Fishing in suburban river for antibiotic resistant coliform bacteria

Screening of Extended-Spectrum Beta-Lactamases (ESBLs)-producing and Carbapenem-Resistant Enterobacteriaceae (CRE)

A study of suburban river water in Johor, Malaysia

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Introduction

- Aquatic environments serve important roles for global evolution and spread of antibiotic resistance.
- Coliform bacteria are subgroup of Gram-negative *Enterobacteriaceae* family that are usually present in digestive tracts and wastes of animals, and they typically serve as indicator organisms for accessing water quality.
- World Health Organisation (WHO) had included ESBLs-producing and carbapenem-resistant *Enterobacteriaceae* (CRE) as critical priority pathogens that pose major antibiotic-resistant threats globally.
- ESBLs-producing *Enterobacteriaceae* are resistant to majority of broad spectrum antibiotics including 3rd generation cephalosporins (cefotaxime).
- CRE are resistant to carbapenem (meropenem) which is often considered as currently last available antibiotic against bacterial infections.

Aims

- To screen for presence of ESBLs- and carbapenemases-producing coliforms in water samples from suburban river.
- To determine susceptibility of chosen isolates against β-lactam antibiotics including ampicillin (AMP), cefotaxime (CTX) and meropenem (MEM).
- To confirm identity of selected isolates based on 16S-rRNA sequencing.

Methods

Water sample was collected in triplicate at 3 different sites of Kampung Sungai Melayu (River Melayu Village), located in southern part of West Malaysia.

Figure 1. Three different sites for water sample collection. Site (A), (B) and (C) correspond to estuary, jetty and mussels farming site.

Water sample from different sites were filtered using 0.45 µm membranes. Membranes were then transferred onto 3 different types of culture media including HiCrome coliform agar (HCA), HCA + AMP + CTX and HCA + MEM. Concentration of antibiotics were determined based on EUCAST epidemiological cut-off values.

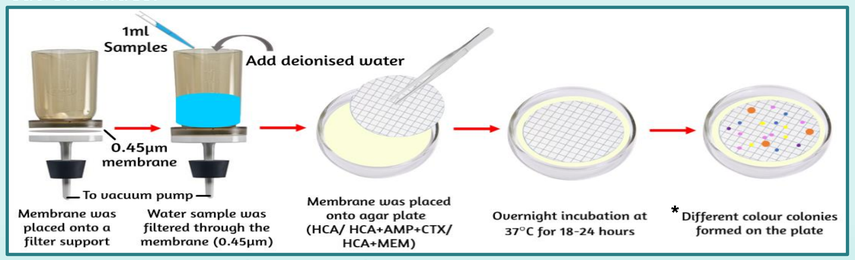
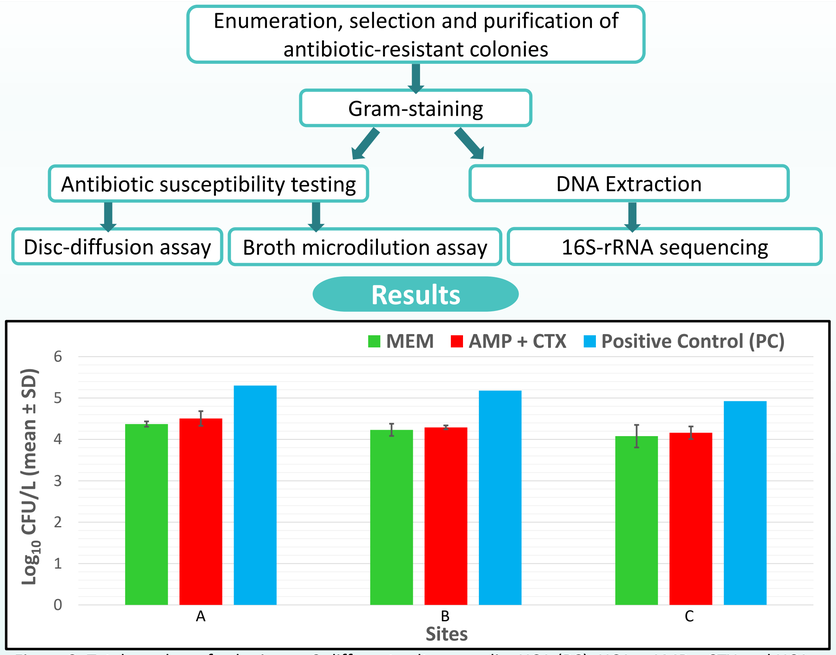


Figure 2. Overall procedures for membrane filtration method.

