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Prevalence of toxic genes of *Vibrio parahaemolyticus* in shrimps (*Penaeus monodon*) and culture environment

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Abstract: *Vibrio parahaemolyticus* is prevalent in tropical marine environment in all seasons and can cause seafood-borne gastroenteritis. A total of 251 suspected isolates were tested including 60 from frozen shrimp, 50 from cultured live shrimp, 67 from sediments of culture ponds and 74 from water were subjected to polymerase chain reaction (PCR) targeting the *tox*R gene for confirmation as *V. parahaemolyticus*. Of the 128 *tox*R positive isolates, 15% of the isolates from culture environment (from live shrimp, sediments and water) and 7% of frozen shrimp samples were positive for the *tdh* and *trh* genes. Since urease production could be a marker of *trh* but not *tdh* in *V. parahaemolyticus,* a total of 189 of the 251 suspected *V. parahaemolyticus* isolates were tested for urease production and 41% of the isolates were found to be positive for urease production. However not all urease positive strains of *V. parahaemolyticus* were positive for either *tdh* or *trh* genes. Detection of virulent strains in shrimp culture environment in Malaysia suggests a probable risk for health of people consuming raw shrimp.

Keywords: *Vibrio parahaemolyticus*, virulent strain, *tox*R-gene, *tdh*-gene, *trh*-gene, black tiger shrimp

Introduction

Shrimp accounts for about 20% of the value of exported fishery products over the past 20 years (CAC, 2002). Imports into developed countries accounted for about 40% of intra-developed countries trade, while about 60% comes from developing countries; out of the exports from developing countries 80% goes to developed countries and only 20% stays in the group (Josupeit, 2005). Also, shrimps are one of the major aquaculture products of export importance from the tropics. Eighty percent of the world's farm raised shrimps is contributed through aquaculture in Asia (Bhaskar *et al*., 1998). The number of detention and rejection cases due to the detection *V. parahaemolyticus* from Asia is increasing.

Vibrio parahaemolyticus is a gram-negative halophilic bacterium distributed in temperate and tropical coastal waters throughout the world (DePaola *et al*., 2000) and some strains can cause gastroenteritis in humans through the consumption of contaminated seafood (Matsumoto *et al*., 2000). *V. parahaemolyticus* is a major cause of food-borne illness in the world (Joseph *et al*., 1983; Wong *et al*., 2000a) and in Asia, approximately half of the food poisoning outbreaks in Taiwan, Japan, and several Southeast Asian countries are due to *V. parahemolyticus* (Chiou *et al*., 1991). Raw or undercooked fish and seafoods have been implicated as major vehicles of *V.* parahaemolyticus infection to humans (Venkitanarayanan and Doyle, 2001). The presence of thermostable direct hemolysin (TDH) is a proven virulence factor which can cause gastroenteritis (Nishibuchi *et al*., 1992). A proposed virulence factor, the TDH-related hemolysin (TRH), encoded by the gene *trh*, and has been discovered in clinical stains of *V. parahaemolyticus* lacking *tdh* (Honda *et al*., 1987; Honda *et al*., 1988).

European Commission concluded that the practice of judging seafood exclusively based on total *V. parahemolyticus* counts without consideration of the virulent factors such as *tdh* and *trh* is not appropriate (EC, 2001). Robert-Pillot *et al*. (2004) also

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mentioned that total *Vibrio* counts are not indicative enough for the presence of pathogenic *Vibrios* as the non pathogenic *Vibrios* can be present in seafood or environmental waters. Therefore the presence of haemolysin genes is always considered as markers of pathogenicity in *V. parahaemolyticus*.

More than 90% of clinical *V. parahaemolyticus* isolates possess *tdh* (DePaola *et al*., 1990; Kaysner *et al*., 1990; DePaola *et al*., 2000; Wong *et al*., 2000b). In contrast, the *tdh* and *trh* genes were rarely detected in the environmental strains of *V. parahaemolyticus* (Shirai *et al*., 1990; Kishishita *et al*., 1992). The incidence of pathogenic *V. parahaemolyticus* has been reported to be less than 1-2% among environmental strains (Kelly and Stroh, 1988; Honda and Iida, 1993), but studies using molecular techniques indicate higher prevalence of pathogenic strains.

