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Identification of *Vibrio parahaemolyticus* isolates by PCR targeted to the *toxR* gene and detection of virulence genes

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Abstract: *Vibrio parahaemolyticus* is a gram negative bacterium and causes gastrointestinal illness in humans. In this study, twenty five out of fifty cockle samples from Padang, Indonesia produced purple colonies when they were grown on selective medium, CHROMagar^{TM} *Vibrio*. Specific–PCR for *toxR* gene detection gave positive results in which a band with 368 base pairs size appeared on the gel for all the isolates that confirmed the presence of *V. parahaemolyticus*. In the virulence properties test, all the isolates showed negative results for *tdh* and *trh* genes detection. The results indicate that the isolates under this study do not contain virulence properties that correlate to the ability of infection and diseases, which means that they are non-pathogenic.

Keywords: *Vibrio parahaemolyticus*, *toxR* gene, PCR, *trh* gene, *tdh* gene

Introduction

V. parahaemolyticus is a bacterium in the same family as those that cause cholera. It lives in brackish saltwater and causes gastrointestinal illness in humans. It is a halophile, or salt-requiring organism. Most people become infected by eating raw or undercooked shellfish, particularly oyster and cockles. At least 12 *Vibrio* spp. are classified as pathogenic strains and become major factor for foodborne diseases. *V. parahaemolyticus* caused about 25% of total foodborne diseases in comparison to other vibrio species (Feldhusen, 2000). Three species of vibrio (from 28 species) that are often associated with *V. parahaemolyticus* in aquatic environmental and seafood are *V. vulnificus*, *V. alginolyticus* and *V. cholerae.*

V. parahaemolyticus is a major cause of foodborne illness such as gastroenteritis in human through consumption of undercooked seafood or wounds exposed to marine animals or warm coastal waters especially in Southeast Asian (Wong *et al.,* 2000). This is because a short warm period (temperature range from 10°C-43°C, optimum is 37°C) is sufficient for *V. parahaemolyticus* which has short generation time (8-9 minutes) to grow until infectious levels (10^6 organisms) (Daniels *et al.,* 2000).

There are many methods used in the detection of *V. parahaemolyticus*. The

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standard selective medium method used for *vibrious* is thiosulfate/citrate/bile salt/sucrose agar (TCBS). *V. parahaemolyticus* colonies are green or blue green on the agar due to sucrose fermentation. Hara-Kudo *et al.* (2003) developed other selective medium, CHROMagar™ *Vibrio* that also confirms the *V. parahaemolyticus* colonies by colour which is purple or violet when grown on this medium agar. The latest technique would be PCR that can be used for detection of *V. parahaemolyticus* in various samples including seafoods or other samples, and this method is faster, easier and more reliable. This technique can be applied because of the presence of *toxR* gene (was first discovered as the regulatory gene of the toxin operon), that appears to be well conserved among *V. parahaemolyticus.* This gene can be used to develop a PCR method for identification of *V. parahaemolyticus* (Kim *et al.*, 1999). Other than *toxR* gene, *gyrB* gene also can be used for detection using PCR because this gene is also found to be well conserved among *V. parahaemolyticus* isolates (Kasthuri *et al*., 1998).

The presence of virulence factors is associated to the pathogenesis of *V. parahaemolyticus*. The virulence properties detected in *V. parahaemolyticus* genome are *tdh* and *trh* genes, which code for haemolysin product namely thermostable direct haemolysin (TDH) and TDH-related haemolysin (TRH) (Honda *et al.*, 1993). Studies using *tdh* or *trh* related hemolysin or both gene probes on *V. parahaemolyticus* strains showed a strong correlation between clinically significant strains and presence of either of these genes, implying that both *tdh* and *trh* genes are virulence factors in *V. parahaemolyticus* (Shirai *et al*., 1990).

PCR was used to detect both genes using DNA primers that are specific for the genes encoding TDH or TRH (Tada *et al*., 1992). The presence of TDH can also be demonstrated on Wagatsuma agar which was referred as Kanagawa phenomenon positive (beta haemolysis) on Wagatsuma blood agar (Kaysner *et al.*, 1992).

The objective of this study is to detect the presence of *V. parahaemolyticus* isolates on the selective medium, CHROMagar™ *Vibrio.* This test was further confirmed by specific-PCR to detect the presence of toxin operon (*toxR)* gene (Kim *et al.*, 1999). Finally, all the isolates were tested for their virulence properties by the presence of *tdh* and *trh* genes.

