Research article

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Isolation of Antibiotic Resistant Bacteria from Rivers in Kelantan, Malaysia

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ABSTRACT

Antibiotic resistant bacteria have been a major concern due to the problems that they may pose in terms of public and environmental health. This present study aimed to investigate the presence of antibiotic resistance among bacterial strains isolated from rivers and to determine the impact of various human activities on the distribution of antibiotic resistant bacteria. Bacteria were isolated from river water samples of Lebir River (Point 1 and Point 2) and Kelantan River (Point 3 and Point 4). A total of 27 bacterial isolates were successfully isolated from the rivers, followed by characterization and identification of the isolates using a series of biochemical tests. All the isolated bacteria were predicted as *Actinomyces* sp., *Arachnia* sp., *Streptomyces* sp., *Streptococcus* sp., *Enterococcus* sp., *Bacillus* sp., *Clostridium* sp., *Neisseria* sp., *Veillonella* sp., *Bacteroides* sp., *Salmonella* sp., *Shigella* sp., *Proteus* sp., *Enterobacter* sp., *Klebsiella* sp., *Escherichia* sp, *Pseudomonas* sp., *Aeromonas* sp., *Vibrio* sp. *Staphylococcus* sp., *Micrococcus* sp., *Actinobacillus* sp., and *Flavobacterium* sp. Antibiotic susceptibility test was conducted on the bacterial isolates against six types of antibiotics (ampicillin, gentamycin, tetracycline, chloramphenicol, rifampicin and ciprofloxacin). Almost all bacterial isolates were resistant to at least one type of antibiotic, whereas more than half of the isolates showed multiple antibiotic resistance. The findings show the presence of antibiotic resistant bacteria in all four samples, indicating the relationship between the resistance of isolates and the human activities within the vicinity of the area. However, further analysis of the antibiotic resistant bacteria is recommended to further identify and understand them due to the potential risk that they may pose to human and environment.

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Introduction

Antibiotic resistant bacteria potentially pose to the public and environmental health, serve as a major concern in the society. The presence of antibiotic resistant bacteria especially in the waterways, for instance a river, may lead to horrible consequences as rivers are commonly used by the nearby residents as water sources for their daily activities. One of the negative consequences would be in the treatment of life-threatening illnesses [1], as the disease-causing bacteria are unable to be combatted with the use of antibiotics.

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Manyi-Loh et al. projected that the intensive farming and antibiotic use in agriculture results in antibiotic residues in the environments. The antibiotic residues and antibiotic-resistant bacteria are considered as environmental pollutants and responsible for the increment of public health crisis linked to antibiotic-resistant bacteria [2]. Review from Martinez and Berendonk et al. reported that the extensive use of antibiotics in human treatment, animal farming and agriculture results in residues that contain antibiotics resistant bacteria that have the potential to pollute the natural environments. In these reviews, stated that apart of its utilization for human therapy, antibiotics are extensively used for agricultural purposes for animal farming. Residues from farms and human environments may contain antibiotics and antibiotic resistance genes that have the potential to be a contaminant natural environment.[3, 4].

Nnadozie and Odume reviewed that the freshwater environments are subjected to possible contamination by residual antibiotics that makes the freshwater environments to become reservoirs and pose public health concern [5]. Al-Badaii and Othman stated that in both polluted and unpolluted surface water, antibiotic resistant bacteria are present in surface water, with the quantity of antibiotic resistant bacteria isolated from polluted water exceeded that of the latter. The inability to assess the quality of the freshwater and avoid future disease outbreaks associated to contaminated water utilization are possibly the impacts when the complete picture of the distribution and frequency of antibiotic resistance in the environment is lacking [6]. As the aquatic ecosystem health pose major impact on all terrestrial ecosystems including humans, animals, and plants, the measurement of water quality variables and heavy metals alone may not be sufficient to assess a river. Residual antibiotics in the freshwater are possibly from different sources such as agriculture, wastewater discharge and animal farming. The residual antibiotics serve as the selective pressure and main concern for the development of antibiotic resistant bacteria. Hence, the measurement of concentration of antibiotics in the natural water is important [7].

