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Population Density and Antibiotic Resistant of Bacteria from Bivalve (*Perna viridis* and *Anadara granosa*)

(Kepadatan Populasi dan Kerintangan Antibiotik oleh Bakteria daripada Bivalvia (*Perna viridis* dan *Anadara granosa*))

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ABSTRACT

*This study was carried out to know the bacteria population density in the blood cockle (*Anadara granosa*) and green lipped mussel (*Perna viridis*), to analyse the bacteria resistance towards antibiotics and antimicrobial activity of isolates against selected pathogen. Samples of blood cockle and green lipped mussel were obtained from five areas in Kedah and Negeri Sembilan. Bacterial population densities in mussels and cockles were* $3 \times 10^2 - 8 \times 10^8$ *CFU/mL and* $5 \times 10^2 - 5 \times 10^8$ *CFU/mL, respectively. A total of 162 isolates were obtained, of which 131 isolates were from mussels and 31 isolates were from cockles.* Vibrio *sp. was the most dominant genus in both types of samples. Antibiotic testing of all isolates showed most were resistant to Penicillin (10 U) and most were sensitive to Ciprofloxacin (5 µg). Most isolates (160/162) showed resistance to at least two antibiotics and 10 isolates were resistant to more than five antibiotics. Multiple antibiotic resistance indices (MAR) were calculated based on the antibiotic resistance results. Most isolates had a MAR index value of 0.2 which indicated the isolates were not contaminated with antibiotic residues. The highest index value was 0.7. Fifteen out of 39 isolates which produced beta-lactamase enzyme were tested for antimicrobial activity against selected pathogen. Results indicated that antimicrobial activity were varies among the isolates. Isolate SMII-Ip produced antimicrobial activity against six out of the nine tested pathogen and none of the isolates active against* Pseudomonas mirabilis.

Keywords: Anadara granosa; *antibiotic; antimicrobial*; *population density;* Perna viridis

ABSTRAK

*Kajian ini dijalankan bagi mengetahui kepadatan populasi bakteria daripada kerang (*Anadara granosa*) serta kupang (*Perna viridis*), menganalisis kerintangan bakteria terhadap antibiotik serta aktiviti antimikrob oleh pencilan terhadap patogen pilihan. Sampel kerang dan kupang telah diperoleh dari lima kawasan perairan Kedah dan Negeri Sembilan. Kepadatan populasi bakteria pada kupang adalah* $3 \times 10^2 - 8 \times 10^8$ *CFU/mL dan kerang* $5 \times 10^2 - 5 \times 10^8$ *CFU/mL Sebanyak 162 pencilan telah berjaya dipencilkan, dengan 131 pencilan adalah daripada kupang dan 31 daripada kerang.* Vibrio *sp. merupakan genus paling dominan daripada kedua-dua sampel. Ujian kerintangan antibiotik terhadap semua pencilan menunjukkan kebanyakan isolat rintang terhadap Penisilin (10 U) dan sensitif terhadap Ciprofloksasin (5 µg). Hampir semua pencilan (160/162) rintang terhadap sekurangnya-kurangnya dua antibiotik dan 10 pencilan rintang terhadap lebih daripada lima antibiotik. Kiraan indeks Antibiotik Pelbagai Rintang (MAR) berdasarkan hasil ujian kerintangan antibiotik telah dijalankan. Kebanyakan isolat mempunyai nilai indeks MAR 0.2 yang bermakna pencilan tidak terdedah kepada pencemaran antibiotik. Nilai indeks MAR tertinggi adalah 0.7. Sebanyak 15 daripada 37 pencilan yang menghasilkan enzim beta-laktamase telah diuji aktiviti antimikrob terhadap mikrob pathogen terpilih. Hasil menunjukkan aktiviti antimikrob yang berbeza bagi pencilan yang berbeza. Pencilan SMII-Ip menghasilkan aktiviti antimikrob terhadap enam daripada sembilan pathogen yang diuji dan tidak terdapat pencilan yang aktif merencat* Pseudomonas mirabilis.

