OCCURRENCE OF *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS* IN RETAIL RAW OYSTERS FROM THE EASTERN COAST OF THAILAND

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Abstract. Occurrence, population density and virulence of *Vibrio parahaemolyticus* and *V. vulnificus* in 240 retail raw oysters collected monthly between March 2010 and February 2011 from Ang Sila coast, Chon Buri Province, Thailand were determined using most probable number (MPN) multiplex PCR. Multiplex PCR detected *V. parahaemolyticus* in 219 raw oyster samples, of which 29 samples contained the virulence *tdh*. MPN values for *V. parahaemolyticus* and pathogenic strains in most samples ranged from 10 to 10^2 and from 3 to 10 MPN/g, respectively. The presence of *V. vulnificus* was found in 53 oyster samples in amounts between 10 and 10^2 MPN/g. Of 1,087 *V. parahaemolyticus* isolates, 14 and 2 isolates carried *tdh* and virulence *trh,* respectively but none with both genes. However, none of the presumptive isolates was shown to be *V. vulnificus*. The detection of pathogenic *V. parahaemolyticus* and *V. vulnificus* in raw oysters has rendered high awareness of risk in consumption of raw or undercooked oysters.

Keywords: *Vibrio parahaemolyticus*, *Vibrio vulnificus*, multiplex PCR, retail oyster

INTRODUCTION

Vibrio parahaemolyticus and *Vibrio vulnificus* are gram-negative halophilic bacteria found ubiquitously in estuarine and marine environments and are recognized as major food-borne pathogens. *V. parahaemolyticus* is a common cause of gastroenteritis associated with consumption of raw or undercooked seafood, especially shellfish (Drake *et al*, 2007). Infection with *V. vulnificus* can occur through direct exposure of a wound to sea water and causes a more serious disease, even progressing to septicemia and fatality (Jone and Oliver, 2009). Illness due to *V. parahaemolyticus* has been reported more widely worldwide (CDC, 2005; Wang *et al*, 2007) including in Thailand (Jatapai *et al*, 2010), whereas *V. vulnificus* is a rare cause of illness, but it incidence underreported (Kiratisin *et al*, 2012).

Pathogenicity of *V. parahaemolyticus* is associated with the thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH), which are encoded by *tdh* and *trh,* respectively (Nishibuchi and Kaper,

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1995). Both TDH and TRH have various biological activities, including hemolytic activity, cardio toxicity, and enterotoxicity (Shimohata and Takahashi, 2010). However, thermolabile hemolysin gene (*tl*) is present in all *V. parahaemolyticus* strains and is used as a molecular marker for species identification (Di Pinto *et al*, 2008).

PCR detection based on *V. vulnificus*-specific hemolysin gene (*vvh*) is used for identification of this pathogen, but *vvh* is present in all strains isolated from clinical and environmental sources (Panicker *et al*, 2004). Pathogenicity of *V. vulnificus* involves many factors and mechanisms that are still poorly understood. From a practical point of view, none of the current analysis methods can reliably distinguish between virulent and non virulent strains of this bacterium.

The increasing numbers of susceptible individuals as well as the development of international trade, occurrence of *V. parahaemolyticus* and *V. vulnificus* in seafood is of great concern. Although there are many reports related to the prevalence of *V. parahaemolyticus* and *V. vulnificus* in oysters from Asia, Europe, and the United States (Wright *et al*, 2007; Lee *et al*, 2008; Cañigral *et al*, 2010), such studies are uncommon in Thailand. The development of PCR-based detection of multiple *Vibrio* species has been previously reported (Izumiya *et al*, 2011). In Thailand a validated multiplex PCR assay for the simultaneous detection of *V. parahaemolyticus* and *V. vulnificus* in oyster and seawater has been developed (Aeamsri, 2012).

In this study, the most probable number (MPN) method coupled with multiplex PCR were employed to determine the prevalence of *V. parahaemolyticus* and *V. vulnificus* in raw oysters for retail sale along Ang Sila coast in Chon Buri Province, Thailand. In addition, direct detection and enumeration of *V. parahaemolyticus* and *V. vulnificus* in oyster samples using pure culture isolations of both target organisms were carried out and subsequently subjected to molecular characterization.

