



# INVESTIGATING BRUNEI'S SEAFOOD MARKETS FOR *VIBRIO PARAHAEMOLYTICUS* USING THE MOST PROBABLE NUMBER-POLYMERASE CHAIN REACTION

ORIGINAL ARTICLE, Vol-5 No.2

Asian Journal of Medical Science, Volume-5(2014)

<http://nepjol.info/index.php/AJMS>

<sup>1</sup>Zaidah Rizidah Murang, <sup>2</sup>Siti Hanna Muharram, <sup>3</sup>Oduola Abiola. Pengiran Anak Puteri Rashidah Sa'adatul Bolkiah Institute of Health Sciences, Universiti Brunei Darussalam, Jalan Tungku Link, BE1410, Negara Brunei, Darussalam.

## CORRESPONDENCE:

Dr. Oduola Abiola  
Pengiran Anak Puteri Rashidah  
Sa'adatul Bolkiah Institute of  
Health Sciences,  
Universiti Brunei Darussalam,  
Jalan Tungku Link, BE1410,  
Negara Brunei, Darussalam.  
Tel: +673 8193134  
Fax: +673 2461081  
Email:  
[oduola.abiola@ubd.edu.bn](mailto:oduola.abiola@ubd.edu.bn)

---

*"V. parahaemolyticus not  
detected in Brunei seafood  
market."*

---

## ABSTRACT

**Background:** *Vibrio parahaemolyticus* (*V. parahaemolyticus*) is the commonest source of seafood poisoning and has a very high incidence in the countries of Asian.

**Aims and Objectives:** This study aims to investigate the presence of *V. parahaemolyticus* in seafood from Brunei seafood market using the Most Probable Number- Polymerase Chain Reaction (MPN-PCR).

**Results:** None of the twenty-three seafood samples that were purchased at random from unselectively chosen wet markets and hypermarkets in Brunei Darussalam yielded *V. parahaemolyticus*.

**Conclusion:** This could be due to any or a combination of: the absence or low level of *V. parahaemolyticus* from where the samples were harvested, inactivation of *V. parahaemolyticus* during the processing and preservation of the samples, possible sampling factors as well as good hygienic practices in Brunei's seafood market

**Keywords:** Most Probable Number-Polymerase Chain Reaction (MPN-PCR),  
Seafood, *Vibrio parahaemolyticus*

## INTRODUCTION

*Vibrio parahaemolyticus* (*V. parahaemolyticus*) is a naturally occurring salt-requiring bacterium of aquatic habitat and frequently isolated from seafood<sup>1</sup> especially of raw or undercooked ones<sup>2</sup> and is responsible for about a quarter of global food borne diseases.<sup>3</sup> It is therefore a leading cause of seafood-borne gastroenteritis, especially in countries where seafood consumption is high<sup>4</sup> as well as being responsible for foodborne disease outbreaks, with fatalities in Japan in 1950,<sup>5,6</sup> India,<sup>2,7</sup> China,<sup>6, 8, 9</sup> Taiwan,<sup>10</sup> Korea<sup>11</sup> and Malaysia.<sup>12</sup>

The mechanism by which *V. parahaemolyticus* infects humans has yet to be entirely determined. However, pathogenic *V. parahaemolyticus* is known to produce either thermostable direct haemolysin (TDH), TDH-related haemolysin (TRH) or both.<sup>13,14</sup> TDH and TRH are encoded by *tdh* and *trh* genes, respectively. Thus, they are recognized as major virulent factors of *V. parahaemolyticus* and either or both of these virulent factors can cause illness. The *toxR* gene is well conserved among *V. parahaemolyticus* which are non-pathogenic in the absence of *tdh* or/and *trh* gene, and consequently used as target for its specific detection.<sup>15</sup>

According to the Food and Agriculture Organization report,<sup>16</sup> seafood constitutes the main source of animal protein for a significant percentage of world population. Therefore, as *V. parahaemolyticus* is known to cause gastroenteritis which could be fatal if complications occur, it is important to determine its degree of contamination of seafood to prevent food poisoning in consumers. In addition as a very high level of contamination with *V. parahaemolyticus* has been reported in bivalves and shrimp culture environment in Malaysia,<sup>12</sup> it is highly likely that seafood in Brunei is also contaminated with *V. parahaemolyticus* due to the proximity of the two countries and importation of seafood into Brunei.

