UNIVERSITI PUTRA MALAYSIA

OCCURRENCE OF PATHOGENIC BACTERIA IN BLOOD COCKLES, *Anadara granosa*

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "**Occurrence of Pathogenic Bacteria in Blood Cockles, *Anadara granosa***", by Nurin Syakirin Jantan and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Project

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DEDICATIONS

Specially dedicated to my parents, family, friends and all seafood lovers….

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Bismillahhirrahmannirahim.

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada kursus VPP4999-Project

KEWUJUDAN BAKTERIA PATOGEN DALAM KERANG,

Anadara granosa

oleh

Nurin Syakirin Jantan

Penyelia : Prof. Madya Dr. Zunita Zakaria

Penyelia bersama : Prof. Dr. Saleha Abdul Aziz

Anadara Granosa juga dikenali sebagai kerang adalah makanan laut yang sangat popular di Malaysia. Pada tahun 2015, terdapat pengurangan drastik dalam musim menuai dan salah satu daripada sebab utama adalah disebabkan oleh kemerosotan kualiti air di persekitaran pembiakan kerang. Kerang adalah terdedah dan berisiko tinggi dicemari oleh mikroorganisma patogen kerana mereka adalah organisma penapis makanan. Kebanyakan kajian mengenai *Anadara Granosa* telah memberi tumpuan kepada hanya beberapa organisma terpilih. Penilaian mikrobiologi keseluruhan kerang yang amat kurang. Oleh itu kajian ini bertujuan untuk

menentukan jenis bakteria patogen yang terdapat di dalam kerang, *Anadara granosa* dan corak kerentanan bakteria terhadap antibiotik . Tiga puluh sampel dikumpulkan daripada *Anadara granosa* yang diperolehi daripada 15 pasar basah dan pasar raya di sekitar Lembah Klang. Semua sampel diproses untuk pengasingan dan pengenalan menggunakan kaedah konvensional standard. Ujian kerentanan antibiotik telah dilaksanakan ke atas pelbagai jenis bakteria diperolehi. Sejumlah 85 jenis bakteria telah berjaya diasingkan dan semua merupakan bakteria Gram negatif. *Aeromonas* spp (23%), *Proteus vulgaris* (20%) dan *Vibrio alginolyticus* (15%) merupakan genus paling dominan yang dikenal pasti. Patogen yang penting kepada manusia juga telah dikenalpasti termasuk *Vibrio parahaemolyticus* (6%), *Vibrio cholerae* (5%) dan *Salmonella* spp. (2%). Semua bakteria didapati rintang kepada antibiotik Ampicilin (10 µg) dan sensitif kepada Trimethoprim / sulfamethoxazole (25µg). Didapati *Aeromonas* spp, *Klebsiella pneumonia* dan *Vibrio parahaemolyticus* tergolong dalam kumpulan bakteria rintang pelbagai antibiotik. Kesimpulannya, kajian ini menunjukkan bahawa kerang yang sangat tercemar dengan bakteria patogen.

Kata kunci - kerang, ujian kepekaan antibiotik, rintangan pelbagai antibiotik

ABSTRACT

An abstract of the project paper presented to Faculty of Veterinary Medicine in partial fulfilment of course VPP4999-Project

OCCURRENCE OF PATHOGENIC BACTERIA IN BLOOD COCKLES, *Anadara granosa*

By

Nurin Syakirin Jantan

Supervisor : Assoc. Prof. Dr. Zunita Zakaria

Co-Supervisor : Profesor Dr. Saleha Abdul Aziz

Anadara granosa (blood cockles), also known as kerang is a very popular seafood in Malaysia. 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. Antibiotic susceptibility test was performed for the different types of bacteria obtained. A total of 85 isolates were successfully isolated and all were gram negative bacteria. *Aeromonas* spp (23%), *Proteus vulgaris* (20%) and *Vibrio alginolyticus* (15%) were the most dominant genus identified. Important human pathogens were also identified which include Vibrio *parahaemolyticus* (6%), *Vibrio cholerae* (5%) and *Salmonella* spp. (2%). All isolates were found to be resistant to Ampicilin (10 µg) and were sensitive to Trimethoprim/sulfamethoxazole (25µg). Among the tested isolates, *Aeromonas* spp, *Klebsiella pneumonia* and *Vibrio parahaemolyticus* were found to be multi-drug resistant. In conclusion, this study suggests that cockles are highly contaminated with pathogenic bacteria and some of the bacteria are multidrug resistant.

