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Short Communication

Detection, Isolation and Antibiotic Testing of *Vibrio cholerae* in seafood

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ABSTRACT

Vibrio cholerae is important water borne human pathogen that associated with cholera disease. The goal of this study was to determine the prevalence and antimicrobial resistance of *V. cholerae* found in seafood. Samples of prawn, cockles and squid samples bought from Kuala Terengganu and Besut markets. The detection of *V. cholerae* in the samples was done using Polymerase Chain Reaction (PCR) assay and plating method. *V. cholerae* isolates were subjected to six antibiotics susceptibility test using Kirby Bauer method. In Besut district, *V. cholerae* was found in cockles (33%), squid (38%) and prawn (44%) in supermarket. For wet market, *V. cholerae* only found in squid (18%) and no detection from prawn samples. For samples collected from Kuala Terengganu supermarket, the prevalence of *V. cholera* in cockle was at 29%, in squid at 56% and prawn at 75%. For samples collected from wet market, *V. cholerae* only present in squid at 18% and no detection in prawn samples. A total of 30 *V. cholerae* isolates were subjected to antibiotic testing. The isolates were completely resistance towards penicillin (100%), vancomycin (80%), tetracyline (23%), gentamycin (7%), norfloxacin (3%) and ciprofloxacin (3%). This study concluded prawn, cockle and squid samples from both district were contaminated with *V. cholerae*. Thus, it might pose risks to consumer if undercooked contaminated seafood were consumed.

Keywords: *Vibrio cholerae*, seafood, Polymerase Chain Reaction (PCR), antibiotic resistance

ABSTRAK

Vibrio cholerae merupakan pathogen bawaan air yang dikaitkan dengan wabak penyakit taun. Kajian ini bertujuan untuk mengetahui prevalen dan rintangan antimikrob *V. cholerae* yang dijumpai di dalam makanan laut. Kaedah Reaksi Polimerasi Berantai (RPB) telah diaplilkasikan ke atas sampel udang, kerang dan sotong yang dibeli dari pasar di Kuala Terengganu dan Besut. Isolat *V. cholerae* diuji kerintangannya terhadap antibiotik dengan menggunakan kaedah Kirby Bauer. Di daerah Besut, *V. cholerae* yang diperolehi di dalam kerang (33%), sotong (38%) dan udang (44%) di didapati di pasar raya. Sampel dari pasar basah, *V. cholerae* hanya ditemui di dalam sampel sotong (18%) dan tidak dijumpai di dalam sampel udang. Bagi sampel yang diperolehi dari pasaraya di daerah Kuala Terengganu, prevalen *V. cholerae* di dalam kerang adalah 29%, dalam sotong adalah 56% dan dalam udang adalah 75%. Bagi sampel yang diperolehi dari pasar basah, *V. cholerae* hanya ditemui di dalam sampel sotong sebanyak 18% sementara sampel udang tidak dikesan kehadiran *V. cholerae*. Kerintangan antibiotic telah dilakukan ke atas 30 *V. cholerae* isolate. Isolat-isolat tersebut didapati rintang sepenuhnya terhadap penicillin (100%), vancomycin (80%), tetracycline (23%), gentamycin (7%), norfloxacin (3%) dan ciproflaxacin (3%). Kajian ini merumuskan sampel udang, kerang dan sotong dari kedua-dua daerah adalah tercemar dengan *V. cholerae*. Oleh itu, ia akan mendatangkan risiko kepada pengguna sekiranya termakan makanan hasil laut tercemar yang tidak masak sepenuhnya.

Katakunci: *Vibrio cholerae,* makanan laut, Reaksi Polimerasi Berantai (RPB), kerintangan antibiotic

INTRODUCTION

Vibrio cholerae is the leading cause for *Vibrio*- associated illness. It is the etiological agent for cholera in which the transmission occurs in various ways, such as contaminated food, raw source of seafood or direct fecal contact with food handler (Faruque et al., 1998). *V. cholerae* is the primary agents of bacterium- associated illness due to the seafood consumption. (Baker-Austin et al., 2010; WHO, 2001). *V. cholerae* is divided into two major groups: the cholera-causing strains of serogroups O1 and O139, and the other *V. cholerae* serogroup non-O1 and non-O139. *V. cholerae* serogroup non-O1 and non-O139 are normal component of the bacterial flora of estuarine and coastal waters and it has shown that they can cause diarrhoea in sporadic cases due to contaminated seafood (Robert-Pillot et al., 2014).