Thus, this study investigated the presence of *V. parahaemolyticus* in seafood from both wetmarkets and hypermarkets in Brunei Darussalam using MPN-PCR method so as to provide insight into the biosafety assessment of *V. parahaemolyticus* in the Sultanate.

## MATERIALS AND METHODS

### 2.1. Bacterial strains

TDH-producing *V. parahaemolyticus* (ATCC 33846), *toxR*-producing *V. parahaemolyticus* (ATCC BAA-239) and *BRCA* gene [*BRCA10A* and *BRCA10BC* primers (Promega)] were used as controls.

### 2.2. Sampling

A total of 23 randomly selected seafood samples were purchased in Brunei between January and April 2013: 11 from wetmarkets and 12 from hypermarkets. They consisted of 9 packets of prawns (6 from wetmarkets, 3 from hypermarkets) 7 bloody clams from hypermarkets, 3 lalas (2 from hypermarkets, 1 from wetmarket), 1 flower clam from wetmarket, 2 mussels from wetmarket and 1 small shrimp from wetmarket. Each sample was placed in a separate, sterile and labelled plastic bag and stored at -80°C prior to investigation.

### 2.3. Most Probable Number

Ten g of each sample was homogenized in a sterile stomacher bag with 90 ml of alkaline peptone water (10% peptone, 5% NaCl, pH 9.2). 40 ml of thoroughly mixed homogenate was aseptically transferred into a sterile Falcon tube and incubated overnight at 37°C without shaking. A three-tube MPN procedure was performed as described by Hara-Kudo *et al.* (2003) (5). Briefly, 0.1, 0.01, 0.001 ml of the homogenate serial dilution was performed in triplicate. 1 ml of each of the overnight culture was then transferred into 9 ml of APW and incubated at 37°C for 6 h with shaking at 200 rpm.

### 2.4. Boiled-cell DNA extraction

One ml from each APW tube was transferred to an Eppendorf tube, centrifuged at 10,000 x g for 5 min and supernatant was discarded. The pellet was suspended in 1 ml of sterile normal saline, vortexed, kept in boiling water (100°C) for 10 min and immediately submerged in ice for 10 min. A further centrifugation was done at 14,000 x g for 10 min and supernatant was used as DNA template for PCR analysis.

### 2.5. PCR analysis

#### 2.5.1. *toxR* PCR

Reaction mixture for *toxR* analysis consisted of 1 µl of the DNA template, 4 µl 5X buffer (Promega), 0.5 µl of 10

mM concentration of dNTPs (Promega), 1.25 µl of MgCl<sub>2</sub> (Promega), 0.25 µl of taq DNA polymerase (Promega), 12.0 µl sterile distilled water and 1 µl of 10pmol/l of each primer (Table 1). 19 µl of PCR master mixture was aliquoted into PCR tubes (20 µl with the DNA template). Amplification conditions of the Thermocycler (Applied Biosystems) were 20 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 63°C for 1.5 min and extension at 72°C for 1.5 min. Initial denaturation and further extension were at 94°C for 1 min and 72°C for 7 min, respectively.

#### 2.5.2. *tdh*/*trh* PCR

PCR reaction mixtures for *tdh* and *trh* genes were prepared as for *toxR*. Primers specific for *tdh* and *trh* genes (1 µl of 10pmol/l of each primer) were used (Table 1). Amplification conditions of the thermocycler (Applied Biosystems) were 30 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min and extension at 72°C for 1.5 min. Initial denaturation and further extension were at 94°C for 1 min and 72°C for 7 min, respectively.

#### 2.6. Electrophoresis

Ten µl of the reaction mixture was mixed with 2.5 µl of DNA loading dye (Promega) and the mixture was resolved by electrophoresis in 1.5% agarose gel (Promega) at 100 V. 50 bp DNA marker ladder (Promega) was used. Electrophoresis gel was stained with ethidium bromide and observed using the UV transilluminator (VersaDoc Imaging System).

### 3. Ethical considerations

Approval was obtained from the Universiti Brunei Darussalam Research and Ethics Committee prior to the commencement of this study. The seafood samples for the study were purchased from several wet markets and hypermarkets without the vendors being informed, to prevent biased result. If pathogenic *V. parahaemolyticus* was to be found in any of the samples being used for the study, the Public Health Department was going to be informed so that they could take appropriate actions to ensure the safety of the public.

