

Multi-Drug Resistance Bacteria: A Case Study in Western Peninsular Malaysia Freshwaters

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ABSTRACT

The emergence of antibiotic resistant bacteria in the aquatic environment has led to an increase in waterborne health risks to an alarming extent. This study attempts to investigate the population of certain antibiotic resistant strains in Peninsular Malaysia. From the samples of 14 rivers from 7 different states, 203 isolates were successfully isolated. These were from rivers in Negeri Sembilan, Melaka, Selangor, Kuala Lumpur, Kedah, Kelantan and Terengganu with 35, 15, 37, 39, 24, 26 and 27 isolates, respectively. The isolates were tested for their susceptibility towards 6 broad spectrums of antibiotics which are gentamicin, ampicillin, rifampicin, chloramphenicol, tetracycline, and ciprofloxacin. Out of the 203 isolates, 117 isolates were identified to have more than 20% MAR index value, with 47 of the isolates possess a minimum 50% MAR index value. Based on MAR index value, 59% of the isolates are high risk threats indicating a serious emergence of antibiotic resistant bacteria in the rivers in Peninsular Malaysia. Isolates with MAR index value of more than 50% were selected for 16S rRNA gene sequencing for further identification. Based on 16s rRNA gene sequencing, the isolates are a mixture of pathogenic and commensals bacteria, implying that the environment, especially rivers, can be a reservoir for genetic jugglery.

Keywords: Antibiotic resistance bacteria, Broad spectrum antibiotics, Multiple antibiotic resistance index (MAR), Western peninsular Malaysia, 16s rRNA gene sequencing

Introduction

Antimicrobial resistance (AMR) refers to the multiplication of pathogenic microorganisms under the presence of antimicrobials. It was initially observed as an ordinary medical problem in hospital-acquired infections, particularly in critically ill and severely immunocompromised patients [1]. Today, AMR has become one of the recognized threats to the human population along with other common bacterial infections that are difficult to treat [2].

Approximately 700,000 people died each year from antimicrobial resistance bacterial infections

[3]. In Malaysia, the Malaysian Action Plan on Antimicrobial Resistance (MyAP-AMR) has been proposed to tackle a steady increase in antibiotic resistance, especially in common organisms. Global consumption of antibiotics by humans has risen by 40% in the first ten years of the 21st century. The BRIC nations (Brazil, Russia, India, China) contributed to three quarters of this growth [3]. Meanwhile, the use of veterinary pharmaceuticals has become vital to the animal food industry. As a perspective, there are approximately 34 million cattle (2015) and 9 billion chickens (2014) in

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the United States alone [4].

The emergence of human and animal antimicrobial resistant pathogens is a result of antimicrobial exploitation in humans and livestock [5]. The World Health Organization (WHO) proposed the elimination of antibiotics for growth promotion in agriculture that were also used in human medication. The European Union (EU) has also initiated several actions, including the removal of all antimicrobials used as a growth promoter in the livestock industry (Regulation EC 1831/2003) [6]. Many major livestock-producing countries have established their own national surveillance systems to ensure the use of antimicrobials in livestock and veterinary medicine is in control and monitor the antimicrobial resistance emergence. United States is one of such countries with the establishment of the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) in 1996 [7].

Antibiotics accumulation in the environment is from municipal and agricultural sources, including faeces, improperly disposed prescriptions, medical waste, discharge from wastewater treatment facilities, leakage from septic systems, and agricultural waste [8, 9]. Inefficient antibiotics degradation in the environment could result in the development and growth of antibiotic resistant microbial populations [10, 11]. This condition could be worsened by the cyclic manure application on the same location that exposes the soil microbes to antibiotic residues and antibiotic resistant bacteria. Both antibiotic residues and antibiotic resistant bacteria could propagate in the environment by surface runoff or leached through soil and reach nearby rivers or lakes [8, 9].

Recent report by O'niell et al. [9] concluded that resistance might also propagate from its sources via water. Animal manure may contain antimicrobial compounds from on-farm livestock management. Antimicrobials in manure and biosolids may enhance the selection of resistant bacteria in the aquatic environment through diffuse pollution pathways [12].

In Malaysia, intensive agriculture, industrial activities, domestic wastewater and urban runoff have caused an impact on water pollution in the freshwater environment. The contamination in the freshwater environment could serve as a hotspot for the development of antibiotic resistance. Alhaj et al. [13] have conducted their study in Malaysia by studying the prevalence antibiotic resistance

among *Escherichia coli* from different sources. Their study concluded that from 70 isolates of *E. coli* isolated from clinical, marine, river, food and animal farming sources, many of it (61.2%) showed resistance towards 10 antibiotics tested. Their study also reflects the water quality and environmental contamination by antibiotics residues and antibiotic resistance bacteria (ARB) in Malaysia. Besides that, a study by Kathleen et al. [14] concluded that the bacterial isolates' multiple antibiotic resistant (MAR) index ranged between 0 and 0.63 in the aquaculture site in the eastern peninsular of Malaysia.

