Occurrence of Pathogenic Bacteria in Blood Cockles, *Anadara granosa*

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Abstract: *Anadara granosa* (blood cockles), also known as *kerang* is a very popular seafood in Malaysia and South East Asia. In 2015, there has been a drastic reduction in the harvest and one of the main reasons is due to the deteriorating water quality in the cockles' breeding environment. Thus, these cockles are exposed and at high risk of being contaminated by pathogenic microorganism because they are filter-feeder organism. Most of the researches on *Anadara granosa* have focused on only few selected organisms. The overall microbiological assessment of the cockles is lacking therefore this study aimed to determine the types of pathogenic bacteria in blood cockles, *Anadara granosa* and their antibiotic susceptibility pattern. Thirty pooled sample of *Anadara granosa* were purchased from 15 wet markets and supermarkets within Klang Valley. All samples were subjected to isolation and identification using standard conventional method. A total of 85 isolates were successfully isolated and all were gram negative bacteria. Antibiotic susceptibility test was performed for the different types of bacteria obtained. All isolates were found to be resistant to *Ampicilin* (10 µg) and were sensitive to Trimethoprim/sulfamethoxazole (25µg). In conclusion, this study showed that cockles are exposed to highly pathogenic bacteria and there is presence of antibiotic resistance.

1 INTRODUCTION

Anadara granosa is shellfish members of the Class Bivalvia, mollusks that enclosed between two shells which are closed together and joined together with elastic hinge ligament (Sauders Veterinary Dictionary, 2nd Edition. Malaysia produced 100,000 tons of cockles for both local consumption and export. The cockles are harvested from the coastlines especially in Selangor, Perak and Johor. They usually distributed at tidal mudflat area with low oxygen content due to presence of hemoglobin that have ability to retain high oxygen content. Cockles are exposed and at high risk of contaminated by multiple organism, for instance bacterial, viral and toxin-producing dinoflagellates because they are filter-feeder organism such as phytoplankton, zooplankton, bacteria, viruses and inorganic materials (Burkhardt & Calci 2000; Rippey, 1994). Consumption of cockles that is harvested from contaminated area can cause illness to human.

According to previous studies, it reveals that aquatic environment is a reservoir for antibiotic resistance due to frequent usage of antimicrobial and antibiotic contamination (Samuel *et al.* 2016; Huang *et al*. 2001). Thus, presence of pathogenic bacteria together with multiple antibiotic resistances found in aquaculture product will become a threat to public health. This study was conducted to determine the types of pathogenic bacteria in blood cockles and determine the antibiotic resistance of selected isolated bacteria.

2 MATERIALS AND METHODS

2.1 Sample and Data Collection

Thirty samples of *Anadara granosa* were purchased from 15 wet markets and supermarkets. The shells were rinsed by 70% alcohol and the meat was removed aseptically. Three grams of the sample was homogenized with 30ml of peptone water by stomacher for 2minutes.

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2.2 Methods

2.2.1 Isolation of Vibrio sp

The shell of *Anadara granosa* was cleaned using 70% alcohol. The meat was removed from the shells and into 3g portions. The samples were then homogenized with 30ml of alkaline peptone water (Oxoid CM1028B) using stomacher for 2 minutes. The homogenized sample was then incubated at 30° C for 24 hours under aerobic condition. After the enrichment process, a lapful of the enriched sample was streaked onto TCBS (CONDA). The plates were incubated at 30°C for 24 hours under aerobic condition.

2.2.2 Isolation of Bacteria Other than Vibrio sp

The shell of *Anadara granosa* was cleaned using 70% alcohol. The meat was removed from the shells and into 3g portions. The samples were then homogenized with 30ml of peptone water (Oxoid CM009) using stomacher for 2 minutes. After homogenization, a lapful of each homogenized sample was streaked onto Blood agar (Oxoid CM0055) and MacConkey Agar (Oxoid CM1169). The plates were incubated at 30°C for 24 hours under aerobic condition.

2.2.3 Identification of Vibrio sp

Presumptive Vibrio sp exhibiting green and yellow colonies on TCBS agar were selected and Gram stained. These colonies were sub-cultured into Triptych Soy (TSA) agar (BD #221283) in order to obtain pure culture. The cultures were subjected to series of biochemical test including oxidase test, ability to growth at different NaCl concentration, *Voges–Proskauer* test (VP), Lysine *Decarboxylase* (LDC) and *Ortho-Nitrophenyl-β-Galactoside* (ONPG) or species identification.

