IDENTIFICATION TARGETED ON *toxR* GENE AND DETECTION OF VIRULENT FACTOR ENCODING GENES ON *Vibrio parahaemolyticus* ISOLATED FROM RAW CHICKEN MEAT

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ABSTRACT

A number of V. parahaemolyticus isolates has been isolated from raw chicken meat samples collected in Pasar raya Padang. Isolation was done using ChromagarTM Vibrio media. Identification of toxR gene was then done to these isolates. toxR is a very specific gene in V. parahaemolyticus species. Detection for the presence of genes encoding virulence factors, in this case were the gene encoding thermostable direct hemolysin (tdh) and TDH related hemolysin (trh) was performed on toxR positive V. parahaemolyticus isolates. Identification of toxR gene and detection of tdh and trh genes were done trough amplification using polymerase chain reaction PCR (method). The results showed that all of the tested V. parahaemolyticus isolates (22 isolates) had toxR gene, but none of isolates has gene encoding the production of virulence factors both tdh and trh.

Keywords: V. parahaemolyticus, toxR, tdh, trh.

1. INTRODUCTION

V. parahaemolyticus is a halophilic gramnegative bacteria, which are usually associated with marine organisms, or living freely in seawater. *V. parahaemolyticus* was first isolated on a food poisoning outbreak in Japan in early 1950s (Kelly and Stroh, 1989). Currently, *V. parahaemolyticus* has become one of the pathogens of food contaminants with the highest prevalence in Asian countries (Pan *et al*, 1997).

Some strain of V. parahaemolyticus has ability in causing gastroenteritis in humans. The main symptoms of gastroenteritis caused by V. parahaemolyticus include abdominal cramps, nausea and vomiting. Most strains of clinical V. parahaemolyticus produce major virulence factor namely thermostable direct hemolysin (TDH) and show β -hemolysis activity on Wagatsuma agar (Kanagawa positive, KP+). Another virulence factor, TDH-related hemolysin (TRH), usually associated with Kanagawa phenomenon negative (KP-) or urease positive strains (Kelly and Stroh, 1989). The ability of V. parahaemolyticus strains in causing diarrhea is highly dependent on the existence of TDH and TRH. TDH and TRH whose production is encoded by tdh and trh genes is the important virulence factors for the development of gastroenteritis (Shirai et al, 1990). Therefore, these genes are referred to as

the important virulence factor coding genes in *V. parahaemolyticus*.

Although V. parahaemolyticus is a marine bacterium that has long been associated with *diarrhea* after eating raw or not cooked perfectly seafood, some recent studies seems successfully in isolating these bacteria from samples which are not coming from sea environment. Research conducted by Marlina et al (2007) has managed to isolate these bacteria from pensi species (*Corbicula moltkiana* prime) collected from lake Singkarak Sumatera Barat. The isolated strains carried hemolysin toxin-producing gene (*tdh* and *trh*). Another study conducted by Zulkifli (2009) also success in isolating on cockles collected from rivers in Padang. Based on those reports, we investigated the existence of V. parahaemolyticus on chicken meat samples collected from traders in Pasar raya Padang, identified the species by amplifying toxR gene and detected the virulent factor coding genes (tdh and trh) on isolates.

The method used to amplifying and detecting *toxR* and virulence genes of V. the parahaemolyticus is polymerase chain reaction (PCR). Polymerase Chain Reaction (PCR) has been widely used to identify genes from different bacteria including V. species of parahaemolyticus. This is due to the high level of sensitivity of this (Paton and Paton, 1998).

2. MATERIALS AND METHODS Equipments and materials

PCR machine (Eppendorf Mastercyclergradient ®), Eppendorf tubes, micro pipette (Eppendorf ®), centrifugator (Eppendorf Minispin ®), laminar air flow (Esco ®), vortex (Mixer ® VM-1000), incubator (Gallenkamp ®), rotary shaker incubator (Bigger Bill Digital ®), colony counter (Stuart scientific ®), the elektrophoresis device, transilluminator, polaroid film, the test sample, V. Parahaemolyticus AQ4037 (positive control for the toxR and trh genes), V. parahaemolyticus AQ3815 (positive control for tdh gene,), Salt Polimixin Broth (SPB), distilled water, NaCl, CHROMagarTM Vibrio (CHROMagarTM), Luria Bertani (LB) Broth, Luria Bertani (LB), 5X Colorless GoTaq Reaction, 10X Ex Tag buffer solution, 2.5 mM dNTP solution, GoTag DNA polymerase, agarose, tris-borate-EDTA (TBE), a blue dye, 100 bp ladder, ethidium bromide, and three pairs of primers, namely:

