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Detection of *Vibrio cholerae* in raw cockles (*Anadara granosa*) by polymerase chain reaction

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Abstract: Aimed of this study was to determine the presence of *Vibrio cholerae* in cockles (*Anadara granosa*) from different coasts in Malaysia and to measure the biosafety of *V. cholerae* in raw cockles at wet market in Malaysia using the polymerase chain reaction (PCR) in combination with the most probable number (MPN) method. A total of 100 samples from 4 different wet markets in the West and East were examined for the presence of *V. cholerae*. The prevalence of *V. cholerae* between the two coasts was not significant different. In fact, the 74% of samples from West coast area was found positive while the 69% for samples collected in the East coast. West coast samples showed a prevalence of 60% for the wet market A=, 64% for B=, 88% for C= and 84% for the market D); East coast samples showed the same percentage with 72% for the wet markets E, F and H, followed by wet market G with 60%.With the MPN-PCR method, using 80 samples of raw cockles obtained from 4 wet markets, the occurrence of *V. cholerae* detected was of 95%. The frequency of *V. cholerae* in raw cockles obtained from wet market I and L was higher (100%) compared to other wet market (Wet market B=, 90%; Wet market C=, 95%).The density of *V. cholerae* detected in all samples ranged from <30 up to >24000 MPN/g, but most of the samples (24 samples) were in category >24000 MPN/g concentration. *V. cholerae* was present in raw cockles in higher number. Hence, these results demonstrate the presence of pathogenic *V.cholerae* in cockles harvested and reveal the potential risk of illness associated with their consumption. This study will be the first biosafety assessment of *V. choleare* in raw cockles in Malaysia and it will provide significant insights about Malaysian scenario.

Keywords: *Vibrio cholerae*, cokles (*Anadara granosa*), most probable number, polymerase chain reaction

Introduction

Vibrio cholerae is a natural inhabitant of the aquatic environment, mostly in estuarine systems, and is the cause of life-threatening diarrhea epidemics (Aneta, 2006). Water plays an important role in the transmission of this pathogen. *V. cholerae* belongs to the family Vibrionaceace. They all gram negative motile rods with curve or comma shape (Nwachuckwu, 2006). *V. cholerae* is the natural component of the bacterial flora of both freshwater and marine environments (Lipp, 2002). Abiotic surface of marine habitat provide to *V. cholerae* microhabitats favorable for survival of *V. cholerae*, protecting it against stressful environmental conditions (Watnick and Kolter, 1999). *V. cholerae* can also replicate within mussels, ameobe and snails, which provide environmental reservoirs for infection (Peterson, 2002). Several studies have demonstrated that *Vibrio* spp. are able to survive in water for long time, and can still cause infections in conditions of lost culturability (Wai *et al*.,1999) .

V. cholerae is the etiological agent of cholera which is spread by contaminated food, water or direct fecal contact with food handlers. It can cause acute intestinal disease characterized by profuse rectal loss of water and electrolytes and if untreated may lead death. The symptom may due to the exotoxin produced which effects the epithelial cells resulting in watery diarrhea (Nwachuckwu, 2006).Cholera epidemics outbreaks have killed millions of people and continue to be the major public health concern worldwide (Faraque *et al*., 2003). From the research by (Ellen *et al*., 2001), undercook seafood continue to account for most US cholera cases. Samples of choice in this study were cockles (*Anadara granosa*), one of the most important aquaculture species in

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Malaysia. Cockle is the common name for a group of small, edible, saltwater clams, marine bivalve mollusks in the family Cardiidae. In Malaysia, the cockles are popular as an ingredient in several types of local foods such as laksa, char kway teow and steamboat. Boiled cockles (sometimes grilled) are also sold at many hawker centers in Malaysia. Raw cockles frequently carry pathogenic *Vibrio* spp. including *V. cholerae* and are often implicated in the transmission of these bacteria. Furthermore, numbers of *Vibrio* spp. including *V. cholerae* may enhance during storage of shellfish especially if refrigeration is not used. Therefore, the aim of this study was to determine the prevalence of *V. cholerae* in cockles from wet market in selected area in Malaysia.

