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Molecular characterization of multi-drug resistant *Escherichia coli* isolates from tropical environments in Southeast Asia

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The emergence of antibiotic resistance among multidrug-resistant (MDR) microbes is of growing concern, and threatens public health globally. A total of 129 *Escherichia coli* isolates were recovered from lowland aqueous environments near hospitals and medical service centers in the vicinity of Kuala Lumpur, Malaysia. Among the eleven antibacterial agents tested, the isolates were highly resistant to trimethoprim-sulfamethoxazole (83.7%) and nalidixic acid (71.3%) and moderately resistant to ampicillin and chloramphenicol (66.7%), tetracycline (65.1%), fosfomycin (57.4%), cefotaxime (57.4%), and ciprofloxacin (57.4%), while low resistance levels were found with aminoglycosides (kanamycin, 22.5%; gentamicin, 21.7%). The presence of relevant resistance determinants was evaluated, and the genotypic resistance determinants were as follows: sulfonamides (*sulI*, *sulII*, and *sulIII*), trimethoprim (*dfrA1* and *dfrA5*), quinolones (*qnrS*), β-lactams (*ampC* and *blaCTX-M*), chloramphenicol (*cmlA1* and *cat2*), tetracycline (*tetA* and *tetM*), fosfomycin (*fosA* and *fosA3*), and aminoglycosides (*aphA1* and *aacC2*). Our data suggest that multidrug-resistant *E. coli* strains are ubiquitous in the aquatic systems of tropical countries and indicate that hospital wastewater may contribute to this phenomenon.

Key Words: antibiotic resistance genes; hospital wastewater; multidrug resistant *E. coli*

Introduction

For the past few decades, antibacterial agents have been frequently used as human and veterinary therapies to fight against bacterial infections, along with applications in animal and crop husbandries for growth promotion and prophylaxis. This excessive and indiscriminate use of antibiotics in various applications is suggested to be a contributing factor in the development of antibiotic resistance and multidrug-resistant bacteria (MDR), which has become an emerging threat to global public health. In Malaysia, the utilization of antibiotics in most hospitals has been steadily increasing over the last several decades. As reported in the National Antibiotic Guideline 2014, a 2013 audit of the usage of antibiotics over the past several years showed an escalating use of fluoroquinolones, aminoglycosides, and β-lactamase inhibitor combinations. The trend of increasing antibiotic use warrants close monitoring to ensure that antibiotics being prescribed are being used properly to avoid the abuse of antibacterial drugs. Antibiotics, or their metabolites, released in hospital wastewater threaten nearby water systems, including nearby rivers. To the best of our knowledge, a major fraction of the antibiotics consumed by humans and animals

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remains unmetabolized, and a significant amount (approximately 50–60%) of the active form of these drugs is released into the environment via excretion, thus contributing to the presence of antibiotic residues in water. Hence, one potential risk is that aquatic ecosystems may provide an ideal setting for the proliferation and dissemination of resistant bacteria due to continuous antibiotic exposure, even at very low concentrations, which may be toxic to the aquatic community and ultimately to public health (Sidrach-Cardona et al., 2014).

Hospitals and healthcare facilities, where antibiotics are intensively used and where antibiotic-resistant bacteria may have a selective advantage over susceptible strains, are viewed as important hotspots for antibiotic-resistance genes (ARGs). Hospital discharge may provide a significant reservoir of resistant bacteria and ARGs due to the constant exposure of the bacterial community to antibacterial residues. A study by Marti et al. (2013) highlighted that ARGs have environmental origins, but the persistent entry and accumulation of antimicrobial agents in the environment facilitates their spread.

Antibiotic-resistant bacteria have been isolated from multiple sources, including hospitals, domestic sewage, drinking water, rivers, and lakes (Alhaj et al., 2007). This could increase the chances of humans being infected with bacteria possessing ARGs, if they are exposed to a polluted environment or ingest contaminated food or water. Apart from that risk, antibiotic-resistant bacteria containing other virulence genes can spread all over the world through global travel, and thereby create significant health and economic impacts (Ram et al., 2009). However, of greater concern is the increased risk that the particularly-resistant bacteria, associated with mobile genetic elements, may heighten the likelihood that ARGs and virulence factors are spread simultaneously, which could lead to the emergence of new pathogens, since resistant bacteria develop rapidly (Ramírez Castillo et al., 2013). In recent years, although substantial research has been carried out in other countries, such as China, India, Poland, and Brazil, regarding MDR bacteria and the development of resistance mechanisms in aquatic systems (Diwan et al., 2010; Korzeniewska and Harnisz, 2013; Rebello and Regua-Mangia, 2014; Xu et al., 2016), there are few studies available concerning the prevalence of MDR enteropathogenic bacteria in aquatic environments in Malaysia. This is despite the fact that the microbiological qualities of river water sources in other countries have been reported (Hamelin et al., 2007; Huang et al., 2016; Olaniran et al., 2009; Ramírez Castillo et al., 2013).