toxR-4: 5'-GTCTTCTGACGCAATCGTTG-3' and toxR-7: 5'-

ATACGAGTGGTTGCTGTCATG-3' for the detection of *toxR* gene, TDH D3: 5'-CCACTACCACTCTCATATGC-3' and TDH D5: 5'-GGTACTAAATGGCTGACATC-3' for detection of *tdh* gene, TRH R2: 5'-GGCTCAAAATGGTTAAGCG-3' and TRH R6: 5'-CATTTCCGCTCTCATATGC-3' for detection of *trh* gene.

Sampling

The test samples in this study were 11 raw chicken meat samples. Samples were taken from traders in Pasar Raya Padang. To avoid contamination, samples were taken in a way directly entered by the merchant into sterile containers, immediately stored in containers and taken to the laboratory for testing.

V. parahaemolyticus Isolation

Each sample (10 gram) was added in to 100 ml Salt Polimixin Broth (SPB) media and incubated at 37° C for 24 hours. These cultures were inoculated using a needle loop onto surfaces of CHROMagarTM Vibrio previously been poured

and allowed to solidify in a petri dish. After incubated for 24 hours at 37°C, purple colonies formed were suspected as colonies of V. *parahaemolyticus*. Each single suspected colony was inoculated into the Luria Bertani (LB) Broth containing 3% NaCl and incubated in a rotary shaker incubator at 37°C for 24 hours.

DNA template preparation

Before the amplification of genes using PCR method, DNA of control and testing bacteria were extracted using boil cell extraction (BCE) method. 1 ml LB broth culture centrifuged at 12,000 rpm for 1 min, the supernatant were removed, while the sediment were added with 500 mL sterile distilled water and then suspended using vortex. The suspension formed was heated for 10 minutes in boiling water and immediately put into the refrigerator with a temperature of -20°C for 10 minutes. Subsequently centrifuged at 12,000 rpm for 3 minutes and the supernatant were transferred into a new Eppendorf tube. The supernatant were the DNA template to be used for amplification of genes by PCR method.

Amplification of *toxR*, *tdh* and *trh* genes

Identification of *toxR*, *tdh* and *trh* genes in *V*. *parahaemolyticus* were done using PCR method. DNA template which had been prepared previously inserted into the Eppendorf tube for PCR machine and combined with other PCR components. The type and amount of each PCR components as shown in table below:

Table 1. PCR component

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Component	Amount(µl)
Buffer solution ^a	5,0° 2,5 ^d
2,5 mM dNTP	2,0
Primer 1 ^b	1,0
Primer 2 ^b	1,0
Aquadest	15,0° 17,4 ^d
GoTaq DNA Polymerase	0,1
DNA Template	1,0

^a5x Colorless GoTaq Reaction Buffer for the amplification of *tdh* and *trh* genes; 10x Ex Taq buffer solution for the amplification of *toxR* gene, ^bPrimer pairs are suitable for each gene, ^cFor the

detection of tdh and trh genes, ^dFor the detection of toxR gene

Eppendorf tube is then inserted into the PCR machine and amplification was performed using program which is suitable for detection of each gene as shown in table below:

cycles)					
Stage	Temperature (°C)	Time (minute)			
Predenaturation	96	5			
Denaturation	94	1			
Annealing	63	1,5			
Extention	72	1,5			
Elongation	72				

Table	2. Stage	for <i>tox-R</i>	gene	amplification	(23
		-			

Table 3. Stage for tdh and trh genes amplification (33 cycles)

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Stage	Temperature (°C)	Time (minute)
Predenaturation	96	5
Denaturation	94	1 3
Annealing	55	1
Extention	72	2
Elongation	72	7

After all of the stages in the PCR process were completed, the results of this amplification were colored using blue dye and then separated along electrophoresis process on 1% agarose gel in 1x TBE. Electrophoresis was performed at 100 V voltage for 20 minutes with 1x TBE as the mobile phase. The 100 bp ladder was used as a marker for determining the size of amplification product. After the electrophoresis process was completed, agarose gel was stained using solution bromide ethidium (0.5)mL/ml). Electrophoresis result which was observed by UV transilluminator will form separate bands which were distinguished by the number of their base pairs (bp). The size of toxR, tdh and trh amplification product were 368 bp, 251 bp and 250 bp respectively. Size estimation of each product was done through comparison with 100 bp ladder. Results were then documented by polaroid film.