Materials and Methods

Sampling and processing of samples

Fifty (50) cockle samples were obtained from Padang, Indonesia. The samples were collected from lake and river in West Sumatra. 25 g flesh of cockles sample (each sample) was homogenized in 225 ml Alkaline Peptone Water (APW), with the addition of 3% NaCl. The samples were allowed to settle down in APW (hereinafter called APW enrichment broth) at room temperature for 15 min. Then, they were incubated at 37°C for 24 h. After that, 1 ml portion of each of the broth culture was transferred into a sterile bottle containing 1 ml salt polymixin broth and incubated at 35-37°C overnight.

Identification on CHROMagar™ *Vibrio*

From the salt polymixin broth culture, a loop full of culture was streaked on the CHROMagar™ *Vibrio* surface and later sealed with parafilm before incubated for overnight. Pure purple colonies (refer to Table 1) were selected and were streaked onto a new CHROMagar™ *Vibrio* surface for purity and then were grown in Luria-Bertani (LB) broth and later on LB agar (as a stock).

Confirmation by specific-PCR (detection of

toxR gene)

The selected colonies from CHROMagar™ *Vibrio* were tested using PCR method to detect the presence of *toxR* gene which appeared highly conserved in *V. parahaemolyticus* isolates (Kim *et al.*, 1999).

Genomic DNA extraction

Test cultures were grown in 3.0 ml Luria-Bertani broth at 37°C for overnight. The cell suspension was transferred to 1.5 ml microfuge tube and was centrifuged at 10,000 rpm, 25°C for 2 min. The supernatant was discarded and 1 ml of sterile distilled water was added and vortexed to mix. The cell suspension was then centrifuged at 10,000 rpm, 25°C for 5 min. The supernatant was discarded and again 1 ml of sterile distilled water was added. After vortex, the cell suspension was incubated at 60°C for 10 min in waterbath. Immediately the tube was immersed into ice and kept for 10 min and after a short spin (10,000 rpm for 15 sec), the supernatant was transferred to a new tube. The supernatant contained genomic DNA and is ready to be used as a template for *toxR, tdh* and *trh* genes detection in *V. parahaemolyticus.*

Specific primers (R: 5'-GTCTTCTGACGCAATCGTTG - 3' and F: 5' – ATACGAGTGGTTGCTGTCATG – 3') correlated to *V. parahaemolyticus toxR* region with 50% GC contents (Kim *et al.*, 1999). The primers were obtained from New England Biolabs Inc. in Gold Oligo grade.

PCR protocol

PCR was carried out using 0.5 ml microfuge tubes. The total volume reaction mixtures was 20.0 µl which contained 14.3 µl sterile distilled water, 2.0 µl 10 x PCR buffer with $MgCl_2$, 1.6 µl 25 mM deoxyribonucleotide phosphates, 0.8 µl of each primer, 0.1 µl of 0.5 units *Taq* DNA and 1.2 µl template DNA. The cycling conditions were as follows; predenaturation at 96°C for 5 min, denaturation at 94°C for 1 min, annealing at 63°C for 1.5 min, and extension at 72°C for 1.5 min, with a final extension at 72°C for 7 min at the end of 20 cycles.

Virulence properties test (specific-PCR)

After confirmation by *toxR* detection, isolates were then tested for their virulence properties (Kim *et al.,* 1999). Specific primers (*tdh* gene, R: 5'-CCACTACCACTCTCATATGC -3' and (F): 5' – GGTCTAAATGGCTGACATC–3'; *trh* gene, (R): 5'-GGCTCAAAATGGTTAAGCG-3' and (F): 5'– CATTTCCGCTCTCATATGC – 3' correlated to *V. parahaemolyticus tdh* and *trh* regions with 50% GC contents (Tada *et al.*, 1992). The primers were obtained from New England Biolabs Inc. in Gold Oligo grade.

PCR-Protocol

PCR (Perkin Elmer) was carried out using 0.5 ml microfuge tubes. The total volume of reaction mixtures was 20.0 µl which consisted of 14.3 µl sterile distilled water, 2.0 µl 10x PCR buffer with $MgCl_2$, 1.6 µl 25 mM deoxyribonucleotide phosphate, 0.8 µl of each primer, 0.1 µl of 0.5 units *Taq* DNA and 1.2 µl template DNA. The cycling conditions were as follows; predenaturation at 96°C for 5 min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min at the end of 35 cycles.