Escherichia coli is a commensal member of the gastrointestinal tract microflora of humans and other warm-blooded animals, and it is a good microbiological indicator for surveillance studies of antimicrobial resistance (Alhaj et al., 2007). Unlike other microorganisms, *E. coli* is able to acquire resistance easily and has been recognized as a contributor to the dissemination of ARGs in natural environments (Alhaj et al., 2007; Kappell et al., 2015). Furthermore, *E. coli* is a health threat in some tropical and subtropical areas, and is one of the leading causes of diseases and death in humans and animals (Rebello and Regua-Mangia, 2014).

Malaysia is known for its tropical climate, and has high temperatures and humidity levels that are stable throughout the year. This type of climate can easily enhance the multiplication of microorganisms and can contribute to the enhanced dissemination of ARGs. The research area in this study was a river near Kuala Lumpur, close to where antibacterial agents, or their metabolites, together with resistant bacteria might be discharged. Thus, the research area is a river that receives wastewater released from nearby hospitals and medical service centers. The target species was *E. coli*, which was obtained using selective media and confirmed by molecular detection methods. Several tests for antibiotic susceptibility and a minimum inhibitory concentration (MIC) were performed to obtain potential resistant isolates. The resistance prevalence of *E. coli* was determined, and MDR isolates were molecularly characterized to validate the existence of relevant conserved resistant genes.

Methods

Sample collection. The research area was the Gombak River near Kuala Lumpur, Malaysia. This was selected as the sampling location because it receives treated and untreated wastewater released from nearby hospitals and medical service centers (3°10′13.2″ N 101°41′41.6″ E). Water samples were collected by using a stainless-steel bucket which was precleaned by rinsing (in sequence) with 70% ethanol, distilled water, and water from the specific location. Samples were stored in 1-L sterile polyethylene bottles and kept cold during transportation to the laboratory. Then, the 1-L water samples were vacuum-filtered within 24 h of collection through a 47-mm glass microfiber filter (GC/C; Whatman) to remove particulate matter. Filtered samples were subsequently stored in the dark at 4°C until further use.

Isolation and selection of E. coli from river water. Water samples (100 ml) were vacuum-filtered through cellulose nitrate membrane filters with a 0.2-μm pore size (Whatman). After filtration, the membrane filters were then sterilely placed into 50-mL Falcon tubes using sterile forceps and washed with 1 ml of 0.7% NaCl to collect the recovered microorganisms. These were then subjected to a ten-fold serial dilution prior to inoculation onto the selective media. Approximately 0.1 ml of the diluted sample was inoculated evenly across the surface of a Chromocult® Coliform Agar plate (CCA; Merck Millipore) using a sterile hockey stick until it dried completely. The inoculated agar plates were then incubated at 37°C for 24 h in the dark.

The following criteria used for the identification of colonies on CCA are as follows: dark-blue to violet-colored colonies were considered to be *E. coli*, whereas both salmon-colored colonies and colorless colonies were considered to be *Enterobacteriaceae*, but were classified as coliforms and non-coliforms, respectively. After overnight incubation, only the dark-blue to violet-colored colonies were counted as being presumed to be *E. coli*. The resulting colonies were repeatedly subcultured onto CCA media as an initial confirmation step, and to obtain pure cultures of the colonies. The presumed *E. coli* isolates were

subjected to additional confirmation by coating the dark-blue colonies on CCA with a drop of Kovacs' indole reagent, which turns *E. coli* colonies cherry red. In addition, eosine-methylene blue (EMB) agar (Becton Dickinson and Company), another selective media designed to isolate, cultivate, and differentiate Gram-negative enteric bacteria from both clinical and nonclinical samples, was used in the study. EMB media was designed to identify and isolate Gram-negative enteric rods, which allows for the distinction between lactose-fermenting and non-lactose-fermenting organisms (Atlas, 2005). The colonies of isolated bacteria were re-streaked onto EMB agar media, and *E. coli* isolates were recognized by the appearance of a green metallic-sheen on colonies on the agar surface after overnight incubation at 37°C in the dark. Hence, only isolates which appeared as dark-blue to violet-colored colonies on CCA media with a positive reaction toward Kovacs' indole reagent, and which were further confirmed by the formation of green metallic-sheen colonies on EMB media, were selected for subsequent analysis and preserved in Luria-Bertani (LB) broth with 20% (v/v) glycerol at –80°C.