Therefore, the present study was conducted to assess the presence of antibiotic resistant bacteria in Kelantan River and Lebir River in Kelantan, by first isolating the antibiotic resistant bacteria from the rivers. Biochemical tests were also done to characterize and identify the antibiotic resistant bacteria that present in the selected rivers, followed by conducting the antibiotic susceptibility test for the bacteria isolates towards the chosen antibiotics; gentamicin, ampicillin, rifampicin, chloramphenicol, tetracycline, and ciprofloxacin.

Material and Methods

Study site and sampling

The sampling process took place at the two selected rivers in Kelantan which were Lebir River and Kelantan River. The sampling sites chosen from the Lebir River was in the Gua Musang district, located near to agricultural area at the coordinate of (4.870728, 102.438744) (Point 1) and the other site was at the residential area at the coordinate of (4.932378, 102.418298) (Point 2).

In a meanwhile, another river that had been selected was Kelantan River, around Kuala Krai area and Tangga Krai area. The first sampling site selected from the Kelantan River was located near to the medical centers area in Kuala Krai at the coordinate of (5.530988, 102.195158) (Point 3) while the second sampling site from Kelantan River was located at Tangga Krai. This sampling site was located near to the residential area at the coordinate of (5.612043, 102.144881) (Point 4). An amount of 500 ml of water sample were taken from surface water of both rivers at two different places each in November 2018 .The water samples collected in sterile glass bottles and the samples then were put in an ice box to maintain its temperature at 4˚C while being transferred to laboratory and stored in the cold room until further analysis [8].

Isolation and Characterization of bacterial isolate by Biochemical Test

A series of serial dilution of water sample was prepared. Then, 1 ml of the diluted water samples was spread on the nutrient agar. The plates were then incubated at 37˚C for 24 hours. The single colonies of different morphology from the spread plates was streaked for subcultures on nutrient agar to obtain the pure colonies of the bacterial isolates. The subcultures of the bacterial pure colonies isolated were characterized based on their morphological characteristics. Then, Gram staining will be performed to further characterize them into Gram negative and Gram positive bacteria [9]. The isolated bacteria were further characterized by the biochemical tests since that bacterial physiology are different from one species to another. In order to determine the biochemical characteristics of bacteria, the biochemical tests performed were catalase, indole, McConkey agar, urease, mannitol salt agar, citrate test, and oxidase test. The results of the biochemical tests were then compared to Bergey's Manual of Determinative Bacteriology to predict the possible bacteria species based on the biochemical activities of the bacteria [10,11].

Antibiotic susceptibility test

In order to determine the antibiotic susceptibility profiles of the bacterial isolates, the bacterial isolates were tested by using the standard Kirby-Bauer disk diffusion method. The antibiotic susceptibility testing was performed by using Mueller-Hinton agar against six types of antibiotics, which are gentamicin, ampicillin, rifampicin, chloramphenicol, tetracycline, and ciprofloxacin. These six antibiotics are the broad-spectrum antibiotics which can be used for both Gram negative and Gram positive bacteria. Each of the antibiotics disk had two different concentrations which were 10 and 30 μg/ml per disk. The freshly grow bacterial isolates were suspended in 4 to 5 ml of sterile nutrient broth for the bacterial inoculum preparation. The turbidity of the broth was adjusted to 0.5 McFarland standards and using sterile cotton swab, it was spread on Mueller-Hinton agar plates. The different antibiotic impregnated disks were placed over the freshly prepared agar (which already had bacterial cultures on it) at appropriate distances from one another. Then, all of the plates were incubated at 37˚C for 24 hours [12]. The zone of inhibition was measured and compared with Clinical and Laboratory Standard Institutes (CLSI) guidelines to classify the bacterial colonies as resistant or susceptible to the antibiotics [13].

