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Incidence and antibiogram of *Vibrio parahaemolyticus* in processed and frozen bivalve mollusks in Kuala Terengganu, Malaysia

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Abstract

This study aimed to investigate the prevalence and antibiogram of *Vibrio parahaemolyticus* in processed bivalve molluscs in Kuala Terengganu. A total of 80 seafood samples, namely mussels (n=20), carpet clams (n=20), cockles (n=20) and scallops (n=20), were subjected to PCR and conventional plating method for the detection of *V. parahaemolyticus*. *V. parahaemolyticus* was found in green mussels (55%), carpet clam (80%), cockles (40%) and scallops (55%). Fifty-five *V. parahaemolyticus* isolates were subjected to 9 types antibiotic sensitivity test using discs diffusion method. All isolates were susceptible to Tetracycline and Gentamycin. Isolates showed high resistance towards Vancomycin (52.73%), Penicillin (45.45%) and Amplicillin (32.73%). Resistance towards Amikacin, Ciprofloxacin and Norfloxacin were found to be 1.82%. It can be concluded that local bivalve molluscs were contaminated with *V. parahaemolyticus* and isolates showed resistance towards certain antibiotics. Therefore, consumption of raw or semi-cooked bivalve molluscs is not advisable.

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Introduction

Bivalve mollusk is a type of shellfish that abundantly found in marine water and they are mainly filter feeding animals. This creature is easily contaminated with *V. parahaemolyticus* especially when the marine water or the surrounding water is contaminated with the pathogen (Bilung *et al*., 2005; Sudha *et al*., 2012). According to Bilung *et al.* (2005), based on the data from Ministry of Health, about 3% of foodborne infection is due to *Vibrio parahaemolyticus* but the exact number is not known as there are many cases that are not reported and limited study is done in Malaysia on *Vibrio parahaemolyticus*. But most of the outbreaks reported were due to consumption of raw or poorly cooked seafood (Bilung *et al.*, 2005; Khan *et al.*, 2007; Vengadesh *et al*., 2012).

V. parahaemolyticus is found ubiquitous in marine environment and has frequently associated with foodborne illness from the consumption of raw, undercooked or contaminated shellfish. It shows possibility in causing gastrointestinal disease which is self limiting and rarely fatal which severity can range from mild to moderate (Ray and Bhunia, 2008).

Bacterial resistance to antibiotics has been an emerging issue threatening the public health due to the wide abuse and misuse of antibiotics without proper prescription (Adeleye *et al.*, 2008). Previous studies have shown that Streptomycin, Rifampicin, Kanamycin, Tetracycline and Polymixin B were active against *Vibrio* spp. but a study by Ottaviani *et al.* (2001) showed that *V. parahaemolyticus* were resistant to Penicillin, Carbenicillin, Ampicillin, Cephalotin, Kanamycin and Rifampicin (Zulkifli *et al*., 2009). Zulkifli *et al.* (2009) also reported the resistance of *V. parahaemolyticus* towards tetracycline.

The objectives of this study were to determine the prevalence and the antibiogram of *V. parahaemolyticus* in processed and frozen bivalve mollusks sold in supermarkets located in Kuala Terengganu. The findings will serve as useful data in future risk assessment for *V. parahaemolyticus*.

Materials and Methods

Sample collection

The study included the analysis of 80 cleaned, deshelled and frozen bivalve mollusk samples collected from three supermarkets in Kuala Terengganu, Malaysia, which were randomly selected. All the samples were bought on the day of analysis and processed upon arrival to the laboratory.

Enrichment

A 10 g portion of each sample was placed in a

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Table 1. Primer sequences for the detection of *V. parahaemolyticus*

Primer	Sequence	Length	Size	Reference
toxR4	5'-GTCTTCTGACGCAATCGTTG-3'	20 bp	368 bp	Kim *et al.* (1999)
toxR7	5'-ATACGAGTGGTTGCTGTCATG-3'	21 bp		

Table 2. Detection of *Vibrio parahaemolyticus* in bivalve mollusks using PCR and conventional plating method