MATERIALS AND METHODS

Reference strains

V. parahaemolyticus DMST 15285 (*tl*$^+$, *tdh*$^+$), *V. parahaemolyticus* ATCC 17802 (*tl*$^+$, *trh*$^+$), and *V. vulnificus* DMST 19346 (*vvh*$^+$), were from the culture collection of the National Institute of Health (NIH), Department of Medical Sciences, Ministry of Public Health, Thailand.

Source of oysters

A total of 240 raw shucked oysters (*Saccostrea cucullata*) cultivated on Ang Sila coast, Chon Buri, Thailand, were purchased monthly from local retailers during March 2010 to February 2011, packed on ice and transported to the laboratory within an hour and analyzed immediately.

Detection and enumeration of *V. parahaemolyticus* and *V. vulnificus* in raw oyster samples

The most probable number-multiplex PCR method, modified from the USA Food and Drug Administration Bacteriological Analytical Manual (FDA, 2004), was used for detection and enumeration of *V. parahaemolyticus* and *V. vulnificus* in the raw oyster samples. In brief, ten-fold serial dilutions of the oyster homogenates were prepared in sterile alkaline peptone water (APW), pH 8.6, for the 3-tube-MPN procedure. Following incubation for 18 hours at 35ºC, 1 ml aliquot of each MPN tube showing growth was centrifuged at 10,000*g* for 5 minutes, pellet washed with sterile TE buffer (10 mM Tris-HCl,

1 mM EDTA, pH 8.0) and DNA extracted as previously described (Aeamsri, 2012). In short, the cell pellet was treated with SDS-proteinase K lysis solution (0.5% (w/v) SDS, and 0.15 mg/ml proteinase K in TE buffer) at 37ºC for 30 minutes. Following centrifugation at 10,000*g* for 5 minutes, DNA was precipitated from the supernatant with isopropanol, washed with 70% cold ethanol and stored in sterile deionized water at -20ºC until used.

Multiplex PCR amplification of *tl*, *tdh* and *vvh* was conducted in a total volume of 50 l consisting of 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 M each primer [those for *tl* and *tdh* from Bej *et al* (1999) and of *vvh* from Panicker *et al* (2004)], 5 l of DNA, and 2.5 U *Taq* DNA polymerase (Vivantis, Shah Alam, Malaysia). Thermocycling (Biometra, Göttingen, Germany) was performed as follows: 95ºC for 15 minutes and then addition of *Taq* DNA pol; followed by 35 cycles of 94ºC for 45 seconds, 63ºC for 30 seconds, and 72ºC for 30 seconds; with a final step at 72ºC for 7 minutes. Amplicons were separated by 1.5% agarose gel-electrophoresis, stained with ethidium bromide and visualized under a UV transilluminator. Positive control of *tl*, *tdh* and *vvh* generated amplicon of 450, 269 and 205 bp, respectively, and negative control contained nuclease-free distilled water. Samples that displayed negative amplification, which implied undetectable level of target bacteria, were subjected to repeat analysis.

MPN values of positive PCR results for a particular bacterium in each set of three replicates were estimated from the MPN table (FDA, 2004). The results are expressed as MPN/g of oysters. The lowest detection limit of this approach was 3 MPN/g and the upper limit was 1,100 MPN/g.

Characterization of *V. parahaemolyticus* and *V. vulnificus* isolates

One loopful of APW-enriched cultures of *V. parahaemolyticus* and *V. vulnificus* was streaked onto CHROMagar Vibrio (CHROMagar Microbiology, Paris, France) and incubated at 37ºC for 24 hours. The isolates, presumptively indicated as *V. parahaemolyticus* (mauve colonies) and *V. vulnificus* (green blue colonies) were subjected to identification tests, viz. Gram staining, oxidase test, growth on triple sugar iron (TSI) agar and motility-indole-lysine test (Farmer *et al*, 1985).