Therefore, this study aimed to investigate the emergence of AMR in Peninsular Malaysia by examining water samples from several rivers in Peninsular Malaysia. Samples were taken from rivers of 7 neighbouring states; Negeri Sembilan, Melaka, Kuala Lumpur, Selangor, Kedah, Terengganu and Kelantan. These rivers were chosen for sampling due to their proximity to densely populated settlements. Selangor ranked as the most populated state with a population of 5.46 million, followed by Kedah at 1.95 million, Kuala Lumpur at 1.67 million, Kelantan at 1.54 million, Terengganu at 1.04 million, Negeri Sembilan and Melaka at a population of 1.02 and 0.82 million, respectively [15]. The isolated bacteria were tested for physiological and biochemical characteristics, in addition to their susceptibility to antibiotics. Finally, the identification of the ARB was performed using 16s rRNA gene. Through this study, the freshwaters were evaluated for their implication of antibacterial resistance organism from river water to the public health and food security, which could contribute to more data on ARBs in the freshwater environment and overcoming the limitation on existing literature. This study could serve as preliminary data and provide potential clues for ARBs and ARGs contamination control.

Material and Methods

Sample collection and bacterial isolation

Two rivers from each seven neighbouring states in Peninsular Malaysia were chosen as sampling sites. These rivers were selected based on their locations near the aquaculture farm and residential area. The details of the sampling areas are tabulated in Table 1, in which samples were collected from Point A (upstream) and Point B (downstream). An amount of 500 ml of water sample was collected in sterile glass bottles from the

Table 1. Tables are numbered with Roman numerals and even at the beginning of a sentence

States	Coordinate	Rivers	Number of isolates
Negeri Sembilan	Point A: 2.720111, 102.022564	Linggi	7
	Point B: 2.657238, 101.994087		12
	Point A: 2.825212, 102.331082	Jempol	10
	Point B: 2.851562, 102.302156		6
Melaka	Point A: 2.194627, 102.248791	Melaka River	5
	Point B: 2.202485, 102.251030		3
	Point A: 2.340697, 102.060471	Tuang river	4
	Point B: 2.338305, 102.060361		3
Selangor	Point A: 3.031978, 101.768573	Langat	9
	Point B: 2.896472, 101.72885		8
	Point A: 3.221031, 101.590368	Chemubong	10
	Point B: 3.221058, 101.583678		10
Kuala Lumpur	Point A: 3.170147, 101.695182	Gombak	9
	Point B: 3.170836, 101.686803		8
	Point A: 3.178345, 101.682919	Batu	6
	Point B: 3.197522, 101.678123		18
Kedah	Point A: 5.564073, 100.428082	Kuala Muda	6
	Point B: 5.582418, 100.374749		9
	Point A: 5.634242, 100.504987	Sungai Petani	7
	Point B: 5.639848, 100.475997		5
Kelantan	Point A: 4.870728, 102.438744	Lebir	4
	Point B: 4.932378, 102.418298		8
	Point A: 5.530988, 102.195158	Kelantan	8
	Point B: 5.612043, 102.144881		7
Terengganu	Point A: 5.280724, 103.141581	Ibai	4
	Point B: 5.278680, 103.168602		9
	Point A: 5.279265, 103.084646	Terengganu	6
	Point B: 5.328204, 103.125040		5

surface water of each river. The samples were then stored at a temperature of 4°C before being transferred to cold room until further analysis. Isolation of bacterial strains from each sample was performed using serial dilution and spread plate on nutrient agar. All the plates were incubated at 37°C for 24 hours. The single colonies of different morphology from the spread plates were streaked on nutrient agar for subcultures to obtain the pure colonies of the bacterial isolates.

Antimicrobial susceptibility test

Mueller-Hinton agar (MHA) was used in this study to grow the bacteria from the samples. This study used six different antibiotics: ampicillin, ciprofloxacin, chloramphenicol, gentamycin, tetracycline, and rifampicin. The concentration of antibiotics was set at 10 µg/ml and 30 µg/ml. The antibiotic discs were prepared using Whatman filter paper no. 1 with a diameter of approximately 6 mm. The colonies of the pure culture were trans-

ferred into 5 ml of nutrient broth medium. The broth culture was then incubated at 37°C until it achieves or exceeds the turbidity of 0.5 MacFarland standard after 2 to 6 hours of incubation. The antibiotics were applied to the sterilized discs the discs were dispensed onto the surface of the inoculated agar plate. The antimicrobial susceptibility test for each isolate was carried out in duplicate. The plates were incubated at 37°C for 24 hours. After 16 to 18 hours of incubation, the diameters of the zones of complete inhibition were measured. The inhibition zones are interpreted by referring to the Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints [16].

Determination of MAR index value

The isolates' Multiple Index Resistance or MAR index value was determined by using the following equation [17].

$$MAR \text{ index value} = \frac{a}{b} \times 100$$

Note:

a : Number of resistant antibiotics

b: Total number of the tested antibiotics

Bacterial identification

The bacterial isolates' DNA was extracted using the heat shock method, also known as the boiling-centrifugation method [14]. Two millilitres of the overnight bacterial cultures were centrifuged at 10,000 rpm for five minutes and the supernatant was discarded. Then, another two millilitres of the bacterial cultures were added to the same micro-centrifuge tube and centrifuged at 10,000 rpm for another five minutes to allow pellet formation. The supernatant was then discarded and the pellet formed was re-suspended in 500 µl of sterile distilled water. The suspension was boiled at 100°C for ten minutes. Next, the boiled suspension was immediately cooled in ice for five minutes and then centrifuged at 10,000 rpm for ten minutes. The supernatant from the centrifuged suspension was collected and used for PCR amplification [14].

