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ISOLATION AND IDENTIFICATION OF *Vibrio parahaemolyticus* FROM FLOWER CRAB (*Portunus pelagicus*) IN NEGERI SEMBILAN AND SELANGOR

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Abstract

This study was conducted to determine the presence of *Vibrio parahaemolyticus* on 30 samples of flower crab (*Portunus pelagicus*) collected from Negeri Sembilan (n = 15) and Selangor (n = 15) from March to July 2014. A total of 193 isolates of *Vibrio* sp. were isolated from the thiosulphate citrate bile-salt (TCBS) agar medium. Of 79 Vibrio isolates were identified as *V. parahaemolyticus* through biochemical testing methods. In *V. parahaemolyticus* identification by detection of regulatory gene *tox*R, 51 (64.6%) of 79 isolates contained *tox*R gene while none of the isolates contained virulence gene of *tdh* dan *trh*. by targeting *tox*R gene which produced amplicons of 368 bp molecular weight. All *V. parahaemolyticus* isolates were negative for *tdh* and *trh* genes.

Keywords: *Vibrio parahaemolyicus*, flower crab, *tox*R, *trh*, *tdh*

1.0 INTRODUCTION

Vibrio parahaemolyticus is a halophilic Gram-negative rod shaped bacteria present naturally in the marine environment andfrequently present from a variety of seafood products. It is the most prevalent seafood- borne gastroenteritis pathogen reported in Asia, Europe and other coastal countries with high rates of seafood consumption (Wong and Lin, 2001). The ingesting of uncooked infected seafood may lead to the transmission of these bacteria to human because of the filter-feeding activity of mollusks that concentrate bacteria into their tissue (Pilot et al., 2004).

As both pathogenic and non-pathogenic strains of *V. parahaemolyticus* exist in seafood, the detection of virulence genes (*tdh, trh*) by use of a PCR method will help in recognition of the pathogenic strains. In Malaysia, *V. parahaemolyticus* outbreaks related to the seafood consumptions have not been reported yet. There are study reported, 65 over 100 cockles were contaminated with *V.parahaemolyticus* where two strains were produced *tdh* genes while 11 strains were produced *trh* genes (Bilung et al., 2005). Also, this bacteria has been reported appeared in black tiger shrimp (*Panaeus monodon*) (Sani et al., 2008). The pathogenic and non-pathogenic strains of *V. parahaemolyticus* are dealing with gastrointestinal illnesses. Diarrhea, abdominal cramps, nausea, vomiting, headache, fever and chills are common clinical symptoms to patient (Pilot et al., 2004).

The aquaculture industry is one of the sectors given attention by the Malaysian Government to generate a lucrative income. Jabatan Kesihatan (2015) state that the fisheries sector had contributed RM11,466.53 million to the nation's economy, showing an increase of 0.23% compared to 2012. According to Oniam et al. (2012) flower crab was easily found along the coastal areas of the tropical western Indian Ocean and eastern Pacific. In Malaysia, the *Portunus pelagicus* species commonly called as flower crab or renjong crab. The crab is obtained through fishery catches that are usually seasonal and inconsistent. Crab meal is also one of the main menus among Malaysians and are cooked in variety methods such as in gravy, fried or eaten raw. Most of our people were include the entire body of crab when cooking.

We studied the occurrence of *V. parahaemolyticus* isolates from flower crabs obtained from six different market in Selangor and Negeri Sembilan, Malaysia. Biochemical tests and PCR method was applied in identifying the *V. parahaemolyticus* in flower crab samples. By using the PCR method, the *tox*R gene is a targeted DNA gene for *V. parahaemolyticus* identification in food and clinical trials (Kim et al., (1999).

2.0 METHODOLOGY

2.1 Sample Collection

Thirty samples (n=30) of flower crab (*Portunus pelagicus*) were collected from several wet market in two states of Malaysia in which 15 samples each from Selangor and Negeri Sembilan. All samples were collected during March until July 2014. Samples were cooled with ice bricks about 7^{o} C to 10^{o} C during transportation and analyzed immediately to maintain the freshness of samples.

2.2 Isolation of *Vibrio parahaemolyticus*

Twenty-five (25) gram of flower crab (whole part) was aseptically added into sterile stomacher bag containing 225ml of alkaline peptone water (APW) and homogenized in stomacher machine for 90 second. The suspension then was transferred into 250ml volume of Schott bottle and incubated overnight at 37^{o} C. 10µl of this enrichment broth was inoculated on TCBS agar using spread plate method. Then, the incubation process were done at 37^{o} C for 24 h.