Seafood is one of major source of protein for human health. However, seafood also known as one of the sources to bacterial contamination in which foodborne pathogens may be present at low levels when fish or shell fish are harvested, and others may be introduced during handling and processing or by unsanitary practices. Risk is further increased if the food is mishandled during processing where pathogens could multiply exponentially under favourable conditions (Adam & Moss, 2000; Badrie et al., 2006; Espiñeira et al., 2010).

Many studies have been carried out on seafood and findings concerning the transmission or distribution of pathogenic *Vibrio* in oysters and mussels are well documented (Roque et al., 2009; Terzi et al., 2009; Collin & Rehnstam-Holm, 2011). However, a very few data are available for detection of *V. cholerae* in the fish samples especially in the East Coast Malaysia.

Possible contamination toward the fresh seafood as *V. cholerae* lives in estuarine and marine environment which are the habitat for aquatic animals. There was a case reported on the ice contaminated with *V. cholerae* was used to preserve the fish (Teh et al., 2012). Consumption of seafood product in Terengganu are higher, thus it may pose risks of food poisoning or diseases implicated by *V. cholerae*. Data of prevalence of *V. cholerae* in the seafood especially raw fresh fish for East Coast of Malaysia is very limited. Thus, the goal of the present study was to detect, isolate and characterize *V. cholerae* in seafood sold in Terengganu.

MATERIALS AND METHODS

Food sample collection

A total of 91 samples consist of raw squids, prawns and cockles were purchased from retail outlets in Terengganu. Each sample was labelled with an identification number to differentiate their place of origin. All samples were transported to the laboratory in coolbox (4± 2°C) and examined within the same day.

Enrichment of *Vibrio cholerae* in seafood

For samples enrichment, a portion of sample weight 10g was added into a sterile stomacher containing Alkaline Peptone Water (APW) (Merck, Denmark). The mixture was then homogenized using the stomacher (Seward, UK) for 1 min at 250 rpm. The homogenized samples were incubated in the incubator (Memmert, Germany) for 22± 2 h at 37°C.

Isolation of *Vibrio cholera*

After overnight incubation of enrichment sample, isolation was then via streak plate onto Thiosulphate citrate bile sucrose (TCBS) agar (Merck, Denmark) and incubated at 37°C for 24 h. The sucrose-fermenting yellow colony was picked for further confirmation using Polymerase Chain Reaction (PCR) assay.

DNA extraction method

The DNA extraction was performed using boiled cell method as described in our previous study (Tang et al., 2014).

PCR amplication

PCR amplication was run in the thermal cycler (Applied Biosystem, Thermal Cycler, Singapore) using GoTaq reagents (Promega, USA). PCR reaction mixture consists of the following composition: 1X GoTaq Flexi Buffer, 2 mM of Mg_2Cl, 0.2 µM of each primers, 2 U of Taq polymerase and 2 µL of DNA template. PCR protocol were performed as follows: initial denaturation at 95°C at 5 minutes; 35 cycles of denaturation at 95 °C (45 s), annealing at 55°C (45 s), extensions at 72°C (45 s); and final extension at 72°C for 5 minutes. Three pairs of primers used to detect the *ctx*B, *tcp*I, and *hly*A genes with the following sequence: *ctx*B-F: ATG AGG CGT TTT ATT CCA TAC A, *ctx*B-R: TAC CAG GTA GTC AAC ATA TAG ATT CA; *tcp*I-F: TGC GTG ATG CTA ATT GGA CT, *tcp*I-R: TTC GGT TTG TTT GCT TGA TG; and *hly*A-F: GGC AAA CAG CGA AAC AAA TAC C, *hly*A-R: CTC AGC GGG CTA ATA CGG TTT A.

Agarose gel electrophoresis

The amplified PCR product will be detected using agarose gel electrophoresis. Total of 5 µL of amplified PCR products were ran through electrophoresis using 1.0% agarose gel electrophoresis and the gel will be stained using GelRed DNA Stain (Biotium, USA). A 100 bp DNA ladder (Promega, USA) was used as molecular size marker. The electrophoresis was ran for 60 mins at 90 V. The PCR product on the agarose gel was visualised using UV transilluminator (Fujitsu, Japan).