The 16S rRNA gene was amplified with PCR using the universal primers-fD1 (5'-AGAGTTT-GATCATGGCTCAG-3') and reverse primer-rP1 (5'-ACGGTTACCTTGTTACGACTT-3'). These are complementary to the 5'-end and 3'-end of prokaryotic 16S rRNA genes. Sequencing of the PCR products was outsourced to Apical Sdn. The nucleotide sequences were analyzed using the BLAST program that compares the nucleotide sequences from the National Center of Biotechnology Information (NCBI) [18] database.

Results and Discussions

Morphological determination of isolates

From the isolation, 35, 15, 37, 39, 24, 26 and 27 pure colonies were isolated from the two rivers in Negeri Sembilan, Melaka, Selangor, Kuala Lumpur, Kedah, Kelantan and Terengganu, respectively (Figure 1). The details on the number of isolates according to the sampling area are tabulated in Table 1. All isolated bacterial colonies were then tested for their susceptibility towards six types of antibiotics, as mentioned previously.

Susceptibility towards antibiotics

Each isolate was subjected to 2 levels of antibiotic concentration (10 µg/ml and 30 µg/ml, since bacteria's response towards antibiotics is concentration-dependent [19]). Figure 2 shows the per-

centage of ARB isolated from all 14 rivers. Based on Figure 2, most of the isolates possess resistance towards: chloramphenicol (91%) followed by tetracycline (76%), ampicillin (74%), rifampicin (73%), gentamicin (43%), and ciprofloxacin (21%) for antibiotics with the concentration of 30 µg/ml. As for 10 µg/ml of tested antibiotics, 54.68%, 53.20%, 48.77%, 47.78%, 46.31%, and 25.62% of isolates are resistance towards ampicillin, chloramphenicol tetracycline, rifampicin, gentamicin, and ciprofloxacin, respectively. Most isolates are resistant to a higher concentration of antibiotics, especially for chloramphenicol, tetracycline, ampicillin, and rifampicin. Antibiotic resistance or tolerance profile may be developed if the bacteria are exposed to antibiotics at non-lethal concentrations. The antibiotics could exhibit antimicrobial activities on susceptible cells at higher concentration [19].

Figure 3 shows the percentage of ARB isolates grouped according to their respective states. Irrespective of location, most isolates showed higher resistance to a lower concentration of antibiotics. However, there are several instances where isolates showed identical resistance towards antibiotics for example Negeri Sembilan (ciprofloxacin), Melaka (gentamycin, tetracycline and ciprofloxacin), Terengganu (ampicillin and ciprofloxacin). This could reflect the pattern of antibiotics use in each respective state.

Of all tested antibiotics, isolates showed the least resistance towards ciprofloxacin. Isolates from Negeri Sembilan showed no resistance to ciprofloxacin, even at 10 µg/ml, which could be due to relatively recent exposure to ciprofloxacin in this particular state. Meanwhile, in Kedah, 65.38% of isolates showed resistance to ciprofloxacin at 10 µg/ml and 53.85% at 30 µg/ml.

Based on Figure 3, most bacterial isolates from both rivers in Negeri Sembilan showed resistance to gentamicin, ampicillin, chloramphenicol, and tetracycline. Only a few of the isolates showed resistance towards ciprofloxacin. No isolates showed resistance towards ciprofloxacin in both rivers in Negeri Sembilan. Only one isolate showed resistance towards this antibiotic in Melaka River, Melaka, which might be due to the late introduction (1987) of ciprofloxacin in human medication compared to the rest of the tested antibiotics. This might suggest that the bacteria are still developing resistance towards this antibiotic.

The types of antibiotics chosen in this study

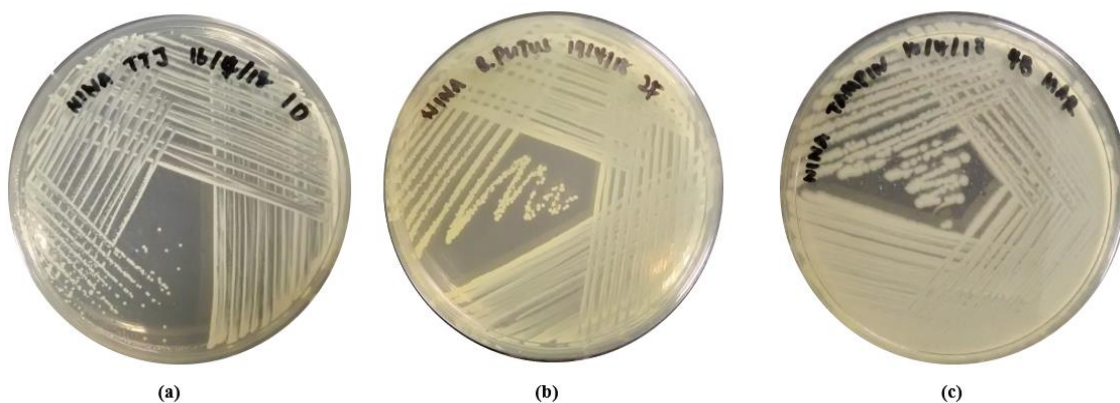


Figure 1. Pure colonies isolated from the Linggi and Jempol in Negeri Sembilan. (a) Isolate 1D; (b) Isolate 2F; (c) Isolate 4B.

