AJAB

Asian J Agric & Biol. 2019; Special Issue: 69-75.

Original Article

Prevalence and antimicrobial susceptibility testing of *Vibrio parahaemolyticus* isolated from peeled blood cockles (*Anadara granosa*) sold in Kuala Terengganu

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Received:
March 10, 2019
Accepted:
November 05, 2019
Published:
December 05, 2019

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Abstract

Blood cockles (*Anadara granosa*) are the natural inhabitants of the marine environment that were harvested from the seawater and can be contaminated with the *Vibrio* species bacteria as well as *Vibrio parahaemolyticus*. *V. parahaemolyticus* is one of the major seafood-borne disease that leads to human acute gastroenteritis due to consumption of raw or undercooked seafood. Peeled blood cockles from five different supermarkets around Kuala Terengganu were sampled. A total of thirty samples of peeled blood cockles were obtained and the *V. parahaemolyticus* were identified. Eleven out of thirty of the samples acquired were positive with *V. parahaemolyticus* when streaked on selected agar (TCBS and CHROMagar™ *Vibrio*) which then confirmed by biochemical test (API 20E strips test) and specific-PCR method (*Vp-toxR* genes). Eleven *V. parahaemolyticus* isolates were tested for susceptibility to various antibiotics using the disk diffusion method according to guidelines set by the National Committee for Clinical Laboratory Standard. Eight types of antibiotics tested which selected randomly from the main group such as Aminoglycosides, β- lactams, Cephalosporins, Glycopeptides, Microlides, Quinolones and Tetracyclines. Antibiotics tested were Streptomycin (25µg), Penicillin (10µg), Cefuroxime (30µg), Teicoplanin (30µg), Erythromycin (10µg), Ciprofloxacin (5µg), Tetracyclines (30µg) and Chloramphenicol (50µg). All the isolates (100%) were found to be resistance to penicillin, chloramphenicol and teicoplanin. However, no isolates (0%) were resistance to streptomycin, erythromycin, ciprofloxacin, tetracyclines and cefuroxime. The results of this study provided useful information in the search for safe and efficient antibiotics in treating food poisoning patient due to *V. parahaemolyticus*.

Keywords: Peeled blood cockles, *Anadara granosa*, Antibiotic susceptibility, *Vibrio parahaemolyticus*, Food poisoning

How to cite this:

Chilek TZT, Yusoff NASM, Ahmad F, Zamri AI, Ismail N and Razak SBA, 2019. Prevalence and antimicrobial susceptibility testing of *Vibrio parahaemolyticus* isolated from peeled blood cockles (*Anadara granosa*) sold in Kuala Terengganu. Asian J. Agric. Biol. Special Issue: 69-75.

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Introduction

Pathogenic bacteria are bacteria that can cause food poisoning or can cause disease to humans. It occurs when we eat foods that have been contaminated by the bacteria or inadequate cooking process to destroy the existing pathogenic bacteria in the food (Ghenem and Elhadi, 2018). Blood cockle have a high ability of poisoning threats to consumer's safety and health because of their eating habits by filtering, indirectly collecting more pathogenic bacteria and contaminants (Hossen et al., 2014). Noorlis et al. (2011) stated that pathogenic bacteria that can pose a threat of food poisoning to human are *Vibrio parahemolyticus*, *Vibrio cholerae*, *Clostridium botulinum*, *Salmonella* spp., *Esherichia coli* and *Shigella* spp. Therefore, blood cockle harvested in areas that exposed to the pollution is capable of providing food poisoning, especially when eating raw or undercooked blood cockle or seafood product (Ghenem and Elhadi, 2018). Since the blood cockle is a filter feeder organism, then the more pathogenic bacteria can be collected. Therefore, the presence of bacteria in the blood cockle is higher than the surrounding water content.

