PREVALENCE, DIVERSITY AND RISK ASSESSMENT OF ANTIBIOTIC RESISTANT *E. COLI* FROM ANTHROPOGENIC IMPACTED LARUT RIVER AND SANGGA BESAR RIVER

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY (OCEAN AND EARTH SCIENCES)

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PREVALENCE, DIVERSITY AND RISK ASSESSMENT OF ANTIBIOTIC RESISTANT *E. COLI* FROM ANTHROPOGENIC IMPACTED LARUT RIVER AND SANGGA BESAR RIVER

ABSTRACT

The rising emergence of rapidly evolving, multi-antibiotic resistant *Escherichia coli* (*E. coli*) remained a global public health concern. Aquatic ecosystems under frequent anthropogenic influence could serve as reservoirs that provide an ideal condition for the acquisition and dissemination of antibiotic resistant genetic determinants. To better understand the impact of anthropogenic wastewater towards the occurrence, genetic diversity and virulence of antimicrobial resistant *E. coli* as well as their resistance genes, surface waters from riverine estuarine waters of Larut River and Sangga Besar River were studied. Six sampling sites situated upstream and downstream of the Larut River including wastewater from zoo, hospital, and slaughterhouse, along with three sites from neighbouring Sangga Besar River were studied. Concentrations and ecological risk of 22 antibiotics from surface water samples were quantified by solid phase extraction and high performance liquid chromatography tandem mass chromatography. Total coliform and *E. coli* were isolated and enumerated by membrane filtration technique. Antibiogram was profiled using antimicrobial susceptibility test (AST), whereas phylogeneticity, virulence gene, and antibiotic resistance gene were determined by multiplex PCR assays. Canonical correlation analysis (CCA) was then performed to analyse the *E. coli* phylogenetic groups against resistance genes *sul* and *tet*, physicochemical parameters, and antibiotic residue concentrations. Sixteen antibiotic residues were detected, with concentrations ranging from limit of detection (LOD) to 1,262.3 ng l^{-1} with residues of

erythromycin, clarithromycin, and ofloxacin in hospital and zoo effluents posing a high risk to algae while tetracycline had low to medium ecological risks toward all the relevant organisms from aquatic environments (algae, invertebrate *Daphnia magna*, and fish). *E. coli* abundance at Larut River ranged from EST 1 to 4.1×10^5 CFU 100 ml^{-1}, whereas Sangga Besar River ranged from EST 1 to 2.5×10^3 CFU 100 ml^{-1}. All phylogenetic groups except B1 had non-homogenous distribution, with phylogenetic group A, found significantly higher in effluents of hospital (58.57 %) and slaughterhouse (49.18 %) followed by S1b (51.67 %). Also, more than half of the *E. coli* isolates from Larut and Sangga Besar River have multiple antibiotic resistances. The prevalence of multiple antibiotic resistance phenotypes of *E. coli* with the presence of *tet* and *sul* resistance genes was higher in wastewater effluents compared to river waters. The phylogenetic composition of *E. coli* and resistance genes were associated with physicochemical properties and antibiotic residues. Findings conclude that sites impacted by wastewater effluents along with antibiotic residues had affected *E. coli* phylogenetic composition, antibiotic resistance phenotype diversifications, and resistance genes distribution in Larut River.

Keywords: *Escherichia coli*, diversity, antibiotic resistance, anthropogenic, virulence.

PREVALENS, KEPELBAGAIAN DAN PENILAIAN RISIKO *E. COLI* YANG MERINTANG ANTIBIOTIK DARI SUNGAI LARUT YANG MENERIMA KESAN ANTROPOGENIK DAN SUNGAI SANGGA BESAR

ABSTRAK

Peningkatan kemunculan *Escherichia coli* (*E. coli*) berevolusi pesat dan berbagai rintangan masih merupakan isu kesihatan awam global yang membimbangkan. Ekosistem akuatik di bawah pengaruh antropogenik yang berterusan akan menjadi takungan umum untuk pemerolehan dan penyebaran penentu genetik rintangan antibiotik. Untuk lebih memahami kesan air sisa antropogenik terhadap kejadian, kepelbagaian genetik, dan virulensi *E. coli* berbagai rintangan antibiotik serta gen rintangannya, air permukaan daripada perairan muara Sungai Larut dan Sungai Sangga Besar telah disiasat. Enam tapak persampelan di hulu dan hilir Sungai Larut termasuk sisa air dari zoo, hospital, dan rumah sembelih serta tiga lagi tapak dari Sungai Sangga Besar yang berdekatan telah disiasat. Kepekatan dan risiko ekologi sampel permukaan air bagi 22 jenis antibiotik telah diukur dengan kaedah pengekstrakan fasa pepejal (SPE) diikuti oleh kromatografi cecair berprestasi tinggi jisim gabungan (HPLC–MS/MS). Jumlah koliform dan *E. coli* telah disingkan dan dikira dengan teknik penapisan membran. Profil antibiogram ditentu melalui ujian kerentanan antimikrob (AST), manakala filogenetik, gen virulensi, dan gen rintangan antibiotik diperolehi dengan ujian multiplex PCR. Analisis korelasi kanonik lalu dilaksanakan untuk menganalisis kumpulan filogenetik *E. coli* terhadap gen rintangan *sul* dan *tet*, parameter fizikokimia, dan kepekatan sisa antibiotik. Bilangan koliform dan *E. coli* di Sungai Larut merangkumi EST 1 hingga 4.1×10^5 CFU 100 ml^{-1}, manakala Sungai Sangga Besar merangkumi EST 1 hingga 2.5×10^3 CFU 100 ml^{-1}. Kesemua kumpulan

filogenetik kecuali B1 memiliki taburan yang tidak homogen, dimana kumpulan filogenetik A (34.39 %), dikesan jauh lebih tinggi dari efluen hospital (58.57 %) dan rumah sembelih (49.18 %) diikuti oleh S1b (51.67 %). Selain itu, lebih daripada separuh isolat *E. coli* dalam Sungai Larut dan Sangga Besar memiliki rintangan antibiotik yang berbagai. Kelaziman fenotip *E. coli* rintangan antibiotik yang berbagai serta pemilikan gen rintangan *tet* dan *sul* adalah lebih tinggi di tapak air sisa berbanding dengan perairan sungai. Komposisi filogenetik *E. coli* dan gen rintangan dikaitkan dengan ciri fizikokimia dan sisa antibiotik. Kesimpulannya, tapak yang terjejas oleh air sisa bersama dengan sisa antibiotik jelas mengubahsuai komposisi filogenetik *E. coli*, kepelbagaian fenotip rintangan antibiotik, dan taburan gen rintangan di Sungai Larut.

Kata kunci: *Escherichia coli*, kepelbagaian, rintangan antibiotik, antropogenik, virulen.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
+	:	Positive
-	:	Negative
/	:	Divide
×	:	Multiply
±	:	Plus–minus
=	:	Equals to
Σ	:	Sum
°C	:	Degree Celcius
–	:	Range
<	:	Less than
≤	:	Less than or equal to
>	:	More than
≥	:	More than or equal to
(σs)	:	Sigma factor
(H)	:	Flagellar antigen
(K)	:	Capsular antigen
(O)	:	Somatic antigen
HPLC—MS/MS	:	High-performance liquid chromatography-tandem mass spectrometry
µg	:	Microgram
µg l^{-1}	:	Microgram per litre
µm	:	Micrometre
µM	:	Micromolar
µl	:	Microlitre

$\chi 2$	:	Chi-square
$^{13}C_3$-caffeine	:	Caffeine-trimethyl
16s rRNA	:	16 Svedberg ribosomal ribonucleic acid
Abbr.	:	Abbreviation
AF	:	Assessment factor
AMC	:	Amoxycillin/Clavulanic acid
AMP	:	Ampicilin
ANOVA	:	Analysis of variance
AP	:	Amphenicol residues
APEC	:	Avian pathogenic *E. coli*
AR	:	Antibiotic resistance
ARB	:	Antibiotic resistant bacteria
ARG	:	Antibiotic resistance gene
AST	:	Antimicrobial susceptibility test
ATTC	:	American Type Culture Collection
AZM	:	Azithromycin
bp	:	Base pair
BOD	:	Biochemical oxygen demand
C	:	Chloramphenicol
CAP	:	Chloramphenicol residue
CCA	:	Canonical correlation analysis
CFU	:	Colony forming unit
CHROMEagar^{TM} ECC	:	Chromogenic Media Focus on *E. coli* and coliform
CIP	:	Ciprofloxacin
CIX	:	Ciprofloxacin residue
CN	:	Gentamicin

COD	:	Chemical oxygen demand
CRO	:	Ceftriaxone
Csp	:	Cold shock protein
CTC	:	Chlortetracycline residue
CTM	:	Clarithromycin residue
DAD	:	Diode array detection
DAEC	:	Diffusely adherent *E. coli*
DDD	:	Defined daily dose
df	:	Degree of freedom
DHPS	:	Deoxyhypusine Synthase
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxynucleoside triphosphate
DO	:	Dissolved oxygen
DOE	:	Department of Environment
E. coli	:	*Escherichia coli*
EAEC	:	Enteroaggregative *E. coli*
EC50/LC50	:	Effective concentration 50 per lethal concentration 50
ECOR	:	*E. coli* reference
EHEC	:	Enterohemorrhagic *E. coli*
EI	:	Enzyme inactivation
EIEC	:	Enteroinvasive *E. coli*
ELISA	:	Enzyme-linked immunosorbent assay
ENR	:	Enrofloxacin
ENRO	:	Enrofloxacin residue
ENX	:	Enoxacin residue
EP	:	Efflux protein

EPEC	:	Enteropathogenic *E. coli*
ERIC	:	Enterobacterial repetitive intergenic consensus
ExPEC	:	Extraintestinal pathogenic *E. coli*
ETEC	:	Enterotoxigenic *E. coli*
ETM-H_2O	:	Dehydrated erythromycin
F	:	Nitrofurantion
F	:	F-value, variance between means
FF	:	Florfenicol residue
FFC	:	Florfenicol
FNR	:	Fumarate and nitrate reductase gene
FQL	:	Fluoroquinolone residues
FIB	:	Faecal indicator bacteria
g	:	Gram
GF/F	:	Glassfibre filters
GPS	:	Global positioning system
h	:	Hour
H	:	Hospital
H'	:	Diversity index
H_2SO_4	:	Sulphuric acid
ha	:	Hectare
HGT	:	Horizontal gene transfer
HLB	:	Hydrophilic-lipophilic balance
HPLC	:	High performance liquid chromatography
hra1	:	Heat resistant agglutination
IMViC	:	Indole, methyl-red, Voges-Proskauer and citrate reaction
INWQS	:	Interim National Water Quality Standards

IPEC	:	Intestinal pathogenic *E. coli*
IPM	:	Imipenem
kb	:	Kilobase
kg $year^{-1}$	:	Kilogram per year
km	:	Kilometre
km^2	:	Square kilometre
KZ	:	Cephazolin
L(E)C50	:	Toxicity value
LC-MS	:	Liquid chromatography with mass spectrometer
LGT	:	Lateral gene transfer
LHR	:	Locus of heat resistance
LOD	:	Limit of detection
log	:	Logarithm
log CFU 100 ml^{-1}	:	Logarithm of Colony-forming unit per 100 millilitre
LQ	:	Limits of quantification
m	:	Metre
M	:	Molarity
MANOVA	:	Multivariate analysis of variance
MAR	:	Multiple antibiotic-resistant
MDS	:	Multidimensional scaling
MEC	:	Measured environmental concentration
MGE	:	Mobile genetic elements
mg	:	Milligrams
mg l^{-1}	:	Milligrams per litre
MIC	:	Minimal inhibitory concentration
min	:	Minute

ML	:	Macrolides
ml	:	Millilitre
ML	:	Macrolide residues
MLEE	:	Multilocus enzyme electrophoresis
ml min^{-1}	:	Millilitre per minute
MLST	:	Multi-locus sequence typing
mm	:	Millimetre
mm $year^{-1}$	:	Millimetre per year
MMFR	:	Matang Mangrove Forest Reserve
mmol l^{-1}	:	Millimole per litre
MNEC	:	Meningitis-associated *E. coli*
mol l^{-1}	:	Mole per litre
MPN	:	Most probable number
MS/MS	:	Tandem mass spectrometry
MSC	:	Minimal selective concentrations
MST	:	Microbial source tracking
n	:	nth term of a sequence of numbers
N	:	Neomycin
N/A	:	Not applicable
NA	:	Nalidixic acid
Na_2EDTA	:	Ethylenediamine tetraacetic acid disodium
NaCl	:	Sodium chloride
$NaHCO_3$	:	Sodium bicarbonate
ng l^{-1}	:	Nanogram per litre
NH_4	:	Ammonium
NMEC	:	Neonatal-meningitis *E. coli*

NO_2	:	Nitrite
NO_3	:	Nitrate
NOEC	:	No observable effect concentration
NOX	:	Norfloxacin residue
NTU	:	Nephelometric turbidity units
NWQS	:	National Water Quality Standards
OA	:	Oxolinic acid
OD	:	Optical density
OFX	:	Ofloxacin
OTC	:	Oxytetracycline residue
p	:	Probability
PAST	:	Paleontological Statistics Software
PCR	:	Polymerase chain reaction
Phylogroups	:	Phylogenetic groups
PNEC	:	Predicted no-effect concentration
PO_4	:	Phosphate
ppt	:	Parts per thousand
R^2	:	R-squared, coefficient of determination
rdar	:	Red, dry, and rough
rep	:	Repetitive element sequence
rep-PCR	:	Repetitive element sequence-PCR
RFLP	:	Restriction fragment length polymorphisms
RPP	:	Ribosomal protection protein
rpm	:	Rotation per minute
RQ	:	Risk quotient
RTM	:	Roxithromycin residue

RT-PCR	:	Reverse transcription-polymerase chain reaction
S	:	Streptomycin
S/N	:	Signal to noise ratio
S1a	:	Sampling site Larut River (a)
S1b	:	Sampling site Larut River (b)
S1c	:	Sampling site Larut River (c)
S2a	:	Sampling site Sangga Besar River (a)
S2b	:	Sampling site Sangga Besar River (b)
S2c	:	Sampling site Sangga Besar River (c)
SA	:	Sulphonamide residues
SAAM	:	Sulfacetamide residue
SDM	:	Sulfadimethoxine residue
SDZ	:	Sulfadiazine residue
SMA	:	Sulfadimidine residue
SMX	:	Sulfamethoxazole residue
s	:	Seconds
SePEC	:	Sepsis-associated pathogenic *E. coli*
SF	:	Sulphafurazole
SH	:	Slaughterhouse
SiO_4	:	Silicate
SPD	:	Sulfapyridine residue
SPE	:	Solid-phase extraction
SPSS	:	Statistical Package for the Social Sciences
STEC	:	Shiga toxin-producing *E. coli*
STP	:	Sewage treatment plant
STZ	:	Sulfathiazole residue

SXT	:	Sulphamethoxazole/trimethoprim residue
sul	:	Sulphonamide resistance gene
TBE	:	Tris/borate/ethylenediamine tetraacetic acid
TC	:	Tetracycline residue
TDS	:	Total dissolved solid
TE	:	Tetracycline
tet	:	Tetracycline resistance gene
TMP	:	Trimethoprim
TRI	:	Trimethoprim residues
UPEC	:	Uropathogenic *E. coli*
UPGMA	:	Unweighted pair group method with arithmetic mean
UV	:	Ultra violet
V	:	Voltage
v/v	:	Volume per volume
VTEC	:	Verocytotoxic *E. coli*
WGS	:	Whole genome sequencing
WHO	:	World Health Organisation
WQI	:	Water Quality Index
WWTP	:	Wastewater treatment plant
Z	:	Zoo

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Escherichia coli (*E. coli*) is ubiquitous in faeces, therefore it has been favoured as an indicator of faecal contamination and as an indicator of hygiene and food safety. However, studies have revealed that there is strong evidence showing trends of increasing ubiquity of *E. coli* in aquatic environments (Byappanahalli et al., 2003; Berthe et al., 2013; Perini et al., 2015). Most *E. coli* strains are harmless but some serotypes could be pathogenic, which may lead to intestinal and extra-intestinal infections. This risk of infection could be significantly increased if the bacterium is resistant to antibiotics (Pereira et al., 2013).

