Identification and Molecular Characterization of Multidrug-resistant *Escherichia coli* Isolated from Aquatic Environment

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Research title

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Summary

The development of antimicrobial resistance among multidrug-resistant (MDR) bacteria is of growing public health concern globally. There is clear relationship between the antibiotic use and the development and persistence of the bacterial resistance. The aquatic environment such as river provides an ideal setting for the dissemination of the antibiotic resistance bacteria (ARB) and may cause the affected water system to serve as a reservoir of MDR bacteria. Malaysia is well known for its tropical climate that has high temperature and humidity all year-round. This type of climate enhances the multiplication of bacteria and contributes to enhance the propagation of antibiotic resistance genes (ARGs). Therefore, it is important to understand more on the availability of antibiotic resistant microorganisms under different climate condition. The emergence of MDR and extensively drug resistant bacteria in recent years has recognized fosfomycin antibiotic as an alternative therapy. *Escherichia coli* (*E. coli*) is well investigated species in the bacterial resistance studies and recognized as one of the contributors to the spread of ARGs in the natural environment. Thus, *E. coli* was selected as target species in this study which was isolated using selective media and confirmed by molecular detection methods. The potential resistant isolates were obtained by several antibiotic susceptibility testing against frequently used antibiotics in healthcare systems. Polymerase Chain Reaction (PCR) and Next Generation Sequencing (NGS) were involved to validate the existence of relevant conserved resistance genes in this study.

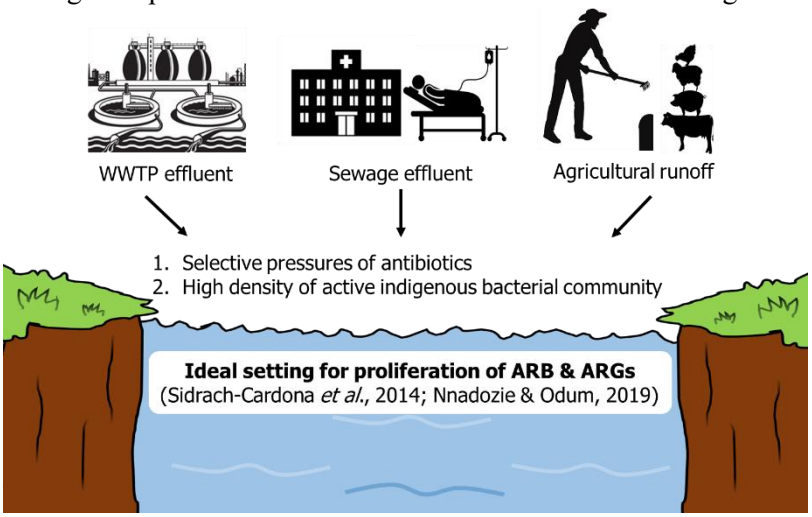

Figure 1 Possible routes of residual antibiotics from various origins reaching to natural environment such as freshwater system and contribute to the proliferation of resistant bacteria and genes which eventually possibly transmitted back to human and animals.

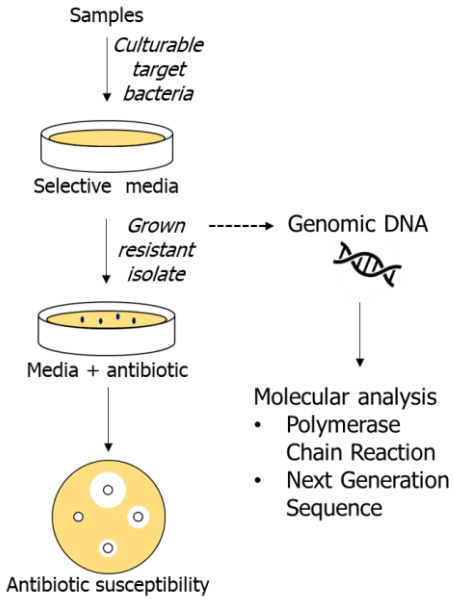

Figure 2 The detection of resistant bacteria in the environment by culture-dependent method.

A high availability of MDR *E. coli* was obtained from the tropical aquatic environment previously, name Gombak River located in an urban city of Kuala Lumpur, Malaysia, therefore a similar study was conducted in different climate region which is temperate region of Japan to compare the availability of resistant Enterobacteria in the environment with aims to isolate the Enterobacteria from temperate aquatic environment and identify the prevalence of antibiotic resistant of isolated Enterobacteria. The recovery of *E. coli* from water samples was done by the membrane-filtration method. The prevalence of resistant *E. coli* was phenotypically assessed against fifteen antibiotics via antibiotic susceptibility test and Kirby-Bauer disc diffusion assay. The combination with molecular techniques which involved the utilization of genomic DNA of the resistant isolates could determine their resistance mechanisms by identifying the resistant genes responsible (Figure 2).

