

## ANTIBIOTIC RESISTANT BACTERIA FROM SEDIMENT OF COASTAL WATER OF PAHANG, MALAYSIA

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### Abstract

This study was undertaken to determine the presence of antibiotic resistant bacteria in sediment of coastal water of Pahang, Malaysia. A total of 125 bacteria were isolated from sediment samples of Tanjung Lumpur (A and B), Teluk Chempedak, Batu Hitam and Balok and tested against 10 different antibiotics by disc diffusion method. In Teluk Chempedak and Balok, the greatest frequencies of resistance in bacteria were found in Polymyxin B and Streptomycin but less common in Chloramphenicol. Meanwhile, high resistance of bacteria against the antibiotic in Tanjung Lumpur existed in Polymyxin B, Penicillin G and Ampicillin, whilst no resistance in bacteria was found in Chloramphenicol, Gentamycin and Erythromycin. Most of the bacteria from Batu Hitam exhibited high resistance to most of the antibiotics tested. Approximately, most of the isolates showed Multiple Antibiotic Resistance (MAR) index value higher than 0.2 (>0.2) which suggest there are high-risk of antibiotics contamination in the areas. Two isolates that showed resistance to most antibiotics were identified as *Stenotrophomonas maltophilia* and *Brevundimonas vesicularis*. Findings from this preliminary study revealed the presence of antibiotic resistance strain of bacteria which may indicate the occurrence of antibiotic contamination s at the coastal water of Pahang, Malaysia and this may pose a potential public health implications.

Keywords: Antibiotic resistance bacteria, sediment, selected coastal water, Pahang

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## 1.0 INTRODUCTION

The discovery of antibiotics was undoubtedly one of the greatest achievements of the twentieth century. They have microcidal or microstatic activity which functions to disrupt the microbial metabolism by a variety mechanism. In recent decades, antibiotics have been intensively and widely used in humans and animals for the prevention and treatment of diseases, and as growth promoters in agriculture and aquaculture thus increasing the proportion of antibiotic resistant bacteria in the environments [1], [2]

These chemical compounds received little attention as pollutants in the aquatic environment [3]. Apart from chemical pollution caused by the antibiotics, the elevated of concentration of antibiotics in the aquatic environment may also accelerate the development of antibiotic resistance genes (ARGs) and the emergence of antibiotic resistance bacteria [4] which shade risks to humans' health and disturb the aquatic ecosystems. Bacteria resistant to antibiotic are

commonly discovered in clinical and veterinary areas but it is not well characterized in aquatic environments [5]. The resistance to antibiotics has become a severe issue in human health and to the ecosystem function. Many antibiotics persist in the sediment and organisms in the aquatic environment for several months following administration [6]. Their residues may affect the biological action on the microbes if they continually emitted to the environment [7].

Coastal environment plays an important role as habitat to a number of plants and animals which serve as breeding and nursery grounds, shelters, sources of food for various marine lives [8]. Recently, coastal waters are facing a wide variety of stressor affecting both the ecosystem and human health caused by domestic wastewater treatment and disposal practices which led to introduction of high levels of nutrients and enteric pathogens. Besides that, the release of antibiotics from hospital wastewater, development of recreational area and discharge of effluent and wastewater from resorts and restaurants

and ballast water from ships along the coastal area also contribute serious damages to the coastal environments.

Currently, several studies relating to the existence of antibiotic resistance bacteria in sediment of coastal waters have been done [5], [9], [10], [11], however studies conducted on antibiotics resistance bacteria in sediment of coastal water in Malaysia are still lacking. Thus, this study presents a preliminary report on the occurrence of antibiotic resistance in bacteria in coastal water of Pahang, Malaysia.

## 2.0 EXPERIMENTAL

### 2.1 Study Areas and Samples Collection

Pahang is the largest state in Peninsular Malaysia and situated in the eastern coastal region. The sediment samples were collected from the study areas by using Eckman grab. The sampling was carried out in triplicate at each location with 35m distance from of each point. Each point was determined by Geographical Positioning System (GPS) coordinate (Table 1). The samples collected were transferred into sterile polyethylene bags and processed immediately.