Materials and Methods

Samples collection

Two different areas East and West cost of Peninsular, Malaysia were chosen for the harvest of cockles. Selangor was chosen as the West coast area, while Pahang representative of the East coast. For West coast area, 100 samples of cockles (*Anadara granosa*), sold in 4 different wet market in Selangor were sampled during the period of August 2008-December 2008 and analyzed for the presence of *Vibrio cholerae*. For East coast, the same method of sampling was used in 4 different wet markets in Pahang between January 2009 and April 2009. In order to evaluate the prevalence of *V. cholerae* using MPN-PCR method, 80 samples were collected in 4 selected wet market markets from July to October 2009. All samples were transported in ice condition and were analyzed directly.

Samples preparation

Twenty grams of each sample were added to 225 ml of sterile buffered Alkaline peptone water (Oxoid, England) that adjust to pH 8.8, homogenized in a stomacher for 3 min. Serial dilutions were plated onto Thiosulphate Citrate Bile Salt (TCBS) agar (Fluka, India). Plates were incubated under aerobic conditions for 24 h at 35°C. Isolates were purified by repeated streaking onto respective growth media such as Trypticase Soy Agar (TSA) (Dmerck,Germany). All isolates were tested for Gram staining and oxidase. Gram-negative and oxidase positive isolates were selected for further test.

Enumeration with most probable number (MPN) technique

Ten grams of meat and fluid of cockles were homogenized with 100 ml of alkaline peptone water for 90 seconds and dilutions of 1:100, 1:1000 and 1:10000 were prepared in triplicate following three -tubes MPN format (Tan *et al*., 2008). All MPN tubes were incubated at 35°C for 24 h. According to MPN technique, turbid tubes were considered positive and subjected to PCR to confirm the presence of *V. cholerae*.

DNA extraction

Template preparation was carried out by the boiling method (Baumler *et al*., 1997). Cultures were grown in alkaline peptone water at 35°C for 24h and centrifuge at 12000 rpm for 1 min. Supernatants were carefully discarded and pellets were resuspended with 500 µl of sterile distilled water, bolied for 15 minutes and immediately incubated in ice for 10 min. The mixture was then centrifuged at 12000 rpm for 3 min and the supernatants were then transferred into sterile tubes to use as DNA template for PCR amplification.

PCR amplification

PCR reactions were performed in a total volume of 20 µl per tube, containing 2 µl genomic DNA, 1.5 mM $MgCl_2$, 10 µl 1x Readymix *Taq* PCR (containing 1.5 U *Taq* DNA polymerase, 10 mM KCl, 0.001% gelatin, 0.2 mM dNTP), and 0.2 µl of primer (5'-CGTAAAGCGCATGCAGGTG-3', 5'-CTTCGCCACCGGTATTCCTT-3') (Narjol.G.E, Axel *et al*., 2005). PCR amplifications were carried out in a ThermoCycler (Perkin Elmer GeneAmp PCR System 2400) with the PCR program consisting of the initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 1 min, at 60°C for 1 min and at 72°C for 1 min, and a final elongation at 72°C for 5 min.

Gel electrophoresis

All amplification products were combined with 4 µl of loading buffer (Bio-Rad) and 10 µl of these mixtures were applied to a horizontal agarose gel (Seakem® LE Agarose, Rockland, ME, USA, 1.2% (w/v)) in 1× TAE Buffer (Bio-Rad) containing 0.5 µg/ml of ethidium bromide. Electrophoretic separation was at 100V for 40 min and a molecular weight marker (100 bp PCR ladder, Eurogentec) was included. The gels were visualized under UV transilluminator and recorded as jpeg file by using Gel Documentation System, Model Gene Genius, GMV20, Syngene (GelDoc2000, Bio-Rad). Image analysis was performed using Quantity One® software (Bio-Rad).

Statistical analysis

Analysis of the comparison between the two coasts analyzed was performed by means of the 1-way analysis of variance (ANOVA) ($p = < 0.05$). The objective was to establish whether there was significant difference between the two coast samples

Results and Discussion

Sampling was done in two different areas, West and East coast, Peninsular, Malaysia. The prevalence of *V. cholerae* from different place of cockles' sources is shown in Table 1(a) and 1(b). The study analyzed 100 samples from each area and the occurrence of *V. cholerae* detected in raw cockles based on statistical analyses showed not significant between two coast samples. Based on the percentage for positive sample, West coast is 74% and 69 % from East coast. For West coast area, wet market D showed the higher detection (88%) of *V. cholerae*, followed by wet market C (84%) and 64% for B, while the wet market A showed the lowest prevalence (60%). The frequency of *V. cholerae* in raw cockles obtained from East coast area was of 72% for the wet markets E, F and G , followed by wet market E (60%).

