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Occurrence of *Vibrio parahaemolyticus* in retailed seafood in The Netherlands

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Abstract: This study was conducted to determine the prevalence of *Vibrio parahaemolyticus* in seafood samples in The Netherlands. In total 200 seafood samples, including fish, shrimp, oyster and mussel, collected from the retail market in The Netherlands were examined for the occurrence of *V. parahaemolyticus* using both a cultural and a direct PCR-based method. Two different selective media, thiosulfate citrate bile salts agar (TCBS) and CHROMagar Vibrio (CV), were evaluated for their efficacy to isolate *V. parahaemolyticus* from seafood samples. The results showed that there were no differences among the two media to isolate *V. parahaemolyticus* from all seafood samples ($P > 0.05$). Using the cultural method, *V. parahaemolyticus* was isolated from 16 (8%) and 27 (13.5%) samples, on TCBS and CV plates respectively. All the positive samples were mussels and oysters. Of the 43 isolates of *V. parahaemolyticus* (on TCBS and CV) obtained, none of the isolates was positive for the genes *tdh* or *trh*. The PCR-based method was performed at 0 (t=0), 6 (t=6), and 18 (t=18) hours after the enrichment step and allowed the detection of *V. parahaemolyticus* in 22 (11%) and 38 (19%) samples, with the DNA extracts prepared from the first enrichment (t=6 h) and the second enrichment (t=18 h) respectively. None of the samples were detected to be *V. parahaemolyticus*-positive when the DNA extracts were prepared from the sample homogenate before the enrichment step (t=0 h).

Keywords: *V. parahaemolyticus*, seafood, retail market

Introduction

Vibrio spp. are Gram-negative, facultative anaerobic, curved rod shape, non spore forming bacteria that test positive for catalase and oxidase (Austin, 2010; Nair *et al*., 20062). These bacteria are motile, have polar flagella, and are inhibited by the vibriostatic compound 0129 (Hofer *et al*., 2001). Vibrios are ubiquitous bacteria that are naturally present in the marine environment, and are particularly resistant to high salt concentrations. Several species within the genus *Vibrio* are associated with foodborne infections and food spoilage. Some species are more specifically pathogenic to humans, such as *Vibrio parahaemolyticu*s and *Vibrio cholerae*, which are causes of severe intestinal diseases (Kass and Riemann, 2006; Nishibuchi and DePaola, 2005). Among the 65 species that have now been described in the genus Vibrio (Twedt, 1989), twelve species (*V. cholerae, V.mimicus, V. metschnikovii, V. cincinnatiensis, V. hollisae, V. damsel, V. fluvialis, V. furnissii, V. alginolyticus, V. parahaemolyticus, V. vulnificus, V. carchariae*) are recognized as human pathogens (Nair *et al*., 2006), with 8 species considered to be directly food associated (Oliver and Kaper, 2007). Of these, 3 species (*V. cholerae, V. parahaemolyticus and V. vulnificus*) are the most important and responsible for most cases of food-borne illness (Austin, 2010; Nair *et al*., 20062; Sakazaki *et al*., 2006).

Vibrio infections are important food-borne diseases of bacterial origin especially in Asia and Africa regions, causing approximately half of the food-related outbreaks in Thailand, Cambodia, India, and other Southeast Asian countries (Ahmed and Shakoori, 2002; Das *et al*., 2009). It is also known as the major cause of gastroenteritis associated with seafood (McLaughlin *et al*., 2005; Mead *et al*., 1999; Ottaviani *et al*., 2005; Wagley *et al*., 2009). *Vibrio* spp. occur naturally in estuarine and marine environments, mainly in warm climate (Deepanjali *et al*., 2005). The increase in seafood consumption and the global warming, resulting in increased ocean surface temperatures, may cause higher prevalence of *Vibrio* spp, and enhance the risk of Vibrio food-borne infections.