Kata kunci: Anadara granosa*; antibiotik; antimikrob; kepadatan populasi;* Perna viridis

INTRODUCTION

Bivalves are a class of marine and freshwater mollusks with laterally compressed bodies enclosed by a shell in two hinged parts. They include clams, mussels, oysters, scallops and numerous other families. It has long been a part of the diets of human populations. In Malaysia, mussels and clams are among the frequent shellfish in food and is one of the country's economic resources for the fisheries sector. Mussel farming is ranked second in terms of production of the commodity after shellfish.

Both clams and mussels are organisms that adhere to substrate and do not move. Thus these organisms are often contaminated by a variety of microorganisms because of the way they obtain their nutrients which is by filtering small particles such as phytoplankton, zooplankton, bacteria, viruses and inorganic materials (Burkhardt &

Calci 2000; Defossez & Hawkins 1997; Dunphy et al. 2006; Lees 2000). According to Cavallo (2009), the density is so high that it can be deadly if the bacteria are pathogenic. Thus besides being a source of food, it is used to monitor the presence of enteric bacteria such as *Escherichia coli* which is the detection of pollution in an area and the bacteria of the genus *Vibrio* and *Aeromonas* which both are pathogenic to animals and humans.

Based on previous studies it showed that aquatic environment is a potential reservoir for antibiotic resistant bacteria (Nonaka et al. 2000). Antibiotic contamination is the main cause of the existence of antibiotic resistant bacterial communities (Huang et al. 2001; Kümmerer 2009). The existence of bacterial community that is resistant to multiple antibiotics can have negative impacts on ecosystems, human health (Huang et al. 2001; Kümmerer 2009) and economy (Lynn & Solotorovsky 1981). Therefore, the objective of this study was to compare the bacterial population density of cockles and mussels samples and investigate the antibiotic susceptibility of those isolates.

Materials and Methods

Sampling, Isolation and Identification of Bacteria

Blood cockles were collected from Sungai Yan Baru, Jeti Sungai Udang and Tanjung Dawai (Figure 1). Lipped mussels were collected from Sungai Merbok and Port Dickson (Figure 1). All the samples were kept in containers at 4°C until processed. The samples were immediately processed upon reaching the laboratory. All samples were rinsed three times with sterile water and one time with 70% ethanol in order to remove the nonattached bacteria. Shell was then opened using a sterile knife. Ten gram samples of mantle tissue were homogenized in a homogenizer and transfer into 90 mL Marine broth (MB). The samples were incubated for 24 h at 28°C. Then, serial dilutions from 10^{-1} - 10^{-7} using saline 0.85% were done. A total of 0.1 mL of each dilution was spread on Marine agar (MA), Thiosulfat sitrat bile salt (TCBS) agar and *Aeromonas* agar (AA). After incubation at 28°C for 24 h for all the plate, all colonies with different pigmentation and morphology were randomly observed and isolated. Identification was done by biochemical test. Tests carried out include Gram staining, oxidase, catalase, sulfide-indole-motility, triple sugar iron and Simon citrate tests.

Antibiotic Resistant Test

Susceptibility to selected antibiotics was tested on Mueller Hinton agar (MHA) plates by the disc diffusion method of Bauer et al. (1966). Briefly, the MHA plates were swabbed with overnight grown cultures of the isolates. Antibiotic discs Oxoid, were aseptically placed on the swabbed plates. The antibiotics used include Ampicillin (10 µg), Aztreonam (30 µg), Ceftriaxone (30 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), Imipenem (10 µg), Kanamycin (30 µg), Neomycin (30 µg), Nalidixic Acid (30 µg), Penicillin (10 U), Polymyxin B (300 U). The plates were incubated at 30°C for 24 h and the clear zone formed around the discs was recorded. The multiple antibiotic resistance (MAR) index (number of antibiotics to which the isolate was resistant/total number of antibiotics tested) was determined for each isolate (Sarter et al. 2007).

$$\text{MAR Index} = \frac{X}{Y \times Z},$$

where X is the total of antibiotic resistance case; Y is the total of antibiotics used in the study and Z is the total of isolates.