Antibiotic susceptibility test and MDR prevalence of E. coli isolates. Pure colonies of the *E. coli* isolates were tested for their resistance and susceptibility toward different classes of antibiotics using the respective concentrations in accordance with the Clinical Laboratory and Standards Institute (CLSI) guidelines (Patel and Franklin, 2014). The eight major antibiotic classes used in this study were: β-lactams: ampicillin (AMP, 10 μg/ml) and cefotaxime (CFX, 30 μg/ml); sulfonamides: sulfamethoxazole (SMZ, 23.75 μg/ml); folic acid: trimethoprim (TM, 1.25 μg/ml); phosphonic acid: fosfomycin (FOF, 200 μg/ml); phenicols: chloramphenicol (CMP, 30 μg/ml); aminoglycosides: kanamycin (KNM, 30 μg/ml) and gentamicin (GMN, 10 μg/ml); and fluoroquinolones: nalidixic acid (NAL, 30 μg/ml), ciprofloxacin (CIP, 5 μg/ml), and tetracycline (TET, 30 μg/ml). The selection of antibiotics was based on those most commonly prescribed in both public and private hospitals in Malaysia.

Prior to the antibiotic testing, fresh cultures of the pure *E. coli* isolates were obtained, and Mueller-Hinton Agar (MHA) containing the selected antibiotics were prepared in advance. In order to prepare the MHA media containing antibiotics, antibiotic stock solutions were first prepared and diluted using the appropriate solvents and diluents. After autoclaving the MHA media, the media was cooled to approximately 55°C using a water bath before proceeding with the addition of the antibiotic solution. After the preparation of the MHA media, the fresh isolates were inoculated onto numbered agar plates using sterile toothpicks and incubated overnight at 37°C in the dark. The growth and appearance of colonies were observed after 24 h to determine the resistance and susceptibility profiles.

The Kirby-Bauer disk diffusion method was used as described in the literature (Ali et al., 2014; Thompson et al., 2013) to evaluate the antimicrobial susceptibility profiles of the isolates using a standardized single disk on MHA media. A total of 10 antibiotics were tested: AMP (30 μg/ml), CFX (5 μg/ml), KNM (30 μg/ml), GMN (10 μg/ml), CIP, (5 μg/ml), NAL (30 μg/ml), TET (15 μg/ml), FOF, (200 μg/ml), CMP (30 μg/ml), and TM-SMZ (1.25/23.75 μg/ml). The stock solutions for each antibiotic were prepared by using the formula $(1000/P) \times V \times C = W$; where, P = potency of the antibiotic base, V = volume in ml required, C = final concentration of the solution, and W = weight of the antimicrobial to be dissolved in V. The antibiotic concentrations were chosen based on the therapeutic concentrations used in most hospitals in Malaysia. A 0.5 McFarland standard suspension of each isolate was prepared, and a lawn culture was grown on an MHA plate. Plates were incubated at 37°C for 18 h and zone diameters were measured using calipers. Zones of inhibition were determined in accordance with procedures of the CLSI standard guidelines (Patel and Franklin, 2014) (CLSI, 2014).

The multiple antibiotic resistance (MAR) index was determined for each isolate by dividing the number of antibiotics to which the organism was resistant by the total number of antibiotics tested (Hinton et al., 1985). This index gives a general indication of the probable source(s) of an organism.

Measurement of Minimum Inhibitory Concentrations (MICs) and disk diffusion test. MIC values were measured and interpreted as R (resistant), I (intermediate), and S (susceptible) according to the classification provided by CLSI guidelines (Patel and Franklin, 2014) (CLSI, 2014). The MICs of the selected isolates were tested using a modified Kirby-Bauer disk diffusion technique with the different antibacterial agents. This disk diffusion test was conducted in triplicate to obtain the mean reading of the diameter of the zone of inhibition. MHA was prepared in advance for the disk diffusion test. Meanwhile, the inoculums were prepared by growing the selected six isolates in 50 ml of LB broth at 37°C with shaking at 160 rpm. The density of the bacterial suspension was standardized by adjusting the optical density at 650 nm to 0.5. Each of the isolates was then inoculated onto the MHA surface using a sterile cotton swab. At the same time, antibiotic discs were prepared by immersing a 6-mm disc supplied by Advantec in different antibiotic solutions with known concentrations. The antibiotic discs were sterilely placed on the surface of the inoculated agar plates using sterile forceps with proper spacing, and the agar plates were then incubated for 24 h at 37°C in the dark.