## RESULTS

Twenty three randomly selected seafood samples from both wet markets and hypermarkets were investigated

for the presence of *V. parahaemolyticus*. In none of the 23 samples was any of *toxR*, *tdh* or/and *trh* gene(s) detected. Figure 1 is representative of electrophoretic gel (sample 8) aimed at detecting *toxR* gene. No bands were detected other than the 50bp DNA ladder that strongly suggested that *V. parahaemolyticus* was not present.

Figure 2 is an electrophoretic gel showing the bands of *BRCA* genes (positive controls). A control that contained no DNA was included to serve as a negative control. For the positive controls, the bands were located between 500 and 550bp, and 400 and 450bp. This is in agreement with an earlier report (17) that determined *BRCA10A* genes to be ~508bp and *BRCA10BC* to be ~455bp.

Figure 3 shows the bands of *ATCC 33846*<sup>TM</sup> and *ATCC BAA-239*<sup>TM</sup> that are positive controls for *tdh* and *toxR* genes. A control that contained no DNA was included to serve as a negative control. For the positive controls, the bands are located between 350 and 400bp, and 250 and 255bp. A previous study (18) also determined *toxR* to be ~368bp and ~251bp for *tdh*.

## DISCUSSION

No strain of *V. parahaemolyticus* was detected in any of the 23 seafood samples from randomly selected wetmarkets and hypermarkets in Brunei Darussalam. This was surprising as *V. parahaemolyticus* is known to be prevalent in Asia (2, 5, 6, 7, 8, 9) and has been reported in Malaysia (12), which has similar environmental and other conditions that foster the proliferation of *V. parahaemolyticus* to that of Brunei. Moreover, a number of the samples were possibly imported into Brunei from Malaysia.

MPN-PCR was used to investigate the presence of *V. parahaemolyticus* in the seafood samples. This method has been used in similar studies and shown to be very specific and sensitive as well as suitable for the detection *V. parahaemolyticus* in seafood samples (8, 15). The two types of controls used produced characteristic electrophoretic gels bands thereby confirming the reliability of the results. Hence, the undetected *V. parahaemolyticus* was most likely not due to methodological insufficiency, rather may have

Table 1. PCR primers used for detection of *V. parahaemolyticus* (Tada *et al.*, 1992) (23).

| Target gene | Primer name | Sequence (5'-3')      |
|-------------|-------------|-----------------------|
| <i>toxR</i> | toxR F      | GTCTTCTGACGCAATCGTTG  |
|             | toxR R      | ATACGAGTGGTTGCTGCTATG |
| <i>tdh</i>  | tdh F       | CCACTACCACTCTCATATGC  |
|             | tdh R       | GGTACTAAATGGCTGACATC  |
| <i>trh</i>  | trh F       | GGCTCAAATGGTTAAGCG    |
|             | trh R       | CATTCCGCTCTCATATGC    |

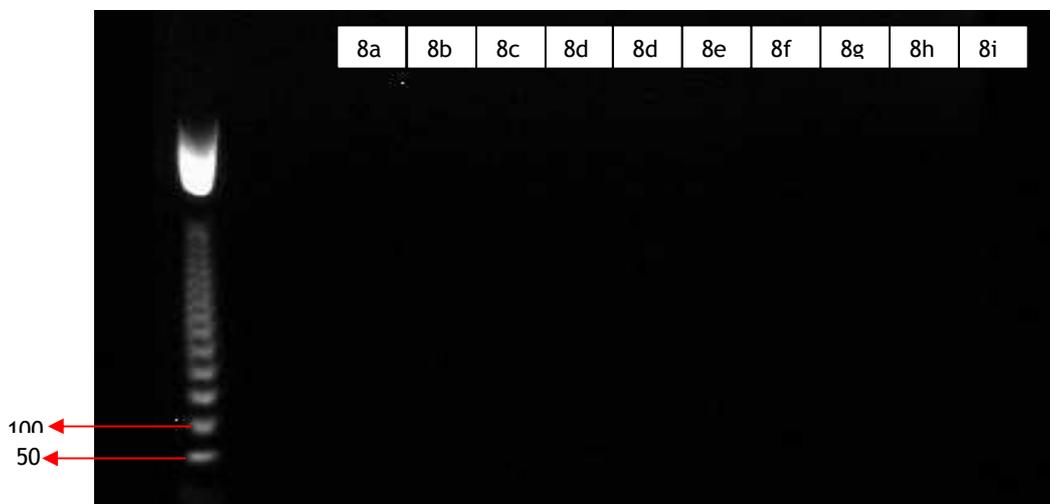


Fig1. This figure shows an image produced by Versadoc system of the gel electrophoresis of sample 8 that aimed at *toxR* gene. It shows a 50bp DNA ladder (Promega). No other bands were detected. 8a, d and g consisted of 0.1 ml of sample homogenate, 8b, e, h 0.01 sample homogenate and 8c, f and i 0.001 ml of sample homogenate as prepared during MPN procedure.