Samples (Local name)	Scientific name	n	PCR		Plating	
			n	%	n	%
Cockles (*Kerang*)	*Anadara granosa*	20	8	40	3	15
Scallops (*Kekapis*)	*Patinopecten yessoensis*	20	11	55	9	45
Green mussel (*Kupang*)	*Perna viridis*	20	11	55	8	40
Carpet clam (*Lala*)	*Paphia textile*	20	16	80	8	40
	TOTAL	**80**	**46**	**58**	**28**	**35**

n = Number of sample
% = Percentage of positive sample from total sample for each type bivalve mollusks.

stomacher bag added with 90 ml of Alkaline Peptone Water (Merck, Darmstadt, Germany) with 3% sodium chloride (Merck) and pummeled in a stomacher (Interscience, France) for 60 s. The homogenized samples were incubated at 37°C for 24 h. Enriched samples were further identified by PCR and plating onto TCBS agar (Merck, Darmstadt, Germany).

Identification with TCBS agar

From the enriched samples, a loopful of culture was streaked on TCBS agar and was incubate at 37°C for 24 h. Presumptive *V. parahaemolyticus* blue to green colony was selected and purified on new TCBS agar. Five presumptive colonies were subjected to colony PCR targeting *toxR* gene (Kim *et al*., 1999) to confirm for *V. parahaemolyticus.*

PCR detection

DNA extraction from enriched samples was carried out by boiling method as described by Tunung *et al.* (2011). The reference of *V. parahaemolyticus* strains (ATCC 17802) was used as positive control for the PCR reaction. PCR amplification was performed in a 25-µl reaction mixture containing components with final concentration of 1X PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM of deoxynucleoside triphosphate mix, 0.4 µM of each primer (Table 2), 0.5 U/µl Taq polymerase, and 2.0 µl of DNA template. All PCR reagents were from Promega (Madison, U.S.A.) and the primers were synthesized by First Base Laboratories Sdn. Bhd., Malaysia. The following cycling conditions were used: predenaturation at 95°C for 5 mins, 30 cycles of denaturation at 94°C for 60 s, annealing at 62°C for 60 s, and extension at 72°Cfor 60 s, and followed by a final extension at 72°C for 5 mins. PCR products (5 µl) was loaded and electrophoresed in 1.0% agarose gel with 100 V and were stained with GelRed and viewed using the AlphaImager HP Gel Documentation (Alpha Innotech, CA, USA).

Antibiotic susceptibility test

Susceptibility of the 55 isolates to antibiotics was determined by the disc diffusion tests based on the guidelines of Clinical Laboratory Standard Institute (CLSI, 2005). All isolates were grown in TSB (Merck, Germany) with 3% NaCl (Merck, Germany) and were incubated at 37°C for 18 to 24 hours. The cultures were swabbed evenly on Mueller-Hinton (MH) agar plates (Merck, Germany) using sterile non-toxic cotton swab to form a uniform lawn of bacterial growth, which were then left to dry for 3 to 5 minutes before placing antimicrobial sensitivity discs. The culture *E. coli* ATCC 25922 was included as a control test in the susceptibility testing.

Nine types antibiotic (Oxoid, Hamphire, United Kingdom) were selected to include those commonly used in human therapy, as well as those allowed and commonly used in agricultural practices. The antibiotics are Penicillin G (10 U), Vancomycin (5 µg), Tetracycline (30 µg), Gentamycin (120 µg), Amikacin (30 µg), Ciprofloxacin (5 µg), Norfloxacin (10 µg), Erythromycin (15 µg), and Ampicillin (10 µg).

Antibiotic impregnated discs with nine types of antibacterial agents were placed on the inoculated plates and incubated at 37°C overnight. The diameter of inhibition zone was measured and compared with zone diameter interpretative chart from Clinical Laboratory Standard Institute (CLSI, 2005) to classify the isolates as susceptible, intermediate or resistant.

Multiple antibiotic resistance (MAR)

Multiple antibiotic resistance (MAR) index of the isolates was determined as a/b, where 'a' represents the number of multiple antibiotics to which the particular isolates are resistant, and 'b' the number of multiple antibiotics to which the particular isolates are exposed (Krumperman, 1983; Gwendellynne, 2005).

