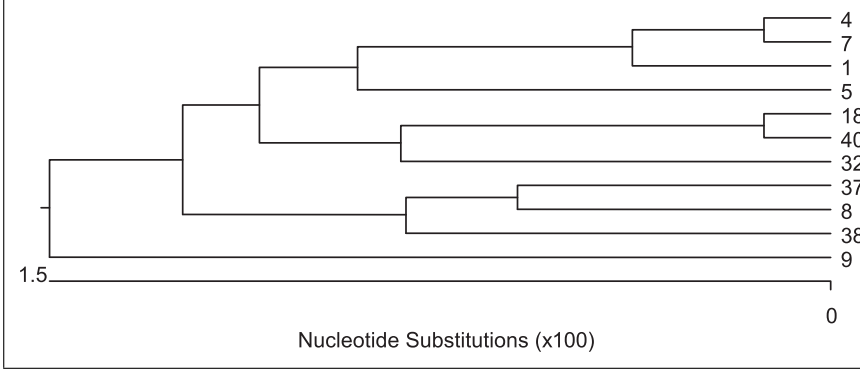

Figure 1–Phylogenetic tree of different *V. parahaemolyticus* isolates based on the partial sequence of 16S *rRNA* gene. Isolates with numbers 1, 4, 7, and 5 in the same cluster; 18, 40 along with isolate 32 in one cluster, isolates 37, 8, and 38 in one cluster and isolate number 9 stands alone in one cluster (isolates 1 and 4: sediment isolates, isolates 5, 7, and 8: water isolates, isolates 9, 18, 32, 37, 38, and 40: fish isolates).

Table 3–Nucleotide similarity percentage among different isolates of *V. parahaemolyticus* 1-R.seq, 4-R.seq (sediment samples); 5-R.seq, 7-R.seq and 8-R.seq (water samples); 9-R.seq, 18-R.seq, 32-R.seq, 37-R.seq, 38-R.seq and 40-R.seq (fish samples).

Percent Identity

Divergence	1	2	3	4	5	6	7	8	9	10	11		
1		96.7	97.7	95.3	96.2	98.6	96.4	98.3	98.8	95.6	95.9	1	1-R.seq
2	2.9		98.3	95.4	95.9	96.2	99.4	97.7	96.3	95.7	96.2	2	18-R.seq
3	2.1	1.5		96.6	97.2	96.7	97.9	99.0	96.8	96.9	97.9	3	32-R.seq
4	2.2	2.2	1.2		96.7	94.7	95.3	96.3	94.7	95.8	96.1	4	37-R.seq
5	2.7	3.1	2.0	0.9		95.4	95.4	97.0	95.7	95.8	95.8	5	38-R.seq
6	0.9	3.4	3.2	2.9	3.5		95.9	97.3	99.1	94.6	95.5	6	4-R.seq
7	3.0	0.2	1.8	2.5	3.5	3.6		97.5	96.3	95.1	96.2	7	40-R.seq
8	1.4	2.0	0.9	1.3	2.0	2.4	2.0		97.5	96.8	97.0	8	5-R.seq
9	0.6	3.2	2.9	2.9	3.2	0.2	3.3	2.0		94.9	95.3	9	7-R.seq
10	3.2	3.3	2.0	1.2	2.3	4.2	3.6	2.0	4.0		96.4	10	8-R.seq
11	4.0	3.9	2.0	2.3	2.6	4.5	3.7	2.8	4.7	3.3		11	9-R.seq
	1	2	3	4	5	6	7	8	9	10	11		

for sediments samples tested in this study do match other studies (4-6%) from developed geographical locations (Gjerde and Boe 1981; Rodriguez-Castro and others 2010) but do not match other studies (0.6%) from tropical locations (Gopal and others 2005) or (8.5%) from developing regions (Chao and others 2009b). More extensive studies taking in consideration the depth level and distance from the coast might be needed to verify their effect on level of contamination. In the nucleotide sequence analysis of the 16S rRNA, no relatedness were observed among the sources of *V. parahaemolyticus* isolates (either from water, sediment or fish) due to the high nucleotide similarity among the isolates. The nucleotide similarity among isolates might be related to cross-contamination during handling and marketing of fish samples where different fish species are marketed together using contaminated utensils or personnel.