The appearance of an inhibition zone surrounding the disc after 24 h of incubation was indicative of sensitivity, which was determined by measuring the zone and comparing with the guidelines provided by CLSI. Resistant isolates did not form any zone of inhibition around the disk. Meanwhile, isolates with intermediate resistance formed smaller inhibition zones. The diameter of the zone was measured and subsequently compared with a standard table in order to categorize whether the isolate was resistant, intermediate, or susceptible toward the antibiotic tested.

DNA extraction and PCR methods for identifying uidA and known ARGs. The overnight liquid culture of the six isolates was prepared by cultivating the isolates in 50 ml

of LB broth at 37°C with shaking at 160 rpm. After overnight incubation, the bacterial suspension of each isolate was transferred into a 50-mL Falcon tube and then centrifuged at 10,000 × *g* for 10 min to collect the bacterial cells. The collected cells were resuspended into 200 μl of sterile distilled water and then centrifuged at 10,000 × *g* for 10 min to wash the remaining media from the cells. After removing the supernatant, the cells were resuspended in 200 μl of 1 × Tris-EDTA [TE; 10 mM Tris-acetate, 1 mM EDTA (pH 7.5)] buffer and mixed by vortexing. Phenol-chloroform (200 μl) was then added, and the solution was mixed by tapping. The resuspended cells were then centrifuged at 15,000 × *g* for 3 min. Phenol-chloroform is immiscible in water, thus, centrifugation produced two phases. The lower organic phase contained dissolved protein, and the upper aqueous phase contained DNA. The aqueous phase was carefully transferred into a tube and stored at –80°C until use.

PCR, targeting the *uidA* gene, was performed to confirm the identities of the *E. coli* isolates (Bej et al., 1991). The forward and reverse primers with the sequences of 5′-TGGTAATTACCGACGAAAACGGC-3′ and 5′-ACGCGTGGTTACAGTCTTGCG-3′ (Molina et al., 2015), respectively, were used to specifically detect the presence of the *uidA* gene with an expected size of 162 bp. The phenotypic antibacterial resistance of each of the selected six *E. coli* isolates was analyzed using the presence of relevant resistance genes, which are β-lactamase encoding genes (*ampC* and *blaCTX-M*), sulfonamide resistance genes (*sulI*, *sulII*, and *sulIII*), folic acid resistance genes (*dfrA1* and *dfrA5*), phosphonic acid resistance genes (*fosA*, *fosA3*, and *fosC2*), phenicol resistance genes (*cmlA1*, *catI*, and *catII*), aminoglycoside resistance genes [*aph(3)-Ia*, *aac(3)-IIa* and *ant(2″)-Ia*], fluoroquinolone resistance genes (*qnrA*, *qnrB*, and *qnrS*), and TET resistance genes (*tetA*, *tetK*, and *tetM*). Primers used to detect these genes are shown in Supplementaly Table S1. The reaction mixture contained 4 μl of 5 × Green GoTaq® Flexi Buffer, 1.2 μl of 25 mM $MgCl_2$ solution, 0.4 μl of 10 mM dNTPs, 0.5 units of GoTaq(r) DNA Polymerase, 0.3 μl each of oligonucleotide primer (Integrated DNA Technologies, Inc.), 1 μl of DNA template, and 12.7 μl of nuclease-free water, to constitute a total reaction volume of 20 μl. All PCR assays were performed on a thermal cycler (ProFlexTM PCR System; Applied Biosystems).

Results

Isolation and selection of E. coli from a tropical environment

We collected water samples four times over two months to avoid the dry/wet seasonal variation of samples. A total of 129 isolates (1st sample: 29 isolates, 2nd sample: 32 isolates, 3rd sample: 43 isolates, and 4th sample: 25 isolates) recovered from the Gombak River in Kuala Lumpur, a tropical aquatic environment, exhibited positive β-glucuronidase activity and appeared as dark-blue to violet-colored colonies on CCA media, revealing the colonies as Gram-negative microorganisms. These colonies were selected as presumptive *E. coli* isolates. Presumptive *E. coli* isolates were also recovered on another selective media, EMB media. The appearance of blue-black colonies with a greenish-metallic sheen in reflected light on EMB media indicates vigorous lactose fermentation and acid production, which precipitated a green-metallic pigment, further confirming that the isolates were *E. coli*. Furthermore, all selected presumptive *E. coli* isolates underwent PCR amplification of the *uidA* gene. All tested strains showed positive for the gene with the specific primers used (data not shown).