Results and Discussion

The prevalence of *V. parahaemolyticus* in cockles and scallops were determined from PCR and plating onto TCBS agar. Table 2 below summarizes the prevalence of *V. parahaemolyticus* in cockles, scallops, green mussels and carpet clam. From the samples analyzed, *V. parahaemolyticus* were detected highest in carpet clam at 80% and lowest in cockles at 40%. Both scallops and green mussels recorded 55% presence of *V. parahaemolyticus.* All plating onto TCBS agar showed lower detection compared to molecular method. The PCR detection of the bivalve mollusk samples are shown in Figure 1.

Contradictory to the most studies that reported high prevalence of *V. parahaemolyticus* in cockles (Bilung *et al.*, 2005), the cockles bought from

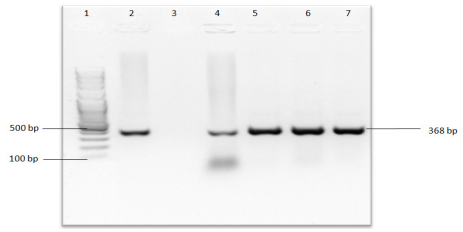

Figure 1. Detection of *toxR* gene of *Vibrio parahaemolyticus* in bivalve mollusk. Lane 1: 100 bp DNA ladder; Lane 2: Positive control (ATCC 17802); Lane 3: negative control; Lane 4 to 7: seafood samples with positive detection of *V. parahaemolyticus*

supermarkets at Kuala Terengganu showed low presence of *V. parahaemolyticus*. According to Sudha *et al.* (2012), different place will have different occurence of *V. parahaemolyticus*. From the study by Zulkifli *et al.* (2009), pathogenicity of the *V. parahaemolyticus* at different geographical location were also different. Many studies show that *V. parahaemolyticus* were highly found in shellfish (Bilung *et al*., 2005; Khan *et al.*, 2007; Vengadesh *et al.*, 2012).

The condition of the sample can greatly affect the growth of *V. parahaemolyticus* as it is sensitive to low temperature and the minimal temperature for growth of *V. parahaemolyticus* is 12.8°C. With only small amount of *V. parahaemolyticus* in shellfish, it can rapidly multiply in only a few hours (Bilung *et al*., 2011; Sudha *et al.*, 2012). The gut of the bivalve shellfish is the place the microorganisms such *Vibrio* multiply and concentrate (FAO/WHO, 2011).

According to Nelapati *et al.* (2012) season also influence the incidence of food poisoning. At temperature below 13 to 15°C, number of *V. parahaemolyticus* is low. But during summer with temperature above 25°C, the number of *V. parahaemolyticus* reported are high (Sakazaki *et al.*, 2006; Nelapati *et al.*, 2012).

Seafood from supermarket usually is more hygiene compare to seafood that is sold at wet market (Vengadesh *et al.*, 2012). But, the way the seafood is handled also can influence the growth of *V. parahaemolyticus*. In this study, frozen samples were displayed and thawed on stainless steel tray with ice flakes covering them. But there are also certain supermarkets that left the samples in cold water from melted ice without replacing with the fresh ice especially in the evening. In addition, the samples were placed on the display next to other types of seafood. These encourage cross contamination to occur and in this case with *V. parahaemolyticus*. From the observation, the same ladle was used to take the sample and other seafood.

From the observation, there are a lot of cross-contamination that can occur due to handling of the supermarket as handling is one of the factor that contribute to cross-contamination. This is supported by other studies (Bilung *et al.*, 2005; Noorlis *et al.*, 2011; Vengadesh *et al*., 2012) that show that mishandling is the main factor that lead to growth of *V. parahaemolyticus* in seafood.

For isolation, two to three presumptive colonies of *V. parahaemolyticus* colony were picked and further confirmed by colony PCR. From the 46 PCR positive, 55 isolates were obtained for both scallops and cockles. Standard zone diameters to classified microorganisms into susceptible, intermediate and resistant for *V. parahaemolyticus* were not available in the Clinical and Laboratory Standard Institute (CLSI) guideline. The zone diameters were interpreted based on zone diameter standard for *Vibrio cholerae* and *Enterobactericiae* (CLSI, 2005; Oralak *et al.*, 2007; Chao *et al*., 2009).