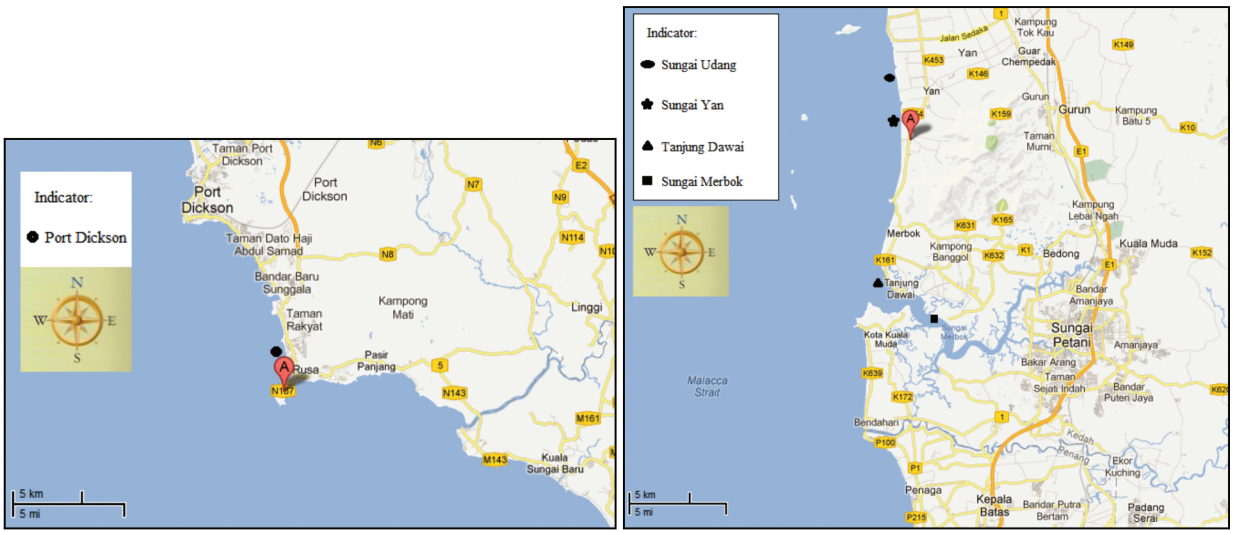

FIGURE 1. Map of sampling locations

BETA LACTAMASE ACTIVITY TEST

This test was done to see the presence of beta-lactamase activity enzyme using asidometrix method. A total of 300 µL aqueous solution (0.2% soluble starch and 1% penicillin benzl) was titrated into the microtiter plate. The isolates were then inoculated into the wells and incubated overnight. In order to determine the release of enzyme, 300 µL Penisiloik acid compounds (0.5% iodine and 1% potassium iodide) was added into each well. Color changes that occurred in the mixture were recorded. Compounds that change color from orange to clear showed positive results.

SCREENING OF ANTIMICROBIAL ACTIVITY

Disk diffusion method was used to screen the antimicrobial activity of positive beta lactamase isolates against Methicillin resistant *Staphylococcus aureus* (N32064), *Staphylococcus aureus* (ATCC11632), *Bacillus subtilis* (ATCC11774), *Eschrichia coli* (ATCC10536), *Serratia marcescens* (ATCC13880), *Proteus mirabilis* (ATCC12453) and *Candida albicans*. Overnight culture of isolates was added into Mueller-Hinton broth (MHB) and 10^6 CFU/mL of the inoculum were spread on Mueller-Hinton agar (MHA). After 5 min, the excess was removed and the Petri dishes were left to dry for 10 min. Tested pathogen which were grown overnight in Nutrient Broth were dropped onto the paper disc. The plates then were incubated at 30°C for 24 h and the clear zone formed around the discs was recorded.