Keywords - cockles, antibiotic susceptibility test, multidrug resistance

1.0 INTRODUCTION

Anadara granosa (blood cockles) also known as *kerang* is a very popular seafood item in Malaysia. Malaysia produced 100,000 tonnes of cockles for both local consumption and export. The cockles are harvested from the coastlines especially in Selangor, Perak and Johor. 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 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).

Previous researches have revealed 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. In addition, cockles can also be used as good bio-indicator for detecting level of toxicity such as heavy metal contamination in aquatic environment (Hassan & Kanakaraju, 2013). Consumption of cockles that is harvested from contaminated area may cause illness to human.

Despite being popular local seafood in Malaysia; less information and only few studies on the microbiology of the cockles that has been carried out on this shellfish.

Hence, this study was undertaken to fulfill the following objectives:

1) to determine the types of bacteria in blood cockles, *Anadara granosa*

2) to determine the antibiotic resistance of selected isolated bacteria.

It is hypothesized that high numbers of pathogens are present in the blood cockles, *Anadara granosa* and the pathogens are resistant to multiple antimicrobials.

2.0 LITERATURE REVIEW

2.1 Biology of Anadara granosa

Anadara granosa, commonly known as cockles, is a Bivalvia, subclass of Pteriomorphia, order of Arcida, super family of Arcoidea and family of Arcidae (Linnaeus, 1758). Bivalves are filter feeders using perforated gills through two opening call as siphons (Figure 1). They burrow on the seafloor with these openings protruding slightly above the mud or sand surface. They mainly feed by filtering small particles such as phytoplankton, zooplankton, bacteria and inorganic materials (Burkhardt & Calci, 2000).

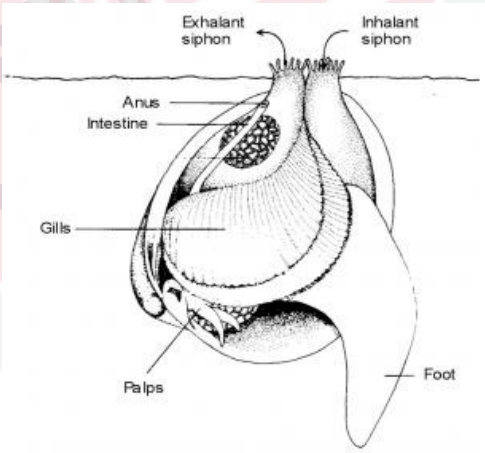

Figure 1. Anatomy of cockles

2.2 Bacterial contamination in cockles

Vibrio spp are ubiquitous and abundant in marine environment in both temperate and tropical regions and the commonest pathogenic bacteria present in cockles (Ahmad et al., 2007). Based on a previous study by Radu et al. (2002), *Vibrio* spp and *Aeromonas* spp are the general pathogenic genus that are mainly isolated from shellfish. Santos and coworkers reported that majority of the bacteria isolated from marine environment are gram negative bacteria. In addition, shellfish are also prone to being contaminated by enteric bacteria due to fecal contamination, mainly *Salmonella* spp, *Shigella* spp as well as *E.coli* (Robert et al, 1990: Radu et.al, 2000).

2.3 Occurrences of antibiotic resistance in aquaculture

Worldwide, antimicrobial agents have been widely used either for therapeutic, prophylactic or other purposes in aquaculture (Shariff *et.al*, 2000; Smith, 2008). Improper usage and overuse of antibiotics will lead to emergence of resistance in the target bacteria, which may results in the transfer of the pathogen to human via food chain (Akinbowale *et al.,* 2006). Antibiotic resistance can spread via direct contact with water, through drinking water and consumption of aquaculture product while indirect by horizontal gene transfer transmission, process of swapping genetic material between bacteria (Heuer, 2009). Mutations in bacterial cells are able to change its original forms and functions which can develop to antibiotic resistance of the bacteria cell (Safayeet, 2008). Nowadays, some of antibiotics used in aquaculture are also being used in human medicine. This will definitely reduce the

antibiotic's efficacy and limits the choice of antibiotics for disease treatments in humans (Kathleen et.al, 2016). Emergence and widespread of multiple antibiotic resistance can be predicted by close monitoring of the monitor antibiotic resistant patterns consistently.

