DETECTION OF *TDH* AND *TRH* GENES IN *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM *CORBICULA MOLTKIANA* PRIME IN WEST SUMATERA, INDONESIA

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Abstract. The occurrence of *Vibrio parahaemolyticus* in raw *Corbicula moltkiana* Prime from Lake Singkarak and Pasar Raya Padang market and in cooked samples in West Sumatera, Indonesia, was studied. Thirteen raw and seven cooked bivalve samples were positive using CHROMAgar™ *Vibrio* medium. All 47 *V. parahaemolyticus* isolates were positive for *toxR* gene but negative for *trh*. However, 36% (17/47) of *V. parahaemolyticus* strains were positive for *tdh* gene. Antibiotic profiling showed that 76% and 38% of isolates from raw and cooked bivalves respectively were resistant to ampicillin. Using RAPD-PCR analysis, most of the strains were clustered according to their source of isolation but some of the strains from raw and cooked samples were clustered together. These results indicate that pathogenic *V. parahaemolyticus* isolates are present in *Corbicula moltkiana* Prime in West Sumatera, Indonesia, suggesting that *V. parahaemolyticus* may also be present in seafood in other regions of Indonesia.

INTRODUCTION

Vibrio parahaemolyticus is an enteric pathogen transmitted to human primarily through consumption of raw or mishandled seafood and there is a strong correlation of pathogenicity with possessions of hemolysin genes, *tdh* and *trh* (Kim *et al*, 1999; Wong *et al*, 1999). PCR methods have been established to identify the presence of *tdh* and *trh* genes from *V. parahaemolyticus* (Tada *et al*, 1992; Lee *et al*, 1993) and PCR assays have been applied to studies of environmental sources and seafood (Robert-Pillot *et al*, 2004). The present study describes the first report on the isolation and detection of the *tdh* gene in *V. parahaemolyticus* from raw and cooked *Corbicula moltkiana* in West Sumatera, Indonesia.

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MATERIALS AND METHODS

Sample isolation and identification

Samples were collected and examined for the presence of *V. parahaemolyticus* by previously described method (Okuda *et al*, 1997). Forty-seven *V. parahaemolyticus* strains were analyzed in this study, including those isolated from raw bivalves (*Corbicula moltkiana*) collected directly at harvesting sites of five different areas in Lake Singkarak, 60 km from

Padang City, and cooked bivalves of the same species, purchased from street vendors from two different sites at Pasar Raya Padang, West Sumatera Province, Indonesia (February 2003-February 2005). *V. parahaemolyticus*, AQ3815 (*tdh*+) and AQ4037 (*trh*+) strains obtained from Prof. Dr. Mitsuaki Nishibuchi, Kyoto University, Japan), were used as positive control for the detection of *tdh* and *trh*.

Antibiotic susceptibility testing

Disk diffusion tests were performed with antibiotic containing disks obtained from Oxoid (Basingstoke, UK) by the method recommended by the National Committee for Clinical Laboratory Standards (NCCLS). The antimicrobial agents tested included ampicillin (10 µg), gentamicin (10 µg), cefuroxime (30 µg), erythromycin (15 µg), sulfametoxazole (25 µg), tetracycline (30 µg), trimethoprim (1.25 µg), ciprofloxacin (30 µg) rifampicin (10 µg) and teicoplanin (200 µg). Bacteria were suspended in saline to the same turbidity as the Mc Farland 0.5 turbidity standard and streaked on Mueller Hinton agar. Plates were incubated for 24 hours at 37ºC. Characterization of strains as sensitive, intermediate or resistant was based on the size of zones of inhibition surrounding the discs and interpreted along with the zone diameter interpretation chart listed in the performance standards for antimicrobial susceptibility testing, NCCLS.