2.2.4 Identification of Bacteria Other than Vibrio sp

Each different type of colony was picked and sub cultured into Blood agar in order to obtain pure colonies for identification. Gram staining (Appendix A) was performed and series of biochemical tests for gram negative and gram positive were carried out. Biochemical tests include blood broth, 6.5%NaCl, *bile, lactose, sorbitol and trehalose* for Gram positive bacteria. Gram negative bacteria were subjected to triple sugar iron (TSI), *Sulphide-Indole-Motility* (SIM), urea and citrate 2.1 Isolation and identification.

2.2.5 Antibiotic Sensitivity Test

Susceptibility of the obtained bacteria to selected antibiotics was tested on Mueller Hinton agar (MHA) plates by the disc diffusion method according to Bauer et al. (1966). One colony from pure culture was emulsified in sterile saline solution until the turbidity was match with standard 0.5 MacFarland solutions. A sterile swab was dipped into the bacterial suspension and then streaked over the entire surface of Mueller-Hinton agar and Blood agar. Six antibiotic discs Oxoid, were aseptically placed on the swabbed plates. The antibiotics discs used include *ampicillin* (10µg), *erythromycin* (15µg), *tetracycline* (30µg), *enrofloxacin* (5µg), *gentamicin* (10µg), *trimethoprim* and *sulfamethoxazole* (25µg), Anti biotic disc used in this study were antibiotics that commonly used in aquaculture as well as in human medicine. The plates were incubated at 30°C for 24 h and the clear zone formed around the discs was measured by using caliper. The growth inhibition zone was compared with zone-size interpretative table as in (CLSI, 2010).

3 RESULTS

The overall isolated bacteria in 30 different wet markets and supermarkets around Klang Valley revealed that a total of 85 isolates were successfully isolated and representing 13 different types of bacteria species (Figure 1). The study showed that *Aeromonas* spp (23%) was the most frequently isolated bacteria from cockles followed by *Proteus mirabilis* (20%), *Vibrio alginolyticus* (15%), *Vibrio parahaemolyticus* (6%), *Photobacterium damsel* (6%), *Vibrio cholera* (5%), *Chromobacterium* sp. (5%), *Proteus vulgaris* (2%), *Salmonella* spp (2%), *Klebsiella pneumoniae* (2%), *Plesiomonas shigelloides* (2%) and *E.coli* (1%).

Antibiotic susceptibility of the isolates was performed using 6 antibiotics ranging from broad-spectrum antibiotics and narrow spectrum. All of the isolates showed resistance to at least one antibiotic. Three isolates were multidrug resistance as they are resistant to more than three types of antibiotics from different classes. The bacteria isolates showed the highest percentages of resistance towards *ampicillin* (68%), followed by *erythromycin* (37%), *tetracycline* (21%), *enrofloxacin* (16%), *gentamicin* (11%) and trimethoprim/ sulfamethoxazole (11%). Isolates showed most

resistant towards ampicillin and most were sensitive to trimethoprim/ sulfamethoxazole (Figure 2).

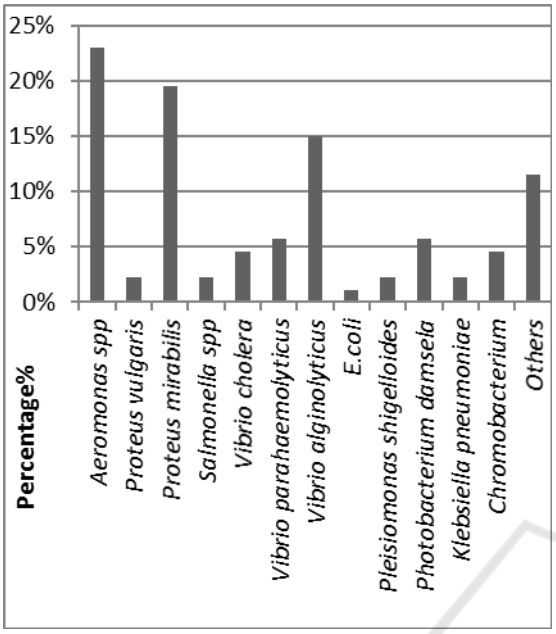

Figure 1: Types of bacteria isolated from blood cockles.