Table 3 summarizes the susceptibility of the isolates towards antimicrobial agents for green mussels, carpet clam, scallops and cockles. No resistance was observed among the isolates towards Tetracycline (30 µg), Gentamycin (120 µg), Amikacin (30 µg), Ciprofloxacin (5 µg), Norfloxacin (10 µg), and Erythromycin (15 µg). The results are comparable with the finding by Khan *et al*. (2007), on antibiotic susceptibility of *V. parahaemolyticus* isolate from shrimps were resistant against vancomycin, penicillin and ampicillin which speculated to be due to antibiotics use in the shrimps farm have increase the resistance of the *V. parahaemolyticus* in shrimps. In the other study by Tunung *et al.* (2012), *V. parahaemolyticus* isolates from vegetable have show different resistant against antimicrobial agents. The isolates were highly resistant towards nalixidic acid.

A low percentage of strains (15%) showed MAR index more than 0.2. MAR indices higher than 0.2 indicated possibility of contamination from high risk sources such as animals and thus posed health risk to human through the food chain (Gwendelynne *et al*., 2005). From the previous study by Khan *et al.* (2007) and Tunung *et al.* (2012), the antimicrobial susceptibility of *V. parahaemolyticus* were varied depends on source of sample obtained. According to Lesley *et al.* (2011), samples that are come from different location will have different resistance towards antibiotic. Sample that was collected from the place where antibiotics were frequently used may contain more resistant strain of *V. parahaemolyticus* due to mutation that have modify the target site or transport mechanism and have cause the antibiotics become inactive on cell (Zulkifli *et al*., 2009). The presence of large plasmids and the ability for the

Table 3. Antibiotic susceptibility testing for isolates from green mussels, carpet clam, scallops and cockles

	Green Mussels		Carpet Clam		Scallops		Cockles	
Multiple Antibiotic Resistance (MAR)	**N**	**n (%)**	**N**	**n (%)**	**N**	**n (%)**	**N**	**n (%)**
MAR Index > 0.2	13	2 (*15*)	12	1 (*8*)	18	1 (*6*)	12	4 (*33*)
MAR Index ≤ 0.2	13	11 (*85*)	12	11 (*92*)	18	17 (*94*)	12	8 (*67*)
Antibiotics	**N**	**R (%)**	**N**	**R (%)**	**N**	**R (%)**	**N**	**R (%)**
Penicillin (P10)	13	13 (*100*)	12	7 (*75*)	18	4 (*22*)	12	12 (*100*)
Vancomycin (VA5)	13	13 (*100*)	12	0 (*0*)	18	17 (*94*)	12	12 (*100*)
Tetracycline (TE30)	13	0 (*0*)	12	0 (*0*)	18	0 (*0*)	12	0 (*0*)
Gentamycin (CN120)	13	0 (*0*)	12	0 (*0*)	18	0 (*0*)	12	0 (*0*)
Amikacin (AK30)	13	0 (*0*)	12	1 (*8*)	18	0 (*0*)	12	0 (*0*)
Ciprofloxacin (CIP5)	13	0 (*0*)	12	1 (*8*)	18	0 (*0*)	12	0 (*0*)
Norfloxacin (NOR10)	13	0 (*0*)	12	1 (*8*)	18	0 (*0*)	12	0 (*0*)
Erythromycin (E15)	13	0 (*0*)	12	0 (*0*)	18	0 (*0*)	12	0 (*0*)
Ampicillin (AMP10)	13	2 (*15*)	12	4 (*33*)	18	5 (*28*)	12	7 (*58*)

N = No. of *V. parahaemolyticus* isolates
n = No. of *V. parahaemolyticus* isolates analyzed for MAR index
R = No. of *V. parahaemolyticus* isolates resistance to the tested antibiotics

plasmids for conjugation process influence the pattern of antibiotic resistance. High molecular weight plasmids can be transconjugated and the presence of the plasmids can produce genes that are resistance to antibiotics and thus increase threat to consumers (Zulkifli *et al*., 2009).

Conclusion

This study showed the presence of *V. parahaemolyticus* might be affected by the processing of the bivalve mollusks such as de-shelling, cleaning, chilling and freeze-thaw cycle. Even though *V. parahaemolyticus* is known to be sensitive to temperature fluctuation, its persistence in shellfish is significant. All isolates also showed resistance towards Penicillin and Vancomycin. Therefore, adequate cooking and proper handling of shellfish are important to reduce foodborne illness.

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