Agarose gel electrophoresis

The PCR products were run on 1.2% agarose gel (Sigma) in 1x Tris-Borate-EDTA (TBE). 15 – 20 µl PCR products were loaded into sample wells and the voltage used was 100 volt for 1 h. The gel was then stained in ethidium bromide (0.5

µg/ml) solution for 1 min and destained in distilled water for 30 min. Then the gel was visualized and photographed under UV transilluminator.

Results and Discussion

When the samples were streaked on the CHROMagar™ *Vibrio,* three types of colonies were observed which were, purple (*V. parahaemolyticus*), blue (*V. cholerae*) and white (*V. alginolyticus*). These results were in agreements with James (2000) who stated three species of vibrious usually associated with *V. parahaemolyticus* in aquatic environmental and seafood were *V. vulnificus*, *V. alginolyticus* and *V. cholerae.* The selection of the isolates were based on the strong colony appeared with the pure purple colour (Results not shown). CHROMagar™ *Vibrio* is a chromogenic medium developed by Hara-Kudo *et al.* (2003) which contains a chromogenic substrate as substrates for beta galactosidase, instead of sugar fermentation (sucrose) for the detection of *V. parahaemolyticus* by purple colony. (Hara-Kudo *et al.*, 2003).

Out of 50 cockle samples from Padang, Indonesia tested on the CHROMagar™ *Vibrio* for the detect of *Vibrio parahaemolyticus*, twenty five samples were found positive (Table 1). This percentage is in agreement with other studies that reported about 50 - 70% of seafood showed the present of *V. parahaemolyticus* (Fletcher, 1985; Annick *et al.*, 2002; Linda *et al*., 2006).

Identifying *V. parahaemolyticus* strains through PCR based method which targets the conserved region of *V. parahaemolyticus* such as *gyrB* and *toxR* gene is more efficient, reliable and faster compare to biochemical test (Kim *et al.*, 1999).

Both genes also exist in other species but with different sequence variation it reflects the phylogenetic relationship among the species (Nishibuchi, 2004). After the identification on the CHROMagar™ *Vibrio* (Results not shown), the PCR assay for *toxR* detection was performed to confirm the presence of *Vibrio parahaemolyticus.* Thirty two (32) isolates were selected for further characterization process as shown in Table 1. For some positive samples, more than one colony was selected because they produced very pure purple and big colony size.

The *toxR* gene fragment (~368 bp) which is specific for *Vibrio parahaemolyticus* was successfully amplified from all the isolates (Figure 1). The toxin operon gene, *toxR* appears to be well conserved among *V. parahaemolyticus*. The degree of homology of the *toxR* gene between *V. parahaemolyticus* and *V. cholerae* is 52% identity. In this study, the primer used for the amplification of *toxR* gene is specific for *V. parahaemolyticus* with *V. parahaemolyticus* WP1 as the positive control.

These results are in agreement with other researchers who reported that this gene was present in all strains of *V. parahaemolyticus* (Kim *et al.*, 1999; Dileep *et al.*, 2003; Sujeewa *et al.*, 2009). The size of *toxR* gene is 368 base pairs and was first discovered as the regulatory gene of the toxin operon, but it was later shown to be involved in the regulation of many genes (Miller *et al.*, 1987; DiRita *et al.*, 1992; Kim *et al*., 1999). Detection of the virulence genes in *V. parahaemolyticus* is only for determination of pathogenic strains whereas *toxR* genes can be used to determine all isolates either pathogenic or non-pathogenic and also functions as regulator for expression of the virulence factor genes in *V. parahaemolyticus* (Sechi *et al.*, 2000).

Detection for *trh* and *tdh* gene fragment in the isolates showed negative results for both *tdh* and *trh* genes (Results not shown). Both genes with 199 and 250

Table 1. Detection of *V. parahaemolyticus* on CHROMagar™ *Vibrio* and number of isolates selected for *toxR, tdh* and *trh* detection

Sample	Positive/ Negative on CHROMagarTM Vibrio	No. of selected isolates for *toxR, tdh and trh* detection
1, 5, 7, 8, 9, 11, 12, 16, 18, 22, 23, 25, 29, 31, 32, 35, 37, 38, 40, 41, 43, 47, 49, 50	Negative	-
2, 3, 6, 10, 14, 17, 19, 20, 24, 26, 27, 28, 33, 36, 42, 44, 45, 46	Positive	1
4, 13, 15, 21, 30, 39, 48	Positive	2