V. parahaemolyticus is widely distributed in marine environment and has been recognized as a major cause of foodborne illness associated with the consumption of raw, undercooked or contaminated shellfish. It can cause mild to moderate gastrointestinal infections, which are usually self limiting and rarely fatal. The pathogenicity factors of *V. parahaemolyticus* are known to be caused by the presence of thermostable direct hemolysin (*tdh*)

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and thermostable direct hemolysin related hemolysin (*trh*) genes (Ray and Bhunia, 2008). Many outbreaks of foodborne infection, especially in Asia countries, have been frequently reported to be due to the presence of these bacteria. Although the incidence of *V. parahaemolyticus* infection is not as frequent as in Asia, several outbreaks have also been reported in the United States and Europe (Mead *et al*., 1999; McLaughlin *et al*., 2005; Ottaviani *et al*., 2005).

The present study was conducted to gain a better understanding of the prevalence of *V. parahaemolyticus* in seafood samples in The Netherlands.

Materials and Methods

Sample collection and preparation

A total of 200 shellfish samples were investigated for the presence of *V. parahaemlyticus*. These samples (included fish, shrimps, oysters, and mussels) were obtained from the local retail markets from various locations in The Netherlands. Samples were collected during the months of June-August 2009. Samples were stored at 5-8°C before analysis, and analyzed within 48 h of collection.

Detection and identification of V. parahaemolyticus

All samples were analyzed following the protocols of the International Organization for Standardization (ISO/TS 21872-1) (Anonymous, 2007).

The samples (10 g) were homogenized in 90 ml alkaline saline peptone water (ASPW) in a sterile polythene stomacher bag for 1 min. Incubation of the first enrichment was done at 41.5°C ± 1°C for 6 h ± 1 h, after which, one milliliter volume of the first enrichment culture (taken from the surface of the broth) was transferred to 9 ml ASPW as the second enrichment broth. Subsequently, a loop (1 µl) of the second enriched broth was streaked onto TCBS and CHROMagarTMVibrio (CV) agar plates. The presence of typical colonies of presumptive V. parahaemolyticus was examined after 24 h ± 3 h incubation of the plates at 37°C. Typical colonies of *V. parahaemolyticus* are green, 2-3 mm and purple, 2-3 mm on TCBS and CV, respectively. Typical colonies of presumptive *V. parahaemolyticus* were subcultured onto Saline Nutrient Agar (SNA), and tested for presumptive identification (oxidase test, Gram staining, morphology, and motility). The colonies being oxidase-positive, Gram-negative, rod curve shaped and motile, were then identified further for the species level using the API 20E system (bioMérieux, Marcy I'Etoile, France). The colonies suspected as *V. parahaemolyticus* were confirmed by PCR assay.

We also did a direct PCR assay of the samples with a DNA template prepared by following the DNA extraction protocol as described by Rosec *et al*. (2009). 500 µl of the sample homogenate (taken before the enrichment step (t=0 h), after the first (t=6 h) and second (t=18 h) enrichment of ASPW) were subjected to the classical PCR for detection of *V. parahaemolyticus*.

A PCR assay targeting on toxR gene was carried out for confirmation of the *V. parahaemolyticus* isolate, while the pathogenicity of the isolates was checked by PCR-detection of *tdh* and *trh* genes. *V. parahaemolyticus* ATCC 17802, *V. parahaemolyticus* WP1 and *V. parahaemolyticus* AQ4037 were used as positive DNA control for *toxR*, *trh*, and *tdh* gene, respectively.

Preparation of DNA template

Strains were grown overnight in 5 ml BHI. One milliliter of the overnight cultures was centrifuged at 3000 x g for 5 min in 1.5 ml Eppendorf tubes. After discharging the supernatant, the pellets were then resuspended in 500 µl RO water. Bacterial suspensions were heat lysed in a thermal block (Thermomixer Compact Eppendorf, Germany) for 10 min at 95°C with shaking at 1400 rpm, and the supernatant was obtained by centrifugation (10.000 x g for 5 min) on a tabletop centrifuge (Centrifuge 5415R or Centrifuge 5424 Eppendorf, Germany). The supernatant was then transferred to a new tube and used as DNA template for the PCR assay or stored at -20°C until the PCR assay.