River pollution is a severe problem in Malaysia. Among 638 rivers, there were 231 rivers classified as slightly polluted while 50 were polluted (DOE 2019a, **Figure 1.1**). The water quality index (WQI) serves as the basis for river assessment in relation to pollution categorisation, health risk, and the designation of classes of beneficial uses as providers under the Interim National Water Quality Standards (INWQS) for Malaysia. The assessment of the WQI conducted by the Malaysian DOE is physicochemical based on six main parameters: biochemical oxygen demand (BOD), dissolved oxygen (DO), chemical oxygen demand (COD), suspended solids, ammonia nitrogen, and pH. It does not include coliform-based indicators. Sewage has been identified as the major source of river pollution in Malaysia (Department of Environment, 2018). The discharge of domestic, animal, sewage treatment plants and industrial wastewaters usually consists of organic matter, inorganic salts, nitrogen, heavy metals, and a wide diversity of microorganisms (bacteria, viruses, and protozoa). *E. coli* is one of the pollutants that affect the water quality in rivers and may pose a serious threat to public health risk.

Antibiotic resistant (AR) bacteria are increasing and are expected to become a global health emergency that could cause 10 million deaths by 2050

(World Health Organisation [WHO], 2019). Thus, the rising emergence of rapid evolution and multiple antibiotic-resistant (MAR) environmental *E. coli* is of great concern (Djordjevic et al., 2013; Harwood et al., 2013). AR *E. coli* may enter the aquatic environment through discharge from livestock production, hospital waste material, and municipal wastewater (Pruden et al., 2006; Pereira et al., 2013). Consequently, rivers could become efficient disseminators of these MAR bacteria and antibiotic resistance genes (ARGs). Furthermore, numerous types of anthropogenic activity, including antibiotic use in hospitals, agriculture, and aquaculture, along with other non-human applications of antibiotics may intensify the spread of ARGs in the environment.

Antibiotics have received increased attention as an emergent micropollutant that diminishes water quality regardless of the compound's concentrations (Sarmah et al., 2006; Allen et al., 2009; Grenni et al., 2018). Under low concentrations, exposure to antibiotics could exert strong selection pressure for ARG adoption among bacteria through lateral gene transfer (LGT) (Stokes & Gillings, 2011) which is also known as horizontal gene transfer (HGT) (Martínez, 2009a; Partridge, 2011; Berendonk et al., 2015). In turn, this promotes the dissemination of resistant bacterial clones that ultimately aids the overall dissemination of resistance genes (Gillings & Stokes, 2012).

Despite that, gaps in monitoring data in many East and Southeast Asian countries are apparent (Anh et al., 2020), with few Malaysian studies contributing surveys on antibiotic resistance in the environment (Rathi et al., 2010; Ghaderpour et al., 2015), A past study did detect antibiotic concentrations in three major rivers in the state of Selangor (Praveena et al., 2018), however, the antibiotics sampled were less comprehensive. To fill this gap, this is the first study in Malaysia that will more comprehensively elucidate the level of contamination caused by commonly used antibiotics towards environmental surface waters, while having their effects on ecological risks and AR *E. coli* distribution characterised. The riverine estuarine water continuum of Larut River which received anthropogenic wastewaters from hospital, zoo, and poultry slaughterhouse was studied. Meanwhile, the less impacted sites from Sangga Besar River were used as a comparison. Twenty—two antibiotics comprising six antibiotic groups (sulphonamides, fluoroquinolones, macrolides, tetracyclines, amphenicols, and diaminopyrimidine) were screened using high-performance liquid chromatography-tandem mass spectrometry (HPLC—MS/MS). Ecological risk quotients were established to assess the degree of ecological impact. Following that, environmental *E. coli* was isolated from each site using selective media and characterised according to phylogenetic group, antibiotic susceptibility, antibiotic resistance genes, and virulence gene to understand prevalence and abundance. Thus, the main objectives of this study were to investigate the anthropogenic impact on (i) occurrence, concentration, and ecological risk of antibiotic residue and (ii) prevalence of a genetically diverse population of *E. coli* with varying antibiotic susceptibility, antibiotic resistance genes, and virulence in Larut River, as compared to Sangga Besar River.

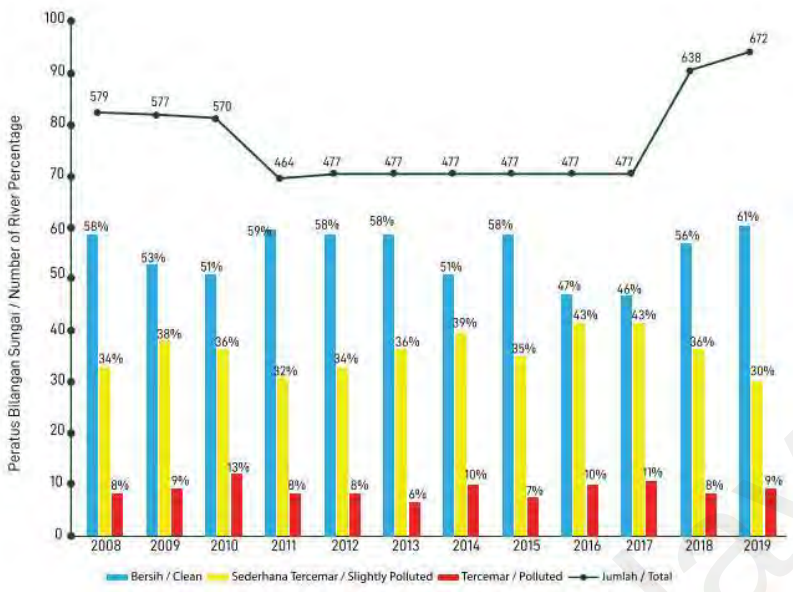

Figure 1.1: Trend of river water quality from 2008-2019 in Malaysia

CHAPTER 2: LITERATURE REVIEW

2.1 *Enterobacteriaceae*

Enterobacteriaceae is a family of Gram-negative bacteria. According to Bergey's Manual of Systematics of Archaea and Bacteria, *Enterobacteriaceae* consists of 44 genera and more than 170 named species of bacteria found inhabiting a multitude of hosts and habitats worldwide (Brenner & Farmer, 2015). Phenotypic similarities shared among *Enterobacteriaceae* include (i) having a straight rod shape, (ii) does not require sodium, heme and/or nicotinamide adenine dinucleotide for growth, and (iii) likely contains enterobacterial common antigen (Brenner & Farmer, 2015). However, certain taxa are non-motile while motile taxa rely on peritrichous or polar flagella for motion (Jorgensen & Pfaller, 2015). Many members of this family regularly inhabit the gastrointestinal microbiota in humans and other animals, while the remainders also inhabit soil, water, fruits, meats, eggs, vegetables, grain, flowering plants, and trees. DNA relatedness is important in defining the relationships of many *Enterobacteriaceae* members (Wayne et al., 1987; Fox et al., 1992; Stackebrandt and Goebel, 1994). A prominent member of the *Enterobacteriaceae* family is *E. coli*. Thus, DNAs from species within most genera of *Enterobacteriaceae* are at least 20 % related to *E. coli* and one another (Brenner & Farmer, 2015).

2.2 *E. coli* and its Importance

E. coli is a gram-negative, facultative anaerobic, oxidase-negative, non-sporulating, rod-shaped, lactose positive, and motile enteric bacillus. The genus *Escherichia* along with its first species *E. coli* had been recognised over a century ago. Since then, the genus underwent considerable reclassification efforts due to advances in genotypic and

genomic diagnostic tools. *Escherichia* once had seven species but now only four species were recognised, which consist of *E. coli*, *E. fergusonii*, *E. albertii*, and *E. marmotae* (Yu et al., 2021).

E. coli was initially believed to mainly inhabit the lower intestinal tract of warm-blooded animals, including humans, and be discharged to the environment through faeces and wastewater treatment plants (Berthe et al. 2013). Thus, *E. coli* is often characterised as a coliform, an informal category of bacteria that includes the genera of non-faecal origin and of faecal origin. Designated as faecal coliform, thermotolerant coliform, or faecal indicator bacteria (FIB), these culturable bacteria were used as an assay for over 150 years to diagnose the potability of water through the detection of faecal contamination (Leclerc et al., 2001; Garrity, 2007; Teaf et al., 2018; Holcomb & Stewart, 2020).

Numerous important advances in the characterisation of *E. coli* genotype and phenotype were on clinical isolates, where emphasis were given to virulence in a few clinically significant lineages infecting human and animal hosts (Hazen et al., 2016; Shah et al., 2018; Jørgensen et al., 2019). That is because *E. coli* is an important causative agent of some intestinal and extraintestinal diseases in humans (Nataro & Kaper, 1998; Blount, 2015). Selective pressure in the gut likely promoted the emergence and maintenance of virulence factors, indicating that commensal strains may function as reservoirs for the evolution of pathogenic *E. coli* (Tenaillon et al., 2010).

Most strains of *E. coli* strains are harmless, some strains are indeed beneficial to their host, e.g. certain strains exhibit substantial probiotic features towards its host by producing vitamin K and vitamin B12, ensuring healthy microbiome development, and playing a role in infection prevention (Bentley & Meganathan, 1982; Lawrence & Roth, 1996; Chang et al., 2004; Tomas et al., 2015). Through indirect

antagonistic effects, *E. coli* Nissle 1917 is one such example of a probiotic that inhibits the invasion by enteroinvasive pathogens (Sonnenborn, 2016).

E. coli is a gram-negative, facultative anaerobic, oxidase-negative, non-sporulating, rod-shaped, lactose positive, and motile enteric bacillus. The genus *Escherichia* along with its first species, *E. coli*, had been recognised over a century ago. Since then, the genus underwent considerable reclassification efforts due to advances in genotypic and genomic diagnostic tools. *Escherichia* once had seven species, but now only four species were recognised, which consist of *E. coli*, *E. fergusonii*, *E. albertii*, and *E. marmotae* (Yu et al., 2021).

E. coli was initially believed to mainly inhabit the lower intestinal tract of warm-blooded animals, including humans, and be discharged into the environment through faeces and wastewater treatment plants (Berthe et al. 2013). Thus, *E. coli* is often characterised as a coliform, an informal category of bacteria that includes genera of non-faecal origin and of faecal origin. Designated as faecal coliform, thermotolerant coliform, or faecal indicator bacteria (FIB), these culturable bacteria were used as an assay for over 150 years to diagnose the potability of water through the detection of faecal contamination (Leclerc et al., 2001; Garrity, 2007; Teaf et al., 2018; Holcomb & Stewart, 2020). Routine water quality assessments still prioritised FIB detection using *E. coli* and intestinal enterococci culture methods because of their relative affordability and straightforwardness (Leclerc et al., 2001; Tallon et al., 2005; Bain et al., 2012; WHO, 2017). The drinking-water quality guidelines set by WHO and the national water quality standards for Malaysia had set *E. coli* or thermotolerant coliform (i.e. faecal coliform) to not be detectable per 100 ml of water sample or contain no more than 10 MPN 100 ml^{-1} of water sample respectively (WHO, 2017; Department of Environment, 2019b).

Numerous important advances in the characterisation of *E. coli* genotype and phenotype were on clinical isolates, where emphasis was given to virulence in a few clinically significant lineages infecting human and animal hosts (Hazen et al., 2016; Shah et al., 2018; Jørgensen et al., 2019). That is because *E. coli* is an important causative agent of some intestinal and extraintestinal diseases in humans (Nataro & Kaper, 1998; Blount, 2015). Selective pressure in the gut likely promoted the emergence and maintenance of virulence factors, indicating that commensal strains may function as reservoirs for the evolution of pathogenic *E. coli* (Tenaillon et al., 2010).

Most strains of *E. coli* strains are harmless, but some strains are indeed beneficial to their host, e.g. certain strains exhibit substantial probiotic features towards its host by producing vitamin K and vitamin B12, ensuring healthy microbiome development, and playing a role in infection prevention (Bentley & Meganathan, 1982; Lawrence & Roth, 1996; Chang et al., 2004; Tomas et al., 2015). Through indirect antagonistic effects, *E. coli* Nissle 1917 is one such example of a probiotic that inhibits the invasion by enteroinvasive pathogens (Sonnenborn, 2016).

2.3 Diversity of *E. coli*

E. coli has diverse phenotypic and genotypic classifications. Initially, *E. coli* population structure was classified into serotypes: somatic (O), capsular (K), and flagellar (H) antigens (Ørskov et al., 1976). Now with the existence of molecular methods, *E. coli sensu stricto* can be classified into one of eight phylogenetic groups (phylogroups) (A, B1, B2, C, D, E, F, and cryptic clade I) (Luo et al., 2011; Clermont et al., 2013). Two additional groups: Phylogroup G and H were recently discovered (Lu et al., 2016; Clermont et al., 2019; Gonzalez-Alba et al., 2019). Two categorical *E. coli* groups exist in accordance to frequency: (i) the major

phylogroups (i.e. A, B1, B2, and D) which are common and (ii) the minor phylogroups (i.e. C, E, F, and cryptic clade I) described to be less common (Clermont et al., 2013; Touchon et al., 2020). *E. coli* strains belonging to different phylogroups have different associations with phenotypic and genotypic traits, metabolic properties, ecotype, lifestyle, and pathogenicity (Clermont et al., 2000; Gordon, 2004; Tenaillon et al., 2010; Méric et al., 2012), i.e. unknown strains are labelled a phylogroup with its accompanying characteristics.