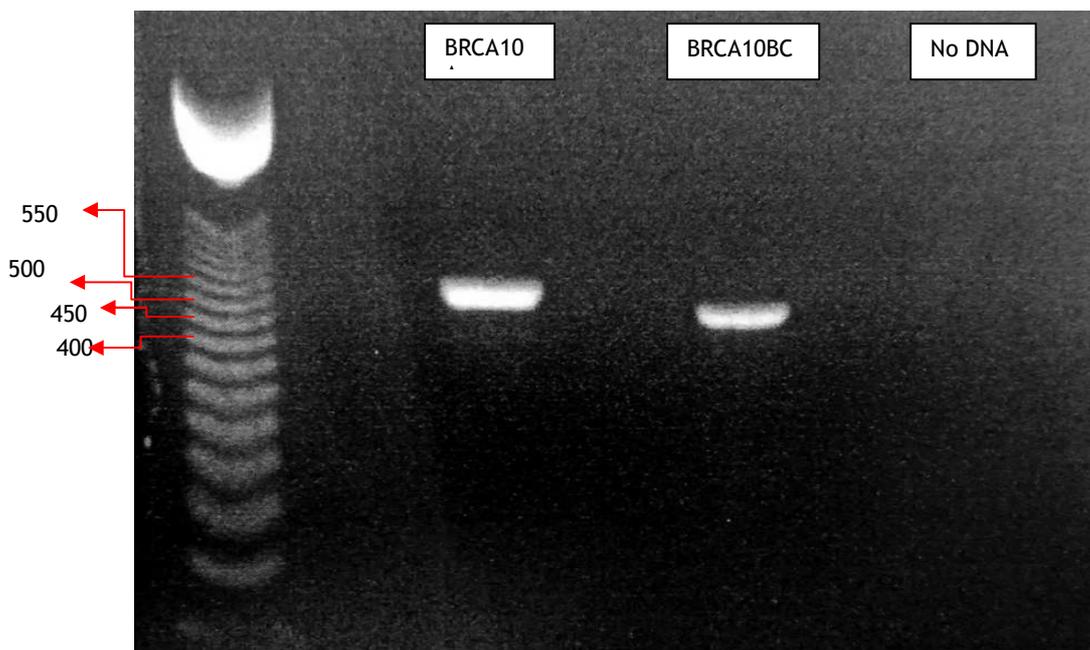


Fig 2. This figure was produced by Versadoc system of the gel electrophoresis of *BRCA10A* and *BRCA10BC* genes and 50bp DNA ladder (Promega). *BRCA10A* lies between 500 and 550, and *BRCA10BC* between 400 and 450.