Antimicrobial susceptibility testing

The antimicrobial susceptibilities of *V. cholerae* were determined by Mueller- Hinton agar by the disk diffusion method (Bauer et al., 1966). The isolates from glycerol stock were revived in Alkaline Peptone Water (APW). The *V. cholerae* suspension was prepared to 0.5 McFarland standard before being inoculated unto Mueller-Hinton Agar (MHA) to form uniform lawn of bacterial growth. The antibiotics used in the study include penicillin (10µg), vancomycin (5µg), tetracycline (30µg), gentamycin (120µg), ciprofloxacin (5µg) and norfloxacin (50µg). The inoculated MHA agar was incubated about 24 hours before the inhibition zone that appear on the antimicrobial susceptibility disk was observed and measured. The diameter of inhibition zones was compared to the standard interpretation chart by Clinical and Laboratory Standard Institute (CLSI) to categorize the isolates media as susceptible or resistance (CLSI, 2005).

RESULTS AND DISCUSSION

Prevalence of *Vibrio cholerae* in samples.

The microbial status of seafood is closely related to the environmental conditions and the microbiological quality of the water (Feldhusen, 2000). As microbiological contamination especially *Vibrio* spp. may cause the various human diseases and became main concern to public health. *Vibrio cholerae* is the etiological agent of cholera which is spread by contaminated food, water or direct fecal contact with food handlers (Suzita et al., 2010). Table 1 summarized the prevalence of *V. cholerae* in squid, cockle and prawn samples obtained in this study.

The present study found the presence of *V. cholerae* in squids from Besut supermarkets at 38% (8/21) and Kuala Terengganu supermarkets at 56% (5/9). Meanwhile, for prawns from Besut and Kuala Terengganu, the percentage of *V. cholerae* was found at 44 % (4/9) and 75% (5/7), respectively. The cockles from Besut and Kuala Terengganu supermarket detected *V. cholerae* at 33% (4/12) and 29% (2/7), respectively. For the wet markets in Besut district, it showed 18% (2/11) of the squids were contaminated and not the prawns. For wet market in Kuala Terengganu, *V. cholerae* was not found in the seafood sampled.

The *tcp*I gene and *ctx*B gene were absent in all isolated *V. cholerae* isolates in seafood samples indicating they were all non-choleragenic. The distribution of virulence gene though seafood sample lack the *ctx*B and *tcp*I gene, they do possess genetic attributes crucial for the organism to cause gastroenteritis of less severe type.

The presence of *V. cholerae* in the raw seafood samples in this study indicates that concern of foodborne illness if these seafoods are consumed in the raw or undercooked state. This high prevalence of *V. cholerae* in seafood samples (Table 1) is of concern because it can cause human illness. Noorlis et al. (2011) reported the high incidence may reflect that the nature of *Vibrio* spp. including *V. cholerae* which is known as a halophilic waterborne bacterium that commonly inhabits environmental water sources worldwide. Previous studies revealed that *V. cholerae* is a very diverse species and is an opportunistic pathogen in aquatic environments that is highly successful in adapting to changing environmental conditions (Song et al., 2013; Thompson et al., 2003).

Table 1 Detection of *Vibrio cholerae* in seafood from Kuala Terengganu and Besut using PCR and plating methods.

Location	N	PCR			Plating		
		*ctx*B(%)	*tcp*I(%)	*hly*A(%)	*ctx*B(%)	*tcp*I(%)	*hly*A(%)
Besut							
SB1	12	2 (17)	2 (17)	4 (33)	0 (0)	0 (0)	4 (33)
SB2	21	0 (0)	0 (0)	11 (52)	0 (0)	0 (0)	8 (38)
SB3	9	0 (0)	0 (0)	9 (100)	0 (0)	0 (0)	4 (44)
WB1	11	0 (0)	0 (0)	6 (55)	0 (0)	0 (0)	2 (18)
WB2	6	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Kuala Terengganu							
ST1	7	0 (0)	0 (0)	7 (100)	0 (0)	0 (0)	5 (75)
ST2	7	0 (0)	0 (0)	7 (100)	0 (0)	0 (0)	2 (29)
ST3	9	0 (0)	0 (0)	4 (44)	0 (0)	0 (0)	5 (56)
WT1	5	0 (0)	0 (0)	5 (100)	0 (0)	0 (0)	0 (0)
WT2	4	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	0 (0)
TOTAL	**91**	**2 (2)**	**2 (2)**	**57 (63)**	**0 (0)**	**0 (0)**	**30 (53)**