Different *E. coli* strains express different phenotypic characteristics. These phenotypic variations could be influenced by ecological factors as evidenced by the fitness of *E. coli* towards its primary and secondary habitats, i.e. host-associated (Macfarlane & Macfarlane, 1997) and extra-host (Savageau, 1983) respectively. The diversity of *E. coli* can be explained by the genomic structure of the microorganism as a result of adaptation to the environment (van Elsas et al., 2011). Genetically, only 20 % of the genes in a typical *E. coli* genome are shared among all strains, making it one of the most genetically diverse bacterial species (Lukjancenko et al., 2010; Tenaillon et al., 2010). In some studies, approximately half of the average genome is conserved across most *E. coli* strains (i.e. core genome) while the rest (i.e. accessory genome) are sporadically found in certain strains, thus the pan-genome (core genome and accessory genome) greatly exceeds the size of the typical genome (Rasko et al., 2008; Touchon et al., 2009; Lukjancenko et al., 2010; Land et al., 2015). Only 2,000 genes from an average of 4,700 genes found in the *E. coli* genome are conserved at the core genome while the remainder belongs to a set of accessory genes (Tenaillon et al., 2010). Overall, the pan-genome in *E. coli* has a high level of plasticity that can only be described as vast, open, and continually growing, provided new strains continually appear (Rasko et al. 2008).

External stresses trigger various genes that encode proteins with specific roles in protecting the cell (Nyström, 2004). From the *E. coli* genome, accessory genes represent a part of the genome that enable adaptations through the acquisition and loss of genes related to cell motility, intracellular trafficking and secretion, carbohydrate transport and metabolism, and secondary metabolism via mobile genetic elements (MGEs) (Touchon et al., 2020). MGEs consist of plasmids, transposons, insertion sequences, integrative and conjugative elements, integrons, prophages, gene cassettes, etc. MGEs promote evolution through HGT (Wiedenbeck & Cohan, 2011) that subsequently provide a range of new functions and capabilities for *E. coli* strains to exploit and survive in certain ecotypes (Lukjancenko et al. 2010). *E. coli* isolates were observed to share genetic compositions reflective of their environmental challenges. *E. coli* isolated from separate wastewater treatment plants from Canada, Switzerland and the U.S. had all shared similar patterns of antibiotic AR genes and virulence genes, and possessed stress-related genes that enhance survivability against wastewater treatment (Zhi et al., 2019). Thus, divergent populations can share an adaptation through HGT which transcends differences in physiological capabilities, cellular structures, and ecological niches (Wiedenbeck & Cohan, 2011).

Virulence is driven by the acquisition of novel genes among pathogenic strains (Touchon et al., 2009; Dobrindt et al., 2010; Leimbach et al., 2013; Juhas, 2015). Presently, molecular methods are used to type the virulence factor, along with the identification of the host's clinical symptoms, *E. coli* strains are classified into pathotypes of diarrhoeagenic/intestinal pathogenic *E. coli* (IPEC) or extraintestinal pathogenic *E. coli* (ExPEC) (Lindstedt et al., 2018). Briefly, IPEC *E. coli* strains are major diarrhoeagenic pathogens that cause gastroenteritis with six well-established intestinal pathotype subgroups: enterohemorrhagic *E. coli* (EHEC) or Shiga toxin-producing *E. coli* (STEC) or verocytotoxic *E. coli* (VTEC) associated with

haemorrhagic colitis and haemolytic-uremic syndrome in humans, enteropathogenic *E. coli* (EPEC) associated with diarrhoea in children, enteroaggregative *E. coli* (EAEC) associated with persistent diarrhoea in humans, enterotoxigenic *E. coli* (ETEC) associated with traveller's diarrhoea, enteroinvasive *E. coli* (EIEC) associated with dysentery in humans and various animals, and diffusely adherent *E. coli* (DAEC) associated with acute diarrhoea, particularly in young children (Scaletsky et al., 2002; Kaper et al., 2004; Daniels, 2006; Van Den Beld & Reubsaet, 2012). Meanwhile, ExPEC consists of three human pathotype subgroups: neonatal-meningitis *E. coli* (NMEC) causing meningitis in newborn infants, uropathogenic *E. coli* (UPEC) causing urinary tract infections, and sepsis-associated pathogenic *E. coli* (SePEC) causing septicemia (Johnson & Russo, 2002; Kaper et al., 2004; Moriel et al., 2010; Wijetunge et al., 2015; Yu et al., 2021). Finally, the non-human pathotype avian pathogenic *E. coli* (APEC) is a strain similar to human ExPEC strains as they share a common repertoire of virulence genes (Mokady et al., 2005; Rodriguez-Siek et al., 2005), the subgroup causes a range of diseases in birds including colibacillosis (Nolan et al., 2013).

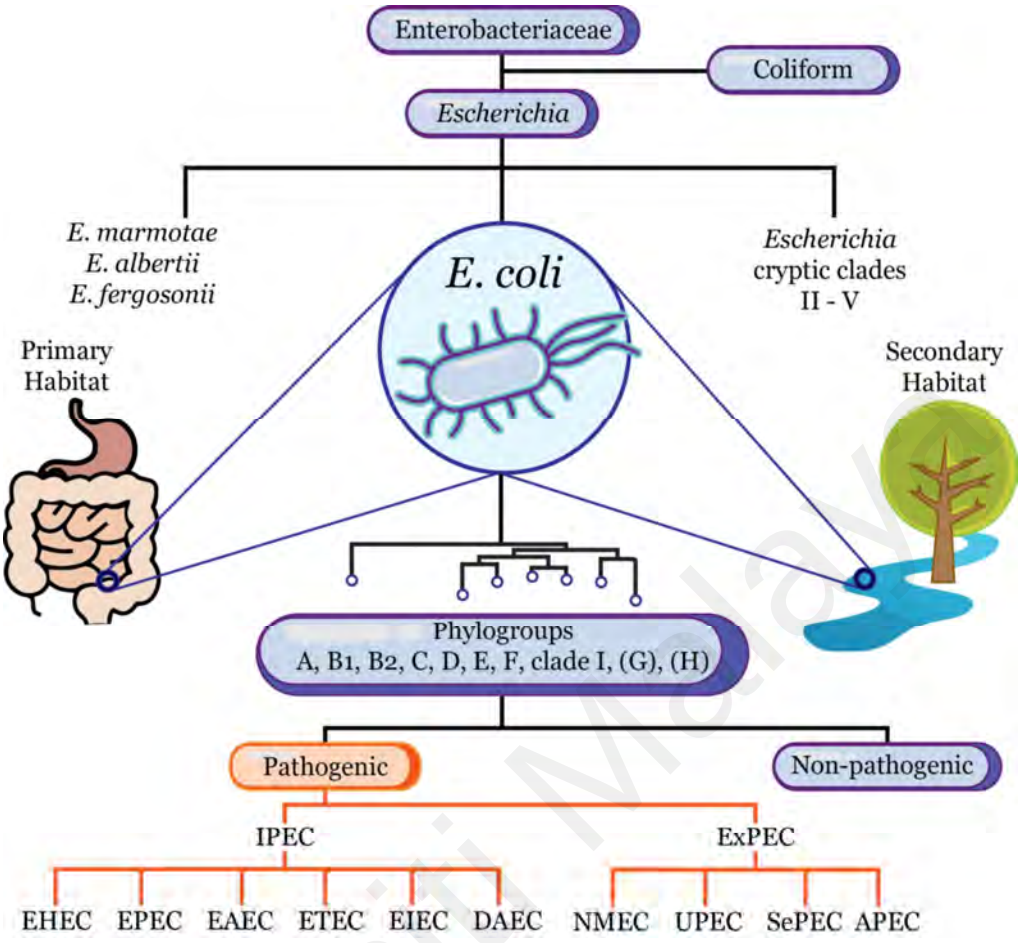

Figure 2.1: The schematic diagram of the current taxonomy of the *Escherichia* genus and the type of phylogenetic lineages under the faecal coliform *E. coli*

Overall, *E. fergusonii*, *E. albertii*, and *E. marmotae* were found to be genotypically similar to *E. coli* and new lineages like the cryptic *Escherichia* clades have been discovered (**Figure 2.1**). The great genetic and biological diversity of *E. coli* may consist of several distinct ecotypes largely divided into primary and secondary habitats. Well-established phylogenetic groups included A, B1, B2, C, D, E, and F along with clade I closely related to *E. coli* whereas phylogroups G and H were the most recently discovered groups. Pathogenic *E. coli* can be classified as IPEC or ExPEC. IPECs can be subdivided into EHEC, EPEC, EAEC, ETEC, EIEC, and DAEC. Similarly, the ExPEC can be subdivided into NMEC, UPEC, SePEC, and APEC.

2.4 Identification and Classification Methods of *E. coli*

E. coli population structure was formed by observing serotypes (O), (K), and (H) antigens using the agglutination technique (Ørskov & Ørskov, 1975; Ørskov et al., 1976). Among all pathotypes, EHEC serotype O157 was the strain of interest that gave rise to 390 outbreaks from 2003-2012 (Heiman et al., 2015). Consequently, species subtyping and identification of *E. coli* phylogeny became a growing priority as it is a major pathogen (Nataro & Kaper, 1998; Blount, 2015) and a FIB for water quality (Leclerc et al., 2001; Tallon et al., 2005; Bain et al., 2012; WHO, 2017). Other methods of *E. coli* strain classification were also applied.

Microbiologists once heavily relied on biochemical and structural phenotypes (i.e. metabolic pathways) for bacterial classification. For instance, the indole, methyl-red, Voges-Proskauer and citrate reaction (IMViC) tests are standard tests used to profile the biochemical properties of *E. coli* (Leclerc, 1962). Advances in genomic and genotypic diagnostic tools have since brought greater discriminative power and helped elucidate the remarkable diversity of *E. coli*.

Early studies on metabolic-enzyme polymorphisms by multilocus enzyme electrophoresis (MLEE) enabled the measurement of variation in the electrophoretic mobility of enzymes of different *E. coli* strains to discern phylogeny (Milkman, 1973; Selander & Levin, 1980). From the pioneering MLEE data, the major phylogenetic groups (A, B1, B2, D), were later identified from the *E. coli* reference (ECOR) collection and validated across studies using different methods such as restriction fragment length polymorphisms (RFLP) (Desjardins et al., 1995) followed by the more recent multilocus sequence typing (MLST) method. Complex DNA fingerprint data (Lim et al., 2009; Ghaderpour et al., 2015; Osińska et al., 2017) soon emerged from a large collection of *E. coli* isolates that could be used to infer genetic diversity. DNA

fingerprint images (i.e. band images) were first generated and then differentiated by comparing the change in spatial location of densitometric curves between each fingerprint before determining the genetic similarity within bacterial populations. Similarity of population structures could be analysed by Pearson's, curve-based, product-moment correlation coefficient, multidimensional scaling (MDS), multivariate analysis of variance (MANOVA), and other forms of discriminant and cluster analyses (Ishii et al., 2006; Ishii & Sadowsky, 2009; Unno et al., 2009). The library-based repetitive element sequence-based PCR (rep-PCR) DNA fingerprinting technique was one such genotypic tool used to identify, track and examine genetic diversity among environmentally important *E. coli* populations (Kon et al., 2009; Lyautey et al., 2010). To produce an array of fingerprints for the characterisation of a bacterial isolate, rep-PCR DNA fingerprinting amplifies conserved intergenic palindromic DNA sequences that are stable, non-coding, and scattered genomewide. Bacterial genome has four such conserved repetitive DNA sequences: (i) enterobacterial repetitive intergenic consensus (ERIC), (ii) BOX, (iii) poly-trinucleotide (GTG) 5, and (iv) repetitive extragenic palindromic (REP) (Versalovic et al., 1994; Mohapatra & Mazumder, 2008). Past literature had successfully applied fingerprinting methods to depict considerable genotypic distinction between *E. coli* strains from aquatic and faecal sources (Goto & Yan, 2011) and also demonstrated spatial and seasonal changes within the bacteria population (Chandran & Mazumder, 2015). Most recently, whole genome sequencing (WGS) was developed and could provide higher discrimination and resolution compared to other methods (Gordon et al., 2008; Touchon et al., 2009; Holmes et al., 2015). Each method has its advantages; a combination of multiple molecular methods is the best approach for a truly robust phylogenetic analysis.

Apart from other recent classifications, the "iterative" quadruplex PCR method by Clermont et al. (2013) was most useful at rapidly assigning *E. coli* strains to specific

phylogenetic groups (phylogroups). Presently, *E. coli* may be classified into eight phylogroups: seven (A, B1, B2, C, D, E, and F) belong to *E. coli sensu stricto*, with the eighth being *Escherichia* cryptic clade I, a phenotypically indistinguishable strain but genetically distinct from *E. coli* (Luo et al., 2011; Clermont et al., 2013). More recently, phylogroup G could be rapidly identified using PCR (Clermont et al., 2019), and H was validated using WGS (Lu et al., 2016; Gonzalez-Alba et al., 2019), increasing species members of *E. coli sensu stricto* to nine main phylogenetic groups. This is followed by the remaining four *E. coli* cryptic clades (II, III, IV, and V) (Walk et al., 2009; Luo et al., 2011; Clermont et al., 2013). The term cryptic clade was used due to the inability of standard phenotypic methods to distinguish novel *E. coli* from existing strains. The relationships of *E. coli sensu stricto* strains were summarised as Phylogroup B2 having almost exclusively intracladed recombination, whereas strains of phylogroups A, B1, and C showed the highest rate of homoplasy (**Figure 2.2**). Specifically, the B2 phylogroup is located a distance apart from the other phylogroups and had the lowest intergroup recombination frequencies (1.6 %), at the opposing end is phylogroup B1 which was highly recombinant (17.7 %). Concurrently, phylogroups C, E, and F probably emerged in relatively modern times by recombination between other phylogenetic lineages, e.g. phylogroup C arises from phylogroups A and B1 while phylogroup F maintains promiscuous interactions with phylogroup D (Turrientes et al., 2014). In comparison, cryptic clades relate distantly to *E. coli sensu stricto* (Gordon, 2013).