RESULTS AND DISCUSSION

The population density of bacterial from mussel was higher than cockle (Table 1). The bacterial population density from mussel on MA was $8 \times 10^4 - 8 \times 10^8$ CFU/mL and cockle was 9×10^2 CFU/mL. The population of the bacteria on selective medium TCBS and mRS was found to be less than MA. On TCBS, bacterial population from mussel was $6 \times 10^2 - 5 \times 10^8$ CFU/mL and cockle $5 \times 10^2 - 5 \times 10^8$ CFU/mL. While for mRS medium, the isolation from mussel was $3 \times 10^2 - 2 \times 10^8$ CFU/mL and cockle $3 \times 10^4 - 3 \times 10^8$ CFU/mL (Table 1). TCBS medium and mRS were used to detect the presence of genus *Vibrio* and *Aeromonas*. These bacteria were often isolated from bivalves (Huss 1997; Oliver 1989). Most species of *Vibrio* and *Aeromonas* were pathogenic to humans and animals. In this study, the bacterial population density of the Port Dickson mussel samples was higher compared with samples from Sungai Merbok except on mRS medium. This means that the possibility of bacterial infection of mussels by *Aeromonas* is higher at Sungai Merbok compared with Port Dickson. For cockle samples, the bacterial population density is higher on samples from Sungai Yan Baru on growth on MA (Table 1). The population of bacteria on both TCBS and mRS media was higher in samples from Tanjung Dawai. This shows that *Vibrio* and *Aeromonas* were higher than the sample from Tanjung Dawai. This condition may be affected by the weather and temperature factors (Elhadi et al. 2004; Lee et al. 2008).

During a 15-month study conducted along the coastal reaches of the Northern Atlantic, Thompson et al. (2004) reported that the total *Vibrio* population reached a maximum concentration of $8.0 \times 10^3 \pm 9.2 \times 10^2$ cells mL^{-1} in June 2002 and a minimum concentration of 37 ± 5 cells mL^{-1}in April 2002. Wright et al. (1996) showed that *V. vulnificus* comprised 0.6% to 17.4% of the total bacterial population in Chesapeake Bay during warmer months. Heidelberg et al. (2002) detected *V. cholerae* and *V. vulnificus* concentrations of 10^3 to 10^4 organisms mL^{-1} of seawater in Chesapeake Bay. According to the study by Brandi et al. (1999) and Holmes et al. (1996), population densities of *Aeromonas* sp. in sea water was $10^{-2} - 10^2$ CFU/mL. While, a study by Veronica (2005) found that population densities of *Aeromonas* sp. the estuary and coastal area near the estuary was between 10^1 and 10^{10} CFU/mL. This means that, in this study the overall density of the bacteria either *Vibrio*, *Aeromonas* or other bacteria are still in the normal range.

In this study, we successfully isolated 162 bacteria isolates from all samples. A total of 131 isolates were from mussel, while 31 isolates were from cockle (Table 1). Based on biochemical tests, 162 isolates were divided into 10 genera (Figure 2). Most bacterial isolates identified were Gram negative (93%). This study was supported by Santos et al. (2010) which states the majority of bacteria from the marine environment are Gram negative bacteria. It was found that *P. viridis* has a more diverse bacterial isolates than *A. garanosa*. *Vibrio* spp. is the most dominant genus in both types of samples especially in mussels. Family Vibrionaceae are often associated with marine bivalves especially oysters and mussels (Castro et al. 2002; Kueh & Chan 1985; Olafsen et al. 1993; Prieur et al. 1990; Pujalte et al. 1999). The population density of *Vibrio*

TABLE 1. Population density of bacteria (CFU/mL) and total strains isolated from lipped mussel and blood cockle

Sample	Location	Medium			Total of isolates
		MA	TCBS	AA	
Lipped mussel	Port Dickson	$2 \times 10^8 - 8 \times 10^8$	$8 \times 10^5 - 4 \times 10^8$	$3 \times 10^2 - 13 \times 10^2$	87
	Sungai Merbok	$8 \times 10^4 - 5 \times 10^8$	$6 \times 10^2 - 5 \times 10^8$	2×10^8	44
Blood cockle	Sungai Yan Baru	$1 \times 10^8 - 4 \times 10^8$	5×10^2	$3 \times 10^4 - 1 \times 10^8$	17
	Jeti Sungai Udang	1×10^8	-	2×10^8	5
	Tanjung Dawai	9×10^2	5×10^8	3×10^8	9