V. parahaemolyticus belongs to the family Vibrionaceae. It is a halophilic bacterium that can grow between 10 °C and 44 °C (optimum 35-37 °C), pH ranges from 5 to 11 and with 3-8% NaCl tolerance (Odeyemi and Stratev, 2016). Its habitat is in the seawater, where the presence is more on summer than other seasons (Chumpol, 2017). *V. parahaemolyticus* usually found in raw seafood especially oysters and blood cockles. *V. parahaemolyticus* was firstly revealed as seafood-borne pathogen in 1951 in Japan (Harakudo et al., 2013). Since then, many foodborne outbreaks due to consumption of contaminated raw or undercooked seafood have been reported.

The bacteria are small in size with 0.5-0.8 µm in width and 1.4-2.6 µm in length (Nurulhuda, 2013). The species are motile, the polar flagella enclosed in a sheath continuous with the outer membrane of the cell wall. Heenatigala and Fernando (2016) stated that *V. parahaemolyticus* is oxidase positive and fermentative without producing gas. *V. parahaemolyticus* fermented glucose, arabinose, maltose, mannitol, and mannose with the production of acids except sucrose, lactose and salicin, and it is able to reduce nitrate to nitrite while requiring sodium ions for growth.

Vibrio parahaemolyticus is one of the most significant bacterial pathogens throughout the world related with seafood. *V. parahaemolyticus* is a halophilic, gram-negative, rod-shaped bacterium with a single polar flagellum that naturally found in coastal marine waters and seafood throughout the world (Vincent et al., 2015). It is one of the 12 known pathogenic species of the genus Vibrio that can cause gastrointestinal illness with diarrhoea, abdominal cramp, nausea and vomiting to human through consumption of raw or undercooked seafood (Hu and Chen, 2016). *V. parahaemolyticus* is present in higher concentrations during summer when the salinity is higher in the aquatic environment (Vincent et al., 2015).

The consumption of raw or undercooked seafood contaminated with *V. parahaemolyticus* can cause acute gastroenteritis and wound infections on occasions (Semenza et al., 2017). It is the leading cause of human gastroenteritis associated with seafood consumption (Nelapati et al., 2012). According to Odeyemi and Stratev (2016), *V. parahaemolyticus* infection was showed by abdominal pain, vomiting, and watery or bloody diarrhoea with an incubation period of 4 to 96 hours. Nowadays, due to rising outbreaks of seafood-borne pathogens, there have been concerns about the microbiological safety of fish and seafood.

The common medium used to isolate *Vibrio* spp. is thiosulfate-citrate-bile-salts-sucrose (TCBS) agar. *V. parahaemolyticus* produce smooth green colonies on TCBS agar when the bacteria are grown onto the medium due to sucrose negative (Nurulhuda, 2013). Letchumanan et al. (2014) mentioned that *V. parahaemolyticus* is acid sensitive and grow best at pH 7.5 to 8.5. Hence, salt tolerance can be used according to their salt requirement for the identification of *Vibrio* spp.

The major cause of losses in the aquaculture industry is vibriosis. It is one of the most persistently diseases that affect fishes, molluscs and crustaceans. Wong et al. (2015) found that the most commonly used antibiotics to treat vibriosis were quinolones, followed by cephalosporins, tetracyclines and penicillin, thus the antibiotics can be used to treat the infections. However, in this study a wide spectrum of antibiotics were used to observe the susceptibility of the *V. parahaemolyticus* strain towards the antibiotics. The *V. parahaemolyticus* is highly resistance to streptomycin, oxytetracycline, chloramphenicol and teicoplanin. According to WHO (2004), a multidose treatment of tetracycline can be administered; in the case of young children, liquid erythromycin is preferred to treat the infections alternatively (WHO, 2004).

V. parahaemolyticus causes watery diarrhoea often with abdominal cramping, nausea, vomiting and fever that will occur within 24 hours of ingestion through eating raw or undercooked seafood (Hu and Chen, 2016; Zulkifli et al., 2009). Patients should drink plenty of liquids to replace the fluids lost during diarrhoea but in severe or prolonged illnesses, antibiotics such as tetracycline, ampicillin or ciprofloxacin was used to treat gastroenteritis (Wong et al., 2015).