*N: Number of sample; SB1: supermarket Besut (1), SB2: supermarket Besut (2), SB3: supermarket Besut (3), WB1: wet market Besut (1), WB2: wet market Besut (2), ST1: supermarket Kuala Terengganu (2), ST2: supermarket Kuala Terengganu (2), ST3: supermarket Kuala Terengganu (3), WT1= wet market Kuala Terengganu (1), WT2= wet market Kuala Terengganu (2)

Temperature and salinity of the environment have been reported as significant parameter influencing the *V. cholerae* distribution (Jiang & Fu, 2001). It is not surprising that the pathogenic bacteria were detected in the seafood harvested in the tropical country like Malaysia because it has been recognized that these organisms are a part of the natural bacterial flora of aquatic environments in areas with warm climates (Hervio-Heath et al., 2002). *V. cholerae* can be easily isolated from estuarine environments during the warm summer months, even in non-epidemic areas. Thus, this factor may contribute to the positive detection of *V. cholerae* in the raw seafood that had been examined.

Previous studies had shown that raw shellfish is well recognized medium that carry pathogenic bacteria including *V. cholerae* which can cause infection to human (Klontz et al., 1993). In other studies also reported that

shellfish is one of the causes for its transmission *V. cholerae* is an autochothous, which is frequently related with phyto- and zooplankton. Thus, specific prevention should be taken to the food handler and food manufacturer to prevent transmission of this pathogen in vehicles of cholera disease such as shellfish (Weber et al., 1994). According to Suzita (2010), in her previous study, it was observed that *V. cholerae* was able to multiply in both shell and non-shell cockles. *V. cholerae* can be transferred easily from the infected cockle to the surrounding water and can survive in the storage condition which are chilled and ambient temperature.

The contamination of *V. cholerae* in raw seafood may occur due to the possible cross contamination occur during the handling process in which the location where samples are exposed, the absence of gloves for handling seafood and the use of contaminated ice and containers during transportation also factors that may contribute to the presence of *V. cholerae* in raw seafood. This is well supported by researchers (Yang et al., 2008; Tunung et al., 2010) who have reported that mishandling and poor hygiene is the main cause of cross-contamination of food at supermarkets. The possibility growth of *V. cholerae* mostly depends on the condition that seafood is subjected during these stages. Pathogenic vibrios can multiply and reach high levels in seafood especially particular concern on live bivalve held with no time temperature control after harvest, which increases the risk of food poisoning incident associated with the consumption of raw or not properly cooked bivalves (Su & Liu, 2007).

Antibiotic Testing

The antimicrobial resistance pattern was summarized in Table 2. This study found that *V. cholerae* isolates were resistance toward penicillin (100%), vancomycin (80%), tetracycline (23%), gentamycin (7%), norfloxacin (3%) and ciprofloxacin (3%). High resistance towards penicillin and vancomycin indicated that these antibiotics were not suitable for treatment in cholera infection. Ninety seven percent of the isolates resistance towards less than 3 types of antibiotics. Only 3% of the isolates were found multi antibiotics resistance. This could be due to the samples were from natural sources and not commercial farm. This finding was in agreement with Tang et al. (2014) in which seafood from natural sources showed low multiple antibiotic resistance.

Table 2 Antibiotic resistance profile for *V. cholerae* isolates from squids, prawn and cockles.

No. of samples	Percentage (%)	Resistance to antibiotics
1	3	PVTeCnCipNor
4	13	PVTe
18	60	PV
2	7	PTe
4	17	P

P: penicillin (10μg), V: vancomycin(5μg), Te: tetracycline(30μg), Cn: gentamycin (120μg), Nor: norfloxacin (50μg), Cip: ciprofloxacin(5μg)

CONCLUSION

These finding concluded that *V. cholerae* were found in raw seafood sold at supermarket and wet market in Terengganu. However, the strains were less sever and non-choleragenic. Thus, hygiene and proper handling of the seafood is important to reduce risk of *V. cholerae* contamination.

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