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OCCURRENCE OF TOXIC GENES IN *VIBRIO PARAHAEMOLYTICUS* ISOLATES FROM SHRIMP(*PENAEUS MONODON*) CULTURE ENVIRONMENT IN MALAYSIA

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ABSTRACT

The occurrence of *Vibrio parahaemolyticus* in water, sediment and shrimps (*Penaeus monodon*) samples from shrimp culture environment was studied. The biochemical identification of *V. parahaemolyticus* was confirmed by Polymerase chain reaction (PCR) targeting the toxR gene and a 387 bp chromosomal locus specific for this species. All the *V. parahaemolyticus* strains isolated were tested for haemolysin genes. PCR with two sets of primers was used to detect the haemolysin genes *tdh* and *trh* in *V. parahaemolyticus* strains. A total of 125 *V. parahaemolyticus* strains were analyzed in this study, including 28 from culture shrimp, 52 from sediments of culture ponds and 45 from water. 58.4% of strains found to be positive for *tox*R. 9.6% of the isolates from culture environment possessed the haemolysin *tdh* gene. None of the isolates found to be positive for *trh*. Our results indicate that pathogenic *V. parahaemolyticus* strains are present in shrimp culture environment in Malaysia.

INTRODUCTION

Vibrio parahaemolyticus is prevalent in tropical marine environment in all season and can cause seafood-borne gastroenteritis. *V. parahaemolyticus* 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, based on their detection of the presence of *V. parahaemolyticus* in the block frozen raw black tiger shrimp imported from Malaysia. In terms of trade, the fish industry is one of the major export and income source of Malaysia and the industry contributed RM1.3 billion (1.5 million ton/year) or 20% to the total food export from Malaysia (Radu et al. 2005). European Commission concluded that the practice of judging seafood exclusively based on total *V. parahemolyticus* counts without consideration of the virulent factors (thermostable direct haemolysin (*tdh*) and *tdh* related haemolysin (*trh*)) is not appropriate (Anon 2001). 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.

MATERIALS AND METHODS

All shrimp farms raised shrimp by the semi-intensive method which is most common in Malaysia. Samples were collected from 3 shrimp farms in Selangor state in Malaysia. Five random samples of shrimps, sediment and water from each culture pond were

aseptically collected. Samples of sediment and shrimps were collected in sterile polythene bags. Water samples from culture ponds were collected in sterile bottles. Three to five water samples from water source for farms were collected into sterile bottles. Samples of shrimp, pond water and pond sediment were taken to microbiology laboratory in University Kebangsaan Malaysia for analysis. Bacteriological analysis consisted of examination for *V. parahaemolyticus*. The whole shrimps were cut aseptically into small dices using sterile scalpel. Sediment was mixed well. From the above mixture 25g of shrimp or sediment were weighed into a sterile stomacher bag and blended in a stomacher with 225ml of alkaline salt peptone water (ASPW) for 2 min. and 1 min. respectively at medium speed level. These blended samples constituted the 1:10 dilution and were serially diluted using 9ml sterile peptone (3%). 0.1 ml aliquots (from 10^{-1} to 10^{-4}) were spread plated onto TCBS agar in duplicate and incubated at 37°C (Anon 1990). The morphology and the number of colonies on TCBS were recoded for all samples. Colonies were sub cultured on NA with 3% sodium chloride and identified using Mini API 20E (BioMerieux France). Salinity (Atago Co. Ltd., Tokyo Japan), temperature and pH (Hanna HI 8424) of water samples were recorded.

PCR was performed separately for *tox*R, *tdh* and *trh* genes for the biochemically identified isolates of *V. parahaemolyticus* as described previously (Kim et al. 1999; Tada et al 1992). Colonies from 3% nutrient agar (which were biochemically confirmed as *V. parahaemolyticus*) were mixed with 500µl sterile distilled water inside Eppendrof tubes. This was mixed well by using vortex mixer. The suspension was heated in a hot bath for 10 min to lyse the cells and snap cooled on ice for rapid release of DNA. The lysate was immediately stored at -20°C until PCR was performed. PCR reaction was performed in a 20µl volume consisting of 2µl of 10X buffer, 1.6µl of 2.5mM concentration of dNTP, 0.8µl of each primer and 0.1µl of *Taq* polymerase. 1.2µl of DNA extract from sample was applied to subsequent PCR reaction. The PCR was performed in a thermal cycler (AB Applied Biosystems USA). The products of PCR were resolved by electrophoresis on 2% agarose gels, stained with ethidium bromide and finally visualized under illumination.