Multiple Antibiotic Resistant (MAR) Index Value

Multiple antibiotic resistant (MAR) index was calculated for each of the bacterial isolates and the antibiotic resistant bacteria that have the MAR index for more than 20% were selected for further analysis. MAR index were calculated using the formula a/b, in which, a is the total number of antibiotics to which the organism is resistant, and b is the total number of antibiotics to which the organism is tested [14]. In this study, the antibiotics used were gentamicin, ampicillin, rifampicin, chloramphenicol, tetracycline, and ciprofloxacin. Equation below explains how the MAR index was calculated for each of the bacteria isolated in this study.

$$MAR\ Index = \frac{total\ number\ of\ antibiotics\ to\ which\ the\ organism\ is\ resistant\ (a)}{total\ number\ of\ antibiotics\ to\ which\ the\ organism\ is\ tested\ (b)}$$

Results and Discussion

Isolation and Characterization of Bacterial Strain from Both Rivers Water Samples

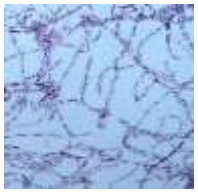
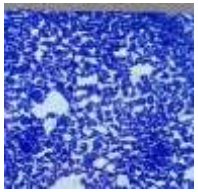
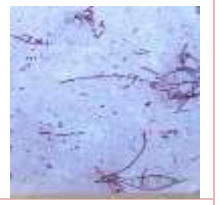
The four river water samples were collected, including Lebir River (Point 1, Point 2) that located near to agricultural area and the residential area, and Kelantan River (Point 3, Point 4) that located near to the medical centers area and residential area (Figure 1). A total of 27 pure colonies were managed to be isolated from the samples from both of the rivers. From Lebir River, 4 and 8 pure colonies were isolated at Point 1 and Point 2, respectively. Meanwhile, from Kelantan River, 8 and 7 pure colonies were successfully isolated from Point 3 and Point 4, respectively. Table 1 shows the morphology characteristics of the bacterial colonies isolated from Lebir River and Kelantan River whereas Table 2 shows the bacterial colonies isolated from both Lebir River and Kelantan River with the Gram stains viewed under 100X magnification. Table 3 tabulates the biochemical tests conducted for all the isolates collected from Lebir River and Kelantan River. Based on the table, it can be concluded that the population for different point in the same river may differ from one another. The human activities near the sampling areas might have contributed to the populations of the bacteria present in both rivers.

Fig 1 Sampling location of Lebir River (Point 1, Point 2) and Kelantan River (Point 3, Point 4)

Table 1 Morphology characteristics of bacterial colonies isolated from Lebir and Kelantan River

Lebir River						Kelantan River					
Isolate	**Form**	**Elevation**	**Opacity**	**Colour**	**Surface**	**Isolate**	**Form**	**Elevation**	**Opacity**	**Colour**	**Surface**
Point 1						**Point 3**					
1A	Irregular	Flat	Opaque	Whitish	Rough	3A	Circular	Raised	Opaque	Light purple	Smooth, glistening
1B	Circular	Flat	Opaque	Yellowish	Smooth	3B	Circular	Raised	Translucent	Yellow	Smooth
1C	Irregular	Flat	Opaque	Whitish	Smooth	3C	Irregular	Flat	Opaque	Whitish	Smooth
1D	Irregular	Umbonate	Opaque	Whitish	Rough	3D	Circular	Raised	Opaque	Yellowish	Smooth
Point 2						3E	Circular	Raised	Opaque	Yellowish	Rough
2A	Circular	Raised	Opaque	Dark purple	Smooth	3F	Circular	Raised	Translucent	Yellowish	Smooth, glistening
2B	Circular	Raised	Opaque	Whitish	Smooth	3G	Irregular	Flat	Opaque	Whitish	Rough
2C	Irregular	Crateriform	Opaque	Whitish	Rough	3H	Circular	Flat	Opaque	Whitish	Smooth
2D	Irregular	Flat	Opaque	Whitish	Smooth, glistening	**Point 4**					
2E	Irregular	Flat	Opaque	Whitish	Smooth	4A	Circular	Raised	Opaque	Dark purple	Smooth
2F	Circular	Raised	Translucent	Yellowish	Smooth, glistening	4B	Circular	Raised	Translucent	Whitish	Smooth
2G	Circular	Raised	Opaque	Yellowish	Smooth	4C	Irregular	Flat	Opaque	Whitish	Smooth
2H	Circular	Raised	Opaque	Yellow	Smooth	4D	Circular	Raised	Translucent	Whitish	Smooth
						4E	Circular	Raised	Opaque	Whitish	Smooth
						4F	Irregular	Flat	Translucent	Whitish	Smooth
						4G	Circular	Raised	Translucent	Yellowish	Smooth