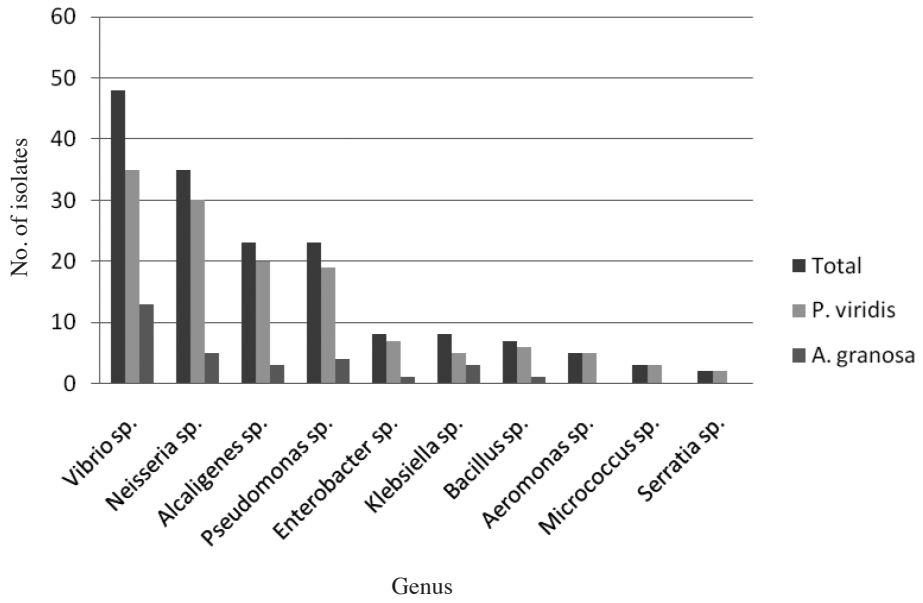

FIGURE 2. Bacterial isolates from *P. viridis* and *A. granosa*

spp. in the marine environment is usually more because Vibrios can occur in a wide range of aquatic environments including estuaries, marine and coastal waters and sediment (Suantika et al. 2001; Thompson et al. 2004; Urakawa et al. 2000; Vandenberghe et al. 2003; Venter et al. 2004). Family Vibrionaceae are also the cause of disease to the mussel either against the larvae, juveniles or adults. These bacteria cause inflammation on the mantle, white spots and cannot stick to the surface (Cai et al. 2007).

Antibiotic susceptibility of the isolates was performed using 12 antibiotics ranging from broad-spectrum antibiotics and narrow spectrum. This study is necessary because of the increasing emergence of antibiotic-resistant pathogens associated with fish and shellfish (Bansemir et al. 2006; Braithwaite & McEvoy 2005; Projan & Bradford 2007). Anviral and antibacterial activity often found in hemolymph of some species of molluscs, including oysters and mussels (Gueguen et al. 2006; Maktoob & Ronald 1997; Olicard et al. 2005; Roch et al. 2008). As a result, the antibiotic resistance test showed all strains were resistant to Penicillin and the number of isolates Ciprofloxacin resistance is the lowest (Table 2). Almost all of the isolates showed resistance to at least two antibiotics except two isolates (Table 2). In addition there were 10 isolates resistant to more than five antibiotics (Table 2). These results were supported from studies by Martinez (2003) which states that more than 90% of bacteria isolated from marine environments resistant to more than one antibiotic and 20% resistant to at least five antibiotics. According to Pinera-Pasquino (2006), resistance to Penicillin frequently found in Gram negative bacteria. This has been proven in this study that the majority of the isolates were resistant to