Blue- green colour from colonies on TCBS agar were presumed as *V. parahaemolyticus*. Each isolate was inoculated into slanting saline nutrient agar with 3% (w/v) NaCl for biochemical tests. The green or blue-

green colonies were presumptively. The incubation process were done at 37°C for 24 h, then conventional bacterial methods were used to examined the isolated strain, including triple sugar iron reaction, lysine iron agar, oxidase, indole and motility. The strains were maintained in saline nutrient broth with 3% (w/v) NaCl containing 20% of glycerol solution at -20°C (Sahilah et al., 2010).

2.3 Extraction of DNA

The DNA of bacterial was extracted by using boiling, chilling and centrifugation method [8,9]. The bacterial cells were matured overnight in 0.0015 L tube of nutrient broth enhanced with 3% (w/v) NaCl at 35°C to 37°C. The suspension was separated using centrifuge at 12 krpm for 1 min. The supernatant that arise was discarded with 0.001 L of distilled water that has been disinfected. The pellet was then washed. To disperse the pellet the new suspension was vortexed thoroughly. Next, it was heated for 10 min at 97°C and promptly was transferred to -20°C for 10 min. The suspension was separated using centrifuge at 10 krpm for 3 min. The supernatant obtained used as a DNA template.

2.4 Determination of *tox*R gene

The determination of *V. parahaemolyticus tox*R gene was conducted as described by (Kim et al., (1999). This primer produced 368 bp amplicon. PCR amplification was performed in 0.05 ml reaction volume prepared in a 0.0002 L disinfected PCR tubes. The mixture containing 25 µl of GreenTaq MasterMix, 0.001 ml of forward and 0.001 ml of reverse oligonucleotide primer,18 µl of nuclease free water (NFW) and the 5 µl of extracted DNA. Positive and negative DNA control were achieved by addition of NFW and *V.parahamaemolyticus* culture purchased from Olipro™ Malaysia company respectively. Amplification was conducted in Eppendorf thermal-cycler.

The cycle for *tox*R gene was as followed; the initial heat activation was held for 5 min at 96°C in temperature program, 20 cycles of the denaturation process at 94°C for 1 min, annealing at 63°C for 1.5 min, elongation at 72°C for 1.5 min and final extension at 72°C for 7 min (Marlina, 2006). The PCR amplification products were fractioned by electrophoresis through 1.5% (w/v) agarose gel 1X TAE buffer at 90 V for 40 min and was stained with Maestrosafe™ Nucleic Acid. Next, 100 bp GeneRulerDNA™ ladder was used as DNA size marker. Lastly, all samples were observed and recorded under UV trans-illuminator Gel Documentation System.

2.5 Detection of *tdh* and *trh* gene

The amplification of *tdh* and *trh* genes were examined with the previous establish method [4,10]. For forward and reverse primer sets, respectively: 5'- CCA CTA CCA CTC TCA TAT GC-3' and 5'- GGT ACT AAA TGG CTG ACA TC- 3' for *tdh* and 5'- GGC TCA AAA TGG TTA AGC G-3'and 5'- CAT TTC CGC TCT CAT ATG C-3' for *trh* (Tada et al., 1992). These primers produced 250 bp and 251 bp molecular sizes, respectively. The 0.05 ml reaction mixture contains 25 µl of GreenTaq MasterMix, 0.001 ml of forward and 0.001 ml of reverse oligonucleotide primer, 0.18 ml of nuclease free water (NFW) and the 0.005 ml of extracted DNA. Positive and negative DNA controls were covers in all virulent tests.

The PCR yields were examined by electrophoresis in a 1.5 % (w/v) agarose in 1X TAE buffer at 90 V for 40 min and stained by Maestrosafe™ Nucleic Acid. All gels were observed and recorded under UV

trans-illuminator Gel Documentation System. 100 bp GeneRulerDNA™ ladder was used as DNA size marker. The PCR product (amplicon) was stored at -20°C for prior further analysis.

3.0 RESULT AND DISCUSSION

3.1 Occurrence of *Vibrio parahaemolyticus*

A total of 30 samples of flower crabs *(Portunus pelagicus)* were screened for the occurrence of *V. parahaemolyticus*. Only 25 samples were positive to *V. parahaemolyticus* colony after isolated on TCBS agar. As many as 193 isolates of *V. parahaemolyticus* were examined by five (5) biochemical test. Table (1) shows the result where 93 *V. parahaemolyticus* strains were positive to triple sugar iron by performing alkaline slant (purple) and acid butt (yellow).