Table 2 The bacterial colonies isolated from both Lebr River and Kelantan River with the Gram stains viewed under 100X magnification

Lebir River						Kelantan River					
Point 1			Point 2			Point 3			Point 4		
Strain		Gram Stain	Strain		Gram Stain	Strain		Gram Stain	Strain		Gram Stain
1A			2A			3A			4A		
1B			2B			3B			4B		
1C			2C			3C			4C		
1D			2D			3D			4D		

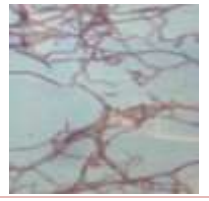
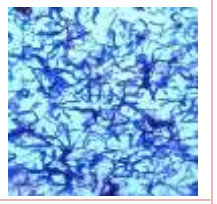
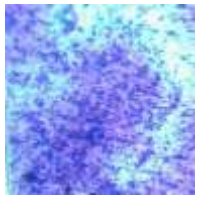
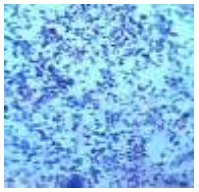
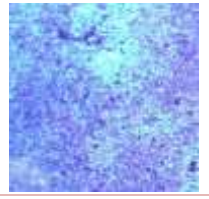
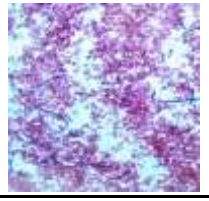
			2E			3E			4E		
			2F			3F			4F		
			2G			3G			4G		
			2H			3H					

Table 3 Biochemical tests conducted for isolates from Lebir River and Kelantan River

Lebir River																	
Point 1									Point 2								
Strain	Biochemical Test							Conclusion (Family Type)	**Strain**	Biochemical Test							Conclusion (Family Type)
	Catalase	Citrate	McConkey Agar	Indol	Urease	Oxidase	Mannitol Salt Agar			Catalase	Citrate	Mc Conkey Agar	Indol	Urease	Oxidase	Mannitol Salt Agar	
1A	-	-	-	-	+	+	-	*Actinomyces* sp. *Arachnia* sp. *Streptomyces* sp.	**2A**	-	-	+	-	-	+	-	*Bacteroides* sp. *Salmonella* sp. *Shigella* sp.
1B	-	-	-	-	+	+	-	*Streptococcus* sp. *Enterococcus* sp	**2B**	-	-	+	-	+	+	-	*Proteus* sp.
1C	-	-	-	-	-	+	-	*Bacillus* sp. *Clostridium* sp.	**2C**	-	-	+	-	-	-	+	*Neisseria* sp. *Veillonella* sp.
1D	+	-	+	-	-	+	+	*Neisseria* sp. *Veillonella* sp.	**2D**	-	-	+	-	-	-	+	*Salmonella* sp. *Shigella* sp.

Kelantan River																	
Point 3									Point 4								
Strain	Biochemical Test							Conclusion (Family Type)	**Strain**	Biochemical Test							Conclusion (Family Type)
	Catalase	Citrate	Mc Conkey Agar	Indol	Urease	Oxidase	Mannitol Salt Agar			Catalase	Citrate	Mc Conkey Agar	Indol	Urease	Oxidase	Mannitol Salt Agar	
3A	+	+	+	-	+	+	+	*Neisseria* sp. *Veillonella* sp.	**4A**	-	+	+	-	-	+	-	*Vibrio* sp. *Pseudomonas* sp. *Aeromonas* sp.
3B	-	-	+	-	-	-	-	*Enterobacter* sp. *Klebsiella* sp.	**4B**	-	+	+	-	-	+	-	*Neisseria* sp. *Veillonella* sp.
3C	-	-	-	-	-	-	-	*Streptococcus* sp. *Enterococcus* sp.	**4C**	+	+	+	-	-	+	+	*Bacteroides* sp. *Actinobacillus* sp. *Flavobacterium* sp.
3D	-	-	-	-	-	+	-	*Staphylococcus* sp. *Streptococcus* sp. *Enterococcus* sp.	**4D**	-	-	+	-	-	-	-	*Neisseria* sp. *Veillonella* sp.