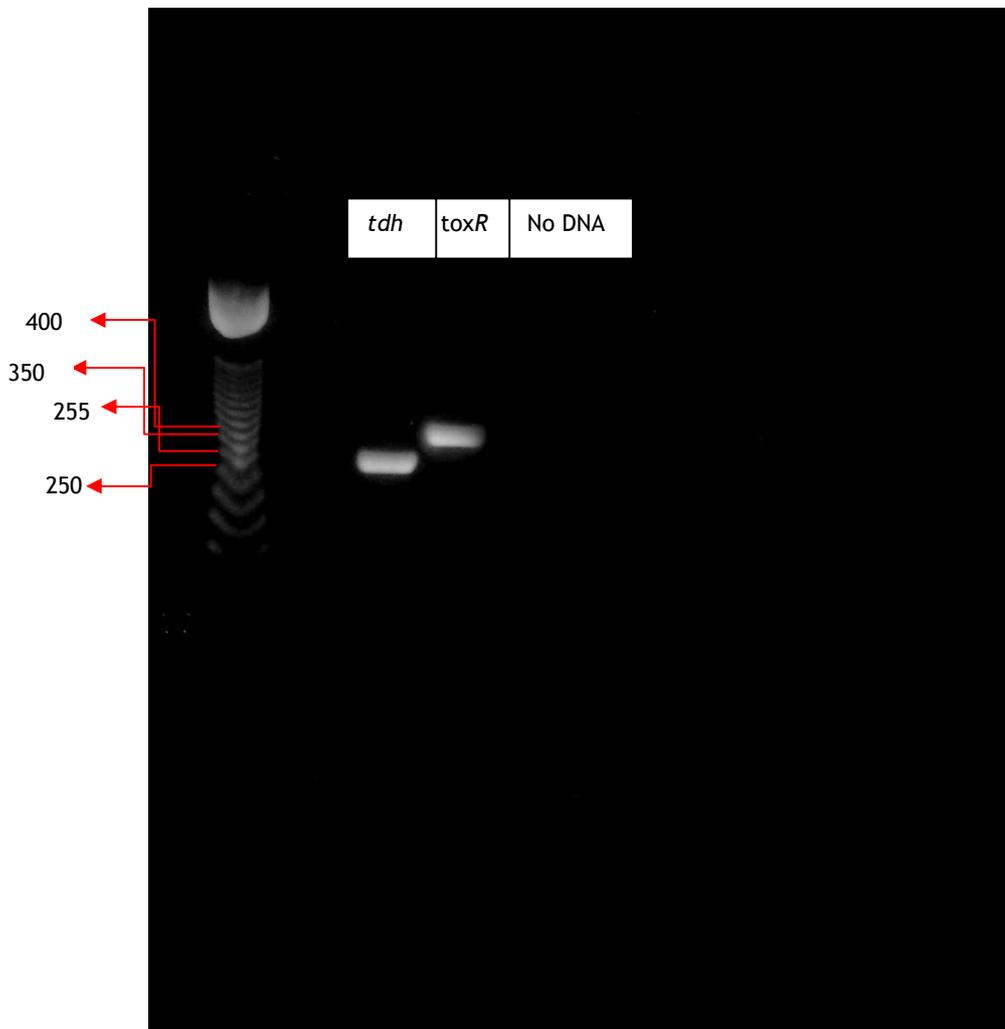


Fig 3. This figure shows 50bp DNA ladder (Promega) and the bands of *ATCC 33846*<sup>TM</sup> and *ATCC BAA-239*<sup>TM</sup> that are controls for *tdh* and *toxR* and genes. The genes' bands are located between 250 and 255 for *tdh* and 350 and 400 for *toxR*.

resulted from other factors. For example, a lack of *V. parahaemolyticus* in the samples could be due to the fact that they may not have been contaminated from Brunei waters or other sources where the samples were harvested. This suggests that their salinity or the temperature may not allow for optimum growth of *V. parahaemolyticus* even if indeed there was a contamination in the first case. It is also possible that the level of *V. parahaemolyticus* be too low to be detected despite the high sensitivity of the MPN-PCR method.

Another possibility is for *V. parahaemolyticus* if present at the time of harvest to have been inactivated

during processing or preservation of the samples in the wetmarkets and/or hypermarkets as well as during the investigation. The processing in the wetmarkets and hypermarkets may have reduced *V. parahaemolyticus* in the samples tested to an undetectable level if not completely removed. This is because preservation techniques, such as low-temperature, drying, smoking or salt curing, are commonly used in seafood processing in hypermarkets to control the growth and survival of spoilage and pathogenic microorganisms including *V. parahaemolyticus* which has the ability to exist in a non-cultivable state (19) due to such treatments. In this study however, the only method of preservation that

apply is the low temperature which has been reported to cause an acquisition of a non-cultivable state by *V. Parahaemolyticus*.<sup>20</sup>

Interestingly, it has been previously reported that different strains of *V. parahaemolyticus* have different capability of adaptation to cold environment, and that the low-temperature adapting strains probably increased the risk of food borne diseases (21). In our opinion, cultivability or otherwise is not an issue with not detecting *V. parahaemolyticus* in any of the 23 samples that we investigated.

This is because the method of detection in this study is PCR based. PCR permits identification of non-cultivable or slow-growing microorganisms and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes.<sup>22</sup> Thus, it could be argued that *V. parahaemolyticus* was not detected in the samples because it was absent or not present in detectable level especially given the rigorous experimental controls that we have employed.