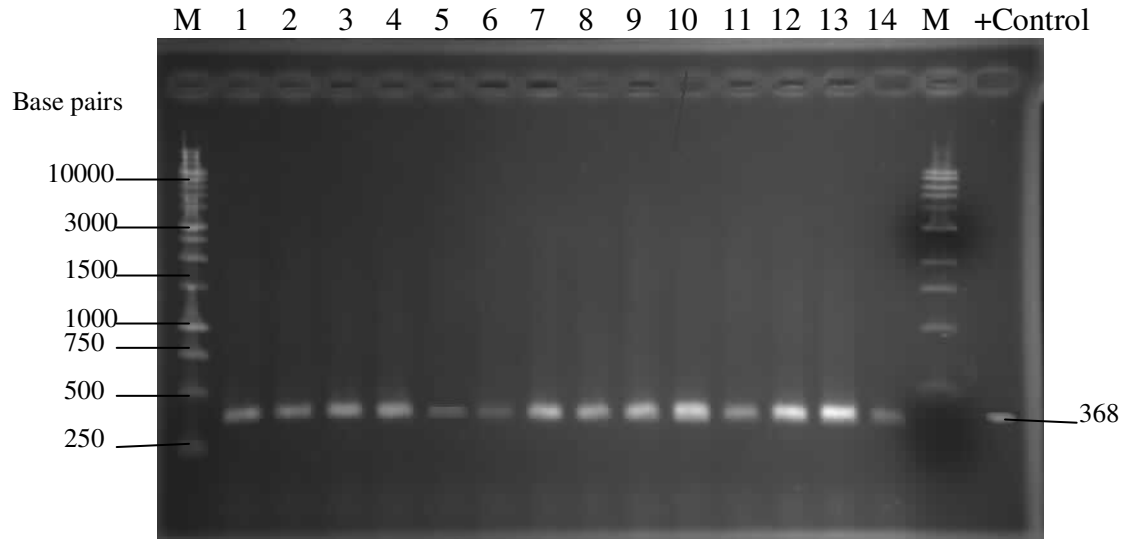

Figure 1. Detection of *toxR* gene in *V. parahaemolyticus* on 1.2 % agarose gel. Lanes : M, 1 kb ladder; 1, VP1; 2, VP2; 3, VP3; 4, VP4; 5, VP5; 6, VP6; 7, VP7; 8, VP8; 9, VP9; 10, VP10; 11, VP11; 12, VP12; 13, VP13; 14, VP14; +Control, Positive control (*V. parahaemolyticus* WP1 isolates). Only VP1 to VP14 were shown as representative results.

base pairs sizes did not appear on the gel while these genes appear in the control positive strains (*V. parahaemolyticus* AQ4034 and *V. parahaemolyticus* AT4, respectively). These results were in agreement with previous studies that reported *trh* and *tdh* genes were found in very low number (1-7%) among environmental and seafood samples (DePaola *et al.*, 1990, 2000; Wong *et al.*, 2000; Lee *et al.*, 2002; Dileep *et al.*, 2003; Nordstrom *et al.*, 2003).

Based on this result, all the isolates under this study do not contain virulence properties that have strong correlation to the ability of causing diseases, which means that they are non-pathogenic.

Detection of *trh* and *tdh* genes is important to study the distribution of pathogenic strains especially in seafood. Most of the seafood from tropical region especially Southeast Asia is known to have high risk of the *V. parahaemolyticus* presence with percentage between 20 - 70% (Wong *et al.*, 1999; Ronald and Santos, 2001). This is because of the the high marine temperature (25 – 35°C) and thus the occurrence and distribution of *V. parahaemolyticus* is all year round. The hot marine water is a major contribution factor to the occurrence of high percentage *V. parahaemolyticus* in the cockle samples.

Although *V. parahaemolyticus* is widely distributed in the environments and seafood all over the world and most of them are not pathogenic to humans, consumer still need to increase their awareness and ensure that their seafood is cooked properly. The prevention of infection depends on handling of raw seafoods and preparation of finished foods in order to reduce or eliminate foodborne hazards. In conclusion, the percentage of pathogenic *V. parahaemolyticus* in this study is 0% but the pathogenic level of this bacterium can be affected by geographical region.

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