Conclusion

In conclusion, the study confirmed the presence of pathogenic *V. parahaemolyticus* in seafood and coastal water and sediment in Jordan at similar rates found in other coastal regions. This study supports previous findings that TCB selective agar can be used as a rapid screening test for detection of *V. parahaemolyticus,* but a confirmatory identification of the presence of *tox*R gene is needed. Detection of virulence genes is necessary for the identification of pathogenic *V. parahaemolyticus.* Further studies linking *V. parahaemolyticus* as a diarrheal causative agent in clinical cases related to sea food consumption in Jordan comes of particular importance.

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References

Abd-Elghany SM, Sallam KI. 2013. Occurrence and molecular identification of *Vibrio parahaemolyticus* in retail shellfish in Mansoura, Egypt. Food Control 33:399–405.

Cabrera-García ME, Vázquez-Salinas C, Quiñones-Ramírez EI. 2004. Serologic and molecular characterization of *Vibrio parahaemolyticus* strains isolated from seawater and fish products of the Gulf of Mexico. Appl Environ Microbiol 70:6401–6.

Chao G, Jiao X, Zhou X, Yang Z, Huang J, Pan Z, Zhou L, Qian X. 2009a. Serodiversity, pandemic O3:K6 clone, molecular typing, and antibiotic susceptibility of foodborne and clinical *Vibrio parahaemolyticus* isolates in Jiangsu, China. Foodborne Pathog Dis 6:1021–8.

Chao G, Jiao X, Zhou X, Yang Z, Huang J, Zhou L, Qian X. 2009b. Distribution, prevalence, molecular typing, and virulence of *Vibrio parahaemolyticus* isolated from different sources in coastal province Jiangsu, China. Food Control 20:907–12.

Gauger EJ, Gomez-Chiarri M. 2002. 16S ribosomal DNA sequencing confirms the synonymy of *V. harveyi* and *V. carchariae*. Dis Aquat Organ 52:39–46.

Gerlach W. 2012. Taxonomic classification of metagenomic sequences [Doctoral dissertation]. Germany: Faculty of Technology, Bielefeld University. Available at: http://pub.unibielefeld.de/luur/download?func=downloadFile&recordOId=2468026&fileOId=2468027. Accessed 2014 October 28.

Gjerde EP, Boe B. 1981. Isolation and characterisation of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* from the Norwegian coastal environment. Acta Vet Scand 22:331–43.

González-Escalona N, Romero J, Espejo RT. 2005. Polymorphism and gene conversion of the 16S rRNA genes in the multiple rRNA operons of *Vibrio parahaemolyticus*. FEMS Microbiol Lett 246:213–9.

Gopal S, Otta SK, Kumar S, Karunasagar I, Nishibuchi M, Karunasagar I. 2005. The occurrence of *Vibrio* species in tropical shrimp culture environments; implications for food safety. Int J Food Microbiol 102:151–9.

Gutierrez West CK, Klein SL, Lovell CR. 2013. High frequency of virulence factor *tdh*, *trh*, and *tlh* genes in *Vibrio parahaemolyticus* strains isolated from a Pristine Estuary. Appl Environ Microbiol 79:2247–52.

International Organization for Standardization (ISO TS 21872-1). 2007. General guidance for the detection of V. parahaemolyticus. Geneva, Switzerland: International Organization for Standardization.

Kagiko MM, Damiano WA, Kayihura MM. 2001. Characterization of *Vibrio parahaemolyticus* isolated from fish in Kenya. East Afr Med J 78:124–7.