3. RESULTS AND DISCUSSION

We successfully isolated 22 suspected V. *parahaemolyticus* colonies from 2 of 11 raw chicken meat samples which were examined. The presence of suspected V. *parahaemolyticus* in samples characterized by the formation of purple colonies on the surface of CHROMAgarTM Vibrio medium.



Figure 1. Suspected V. parahaemolyticus colonies on ChromagarTM Vibrio

CHROMagar [™] Vibrio is a selective media for identification of V. parahaemolyticus with a higher level of differentiation compared to TCBS medium (Kudo et al, 2001). From 2 of 11 samples which were examined, we successfully isolated 22 suspected V. parahaemolyticus. Identification targeted on toxR gene was performed to all of these isolates using PCR method. toxR gene is a gene that is very specific on the V. parahemolyticus species. The PCR method to detect this gene has been reported (Lee et al, 1995) as a very useful method to confirm the presence of this species in samples. Dileep et al (2003) also states that the detection of toxRdetect gene by PCR method to V_{\cdot} parahaemolyticus is more sensitive than biochemical identification.

From out of total of 22 tested isolates in this research, all (100%) showed toxR positive results. toxR positive isolates showed 368 bp band in electrophoresis gel. The same result have been reported by Zulqifli *et al* (2009), all of CHROMagarTM Vibrio isolates from cockles

gave positive results on testing of toxR using PCR method.



Figure 2. Electrophoresis gel of toxR positive V. parahaemolyticus isolates. From left to right: lane 1-11 were positive toxR isolates, lane 12 was positive control, lane 13 was 100 bp ladder.

Previously, V. parahaemolyticus has been isolated from various places around the world. Tanil et al (2005) succeeded in isolating V. parahaemolyticus from seawater in Peninsular, Malaysia. V. parahaemolyticus also been isolated from coastal waters of western United States (Okuda et al, 1997). A study recently conducted by Sujeewa et al (2009) succeeded in isolating V. parahaemolyticus from shrimp samples in Malaysia. Generally, V. parahaemolyticus were isolated from marine waters or food samples from the sea, because V. parahaemolyticus is a bacteria that normally lives in this habitat (DePaola et al, 2000).

Results of this research is interesting for further explored, because *V. parahaemolyticus* isolates were isolated in samples which were not derived from the sea environment. Furthermore, studies are necessary to investigate whether the presence of *V. parahaemolyticus* in samples is the result of bacterial adaptation capabilities on low-salt environment, or is the result of crosscontamination when samples are marketed in the market.

Similar results have also found in other study (Marlina *et al*, 2007), where a number of *V*. *parahaemolyticus* isolates carrying *tdh* gene were isolated from *Corbicula* moltkiana, a species which lives in lake Singkarak West Sumatera. Similarly, other publication also showed that *V*. *parahaemolyticus* was isolated from cockles live

in rivers around Padang, Indonesia (Zulkifli *et al*, 2009).

Major virulence factor coding genes in V. parahaemolyticus are tdh and trh. The presence of these genes are represent the level of pathogenicity of V. parahaemolyticus isolates. In this study, 22 toxR positive isolates were detected for the present of *tdh* and *trh* genes using PCR method. As a result, none of the isolates has tdh or trh gene. This result suggests that V. parahaemolyticus isolates in this study are not virulent isolates. These such results also seen in V. parahaemolyticus previously isolated from cockle samples in Padang, where the overall toxRpositive isolates have no *tdh* or *trh* gene (Zulkifli et al, 2009). However, some other studies seem to successfully detect the presence of these virulence genes in environmental samples (Sujeewa et al, 2009; Marlina et al, 2007).

4. CONCLUSION

All of 22 V. *parahaemolyticus* isolates isolated from chicken meat samples marketed in Pasar raya Padang were having toxR gene, but none of them has *tdh* or *trh* gene.

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