Antibiotics can reduce the severity of symptoms by decreasing the volume of diarrhoea, and the amount of fluids required to maintain the hydration (Kitaoka et al., 2011). Nowadays, chloramphenicol-resistant strains have emerged in many pathogens, hence the antibiotic of choice cannot be chloramphenicol. Additionally, many strains have developed resistance to antibiotics, which is considered appropriate alternatives to chloramphenicol, such as ampicillin and trimethoprim/sulfamethoxazole (Tuan Zainazor, 2006). Though there are many benefits to individuals who were treated with antibiotics, the World Health Organization (WHO) does not recommend their general use because antibiotics contribute to increasing antimicrobial resistance (Kitaoka et al., 2011).

Material and Methods

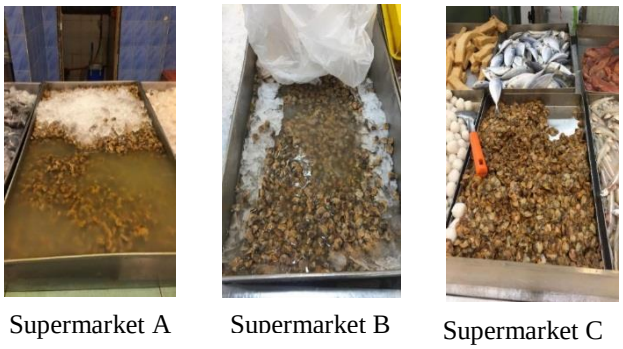
Sample collection

The peeled blood cockles were purchased at selected markets in Kuala Terengganu. The samples were collected from five different supermarkets. The duration of sampling is about two months. The samples were taken twice a week from July 2018 until September 2018. A total of thirty samples of peeled blood cockles (*Anadara granosa*) from each supermarket were obtained.

Sterile suitable sampling apparatus was used to collect the samples. The samples were placed and chilled it in cool box containing with small ice cubes to maintain the transportation temperature between 0 to 4 ºC. Then, the samples were subjected for the enumeration of *V. parahaemolyticus* in the laboratory.

Identification of *V. parahaemolyticus*

Enriched samples were streaked on both thiosulfate-citrate-bile salt-sucrose agar (TCBS) and CHROMagar™ *Vibrio* and incubated at 37℃ for 18-24 hours. A minimum three to five typical colonies of *V. parahaemolyticus* were purified on nutrient agar (NA) supplemented with 3% NaCl and incubated at 37℃ overnight. Colonies were biochemically confirmed as *V. parahaemolyticus* using gram stains and oxidative tests. Phenotypic characterisation of *V. parahaemolyticus* was confirmed by API 20E strips test.



Supermarket A Supermarket B Supermarket C

Supermarket D Supermarket E

Figure-1: Sample from sources of contamination of peeled blood cockle from five different supermarkets.

Antibiotics susceptibility

Vibrio parahaemolyticus were tested for susceptibility to various antibiotics using disk diffusion method. The antimicrobial susceptibility test was performed essentially by the disc diffusion method, with disc containing antibiotics. Eight types of antibiotics tested which selected randomly from the main group such as Aminoglycosides, β- lactams, Cephalosporins, Glycopeptides, Microlides, Quinolones and Tetracyclines. Antibiotics tested are Ampicillin (10 µg), Cefuroxime (30 µg), Ciprofloxacin (5 µg), Chloramphenicol (50 µg), Erythromycin (10µg), Penicillin (10 µg), Streptomycin (25 µg), Teicoplanin (30 µg), and Tetracyclines (30 µg). Eight groups of antibiotics used in this study were summarized in Table 3.4.