Most of the cockles' sources from West coast area were from landing site in Sabak Bernam, Sungai Besar and Pantai Remis. This area's environment is brackish and muddy bottom compared with East coast which is sandy and less brackish. So, West coast area is a suitable environment for cockle's habitat. The slight different percentages of positive samples between East and West coast showed that the different physical environment for cockles' habitat did not influence for transmission of *V. cholerae*. The different environment could be only important for cockles reproduction. Furthermore *V. cholerae* is able to survive and grow in freshwater samples (Vital *et al*., 2007). Salinity and temperature are reported to be important parameters to control *V. cholerae* growth in the aquatic system (Thomas *et al*., 2006).On average, seawater in the world's oceans has a salinity of about 3.5%. *V. cholerae* can be present in both free living state or attached to copepods, zooplankter and algae (Worden *et al*., 2006), phytoplankton, and to the carapaces of larger crustaceans such as shrimp and crab (Castro-Rosas and Escartín, 2002). Some researchers have demonstrated that abiotic surfaces in the marine habitat can provide microhabitats favorable for survival of *V. cholerae*, protecting it against stressful environmental conditions (Watnick and Kolter, 1999).

The slight differences the percentage of positive samples between wet market in same coast area (West coast sample A=60%, B=64%, C=84% and D=88%), (East coast sample A=72%, B=72%, C=60% and D=72%) it could be due by the duration of cockles keeping before selling in market. They are cultured in coastal waters, which are normally not depurated after harvest and are kept and sold at local markets. In addition numbers of *Vibrio* spp., including *V. cholerae*, may increase during storage of shellfish (Kolvin and Robert, 1982).Relatively high levels of *Vibrio* spp. were observed ranging from 3.05±0.35 to 5.07±0.16 log cfu/g for West coast samples and 2.03±0.59 to 4.54±0.01 log cfu/g, suggesting that raw cockles are involved in the transmission of this pathogen. Several species of *Vibrio* can be detected from cockles such as *V. parahemolyticus*, (Zulkifli *et al*., 2009) *V. vulnificus* (Son *et al*., 1998), *V. alganoliticus* (Bilung *et al*., 2008), *V. mimicus* and *V. fischeris* (Liew *et al*., 1998), or in shrimp and the environment (Sujeewa *et al*., 2009). Based on previous research by Elhadi *et al*. (2004), the overall percent incidence of *Vibrio* spp. was highest (82%) in cockles (*Anadara granosa*) among the seafoods examined.

A combination of Most Probable number-Polymerase Chain Reaction (MPN-PCR) method was applied to detect the presence of *V. cholerae* and to enumerate their density in the food samples. Out of 80 samples from 4 wet markets around Selangor in a period of 2 month from August to September 2009, *V. cholerae* was found in the 95% of raw cockles. Overall, the higher prevalence of *V. cholerae* in raw cockles has previously been associated with infection of cholera. This is supported by the study of Donatella *et al.* (2009), indicating that a higher prevalence of *V. cholerae* in shellfish includes prawns (16.6%) and mussels (7.6%). In addition, an outbreak of cholera in LA,USA in 1978 was associated with shellfish caught in the Gulf of Mexico (Blake *et al*., 1977).The frequency of *V. cholerae* in raw cockles obtained from wet market I and L was higher (100%) compared to other wet markets (Wet market J=95%; Wet market K=90%). The density of *V. cholerae* detected in all samples from wet market I and L was ranged from 110 up to >24000 MPN/g, <30 up to >24000 for wet market J and K. Most of the samples (24) were in category >24000 MPN/g concentration. It is well recognized that raw shellfish frequently carry pathogenic *Vibrio* spp., including *V. cholerae* and are frequently involved in the transmission of these bacteria (Klontz *et al*., 1993). Primer that detected the 16s RNA genes in *Vibrio cholerae* was chosen. Figure 1 is representative of the gel image for polymerase chain reaction results for *V. cholerae* (162bp) (Narjol *et al*., 2005). The higher frequency of *Vibrio cholerae* in cockles suggests that it is