									2E	+	-	+	-	-	-	+	*Enterobacter* sp. *Klebsiella* sp.	**3 E**	+	-	+	-	+	-	-	*Actinomyces* sp. *Streptomyces* sp.	**4 E**	-	-	+	-	+	+	-	*Actinomyces* sp. *Streptomyces* sp.
									2F	-	-	+	+	-	+	+	*Escherichia* sp.	**3 F**	-	+	-	-	-	+	-	*Streptococcus* sp. *Micrococcus* sp.	**4 F**	+	+	+	-	+	-	-	*Micrococcus* sp. *Staphylococcus* sp.
									2G	-	+	+	-	-	+	-	*Pseudomonas* sp. *Aeromonas* sp. *Vibrio* sp.	**3 G**	-	-	+	-	-	+	+	*Staphylococcus* sp. *Micrococcus* sp.	**4 G**	-	-	+	-	+	+	-	*Vibrio* sp. *Pseudomonas* sp. *Aeromonas* sp.
									2H	-	-	-	-	+	+	-	*Pseudomonas* sp. *Aeromonas* sp. *Vibrio* sp.	**3 H**	+	-	+	-	+	+	-	*Neisseria* sp. *Veillonella* sp.									

Antibiotic Susceptibility Testing

The result obtained for the antibiotic susceptibility tests using disk diffusion method were as listed in Table 4 and Table 5. Based on the results obtained, out of 27 bacterial isolates, almost all bacterial isolates were resistant to at least one type of antibiotic, whereas more than half of the isolates showed multiple antibiotic resistance (resistance to more than one type of antibiotics).

Among all of the species predicted, only *Neisseria* sp. and *Veillonella* sp. that could be found in both Point 1 and Point 2, however at different population sizes. The population sizes of both species were higher in Point 1, which may indicate that the niche is more suitable for the species to live in. With that apart, the bacterial population for both sampling points were different, probably due to the higher nitrogen, potassium and heavy metals from the fertilizers used for the agriculture (Point 1) and other elements from human activities (Point 2), which made the river water there unsuitable for certain species to inhabit. On the other hand, *Pseudomonas* sp., *Actinobacillus* sp., *Flavobacterium* sp., *Aeromonas* sp., *Vibrio* sp. and *Bacteroides* sp. were found at Point 4 (residential area) but not at Point 3 (medical centres area), which might indicate that those species were not resistant to the antibiotic residues that present in the river water of Point 3, hence could not survive as well as they could at Point 4.

Lebir River and Kelantan River were chosen for this study because there is a concern of Kelantan River being polluted due to the various human activities within the vicinity of the area [15]. In the other hand, Lebir River is claimed to have a good water quality despite the fact that Lebir River is not completely free from human activities either, hence there is still a possibility that both rivers are contaminated with antibiotic residues which lead to the prevalence of antibiotic resistant bacteria [16]. With regard of the pollution status of the rivers, this study had found that antibiotic resistant bacteria are present in both polluted river (Kelantan River) and unpolluted river (Lebir River), which is similar to what Al-Badaii and Othman (2015) reported [6].