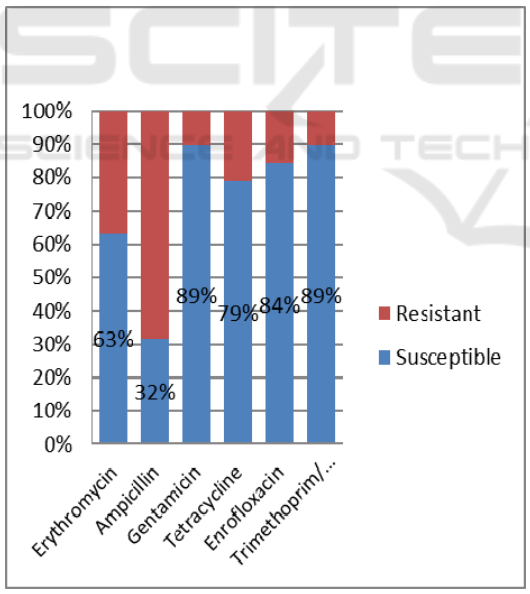

Figure 2: Percentage of resistant bacteria against different antibiotics.

4 DISCUSSION

All isolated bacteria consisted of gram negative bacteria. This concurs with the results obtained by Santos *et al.* (2010), which stated that majority of bacteria within marine environment are gram negative bacteria. To date, very limited studies have been carried out on the microbiology assessment and antibiotic resistance in bivalves in Malaysia. One of those studies revealed that 93% of the isolated bacteria were gram negative bacteria (Ahmad, 2014).

Bacteria obtained from this study can be categorized into 2 family groups which are *Vibrionacea* and *Enterobacteriae*. These two groups can be easily differentiated by oxidase test for which the *Vibrionaceae* will give positive result. These two groups of family are mostly pathogens that usually cause gastroenteritis in human. *Aeromonas* is the most abundant isolated bacteria in this study. Gastrointestinal infections caused by Aeromonads are mostly self-limiting, and antibiotic therapy is required only in chronic cases of immunosuppressed hosts (Igbinosa, 2012). The least isolated bacteria were from family of Enterobacteriaceae which are *E.coli* (1%), *Salmonella* spp (2%), *Klebsiella pneumonia* (2%), *Plesiomonas shigelloides* (2%), *Proteus vulgaris* (2%) and *Proteus mirabilis* (20%). Increase in bacterial contamination at beaches along many coastlines usually occurred during heavy rainfall or rainy season (Gregory, 2009). In this study, sampling was done on the dry season in the West Coast of Peninsular Malaysia, therefore it is predicted more contaminants might be seen if it is the rainy season.

According to Letchumanan (2014), Malaysia is one of the Asian countries that often suffer food borne outbreaks mainly caused by *Vibrio* sp. Other countries include Japan, India, China, Taiwan and Korea. Three members of the Vibrio genus were isolated in this study were *Vibrio alginolyticus, Vibrio cholera* and *Vibrio parahaemolyticus.* This finding is in agreement with the studies of Ahmad (2014) and Thompson (2014) which stated that *Vibrio* sp is the most dominant genus present in cockles. *Vibrio* sp have unique ability whereby they are hardy organism as they able to withstand harsh environment. They can be found in a wide range of environment; from estuaries, coastal, marine water and even sediment. In a previous study conducted by Wan and Nor (2004) on bacterial quality of some shellfish revealed that *Vibrio* spp. are commonly isolated from cockles compared to other shellfish.

Some limitation on isolation and identification of bacterial pathogens by using conventional methods is lack of sensitivity as stated in Law et.al, 2014 and this may lead to false negative e results. In addition, marine organisms might occur in a state which is viable but non-cultural. In this study approximately

11% of bacteria cultures were not able to be identified, and this may be attributed to the limitations of the conventional identification system employed.

Multidrug resistance is defined as bacteria that are resistant to 3 or more antimicrobial classes. In this study, 3 isolates namely *Aeromonas* sp., Vibrio *parahaemolyticus* and *Klebsiella pneumoniae* were found to be multidrug resistant (MDR). Based on one study done by Ghaderpour et al. (2015), emergence of resistance of bacteria is associated with anthropogenic pollution in Matang estruary, Kuala Sepetang. This estuary is one of the major cockles producing area. This estuary was contaminated with untreated silages that contain organic materials, household chemicals and pathogens therefore contaminated the cockles breeding environment. The presence of these MDR is alarming as infections caused by these resistant organisms are difficult to treat.

5 CONCLUSIONS

This study suggests that *Anadara granosa* have poor microbiological quality and harbor various pathogenic bacteria. *Trimetoprim/ sulfamethoxazole* and *gentamicin* are most effective in eliminating bacteria in cockles. A number of the pathogenic bacteria obtained namely *Aeromonas sp., Vibrio parahaemolyticus* and *Klebsiella pneumoniae* exhibited multidrug resistant trait.

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