Identification of both organisms was confirmed by PCR. In brief, cultures of *V. parahaemolyticus* and *V. vulnificus* were grown overnight at 35ºC on trypticase soy agar (TSA) (Difco, Detroit, MI) supplemented with 3% NaCl. DNA was extracted from bacteria by boiling for 10 minutes, followed by centrifugation at 10,000*g* for 10 minutes and supernatant was stored at -20ºC until used for monoplex PCR analysis. The species-specific primers targeting the *tl* and *vvh* of *V. parahaemolyticus* and *V. vulnificus* were used to confirm the respective species. In addition to the species specific gene marker, pathogenic *V. parahaemolyticus* was indicated by the presence of *tdh* (269 bp amplicon) and/or *trh* (500 bp amplicon) (Bej *et al*, 1999) according to monoplex PCR assays.

RESULTS

Occurrence and density of *V. parahaemolyticus*, *tdh*$^+$ *V. parahaemolyticus* and *V. vulnificus* in raw oysters

Multiplex PCR detection of *V. parahaemolyticus* and *V. vulnificus* in raw oysters developed in our previous study was adopted as a tool in this survey. The detection limit of the technique after 4-6

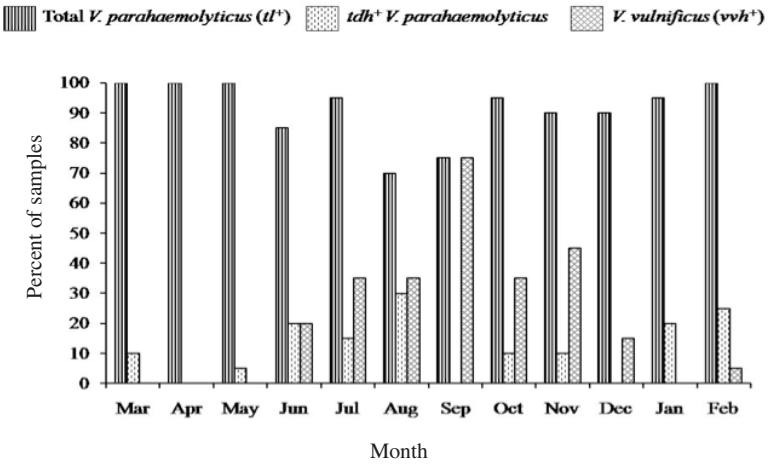

Fig 1–Monthly occurrence of *V. parahaemolyticus* and *V. vulnificus* in raw oysters sampled from Ang Sila coast, Chon Buri, Thailand.

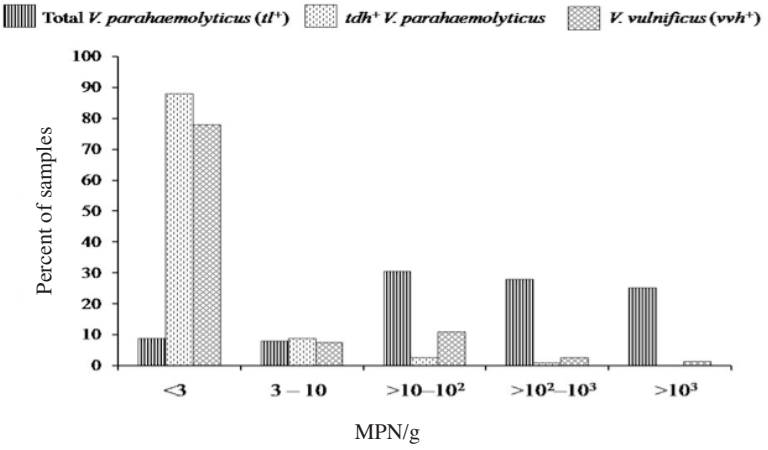

Fig 2–Density of *V. parahaemolyticus* and *V. vulnificus* in raw oysters sampled from Ang Sila coast, Chon Buri, Thailand.

hours of enrichment was 100 cfu/g in spiked oyster samples. Comparison of the multiplex PCR and the conventional culture method was made to validate our technique, and the values of relative accuracy, relative specificity and relative sensitivity of the multiplex PCR were 96-100% (Aeamsri, 2012).