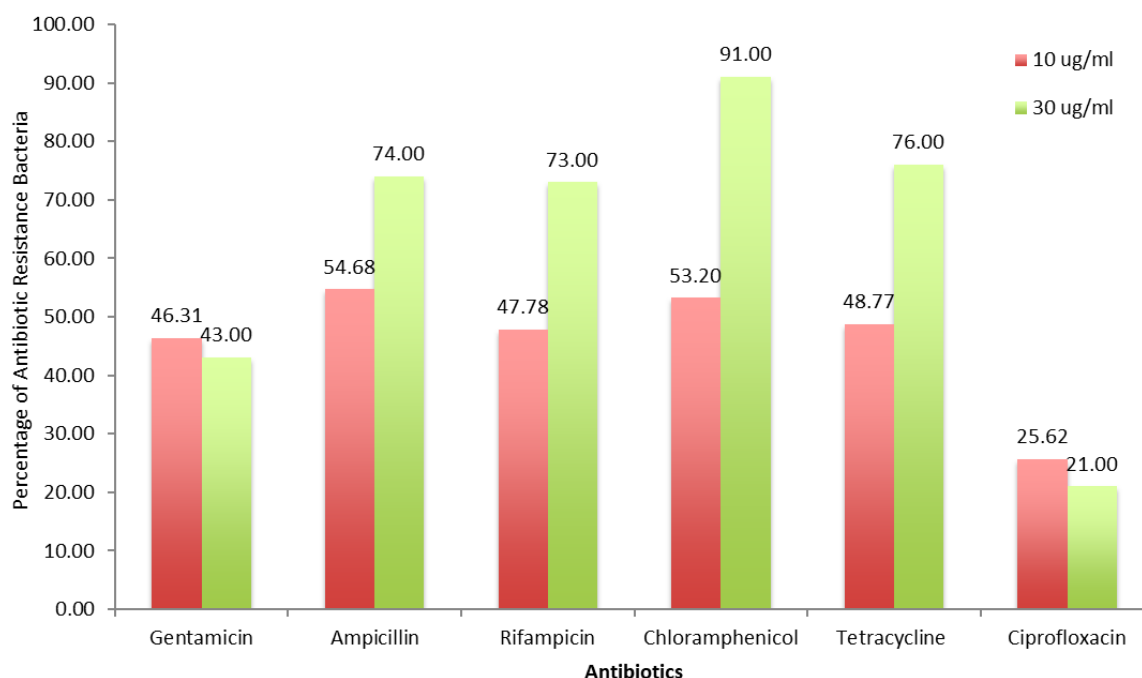


Figure 2. Percentage of isolates resistance towards tested antibiotics

were the most commonly used antibiotics in Malaysia. For example, rifampicin is commonly used to treat several types of bacterial infections, including tuberculosis (TB) and leprosy. Since National TB Control Programme (NTP) in 1961, the case of TB has dropped to below 10 cases [20]. Meanwhile, the launch of National Leprosy Control Centre (NLCC) has reduced leprosy over the years. Due to the relatively small number of TB and leprosy cases, it is expected rifampicin usage would decrease accordingly. Also, the number of resistant strains to rifampicin should be lower than any other strains due to its low usage.

However, from Figure 3, apparently the number isolates that were resistant towards rifampicin

is on par with other antibiotics such as gentamicin, chloramphenicol and ampicillin, in which all of these antibiotics were broad-spectrum. This is particularly true for Selangor, Kuala Lumpur and Terengganu. Others district, including Negeri Sembilan, Melaka, Kedah and Kelantan, use rifampicin in a bigger volume to treat TB and leprosy which indicates increase cases of TB and leprosy in Malaysia (for Selangor and Kuala Lumpur). It could be linked to immigrants from countries that have been declared as TB burden countries by WHO such as Bangladesh, Pakistan and Indonesia [21]. With a total of 783,574 people Selangor and Kuala Lumpur contribute to about 44.6% of total immigrants residing in Malaysia [22].

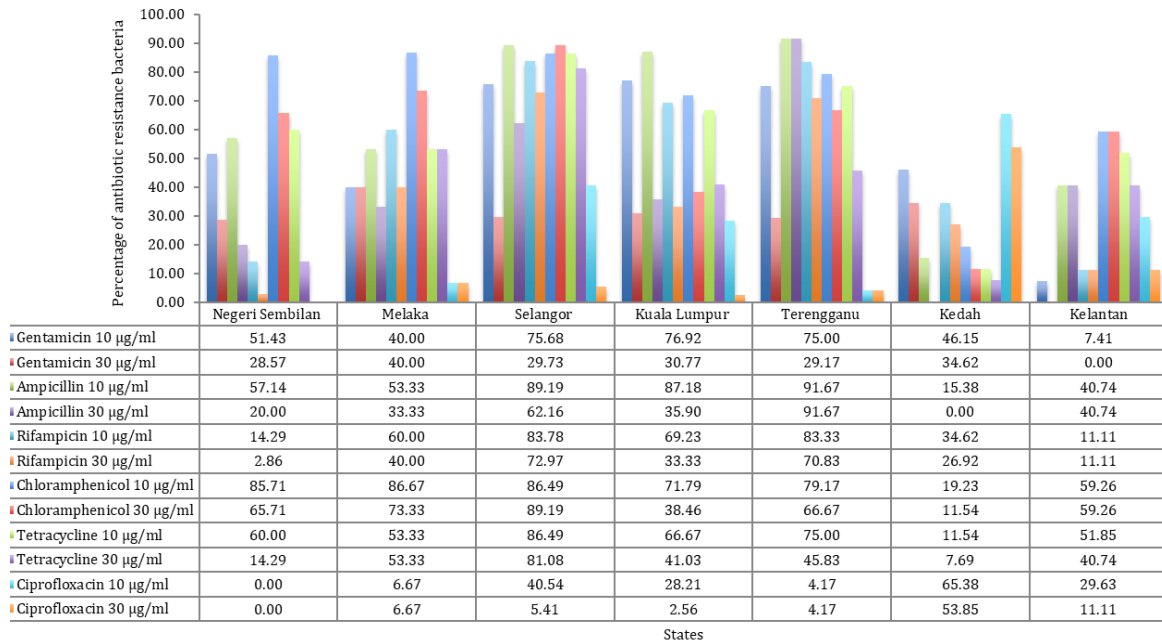


Figure 3. The number of antibiotic resistance bacteria according the sampling area