Detection of *tox*R, *tdh* and *trh* genes

A colony of *V. parahaemolyticus* was picked up from CHROMagar™ *Vibrio* medium and inoculated into Luria Bertani (LB) broth containing 3% NaCl. The isolate was grown in LB broth with shaking at 180 rpm for 18-24 hours at 37ºC. Crude bacterial genomic DNA was isolated using boiling cell extraction method and PCR assay was performed to detect the presence of *tox*R, *tdh* and *trh* genes as described by Tada *et al* (1992), using three pairs of oligonucleotide primer (*tox*R-4: 5′-GTCTTCTGACGCAATCGTTG-3′, *tox*R-7: 5′-ATACGAGTGGTTGCTGTCATG-3′, *tdh* D3: 5′-CCACTACCACTCTCATATGC-3′, *tdh* D5: 5′-GGTACTAAATGGCTGACATC-3′); (*trh* R2: 5′-GGCTCAAAATGGTTAAGCG-3′, *trh* R6: 5′-CATTTCCGCTCTCATATGC-3′). The resultant amplicon was 368 bp, 251 bp and 250 bp for *tox*R, *tdh* and *trh,* respectively. An aliquot of 20 µl of PCR mixture contained 30 ng of template DNA, 10 pmol of each oligonucleotide primer (2 µl each), 1.6 µl of 25 mM $MgCl_2$, 0.8 µl of 2.5 mM of each dNTP and 0.5 U of *Taq* DNA polymerase (Bioron, USA). PCR reaction was carried out in a PTC200 thermocycler (MJ Research) using the following conditions: predenaturation at 94ºC for 5 minutes, 35 cycles of denaturation at 94ºC for 1 minute, primer annealing at 55ºC for 1 minute, and primer extension at 72ºC for 1 minute, a final extension at 72ºC for 7 minutes. The PCR products were seperated by electrophoresis in 1.8% agarose gel, stained with ethidium bromide (10 mg/ml) and recorded using a gel documentation system (Syngene, USA).

Random amplified polymorphic DNA (RAPD) analysis

RAPD PCR for*V. parahaemolyticus* was carried out using the 10-mer primers, Gold Oligo PAR 3 (5′-CTT GAG TGG A-3′), Gold Oligo PAR 4 (5′-TCC TCAAGA C-3′) and Gold Oligo PAR 8 (5′-GAG ATG ACG A-3′) as they provided reproducible and discriminatory patterns. Two microliters of the boiled cell supernatant were mixed in a 23 µl of reaction mixture containing 2.5 µl of 10xPCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton® X-100), 1.5 µl of $MgCl_2$ (50 mM), 1.0 µl of each deoxynucleoside triphosphates (10 mM each of dATP, dCTP, dGTP, dTTP), 2.0 µl of each forward and reverse primers (10 µM), 1.0 µl of 2.5 units of AmpliTaq DNA polymerase and 13 µl of sterile distilled water. PCR was performed using a thermal cycler (Perkin–Elmer, Cetus, USA) with an initial denaturation step (94ºC, 5 minutes), followed by 45 cycles of denaturation (94ºC, 1 minute), annealing

(36ºC, 1 minute 30 seconds), and extension (72ºC, 2 minutes and 30 seconds), and a final extension (72ºC, 10 minutes). The amplified products were electrophoresed at 75 V through 1.5% agarose gel. The amplified bands were visualized after staining with ethidium bromide (10 mg/ml) and photographed under UV light.

RESULTS

The 47 isolates of *V. parahaemolyticus* were obtained from 13 out of 35 raw and 7 out of 12 cooked *Corbicula moltkiana* samples tested and all demonstrated the presence of *toxR* gene (Fig 1). Detection of *tdh* and *trh* genes using PCR showed that 17 out of 47 *Vibrio parahaemolyticus* were positive for *tdh* (Fig 2), but all the isolates, both from raw and cooked food, were negative for *trh.*

Dendrogram generated from the RAPD analysis revealed seven clusters and a single isolate at 16% similarity. As shown in Fig 3, cluster A and G were formed by isolates from the raw food samples. On the other hand, there were four clusters (cluster B, C, D and F) generated from cooked food samples. Cluster E consisted of mixed isolates from raw and cooked food samples.

The antibiotic resistance profiles of the *Vibrio parahaemolyticus* isolates from raw and cooked *Corbicula moltkiana* Prime showed that all isolates exhibited resistance to three or more of the antibiotics tested (Table 1). More than 76% isolates from raw food samples were resistant to ampicillin and only 38% for isolates from cooked food samples. Twenty-three percent and 8% of the isolates from raw and cooked samples respectively were sensitive to tetracycline, the second drug of choice for treatment of patients with diarrhea symptoms.