Table 4 Antibiotic susceptibility tests for the bacterial colonies isolated from Point 1 and Point 2 of Lebir River

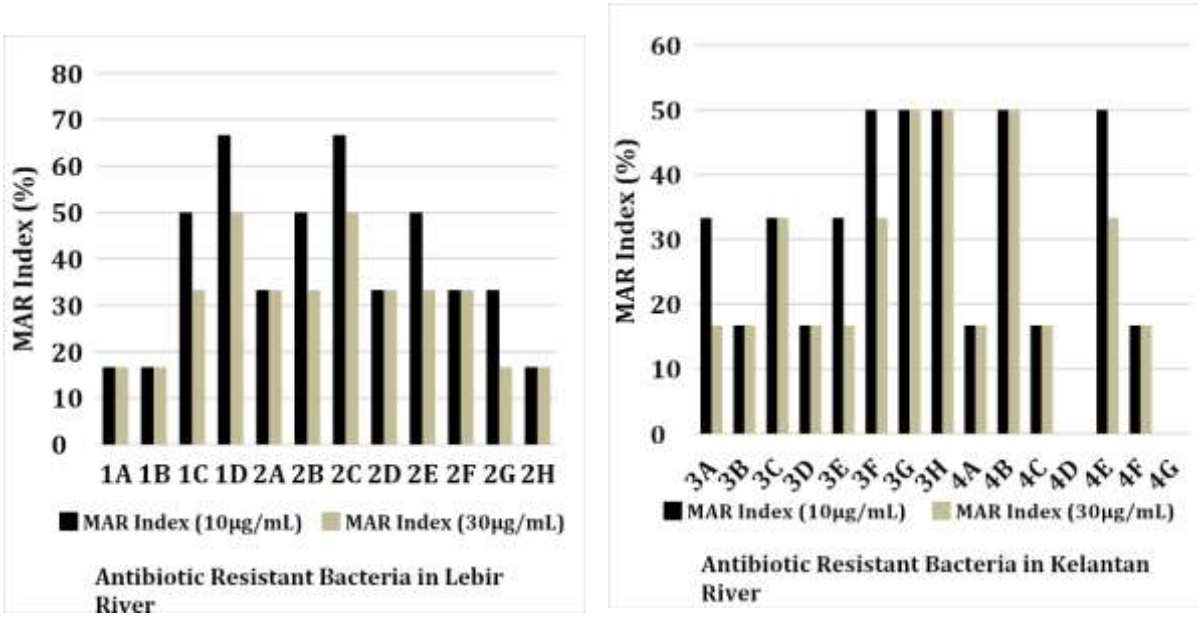
	Antibiotics	**Ampicillin**		**Gentamycin**		**Chloramphenicol**		**Tetracycline**		**Rifampicin**		**Ciprofloxacin**	
	Bacterial colonies	**10 µg/ml**	**30 µg/ml**	**10 µg/ml**	**30 µg/ml**	**10 µg/ml**	**30 µg/ml**	**10 µg/ml**	**30 µg/ml**	**10 µg/ml**	**30 µg/ml**	**10 µg/ml**	**30 µg/ml**
Point 1	1A	R	R	S	S	S	S	S	S	S	S	S	S
	1B	S	S	S	S	S	S	R	R	S	S	S	S
	1C	R	R	S	S	R	R	S	S	S	S	R	S
	1D	R	R	R	S	R	R	R	R	S	S	S	S
Point 2	2A	R	R	S	S	R	R	S	S	S	S	S	S
	2B	R	R	S	S	S	S	R	R	S	S	R	S
	2C	R	R	S	S	R	R	R	R	S	S	R	S
	2D	S	S	S	S	R	R	S	S	R	R	S	S
	2E	S	S	S	S	R	R	R	R	S	S	R	S
	2F	R	R	S	S	S	S	S	S	R	R	S	S
	2G	S	S	S	S	S	S	R	R	S	S	R	S
	2H	R	R	S	S	S	S	S	S	S	S	S	S

Table 5 Antibiotic susceptibility test for the bacterial colonies isolated from Point 3 and Point 4 at Kelantan River