2.4 Significance to public health

Aquaculture is an important sector and currently growing rapidly to meet the world demands for protein source (Kathleen et.al, 2016). However, this sector is challenged with diverse type of diseases especially the shellfish. For instance, cockles are benthic filter feeder, thus they are more prone to contamination by bacteria, virus, parasite and even heavy metal (Yap et.al, 2009; Rippey, 1994). According to Potasman (2002), these microorganisms that can be transmitted to human may cause gastroenteritis and the prognosis of shellfish-associated infection is typically good. Most of the diseases caused are hepatitis A, vibriosis, salmonellosis and giardiasis (Iwamoto, Ayers, Mahon, & Swerdlow, 2010).

3.0 MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

A total of 30 pooled cockles were purchased from 3 vendors from 10 markets in different locations in Klang Valley a period of 2 weeks starting from 12th January until 22nd of January 2016.The samples were placed in an ice box and transported to Veterinary Bacteriology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia within 6 hours for further processing.

3.2 ISOLATION OF BACTERIA

3.2.1 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 loopful of the enriched sample was streaked onto TCBS (CONDA). The plates were incubated at 30°C for 24 hours under aerobic condition.

3.2.2 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 loopful 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.

3.3 IDENTIFICATION OF BACTERIA

3.3.1 Identification of *Vibrio* sp.

Presumptive *Vibrio* sp exhibiting green and yellow colonies on TCBS agar were selected and Gram stained. These colonies were subcultured into Tryptic 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 (Appendix B).

3.3.1 Identification of bacteria other than *Vibrio* sp.

Each different type of colony were picked and subcultured 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 (Appendix B).

3.4 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),Antibiotic 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) (Appendix C).

4.0 RESULTS

4.1 Bacterial Isolation and Identification

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 2). 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 pneumonia* (2%), Plesiomonas *shigelloides* (2%) and *E.coli* (1%).