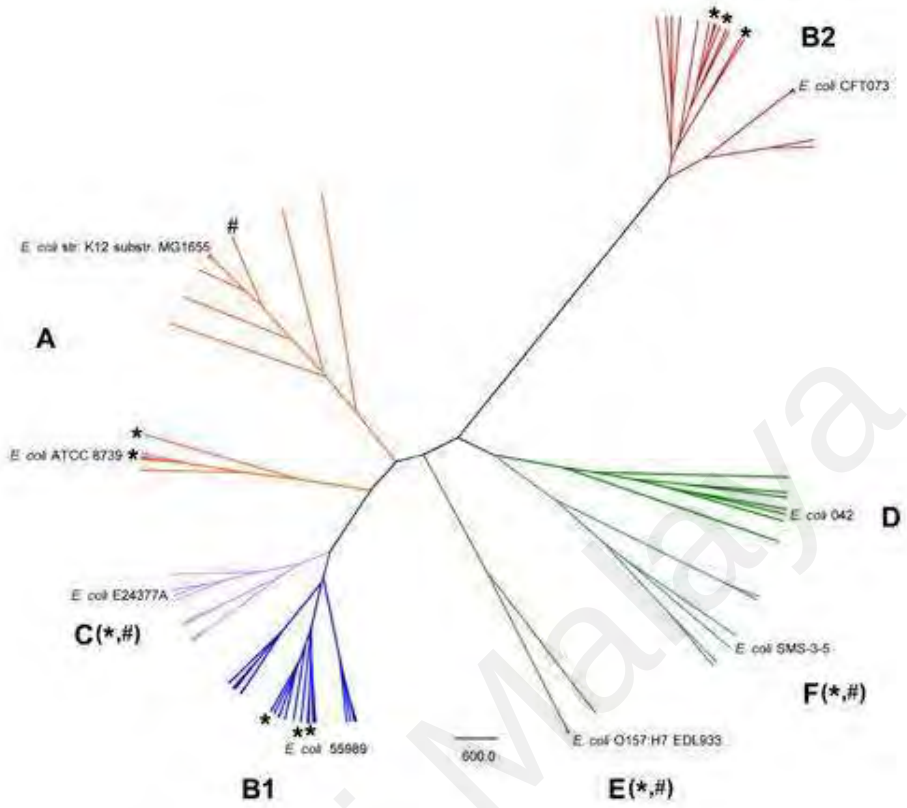

Figure 2.2: Phylogenetic trees of *E. coli* showing the relationship between phylogroups (Turrientes et al., 2014)

2.5 *E. coli* in Environment

By the early 2000s, a growing number of reports found *E. coli* populations persisting and replicating for extended periods in diverse environmental matrices. Some matrices include soil/sediment from subtropical and temperate regions (Lee et al., 2006; Ishii & Sadowsky, 2008; Ishii et al., 2009; Pote et al., 2009; Byappanahalli et al., 2012; Jang et al., 2015), algal mats (Badgley et al., 2011), water column from freshwater (Jang et al., 2011, 2015), marine water (Lee et al., 2006), estuary water (Berthe et al., 2013), and even treated drinking water (Blyton & Gordon, 2017). Each environment would affect the distribution and diversity of *E. coli* differently. Touchon et al. (2020) conducted a continent-scale genomic study of *E. coli* in Australia from 1993–2015, although they have observed that all phylogroups recovered could be isolated from multiple sources, certain phylogroups are, however, over-represented in some while under-represented in others.

In the overall aquatic environments, the factors that influence the predominance of certain phylogroups are generally categorised under current land use, hydrological conditions, and a potential site of contamination (Hamelin et al., 2007; Sabaté et al., 2008; Ratajczak et al., 2010; Mokracka et al., 2011). A change in phylogroup distribution after rainfall at a small rural watershed containing pasture and human occupation has been found to be related to increased faecal contamination. The change in *E. coli* phylogroup population structure (e.g. ratio of phylogroup A to B1) was thus proposed to represent a change in land use and natural hydrological conditions (Ratajczak et al., 2010). Wastewater effluent contains faecal coliform. A worldwide database on *E. coli* phylogroup frequency isolated from wastewater had established a strong positive correlation between *E. coli* phylogroup distribution in wastewater and in human hosts (Stoppe et al., 2017). Thus, studies have compared wastewater effluent impacted sites to non-impacted sites to identify patterns in the distribution of *E. coli*

phylogroups (Zhi et al., 2016; Martak et al., 2020). Strong spatial modification in *E. coli* phylogroup structure was observed when sampling sites transitioned from rural to urban areas along an aquatic continuum (Petit et al., 2017). Although phylogroups A and B1 are generally predominant and prevalent among water isolates (Walk et al., 2007; Ratajczak et al., 2010; Petit et al., 2017; Bong et al., 2020), studies that focused on bacterial communities in urban streams had shown that urbanisation had an impact on bacterial population structure (Wang et al., 2011; Hosen et al., 2017). Notably, phylogroup A was likely over-represented in waters near urban areas or from wastewater. Studies supporting this trend include the Rhine River (Stange et al., 2016), Tagus Estuary (Pereira et al., 2013), municipal wastewater and urban surface water of the Porto region (Figueira et al., 2011), waters of Rio de Janeiro (De Luca Rebello & Regua-Mangia, 2014), and a coastal lagoon of Venice (Perini et al., 2015). In comparison, phylogroup B1 strains were reported to survive and persist better in natural water environments compared to other phylogroups (Walk et al., 2007; Ratajczak et al., 2010; Berthe et al., 2013; Touchon et al., 2020;). Studies that support this included blooms from lakes (Power et al., 2005), a water catchment with mixed land use (Cho et al., 2018), a rural watershed composed of pasture and human occupation (Ratajczak et al., 2010), water canals of food crops irrigation (Jokinen et al., 2019), mangrove estuaries (Ghaderpour et al., 2015), water from the river and adjacent marine coast (Bong et al., 2020), and wetland waters with a dominant crow population (Sen et al., 2019). Conversely, phylogroups B2, and to a lesser extent, D were normally under-represented in aquatic environments (Walk et al., 2007; Petit et al., 2017; Ratajczak et al., 2010). Finally, cryptic clade strains cover 8.5 % and 14 % of the total surface water samples of an estuary (Berthe et al., 2013) and wetlands (Martak et al., 2020). Early data suggests cryptic clades are environmentally adapted (Luo et al., 2011; Oh et al., 2012). More studies are

needed to further verify the ecological distinction of cryptic clades along with minor phylogroups C, E, and F.

Among the *E. coli* population found in natural environments, certain strains are potentially pathogenic. A case of waterborne gastroenteritis outbreak by diarrhoeagenic O157:H7 *E. coli* was suspected to be caused by sewage contamination in drinking water (Swerdlow et al., 1992). Generally, strains harbouring virulent factors concentrate in phylogroups B2, D, E, and F, while the least abundant belong to A (Touchon et al., 2020; Nowicki et al., 2021). Therefore, phylogroup identification could be a simple but useful tool to narrow down the identity of a pathogenic strain (Anastasi et al., 2010) due to a relationship formed between the genetic background and virulence factors (Escobar-Páramo et al., 2004a).

2.6 Survival of *E coli* in Environment

The survival of *E. coli* is influenced by biotic and abiotic factors in natural environments (Rochelle-Newall et al., 2015). The abiotic factors include solar insolation, hydrologic conditions, suspended solids, temperature, pH, oxygen availability, general water chemistry, and nutrient availability. Biotic factors include predation and competition from microbiota, biofilm formation, and differential growth/survival ability among phylogroup. Factors to be discussed are as follows:

Solar insolation is the predominant abiotic factor causing a decrease in *E. coli* count on surface waters of freshwater (Jozi et al., 2014) and marine environments (Whitman et al., 2004). Light in the lower wavelengths, such as ultraviolet (UV) light, directly causes DNA damage to cells (photobiological mechanism) and/or oxidation of cellular contents (photooxidative mechanism). However, light is only effective in

surface waters where it can reach (Whitman et al. 2004). As such, turbidity of the water environment plays a significant role.To counteract UV radiation and desiccation from insolation at water surface. Light in the lower wavelengths, such as ultraviolet (UV) light, directly causes DNA damage to cells through a photobiological mechanism. To counteract UV radiation and desiccation from insolation, *E. coli* could enhance production of group 1 capsule to improve survivability (Power et al., 2005).

Hydrologic conditions, such as stormwater discharge, cause increased pollutant transport into aquatic environments. Surface water quality would subsequently deteriorate through increased turbidity, suspended solid concentrations, organic matter, and faecal contamination. Compared to baseflow, stormflow condition recorded higher *E. coli* concentrations (Rochelle-Newall et al., 2016) and modified the phylogenetic composition among *E. coli* population to promote adaptive strains (Ratajczak et al., 2010) in watersheds.

Suspended solids, not only do they provide organic and inorganic nutrients but also protect against environmental factors such as UV radiation, metal toxicity, grazing, and attack by bacteriophages (Medema et al., 2003) to microbes. *E. coli* adjusts its cell surface properties for better attachment to these suspended solids, namely through a complex process involving outer membrane proteins, hydrophobicity, and surface charge (Goulter et al., 2009). To support this, Bong et al. (2020) observed *E. coli* abundance had correlated positively with total suspended solids, suggesting that *E. coli* were distributed in rivers bounded to particulate matter while under indirect influence from precipitation.

Temperature, such that the environment experiences generally lower temperature with wider fluctuations. For example, *E. coli* isolates from bodies of water generally maintain growth at 20 °C, but a minority could still tolerate low temperatures of 7 °C

(Berthe et al., 2013). As such, a significant difference in survival rate between environmental compartments was observed, including rivers and agricultural waters, wastewaters and agricultural waters, rivers and lakes, and wastewater and lakes (Blaustein et al., 2013). To explain this, water adaptation among *E. coli* population thus varies by genotype, i.e. strains with better temperature tolerance likely harbour the genomic island locus of heat resistance (LHR) (Zhi et al., 2016) and/or the cold shock protein (Csp) superfamily (Yu et al., 2019).

pH, where the range between 6 and 8 does not inhibit *E. coli* (Foppen & Schijven, 2006). However, growth is limited at approximately pH 4, while survival at pH 2 to 3 only lasts several hours at stationary phase (Small et al., 1994). *E. coli* were thus suggested to possess a strong ability to survive in alkaline water due to a positive correlation found between pH and bacterial abundance according to a study on an aquatic environment with submerged vegetation (Gu et al., 2020). However, there are exceptions among *E. coli* strains. For example, several *E. coli* O157:H7 strains survive better at lower pH than higher pH, similar to acidophiles (van Elsas et al. 2011). This suggests that specific *E. coli* strains survive selectively depending on the local pH of the environment.

Oxygen availability, *E. coli* is a facultative anaerobe that can adapt to anoxic and oxic natural environments. The transition between anaerobic and aerobic respiration is driven by extensive changes in gene expression and protein synthesis, resulting in changes in metabolic fluxes and metabolite concentrations. The oxygen response of *E. coli* is mainly controlled by the global transcriptional regulators FNR and ArcA (Bettenbrock et al., 2014).

Water chemistry, various inorganic and organic chemicals present in the aquatic environment could inhibit *E. coli* growth at varying concentrations. The two major

factors to consider are salinity and chlorine concentrations for *E. coli*. Salinity influence desiccation/hydration of *E. coli* and is a type of common stress the bacteria face when in natural environments (Evans and Wallenstein 2012; van Elsas et al. 2011). The general minimum growth inhibitory concentrations are 20 % NaCl in nutrient-rich media and 3.5 % NaCl in nutrient-depleted media for salinity (Hrenovic & Ivankovic, 2009), whereas chlorine is 1.5 mg l^{-1} (Owoseni et al., 2017). Nearly every wastewater treatment plant uses chlorination to disinfect wastewater, followed by a dechlorination process before the water is safely sent back into the environment.

Nutrient availability, i.e. concentration of carbon, nitrogen, and phosphorus affects the survival and growth of *E. coli* in the environment. Specifically, under nutrient-starved conditions, such as low nitrogen (Chubukov & Sauer, 2014) and phosphorus (Juhna et al., 2007) levels, *E. coli* normally experience low metabolic rate with greater expression of stress defence genes while entering longer durations of stationary growth (Vital et al., 2015). The sigma factor (σs) is one such central regulator for stress in *E. coli*, which is strongly expressed during stationary phase (Lange & Hengge-Aronis, 1991; Patten et al., 2004). Under long-term stressful survival conditions, the common phenotypic characteristic of a (σs) dependent *E. coli* strain is an adhesive extracellular matrix comprising cellulose, curli fimbriae, and other polysaccharides (Zogaj et al., 2001; Römling, 2005), which can be identified by the formation of 'red, dry, and rough (rdar) morphotype' multicellular colonies on solid media dyed with Congo red (White et al., 2011).

Predation and competition from microbiota, *E. coli* population interacts with other microbes in nature. The decline in *E. coli* persistence in water is influenced by interactions like predation, lysis, and competition by protozoa, phages, and indigenous microbiota respectively. Generally, studies found *E. coli* survival was enhanced when

river water sample was sterilised before inoculation (Flint, 1987), indicating a potential influence of indigenous microbiota (van Elsas et al. 2011). Generally, studies found enhanced *E. coli* survival in a sterile river water sample (Flint, 1987). In contrast, survival in a sample containing a diverse indigenous microbiota was observed to be inversely proportional (van Elsas et al., 2011), indicating that species diversity may inhibit the dissemination of *E. coli* strains.

Biofilms serve to protect bacteria from hostile environmental factors such as UV radiation, desiccation, protozoan predation, and chemical exposures (McDougald et al., 2012). Protection can be due either to physical inhibition in antimicrobial diffusion or direct binding of the antibiotics in the biofilm, thus allowing most bacteria in nature to persist in coordinated, spatially organized, and metabolically integrated biofilm communities (Dufour et al., 2010). The heat resistant agglutination (hra1) gene harboured by *E. coli* encodes an accessory intestinal epithelial cell colonisation factor responsible for conferring biofilm formation characteristics (Bhargava et al., 2009).

Differential survival/growth ability among phylogroup, where certain phylogroups within the *E. coli* species were over-represented for several adaptation processes. Notably, B1 phylogroup likely facilitates survival in stressful environmental niches and possess adaptions towards aromatic compound utilisation for aquatic plant colonisation (Méric et al., 2012), salinity tolerance (Bong et al., 2020), growth in low temperatures (Berthe et al., 2013), and formation of an extracellular matrix, i.e. biofilm (Di Sante et al., 2018). In agreement with these observations, prevalence of the aforementioned rdar morphotype, a phenotype associated with survival, was significantly higher among B1 isolates (White et al., 2011; Di Sante et al., 2018). In comparison, Bong et al. (2020) suggest that phylogroup A adapts better in deteriorated

water quality with high ammonium and nitrite concentrations, phylogroup B appears to thrive in water with higher dissolved oxygen, while phylogroup D is abundant in waters with nitrate. Whereas the *Escherichia* cryptic clade strains (excluding clade I) have phenotypes showing the rdar morphology, enhanced abilities to form biofilms, and tolerate low growth temperatures (Ingle et al., 2011; Di Sante et al., 2018).