Antibiotic resistance phenotypic profiles of E. coli isolates

The antibiotic resistance and susceptibility of the 129 *E. coli* isolates were profiled for their probable phenotypic resistance to eleven antibacterial agents from eight different antibacterial classes. A total of 108 isolates (83.7%) were resistant to TM-SMZ. The second most prevalent antibiotic resistance identified was toward NAL (71.3%; $n = 92$). High levels of resistance were observed against AMP and CMP (66.7%; $n = 86$), TET (65.1%, $n = 84$), FOF and CFX (63.6%, $n = 82$), and CIP (57.4%; $n = 74$). Conversely, high sensitivities were detected to KNM (77.5%) and GMN (78.3%), with only 22.5% ($n = 29$) and 21.7% ($n = 28$) of the isolates being resistant to KNM and GMN, respectively.

MDR *E. coli* strains are characterized by resistance (non-susceptibility) to at least three or more classes of antibiotic. It was observed that almost 100% of the isolates were resistant to at least one of the antibacterial agents tested; 96.12% ($n = 124$) were resistant to at least two antibiotic classes, and 90.7% ($n = 117$) were resistant to three and more classes of antibiotics, and thus categorized as MDR. Among isolates with the MDR phenotype, 15.5% ($n = 20$) were resistant to five and six antibacterial agents; 13.95% ($n = 18$) and 13.18% ($n = 17$) were resistant to seven and eight antibacterial agents, respectively; followed by 10.08% ($n = 13$) to nine, 10.08% ($n = 13$) to four antibacterial agents, and 7.75% ($n = 10$) to three antibacterial agents. It is worth noting that 4.65% ($n = 6$) of the isolates exhibited resistance to ten antibacterial agents tested. Sensitivity to all the drugs tested was found in 0.78% ($n = 1$) of the isolates. In addition to the percentage of MDR *E. coli* in this study, there were also potential trends in the number of MDR isolates, which could be clustered in MDR bacteria (resistance to four or fewer antibiotics) and highly MDR bacteria (resistance to four or more antibiotics). Approximately 83% of the isolates exhibited high MDR properties because of the high resistance rates to TM-SMZ, NAL, AMP, and TET.

MICs test - Disk diffusion method

The MIC of each antibacterial agent is defined to be the lowest concentration that inhibited the visible growth of a microorganism (Korzeniewska and Harnisz, 2013). Six isolates, named GR1 to GR6, that were resistant to ten antibacterial agents were selected for further MIC testing by the Kirby-Bauer disc diffusion method. The diameter of the inhibition zone was measured to the nearest millimeter. The mean reading of the diameter zones were measured, recorded, and then interpreted by comparing with the guideline provided by CLSI (CLSI, 2014). Based

on the results, of the antibiotics tested, there was 100% resistance toward AMP, KNM, GMN, CIP, NAL, TET, CMP, and TM-SMZ. Intermediate levels of resistance of three and two were found toward CFX and FOF, respectively (Table 1). Meanwhile, it was found that two of the isolates were resistant to all the antibiotics tested, and four showed reduced susceptibility (intermediate) to those two antibiotics (CFX and FOF).

Table 1. MIC test result for six selected MDR *E. coli* strains isolated.

Antibiotics	No. of Isolates (n = 6); Interpretation* (S, I, R)					
	GR1	GR2	GR3	GR4	GR5	GR6
AMP	R	R	R	R	R	R
CFX	I	R	I	R	I	R
KNM	R	R	R	R	R	R
GMN	R	R	R	R	R	R
CIP	R	R	R	R	R	R
NAL	R	R	R	R	R	R
TET	R	R	R	R	R	R
FOF	I	R	R	I	R	R
CMP	R	R	R	R	R	R
TM/SMZ	R	R	R	R	R	R

*S, susceptible; I, intermediate and R, resistant.
The zones of inhibition were measured according to CLSI 2014 standard.

Resistance gene determinants of selected isolates

The highly MDR *E. coli* isolates were subjected to subsequent microbiological analysis by PCR amplification to assess the presence of relevant resistance genes. The selection of the resistance genes evaluated was based on their frequent occurrence in phenotypically resistant *E. coli* isolates. Therefore, 22 resistance genes responsible for resistance to 8 antibiotic families (β-lactams, sulfonamides, folic acid, phosphonic acid, phenicols, aminoglycosides, TET, and fluoroquinolones) were assessed for detection of the prevalence and distribution of resistance determinants among the *E. coli* isolates. These genes were chosen from among the most prevalent and transferable antibiotic resistance genetic determinants in *E. coli*, considering both clinical and environmental relevance (Pereira et al., 2013). The pattern and frequency of resistance gene determinants in the isolates are presented in Table 2.