	Antibiotics	**Ampicillin**		**Gentamycin**		**Chloramphenicol**		**Tetracycline**		**Rifampicin**		**Ciprofloxacin**	
	Bacterial colonies	**10 µg/ml**	**30 µg/ml**	**10 µg/ml**	**30 µg/ml**	**10 µg/ml**	**30 µg/ml**	**10 µg/ml**	**30 µg/ml**	**10 µg/ml**	**30 µg/ml**	**10 µg/ml**	**30 µg/ml**
Point 3	3A	S	S	R	S	R	R	S	S	S	S	S	S
	3B	S	S	S	S	R	R	S	S	S	S	S	S
	3C	S	S	S	S	R	R	R	R	S	S	S	S
	3D	S	S	S	S	S	S	R	R	S	S	S	S
	3E	S	S	S	S	R	R	R	S	S	S	S	S
	3F	R	R	S	S	R	R	R	S	S	S	S	S
	3G	R	R	S	S	S	S	R	R	S	S	R	R
	3H	S	S	S	S	R	R	R	R	S	S	R	R
Point 4	4A	S	S	S	S	S	S	S	S	S	S	R	R
	4B	S	S	S	S	R	R	R	R	R	R	S	S
	4C	S	S	S	S	R	R	S	S	S	S	S	S
	4D	S	S	S	S	S	S	S	S	S	S	S	S
	4E	R	R	S	S	R	R	R	S	S	S	S	S
	4F	S	S	S	S	R	R	S	S	S	S	S	S
	4G	S	S	S	S	S	S	S	S	S	S	S	S

MAR Index Analysis

Based on Figure 2 (a), it can be seen that almost all isolates from Lebir River have MAR index of >20%. Half of the four isolates from Point 1 has MAR index of >20% whereas another half of them has MAR index of < 20%. For Point 2 isolates, six out of eight isolates have MAR index of >20% for both antibiotic concentrations tested. Only one isolate has MAR index of <20% for both antibiotic concentrations and also only one isolate that has MAR index of >20% for one antibiotic concentration tested. Figure 2 (b) illustrates that 50% of Point 3 isolates have MAR index of >20% for both antibiotic concentrations tested. In the meantime, only two isolates (4B and 4E) out of seven isolates from Point 4 have MAR index of >20% for both antibiotic concentrations tested.



(a) (b)

Fig 2 MAR index value for bacteria isolated from a) Lebir River and b) Kelantan River.

Conclusion

In this study, the antibiotic resistant bacteria were managed to be isolated from the water samples of all four sampling points of Lebir River and Kelantan River in Kelantan, summed up to a total of 27 bacterial isolates (Point 1: 4 isolates; Point 2: 8 isolates; Point 3: 8 isolates; Point 4: 7 isolates). Based on the results, the isolates were predicted as *Actinomyces* sp., *Arachnia* sp., *Streptomyces* sp., *Streptococcus* sp., *Enterococcus* sp., *Bacillus* sp., *Clostridium* sp., *Neisseria* sp., *Veillonella* sp., *Bacteroides* sp., *Salmonella* sp., *Shigella* sp., *Proteus* sp., *Enterobacter* sp., *Klebsiella* sp., *Escherichia* sp, *Pseudomonas* sp., *Aeromonas* sp., *Vibrio* sp. *Staphylococcus* sp., *Micrococcus* sp., *Actinobacillus* sp., and *Flavobacterium* sp. The antibiotic susceptibility testing using disk diffusion method against six types of antibiotics (gentamicin, ampicillin, rifampicin, chloramphenicol, tetracycline, and ciprofloxacin) were performed towards all of the bacterial isolates and the MAR index values for each of the isolates were successfully calculated. The prsent study is expected to provide a baseline data on the MAR in the water environment. The result obtained showed that both polluted and unpolluted rivers consist of antibiotic resistant bacteria. Almost all isolates in Lebir river are claimed to be MAR bacteria, indicating that the human activities have strong correlation with the MAR in the freshwater environment. The isolated MAR bacteria could be potentially human pathogenic bacteria and pose high risk to human, animal, and environmental health. Therefore, further identification on the bacterial species using molecular method to justify the potential virulence of the MAR bacteria in the freshwater environment are strongly recommended.

Abbreviations
AR: Antibiotic Resisrance; ARB: Antibiotic resistance bacteria, AMR: antimicrobial resistance; CLSI: Clinical and Laboratory Standard Institute; MAR: Multiple antibiotics resistance; sp: species

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Availability of data and material
Please contact the corresponding author for any data request.

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