* There are 5 different possible colour of colonies that can form on HCA and different colour of colonies represent different bacterial species. (**Blue & Violet** – *Escherichia coli*; **Light pink** – *Klebsiella pneumoniae*; **Salmon red** – *Citrobacter freundii/ Enterobacter cloacae*; **White** – *Salmonella enteritidis/ Shigella flexneri*) The above description is based on product information from Sigma-Aldrich, USA.



Results

Figure 3. Total number of colonies on 3 different culture media: HCA (PC), HCA + AMP + CTX and HCA + MEM, based on water samples collected from site A, B and C.

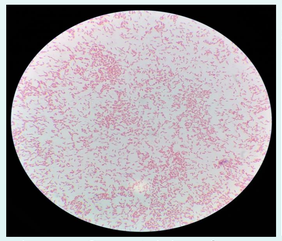


Figure 4. Gram staining of isolates CTX/A1/V1. The bacteria appear as Gram-negative rods.
Pink – Gram-negative
Purple – Gram-positive

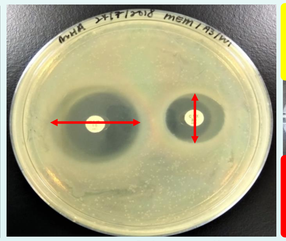


Figure 5. One of the Mueller-Hinton agar used in disc-diffusion assay.
Arrow indicates diameter of inhibition zone.

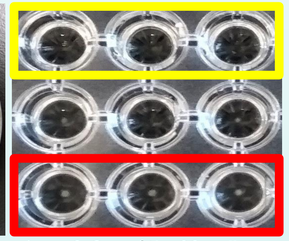


Figure 6. One of the 96-wells plate used in broth microdilution assay.
Yellow box – absence of bacterial growth
Red box – presence of bacterial growth

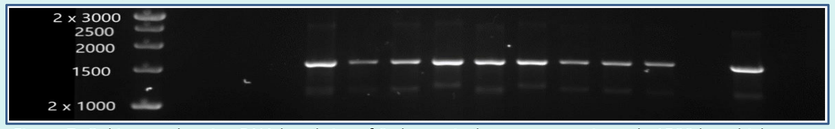


Figure 7. Gel image showing DNA band size of 9 chosen isolates at approximately 1500 bp which indicate presence of bacterial 16S-rRNA gene in the extracted DNA.

Table 1. Listed features of 9 chosen isolates and their identity determined from 16S-rRNA sequencing.

Isolates	Colour of colonies	Gram-stain	Shape	Species
CTX/A1/S1	Salmon red	Negative	Rod	*Enterobacter cloacae*
CTX/A1/V1	Violet	Negative	Rod	*Chromobacterium violaceum*
CTX/A3/W1	White	Negative	Rod	*Aeromonas spp.*
CTX/A3/P1	Light pink	Negative	Rod	*Acinetobacter baumannii*
CTX/B1/B2	Blue	Negative	Rod	*Escherichia coli*
MEM/A3/W1	White	Negative	Rod	*Acinetobacter spp.*
MEM/B1/W2	White	Negative	Rod	*Pseudomonas spp.*
MEM/C2/W3	White	Negative	Rod	*Pseudomonas spp.*
MEM/C1/S3	Salmon red	Negative	Rod	*Pseudomonas boreopolis*

Table 2. Inhibition zone sizes and susceptibility of 9 chosen isolates and *E. coli K12* against 30 µg cefotaxime and 10 µg meropenem determined from disc-diffusion test.