Discussion

E. coli is one of the most broadly investigated bacteria and it is highly prevalent in the environment. The isolates obtained in this study substantiate the results of previous studies concerning the presence of *E. coli* in aquatic environments. *E. coli*, along with other *Enterobacteriaceae*, were also detected in various aquatic sources in other parts of Malaysia, such as the Kayu Ara River, Pencala River, and Semenyih River (Al-Badaii and Shuhaimi-Othman, 2015; Tissera and Mae Lee, 2013), the Pearl Rivers in South China (Tao et al., 2010), Dutch surface water (Blaak et al., 2014), the Vistula River in Poland (Kotlarska et al., 2015), and the Bernesga River in Northwest Spain (Sidrach-Cardona et al., 2014). The frequent presence of these microorganisms, potentially carrying resistance or virulence factors, in the aquatic system is potentially a risk to public health, either through consumption or contact with the water.

X-glucuronide is responsible for the detection of *E. coli* by the production of β-glucuronidase, while salmon-GAL is responsible for the detection of other total coliforms by the production of β-galactosidase. Molecular identification of *E. coli* by PCR amplification is based on β-glucuronidase enzyme activity detection. β-glucuronidase, which is encoded by the *uidA* gene, is an enzyme involved in the metabolic activity of *E. coli* strains and this characteristic of *E. coli* is widely utilized for the molecular detection of this species. This enzyme was chosen in this study because it was found to be specific and has been observed in more than 97% of *E. coli* isolates (Maheux et al., 2009), and has become an important marker for the detection of *E. coli*. Several studies also reported that approximately 94–96% of the β-glucuronidase enzyme is observed in *E. coli* strains; thus, the presence of this enzyme is useful in identifying this species (Horakova et al., 2008; Perin et al., 2010).

The current study showed that the highest resistance rate was toward TM-SMZ of the antibacterial agents tested. Humans excrete approximately 40–60% of the TM ingested via urine and feces; thus, residual and related metabolites end up in wastewater causing water pollution. It is suspected that the high resistance incidence in our finding is due to the persistent clinical use and the presence of low concentrations, of the drugs in the environment (Ji et al., 2016; Oros-Ruiz et al., 2013). Suzuki et al. (2013) reported that the bacterial resistance rate to sulfonamides was between 2–90% in the aquatic environment, which was higher than the resistance to TET (0.07–0.18%) and quinolone (0.1–15%). The sulfonamides that are released into the aquatic environment remain active against bacteria due to their chemical properties, such as low chelating ability, low binding constants, and high water solubility and stability, thus accelerating the development of antibiotic-resistant bacteria within natural bacterial communities. It can be inferred that both of these drugs remain in their active forms once in the environment and appear to be causative agents of bacterial resistance. It can be speculated that the high resistance level toward TM-SMZ, detected in this study, indicates that waste or discharge from hospitals can exert a selective pressure on bacterial communities and lead to the development of antibiotic resistance in the receiving river (Hocquet et al., 2016). The comparatively high resistance toward β-lactams found among the isolates in this study might be due to the presence of natural populations of β-lactam-resistant bacteria, as low levels of β-lactam drug resistance are known to be intrinsically present in some Gram-negative environmental isolates (Tao et al., 2010). *E. coli* is the major etiological agent in urinary tract infections (UTIs), which account for up to 90% of *E. coli* infections (Lee et al., 2008). As the rates of resistance against agents commonly used to treat UTIs, such as TM-SMZ and CIP, increase, FOF has been identified as another treatment option. FOF is broadly used to treat infections by urinary pathogens, mostly those caused by *E. coli* (Michalopoulos et al., 2011). Thus, the existence of this

Table 2. Prevalence and distribution of resistance genes among 6 selected MDR *E. coli* strains.