TABLE 2. Antibiotic resistant of isolates from different sampling location

Antibiotic	Location					Total
	Port Dickson	Sungai Merbok	Sungai Yan Baru	Jeti Sungai Udang	Tanjung Dawai	
AM-10	66	31	11	2	8	118
ATM-30	19	9	3	3	1	35
CRO-30	3	5	2	0	0	10
CIP-5	2	0	0	0	0	2
C-30	11	4	1	1	2	19
E-15	10	9	1	1	2	23
IPM-10	6	5	0	1	1	13
K-30	8	6	0	1	0	15
N-30	9	1	0	1	0	11
NA-30	8	1	1	2	1	13
P-10	84	37	14	3	9	147
PB-300	4	1	1	0	1	7

Indicator: AM 10- Ampicillin (10 µg), ATM 30- Aztreonam (30 µg), C 30- Ceftriaxone (30 µg), CIP 5- Ciprofloxacin (5 µg), C 30- Chloramphenicol (30 µg), E 15- Erythromycin (15 µg), IPM 10- Imipenem (10 µg), K 30- Kanamycin (30 µg), N 30- Neomycin (30 µg), NA 30- Nalidixic Acid (30 µg), P 10- Penicillin (10 U), PB 300- Polymyxin B (300 U)

Penicillin (Figure 3). High resistance to Penicillin caused by the use of antibiotics has long been in clinical use since 1940 (Thavasi et al. 2007). Almost 72% of the isolates showed resistance to Ampicillin (Figure 3). According to Salyers et al. (2004) and Wang et al. 2008, resistance to Ampicillin is often available for the isolates from the environment.

The majority of the isolates had resistance values diversity index (MAR index) 0.2 and the highest value was 0.7 with a frequency of less than 10 (Figure 4). Majority of the isolates were MAR index of 0.2 which showed that the isolates were not exposed to antibiotics (Chitanand et al. 2010). High resistance against a variety of antibiotics is not only caused by antibiotic contamination factor, it may also caused by bacterial genetic ecology itself either through mutation, selection or genetic information flux between bacteria (Mazel & Davies 1999). Plasmid is one cause of the spread of resistance genes between bacteria (Smith et al. 1993). According to Ronald et al. (2002), many organisms resistant to Ampicillin or more than one antibiotic has plasmid. This was because the antibiotics Ampicillin resistance usually results from a plasmid (Ronald et al. 2002).

Out of all isolates, 39 were beta-lactamase enzymes positive and the mussel isolates from Sungai Merbok showed the highest percentage (Table 3). The isolates with multiple antibiotic resistant do not necessarily produce the enzyme beta-lactamase. The majority of isolates that produced beta-lactamase positive was of the genus *Pseudomonas* and *Nesseria*. The production of beta-lactamase enzyme was a major factor that contributed to resistance to beta-lactamase antibiotic in Gram negative bacteria (Medeiros 1997). None out of the fifteen isolates with beta-lactamase positive which were tested for their antimicrobial activity against different pathogen (Table 4). Isolate SMII-Ip produced antimicrobial activity against six out of the nine pathogen tested.