In addition, 148 typical *V. parahaemolyticus* gave positive lysin decarboxylate by performing alkaline reaction (purple colour) throughout the medium. 160 strains of *V. parahaemolyticus* indicates oxidase positive by developing dark purple colour within 10 sec. Meanwhile, 137 strains were shown positive result to motility and 152 strains were performed indole test, which formed red layer at top surface of the liquid suspension. As a total result, only 79 out of 193 strains were presumptively detected as *V. parahaemolyticus*. All the presumptive *V. parahaemolyticus* strains were subjected to PCR analysis for further confirmation.

Table 1 Results of Biochemical Test

Biochemical test	Reaction	No. of isolates with same reaction as *Vibrio parahaemolyticus*[a]
Triple Sugar Iron (TSI)	K/A[b]	93
Lysin decarboxylate	+	148
Oxidase	+	160
Indol	+	152

[a] A total of 193 bacterial isolates were subjected to each biochemical test
[b] K/A, alkaline slant (purple) and acid butt (yellow)

3.2 PCR confirmation of *tox*R, *tdh* and *trh* genes

The presumptive isolates of *V. parahaemolyticus* (n=79) were then defined by targeting specific *tox*R gene of *V. parahaemolyticus* as a maker gene for identification to this bacteria (Kim et al., (1999). Based on the PCR method, 51 strains were found to have a positive result to *toxR*-gene by producing amplicon of 368 bp molecular size. This was consistent in agreement with (Kim et al., (1999). Figure 1 to 4 shows the results of *V. parahaemolyticus* that positively amplified to 368bp of *tox*R gene isolated from Selangor (strains VPS1- 30) and Negeri Sembilan (strains VPN31-51) with DNA ladder 100bp.

The 51 *V. parahaemolyticus* isolates were also studied for virulence related gene of *tdh* and *trh* to detect any pathogenic strains obtained from the environmental sample. Nevertheless, absent of strains were found to have positive result for both virulence genes. This finding was estimated due to the occurrence of virulence genes in environmental samples was reported to be very low in comparison to clinical strains (Vimala, 2010).

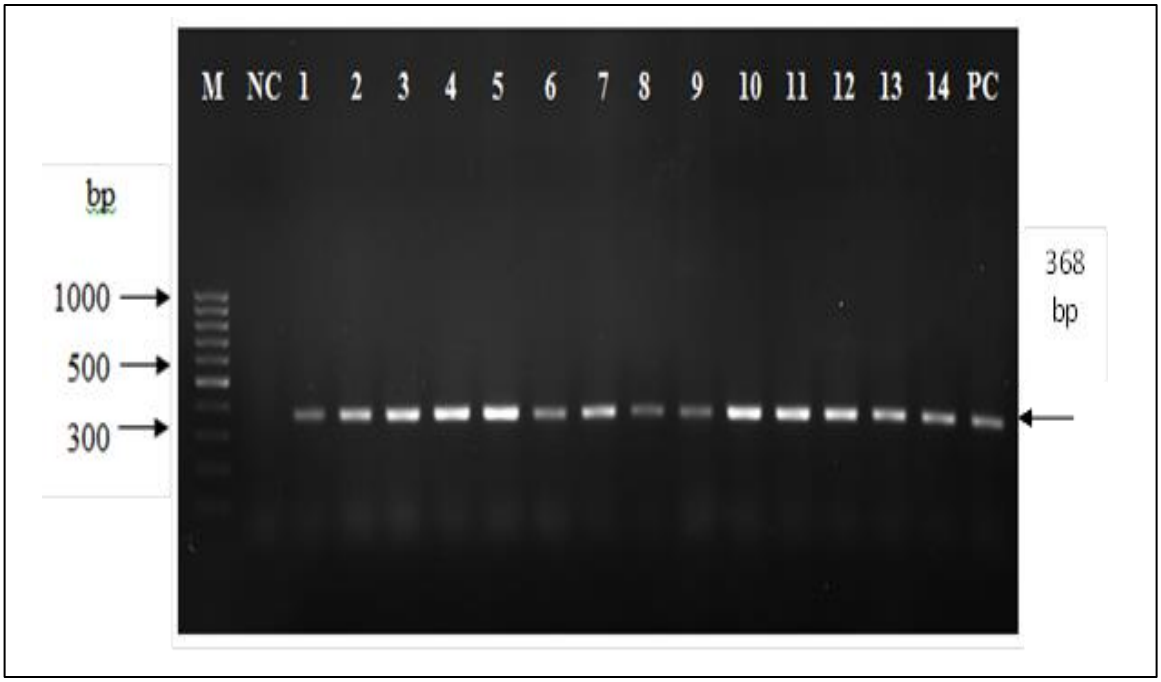

Figure 1 Electrophoresis of the *toxR* g e n e (368bp) detection of *Vibrio parahaemolyticus* from Selangor on 1.5% (w/v) agarose gel. NC: Negative control, Lanes 1 - 15: VPS1- VPS15