Employing multiplex PCR assay technique, *V. parahaemolyticus* was detected in retail raw oyster samples throughout the year of collection, 219/240 (91%) of the samples tested (Fig 1). Percent *V. parahaemolyticus*-positive samples in each month varied from 70% to 100%. Pathogenic strains of *V. parahaemolyticus* (harboring the *tdh*) could be detected in only 29 samples (12%), most frequently in the rainy season (June-October). *V. vulnificus* was found in 53 (22%) of oyster samples and was most common in the rainy season, with no positive detection during the summer months (March-May).

Density of the target organisms varied from below detection limit (3 MPN/g) to more than 1,100 MPN/g (Fig 2). The density range of *V. parahaemolyticus*, *tdh*$^+$ *V. parahaemolyticus* and *V. vulnificus* was 10-10^2, 3-10 and 10 to 10^2 MPN/g, respectively. The highest mean level of *V. parahaemolyticus* was both in summer (307.7 MPN/g) and in winter (November to February) (303.1 MPN/g), while the highest mean levels of *tdh*$^+$ *V. parahaemolyticus* and *V. vulnificus* was observed in rainy season (46.6 and 92.7 MPN/g, respectively).

Table 1
Distribution of *V. parahaemolyticus* and *V. vulnificus* in raw oysters.

Vibrio sp	*N*	*n*	%	Min-Max (MPN/g)	Mean (MPN/g)		
					Summer[a]	Rainy season[b]	Winter[c]
V. parahaemolyticus	240	219	91	3-1,100	307.7	233.9	303.1
tdh$^+$ *V. parahaemolyticus*	240	29	12	3-210	3.2	46.6	25.2
V. vulnificus	240	53	22	3-1,100	ND	92.7	46.2

N, number of raw oyster samples; *n*, number of positive PCR results; ND, not detectable.
[a] March-May, [b]June-October, [c]November-February.

Chacterization of *V. parahaemolyticus* and *V. vulnificus* isolates

Of 1,168 expected *V. parahaemolyticus* isolates obtained from the raw oyster samples, 1,087 (93.1%) isolates displayed positive PCR (*tl*$^+$), confirming the presence of *V. parahaemolyticus*. Among the *tl*$^+$ strains, 14 (1.3%) isolates carried *tdh* and 2 (0.2%) isolates possessed *trh*. However, no *V. parahaemolyticus* isolate having both *tdh* and *trh* was detected. On the other hand, all of expected *V. vulnificus* (191 isolates) showed negative results for *vvh*-targeted PCR.

DISCUSSION

To date, there is no information regarding the occurrence of non-pathogenic and pathogenic strains of *V. parahaemolyticus* and *V. vulnificus* in raw shucked oysters from the eastern coast of Thailand, despite the Ang Sila coast being recognized as not only one of the most attractive recreation destinations but also a large commercial site for oyster cultivation in this region of Thailand. In the present study, high levels of contamination (70-100%) by *V. parahaemolyticus* in raw oyster samples were evident throughout the year of study (March 2010 - February 2011), indicating the ubiquitous nature of this organism in marine environment of this region of Thailand. The frequency of occurrence of *V. parahaemolyticus* found in this study is similar to previous studies of Pacific oysters (*Crassostrea gigas*) in South China showing 89.3% contaminated with *V. parahaemolyticus* (Chen *et al*, 2010). Furthermore, occurrences of pathogenic *V. parahaemolyticus* (12%) and *V. vulnificus* (22%) in the current study were higher than those reported by Kirs *et al* (2011) who found that the *tdh*$^+$ *V. parahaemolyticus* and *V. vulnificus* strains in Pacific oysters from New Zealand to be 3.4% and 17.2%, respectively. Although no significant correlation between environmental parameter and evidence of *V. parahaemolyticus* and *V. vulnificus*, the researchers remarked that the temperature and salinity of *tdh*$^+$ *V. parahaemolyticus* samples are above 35.5°C and 35.9 ppt, respectively. *Vibrio* infections after consumption of raw oysters are more common in tropical and temperate regions. We noted that the temperature of coastal water of the Southeast Asian region is always warm throughout the year, thereby it may be a factor influencing the abundance of these bacteria in

marine environment of Thailand.