Briefly, organisms were grown at 37℃ in Mueller

Hinton Broth. 10µl of overnight culture were used to inoculate a fresh 3ml Mueller Hinton broth followed by incubation at 37℃ with shaking until a 0.5 McFarland turbidity standard was obtained. A sterile swab was dipped into this culture and used to inoculate the surface of a fresh Mueller Hinton Agar (MHA) plates and allowed to dry for 2 to 5 minutes. The antibiotics disc were spaced out onto the plates and were incubated at 37℃ for 24hours. The diameter of the clear zone for each antibiotics discs were interpreted as susceptible or resistance categories. The diameter of the clear zone for each antibiotic disc was interpreted as susceptible or resistant categories according to the guideline recommended by the Clinical and Laboratory Standard Institute (2016).

Results and Discussion

The distribution of *V. parahaemolyticus* in the peeled blood cockles from five different supermarkets shown in Table 1.

Table-1: Distribution of *Vibrio parahemolyticus* in the peeled blood cockles

Supermarket	Sample (n)	*Vibrio parahaemolyticus*
A	6	4
B	6	1
C	6	3
D	6	1
E	6	1

Ten out of thirty samples tested were contained *V. parahaemolyticus*. The results of antibiotic susceptibility testing of *V. parahaemolyticus* isolates obtained from peeled blood cockles (*Anadara granosa*) in Kuala Terengganu were shown in Table 2. Out of eleven isolates tested, no isolates were resistant to streptomycin (0%) under the aminoglycosides group. In this study, penicillin (beta-lactams group) was observed that 100% of the isolates resistance to this antibiotic. All isolates were also found to be resistant to chloramphenicol and teicoplanin. However, no isolates (0%) was found to be resistant to erythromycin and cefuroxime. The antibiotics were also tested for quinolones namely ciprofloxacin. The results showed that no of them (0%) was resistant to ciprofloxacin. All eleven isolates were found to be susceptible to tetracyclines (Table 2). The distribution of antimicrobial resistance of *Vibrio parahaemolyticus* for eleven isolates obtained from peel blood cockles shown in Table 3.

Table-2: Antibiotic susceptibility testing

Colony	P 10	C 50	S 25	E 10	CIP 5	TEC 30	CRM 30	TE 30
Positive Control	S	R	S	S	S	R	S	S
1	S	R	S	S	S	R	S	S
2	S	R	S	S	S	R	S	S
3	S	R	S	S	S	R	S	S
4	S	R	S	S	S	R	S	S
5	S	R	S	S	S	R	S	S
6	S	R	S	S	S	R	S	S
7	S	R	S	S	S	R	S	S
8	S	R	S	S	S	R	S	S
9	S	R	S	S	S	R	S	S
10	S	R	S	S	S	R	S	S
11	S	R	S	S	S	R	S	S

*S = sensitive, R= resistance

Table-3: Distribution of antimicrobial resistance of *Vibrio parahaemolyticus* for 11 isolates obtained from peeled blood cockles in Kuala Terengganu

Antibiotics	No (%) of *V. parahaemolyticus* resistant to selected antibiotics
Aminoglycosides Streptomycin (S25)	0 (0)
β-lactams Penicillin (P10)	11 (100)
Cephalosporins Cefuroxime (CXM30)	0 (0)
Glycopeptides Teicoplanin (TEC30)	11 (100)
Macrolides Erythromycin (E10)	0 (0)
Quinolones Ciprofloxacin (CIP5)	0 (0)
Tetracyclines Tetracycline (TE30)	0 (0)
Others Chloramphenicol (C50)	11 (100)

V. parahaemolyticus is the causative agent of gastroenteritis that related to the consumption of contaminated seafood (Ghenem and Elhadi, 2018). Center for Disease Control and Prevention (2006) mentioned that most of the cases of *V. parahaemolyticus* infection treatment are not necessary, thus to avoid dehydration caused by diarrhoea, the patient must drink plenty of fluid. In a few cases, antibiotics, such as tetracycline and

ciprofloxacin, can be used based on the antimicrobial susceptibilities of organisms (Center for Disease Control and Prevention, 2006) (Saifeddin et al., 2016). In this study, all *V. parahaemolyticus* isolates were resistant to two out of eight antibiotics tested. However, most of the isolates were sensitive towards penicillin, streptomycin, erythromycin, ciprofloxacin, cefuroxime and tetracycline (Table 2). Only two isolates were resistant to chloramphenicol and teicoplanin (Table 2). Meaning that, *V. parahaemolyticus* isolates were susceptible to the aminoglycosides, cephalosporins, macrolides, quinolones and tetracycline groups of antibiotics. The results obtained in this study confirm other results reported elsewhere (Wong et al., 2015), the most commonly used antibiotics to treat vibriosis were quinolones, followed by cephalosporins, tetracyclines and penicillin, which *V. parahaemolyticus* were isolated from food and environment having similar susceptibility pattern.