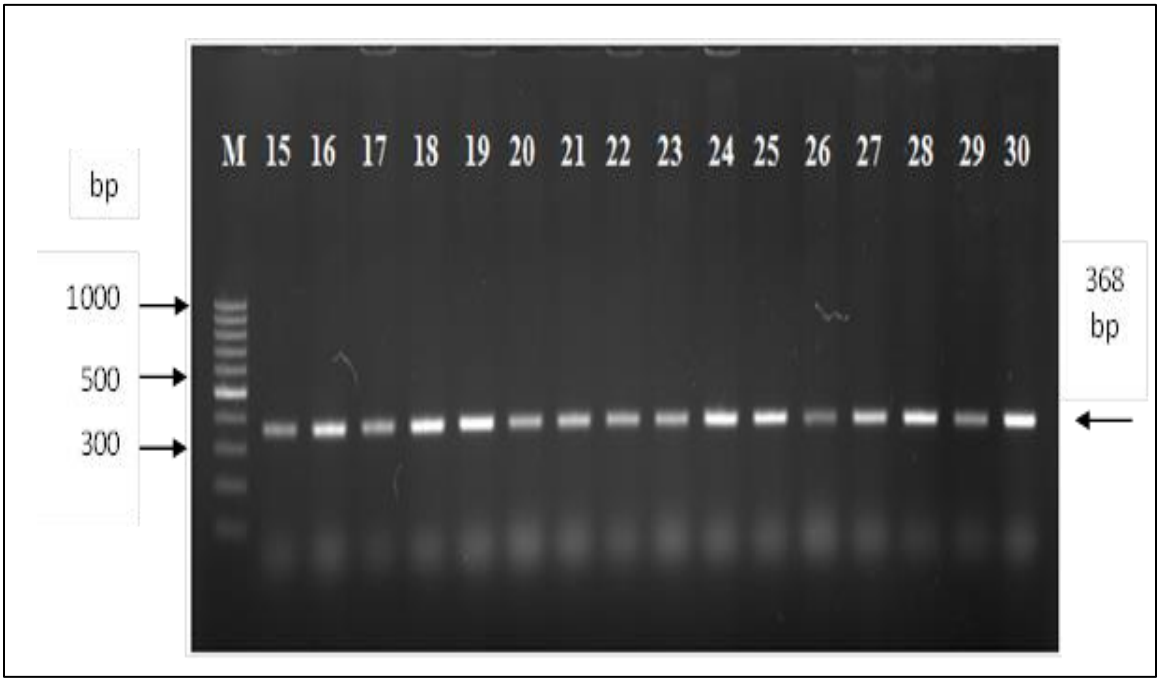

Figure 2 Electrophoresis of the *toxR* g e n e (368bp) detection of *Vibrio parahaemolyticus* from Selangor on 1.5% (w/v) agarose gel. Lane NC: Negative control, Lanes 16 - 30: VPS16- VPS30, Lane PC: Positive control