Primers and PCR conditions

The primers used for detection of *V. parahaemolyticus*, *tdh* and *trh* are listed in Table 1. The PCR was run in a 25 µl volume of the reaction mixture consisting of 2.5 µl of the DNA template and 22.5 µl PCR mix using the following reaction component (in final concentration): 2.5 µl of 10x Faststart buffer (Roche), 2 µl of 10 µM dNTP's (Roche), 6 µl of 5 µM MgCl2 (Roche), 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 0.2 µl of Faststart Taq DNA polymerase (Roche), and 9.8 µl sterile water. PCR assay was performed using Eppendorf Mastercycler Gradient, Germany. For the toxR primer, the PCR was performed with the following program: one cycle of initial denaturation at 94°C for 10 min, 30 cycles consisting of denaturation at 94°C for 60 sec, annealing at 63°C for 90 sec, extension at 72°C for 90 sec, one cycle of additional extension at 72°C for 5 min, and final step at 14°C. For the tdh and trh primers, the PCR was performed with the following program: one cycle of initial denaturation at 94°C for

10 min, 35 cycles consisting of denaturation at 94°C for 60 sec, annealing at 55°C for 60 sec, extension at 72°C for 60 sec, one cycle of additional extension at 72°C for 5 min, and final step at 14°C.

Table 1. PCR primers used for detection of *V. parahaemolyticus*, the thermostable direct hemolysin gene (*tdh*), and the thermostable direct hemolysin-related hemolysin gene (*trh*)

Target gene	Primer name	Sequence (5'-3')	Product size (bp)	Reference
toxR	Vp toxR1 F Vp toxR2 R	GTCTTCTGACGCAATCGTTG ATACGAGTGGTTGCTGTCATG	368	Kim *et al*., 1999
tdh	tdh-1 F tdh-2 R	CCACTACCACTCTCATATGC GGTACTAAATGGCTGACATC	251	Tada *et al*., 1992
trh	trh-3 F trh-4 R	GGCTCAAAATGGTTAAGCG CATTTCCGCTCTCATATGC	250	Tada *et al*., 1992

Amplified products were separated by electrophoresis in 1.5% agarose gel with TBE buffer at 100 V for 50 min, and documented using Optigo Isogen Life Science using Opticom software. A 100 bp DNA ladder marker XIV (Roche Diagnostics) was used as molecular marker.

Statistical analysis

Statistical analysis of results was performed with Chi-square test and Fisher's exact two–tailed test using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). A value of $P<0.05$ was considered statistically significant for comparison.

Results

Two hundred seafood samples were investigated for the presence of *V. parahaemolyticus*. Table 2 summarizes the results of the prevalence study. *V. parahaemolyticus* was detected in 16 (8%) and 27 (13.5%) samples, on TCBS and CV plates respectively, when the detection was conducted by the cultural method. *V. parahaemolyticus* was isolated most frequently from oysters and mussels. Of 10 samples of oysters, 2 (20%) and 3 (30%) samples, on TCBS and CV plates respectively, were positive for *V. parahaemolyticus*. Of 30 samples of mussels, 14 (47%) and 24 (80%) samples, on TCBS and CV plates respectively, were positive for *V. parahaemolyticus.* Of the 43 isolates of *V. parahaemolyticus* obtained, none was positive for *tdh* or *trh.*

Moreover, the PCR assay with the DNA extracts prepared from the second enrichment (t=18h) could identify *V. parahaemolyticus*-contaminated samples that were negative using the cultural method. Direct PCR assay allowed the detection of *V. parahaemolyticus* in 22 (11%) and 38 (19%)

Table 2. Prevalence of *V. parahaemolyticus* isolated from seafood samples in The Netherlands by cultural method and direct PCR method