DISCUSSION

Corbicula moltkiana Prime is one of the famous bivalve's delicacies in West Sumatera, Indonesia, especially for people who stay near the lake and river shore. In West Sumatera, *C. moltkiana* is usually found at Lake Singkarak and Lake Maninjau, and is usually eaten semi-cooked mostly sold by street vendors. *V. parahaemolyticus* has been reported in Japan, United States, Europe and other country in

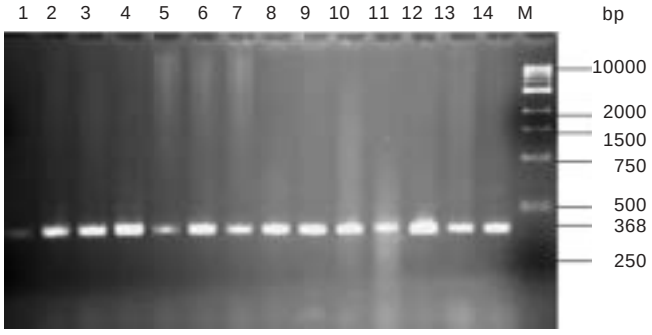

Fig 1–Representative gel electrophoresis picture of PCR detection of *toxR* gene of *V. parahaemolyticus* with product size of 368 bp. Lane M, 1 kb molecular weight marker; lane 1, negative control; lanes 2-13, *V. parahaemolyticus* isolates; lane 14, positive control.

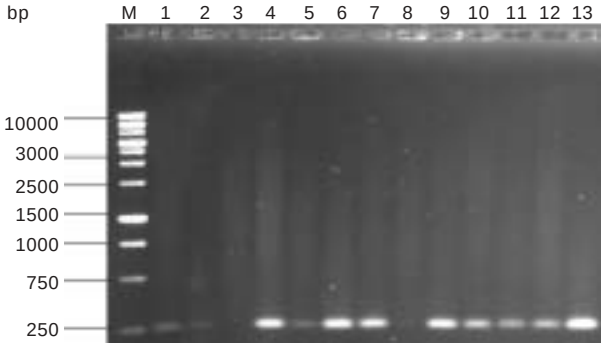

Fig 2–Representative gel electrophoresis picture of PCR detection of *tdh* gene in *V. parahaemolyticus* with product size of 251 bp. Lane M, 1 kb molecular weight marker; lanes 3 and 8 , negative control; 1, 2, 4-7, 9-12, *V. parahaemolyticus* isolates; lane 13, positive control.