### 2.2 Isolation of Bacteria

Bacteria from sediment samples were isolated using spread plate technique. One gram of the sediment samples were mixed with 10 ml autoclaved saline. The homogenized samples were diluted 1:10 v/v with saline followed by serial dilutions ( $10^{-2}$  to  $10^{-6}$ ). About 100 $\mu$ l of dilutions  $10^{-3}$  to  $10^{-6}$  were plated on nutrient agar and incubated for 36hr at 37°C. After incubation, representative colonies were chosen randomly and purified on nutrient slant agar medium for further study. Gram positive and Gram negative bacteria were identified using gram staining

### 2.3 Antibiotic Resistant Test

Antibiotic resistance bacteria were determined using disc diffusion method following the procedures of standardize agar diffusion method by National Committee for Clinical Laboratory Standards (NCCLS) for antibiotic susceptibility tests [12]. The isolates were inoculated in Mueller-Hinton Broth and incubated for 18hr at 37°C. The turbidity of the bacterial suspension was adjusted and compared with 0.5 Mc Farland Standard using spectrophotometer at 625 nm. The amount of the bacteria was equivalent to  $1.5 \times 10^8$  cfu/ml. About 100 $\mu$ l of bacterial suspension was spread on agar plate and left to dry before placing the antibiotic disc on the surface of the plate. All the samples were treated with 10 different antibiotics to test their susceptibility towards the antibiotics. The antibiotics used were namely Tetracycline (30  $\mu$ g), Rifampicin (30  $\mu$ g), Streptomycin (10  $\mu$ g), Vancomycin (30  $\mu$ g), Penicillin G (10 units), Ampicillin (10  $\mu$ g), Chloramphenicol (30  $\mu$ g), Gentamycin (10  $\mu$ g),

Erythromycin (15  $\mu$ g) and Polymyxin B (300 units) which are available from Oxoid in disc form. The results were documented by measuring the inhibition zone of the colonies that formed on the plates after 24hr of incubation at 37°C. The diameter of inhibition zones was measured to the nearest mm and the isolates are classified as resistant ( $R \leq 16$ mm) [12].

**Table 1** Coordinates of sampling areas

	Point 1	Point 2	Point 3
<b>Tanjung Lumpur A</b>	N 03' 47.300E E 103' 19.576	N 03' 47.382 E 103' 19.653	N 03'47.568 E 103' 19.617
<b>Tanjung Lumpur B</b>	N 03' 47.984 E 103' 19.817	N 03' 48.077 E 103' 19.620	N 03' 48.166 E 103' 19.181
<b>Teluk Chempedak</b>	N 03' 48.770 E 103' 22.633	N 03' 48.650 E 103' 22.573	N 03' 48.465 E 103' 22.547
<b>Batu Hitam</b>	E 03' 53.288 N 103' 22.337	N 03' 53.115 E 103' 22.283	N 103' 22.192 E 03' 52.955
<b>Balok</b>	N 03' 55.768 E 103'23.395	N 03' 56.115 E 103' 23.536	N 03' 56.397 E 103' 23.660

### 2.4 Determination of MAR Index

The Multiple Index Resistance (MAR) index values of each isolates were determined by dividing the number of antibiotics to which isolate is resistant by the total of antibiotics tested [13], [14]:

$$\text{MAR index value} = a/b$$

Where 'a' represents the number of antibiotics that isolate bacteria show resistant to and 'b' represents the total number of antibiotics the isolate bacteria show non-resistant. If the MAR index value of isolate is  $> 0.2$ , it indicates high risk of contamination of several antibiotics used in the environment of the isolate originated [5], [11].

### 2.5 API 20NE Bacteria Identification Test

Isolates that displayed the highest value of MAR index were identified by using API 20NE kit. The API strips were inoculated with suspensions prepared from nonselective media in 5ml of 0.85% sterile saline approximating a 0.5 McFarland standard and incubated for 24 or 48 hr. The addition of reagents and the interpretation of reactions were done according to the manufacturer's directions (bioMérieux, Marcy l'Etoile, France). The 20 biochemical reactions on the test strip were converted into an octal profile number and decoded by using Analytical Profile index (ApiWeb™ identification software).