While sampling could be a factor that may have led to the undetected *V. parahaemolyticus* in this study, the different species of seafood samples were randomly purchased from several wetmarkets and hypermarkets to minimise error. Less shellfish was tested as few were available in Brunei due to the presence of red tides during the period of study. In addition, a previous study<sup>23</sup> concluded that the presence of *V. parahaemolyticus* may not be specifically associated with the type of seafood; rather it could be significantly influenced by preservation method or indeed other unknown factors. This suggests that the sampling technique in this study was not likely to be responsible for the lack of detection of *V. parahaemolyticus*. Hygienic practices in wetmarkets and hypermarkets may also play a role in the result obtained. The hypermarkets were hypothesized to be 'cleaner' than wetmarkets, because according to Tunung *et al.* (2010)<sup>12</sup>, the handling, washing and packaging of materials at wet markets are unhygienic and without gloves and hence the risk of cross-contamination between samples of different origins is higher in wet markets than hypermarkets.

Given this hypothesis, it could be expected that

in this study, a difference between the locations where the samples were purchased would be observed. Nonetheless, this study showed that the samples taken from both hypermarkets and wetmarkets did not detect *V. parahaemolyticus* suggesting hygienic practices in both market types in Brunei may be comparable.

In conclusion, *V. parahaemolyticus* is not present in randomly selected seafood samples purchased from both wetmarkets and hypermarkets in Brunei Darussalam. In our opinion our finding was not due to insufficiency or flaw in the method of investigation. Other considered possibilities are sample size, absence of *V. parahaemolyticus* from where the samples were harvested, inactivation of *V. parahaemolyticus* during the processing and preservation of the samples as well as the hygienic practices in wetmarkets and hypermarkets in Brunei. Further studies are therefore needed to investigate these possible causes.

#### Future studies

Given the relatively small sample size studied in absolute terms, we intend in the to use a larger sample size and wider varying types of seafood to further investigate the *V. parahaemolyticus* in Brunei Darussalam's seafood market as well as water samples from various sources of seafood in Brunei.

**Acknowledgment:** Dr Mas Rina Wati Hj Abdul Hamid for donating BRCA genes primers and PCR reagents.

## REFERENCES

1. Liston J. Microbial hazards of seafood consumption. Food. Tech.1990; 44: 562.
2. Gopal S, Otta SK, Karunasagar I, Nishibuchi M and Karunasagar I. The occurrence of *Vibrio* species in tropical shrimp culture environments; implications for food safety. Int. Food. Microbiol.2005; 102: 151-159.
3. Feldhusen F. The role of seafood in bacterial food borne diseases. Microbes Infect 2000; 2: 1651-1660.
4. Trison DL and Kelly M T. *Vibrio* species of medical importance. Diagn. Microbiol. Infec. Dis.1984; 2: 263-276.
5. Hara-Kudo Y, Nishina T, Nakagawa H, Konuma H, Hasegawa J, and Kumagai S. Improved Method for detection of *Vibrio parahaemolyticus* in seafood. Appl. Environ. Microbiol. 2001; 67(12): 5819-5823.
6. Yang Z, Jiao X, Zhou X, Cao G, Fang W, and Gu R. Isolation and molecular characterization of *Vibrio parahaemolyticus* from fresh, low-temperature preserved, dried and salted seafood