Table 3 showed that the eleven isolates of *V. parahaemolyticus* were 100% resistant to teicoplanin and chloramphenicol. However, the isolates were susceptible to the aminoglycosides, cephalosporins, macrolides, quinolones and tetracycline groups of antibiotic. These results agreed with the a few researchers found that *V. parahaemolyticus* is susceptible to quinolone and tetracycline groups of antibiotics (Wong et al., 2015; Yu et al., 2016) which recommended as treatment for *Vibrio* infections.

The mechanism of antimicrobial resistance was complicated, subsequently many researchers have made claim that we have to reduce and better use of antimicrobial to control the infection (Saifeddin et al., 2016). However, Saifeddin et al. (2016) also mentioned the bacteria might be already inherited the resistance by transferable genetic elements so that the reduction use of antimicrobial cannot be a solution. Consideration all factors that caused increasing of bacterial resistance must be taken up, whereas according to Liu et al. (2013), *V. parahaemolyticus* strains that carried a novel plasmid with multidrug resistance genes, which can speed up the emergence of multidrug resistance in *Vibrio* because of these genes were most likely rendered transferable by genetic elements in *Vibrio* spp.

The penicillin family is one of the most significant groups of antibiotics in primary care. They are bactericidal, well distributed and highly efficacious against susceptible organisms (Tuan Zainazor, 2006). A synthetic penicillin, which is an outstanding agent in terms of safety and efficacy, has been developed aiming for broadened the spectrum of activity and enhanced the efficacy of these medications. However, the usefulness of penicillin in recent years has been limited due to emergence of resistant bacterial strains (Saga and Yamaguchi, 2009).

Although antimicrobial resistance could be a significant problem for therapy directed against the organisms, the role of antibiotics in the management of human infections cause by *Vibrio* spp. has not been stated yet. Appropriate antimicrobial therapy is needed for more effective management of severe infections caused by *V. vulnificus*, *V. parahaemolyticus*, and *V. alginolyticus* (Zulkifli et al., 2009). The results obtained were proposed that *Vibrio* spp. strains should be observed carefully to detect those with antibiotic resistance potential. The results of this study provide useful information in the search for safe and efficient antibiotics. In addition, it is also give some insight into the problems faced by food

Conclusion

The result of antibiotic resistance of *V. parahemolyticus* showed out of eleven isolates tested, no isolates (0%) were resistance to streptomycin, erythromycin, ciprofloxacin, tetracyclines and cefuroxime. Penicillin was observed that 100% of the isolates resistance to this antibiotics. All the isolates (100%) were also found to be resistance to chloramphenicol and teicoplanin. Meaning that they were susceptible to aminoglycosides, cephalosphorins, macrolides, quinolones and tetracycline groups of antibiotics.

Acknowledgement

This research was financially supported by Talent and Publication Enhancement-Research Grant (TAPE-RG) 1/2018, Grant No. 55176. The authors sincerely thank the Food Microbiology Laboratory, Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu for their technical support.

Contribution of Authors

Chilek TZT: Conducted the study and prepared the manuscript draft

Yusoff NASM: Data collection and manuscript writing

Ahmad F: Data interpretation
Zamri AI: Data interpretation
Ismail N: Data interpretation
Razak SBA: Manuscript final reading and approval

Disclaimer: None.
Conflict of Interest: None.
Source of Funding: Talent and Publication Enhancement-Research Grant (TAPE-RG) 1/2018, Grant No. 55176.

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