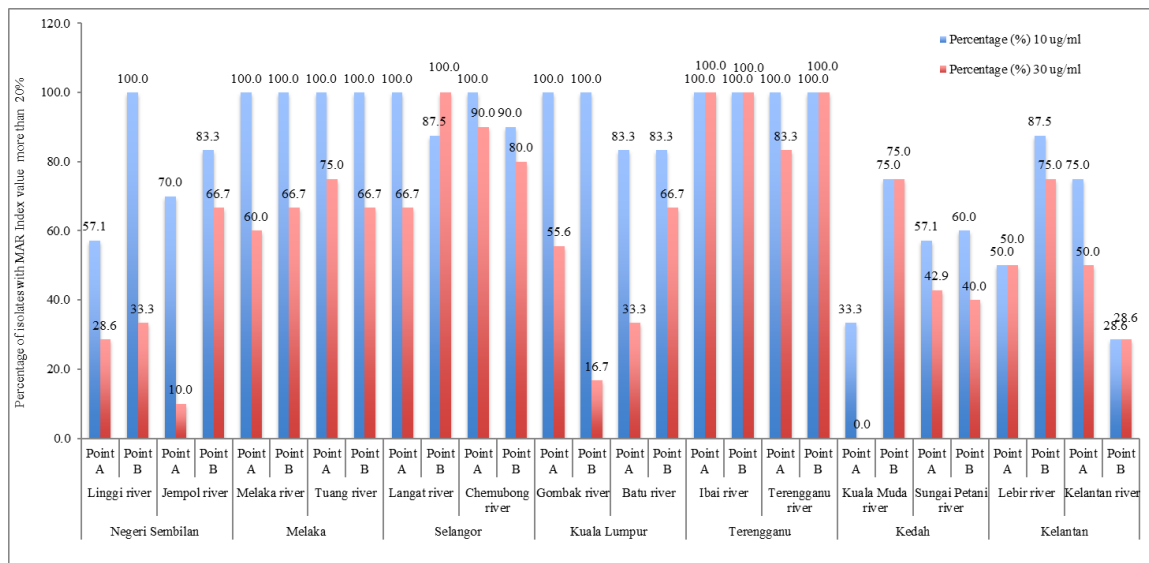


Figure 4. Multiple Antibiotic Resistance (MAR) Index value in sampling area

MAR index value

Figure 4 shows the MAR index for each sampling point in two different rivers of each state. Bacterial isolates from Terengganu scored the highest MAR value, followed by Kuala Lumpur, Selangor, Kelantan, Negeri Sembilan, Melaka and Kedah. The MAR bacteria were observed to have higher resistance for lower concentration of antibiotics. In Terengganu, the isolates showed the highest resistance towards ampicillin (10 and 30 µg/ml) and tetracycline (10 µg/ml). In Melaka, almost similar trends were observed in the highest resistances towards chloramphenicol (10 µg/ml

isolates possessed the highest resistance towards ampicillin (10 µg/ml), followed by 10 µg/ml gentamicin and 10 µg/ml chloramphenicol. The trend is quite different in Selangor as the isolates showed resistance towards 10 µg/ml chloramphenicol followed by chloramphenicol (30 µg/ml) and rifampicin (10 µg/ml). In Negeri Sembilan, the highest resistance was towards chloramphenicol (10 µg/ml), followed by chloramphenicol (30 µg/ml) and tetracycline (10 µg/ml). In Melaka, almost similar trends were observed in the highest resistances towards chloramphenicol (10 µg/ml

and 30 µg/ml) that is followed by resistance towards 10 µg/ml of rifampicin.

Based on Figure 4, most bacterial isolates from both rivers in Negeri Sembilan showed resistance to gentamicin, ampicillin, chloramphenicol, and tetracycline. Only a few of the isolates showed resistance towards ciprofloxacin. No isolates showed resistance towards ciprofloxacin in both rivers in Negeri Sembilan, and only one isolate showed resistance towards this antibiotic in Melaka river, Melaka due to the late introduction (in 1987) of ciprofloxacin in human medication compared to the rest of the tested antibiotics. This might suggest that the bacteria are still developing resistance to this antibiotic

Kuala Lumpur and Selangor have scored the highest percentage of ARB, followed by Negeri Sembilan and Melaka. Increased antibiotic resistance was observed for lower concentration of antibiotics. In Kuala Lumpur, the isolates possessed highest resistance towards ampicillin (10 µg/ml), followed by 10 µg/ml gentamicin and 10 µg/ml chloramphenicol. The trend is quite different in Selangor. The isolates showed 10 µg/ml chloramphenicol resistance, followed by chloramphenicol (30 µg/ml) and rifampicin (10 µg/ml). In Negeri Sembilan, the highest resistance was observed in chloramphenicol (10 µg/ml), followed by chloramphenicol (30 µg/ml) and tetracycline (10 µg/ml). In Melaka, almost similar trends were observed as the highest resistances are towards 10 µg/ml and 30 µg/ml of chloramphenicol. This is followed by resistance towards 10 µg/ml of rifampicin.