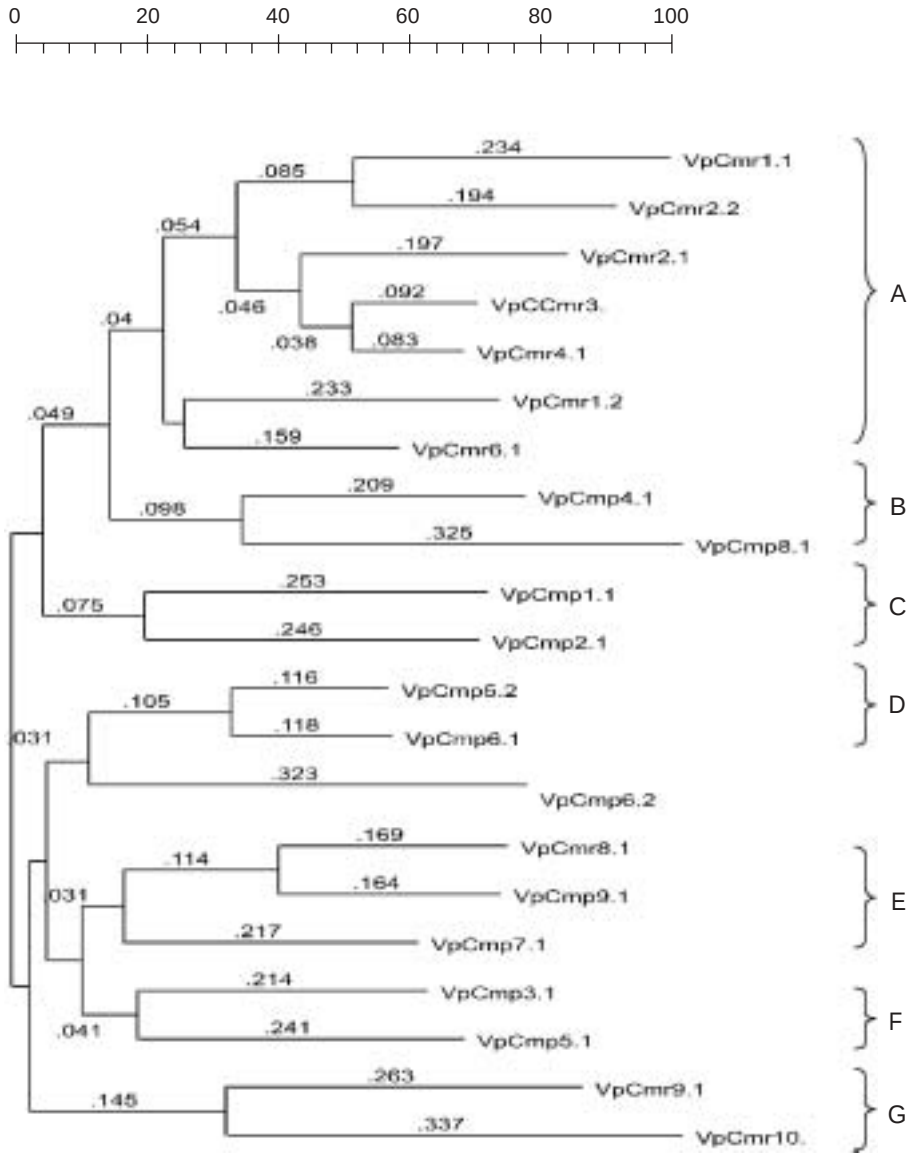

Fig 3–Dendrogram showing the clustering of RAPD patterns for *V. parahaemolyticus* isolates from raw and cooked *Corbicula moltkiana* Prime. Primer Gold Oligo PAR 3, Gold Oligo PAR 4 and Gold Oligo PAR8 were employed, and the dendrogram was generated using RAPD Distance Software.

Asia (Barbieri *et al*, 1999; Wong *et al*, 1999; Daniels *et al*, 2003), and to the best of our knowledge this is the first reported case in *C. moltkiana* in West Sumatra, Indonesia. Reports of the incidence of *V. parahaemolyticus* in products from Hong Kong and Thailand were found to be markedly higher than the incidence in products from Indonesia and Vietnam (Wong *et al*, 1999).

ToxR gene was detected in all the 47 isolates using PCR assay, but all isolates were negative for the *trh* gene. It has been reported

Table 1
Occurrence of *toxR*, *tdh* and *trh* genes and antibiogram in *V. parahaemolyticus* isolated from *Corbicula molktiana* Prime.

Strains	*toxR*	*tdh*	*trh*	Multiple antibiotic resistance
Cmr1.1	+	-	-	AmpCxmERdRlSTeTec
Cmr1.2	+	-	-	CipCxmERdRlSTec
Cmr1.3	+	-	-	AmpCxmpNaRdRlESTeTec
Cmr2.1	+	-	-	AmpCENaRdRlSTeTec
Cmr2.1	+	-	-	AmpCnCxmRlRdSTeTec
Cmr3.1	+	-	-	AmpCCipENaRdRlSTeTec
Cmr3.2	+	-	-	AmpCCipENaNorRdRlSTeTec
Cmr4.1	+	-	-	CxmENaRdRlSTeTec
Cmr4.2	+	-	-	AmpCnERdSTeTec
Cmr5.1	+	-	-	AmpCCxmENaRdRlSTeTec
Cmr5.2	+	-	-	AmpCxmERdRlSTeTec
Cmr6.2	+	-	-	CipCxmERdRlSTec
Cmr7.1	+	-	-	AmpCipCxmNaRdRlSTeTec
Cmr7.2	+	-	-	AmpCENaRdRlSTeTec
Cmr8.1	+	-	-	AmpCCipCxmNaRdRlSTeTec
Cmr8.2	+	-	-	AmpCnERdSTeTec
Cmr9.1	+	-	-	CipCxmERdRlSTec
Cmr9.2	+	-	-	AmpCxmCipNaRdRlSTeTec
Cmr10.1	+	-	-	AmpCCipENaNorRdRlSTeTec
Cmr10.2	+	-	-	CipCxmERdRlSTec
Cmr11.1	+	-	-	AmpCipCxmERdRlSTec
Cmr11.2	+	-	-	CipCxmNaRdRlSTeTec
Cmr12.1	+	-	-	AmpCxmRdRlSTe
Cmr12.2	+	-	-	AmpCnERdSTeTec
Cmr13.1	+	-	-	AmpCCipENaRdRlSTeTec
Cmr13.2	+	-	-	AmpCnERdSTec
Cmp1.1	+	+	-	CnCxmSTeTec
Cmp1.2	+	+	-	CipCnCxmERdSTeTec
Cmp1.3	+	-	-	AmpCnCxmERdRlSTec
Cmp1.4	+	+	-	CipCxmERdSTeTec
Cmp2.1	+	+	-	AmpCCipCnCxmERdRlSTeTec
Cmp2.2	+	+	-	AmpCnCipCxmERdRlSTeTec
Cmp2.3	+	+	-	CipCnCxmERdRlSTeTec
Cmp3.1	+	-	-	CipCxmERdRlSTeTec
Cmp3.2	+	+	-	AmpCxmESTeTec
Cmp3.3	+	+	-	AmpCCipCxmERdRlSTeTec
Cmp4.1	+	+	-	AmpCnCipCxmERdRlTeTec
Cmp4.2	+	+	-	CipCnCxmERdSTeTec
Cmp4.3	+	+	-	AmpCxmERdSTeTec
Cmp5.2	+	+	-	CipCnCxmERdRlSTeW
Cmp5.3	+	+	-	ERlSTeTecW
Cmp6.1	+	+	-	CipCxmERdSTeTecW
Cmp6.2	+	+	-	AmpCnCxmERlTeTec
Cmp6.3	+	-	-	CipCnCxmERdRlSTeW
Cmp7.1	+	-	-	CipCnERlSTeTec
Cmp7.2	+	+	-	CnCxmERlSTecW
Cmp7.3	+	+	-	ERlSTecW