### 3.0 RESULTS & DISCUSSION

In total of 125 bacteria (25 isolates for each location) were randomly selected and examined for antibiotic resistance test.

The results from Figure 1 shows that most of the bacteria tested have high resistance against Polymyxin B at all of the study location. Apart from that, the greatest frequencies of resistance in bacteria were also found in Vancomycin, Streptomycin and Penicillin G at Teluk Chempedak and Balok. The resistance in bacteria to Chloramphenicol, Gentamycin and Rifampicin were less common in these locations. High occurrences of resistance in bacteria against the antibiotics in Tanjung Lumpur were also existed in Penicillin G and Ampicillin, whilst no resistance in bacteria was found in Chloramphenicol, Gentamycin and Erythromycin. Interestingly, all isolates from Batu Hitam displayed high resistance to all antibiotics tested.

The multiple antibiotics resistance (MAR) index value of each isolates was calculated to determine the resistance of bacteria to multiple antibiotics. The MAR index value ranged from 0.1 to 1.0. From the findings, multiple antibiotic resistances were observed in isolates from all locations (Figure 2). Most of the bacteria isolated from Tanjung Lumpur B (64%) and Balok (44%) showed resistance to 4 antibiotics compared to Teluk Chempedak (52%) which had only 3 antibiotics. Meanwhile in Tanjung Lumpur A, 24% of isolates showed resistance to 3 antibiotics and another 24% showed resistance to 5 antibiotics. In Batu Hitam, most of the bacteria (68%) displayed resistance to all antibiotics tested. Figure 3 further demonstrated high percentage of bacteria displaying multiple resistances to antibiotics for all locations.

Two isolates were chosen for identification using API 20NE based on their high resistance towards multiple antibiotics. One isolate was from Batu Hitam with resistance to 10 antibiotics and another one from Balok with resistance up to 8 antibiotics. The results presented high % of ID of the isolates which were *Stenotrophomonas maltophilia* (99.4%) from Balok and *Brevundimonas vesicularis* (91.4%) from Batu Hitam.

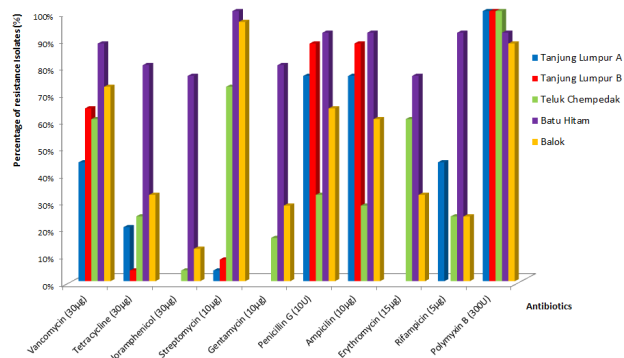
The differences in percentage of bacterial resistance to various antibiotics in this study could reflect the antibiotic contamination in the area and could be considered as a bioindicator to initiate the monitoring programs for antibiotic pollution [16]. Findings from this study revealed a very significant indicator of current incidents associated with antibiotic resistance pollution in aquatic environment.

Majority of the isolates were found to be resistant to polymyxin B. It is assumed that wide use of polymyxin B may lead to the development of resistant bacteria to

polymyxin B. This antibiotic is commercially available as polymyxin B sulfate which clinically used as a topical medication (skin, ears and eyes), parenteral (intravenous and intrathecal administration) and for respiratory tract disease [17], [18]. Polymyxin B is commercially used in ointments to treat soft tissue infections which known as Neosporin [19]. On the other hand, very low percentage was obtained for bacteria exhibiting resistance towards antibiotics chloramphenicol and gentamycin which may indicate the less usage of these antibiotics in in Kuantan.

This finding is also in coherent with previous studies conducted in Malaysia that showed low percentage of bacteria resistance to chloramphenicol [9], [10].