At higher concentration, antibiotics exhibited antimicrobial activities on susceptible cells [19]. At lower sub-inhibitory concentrations, the biological response in bacteria will be induced. Bacteria may develop a resistance or tolerance profile if it is exposed to antibiotics at non-lethal concentrations. It could trigger different cellular responses that enable bacteria to defend themselves. Antibiotic-mediated interaction between species may play a substantial role since the microbes are in polymicrobial communities in the natural environment.

Based on the antibiotic susceptibility tests, the MAR index value was calculated for each bacterium to determine the score of each isolate in terms of their ability to resist multiple antibiotics. The MAR index value was calculated using the MAR

equation as mentioned previously in the methodology section. MAR refers to bacteria resistance to at least three different antibiotics [17]. A value of the MAR Index value exceeding 20% represented high risk threats towards the environment [17]. A higher MAR index value is usually observed in areas with a higher accumulation of antibiotics [23, 24].

Identification of the antibiotic resistant bacteria via 16S rRNA gene sequencing

The rRNA gene was used for identification because it is the most conserved region in all cells, even from distantly related organisms, enabling a more precise sequence alignment for easy comparison between the organisms [25]. For this reason, the 16S rRNA genes have been used extensively for the taxonomy, phylogeny, and the rate of divergence determination among the bacterial species [18].

Table 2 (Supplementary 1) showed the 16S rRNA gene sequencing for the chosen isolates. Isolates 1F, 2A and 2L isolated from Linggi River, Negeri Sembilan were *Ralstonia pickettii* strain NRBC 102503, *Ochrobactrum ciceri* strain Ca-34 and *Staphylococcus kloosii* strain ATCC 43959, respectively. As for Jempol River, Negeri Sembilan, isolates 2A and 2L were recognized as *O. ciceri* strain Ca-34 and *Bacillus paramycooides* strain MCCC 1A04098. *O. ciceri* strain Ca-34 isolates Jempol and Linggi River, which showed resistance towards different antibiotics. In addition, isolates with more than 50% MAR Index value can be found at point 2 (Linggi River) and point 4 (Jempol River).

Results also showed that isolates M5 and M6 from Melaka River, Melaka were identified as *Burkholderia vietnamiensis* strain TVV75 and *Enterobacter xiangfangensis* strain 10-17, respectively. As for Tuang River, Melaka isolate T1 – T4 were identified as *E. xiangfangensis* strain 10-17, *Escherichia marmotae* strain HT073016, *Citrobacter freundii* strain ATCC 8090 and *Bacillus wiedmannii* strain FSL W8-0169, respectively. The common isolates from these rivers also showed resistance towards different antibiotics.

In Langkat River, Selangor, isolates LA6, LA7 and LB4 were identified as *Staphylococcus hominis* subsp. *novobiosepticus* strain GTC 1228, *Vogesella perlucida* strain DS-28 and *Burkholderia contaminans* strain J2956, respectively. Meanwhile, isolates CA4, CA8, CA9, CB5, CB6

and CB10 were identified as *B. vietnamiensis* strain LMG 10929, *B. contaminans* strain J2956, *B. contaminans* strain J2956, *Bacillus cereus* ATCC 14579, *B. vietnamiensis* strain LMG 10929 and *Pseudomonas otitidis* strain MCC10330, respectively. In terms of geographical distribution, *B. contaminans* strain J2956 was found in both Langat and Chemubong river. The strain isolated from the same point at Chemubong river also showed resistance towards different antibiotics.

As for Gombak river, Kuala Lumpur, isolates A1, and B1 were identified as *P. plecoglossicida* strain NBRC 103162. Meanwhile, A3 and B4 were identified as, *Acinetobacter baumannii* strain DSM 30007 and *Pseudomonas plecoglossicida* strain NBRC 103162, respectively. Even though A1 and B1 were identified as the same bacteria, these two strains may show resistance towards different antibiotics. Based on this study, A1 and B1 showed resistance towards different types of antibiotics. This is because the same strain was exposed to different antibiotics for a certain period of time as bacteria will develop resistance to counteract the effect of antibiotics that it was exposed. There is also a possibility that the susceptible isolates are still developing resistance towards the other type of antibiotics [26]. Meanwhile, from Batu river, Kuala Lumpur, isolate D6 and D8 both were identified as *Acinetobacter baumannii* strain DSM 30007 and *Ralstonia pickettii* strain NBRC 102503.

For Kedah, isolates 2B, 2C and 2D from Kuala Muda river, were identified as *Bacillus wiedmannii* strain FSL W8-0169, *Lysinibacillus fusiformis* strain DSM 2898 and *Atlantibacter hermannii* strain CIP 10376, respectively. Meanwhile, in Sungai Petani, isolate 3D, 4B and 4E were identified as *B. cereus* ATCC 14579, *Achromobacter insuavis* strain LMG 26845 and *Enterobacter cloacae* strain LMG 2683, respectively.