Symbols for antimicrobial resistance: Amp, ampicillin (10 µg); Cn, gentamicin (10 µg); Cxm, cefuroxime (30 µg); E, erythromycin (15 µg); Rl, sulfametoxazole (25 µg); Te, tetracycline (30 µg); Cip, ciprofloxacin (30 µg), Rd, rifampicin (10 µg), Tec, teicoplanin (200 µg); W, trimethoprim (1.25 µg).

that none of the *V. parahaemolyticus* isolates in imported food from Indonesia possessed the hemolysin *tdh* and *trh* genes (Wong *et al*, 1999), but in this study 36% of the isolates were positive for the *tdh* gene. *V. parahaemolyticus* has been isolated from patients with acute watery diarrhea in North Jakarta, Indonesia, and all the strains were found to be Kanagawa positive, indicating their virulence potential (Tjaniadi *et al*, 2003). However, the isolates were not examined for the presence of the *tdh* and *trh* virulence genes.

All strains of *V. parahaemolyticus* isolated from raw and processed in bivalves from *C. moltkiana* showed multiple antibiotic resistance, with 22% of the strains resistant to 11 antibiotics tested. In a previous investigation, 100% of *V. parahaemolyticus* strains in Indonesia were reported to be resistant to ampicillin and 94% to cephalothin (Tjaniadi *et al*, 2003). In the present investigation, 77% of the strains from raw bivalves were resistant to ampicillin, but only 36% for cooked samples. On the other hand, previously all the clinical *V. parahaemolyticus* isolates from Jakarta, Indonesia were susceptible to ciprofloxacin (Tjaniadi *et al*, 2003), but results showed that 48% and 35% of isolates from raw and cooked bivalves respectively were resistant to ciprofloxacin. Some 19% of the strains from raw samples were resistant to gentamicin, whereas 44% in cooked bivalves were resistant. Earlier studies revealed that about 8% of shellfish and 4% of prawn isolates recovered from seafood outlets in South India were resistant to gentamicin suggesting that geographical location and local drug selective pressure influence the level of antibiotic resistance. Like enteric gram-negative bacilli, the emergence of resistance among aeromonads is accelerated by inappropriate clinical use of antibiotics. The release of multiple antibiotic-resistant organisms through feces may ultimately pave the way for the contamination of fish and shellfish in the aquatic environment.

PCR genotyping based on RAPD analysis was used to characterized 21 of the *V. parahaemolyticus* isolated from raw (n=10) and cooked (n=11) *C. molktiana*. The two groups of samples were discriminated into seven main clusters at 16% similarity with two and four distinct clusters comprising solely isolates from the raw and cooked samples respectively. RAPD method has been used in the molecular subspecies typing of *V. parahaemolyticus* (Wong *et al*, 1999).

In summary, this study demonstrated that 36% of *V. parahaemolyticus* isolated from *C. molktiana* Prime collected from West Sumatera region possessed the *tdh* gene. As these isolates can cause infectious disease in humans (DePaola, *et al*, 2003; Robert-Pillot *et al*, 2004), the consumption of seafood contaminated with these bacteria pose a great risk for public health. The magnitude of the risk becomes clearer when the eating habit in the world towards raw or undercooked seafood is taken into consideration (Korniushin *et al*, 2003). Therefore, surveillance of contamination of *V. parahaemolyticus* in harvested bivalves and cooked food in Indonesia is crucial for the maintenance of good public health.

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