2.7 Antibiotic Resistant *E. coli*

Antibiotic resistant bacteria (ARB) could be defined as extensively resistant (broadly resistant or susceptible to only one or two categories of antibiotic agents), multidrug resistant (resistant to ≥ 3 therapeutic groups each with ≥ 1 agent), and pandrug resistant (resistant to all available antibiotic agents) (Magiorakos, 2012). Antibiotic resistance genes (ARGs) are auto-replicating elements and are spread among various bacterial species and habitats (Grenni et al., 2018). Although AR is mostly a clinical problem, evidence points toward an environmental origin (Allen et al., 2010; Forsberg et al., 2012). The presence of native ARGs in environmental bacteria communities found within pristine, remote, or extreme environments (e.g. caves or springs) with zero anthropogenic influence constitutes a prime example of the ubiquity of genes conferring AR naturally (Brown & Balkwill, 2009). An environmental setting (e.g. water) can influence the cycling of AR in nature through AR mechanisms either introduced by environmental bacteria or by contamination from human and animal commensals and pathogens (Riesenfeld et al., 2004; Baquero et al., 2008; Zhang et al., 2009; Allen et al., 2010; Perry & Wright, 2013). For the latter, the emergence of ARB in the environment mainly began with the overuse and misuse of antibiotics or co-selecting agents such as biocides, metals, and nanomaterial stressors (Taylor et al., 2011; Qiu et al., 2012). Therefore, ARGs are only considered a

pollutant when the fraction of resistant microorganisms increases above the normal AR levels of a given environment under the following proposed conditions: (i) Gene-transfer units integrated with ARGs are observed to disseminate in natural ecosystems due to the presence of antibiotic selective pressure or (ii) residues from locations under the anthropogenic influence are observed to contain bacteria that carry AR determinants (Martínez, 2009b).

Studies have shown that anthropogenic activities could exacerbate the acquisition of AR by microbial communities. Environments were generally more contaminated by antibiotics, resistant microorganisms, and resistance genes the closer to the anthropogenic source (Oberlé et al., 2012; Xu et al., 2015; Lye et al., 2019). To support this, a study on the trend of antibiotic usage found its application as supplements in livestock had increased over the years, which resulted in higher antibiotic residue detection in faecal and meat samples (Nisha, 2008; Zhao et al., 2010; Bakar et al., 2013), while another study found consistently rising levels of ARG abundance in soil samples over 68 years from the Netherlands (Knapp et al., 2010). Also, studies had reported significant positive correlations between ARGs, MGEs, concentrations of antibiotics, and severity of anthropogenic pollution (Pruden et al., 2012; Chen et al., 2013; Tao et al., 2016). Thus, AR hotspots are likely to be found in aquatic environmental compartments that are subjected to heavy anthropogenic pressure, e.g. municipal wastewater systems, medical facilities, pharmaceutical manufacturing effluents, aquaculture facilities, slaughterhouses, and animal husbandry facilities (Cabello, 2006; Adelowo et al., 2014; Penders et al., 2013; Berendonk et al., 2015; Economou & Gousia, 2015; Lien et al., 2017). An exemplary study focused on determining ARG sources in a river system in rural Nova Scotia, Canada, had categorised tertiary-level WWTP as a point source pollution while land use activities were categorised as a nonpoint source pollution (McConnell et al., 2018).

Predictably, the therapeutic management of AR *E. coli* infections has become challenging. For example, a 2013 survey data consisting of 17 European countries showed that 85 %–100 % of *E. coli* strains were resistant (Gelband et al., 2015). In addition, an increasing trend in AR *E. coli* detection in local natural environments is becoming a significant concern. For example, Ghaderpour et al. (2015) had reported the prevalence of diverse nonpathogenic AR *E. coli* in estuarine waters of the largest mangrove forest in Malaysia and postulated that neighbouring anthropogenic sources were the major contributor to antibiotic resistance. To mitigate evolution and acquisition of resistance from escalating in the environment, high risk antibiotic classes should be targeted for surveillance. ARGs conferring resistance to older first-line antibiotic classes broadly have high abundances across various works, with tetracyclines and sulphonamides widely reported globally across various aquatic environments (**Table 2.1**). The trend of increased resistance to older antibiotics such as tetracycline has been well understood for the past 30 yr (Roberts & Schwarz, 2016). Also, aquatic environments that were impacted by anthropogenic activities had an abundance of these ARGs, e.g. discharged livestock wastewater reported by Biao Chen et al. (2015) had expectedly high quantities of tetracyclines and sulphonamides genes compared to other ARGs, 1.92×10^1 copies/16S rRNA and 2.10×10^2 copies/16S rRNA respectively. Other studies also corroborated this trend, making these ARGs evidently important for monitoring purposes.

Table 2.1: Range of normalized concentration of ARGs (relative to the total 16S rRNA gene copy number) detected from surface waters adjacent to various sources using quantitative RT-PCR

Major antibiotic Class	Antibiotic resistance genes	Water Source	Antibiotic resistance genes range (copies/16srRNA gene)	Reference
Tetracyclines	*tet*A, *tet*C, *tet*E, *tet*G, *tet*M, *tet*O, *tet*Q, *tet*T, and *tet*W	Discharged Livestock wastewater	2.52×10^{-2}–1.92×10^{1}	(Biao Chen et al., 2015)
	*tet*A, *tet*C *tet*G, *tet*O, *tet*M, *tet*Q, and *tet*X	River water receiving urban aquaculture and agricultural effluent	9.65×10^{-7}–1.32×10^{1}	(Ling et al., 2013)
	*tet*A, *tet*C, *tet*G, *tet*M, *tet*L, *tet*O, *tet*Q, *tet*W, *tet*X	Pharmaceutical industries wastewater (antibiotic)	5.20×10^{-5}–3.70×10^{0}	(Liu et al., 2014)
	*tet*M	Aquaculture farms	10^{-7}–10^{0}	(Thiang et al., 2021)
	*tet*Q	Hospital wastewater	2.80×10^{-1}–7.47×10^{-1}	(Li et al., 2016)
	*tet*C, *tet*G, *tet*M, *tet*W, and *tet*O	Discharged Livestock wastewater	5.76×10^{-6}–6.49×10^{-1}	(Ben et al., 2017)
	*tet*A, *tet*B, *tet*C, *tet*G, *tet*M,and *tet*Q	River water receiving effluent from wastewater treatment plant, hospitals, husbandry, and industrial area of machinery manufacturing	3.90×10^{-5}–4.60×10^{-2}	(Yan et al., 2018)
	*tet*A, *tet*B, *tet*C, *tet*O, and *tet*W	Hospital wastewater	2.32×10^{-7}–1.09×10^{-2}	(Szekeres et al., 2017)
	*tet*M, *tet*O, *tet*W, *tet*S, *tet*Q, *tet*X, and *tet*BP	Aquaculture farms	2.10×10^{-5}–3.10×10^{-3}	(Xiong et al., 2014)
	*tet*M, *tet*O, *tet*W, *tet*S, *tet*Q, *tet*X, and *tet*BP	River water receiving urban and agricultural effluent	1.60×10^{-5}–3.10×10^{-3}	(Xiong et al., 2014)
	*tet*B, *tet*M, *tet*O, *tet*W, and *tet*X	River estuary receiving aquaculture and tourism	1.93×10^{-5}–2.87×10^{-4}	(Niu et al., 2016)
	*tet*A, *tet*B, *tet*C, *tet*G, *tet*M,and *tet*Q	Urban lake water	10^{-6}–10^{-4}	(Yang et al., 2017)

Table 2.1, continued

Sulphonamides	*sul*1, *sul*2, and *sul*3	Discharged Livestock wastewater	4.64×10^{-2}–2.10×10^{2}	(Biao Chen et al., 2015)
	*sul*1, *sul*2, and *sul*3	Aquaculture farms	10^{-7}–10^{0}	(Thiang et al., 2021)
	*sul*1 and *sul*2	River water receiving effluent from wastewater treatment plant, hospitals, husbandry, and industrial area of machinery manufacturing	8.60×10^{-4}–7.00×10^{-1}	(Yan et al., 2018)
	*sul*1, *sul*2, and *sul*3	Hospital wastewater	7.33×10^{-2}–6.67×10^{-1}	(Li et al., 2016)
	*sul*1 and *sul*2	River water receiving WWTPs, agricultural feedlots, dairies, and fishponds	4.62×10^{-4}–2.69×10^{-1}	(Luo et al., 2010)
	*sul*1, *sul*2, and *sul*3	Rural river water	6.90×10^{-10}–2.00×10^{-1}	(Jiang et al., 2021)
	*sul*1, *sul*2, and *sul*3	River estuary receiving urban and industrial, agricultural and aquaculture	2.01×10^{-4}–1.58×10^{-1}	(Baowei Chen et al., 2015)
	*sul*1 and *sul*2	Hospital wastewater	8.60×10^{-5}–1.94×10^{-1}	(Szekeres et al., 2017)
	*sul*1 and *sul*2	Coastal seawater	7.14×10^{-5}–1.16×10^{-1}	(Na et al., 2014)
	*sul*1 and *sul*2	Discharged Livestock wastewater	6.63×10^{-4}–1.14×10^{-1}	(Ben et al., 2017)
	*sul*1	WWTP influent and effluent	5.13×10^{-3}–7.59×10^{-2}	(Rafraf et al., 2016)
	*sul*1 and *sul*2	Urban lake water	1.40×10^{-4}–2.79×10^{-2}	(Yang et al., 2017)
	*sul*1, *sul*2, and *sul*3	Aquaculture farms	3.40×10^{-4}–7.50×10^{-3}	(Xiong et al., 2015)
	*sul*1 and *sul*2	River water receiving urban aquaculture and agricultural effluent	2.60×10^{-4}–7.09×10^{-3}	(Ling et al., 2013)
	*sul*1, *sul*2, and *sul*3	River water receiving urban and agricultural effluent	1.20×10^{-4}–6.90×10^{-3}	(Xiong et al., 2014)
	*sul*1 and *sul*2	River estuary receiving aquaculture and tourism	2.42×10^{-5}–3.83×10^{-3}	(Niu et al., 2016)

Table 2.1, continued

Plasmid-mediated quinolone resistance	*oqx*A, *oqx*B, aac(6')-Ib, *qnr*S, and *qep*A	Aquaculture farms	1.30×10^{-5}–7.30×10^{-2}	(Xiong et al., 2015)
	*oqx*B, *qnr*S,and *qnr*D	Discharged Livestock wastewater	7.41×10^{-5}–5.45×10^{-1}	(Biao Chen et al., 2015)
	*qnr*A and *qnr*S	WWTP influent and effluent	9.55×10^{-6}–1.45×10^{-2}	(Rafraf et al., 2016)
	*oqx*A, *oqx*B, aac(6')-Ib, *qnr*S, and *qep*A	River water receiving urban and agricultural effluent	4.40×10^{-5}–7.30×10^{-3}	(Xiong et al., 2014)
	*qnr*D	River water receiving effluent from wastewater treatment plant, hospitals, husbandry, and industrial area of machinery manufacturing	5.0×10^{-5}–3.90×10^{-3}	(Yan et al., 2018)
	*qnr*A and *qnr*D	Rural river water	3.50×10^{-9}–2.70×10^{-3}	(Jiang et al., 2021)
	*qnr*S	River estuary receiving urban and industrial, agricultural and aquaculture	9.74×10^{-5}–9.23×10^{-4}	(Baowei Chen et al., 2015)
	*qnr*D	Urban lake water	1.52×10^{-5}–7.33×10^{-5}	(Yang et al., 2017)
	*qeq*A, *qnr*A, *qnr*D, and *qnr*S	Hospital wastewater (treated and untreated)	2.25×10^{-9}–1.75×10^{-5}	(Yao et al., 2021)
Macrolide-lincosamide-streptogramin B	*erm*B and *erm*C	Discharged Livestock wastewater	1.15×10^{-2}–3.60×10^{0}	(Biao Chen et al., 2015)
	*erm*B	WWTP influent and effluent	5.13×10^{-4}–5.62×10^{-2}	(Rafraf et al., 2016)
	*erm*A, *erm*B and *erm*C	Rural river water	1.7×10^{-9}–5.00×10^{-3}	(Jiang et al., 2021)
	*erm*A and *mef*A	Hospital wastewater	1.89×10^{-6}–1.67×10^{-3}	(Szekeres et al., 2017)
	*erm*B	River estuary receiving urban and industrial, agricultural and aquaculture	3.00×10^{-4}–1.27×10^{-3}	(Baowei Chen et al., 2015)
	*erm*B	River estuary receiving aquaculture and tourism	3.46×10^{-6}–7.76×10^{-5}	(Niu et al., 2016)

Table 2.1, continued

β-lactams	*bla*VIM and bla_{SHV}	Hospital wastewater	3.86×10^{-3}–4.39×10^{-3}	(Szekeres et al., 2017)
	*bla*TEM-1 and bla_{CTX-M}	WWTP influent and effluent	1.74×10^{-6}–5.75×10^{-3}	(Rafraf et al., 2016)
	bla_{OXA-1}, bla_{TEM-1}, bla_{GES-1}, bla_{OXA-10}, bla_{SHV-1}, and bla_{DHA-1}	Hospital wastewater (treated and untreated)	2.00×10^{-9}–1.60×10^{-3}	(Yao et al., 2021)
	*bla*OXA-58	Coastal seawater	2.80×10^{-6}–2.46×10^{-4}	(Xin et al., 2019)
Phenicol	*cf*R and *flo*R	Rural river water	6.20×10^{-9}–5.00×10^{-3}	(Jiang et al., 2021)
	*cat*A1 and *flo*R	Hospital wastewater	1.97×10^{-5}–2.63×10^{-3}	(Szekeres et al., 2017)
Amino-glycosides	*aph*, *aad*D,and *aac*	Discharged Livestock wastewater	9.90×10^{-4}–1.43×10^{0}	(Biao Chen et al., 2015)
	aac	Hospital wastewater	1.34×10^{-3}–9.42×10^{-3}	(Szekeres et al., 2017)
Mobile genetic elements	*intI*1 and Tn916/1545	Pharmaceutical industries wastewater (antibiotic)	6.70×10^{-4}–1.70×10^{0}	(Liu et al., 2014)
	*intI*1	Rural river water	4.10×10^{-5}–7.00×10^{-2}	(Jiang et al., 2021)
	*intI*1	WWTP influent and effluent	2.88×10^{-3}–5.89×10^{-2}	(Rafraf et al., 2016)
	*intI*1	River water receiving WWTPs, agricultural feedlots, dairies, and fishponds	2.40×10^{-6}–2.00×10^{-3}	(Luo et al., 2010)
	*intI*1	Hospital wastewater (treated and untreated)	2.65×10^{-5}–2.25×10^{-4}	(Yao et al., 2021)
Transposon-related element	*tnp*A	Hospital wastewater	1.75×10^{-3}–3.55×10^{-2}	(Szekeres et al., 2017)
	*tnp*A	Rural river water	5.40×10^{-7}–1.70×10^{-3}	(Jiang et al., 2021)
Quaternary ammonium compounds	*qac*EΔ1	Hospital wastewater	1.94×10^{-2}–4.96×10^{-2}	(Szekeres et al., 2017)
Multidrug resistance	*acr*A and *acr*B	Discharged Livestock wastewater	1.95×10^{-6}–9.62×10^{-2}	(Biao Chen et al., 2015)