Samples	Number (%) of samples positive using cultural method		Number (%) of samples positive using direct PCR method		
	On TCBS	On CV	t = 0 h	t = 6 h	t = 18 h
Fish (*n* = 107)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Salmon (*n* = 13)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Atlantic code (*n* = 17)	0 (0)	0 (0)	0 (0)	0 (0)	1 (6)
Pangasius (*n* = 16)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Tilapia (*n* = 16)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Pollock (*n* = 8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Plaice (*n* = 7)	0 (0)	0 (0)	0 (0)	1 (3)	1 (3)
Others (*n* = 30)	0 (0)	0 (0)	0 (0)	1 (2)	1 (2)
Shrimps (*n* = 53)	2 (20)	3 (30)	0 (0)	4 (40)	7 (70)
Oysters (*n* = 10)	14 (47)	24 (80)	0 (0)	16 (53)	28 (93)
Mussels (*n* = 30)	**16 (8)**	**27 (14)**	**0 (0)**	**22 (11)**	**38 (19)**
Total (*n* = 200)					

* Results expressed as the number of positive sample; the numbers in bracket indicate the percentage.

first enrichment (t=6 h) and the second enrichment (t=18 h) respectively. None of the samples was detected to be *V. parahaemolyticus*-positive when the DNA extracts were prepared from the sample homogenate before the enrichment step (t=0 h). With direct PCR, *V. parahaemolyticus* was also detected in one shrimp and two fish sample/samples (Table 2). However, statistical analysis revealed no significant differences between the cultural method using TCBS and CV, and the three direct PCR protocols.

Discussion

In the present study, the investigation on the prevalence of *V. parahaemolyticus* in seafood samples was performed using two methods, a cultural and a PCR-based method. As shown in Table 2, it was suggested that direct PCR method was more sensitive, compared to cultural method, in detecting *V. parahaemolyticus*. This was indicated by its ability in detecting more *V. parahaemolyticus*-contaminated samples that were negative using the cultural method. The same to what has been reported in previous studies (Blackstone *et al*., 2003; Blanco-Abad *et al*., 2009; Kim *et al*., 2008; Tyagi *et al*., 2009). Furthermore, as observed in this study, the application of PCR assay allows for a rapid detection of *V. parahaemolyticus* (took only 3 hours) compared to cultural method which took 2 days before the results were obtained. Although the PCR-based method following the two-stage enrichment was found to be the most rapid, reliable and sensitive method for detection of *V. parahaemolyticus*, for further examination it is required to keep and to store the bacterial specimens, which can only be obtained by a culture method. Hence, these two methods of

detection and identification are complementary to each other.

Overall, 19% of all seafood samples were *V. parahaemolyticus*-positive (38 positive samples out of 200). This prevalence is lower than that reported from China (47.2%) (Chao *et al*., 2009), India (55%) (Chakraborty *et al*., 2008), and Italy (24.3%) (Ottaviani *et al*., 2005). The variation in the prevalence of *V. parahaemolyticus* is conceivably due to seasonal effects, different species of seafood examined, different analytical methods used and possibly the different hygienic practices applied during the handling of seafood products. Of all samples, mussels and oysters samples were most frequently contaminated with these enteropathogenic bacteria with isolation rates of 93% and 70%, respectively. The higher prevalence of *V. parahaemolyticus*-positive samples in the oysters are comparable with that reported from India in which *V. parahaemolyticus* was detected in 93.87% of the samples (Deepanjali *et al*., 2005). However, in contrast to that study, none of the *V. parahaemolyticus* from oysters were positive as determined by PCR for the presence of the *tdh* and *trh* genes.

Comparative analysis of two different selective media (TCBS and CV) revealed CV to be a better choice for isolation and identification *V. parahaemolyticus*, although no significant differences were found between these two media. CV can differentiate various species of *Vibrio* spp., as well as non-*Vibrio* bacteria based on the various colours of the colonies, while TCBS only gave 2 different colours of colony (yellow and green). The colour change of the colony after some extent of incubation on TCBS, as reported by Hara-Kudo *et al*. (2001) was not observed in this study. Furthermore, CV can inhibit the growth of other interfering bacteria more than TCBS, which makes it more easy to isolate the *V. parahaemolyticus* typical colony from the plate.

In conclusion, the presence of *V. parahaemolyticus* in mussels and oysters determined in this study, suggests that mussels and oysters may be potential sources of *V. parahaemolyticus* in The Netherlands. Hence, the data obtained in this study are expected to give valuable information on the microbiological safety of seafoods in The Netherlands.

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