Majority of the isolates were Gram negative bacteria and they exhibited higher resistance than Gram positive bacteria towards the antibiotics tested (Table 2) Most of Gram negative bacteria are pathogenic to human. Previous study reported that plasmids encoded with antibiotic resistance genes were transferred between the pathogenic and non-pathogenic Gram negative bacteria in the environment which presumed the high resistance activity towards the antibiotics [20]. Besides, these findings also implied that the pathogenic bacteria had numerous opportunities to become resistant to antibiotics, thus making it more difficult to prevent bacterial disease.



**Figure 1** Percentage of bacterial resistance to different antibiotics Tanjung Lumpur (A and B), Teluk Chempedak, Batu Hitam and Balok (%)

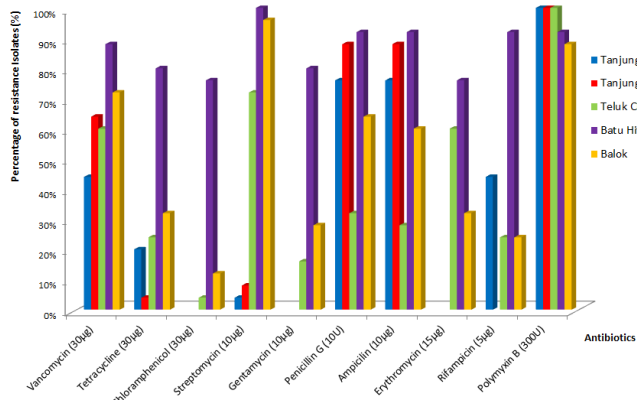


Figure 2 Percentage of bacterial resistance to different antibiotics Tanjung Lumpur (A and B), Teluk Chempedak, Batu Hitam and Balok (%)

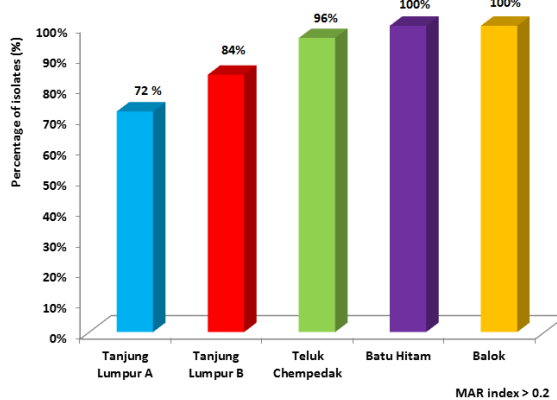


Figure 3 Percentage of frequency MAR index value >0.2 (%)

Table 2 Multiple antibiotic resistance (MAR) of Gram Negative and Gram Positive bacteria

MAR index	Frequency of MAR index									
	Tanjung Lumpur A		Tanjung Lumpur B		Teluk Chempedak		Batu Hitam		Balok	
	Gram-ve	Gram+ve	Gram-ve	Gram+ve	Gram-ve	Gram+ve	Gram-ve	Gram+ve	Gram-ve	Gram+ve
0.1	3	2	1	-	-	-	-	-	-	-
0.2	1	-	2	1	1	-	-	-	-	-
0.3	5	2	4	-	7	6	-	-	1	1
0.4	2	-	15	1	2	2	3	1	11	-
0.5	5	1	1	-	-	2	-	-	4	1
0.6	3	1	-	1	-	-	-	1	1	3
0.7	-	-	-	-	2	-	1	-	-	1
0.8	-	-	-	-	2	1	-	-	2	-
0.9	-	-	-	-	-	-	2	-	-	-
1.0	-	-	-	-	-	-	17	-	-	-
	19	6	22	3	14	11	23	2	19	6

Furthermore, the bacteria might have developed resistance towards several antibiotics due to indiscriminate use of antibiotics. The presence of antimicrobial agents at low concentration through leaching or continued usage may lead to the development of drug-resistant strains and multiple antibiotic resistances in bacteria, which result in the transfer of resistance to pathogenic bacteria and reduced efficacy of antibiotic treatment for human and animal diseases [21]. The multiple resistance or 'multi-resistance' is considered to be the key indicator of problematic bacterial strains because it undermines the empirical treatment regimens which delay the administration of the appropriate antibiotic therapy, and it also reduces the options of treatments that are appropriate [22].