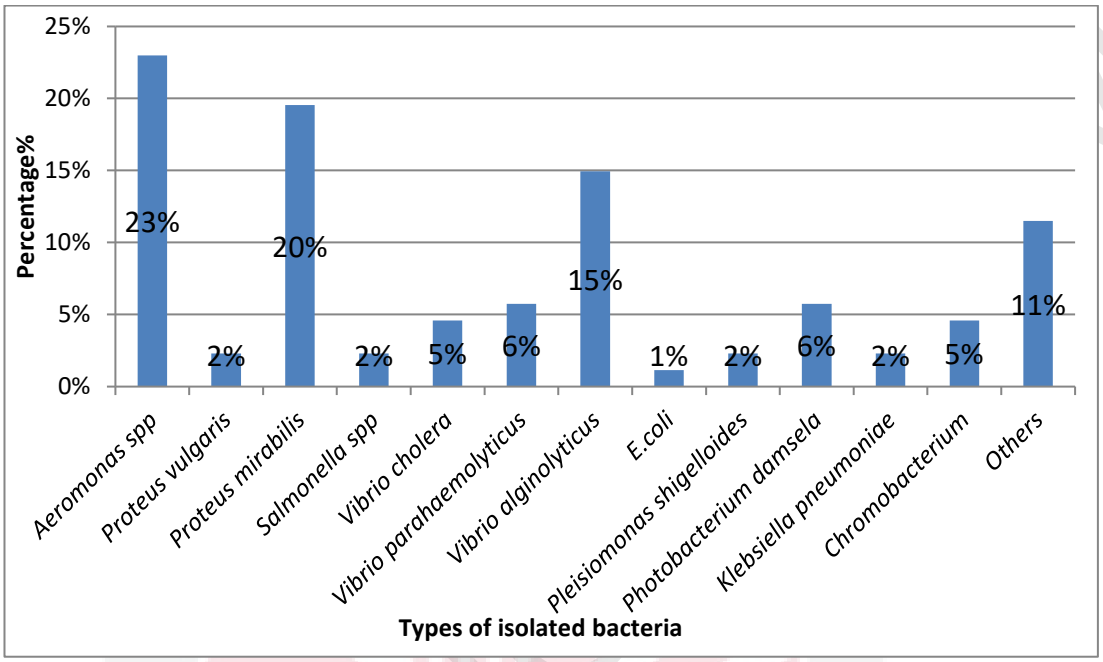

Figure 2 : Types of bacteria isolated from blood cockles

There was no significant association found between most of the isolated bacteria with sample from wet markets or supermarkets ($p > 0.05$) except for *Chromobacterium* sp.

4.2 Antibiotic Susceptibility Profile

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 (Table 1). 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 3).

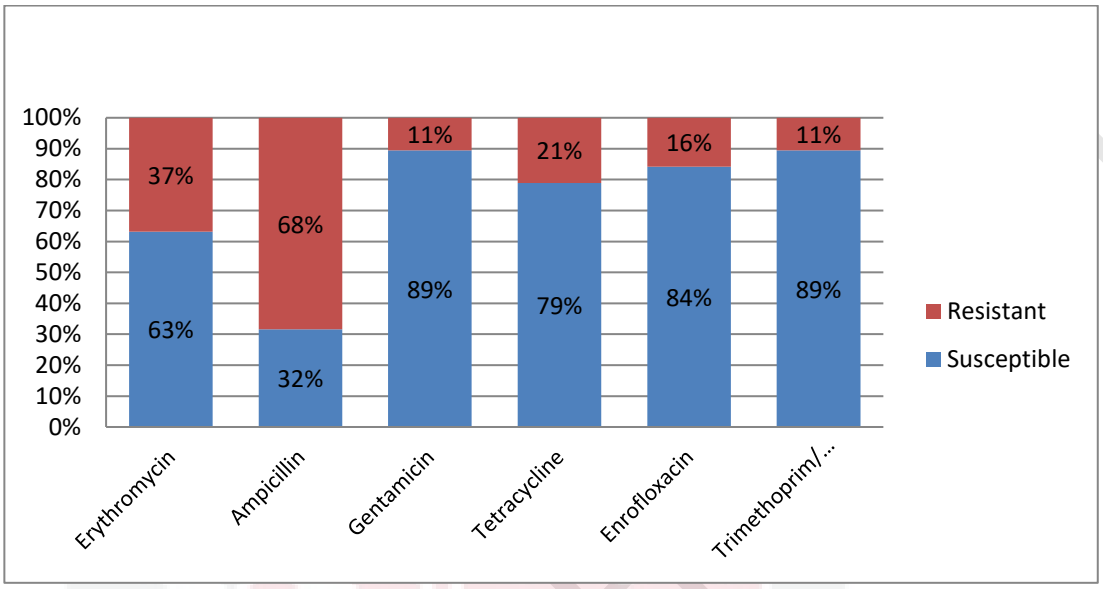

Figure 3: Percentage of resistant bacteria against different antibiotics

Table 1: Resistance profile of bacteria against antibiotics

	Erythromycin	Gentamicin	Tetracycline	Enrofloxacin	Ampicilin	Trimethoprim/ sulfamethoxazole	Antibiogram	MDR
Aeromonas spp	R	R	R	R	R	R	E,A,G,T,EN,SXT	1
Proteus vulgaris	R	S	S	S	R	S	E,EN	0
Proteus mirabilis	S	S	S	S	S	S	-	0
Salmonella spp	S	S	R	S	R	S	T,A	0
Vibrio cholera	R	S	S	S	S	S	E	0
Vibrio parahaemolyticus	R	S	R	R	R	S	E,T,EN,A	1
Vibrio alginolyticus	S	S	S	R	R	R	E,A,SXT	0
Klebsiella pneumonia	R	R	R	R	R	R	E,A,G,T,EN,SXT	1
Chromobacterium	S	S	S	S	R	S	A	0
Photobacterium damsela	R	S	S	S	R	S	E,A	0

5.0 **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 suffers 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 estruaries, 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-culturable. In this study approximately 11% ofn 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 sullages 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.