Isolates	Diameter of inhibition zone (mm)		Susceptibility	
	Cefotaxime (30 µg)	Meropenem (10 µg)	Cefotaxime (30 µg)	Meropenem (10 µg)
E. coli K12 (NC)	34.5 ± 2.12	30 ± 1.41	Susceptible	Susceptible
CTX/A1/S1	19.5 ± 0.71	21 ± 0	Resistant	Intermediate
CTX/A1/V1	10 ± 1.41	27.5 ± 1.06	Resistant	Susceptible
CTX/A3/W1	13 ± 5.66	23 ± 0	Resistant	Susceptible
CTX/A3/P1	18.5 ± 0.71	27 ± 0.71	Resistant	Susceptible
CTX/B1/B2	18 ± 1.41	25.5 ± 1.06	Resistant	Susceptible
MEM/A3/W1	18.5 ± 0.71	25 ± 0	Resistant	***
MEM/B1/W2	19 ± 0	10.5 ± 1.06	Resistant	Resistant
MEM/C2/W3	20.5 ± 2.12	18 ± 0	Resistant	Resistant
MEM/C1/S3	12.5 ± 2.12	0 ± 0	Resistant	Resistant

Data shown are in mean ± standard deviation. Susceptibility of bacterial isolates against CTX and MEM were interpreted based on CLSI guidelines. *E. coli K12* serve as negative control (NC) of the test.

Table 3. Minimum inhibitory concentration (MIC) and susceptibility of 9 chosen isolates against ampicillin, cefotaxime and meropenem determined from broth microdilution method.

Isolates	MIC (mg/L)			Susceptibility		
	Ampicillin	Cefotaxime	Meropenem	Ampicillin	Cefotaxime	Meropenem
CTX/A1/S1	>500	6.25	0.78	Resistant	Resistant	Susceptible
CTX/A1/V1	125	12.5	0.39	Resistant	Resistant	Susceptible
CTX/A3/W1	125	12.5	0.78	Resistant	Resistant	Susceptible
CTX/A3/P1	500	6.25	<0.098	Resistant	Resistant	Susceptible
CTX/B1/B2	>500	6.25	<0.098	Resistant	Resistant	Susceptible
MEM/A3/W1	31.25	12.5	1.56	Resistant	Resistant	***
MEM/B1/W2	125	25	12.5	Resistant	Resistant	Resistant
MEM/C2/W3	125	6.25	3.125	Resistant	Resistant	***
MEM/C1/S3	500	25	>12.5	Resistant	Resistant	Resistant

MIC of isolates were determined based on presence of button-like suspension in the wells and absorbance values of wells at 600 nm wavelength against negative and positive control wells. Data were interpreted based on both CLSI and EUCAST guidelines.

****indicates result needs to be further interpreted and susceptibility of isolates cannot be concluded at this stage*

Discussion

- Proportion of ESBLs-producing coliform bacteria appeared to be greater than meropenem-resistant coliform bacteria at all 3 sites of sample collection.
- All 9 chosen isolates were proven to be resistant to AMP and CTX based on results from disc-diffusion and broth microdilution assay.
- 5 isolates were proven to be non-resistant to meropenem while 2 isolates were proven to be resistant to meropenem in antibiotic susceptibility testing.
- Susceptibility of MEM/A3/W1 and MEM/C2/W3 isolates against meropenem can only be determined after further testing being carried out.
- This is because MIC of both isolates and inhibition zone size of MEM/A3/W1 were within EUCAST screening cut-off range but not clinical resistant range.
- Growth of bacterial species apart from coliform group indicated that HCA is not highly specific to only growth of coliform bacteria.

Conclusion

- Multidrug-resistant coliforms are present in Malaysia suburban river water.
- Coliform bacteria in river water more commonly develop resistance towards ampicillin and cefotaxime than towards carbapenem.
- 16S-rRNA sequencing allow confirmation of bacterial identities but not presence of antibiotic resistance genes and thus further investigation (eg: bacterial whole genome sequencing) is recommended.

Acknowledgement

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