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. Isolates were confirmed as *V. parahaemolyticus* by Polymerase chain reaction (PCR) targeting the *tox*R gene and a 368 bp chromosomal locus specific for this species. All 128 isolates (DNA templates) which gave positive results for *tox*R were examined for the presence of the thermostable direct hemolysin (*tdh*) and related hemolysin (*trh*) genes. A total of 189 suspected *V. parahaemolyticus* isolates were tested for urease production. Fifty one percent (51 %) isolates were confirmed to be *V. parahaemolyticus* by the *tox*R PCR test whereas 8.4% of the isolates from the culture environment (from live shrimp, sediments and water) possessed the *tdh* and *trh* genes. Seven percent (7%) of frozen shrimp samples were positive for these two virulent genes. About 41% of isolates were found to be positive for urease production. Urease production could be a marker of *trh* but not *tdh* in *V. parahaemolyticus*. However not all urease positive strains of *V. parahaemolyticus* possess either *tdh* or *trh*. Detection of virulent strains in shrimp culture environment in Malaysia suggests a probable risk for health of people consuming raw shrimp.

Introduction

V. parahaemolyticus contamination in black tiger shrimp is a priority issue in the shrimp culture industry in Malaysia due to the increased rejection of block frozen raw black tiger shrimp from Malaysia by certain importing countries (Mohammed *et al.*, 2005).

V. 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). The presence of the 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.* 1988).

More than 90% of clinical *V. parahaemolyticus* isolates possess *tdh* (DePaola *et al.*, 2000). In contrast, the *tdh* and *trh* genes were rarely detected in the environmental strains of *V. parahaemolyticus* (Kishishita *et al.*, 1992). As only a small proportion of the

environmental strains are virulent, to determine shrimp safety, it would be important to detect these strains in the shrimps and culture environment.

Materials and Methods

A total of 241 suspected isolates of *V. parahaemolyticus* were analysed for toxic genes. These isolates consisted of 50 from cultured raw/live shrimp, 67 from the sediments of culture ponds and 74 from the water (from culture ponds and water sources). 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 50 cultures from frozen shrimp (ISO, 1990). All isolates were grown in tryptone soy agar supplemented with 3% NaCl (TSA+3% NaCl) and streaked for isolation of single colonies. Four to five colonies were mixed with 500 µl of sterile deionized water in 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 (Kim *et al.*, 1999; Tada *et al.*, 1992).

For *tox*R analysis the master mixture was prepared based on the number of samples. For each sample, 11.9 of deionized water, 2 µl of 10X buffer (Bioron), 1.6 µl of 2.5 mM concentration of dNTP (Bioron), Magnesium Chloride (Bioron) and 0.8 µl of each primer (Oligo, sequences are Fwd 5'-GTCTTCTGACGCAATCGTTG-3' and Rev 5' -ATACGA GTG GTT GCT GTC ATG-3') was mixed. A 18.8 µl volume of this PCR master mixture was aliquot into PCR tubes (20 µl). After that the DNA sample (1.2 µl) were added into the PCR tubes. For the control, 1.2 µl of distilled water was added to the reaction mixture instead of the sample. Finally 0.1 µl of taq DNA polymerase was added. PCR tubes were placed onto the PCR thermocycler (Eppendorf) and appropriate amplification conditions were applied.

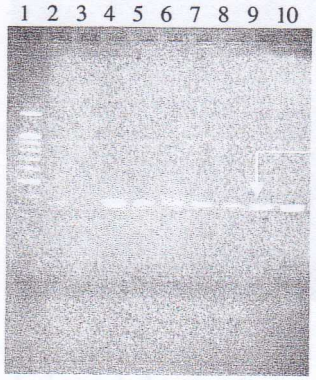
All isolates (128 DNA templates) which gave positive results for *tox*R were examined for *tdh* (251 bp) and *trh* (250 bp) genes. The PCR reaction mixtures for *tdh* and *trh* genes were prepared as for *tox*R. Primers specific for *tdh* (sequences are Fwd 5'-CCA CTA CCA CTC TCA TAT GC-3' and Rev 5'-GGT ACT AAA TGG CTG ACA TC-3') and *trh* (sequences are Fwd 5'-GGC TCA AAA TGG TTA AGC G-3' and Rev 5'-CAT TTC CGC TCT CAT ATG C-3') genes were used. PCR tubes were placed onto the PCR thermocycler and it was run under appropriate amplification conditions. The products of PCR were resolved by electrophoresis on 2% agarose gels, stained with ethidium bromide and finally visualized under uv illumination.

A total of 183 suspected *V. parahaemolyticus* isolates were tested for urease production. Slants of urea agar were prepared in fresh and a heavy inoculum of cultures was streaked

on the surface of slants. It was 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 *tox*R gene at 368 bp specific for this species. One hundred and twenty eight (128) out of 251 (51 %) isolates were confirmed to be *V. parahaemolyticus* by the presence of *tox*R (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.



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

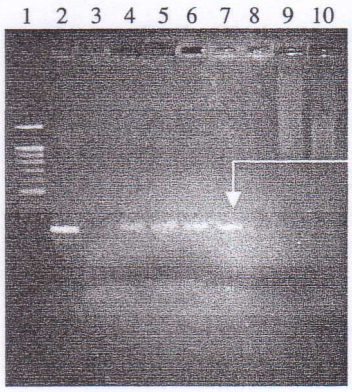
Figure 1: Confirmation of pathogenic *V. parahaemolyticus* by the detection of *tox*R-*ge*ne

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). In this study 8.4 % and 7 % of the isolates from culture environment and frozen shrimps possessed the haemolysin *tdh* and *trh* genes. 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). Detection of virulent strains in shrimp culture environment in Malaysia suggests a probable risk for health of people consuming raw shrimp.

A total of 183 *V. parahaemolyticus* isolates were tested for urease production, and 48% were 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 possess either *tdh* or *trh*.

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 *tdh-gene* in *V. parahaemolyticus* isolates



Lane 1: 100-bp molecular size marker
Lane 2: reference strain (*V. parahaemolyticus* with positive *trh-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 *trh-gene* in *V. parahaemolyticus* isolates

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