6.0 **CONCLUSION**

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.

7.0 **RECOMMENDATION**

It is suggested that the research is expanded to include larger population size in order to determine the prevalence of pathogens. Enumeration of the coliform is advisable in the microbiology assessment quantitatively. Molecular detection of the pathogens is also highly recommended as it will enable bacteria to be identified more rapidly and accurately.

As *Anadara granosa* is considered as popular local seafood in Malaysia, the level of public awareness regarding the risks of consuming this kind of seafood must be enhanced. The general public needs to be educated to ensure that cockles are not to be consumed undercooked.

8.0 **APPENDICES**

Appendix A: Bacteria profile obtained from markets within Klang Valley

Pathogens	Market																												
	S1	S2	K1	K2	K3	P1	B1	B2	B3	SH1	SH2	SH3	KL1	A1	A2	T1	T2	E1	BI1	D1	PP1	PP2	PM1	PM2	M1	M2	M3	MY	H1	G1
Aeromonas spp.	+	+	_	+	+	_	+	+	+	+	+	_	+	_	+	+	_	_	_	+	_	+	+	+	+	+	+	_	+	_
Proteus mirabilis	_	_	_	+	_	+	+	+	+	_	_	_	_	+	+	+	_	+	_	+	+	+	_	+	+	+	+	+	+	_
Proteus vulgaris	+	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	+	_	_	_	_	_	_	_	_	_	_	_
E.coli	_	_	_	+	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Vibrio cholera	_	_	+	_	_	_	_	_	_	+	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Salmonella spp.	_	+	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	+	_	_	_	_	_	_	_	_	_	_	_
Photobacterium damsela	_	_	_	_	_	_	_	_	_	_	+	_	_	_	_	_	_	_	_	+	_	+	+	_	_	_	_	_	_	+
Chromobacterium sp	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	+	_	_	_	_	_	_	_	_	_	_	+	_
Plesiomonas shigelloides	_	_	_	_	_	+	_	_	_	_	+	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Klebsiella pneumonia	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	+	+	_	_	_	_	_	_
Vibrio parahaemolyticus	_	_	+	_	+	_	_	_	_	_	_	_	+	+	_	_	_	+	_	_	_	+	_	_	_	_	_	_	_	+
Vibrio alginolyticus	_	_	_	_	+	_	_	_	_	+	+	+	_	_	_	+	+	+	_	_	+	_	+	_	_	_	_	_	_	_
Others	_	_	+	_	_	_	+	+	+	_	_	+	_	_	_	_	_	+	_	+	_	_	_	_	+	_	_	+	_	_

Appendix B: Biochemical Tests for Identification of Bacteria

Triple Sugar Iron

1. Inoculate TSI Agar by stabbing through the centre of the medium to the bottom of the tube and then streaking on the surface of the agar slant.
2. Incubate the tube at 30°C in 24 hours.
3. Examine the colour changes at butt and slant, presence of sulphide and bubbles.

Sulphide-Indole-Motility (SIM)

1. Inoculate with a straight wire, making a single stab down the center of the tube to about half the depth of the medium.
2. Incubate at 30°C for 24 hours.
3. Add Kovac's reagent for indole test.

Urea

1. The medium is inoculated with a loopful of a pure culture of the test organism; the surface of the agar slant is streaked with the test organism.
2. Incubate at 30°C for 24 hours
3. Observe the development of pink colour changes.

Citrate

1. Inoculate simmons citrate agar lightly on the slant .
2. Incubate at 30°C for 24 hours
3. Observe the development of blue color.