PCR based on *tox*R and on a chromosomal locus of unknown function reported to be specific for *V. parahaemolyticus* (Lee *et al*., 1995) has been found to be useful for confirmation of this species (Karunasagar *et al*., 1997; Kim *et al*., 1999). It has been demonstrated that the PCR technique can detect a low number of specific bacteria against a large background of other prokaryotic and eukaryotic cells and organic materials which may present in the samples (Tsai and Olson, 1992; Thiem *et al*., 1994; Laser *et al*., 1995). Those properties make PCR a suitable method for analyzing environmental samples.

As only a small proportion of the environmental strains are virulent, to determine shrimp safety, it would be important to specifically detect these strains in the shrimps and culture environment. The objective of this study was to determine the prevalence of virulent strains of *V. parahaemolyticus* in frozen shrimp which was ready for human consumption and in shrimp culture environment in Malaysia.

Materials and methods

Bacterial isolates for PCR analysis

A total of 251 suspected isolates of *V. parahaemolyticus* were analysed for toxic genes. Those isolates consisted of 50 from cultured live shrimp, 67 from sediments of culture ponds, 74 from water (from culture ponds and water sources) and 60 from frozen shrimp. Standard culture methodology utilizing alkaline salt peptone water (ASPW) enrichment following spreading and isolation on thiosulfate citrate bile salts sucrose (TCBS) agar plates was used to obtain these *V. parahaemolyticus* isolates (ISO, 1990). Enrichment in ASPW (MPN) and streaking on TCBS agar were used to isolate 60 cultures from frozen shrimp (ISO, 1990). A clinical strain of O3:K6 which was *tdh*-gene positive and another *trh* positive strain which was obtained from University Putra Malaysia were used as reference strains in this study. All isolates were grown in tryptone soy agar supplemented with 3% NaCl (TSA+3% NaCl) and streaked for isolation of single colonies. These single colonies were used for extraction of DNA.

Extraction of DNA

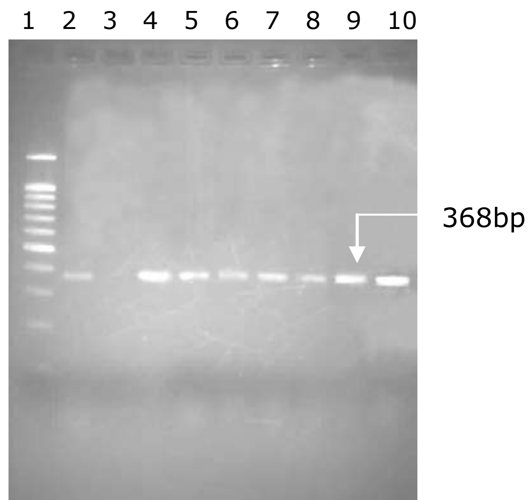
Colonies of bacterial isolates from TSA+3% NaCl were mixed with 500 µl of sterile de ionized water inside Eppendorf tubes. This was mixed well by using vortex mixer. The suspension was heated for 10 min in a heat block (Thermo mixer 5436) and then cooled on ice immediately. Cell debris of these cell lysates was pelleted by centrifugation (at 13000 rpm for 2 min) and the supernatants were used as DNA templates in this PCR assay. PCR was performed separately for *tox*R, *tdh* and *trh* genes for suspected isolates of *V. parahaemolyticus* as described previously (Tada *et al.,* 1992; Kim *et al.,* 1999).

PCR analysis

The reaction mixture for *tox*R analysis consisted of 1.2 µl of the DNA templete, 11.9 µl of de ionized water, 2 µl of 10X buffer (Bioron), 1.6 µl of 2.5 mM concentration of dNTP (Bioron), 1.6 µl of Magnesium Chloride (Bioron), 0.1 µl of taq DNA polymerase and 0.8 µl of each primer (Oligo 10pmol/ µl). 18.8 µl of PCR master mixture was aliquot into PCR tubes (20 µl). The amplification conditions were 20 cycles of amplification consisting of denaturation at 94^{0}C for 1 min, annealing at 63^{0}C for 1.5 min and extension at 72^{0}C for 1.5 min.

All 128 isolates (DNA templates**)** which gave positive results for *tox*R were examined for *tdh* and *trh* genes targeting chromosomal locus at 251 and 250 bp. The PCR reaction mixtures for *tdh* and *trh* genes were prepared as for *tox*R. Primers specific for *tdh* and *trh* genes (0.8 µl of each primer Oligo 10pmol/ µl) were used. The amplification conditions were 35 cycles of amplification consisting of denaturation at 94^{0}C for 1 min, annealing at 55^{0}C for 1 min and extension at 72^{0}C for 1min.