Among *E. coli* strains, the class 1 integrase gene was commonly carried, whereby the gene contains ARG cassettes for antibiotics groups such as sulphonamide, aminoglycosides, and trimethoprim were in the same genetic element. Furthermore, tetracycline resistant *E. coli* carry this gene on plasmids or transposons (Roberts, 2005). Given tetracyclines and sulphonamides' long historical use in human and veterinary clinics, these antibiotics are foreseeably located in MGEs (Garcillán-Barcia et al., 2011; Partridge, 2011); a segment of the genome where a diverse combination of AR tend to be co-selected from a single antibiotic (Fernández-Alarcón et al., 2010; Ou et al., 2015). A meta-analysis study covering human and animal samples by Tadesse et al. (2012) supports this, they found *E. coli* resistance towards tetracyclines and sulphonamides showed a significant upward trend from 1950–2002. By using sulphonamide resistance genes as a model, *sul*1 was usually the most abundant gene in well-zoned and pristine Poudre River (Pei et al., 2006; Pruden et al., 2006), while *sul*2 gene is most abundant in fish farms in various locations in China (Gao et al., 2012) whereas *sul*3 is found in high frequencies in porcine *E. coli* populations in North America (Boerlin et al., 2005). In comparison, genes encoding resistance to tetracyclines are not as straightforward in their deconstruction. For example, certain tetracycline resistance genes code for certain *tet* resistance mechanisms: efflux protein *tet* [(A), (B), (C), (D), (E), (G), (K), (L), and A(P)], ribosomal protection protein *tet* [(M), (O), (S), and (Q)] and enzyme inactivation *tet*(X). Yet, 22 out of 38 tetracycline resistance genes were detected in bacterial isolates from aquatic environments (Dancer et al., 1997; Thompson et al., 2007).

Some authors pointed out that AR phenotype of *E. coli* strains can also be influenced by phylogenetic background, regardless of acquisition of resistance (Ghaderpour et al., 2015); while others found AR patterns to be phylogenetically

unrelated (Mokracka et al., 2011; De Luca Rebello & Regua-Mangia, 2014). For example, phylogroups A and B1 were described as most frequently resistant to antibiotics (Escobar-Páramo et al., 2004a; Anssour et al., 2016). Conversely, B2 strains are less resistant to antibiotics, regardless of the molecular mechanism involved in the acquisition of resistance (Ochman & Selander, 1984; Johnson et al., 1994).

Multidrug resistance in *E. coli* had shown an increasing trend from 7.2 % during the 1950s to 63.6 % in the 2000s (Tadesse et al., 2012). MAR index was used to distinguish between *E. coli* originating from high-AR-risk environments as per Krumperman's (1983) method. This is done by dividing the aggregate antibiotic score of all the isolates from a particular site by the number of antibiotics tested multiplied by the number of isolates from a site. The MAR index is a useful tool to analyse the relative prevalence of resistant bacteria found in the environment. Isolates that are exposed to high-risk sources of contamination indicate frequent use of antibiotics, usually identified with a MAR index value of > 0.2, whereas infrequent use of antibiotics will be $\leqslant 0.2$ (Scott et al., 2002; Poonia et al., 2014). According to Kaneene et al. (2007) and Poonia et al. (2014), the water sources with a MAR index of more than 0.4 are usually from human faecal origin while MAR index of less than 0.4 is of non-human faecal sources. Most MAR *E. coli* were incidentally isolated in waters likely contaminated by faecal bacteria of human origin (Berthe et al., 2013). Prevalence of AR *E. coli* phenotypes reflected the particularities of geographical regions, sources of faecal contamination, and expected exposure of bacteria to antibiotic selective pressure (Mokracka et al., 2011; Ghaderpour et al., 2015; Hernández et al., 2019).

Adoption of AR in pathogenic *E. coli* is common (Koczura et al., 2013; Pereira et al., 2013) due to virulence and AR properties both being encoded on plasmids, bacteriophages, or pathogenicity islands (Djordjevic et al., 2013). *E. coli*

resistance to carbapenem, a last-resort antibiotic, was relatively uncommon before 2000 but has since doubled its prevalence over the years among healthcare-associated infections (Gupta et al., 2011). Without an alternative treatment for infected patients, the mortality rate could increase dramatically. As such, the accidental release of MAR pathogenic *E. coli* harbouring virulence traits into water bodies could be the making of a devastating public health disaster (De Luca Rebello & Regua-Mangia, 2014). Establishing these resistance baseline data in aquatic ecosystems can be an indicator of resistance evolution in different ecosystems (Alexander et al., 2010). Although much was done to suppress AR infections in clinics via AR action plans (European Commission, 2011; WHO, 2015, 2018), there is still a significant lack of environmental focus (O'Neill, 2016). It can be said that current AR action plans do not consider all potentially relevant pathways and drivers of AR in the environment (Singer et al., 2016). For example, Ghaderpour et al. (2015) reported the prevalence of diverse AR *E. coli* in estuarine waters of the largest mangrove forest in Malaysia and suspected anthropogenic sources as the major contributor to antibiotic resistance. Thus, research activities should focus on nonpathogenic environmental microorganisms, such as aquatic environments, that could be potential sources for these ARGs.

2.8 Antibiotic Contamination in River Water and Their Ecological and Public Health Risks

Water bodies featuring rivers, lakes, wetlands, and oceans form a part of a larger system: a river basin, where precipitation and groundwater on land are drained by the main river and its tributaries into a shared outlet. The outermost limit that divides neighbouring river basins is known as a watershed. Surface water source from a river basin often starts from a higher altitude location, e.g. highlands that are either fed by an underground spring, runoff from rain, snowmelt, or glacial melt. This natural boundary classification led to the longstanding and widespread acknowledgement of river basins as important natural units for natural resource management (White, 1957; Teclaff, 1967; Montgomery et al., 1995; McGinnis, 1999; Koehler & Koontz, 2008; Newson, 2009). As such, river basins are usually viewed as a convenient scale for analysis of region-wide social-ecological systems (Biggs et al., 2010; March et al., 2012). So, their boundaries and flow paths organise hydrological and biogeochemical processes that underpin ecosystem services, e.g. water supply, flood protection, and food production (Carpenter et al., 2015). Historically, human settlements were located in sites around water reservoirs, generating anthropogenic. These adverse or persistent anthropogenic disturbances could impact ecosystems negatively (Palmer & Filoso, 2009; Bullock et al., 2011). Water pollution is the presence of excessive pollutants where water becomes unsuitable for drinking, bathing, cooking, or other uses (Owa, 2013) within domestic, agricultural, and industrial sectors (Puri et al., 2008). Asia's rivers are amongst the most polluted, with up to three times as much bacteria from human waste and a faecal count 50 times above the WHO guidelines (ESCAP, UN, 2000). In Malaysia, pollution in rivers is mainly related to anthropogenic activities and the sources of contamination are largely from industrial areas, sewages, workshops, residential areas, animal and agricultural farming activities

(Department of Environment, 2018, 2019a). Anthropogenic factors were linked to a decrease in water quality, i.e. higher concentrations of heavy metals, mercury, coliforms, and nutrient loads (Khatri and Tyagi, 2015).

One such pollutant is antibiotic waste. By the 1950s, chemotherapy became the forefront method to treat communicable diseases caused by bacteria infections, the development of the earliest commercially viable antibiotic had ushered in the golden age of the modern "antibiotic era". Antibiotics were primarily used in clinical therapeutic treatment for humans but also enjoyed notable applications in (i) growth promoters and prophylaxis for animal husbandry, (ii) veterinary, (iii) agriculture, (iv) aquaculture, and (v) biocide in domestic cleaning products (Nisha, 2008; Davies & Davies, 2010; Meek et al., 2015). Antibiotics are thus grouped into classes depending on their mode of action, chemical structure, and mechanism of treating infectious bacteria, classes include Beta-lactam, tetracyclines, macrolide, aminoglycosides, quinolones, glycopeptides, lincosamides, oxazolidinones, and sulphonamides. Time has proven the commercial success of antibiotics, where they have become cheaper, plentiful, and extensively accessible. In 2015, a study with 76 participating countries found that antibiotic consumption is at 42 billion Defined Daily Doses (DDDs), and by 2030 it is projected that consumption will increase by 200 % as driven by gross domestic product per capita growth (Klein et al., 2018). Due to the popularity of antibiotics, they were quickly abused, misused, and overused with little to no regulation across industries by many nations over many years (Davidson et al., 2008). Antibiotic pollution shares some aspects to heavy metal pollution, which like heavy metals; they are natural compounds present across different ecosystems but having their bioavailability expanded from human overuse (Martínez, 2009b), i.e. excessive manufacture emission paired with consumption of antibiotics contributed to the contamination of antibiotic residues

(Lundborg & Tamhankar, 2017). Analytical methods were developed to detect antibiotic residues contaminating the environment, which include Liquid chromatography with mass spectrometer (LC−MS) (Karthikeyan & Meyer, 2006; Fatta et al., 2007; Van Boeckel et al., 2015), simple direct sample injection by high performance liquid chromatography (HPLC) using diode array detection (DAD) (Teixeira et al., 2008), and by enzyme-linked immunosorbent assay (ELISA) (Praveena et al., 2018).

According to conservative estimates, up to half of the still biologically active antibiotics consumed by humans were released into sewage effluent through excretion (Pei et al., 2006; Dolliver & Gupta, 2008; Ashbolt et al., 2013). For aquatic environments, both high concentrations and low concentrations of antibiotics were detected (**Table 2.2**). Tetracycline and sulphonamide classes were again the majority detected antibiotics residues. Many studies chose sulphonamides due to their widespread use, high excretion rate, high solubility, and persistence in the environment (Lamshöft et al., 2007). As synthetic antibiotics, sulphonamides detected in aquatic compartments are unlikely to originate from natural sources (Ou et al., 2015). A study found maximum concentrations ranging between 0.63 µg l-1 and 211 µg l-1 for different sulphonamides in animal wastewater and surface water around farms (Wei et al., 2011). Another study found that approximately 12 tons of sulphamethoxazole per year were released to the sea and that sulphonamides were predominant in rivers and coastal waters across five tropical Asian countries (Shimizu et al., 2013). Compared to sulphonamides, tetracyclines are weak persistors (Boreen et al., 2003; Oka et al., 1989; Pils & Laird, 2007; Sanderson et al., 2005; Sassman & Lee, 2005). Even so, tetracycline is a reliable indicator given its strong affinity to soils and sediments, where its detection correlates well with its respective antibiotic contamination sources (Huang et al., 2001). However, these antibiotics are

still prevalent in human and veterinary medicine due to their broad-spectrum activity, relative safety, and low cost (Harnisz et al., 2011). Unsurprisingly, high concentrations ranging from 3.79 μg l-1 to 72.9 μg l-1 were still detected in surface waters (Wei et al., 2011). Having concentrations of antibiotics corresponding to their respective ARGs indicates that the fate of ARGs is related to the presence of particular antibiotics in the environment (Larsson, 2014). Overall, the largest amount of antibiotic residues are frequently found in areas with high anthropogenic pressures, such as hospital effluents, and wastewater influents and effluents (Patrolecco et al., 2015; Verlicchi et al., 2015; Ory et al., 2016). A European Commission study conducted across 13 countries worldwide found that among 45 antibiotics, sulfamethoxazole, trimethoprim, and ciprofloxacin were the three most frequently detected in waters that flow out of wastewater treatment plants (WWTPs) (Sanseverino et al., 2018). Meanwhile, certain Asian nations recorded antibiotic concentrations up to several mg l^{-1} in WWTP receiving pharmaceutical waste effluents for single compounds whereby normal levels are supposedly < 1 ng l^{-1} to a few μg l^{-1} (Larsson et al., 2007; Li et al., 2008a, 2008b; Shimizu et al., 2013).