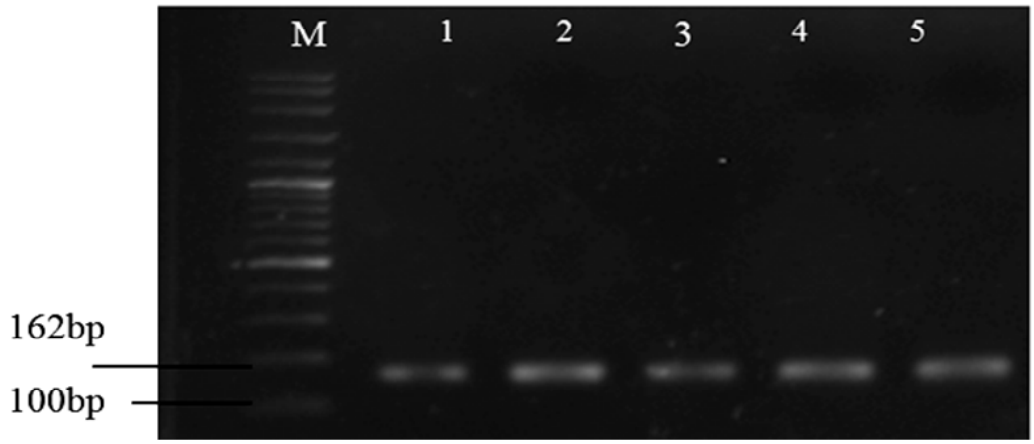

Figure 1. Gel image for PCR detection of *Vibrio cholerae*. Lane M shows the molecular marker 100bp ladder; Lane 1 is the positive control of *Vibrio cholerae*; Lane 2-Lane 5 are PCR amplicons (162bp) specific for *V. cholerae*

Table 1(a). The prevalence of *Vibrio cholerae* in raw cockles from West coast samples

Wet market	[a]n	*Vibrio* spp. (log cfu/g)		[d] PCR positive	%
		[b]min	[c]max		
A	25	3.09±0.31	4.14±0.34	15	60
B	25	3.98±0.90	4.92±0.09	16	64
C	25	3.37±0.44	5.07±0.16	21	84
D	25	3.05±0.35	4.13±0.31	22	88
Total	100			74	74

Table 1(b). The prevalence of *Vibrio cholerae* in raw cockles from East coast samples

Wet market	[a]n	*Vibrio* spp. (log cfu/g)		[d] PCR positive	%
		[b]min	[c]max		
E	25	2.03±0.59	4.54±0.01	18	72
F	25	3.92±0.06	4.18±0.05	18	72
G	25	2.14±0.43	4.52±0.02	15	60
H	25	3.76±0.49	4.49±0.01	18	72
Total	100			69	69

[a]n = Number of sample
[b]min = Minimum MPN/g value
[c]max = Maximum MPN/g value
[d]PCR positive = Number of sample that positive *Vibrio cholerae*

Table 2. Frequency of occurrence of *Vibrio cholerae* in raw cockles sample using MPN- PCR method

Wet market	[a]n	PCR positive	%
A	20	20	100
B	20	18	90
C	20	19	95
D	20	20	100
Total	60	57	95

[a]n = Number of sample

Table 3. *Vibrio cholerae* density (MPN/g) in raw cockles using MPN-PCR method

Wet market	[a]min	[b]med	c max
A	110	480	>24000
B	<30	440	>24000
C	<30	395	>24000
D	110	380	>24000

[a]min = Minimum MPN/g value
[b]med = Median MPN/g value
[c]max = Maximum MPN/g value

necessary a proper procedure of cooking before its consumption.

The combined of MPN-PCR method used in proved that is effectiveness for the detection of *V. cholerae* using specific primers as long as its density in the sample compared with identification by conventional plating and biochemical test. With MPN-PCR, isolation and enumeration of density can carried out directly. Identification by plating on TCBS agar and confirmed by PCR, only give the isolation results. Enumeration of *V. cholerae* by counting on the TCBS plate was not accurate because *V. cholerae* cannot differentiate straightly. Besides *V. cholerae* there is other *Vibrio* sp. have the same morphology on TCBS agar include *V. alganolyticus* and *V. vulnificus*. Detection of *V. cholerae* is very essential when there is an outbreak where the source of contamination has to be determined in short period. However, PCR method also have some disadvantage include false positive results maybe obtained by the contamination and false negative results because of enrichment medium (Nierop *et al*., 2005).

Therefore, cockles (*Anadara granosa*) provided a suitable background for cholera outbreaks. Accordingly, proper cooking, storing and re-heating of foods before eating are considered to be the main safety measures to prevent food-borne transmission of cholera. It was recommended to reconsider this mode of transmission for cholera again as source of cholera epidemics. Besides that, MPN-PCR constitutes the most useful method for the detection of the pathogen in foods compared to conventional methods, including plating and biochemical test.

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