APPENDIX C: Zone of inhibition diameter of antibiotic sensitivity test

	Erythromycin	Gentamicin	Tetracycline	Enrofloxacin	Ampicillin	Trimetoprim/ sulfamethoxazole
Klebsiella pneumonia	7.44mm	19.24mm	25.56mm	26.22mm	-	22.6mm
	7.2mm	27.44mm	-	25.5mm	-	-
Aeromonas sp	28.4mm	-	8.08mm	21.5mm	12.74mm	8.54mm
	-	29.74mm	45.0mm	43.02mm	-	37.1mm
Proteus vulgaris	34.0mm	21.14mm	23.34mm	33.56mm	12.74mm	8.54mm
	-	24.78mm	27.74mm	31.68mm	-	37.1mm
Proteus mirabilis	28.46mm	31.34mm	38.8mm	42.12mm	14.86mm	23.94mm
Vibrio parahemolyticus	30.52mm	19.34mm	25.6mm	27.08mm	14.08mm	21.82mm
	12.3mm	21.76mm	9.46mm	29.54mm	-	17.32mm
Vibrio alginolyticus	19.4mm	21.24mm	24.8mm	26.56mm	10.16mm	22.24mm
	21.38mm	20.9mm	23.62mm	19.3mm	13.06mm	20.94mm
Vibrio cholera	16.86mm	25.08mm	32.5mm	30.74mm	26.4mm	21.26mm
	17.32mm	23.86mm	31.08mm	34.2mm	-	22.38mm
Photobacterium damsela	23.54mm	25.84mm	34.9mm	34.76mm	-	26.84mm
	-	-	23.72mm	33.24mm	-	27.82mm
Chromobacterium	25.82mm	25.46mm	27.44mm	31.46mm	40.4mm	21mm
	18.34mm	20.26mm	22.22mm	29.82mm	11.64mm	20.7mm

APPENDIX D

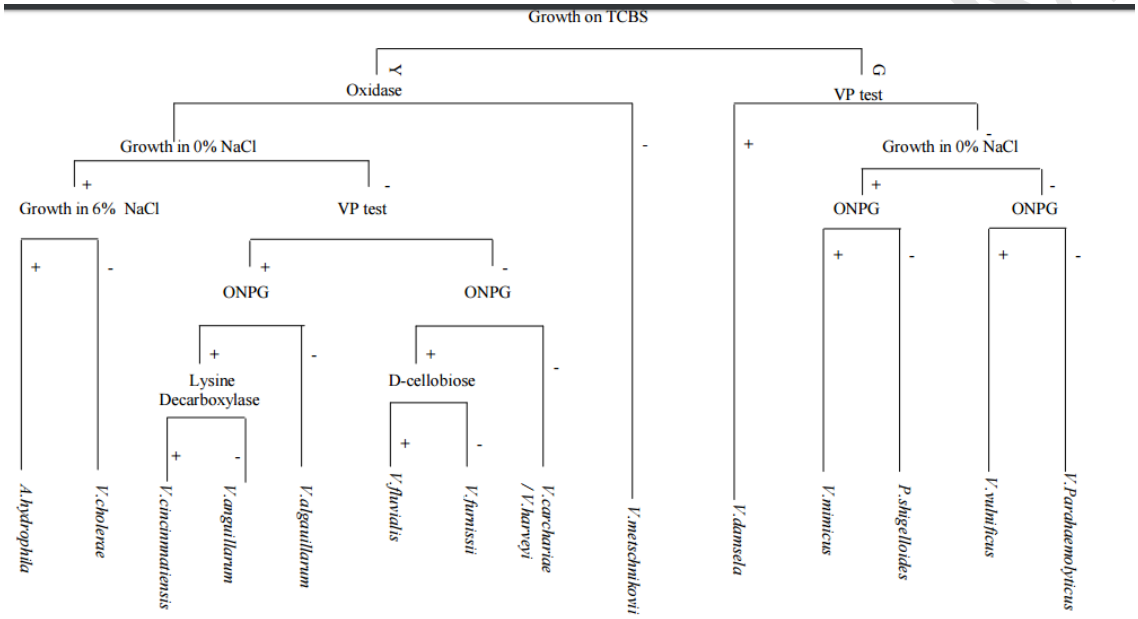

Figure. 1 Biochemical Key for the Identification of *Vibrio* Species

(Jayasinghe, Ahmed, & Kariyawasam, 2005)

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