Table 2.2: Maximum concentrations of antibiotic residue from water sources found in past literature

Major Groups	Antibiotic Name	Water Source	Maximum Concentration (ng l^{-1})	Reference
Penicillin	Penicillin G	Wastewater treatment	153	(Li et al., 2008b)
β-Lactams	Lincomycin	Surface water	248.9	(Calamari et al., 2003)
Amino-glycosides	Gentamicin	Sewage plant export	1,300	(Löffler & Ternes, 2003)
Macrolides	Azithromycin	Sewage plant export	130	(Shimizu et al., 2013)
	Erythromycin-H2O	Surface water	1,700	(Kolpin et al., 2002)
	Clarithromycin	Surface water	260	(Hirsch et al., 1999)
	Roxithromycin	Surface water	560	(Hirsch et al., 1999)
Tetracyclines	Tetracycline	Livestock farm export	10,300	(Wei et al., 2011)
		Underground water	3.8	(Tong et al., 2009)
	Oxytetracycline	Wastewater treatment	920,000	(Li et al., 2008a)
		Livestock farm export	72,900	(Wei et al., 2011)
		Surface water	340	(Kolpin et al., 2002)
	Chlortetracycline	Livestock farm export	3,670	(Wei et al., 2011)
		Surface water	690	(Kolpin et al., 2002)
Sulphonamides	Sulfacetamide	Surface water	7.92	(Ou et al., 2015)
	Sulphamethoxazole	Livestock farm export	63,600	(Wei et al., 2011)
		Sewage plant export	4,330	(Shimizu et al., 2013)
		Surface water	1,900	(Kolpin et al., 2002)
		Underground water	470	(Hirsch et al. 1999)
	Sulfathiazole	Surface water	0.92	(Prasanna et al. 2015)
	Sulphapyridine	Wastewater treatment	1.4	(Hanna et al., 2018)
		Surface water	1.45	(Hanna et al., 2018)
	Sulphamethizole	Surface water	130	(Kolpin et al., 2002)
	Sulphadimethoxine	Livestock farm export	11,000	(Watanabe et al. 2010)
	Sulphadimidine	Livestock farm export	211,000	(Wei et al., 2011)
		Surface water	4,660	(Wei et al., 2011)
		Underground water	160	(Hirsch et al., 1999)
	Sulphadoxine	Livestock farm export	17,000	(Wei et al., 2011)
		Surface water	460	(Wei et al., 2011)
	Sulphadiazine	Aquaculture surface water	108	(Nguyen Dang Giang et al., 2015)

Table 2.2, continued

Quinolones	Ciprofloxacin	Wastewater treatment	29,500,000	(Larssonet al., 2007)
		Sewage plant export	260	(Kostich et al., 2014)
		Surface water	185	(Bai et al., 2014)
	Norfloxacin	Wastewater treatment	405,000	(Larsson et al., 2007)
		Surface water	208	(Xu et al., 2007)
	Ofloxacin	Wastewater treatment	155,000	(Larsson et al., 2007)
		Surface water	89	(Xu et al., 2007)
		Sewage plant export	210	(Guerra et al., 2014)
	Enrofloxacin	Wastewater treatment	840,000	(Larsson et al., 2007)
		Livestock farm export	680	(Andrieu et al., 2015)
		Underground water	3	(Tong et al., 2009)
	Enoxacin	Wastewater treatment	200,000	(Larsson et al., 2007)
2,4-Diamino-pyrimidine	Trimethoprim	Sewage plant export	1,808	(Shimizu et al., 2013)
		AquaC surface water	330	(Nguyen Dang Giang et al., 2015)
Phenicol	Chloramphenicol	Sewage plant export	75	(Choi et al., 2008)
		Sewage plant export	0.99	(Hanna et al., 2018)
	Florfenicol	Wastewater treatment	3.9	(Hanna et al., 2018)
		Surface water	3.9	(Hanna et al., 2018)

At initial high concentration (e.g. > μg l^{-1}), antibiotics produce bactericidal and bacteriostatic effects on susceptible microorganisms. This raises major concerns regarding the toxicity of antibiotics to non-target organisms, e.g. several studies found tetracycline to be toxic to green algae (González-Pleiter et al., 2013; Havelkova et al., 2016). The direct risk of antibiotic residue as a micropollutant entering natural aquatic environments concerns its ability, as an ecological factor, to influence change in the structure of natural communities via the antibiotic's ability to typically remove or inhibit microorganisms; some of these microorganisms could potentially be involved in key ecosystem functions (Sarmah et al., 2006; Allen et al., 2009; Grenni et al., 2018). Therefore, this concerns the ecotoxicological effects of antibiotics on different levels of the biological hierarchy, from bacteria (target organism) to the entire biosphere (non-target organisms such as algae, invertebrates, and fish).

Also, antibiotics were observed to degrade, albeit at different rates according to the types of antibiotic group by process of sorption (Li & Zhang, 2010), hydrolysis (Dolliver & Gupta, 2008; Kümmerer, 2009), chlorination (Dodd & Huang, 2007), oxidation (Hubicka et al., 2013; Li et al., 2014), catalytical degradation (Chatzitakis et al., 2008), photodegradation (Leal et al., 2016), and biodegradation (Jiang et al., 2010; Gros et al., 2014) which are temperature, salinity, moisture, chemical and microbiota dependent. Subsequently, the antibiotic residues would exist in low concentrations (e.g. $ng\ l^{-1} - \mu g\ l^{-1}$) as metabolites that remain bioactive in the environment (Huang et al., 2014). Selection of antibiotic resistance would then happen under these sub-lethal (Gullberg et al., 2011; Andersson & Hughes, 2012; Hughes & Andersson, 2012; Martínez, 2017) and sub-inhibitory concentrations (Martínez, 2017). Importantly, having concentrations of antibiotics corresponding to their respective ARGs indicates that the fate of ARGs is related to the presence of particular antibiotics in the environment (Larsson, 2014). Unsurprisingly, antibiotic concentrations below the minimal inhibitory concentration (MIC) have long been known to naturally select for antibiotic resistance since the 1940s, e.g. β-lactamase-producing strain of Staphylococcus aureus (Levy, 2002; French, 2006; Goodman et al., 2011). Resistance mechanisms such as enzymatic drug modification, alteration of drug targets, reduced cell membrane permeability, and transporters-mediated drug efflux were selected (Blair et al., 2015). Apart from that, exposure to low-level antibiotics exposes negative impacts on the bionts inhabiting aquatic ecosystems (Sarmah et al., 2006).

2.9 Consideration of Antibiotics and AR *E. coli* as a Routine Water Quality Monitoring Standard

As mentioned insufficient environmental focus in AR infection control exists (O'Neill, 2016) with strategies specifically designed for clinical environments via AR action plans (European Commission, 2011; WHO, 2015, 2018). Basically, AR action plans of the past had not consider all potentially relevant pathways and drivers of AR in the environment (Singer et al., 2016). As such, a consensus on the potential dangers (WHO, 2014) and the need to limit AR presence in the environment have since been established (Berendonk et al., 2015; Williams-Nguyen et al., 2016; Allen et al., 2010; Pruden et al., 2013). More recently, WHO had proposed an integrated surveillance system that applies a 'One Health' approach to monitoring extended-spectrum-beta-lactamase (ESBL)-*E. coli* in the environment along with humans and the food chain. It is the first significant collective endeavour that pushes the implementation of a truly comparable, statistically valid, and robust AR database (WHO, 2021).

The research effort in AR characterisation is ongoing, with recent developments showing a positive trend. For example, sulphamethazine and sulphachlorpyridazine had both demonstrated location specificity and were proposed as suitable indicators for aquatic environments contaminated by livestock sources using various liquid chromatography methods (Jiang et al., 2013; Luo et al., 2011; Managaki et al., 2007). Apart from that, culture-based methods are still being used. Bacterial targets for water quality typically avoid pathogens because pathogen concentrations equivalent to tolerable levels of risk are usually < 1 organism per 10^4–10^5 litres (WHO, 2017). As such, bacterial indicators that are already in use and on a subset of resistance determinants were recommended (Berendonk et al., 2015). The absolute number of ARB present in a particular aquatic sample were not overlooked as its importance in

evaluating human health risks as a contamination indicator still remains (Amarasiri et al., 2020). Specifically, current routine water quality monitoring still prioritised FIB detection using intestinal enterococci culture methods because of their relative affordability and straightforwardness (Leclerc et al., 2001; Tallon et al., 2005; Bain et al., 2012; WHO, 2017) which could be used to complement AR phenotype detection strategies. In comparison, standards for molecular indicators and methods are currently being improved and require contributions toward a more comprehensive ARG data. Common molecular methods used to characterise AR includes PCR, quantitative PCR (qPCR), digital PCR (dPCR), loop-mediated isothermal amplification (LAMP), microarrays, and metagenomics. Notably, more robust ARG indicators need to be identified because there is no one-size-fits-all solution. Similarly, established ARG detection and quantification methods require additional refinement and environmental testing to increase their accuracy and reliability else routine monitoring remains impractical (Amarasiri et al., 2020).

Admittedly, more considerations are needed to further justify the implementation of AR prevalence as a valid process to further characterise microbial water quality (Pachepsky et al., 2018). One of the current challenges with AR lies in the absence of an agreed set of antibiotics, ARB and/or ARGs, which can act as a molecular/chemical indicator that could consistently evaluate a contamination incident for each AR contaminant type (antibiotics, ARB, and ARGs) in aquatic environments (Amarasiri et al., 2020). Types and concentrations of antibiotics used in different countries and geographical regions also vary, and as a consequence, detectable antibiotics, ARB and ARGs in aquatic environments will also be location-specific with varying compositions (Shimizu et al., 2013). Routine monitoring should be rapid, specific, sensitive, economical, and easy to use. Hence a suitable AR contamination indicator needs to meet those requirements.

Nevertheless, current routine data collection on ARGs could still help reveal factors driving resistance and subsequently form conceptual models for how resistance emerges and is disseminated (Bengtsson-Palme et al., 2017). These may include distribution, prevalence, temporal trends and geographical trends (Berendonk et al., 2015). The establishment of AR baseline data in an aquatic ecosystem from various studies could at least contribute as an indicator of resistance evolution among different ecosystems (Maal-Bared et al., 2013; Alexander et al., 2010; Mudryk et al., 2010). As such, the effectiveness of environmental management and mitigation practices could still be measured even though the source of individual bacteria or genes may not have been identified (Durso & Cook, 2019). For example, a year-long microbial monitoring period by Vivant et al. (2016) had demonstrated that a decrease in concentration and diversity of AR *E. coli* and the *bla* ARG were attributed to the design of their constructed (man-made) wetland. This shows that when expanded to include a more robust analysis of ARGs, traditional FIB monitoring methods could still help ascertain the particular effectiveness of a mitigation strategy. Continual routine water quality monitoring of ARB and ARGs could fill gaps in the literature. In time, this will lead to the establishment of a more formal risk-based water safety management framework such as the quantitative microbial risk assessment (QMRA) to better support water safety management decisions (WHO, 2016).

CHAPTER 3: METHODOLOGY

3.1 Study Site

Larut River and Sangga Besar River belong in part to two distinct major river basin systems located within the Larut, Matang, and Selama District of the state of Perak (population: 352,600) (Department of Survey and Mapping Perak, 2016) (**Figure 3.1**). The climate of the area is characterised by uniform temperature (average 32 °C), high humidity (80–90 %), and high average annual rainfall (4,000 mm $year^{-1}$) (Samuding et al., 2012). Larut River is approximately 20.9 km long, draining a basin area of approximately 125 km^2 flowing from the Larut Hill Forest Reserve with a steep upper catchment (elevation: 1,250 m) towards a flat middle catchment made up of the Larut, Matang, and Selama district before entering the Straits of Malacca through the low lying Larut Estuary towards the southwest (Ahmad & Abu, 2011). Passing through the river is the Taiping township, which has a population of 245,182 based on a 2013 census and is the second largest town in the state of Perak, with a total area of 186.46 km^2. In regards to land use, Larut River is exposed to various anthropogenic activities in Taiping near the midstream, which includes a zoo, poultry slaughterhouse, and a public hospital that houses 176 beds (Tan et al., 2013). In comparison, Sangga Besar River is located northwest of Larut River with a length of approximately 10 km and a catchment area of 58 km^2 flowing past Kuala Sepetang, a coastal township (population: 31,800) (Forestry Department of Perak, 2010), towards a mangrove forest reserve to similarly meet at the Straits of Malacca to the northwest. Apart from serving as a waterway for fishing boats, Sangga Besar River has cage aquaculture and prominent fishing activities (Annual Fisheries of Perak, 2000). Similarities shared by both rivers include being nestled around the 21,069 ha Kuala Sepetang Forest

Management Zone within the 40,466 ha protected Matang Mangrove Forest Reserve (MMFR) downstream. The MMFR is described as a crescent-shaped embayment that stretches for approximately 52 km along the coast bordering the Strait of Malacca and has a maximum width of 13 km (Gan, 1995). The MMFR clearly received minimal anthropogenic pressure due to the exemplary forestry management system that was put in place (Muda et al., 2005).

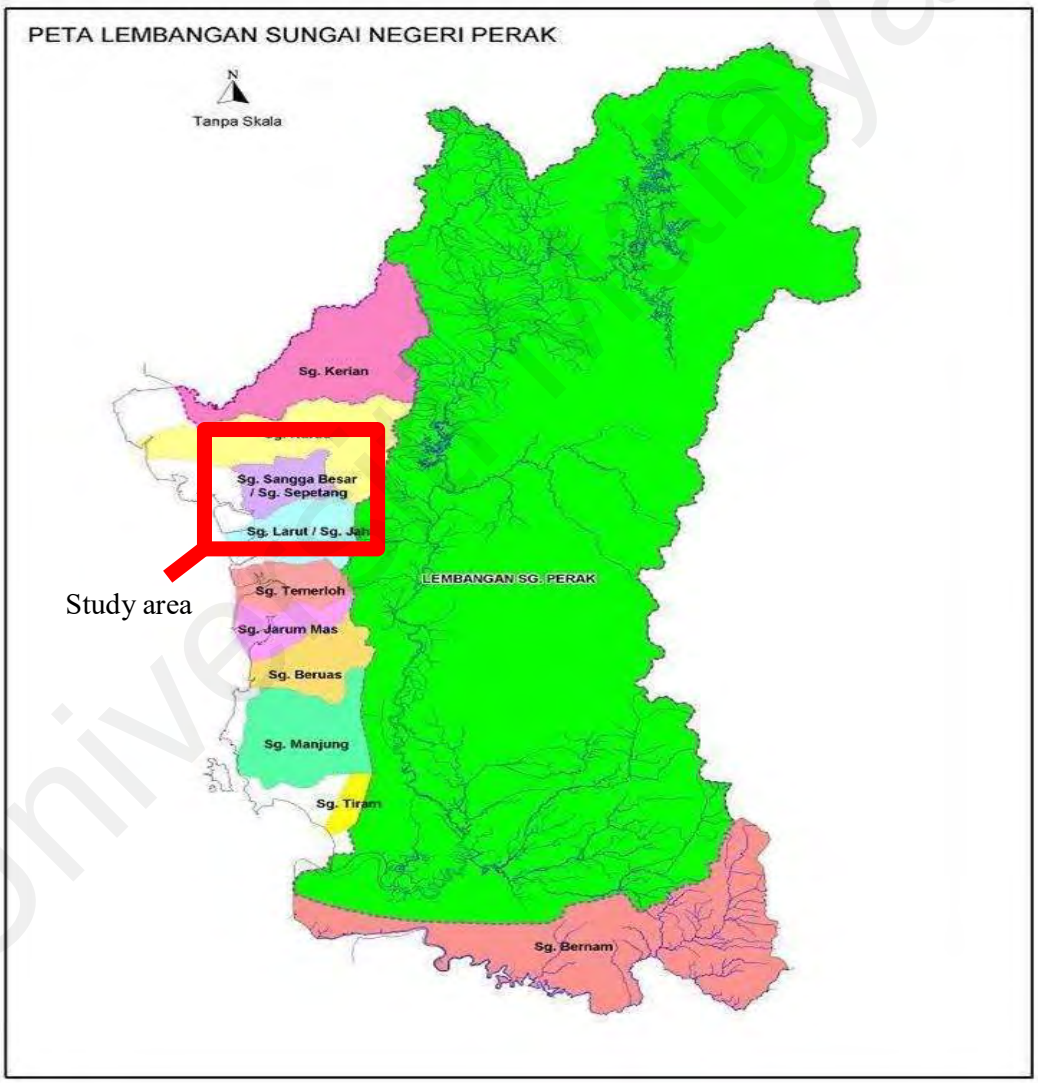

Figure 3.1: Map of Perak with several major river basins where study area falls under the two major basins above

3.2 Sample Collection

Water samples were taken from six sites located upstream, midstream, and downstream of Larut River and three sites from Sangga Besar River over three sampling dates. Surface water was collected using an acid-washed bucket into sterile glass bottles for culture-based and PCR assays, while acid-cleaned plastic bottles were used for other assays. Samples were stored in an ice-filled chest for no more than 24 hours prior to being processed in the laboratory. Larut River receives greywater effluents from hospital, zoo, and poultry slaughterhouse, whereas Sangga Besar River is less polluted (**Figure 3.2**). Sampling was conducted at nine sampling sites located in the upstream, middle, and downstream of Larut River and Sangga Besar River. At Larut river, water samples were collected from upstream (S1a, 04°51.158'N, 100°45.737'E), at the reserve forest Larut Hill (elevation: 1,250 m) followed by the middle where the river water received wastewater discharges from a zoo (04°51.101'N, 100°45.045'E), a public hospital (04°51.149'N, 100°44.018'E), and a slaughterhouse (04°50.238'N, 100°44.709'E) before passing through downstream Larut (S1b, 04°50.535'N, 100°43.925'E) and finally reaching the Larut Estuary (S1c, 04°50.140'N, 100°37.583'E). Based on data collected in 2018 by the Taiping municipality (**Table 3.1**), about 2,699.93 km^2, or half of the total study area, is covered by forest, these consisted of sites S1, S2a, S2b, and S2c. By land use, a combination of housing with institutions and public services together covered 296.27 km^2 (5.48 %) which included hospital site. Meanwhile, industry only makes up 30.18 km^2 (0.56 %), whereby slaughterhouse belonged. Finally, parks and recreation is only 14.05 km^2 (0.26 %) that consisted of zoo.