RESULTS AND DISCUSSION

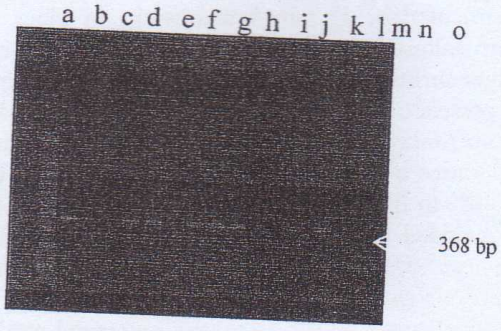

FIGURE 1 Agarose gel electrophoresis of *tox*R PCR products

Lane a: 100-bp molecular size marker
b: reference strain (368bp)
c: control
d-g: isolates from water samples
h-k: isolates from shrimp samples
l-o: isolates from sediment samples

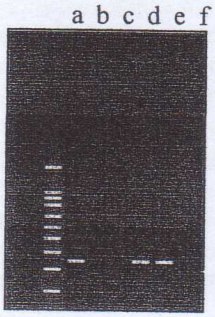

Figure 2: Detection of *tdh* gene by PCR
Lane a: 100-bp molecular size marker
b: reference strain (251bp)
c: control
d: isolates from water samples
e: isolates from shrimp samples
f: isolates from sediment samples

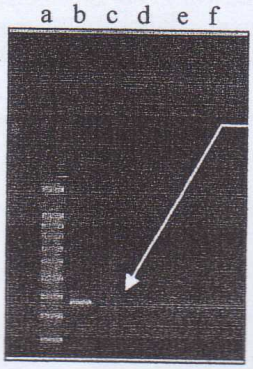

Figure 2: Detection of *trh* gene by PCR
Lane a: 100-bp molecular size marker
b: reference strain for *tdh* (251bp)
c: control
d: isolates from water samples
e: isolates from shrimp samples
f: isolates from sediment samples

Seventy three of 125 cultures showing typical biochemical reactions 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 irrespective of their ability to produce *tdh* or *trh*. Kim et al. (1999) stated that the PCR method targeted to the *tox* R gene can be used as a method for identification at the species level. The *tox*R gene is found only in *V. parahaemolyticus* and not in other bacteria and is used to identify this bacterium. *tdh* or *trh* gene is only present in virulent strain but not in the non-virulent strain of *V. parahaemolyticus*. It is noteworthy 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 & Kaper 1995). Thus their presence may explain the high level of pathogenic *V. parahaemolyticus* detected. For this reason *tdh* and *trh* genes were detected only in isolates that have previously been identified as *V. parahaemolyticus*.

All 73 samples were examined for *tdh* and *trh* positive *V. parahaemolyticus* targeting the *tdh* and *trh* at 251 and 250 bp chromosomal locus. Four and 8 strains isolated from shrimp and sediment respectively were positive for *tdh* (Figure 2). None of the isolates found to be positive for *trh* (Figure 3). Early work of Sakazaki et al. (1968) and Thompson & Vanderzant (1976) showed that Kanagawa *tdh* positive strains of *V. parahaemolyticus* account only 1-2% of environmental strains. But in this study 9.6% of the isolates from culture environment possessed the haemolysin *tdh* gene. Current published reports show an increase of the environmental strains carrying the *tdh* and/or *trh* genes (Wong et al. 1993; Hervio-Heath et al. 2002).

The water temperature ranged between 28.5^{0}C and 31^{0}C. The salinity of the water was 21-33‰ and pH was 6.7-8.6. With these values of the parameters that influence the growth of *V. parahaemolyticus,* it is no surprise that the prevalence rates of *V.* parahaemolyticus at all levels examined were high, and in most cases were 100% positive.

CONCLUSIONS

The detection of the *tdh* gene in 12 strains of *V. parahaemolyticus*, 4 isolated from shrimp and 8 from pond sediment, in shrimp culture environment in Malaysia suggests a probable risk for health of people consuming raw shrimp. Our 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.

ACKNOWLEDGEMENTS

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