Kaysner CA, DePaola A Jr. 2004. *Vibrio*, Chapter 9. In: Hammack T, Davidson M, Feng P, Gharst G, Ge B, Jinneman K, Regan PM, Kase J, Orlandi P, Burkhardt W, editors. Bacterial analytical manual. Washington, DC: Food and Drug Administration. Available at: http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm070830.htm. Accessed 2014 October 28.

Kim YB, Okuda J, Matsumoto C, Takahashi N, Hashimoto S, Nishibuchi M. 1999. Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the *toxR* gene. J Clin Microbiol 37:1173–7.

Kita-Tsukamoto K, Oyaizu H, Nanba K, Shimidu U. 1993. Phylogenetic relationships of marine bacteria, mainly members of the family *Vibrionaceae*, determined on the basis of 16S rRNA sequences. Int J Syst Bacteriol 43:8–19.

Kubota K, Iwasaki E, Inagaki S, Nokubo T, Sakurai Y, Komatsu M, Toyofuku H, Kasuga F, Angulo FJ, Morikawa K. 2008. The human health burden of foodborne infections caused by *Campylobacter*, *Salmonella*, and *Vibrio parahaemolyticus* in Miyagi Prefecture, Japan. Foodborne Pathog Dis 5:641–8.

Leal NC, da Silva SC, Cavalcanti VO, Figueiroa ACTD, Nunes VVF, Miralles IS, Hofer E. 2008. *Vibrio parahaemolyticus* serovar O3:K6 gastroenteritis in northeast Brazil. J Appl Microbiol 105:691–7.

Lin Z, Kumagai K, Baba K, Mekalanos JJ, Nishibuchi M. 1993. *Vibrio parahaemolyticus* has a homolog of the *Vibrio cholerae* toxRS operon that mediates environmentally induced regulation of the thermostable direct hemolysin gene. J Bacteriol 175:3844–55.

Liston J. 1990. Microbial hazards of seafood consumption. Food Technol 44:56–62.

Lozano-León A, Torres J, Osorio CR, Martínez-Urtaza J. 2003. Identification of *tdh*-positive *Vibrio parahaemolyticus* from an outbreak associated with raw oyster consumption in Spain. FEMS Microbiol Lett 226:281–4.

McLaughlin JB, DePaola A, Bopp CA, Martinek KA, Napol NP. 2005. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. N Engl J Med 353:1463–70.

Mohammad AR, Hashim JK, Gunasalam J, Radu S. 2005. Microbiological risk assessment: risk assessment of Vibrio parahaemolyticus in Black Tiger Prawn (Penaeus monodon). Technical report. Putrajaya: Food Safety and Quality Division, Ministry of Health Malaysia.

Nishibuchi M, Kaper JB. 1995. Minireview. Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: a virulence gene acquired by a marine bacterium. Infect Immun 63:2093–9.

Oliver JD, Kaper JB. 1997. *Vibrio* species. In: Doyle MP, Beuchat LR, Montville TJ, editors. Food microbiology-fundamental and frontiers, Washington DC: ASM Press. P 228–64.

Ottaviani D, Leoni F, Rocchegiani E, Santarelli S, Canonico C, Masini L, DiTrani V. 2008. First clinical report of pandemic *Vibrio parahaemolyticus* O3:K6 infection in Italy. J Clin Microbiol 46:2144–5.

Paydar M, Teh CSJ, Thong KL. 2013. Prevalence and characterisation of potentially virulent *Vibrio parahaemolyticus* in seafood in Malaysia using conventional methods, PCR and REP-PCR. Food Control 32:13–18.

Rajapandiyan S, Sudha K, Arunachalam KD. 2009. Prevalence and distribution of *Vibrio vulnificus* in fishes caught off Chennai, Indian Ocean. Afr J Microbiol Res 3:622–5.

Robert-Pillot A, Guenole A, Lesne J, Delesmont R, Fournier JM, Quilici ML. 2004. Occurrence of the *tdh* and *trh* genes in *Vibrio parahaemolyticus* isolates from waters and raw shellfish collected in two French coastal areas and from seafood imported into France. Int J Food Microbiol 91:319–25.