Antibiotics	Resistance gene	Isolates					
		GR1	GR2	GR3	GR4	GR5	GR6
AMP	*ampC*	+	+	+	−	+	+
CFX	*bla*$_{CTX\text{-}M}$	−	+	+	−	−	−
SMZ	*sulI*	−	−	−	+	−	−
	sulII	−	−	+	−	+	−
	sulIII	−	−	+	+	−	−
TM	*dfrA1*	−	+	−	−	−	−
	dfrA5	−	−	−	−	+	−
FOF	*fosA*	−	−	−	−	−	−
	fosA3	−	+	−	−	−	−
	fosC2	−	−	−	−	−	−
CMP	*cmlA1*	−	−	+	−	−	−
	cat1	−	−	−	−	−	−
	cat2	−	−	−	+	−	−
KNM & GMN	*aphA1*	+	−	+	+	+	−
	ant(2″)-Ia	−	−	−	−	−	−
	aacC2	−	−	−	+	+	−
NAL & CIP	*qnrA*	−	−	−	−	−	−
	qnrB	−	−	−	−	−	−
	qnrS	+	+	+	+	−	−
TET	*tetA*	+	+	+	+	+	−
	tetK	−	−	−	−	−	−
	tetM	−	−	+	+	−	−

+, denotes presence; –, denotes absence.

drug for over four decades and its extensive use in Europe and Asia explains the relatively high resistance to FOF detected in this study (Hirsch et al., 2015; Sastry and Doi, 2016). The low cost and low toxicity of TET led to their wide application in the clinical world (Borghi and Palma, 2014), and, thus, relatively high resistance rates to this antibacterial agent are observed. Apart from that, regardless of selective pressure, β-lactams, FOF, and TET resistance may be preserved in bacterial populations due to the long historical usage, which may bring about an overall increase in antibiotic resistance over time. Hence, exposing a bacterial population to one or more antibiotics may give rise to resistance to other antibiotics without any prior exposure. (Al-Badaii and Shuhaimi-Othman, 2015; Ho et al., 2013). Despite being inexpensive and easy to access, the use of CMP in human medicine is restricted due to its carcinogenic effects and potentially harmful side effects, including aplastic anemia, due to the depression of the bone marrow function (Hanekamp and Kwakman, 2010; Ng et al., 2014). In fact, the use of CMP is currently banned in food production. However, a moderately high resistance was observed against this antibiotic, suggesting that chloramphenicol is still commonly used in human therapeutics in Asian countries along with its extensive use in treating ophthalmic infections (Hanekamp and Bast, 2015). For instance, all *E. coli* strains isolated from environmental water in Sarawak were found to be resistant toward CMP (Ng et al., 2014).

In this study, slightly lower resistance rates were observed for CIP (57.4%) compared with first-generation quinolones, such as NAL (71.3%). In a separate study on the prevalence of antibiotic resistance among *E. coli* (Alhaj et al., 2007), low resistance levels toward CIP, with the exception of NAL, were detected, which is consistent with the findings in this study. However, the resistance to quinolones was found to be higher, particularly for CIP, which was two-fold higher than the previous study (CIP 24.3%). The increase in resistance to quinolones could be attributed to the increase in use of these antibiotics.

Data submitted on antibiotic usage in hospitals, including private hospitals, indicates low usage of aminoglycosides (below 200 DDD (defined daily doses)/1000 PD (Parkinson's disease)) (Pharmaceutical Services Division, 2014) over the past three years compared with other groups of antibiotics. The reduced use of this class of antibiotics may explain the low resistance in the iso-

lates tested in this study. Thus, the low resistance observed could be ascribed to the controlled usage of the aminoglycosides.

Gombak River yielded strains with resistance profiles ranging from no resistance to resistance to one up to ten antibacterial agents out of the eleven tested and this group of strains was dominated by strains with resistance to five and six antibacterial agents. The current study demonstrated MDR with a number of antibiotics from three to ten. The frequency of MDR in this study was greater than that found in isolates from rivers in the Osun-State, South-Western Nigeria (Titilawo et al., 2015) and the San Pedro River, Mexico (Ramírez Castillo et al., 2013), and these studies revealed MDR ranging from three to seven and nine antibiotics out of thirteen and twenty antibacterial agents tested, respectively. In contrast, the MDR frequency in the current study was lower compared with that of *E. coli* isolates recovered from urban waterways of Milwaukee, WI, USA (Kappell et al., 2015), which found multidrug-resistance ranging from 3 to all of the 17 antibiotics tested. Together, these results justify the viewpoint that the variances in the MDR patterns may be attributed to the sources of these isolates, which are exposed to different doses, exposure frequencies, and/or types of antibiotics (Wang et al., 2013).