In Kelantan, isolate 2C from Lebir river was identified as *Bacillus licheniformis* strain DSM 13. Isolates 3G, 3H and 4B isolated from Kelantan river were identified as *Microbacterium testaceum* strain DSM 20166, *Lysinibacillus macroides* strain LMG 18474 and *B. cereus* ATCC 14579, respectively.

In Ibai river, Terengganu, isolates 2A, 2B, 2C and 2I were identified as *Chromobacterium violaceum* strain ATCC 12472, *Enterobacter tabaci* strain YIM Hb-3, *B. cereus* ATCC 14579 and *A. baumannii* strain DSM 30007, respectively.

Meanwhile in Terengganu river, isolates 3A, 3B, 4A, 4C, 4D and 4E were identified as *Klebsiella quasipneumoniae* subsp. *similipneumoniae* strain 07A044, *Chromobacterium aquaticum* strain CC-SEYA-1, *Cupriavidus metallidurans* strain CH34, *Weeksella massiliensis* strain FF8, *Cupriavidus necator* strain N-1 and *Acinetobacter nosocomialis* strain RUH 2376, respectively. Isolate 4A seems to be an important bacterium in antibiotic resistance gene transfer as it is a metal resistance bacterium and the correlation between metal resistance bacteria and antibiotic resistance gene transfer has been proposed [27].

As depicted in Table 2 (Supplementary 1), the isolates comprised pathogenic and non-pathogenic bacteria. A study conducted by von Wintersdorff et al. [28] concluded that many clinically relevant resistance genes could originate from non-pathogenic bacteria. This could happen via horizontal gene transfer (HGT) that causes antibiotic resistance to spread from commensal and environmental species to pathogenic ones. These commensal strains could act as vectors in spreading the ARGs among both the pathogenic and the pathogenic bacteria. Therefore, it is possible that the pathogenic bacteria which originally non-resistant could also develop antibiotic resistance due to prolonged exposure to ARGs and their vectors in a resistome or ARGs reservoir.

Conclusion

A total of 203 bacteria have been successfully isolated from 14 rivers in 7 different states of Western Peninsular Malaysia (Negeri Sembilan, Melaka, Selangor, Kuala Lumpur, Kedah, Kelantan and Terengganu). About 57.6% of these isolates showed MAR Index value of more than 20%. Selangor demonstrated the highest percentage of ARB. This suggested that the rivers in Selangor (particularly Chemubong river) are highly contaminated with antibiotics. Based on 16s rRNA gene sequencing, the isolates obtained were from a mixture of pathogenic and commensals bacteria. This also implies that the environment, especially rivers can be a reservoir for genetic jugglery, including the transfer of antibiotic resistance genes.

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Supplementary 1

Table 2. Details of 16s rRNA gene sequencing for the chosen isolates

States	Rivers	Iso-lates	Base pairs	e-value	Query cover (%)	Accession number	MAR Index Value (%)	Identification Percentage	Pathogenicity
Negeri Sembilan	Linggi	1F	1399	0.0	100	MK282223	50.0	<i>Ralstonia pickettii</i> strain NRBC 102503 100%	Nasocomial infection [29]
		2A	1392	0.0	99	MK282224	83.3	<i>Ochrobactrum ciceri</i> strain Ca-34 97%	-
		2L	1413	0.0	99	MK282225	50.0	<i>Staphylococcus kloosii</i> strain ATCC 43959 99%	-
	Jempol	4B	1387	0.0	99	MK282226	50.0	<i>Ochrobactrum ciceri</i> strain Ca-34 97%	-
		4C	1397	0.0	100	MK351231	50.0	<i>Bacillus paramycoides</i> strain MCCC 1A04098 100%	-
Melaka	Melaka	M5	1305	0.0	100	MK282227	50.0	<i>Burkholderia vietnamiensis</i> strain TVV75 100%	Opportunistic pathogen in patients with cystic fibrosis [30]
		M6	1344	0.0	100	MK282228	66.7	<i>Enterobacter xiangfangensis</i> strain 10-17 100%	-
	Tuang	T1	1149	0.0	100	MK282229	83.3	<i>Enterobacter xiangfangensis</i> strain 10-17 99%	-
		T2	1149	0.0	100	MK282230	66.7	<i>Escherichia marmotae</i> strain HT073016 99%	-
		T3	1383	0.0	100	MK282231	50.0	<i>Citrobacter freundii</i> strain ATCC 8090 99%	Related to chronic complicated urinary tract infection [31]
		T4	1397	0.0	100	MK282232	83.3	<i>Bacillus Wiedmannii</i> strain FSL W8-0169 99%	Gastrointestinal illness [32]
Langkat	LA6	1394	0.0	100	MK282214	83.3	<i>Staphylococcus hominis</i> subsp. <i>novobiosepticus</i> strain GTC 1228 99%	Causative agent of septicaemia in cancer patients	
	LA7	1396	0.0	98	MK282215	83.3	<i>Vogesella perlucida</i> strain DS-28 98%	-	
	LB4	1378	0.0	100	MK282216	83.3	<i>Burkholderia contaminans</i> strain J2956 99%	Opportunistic pathogens that lead to debilitating lung infections with a high risk of developing fatal septicemia in cystic fibrosis (CF) patients [34]	
Selangor	Chemubong	CA4	1366	0.0	100	MK282217	50.0	<i>Burkholderia vietnamiensis</i> strain TVV75 100%	See M5
		CA8	1381	0.0	100	MK282218	50.0	<i>Burkholderia contaminans</i> strain J2956 99%	See LB416
		CA9	1385	0.0	100	MK282219	100.0	<i>Burkholderia contaminans</i> strain J2956 99%	See LB4
	Chemubong	CB5	1372	0.0	100	MK282220	100.0	<i>Bacillus cereus</i> ATCC 14579 100%	Opportunistic pathogen causing food poisoning manifested by diarrhoeal or emetic syndromes [35]
		CB6	1365	0.0	100	MK282221	83.8	<i>Burkholderia vietnamiensis</i> strain TVV75 100%	See M5
		CB10	1375	0.0	100	MK282222	83.3	<i>Pseudomonas otitidis</i> strain MCC10330 99%	Association with otic infections in humans [36]
Kuala Lumpur	Gombak	A1	1384	0.0	100	MK282210	50.0	<i>Pseudomonas plecoglossicida</i> strain NBRC 103162 99%	Causative agent of bacterial haemorrhagic ascites of ayu, <i>Plecoglossus altivelis</i> [37]
		A3	1357	0.0	100	MK282211	66.7	<i>Acinetobacter baumannii</i> strain DSM 30007 99%	Nasocomial infection [38]
		B1	1385	0.0	100	MK282212		<i>Pseudomonas plecoglossicida</i> strain NBRC 103162 99%	See A1