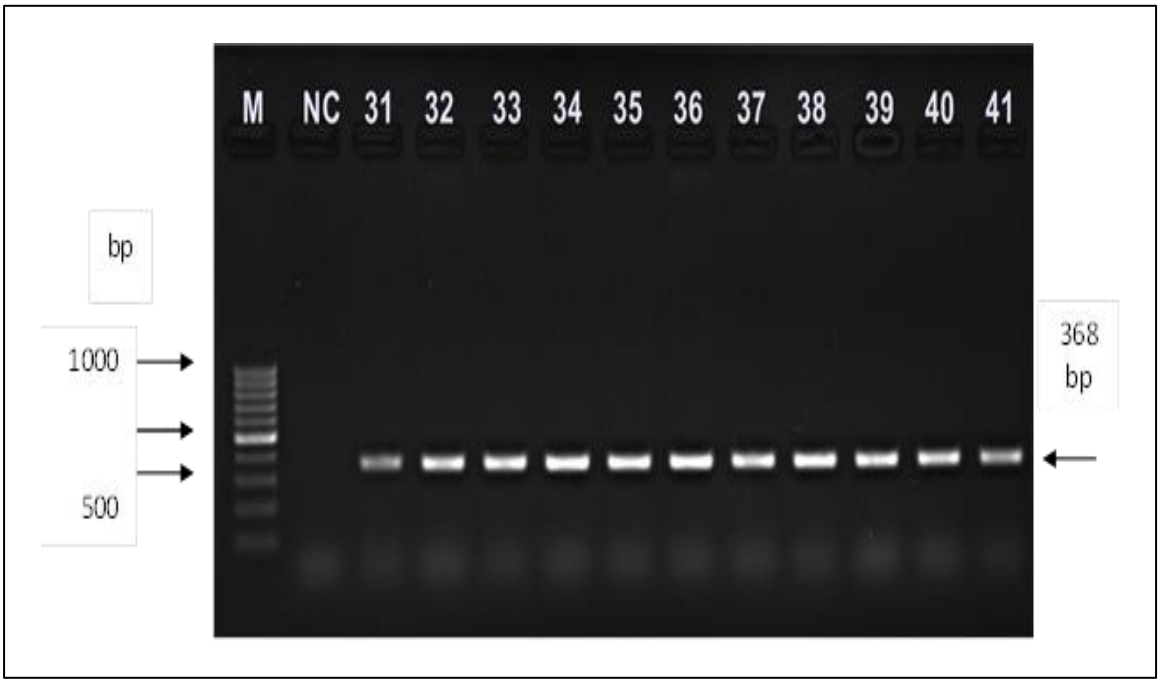

Figure 3 Electrophoresis of the toxR gene (368bp) detection of Vibrio parahaemolyticus from Selangor on 1.5% (w/v) agarose gel. Lane NC: Negative control, Lanes 31- 41: VPN31- VPN41

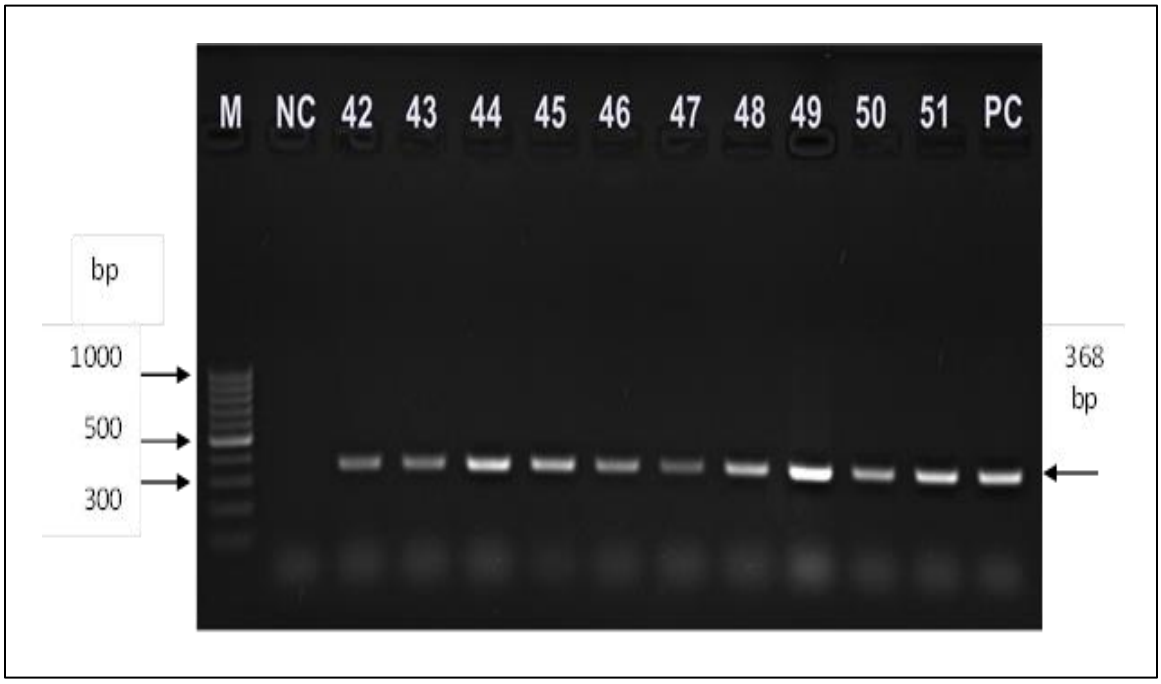

Figure 4 Electrophoresis of the toxR gene (368bp) detection of Vibrio parahaemolyticus from Selangor on 1.5% (w/v) agarose gel. Lane NC: Negative control, Lanes 42- 51: VPN42- VPN51, Lane PC: Positive control

4.0 CONCLUSION

In conclusion, PCR technique was more reliable because of the detection of specific target gene and virulent-related gene in the bacterium. This study provide new information regards to the *V.parahaemolyticus* isolated from flower crabs and associated with the risks of assessment to this bacteria in other regions with similar characteristics.

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