Nine microliters of the reaction mixture was mixed with 1 µl of gel loading buffer and the mixture was resolved by electrophoresis in 2% agarose gel. Electrophoresis gel was stained with ethidium bromide



Lane 1: 100-bp molecular size marker
Lane 2: reference strain (*V. parahaemolyticus* with positive *toxR-gene*, 368 bp)
Lane 3: blank
Lanes 4-5: isolates from the frozen product
Lanes 6-7: isolates from the live shrimp
Lanes 8-9: isolates from the pond sediment
Lane 10: isolates from the pond water

Figure 1. The presence of pathogenic *V. parahaemolyticus* by detection of *tox*R-gene

Lane 1: 100-bp molecular size marker
Lane 2: reference strain (*V. parahaemolyticus* with positive *tdh-gene*, 251 bp)
Lane 3: blank
Lanes 4-5: isolates from the frozen product
Lanes 6-7: isolates from the live shrimp
Lanes 8-9: isolates from the pond sediment
Lane 10: isolates from the pond water

Figure 2. The presence of *V. parahaemolyticus* virulent strain by detection of *tdh-gene*

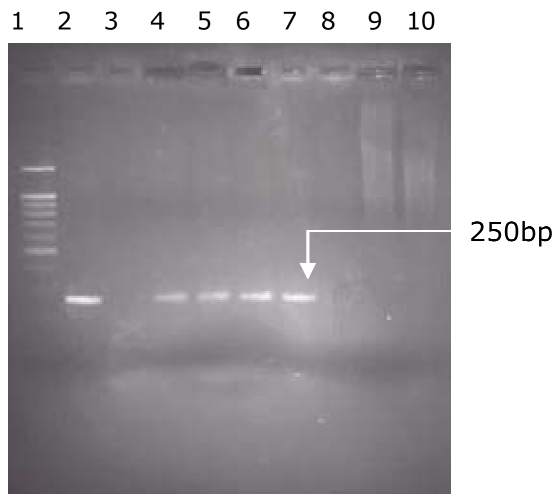
for 10-15 seconds and destained under running tap water for 10-15 minutes. The electrophoresis gel was placed centrally on the transilluminator and observed. The presence of the goal band was compared with the DNA molecular weight standard (100 bp marker). If the gene was present, a DNA band was visualized on an agarose gel.

Urease test

A total of 183 suspected *V. parahaemolyticus* isolates were tested for urease production. Isolates which were grown in TSA+3% NaCl for 20 h were used for this test. Slants of urea agar were prepared in fresh and a heavy inoculum of cultures was streaked on the surface of slants and incubated at 37°C for 20 h. Production of urease was determined by a pink colour (alkaline) to the medium. Negative cultures were incubated an additional 3-4 days for the slow urease producing strains.

Results and discussion

V. parahaemolyticus was confirmed by PCR targeting the toxR gene at 368 bp chromosomal locus specific for this species. One hundred and twenty eight (128) out of 251 (51 %) isolates were confirmed to be *V. parahaemolyticus* by the *tox*R using PCR (Figure 1). *V. parahaemolyticus* strains possess a regulatory gene, *tox*R, which is present in all the strains (Lin *et al*., 1993) irrespective of their ability to produce *tdh* or *trh*. Kim *et al*. (1999) also stated that the PCR method targeted to the *tox*R gene can be used as a method for identification of *V. parahaemolyticus* at the species level. Further, Lee *et al*. (1995) noted that the sequence of a cloned fragment of chromosomal DNA of *V. parahaemolyticus* was specific for this species and Karunasagar *et al*. (1997) reported that PCR amplifying this portion could be used to detect *V. parahaemolyticus* in fish and shellfish. Dileep *et al.*



Lane 1: 100-bp molecular size marker
Lane 2: reference strain (*V. parahaemolyticus* with positive *trdh-gene*, 250 bp)
Lane 3: blank
Lanes 4-5: isolates from the frozen product
Lanes 6-7: isolates from the live shrimp
Lanes 8-9: isolates from the pond sediment
Lane 10: isolates from the pond water

Figure 3. The presence of *V. parahaemolyticus* virulent strain by detection of *trh-ge*ne

(2003) stated that the *tox*R targeted gene PCR method is more sensitive in detecting *V. parahaemolyticus* than the method based on isolation and biochemical identification.