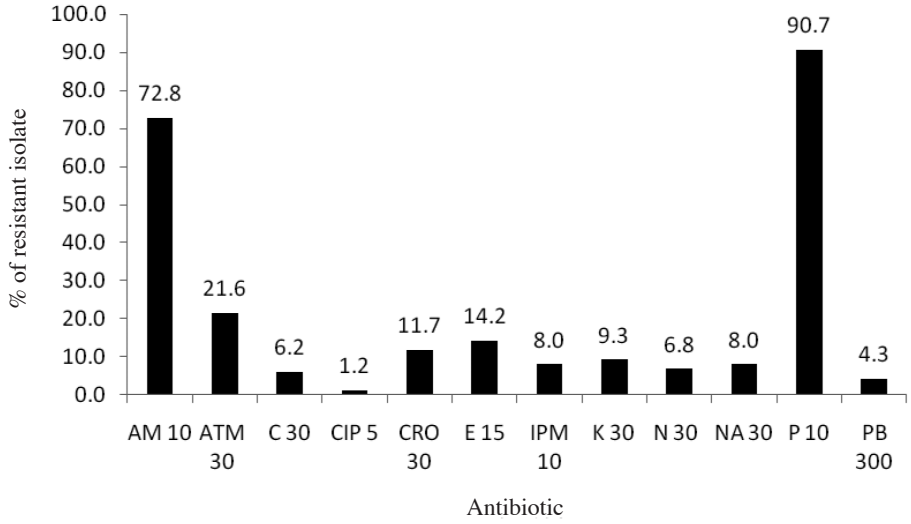

FIGURE 3. Percentage frequencies of isolates resistant to antibiotic

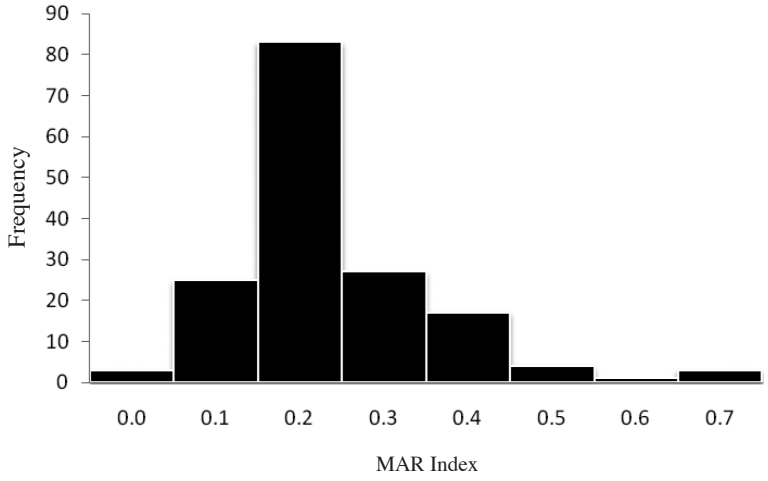

FIGURE 4. MAR index frequency for each isolates

TABLE 3. Beta lactamase activity base on different location and sample

Sample	Location	Total of positive isolates
Lipped mussel	Port Dickson	(22%) 19/87
	Sungai Merbok	(36%) 16/44
Blood cockle	Sungai Yan Baru	(18%) 3/17
	Jeti Sungai Udang	(0%) 0/5
	Tanjung Dawai	(11%) 1/9

TABLE 4. Antimicrobial activity against different microbial pathogens

No. isolates	Pathogens								
	BS	SA	MRSA	VP	PM	SM	EC	AH	CA
1-1b	-	-	-	-	-	+	-	+	-
1-1h(ii)	-	-	-	-	-	++	-	-	-
2-4j	++	-	-	-	-	-	-	+++	-
2-4k	+	-	-	-	-	-	-	-	-
2-4t	-	-	-	-	-	+	-	-	-
3-1a	-	-	-	-	-	+	-	-	-
3-1d	-	-	-	-	-	++	-	++	-
4-1f(ii)	-	-	-	-	-	+	-	+	-
5-1a	++	-	-	-	-	-	-	-	-
SYBI-7b	-	-	-	-	-	-	-	+	-
SMI-Ij	-	++	-	++	-	-	-	++	-
SMI-Ip	+	+	+	-	-	+	+	-	+
SMI-3h	-	-	-	-	-	++	-	-	-
SMII-3J	-	+	+	+	-	++	-	-	+
SMII-7h	+	-	-	-	-	+	-	-	-

Indicator: BS- *B. subtilis*; SA- *S. aureus*; MRSA- Meticillin Resistant *S. aureus*; VP- *V. parahaemolyticus*; PM- *P. mirabilis*; SM- *S. marcescens*; EC- *E. coli*; AH- *A. hydrophila*; CA- *C. albican*; - = 0mm; + = >8 to 10mm; ++ = 10 hingga 20mm; +++ = 20 to 30 mm

CONCLUSION

In conclusion, population density of bacterial from mussel was higher than cockle but the density of the population was normal for both bivalves. Bacterial isolates showed resistance to diverse nature of broad spectrum antibiotics and narrow spectrum. The majority of isolates were resistant to more than two types of antibiotics, particularly Penicillin and Ampicillin. Although the density of the bacterial population was normal in all the samples, constant monitoring on mussel and cockle farm is always required.

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