- products in two coastal areas of eastern China. *Int. Food Microbiol.*2008; 125: 279-285.
7. Chakraborty RD, Surendran PK, and Joseph TC. Isolation and characterization of *Vibrio parahaemolyticus* from seafoods along the southwest coast of India. *World. J. Microbiol. Biotechnol.*2008; 24: 2045-2054.
  8. Luan X, Chen J, Liu Y, Li Y, Jia J, Liu R and Zhang XH. Rapid quantitative detection of *Vibrio parahaemolyticus* in seafood by MPN-PCR. *Curr. Microbiol.*2008; 57: 218-221.
  9. Chen Y, Liu XM, Yan JW, Li XG, Mei LL, Fa QM, and Fa Y. Foodborne pathogens in retail oysters in south China. *Biomed. Environ. Sci.*2010; 23: 32-36.
  10. Hara-Kudo Y, Sugiyama K, Nishibuchi M, Chowdhury A, Yatsuyanagi J, Ohtomo Y. Prevalence of pandemic thermostable direct hemolysin-producing *Vibrio parahaemolyticus* O3:K6 in seafood and the coastal environment in Japan. *Appl. Environ. Microbiol.*2003; 69: 3883-3891.
  11. Lee J, Jung D, Eom S, Oh S, Kim Y, Kwak H and Kim K. Occurrence of *Vibrio parahaemolyticus* in oysters from Korean retail outlets. *Food Control.* 2008; 19: 990-994.
  12. Tunung R, Ghazali FM, Noranizan MA, Hareesh KK, Lesley MB, Nakaguchi Y. Rapid detection and enumeration of pathogenic *Vibrio parahaemolyticus* in raw vegetables from retail outlets. *Int. Food Res. J.* 2010; 18: 67-78.
  13. Honda T and Iada T. The pathogenicity of *Vibrio parahaemolyticus* and the role of thermostable direct haemolysin and related haemolysin. *Rev. Med. Microbiol.* 1993; 4: 106-113.
  14. Nishibuchi M and Kaper JB. Thermostable direct haemolysin gene of *Vibrio parahaemolyticus*: A virulence gene acquired by marine bacterium. *Infect. Immun.* 1995; 63: 2093-2099.
  15. Bej AK, Patterson DP, Brasher CW, Vickery MC, Jones DD and Kaysner CA. Detection of total and hemolysin-producing *V. parahaemolyticus* in shellfish using multiplex PCR amplification of *tlh*, *tdh* and *trh*. *J. Microbiol. Methods.* 1999; 36: 215-225.
  16. Global seafood consumption hits all-time high, from: <http://www.seafoodsource.com/newsarticledetail.aspx?id=9064>. Accessed in 20th March 2013.
  17. Sanz DJ, Acedo A, Infante M, Duran M, Perez-Cabornero L, Esteban-Cardenosa E, Lastra E, Velasco EA. A high proportion of DNA variants of BRCA1 and BRCA2 is associated with aberrant splicing in breast/ovarian cancer patients. *Clin. Cancer Res.* 2010; 16 (6): 1957-1967.
  18. Zulkifli Y, Alitheen NB, Son R, Yeap SK, Lesley MB and Raha AR. Identification of *Vibrio parahaemolyticus* isolates by PCR targeted to the *toxR* gene and detection of virulence genes. *Int. Food Res. J.*2009; 16: 289-296.
  19. Wong HC, Lui SH, Wang, Lee CL, Chiou CS, Liu DP, Nishibuchi M and Lee BK. Characteristics of *Vibrio parahaemolyticus* O3:K6 from Asia. *Appl. Environ. Microbiol.*2000; 66: 3981-3986.
  20. Bates T C and Oliver JD. The viable but non-culturable state of Kanagawa positive and negative strains of *Vibrio parahaemolyticus*. *J. Microbiol.* 2004; 4: 74-79.
  21. Matches JR, Liston J and Daneault LP. Survival of *Vibrio parahaemolyticus* in fish homogenate during storage at low temperatures. *Appl. Microbiol.*1971; 21: 951-952.
  22. Salis AD. (2009). "Applications in Clinical Microbiology". Real-Time PCR: Current Technology and Applications. Caister Academic Press. ISBN 978-1-904455-39-4.
  23. Tada J, Ohashi T, Nishimura N, Shirasaki Y, Ozaki H, Fukushima S, Takano J, Nishibuchi M and Takeda Y. Detection of thermostable direct hemolysin gene (*tdh*) and thermostable direct-related hemolysin gene (*trh*) of *Vibrio parahaemolyticus* by polymerase chain reaction. *Mol. Cell. Probes.*1992; 64: 477-487.

#### Authors Contributions:

**ZRM: Conceptualized study, performed experiments and prepared first draft of manuscript.**

**SHM: Participated in design of study and revised draft of manuscript.**

**OA: Participated in design of study, supervised experiments and finalized the manuscript.**

**Conflict of Interest: None**

**Research Grants: This study was funded by PAP RSB Institute of Health Sciences, Universiti Brunei Darussalam (K20394/005) awarded to ZRM.**

**Date of Submission: 15.10.2013**

**Date of Peer review: 22.10.2013**

**Date of submission of revised version: 22.11.2013**

**Date of peer review: 29.11.2013**

**Date of Acceptance: 30.11.2013**

**Date of Publication: 10.1.2014**