Continue...

States	Rivers	Iso-lates	Base pairs	e-value	Query cover (%)	Accession number	MAR Index Value (%)	Identification Percentage	Pathogenicity
Kedah	Batu	B4	1159	0.0	100	MK351228	66.7	<i>Pseudomonas taiwanensis</i> strain BCRC 17751 100%	-
		B7	1385	0.0	100	MK351229		<i>Acinetobacter baumannii</i> strain DSM 30007 99%	See A3
		D6	1390	0.0	100	MK351230	83.8	<i>Acinetobacter baumannii</i> strain DSM 30007 99%	See A3
		D8	1381	0.0	100	MK282213	50.0	<i>Ralstonia pickettii</i> strain NBRC 102503 99%	See 1F
	Kuala Muda	2B	1362	0.0	100	MN117669	33.0	<i>Bacillus wiedmannii</i> strain FSL W8-0169 100%	See T4
		2C	2525	0.0	99	MN598655	33.0	<i>Lysinibacillus fusiformis</i> strain DSM 2898 98.59%	Tropical ulcers, severe sepsis, and respiratory illnesses [39]
		2D	1388	0.0	100	MN117670	50	<i>Atlantibacter hermanii</i> strain CIP 10376 100%	-
		3D	1367	0.0	100	MN117671	33	<i>Bacillus cereus</i> ATCC 14579 99.7%	Diarrhoeal disease [40]
Sungai Petani	4B	1319	0.0	100	MN117672	33	<i>Achromobacter insuavis</i> strain LMG 26845 97.19%	-	
	4E	1349	0.0	99	MN117673	33	<i>Enterobacter cloacae</i> strain LMG 2683 99.85%	-	
	2C	1430	0.0	100	MN117660		<i>Bacillus licheniformis</i> strain DSM 13 92.73%	See 1D	
	3G	1402	0.0	99	MN117661	50.0	<i>Microbacterium testaceum</i> strain DSM 20166 97.08%	Food poisoning, gastroenteritis, meningitis, septicemia, gingival and ocular infection [41]	
Kelantan	3H	1436	0.0	98	MN117662	50.0	<i>Lysinibacillus macroides</i> strain LMG 18474 96.6%	-	
	4B	1438	0.0	99	MN117663	50.0	<i>Bacillus cereus</i> ATCC 14579 96.59%	Food poisoning, gastroenteritis, meningitis, septicemia, gingival and ocular infection [41]	
	2A	1301	0.0	100	MN117664	33.0	<i>Chromobacterium violaceum</i> strain ATCC 12472 99.2%	Neonatal septicemia infection [42]	
Ibai	2B	1799	0.0	99	MN598656	67.0	<i>Enterobacter tabaci</i> strain YIM Hb-3 96.59%	-	
	2C	2623	0.0	99	MN598657	33.0	<i>Bacillus cereus</i> ATCC 14579 99.72%	Food poisoning [43]	
	2I	1290	0.0	100	MN117674	33.0	<i>Acinetobacter baumannii</i> strain DSM 30007 100%	See A3	
Terengganu	3A	2519	0.0	100	MN598658	67.0	<i>Klebsiella quasipneumoniae</i> subsp. <i>similipneumoniae</i> strain 07A044 99.42%	Human and animal infection [44]	
	3B	1303	0.0	100	MN117665	50.0	<i>Chromobacterium aquaticum</i> strain CC-SEYA-1 100%	-	
	4A	1297	0.0	100	MN117666	50.0	<i>Cupriavidus metallidurans</i> strain CH34 100%	Metal-resistance bacterium [45]	
	4C	2501	0.0	100	MN598659	33.0	<i>Weeksella massiliensis</i> strain FF8 99.93%	-	

Continue...

States	Rivers	Iso-lates	Base pairs	e-value	Query cover (%)	Accession number	MAR Index Value (%)	Identification Percentage	Pathogenicity
		4D	1318	0.0	100	MN117667	67.0	<i>Cupviadus necator</i> strain N-1 100%	-
		4E	1313	0.0	100	MN117668	67.0	<i>Acinetobacter nosocomialis</i> strain RUH 2376 100%	Nasocomial pneumonia [46]

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