The densities of *V. parahaemolyticus* in oyster samples varied greatly (from < 3 MPN/g to >1,100 MPN/g), with the higher population densities encountered during the winter months (data not shown). The population densities reported here were higher than in other studies; for example, the numbers of *V. parahaemolyticus* in raw oysters from Seoul, South Korea increase in summer (10^3 MPN/g) and decrease in fall (< 10 log MPN/g) and to an undetectable level in winter (Lee *et al*, 2008). Pathogenic *tdh*$^+$ *V. parahaemolyticus* population density was relatively lower (3-210 MPN/g) with higher levels occurring in the rainy season. Likewise, population densities of *V. vulnificus* (< 3 to > 1,100 MPN/g) were higher in the rainy season, but *V. vulnificus* was not detected in summer. The population density of pathogenic *V. parahaemolyticus* in oysters from Yaquina (5.6%) and Tillamook (9.1%) Bay, Oregon, USA is very low (≤ 3.6 MPN/g), being detected in July and August (Duan and Su, 2005). However, Chen *et al* (2010) reported high incidence (54.9%) of *V. vulnificus* in oyster samples with high population densities (> 10^4 MPN/g) dominate during fall season similar to those obtained in our study. This study emphasized that the rainy season may be a suitable condition for growth and survival of pathogenic *Vibrio* spp, and therefore leading to their accumulation in growing oysters.

To explain the absence of *V. vulnificus* in summer, we hypothesize that summer conditions are not favorable to the growth of pathogenic *Vibrio* strains. Although *Vibrio* spp grows well in warm saline water, other factors influencing distribution of both *Vibrio* species may include salinity, pH, nutrient levels and pollutants.

Moreover, prevalence of both *Vibrio* species in retail oysters may depend on conditions of harvest, transport, and temperature during temporary preservation at each beach kiosk. Inappropriate storage during post-harvest processing and marketing, especially refrigeration, is the major cause of oyster samples being exposed to high ambient temperature, which allows microorganisms to multiply. According to the US FDA guidelines, post-harvest processed fresh and frozen seafood including oysters that are labeled "processed to reduce *V. parahaemolyticus* and *V. vulnificus* to non-detectable levels" must present evidence that the levels of both pathogens are < 30 MPN/g (FDA, 2011).

As regards the detection of low proportion of pathogenic *V. parahaemolyticus* strains (1.3% and 0.2% for *tdh* and *trh* strain, respectively) in this study, this was in agreement with other reports. Costa Sobrinho *et al* (2010) found that only 1 (0.04%) amongst 2,243 oyster-derived isolates from oysters harvested in the southern coast of Sao Paulo State, Brazil, were identified as containing *tdh*$^+$ *V. parahaemolyticus*. It is well known that only 1-5% of environmental *V. parahaemolyticus* strains possess the *tdh* and/or the *trh* (Nishibuchi and Kaper, 1995). However, we were unsuccessful in isolating *V. vulnificus* from samples of raw oysters, possibly due to the initial proportion of *V. vulnificus* in oyster samples was too low to culture and/or the presence of viable but non-culturable cells.

In summary, this is the first study indicating that *V. parahaemolyticus* was present in the majority of retail raw oysters cultivated on Ang Sila coast of Chon Buri, Thailand, while pathogenic *V. parahaemolyticus* and *V. vulnificus* were less

commonly found. Although pathogenic *Vibrio* spp was detected in oysters at low population density, infection of these bacteria can cause severe symptoms in humans consuming raw or undercooked oysters. Thus, recognition of hazards from consumption of raw oysters is of concern. Data from this study should be useful for risk assessment plans and prevention of disease outbreaks in this coastal area of Thailand.

ACKNOWLEDGEMENTS

This study was supported by the Center of Excellence on Environment Health, Toxicology and Management of Chemicals (EHT), Thailand.

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