Rodriguez-Castro A, Ansede-Bermejo J, Blanco-Abad V, Varela-Pet J, Garcia-Martin O, Martinez-Urtaza J. 2010. Prevalence and genetic diversity of pathogenic populations of *Vibrio parahaemolyticus* in coastal waters of Galicia, Spain Environ Microbiol Rep 2:58–66.
Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–25.
Sanjeev S. 2002. Pathogenic halophilic *vibrios* in seafoods. In: Gopalakrishna Iyer TS, Kandoran MK, Thomas M, Mathew PT, editors. Quality assurance in seafood processing. Cochin: Central Institute of Fisheries Technology and Society of Fisheries Technologists (India). p 252.
Su Y-C, Liu C. 2007. *Vibrio parahaemolyticus*: a concern of seafood safety. Food Microbiol 24:549–58.
Suffredini E, Mioni R, Mazzette R, Bordin P, Serratore P, Fois F, Piano A, Cozzi L, Croci L. 2014. Detection and quantification of *Vibrio parahaemolyticus* in shellfish from Italian production areas. Int J Food Microbiol 184:14–20.
Sujeewa AKW, Norrakiah AS, Laina M. 2009. Prevalence of toxic genes of *Vibrio parahaemolyticus* in shrimps (*Penaeus monodon*) and culture environment. Int Food Res J 16:89–95.
Suthienkul O, Ishibashi M, Iida T, Nettip N, Supavej S, Eampokalap B, Makino M, Honda T. 1995. Urease production correlates with possession of the *trh* gene in *Vibrio parahaemolyticus* strains isolated in Thailand. J Infect Dis 172:1405–8.
Tada J, Ohashi T, Nishimura N, Shirasaki Y, Ozaki H, Fukushima S, Takano J, Nishibuchi M, Takeda Y. 1992. Detection of thermostable direct hemolysin gene (*tdh*) and thermostable direct-related hemolysin gene (*trh*) of *Vibrio parahaemolyticus* by polymerase chain reaction. Mol Cell Probes 64:477–87.
Taniguchi H, Hirano H, Kubomura S, Higashim K, Mizuguchi Y. 1986. Comparison of the nucleotide sequences of the genes for the thermolabile hemolysin from *Vibrio parahaemolyticus*. Microb Pathog 1:425–32.
Tilburg JJ, Zwartkruis-Nahuis JTM, van den Berkmortel D, Rombout J, Jonker KM, de Boer E. 2000. Presence of *Vibrio* species in shellfish. Report of the Inspectorate for Health Protection and Veterinary Public Health, de Stoven 22, 7206 AX, Zutphen, The Netherlands.
Urdaci MC, Marchand M, Grimont PA. 1988. Espèces du genre *Vibrio* associées aux produits marins du bassin d'Arcachon [Abstract]. Ann Inst Pasteur Microbiol 139:351–62.
Venkateswaran K, Dohmoto N, Harayama S. 1988. Cloning and nucleotide sequence of the *gyrB* gene of *Vibrio parahaemolyticus* and its application in detection of this pathogen in shrimp. Appl Environ Microbiol 64:681–7.
Wang TK, Ho SI, Tsai JL, Pan TM. 1996. K-serotype analyses of *Vibrio parahaemolyticus* isolated in Northern Taiwan, 1983 through 1993 [Abstract]. Chinese J Microbiol Immun 29:210–24.
Xu X, Wu Q, Zhang J, Cheng J, Zhang S, Wu K. 2014. Prevalence, pathogenicity, and serotypes of *Vibrio parahaemolyticus* in shrimp from Chinese retail markets. Food Control 46:81–5.
Zhao F, Zhou D, Cao H, Ma L, Jiang Y. 2011. Distribution, serological and molecular characterization of *Vibrio parahaemolyticus* from shellfish in the eastern coast of China. Food Control 22:1095–1100.