The present study outlined the phenotypic MDR patterns of the isolates after testing with AMP, TM-SMZ, TET, NAL, and CMP. Likewise, in a separate study by Amaya et al. (2012), *E. coli* isolates from hospital sewage showed MDR patterns, with resistance to AMP, CMP, CIP, NAL, and TM-SMZ, which was found to be the most common MDR profile in the isolates. One possible interpretation of the results includes the likelihood that the Gombak River flowing through Kuala Lumpur is a reservoir of MDR *E. coli*, and resistant bacteria in this environment are on the rise, as 90.76% of the isolates exhibited resistance to three or more antibiotic classes, which is definitely of great concern. The development of MDR, and decreasing levels of susceptibility (intermediate) observed in this study among isolates of *E. coli* to a broad-spectrum of antibiotics, is a matter of concern as it may limit the future availability of antibiotics for clinical management, particularly in the case of waterborne outbreaks.

Patterns of multiple resistance determinants were detected among the tested MDR *E. coli* isolates as shown in Table 2. Dual *tetA-tetM* was carried by two of the TET-resistant isolates, and three of the isolates carried only the *tetA*. All of the TET-resistant isolates were found to be lacking the *tetK* gene examined, and one was not found to contain any of the TET resistance genes evaluated. Five of the AMP-resistant isolates were *ampC* positive, two of the CFX-resistant isolates harbored the $bla_{CTX\text{-}M}$ gene, and two of the β-lactam-resistant isolates carried both the *ampC* and $bla_{CTX\text{-}M}$ genes. It was found that four of the CFX-resistant isolates, and one of the AMP-resistant isolates, tested negative for the β-lactam resistance genes examined. Similarly, one of the β-lactam-resistant isolates carried none of the β-lactam resistance determinants. Out of the three resistance genes for sulfonamides examined in this study, one of the isolates resistant to the combination of TM-SMZ carried only the *sulII* gene. One of the isolates carried dual *sulI–sulIII*. Similarly, one strain with sulfamethoxazole-resistance harbored dual *sulII–sulIII*. Moreover, three of the isolates did not carry any of the SMZ-resistance genes examined. It was found that one of the TM/SMZ-resistant isolates carried only *dfrA1*, and one carried only *dfrA5*, encoding resistance to trimethoprim. Four of the isolates did not carry *dfrA1* or *dfrA5*. Of the TM/SMZ-resistant isolates, two isolates did not carry any of the SMZ- or TM-resistance determinants. The *aphA1* gene was detected in four of the KNM-resistant isolates. Meanwhile, two of the GMN-resistant isolates carried *aacC2*, and none of the GMN-resistant isolates were found to be positive for *ant(2″-Ia)*. Of the three resistance genes encoding resistance to fluoroquinolones examined in this study, only *qnrS* was detected in four of the fluoroquinolone-resistant isolates, with all the strains lacking both *qnrA* and *qnrB*, and two of the isolates negative for *qnrA*, *qnrB*, and *qnrS*. As for FOF-resistant isolates, only one of the isolates carried the *fosA3* gene. All of the FOF-resistant isolates were found to be negative for both *fosA* and *fosC2*. Five of the FOF-resistant isolates had no observable genotypic features, as *fosA*, *fosA3*, and *fosC2* were not detected. Among the CMP-resistant isolates screened, one of the isolates had the *cmlA1* gene, and one of the isolates were found to have the *cat2* gene. The present study found that all six of the CMP-resistant isolates were negative for *cat1*, and four of the CMP-resistant isolates carried none of the three genes tested.

In this study, a total of 129 *E. coli* isolates were successfully recovered from a tropical aquatic environment in Southeast Asia. Approximately 90.7% of the isolates displayed resistance to multiple classes of antibiotics with isolates showing resistance to three to ten out of the eleven antibacterial agents tested. These strains were further categorized as MDR *E. coli*. The phenotypic resistance profiles and high levels of MDR strains reported in this study suggest a wide distribution and dissemination of resistance among *E. coli* strains, as well as among the entire bacterial community of the aquatic system. However, the current study, together with a previous study for *Staphylococcus aureus*, revealed a contradiction between the phenotypic resistance profiles and the genotypic features with regard to the resistance genes examined (Zulkeflle et al., 2016). It is worth noting the high frequency of the resistant isolates testing negative for the resistance genes examined. The high percentage of non-conserved genes in the resistant isolates observed in the present study merits further investigations, because this finding further suggests that there may be an underlying mechanism influencing the selection and evolution of MDR isolates in the environment, particularly in an aquatic system. As bacteria have developed resistance to broad-spectrum antibiotics, which may develop through novel mechanisms, quick action is crucial to diminish the development and spread of antibiotic resistance.

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Supplementary Materials

Supplementary table is available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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