In this study, all of the locations exhibited high percentage of MAR index value >0.2 (Figure 3) that suggested a variety of antibiotics are being disposed at these areas. From these findings, it can be

summarized that the existence of multiple antibiotics resistance are influenced by several sources of contamination. The emission of antibiotics contaminants might be from the human activities on the beach or water. Besides that, the discharge of effluent and waste waters from the resorts and restaurant which located along the coast of Batu Hitam, Balok and Teluk Chempedak might also be the source of the antibiotics contaminations of the areas.

Also, the widespread of antibiotic resistant bacteria in Tanjung Lumpur (A & B) might due to the release of hospital waste around these areas. It is expected that waste from hospital effluent contain antibiotics residues which will be directly released into the aquatic bodies. Poor management of medical waste which contains antibiotics may lead to the excretion of antibiotics directly into the aquatic environment without considering the future implication to the ecosystem.

Marine recreational waters and sands had received domestic sewage which may contribute to the establishment of distribution routes by microorganism that carrying antimicrobial resistance gene [23], [24]. Furthermore, the existence of antibiotic resistant gene in bacteria may be influenced by human urban and agricultural activities, including sewage discharge, animal husbandry, and fish breeding, as well as natural environmental changes [25]. Human intestinal bacteria are reservoirs for antibiotic resistance genes. They were not only exchange resistance genes among themselves but might also interact with bacteria that are passing through the colon, causing these bacteria to acquire and transmit the antibiotic resistance genes to the environment through fecal excretion [26]. These factors not only lead to the occurrence of antibiotic resistant bacteria carrying antibiotic resistant genes encoding resistance to some kinds of antibiotics directly, but also change the water parameters to influence the growth conditions of microorganisms, resulting in changes in the structure of the microbial community. Besides, the key factor in the development of antibiotic resistance bacteria is that bacteria have the ability to adapt quickly to the new changes in the environment conditions and survive [27]. Therefore, a single genetic mutation in bacteria such as development of resistance genes can greatly affect their ability to cause disease. As the bacteria could reproduce by dividing every few hours and evolve rapidly, this could be the reason of the spreading of resistance genes in the bacteria population in the area. Antibiotic resistance in bacteria is considered to be major problem because many disease causing bacteria are becoming more resistant to commonly use antibiotics. The overuse of antibiotics and the lack of monitoring waste water discharge into the environment might leads to the development of antibiotic resistance bacteria.

From this study, isolates which showed the highest value of MAR index were identified as *Stenotrophomonas maltophilia* and *Brevundimonas vesicularis*. Several studies reported that both bacteria are known to be resistance to multiple antibiotics [28]–[31]. These bacteria are gram negative bacilli. *S. maltophilia* can be found mainly in the soil, plant rhizosphere, surface water, and wastewater; but also in food, drinking water or contaminated medical care fluids. It generally associated with human infections [32]. Meanwhile, *B. vesicularis* can be isolated from the human end cervix, natural soil environments, bottled water and hospital environments [30], [31]. This bacterium can cause arthritis, endocarditis, meningitis and primary bloodstream infection in immunocompromised as well as immunocompetent patient [30]. The presence of these harmful bacteria in marine sediment might be through disposal of wastewater or clinical waste into the aquatic environment. Furthermore, these harmful isolates exhibited high resistance to multiple antibiotics. Therefore, it can be point out that there were multiple antibiotic contaminations that contributes to resistance in bacteria.

## 5.0 CONCLUSION

This study demonstrates the incidence of antibiotic contamination in selected area of Pahang coastal waters as indicated by the presence of antibiotic resistance bacteria in the sediment. Majority of the isolates exhibited multiple antibiotic resistances which further placed the Pahang coastal water as a whole at risk of being more exposed to severe antibiotic contamination if no measures or actions are taken to circumvent the problem. The development of antibiotic resistance in bacteria are the issues that need to be investigated in greater depth, suggesting the importance of wise use of antibiotics and their risk to human health and the environment in general. Thus, an urgent need is required to monitor antibiotic drugs that are used in order to reduce the risk of antibiotic residues contamination and bacterial resistance at the coastal water. This study could also be used as a basis for more extensive study which can provide further understanding on the status of antibiotic contamination.

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