Table 1 Summary of recovered *E. coli* strains from Gombak River (Tropical) and Tomoe River (Temperate).

	Tropical	Temperate	
		Spring	Summer
Population no. of *E. coli*	129	40	72
No. of potential MDR strains (%)	117 (90.7%)	9 (22.5%)	27 (37.5%)

Tomoe River yielded lower total *E. coli* strains than total number recovered in Gombak River. MDR was characterized by their resistance to at least three or more antibiotic classes. About 90.7% of tropical isolates are characterized as MDR which is significantly higher than temperate isolates at proportion of 22.5% and 37.5% of the total isolates recovered in spring and summer season, respectively (Table 1). In addition, the resistance prevalence activities of *E. coli* against different types of antibiotics were observed. High resistance activities towards sulfonamides, beta-lactams, tetracyclines and fosfomycin was observed among temperate isolates. Similarly, among tropical isolates including trimethoprim, fluoroquinolones and phenicol antibiotics. Meanwhile, low frequency of MDR *E. coli* together with known ARGs confirmed by PCR analysis; beta-lactams (*ampC*, bla_{TEM}), sulfonamide (*sul1*, *sul2*, *sul3*) and tetracyclines (*tetA*, *tetM*) detected in Japanese aquatic environment particularly Tomoe River could demonstrate the presence of other potential pathogens as well and representing resistance to most widely used antibiotics in humans and animals. Since a high availability of environmental MDR has been isolated from aquatic environment and showed resistance to wide spectrum of antibiotics, it is highly noteworthy to evaluate the susceptibility of MDR isolates against critically important antibiotic by determining the minimum inhibitory concentrations (MICs) of fosfomycin via agar-dilution method and evaluate the fosfomycin-inactivating enzyme (FosA) activity via disc potentiation and interpretive susceptibility test with the addition of sodium phosphonoformate (PPF) on the fosfomycin disc as the enzyme inhibitor. Results indicated that tropical MDR *E. coli* namely GR2 and GR3 are fosfomycin-resistant, while other tropical isolates and all temperate isolates remained negative for both susceptibility screening. Following the addition of PPF (FosA inhibitor) in disc potentiation test, paired samples *t*-test was used to evaluate the significant difference of the inhibition zones diameter. Both GR2 and GR3 were found highly significant ($p < 0.01$) and considered as truly fosfomycin-resistant due to high MIC value at 512 µg/mL. It is worth noting other tropical isolates were found significantly difference ($p < 0.05$) but less prominent as compared to GR2 and GR3 could be due low MIC values.

NGS technology was used to obtain the genome sequences of selected tropical MDR isolates. BLAST performed against 16S rRNA and custom databases showed GR2, GR3 and GR5 (negative control strain) have high similarity percentage to *E. coli* species. AMRFinder, one of the bioinformatic tools identified genes that confer resistance to beta-lactams ($bla_{\text{TEM-1}}$, $bla_{\text{TEM-176}}$, $bla_{\text{CTX-M-65}}$, $bla_{\text{CMY-2}}$), tetracycline (*tetA*, *tetX*, *tetM*), aminoglycoside (*aac(3)-Iva*, *aac(3)-IIe*, *aadA1*, *aadA2*, *aph(3')-Ia*, *aph(3'')-Ib*, *aph(4)-Ia)*, *aph(6)-Id*), sulfonamide (*sul2*, *sul3*), phenicol (*florR*, *cmlA1*), quinolone (*qnrS1*), trimethoprim (*dfrA1*, *dfrA12*, *dfrA14*). Most importantly, has validated the existence of fosfomycin-inactivating enzymes (*fosA3* and *fosA4*) in GR2 and GR3, respectively. To sum up, the evidence discussed in this study have presented the comparison of *E. coli* and antibiotic resistance from tropical and temperate region where this study demonstrated high prevalence in tropical aquatic systems together with their significant genetic determinants and indicate hospital wastewater along with human practices against antibiotic may contribute to this incidence. Therefore, the detection of MDR enterobacteria, particularly *E. coli* in this study reflects the ability of MDR strains to survive in the environment, particularly tropical aquatic systems create public health implications especially tropical infections that thrive in hot and humid conditions and becomes a major concern because antibiotic resistance is circulating among humans, animals and the environment. The presence of resistant *E. coli* strains to broad-spectrum of antibiotics warning a standard antibiotic therapy may no longer be effective in the future to treat bacterial infections for example gastrointestinal/extra intestinal diseases due to pathogenic *E. coli* strains in Malaysia as well as Southeast Asia.

Figure 3 Infographic of possible routes how ARB can spread between human and environment