Toxic genes (*tdh* and *trh*) are only present in virulent strains but not in the non-virulent strains of *V. parahaemolyticus*. It is noteworthy to mention that *tdh* and *trh* like genes have been found in some strains of other *Vibrio* species such as *V. mimicus*, *V. cholerae* and *V. hollisae* (Nishibuchi and Kaper, 1995). Thus their presence may explain the high level of pathogenic *V. parahaemolyticus* than actual. For this reason in our study *tdh* and *trh* genes were detected only in isolates that have previously been identified as *V. parahaemolyticus* by detecting *tox*R.

All 128 isolates which gave positive results for *tox*R were examined for *tdh* and *trh* genes targeting chromosomal locus at 251 and 250 bp. Five, 8 and 1 strain isolated from live shrimp, sediment and frozen shrimp respectively were positive for *tdh* gene (Figure 2). Among 128 isolates tested 2, 1 and 1 number of strains isolated from live shrimp, pond water and frozen shrimp respectively were positive for *trh* gene (Figure 3).

However, in this study about 15% and 7% of the isolates from culture environment and frozen shrimps possessed the haemolysin *tdh* and *trh* genes. Mohammad *et al*. (2005) also reported the prevalence of virulent genes in shrimp culture environment in Malaysia was 8%, 11% and 17% of frozen shrimp, raw shrimp and pond water respectively. Current published reports also show an increase of the environmental strains carrying the *tdh* and/or *trh* genes (Wong *et al*., 1993; Hervio-Heath *et al*., 2002). DePaola *et al*. (2003) also reported that 12.8% of Alabama oysters were positive for *tdh*+ *V. parahaemolyticus*. It has also been reported that 1-5% of environmental *Vibrio* isolates possess the *tdh* or the *trh* gene (Nishibuchi and Kaper, 1995; Hervio-Heath *et al*., 2002; Robert-Pillot *et al*., 2004). Some other researches reported that the frequency of *tdh* detection in environmental samples and seafoods ranges from 0 to 6% (Kiiyukia *et al*., 1989; Ogawa *et al*., 1989; Cook *et al*., 2002).

In this experiment enrichment in ASPW and MPN method was used to isolate *V. parahaemolyticus* from frozen shrimp whereas direct plating method was used to isolate *V. parahaemolyticus* from raw shrimp, pond sediment and water samples. Pathogenic *V. parahaemolyticus* strains were detected by both the direct-plating and enrichment methods.

A total of 189 *V. parahaemolyticus* isolates were tested for urease production, and 41% revealed urease positive. Contrary to the report presented by Kaysner *et al*. (1994) the presence of *tdh* did not correspond to a strain's ability to hydrolyze urea in most of our isolates; of the 14 *tdh* carrying strains, 12 were found to be negative for urea hydrolysis and only 2 strains were urea positive. By contrast, the presence of *trh* corresponded to the ability to hydrolyze urea; all *trh* carrying strains were urease positive. However not all urease positive strains produce either *tdh* or *trh*. Urease production could be a marker of *trh* in *V. parahaemolyticus* strains, but it does not systematically correlate with the possession of the *trh* gene (Suthienkil *et al*., 1995; Kaufman *et al*., 2002). Osawa *et al*. (1996) also examined 132 strains of *V. parahaemolyticus* and found urea hydrolysis not to be a reliable marker for the production of TDH.

Conclusion

The detection of the *tdh* virulent gene in 14 strains of *V. parahaemolyticus*, one isolated from frozen shrimp, five from live shrimp and eight from pond sediment and also the detection of *trh* gene in four strains*,* two from live shrimp, one from water and one from frozen shrimp in shrimp culture environment in Malaysia suggests a probable risk for health of people consuming raw shrimp. Urease production could be a marker of *trh* but not *tdh* in *V. parahaemolyticus*. However not all urease positive strains of *V. parahaemolyticus* produce either *tdh* or *trh*. Those results suggest that the long term monitoring programme should be initiated to detect pathogenic *V. parahaemolyticus* isolates in the shrimp culture environment as its recovery may affect shrimp export industry in Malaysia.

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