Table 3.1: Land use category in 2018 within the municipality of Taiping, Perak

Land Use Category	Land use area (km^2)	%
Forest	2,699.93	49.94
Agriculture	1,763.60	32.62
Unused Land	98.98	1.83
Parks and Recreation	14.05	0.26
Housing	242.81	4.49
Institutions and Public Services	53.46	0.99
Industrial	30.18	0.56
Commercial	12.10	0.22
Mixed Development	0.00	0.00
Transport	138.81	2.57
Infrastructure and Utility	44.10	0.82
Water	308.19	5.70
Beaches	99.00	0.00
Sum	5,406.22	100.00

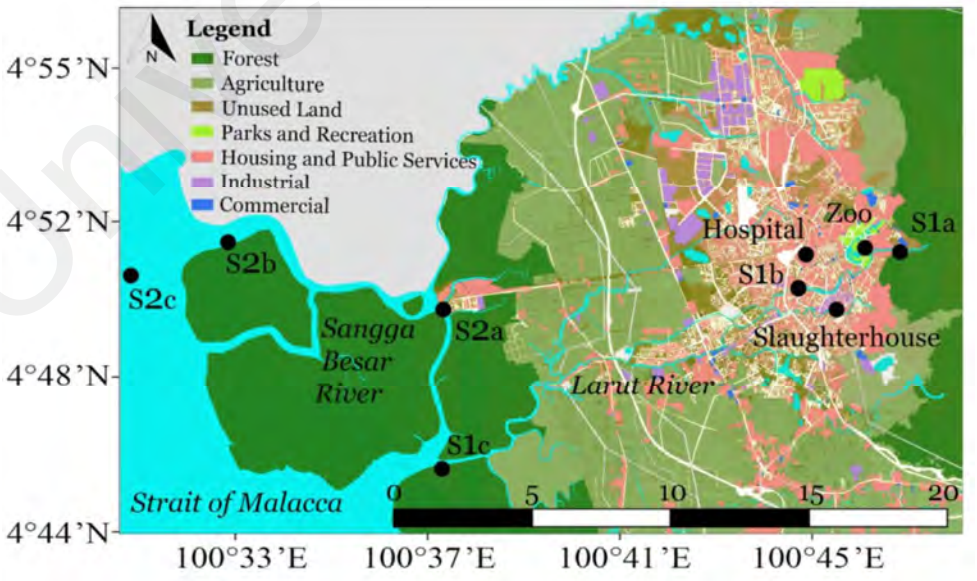

Figure 3.2: Land use map of Taiping, Perak

3.3 In-situ Water Physicochemical Parameters

Temperature, salinity, pH, turbidity, dissolved oxygen (DO), and water depth were recorded in triplicates for each sampling point. A handheld multiparameter probe (model: YSI 556 MPS) was placed just below the surface water to measure the above parameters.

3.4 Inorganic Nutrient Concentrations

For each sampling point, dissolved inorganic nutrients, including ammonium (NH_4), nitrate (NO_3), nitrite (NO_2), silicate (SiO_4), and phosphate (PO_4) concentrations were measured in triplicates using a spectrophotometer in accordance with Parsons et al. (1984) and Lee et al. (2009).

To determine NH_4, 5 ml water sample was added into a glass vial cleaned with dichromate acid and rinsed thoroughly with distilled water. First, 0.2 ml phenol solution was added, followed in sequence by 0.2 ml of sodium nitroprusside and 0.5 ml of sodium hypochlorite solution as an oxidising solution; swirl the vial to mix after the addition of each solution. Allow the vial to stand at room temperature between 20–27 °C for 1 h in the dark. During this period, parafilm was used to cover the top of the vial. Colour change was noted. The absorbance of the solution was measured in triplicates at 640 nm using a spectrophotometer using a 10 cm cell length. Meanwhile, a standard was carried out using 0.535 g analytical grade ammonium sulphate dissolved in 1,000 ml of deionised water, and 1 ml chloroform was subsequently added as a preservative. A volumetric flask was filled with 1 ml solution and topped up with ammonium-free seawater to a volume of 500 ml. The diluted standard was processed similarly to the water sample. The measured extinction for the reagent blank was corrected, and the NH_4 value was calculated from the following expression:

$$\mu\text{g-at N/l} = F \times E \tag{3.1}$$

where E is the corrected extinction and F is the factor as determined below:

$$F = \frac{3.0}{E_s} \tag{3.2}$$

where E_s is the corrected extinction. The F value should be about 6.5.

To determine NO_3, 0.5 ml of concentrated ammonium chloride was added to a 25 ml sample in a flask. The solution was mixed and approximately a 20 ml was poured onto the top of a cadmium column and allowed to pass through. The remainder of the sample was added to the column and drained into a flask under the collection tube. Approximately 10 ml was collected and subsequently discarded; 5 ml was collected in a measuring cylinder and dispensed into the flask, which contained the original sample. Before the following 5 ml sample was added, the column was drained. Then, 0.1 ml sulphanilamide solution was added to the sample, and the reagent was mixed to allow reaction between 2 min to 8 min. Next, 0.1 ml naphthylethylenediamine solution was added and mixed immediately. Let stand for at least 10 min, but no longer than 2 h. The absorbance of the solution was measured in triplicates at 543 nm using a spectrophotometer using a 1 cm cuvette against distilled water. The observed extinction was corrected by that of the reagent blank, and nitrate was calculated from the following expression:

$$\mu\text{g-at N/l} = (\text{corrected extinction} \times F) - 0.95\,C \tag{3.3}$$

where C is the concentration of nitrite in the sample.

To determine NO_2, 0.1 ml of sulphanilamide solution was added to a 5 ml sample, and the reagent was mixed and allowed to stand for more than 2 min but less than 10

min. Next, 0.1 ml of naphthylethylenediamine reagent was added and immediately mixed. The extinction of the solution was measured between 1 min and 2 h afterwards using a 10 cm cuvette at a wavelength of 543 nm. Meanwhile, standard nitrite was prepared using 0.345 g anhydrous dried analytical grade sodium nitrite dissolved in 1,000 ml distilled water preserved with 1 ml chloroform. Diluted standards were prepared using 10 ml of standard solution diluted with 1,000 ml distilled water. Triplicate 2 ml dilute standards were transferred to volumetric flasks; volume was topped up to 50 ml with distilled water, mixed, and transferred to 3 glass vials. The diluted standards were processed similar to the water sample. Finally, the measured extinction was corrected for the reagent blank, and the nitrite concentration was calculated from the following expression:

$$\mu\text{g-at N/l} = \text{corrected extinction x } F \tag{3.4}$$

F is the factor as determined below:

$$F = \frac{2.0}{E_s} \tag{3.5}$$

where E_s is the mean extinction of 3 standards, corrected for the blank. The *F* value should be about 2.

To determine PO_4, water sample was first allowed to stand at room temperature of 15–30 °C. Turbidity of the water sample was measured, a value greater than 0.01 would have the final extinction value corrected. A 20 ml sample was prepared, followed by 2 ml of mixed reagent added and immediately mixed. After the sample mixture was allowed to stand for 20 min, extinction was measured in a 10 cm cell against distilled water at 880 nm. Meanwhile, standard phosphate was prepared using 0.816 g anhydrous potassium dihydrogen phosphate dissolved in 1,000 ml distilled water preserved with

1 ml chloroform. Diluted standards were prepared using 10 ml of standard solution diluted with 1,000 ml distilled water. Triplicate 5 ml dilute standards were transferred to volumetric flasks. Volume was topped up to 100 ml with distilled water, mixed, and transferred to 3 glass vials. The diluted standards were processed similar to the water sample. The extinction was corrected with the reagent blank (and turbidity blank if needed), and phosphate concentration was calculated from the following expression:

$$\mu\text{g-at P/l} = \text{corrected extinction x } F \tag{3.6}$$

F is the factor as determined below:

$$F = \frac{3.0}{E_s - E_b} \tag{3.7}$$

where E_s is the mean extinction of 3 standards and E_b is the mean extinction of the reagent blank. The F value should be about 5.

To determine SiO_4, the water sample was first allowed to stand at room temperature of 18–25 °C. Plastic vials containing 10 ml molybdate solution were prepared. Then, a 25 ml water sample was added to the vial, stoppered, mixed, and allowed to let stand for 10–30 min. A reducing reagent was added rapidly to make 50 ml and immediately mixed. The solution was allowed to stand for 2–3 h. The extinction was measured at 810 nm using a 1 cm cell for concentrations >15 µg-at/l. Meanwhile, standard silicate was prepared using 0.960 g silicofluoride dissolved in 100 ml distilled water. Diluted standards were prepared in a polyethylene container by having the solution further diluted with distilled water up to a volume of 1,000 ml and mixed. The diluted standards were processed similar to the water sample. The measured extinction for the blank using either 1 or 10 cm cell length was measured, and reactive silicate was measured from the following expression:

$$\mu g\text{-at Si/l} = \text{corrected extinction x } F \quad (3.8)$$

F is the factor as determined below:

$$F_{1\ cm} = \frac{100}{E_s - E_b} \quad (3.9)$$

where E_s is the extinction of the standard and E_b is the extinction of the blank. The F_{1cm} value should be about 100 while $F_{10\ cm}$ value should be about 10.

3.5 Antibiotic Residues Measurement

Twenty-two target antibiotics were obtained from Sigma-Aldrich (Saint Louis, Missouri, USA), consisting of six major antibiotic classes: macrolides (roxithromycin, RTM; clarithromycin, CTM; azithromycin, AZM; erythromycin-H_2O, ETM-H_2O), fluoroquinolones (enoxacin, ENX; enrofloxacin, ENRO; norfloxacin, NOX; ofloxacin, OFX; ciprofloxacin, CIX), tetracyclines (chlortetracycline, CTC; oxytetracycline, OTC; tetracycline, TC), amphenicols (florfenicol, FF; chloramphenicol, CAP), diaminopyrimidine (trimethoprim, TMP), sulphonamides (sulfacetamide, SAAM; sulfathiazole, STZ; sulfadimethoxine, SDM; sulfadimidine, SMA; sulfapyridine, SPD; sulfadiazine, SDZ; sulfamethoxazole, SMX). The surrogate standard, $^{13}C_3$-caffeine solution (1 mg ml^{-1} with methanol), from Sigma-Aldrich was dissolved in methanol before cold storage at -20 °c.

All solvents used in this work were in HPLC grade. Erythromycin-H_2O standard stock was prepared by acidifying erythromycin with 3 M H_2SO_4 in accordance with McArdell et al. (2003). As such, erythromycin in its dehydration product was detected, ETM-H_2O, due to it being readily dehydrated by the loss of one water molecule

(Göbel et al., 2005). Methanol, acetonitrile, and ethylenediamine tetraacetic acid disodium (Na_2EDTA) were obtained from Merck (Darmstadt, Germany). Formic acid was obtained from CNW (Germany). Water was deionized using the Milli-Q water purification system (Millipore, Bedford, Massachusetts, USA).

The internal standard curve was applied to calculate the concentrations of the twenty-two antibiotics, i.e. concentrations of the analytes were plotted against the peak area ratio. For each compound, concentrations with a signal to noise (S/N) ratio of 10 were set to be the limits of quantification (LQ). The range of LQs for the selected antibiotic was between 0.02–36.50 ng l^{-1}, while limits of detection (LOD) were between 0.01–10.95 ng l^{-1}. The recoveries of $^{13}C_3$-caffeine were in the range of 0.0–176.7 % in all the water samples.

3.6 Detection and Quantification of Antibiotic Residues

For antibiotic detection and quantification, two litres of water samples from each sampling site were filtered using precombusted 0.7 µm glass fibre filters (GF/F, Sartorius, Göttingen, Germany). Filtrates were adjusted to pH 3 using 3.0 mol l^{-1} sulphuric acid to avoid oxidation of antibiotics with the addition of 0.2 g of Na_2EDTA as a chelating agent before 100 ng of $^{13}C_3$-caffeine was used as a surrogate standard to monitor the recovery rate as described by Zhang et al. (2013). Briefly, Oasis Hydrophilic-lipophilic balance (HLB) cartridges (500 mg, Waters, UK) were pre-treated with 6 ml of deionized water, 6 ml of 10 mmol l^{-1} acidified Na_2EDTA buffer and 6 ml of methanol. Target antibiotics were then concentrated by solid-phase extraction (SPE) by the Visiprep SPE system (Bellefonte, Pennsylvania, USA). The loading rate for each filtrate that passed through a cartridge was set at 10 min ml^{-1}. Cartridges loaded with filtrate were washed using 10 ml of acidified deionized water before vacuum dried for

10 min. The analytes were then eluted three times using 2 ml of methanol, concentrated to a volume of about 20 µl using nitrogen gas, and finally dissolved to a final volume of 1.0 ml with 90 % aqueous methanol.

Analysis of the twenty-two extracted antibiotics was completed using Agilent 1200 series (Palo Alto, California, USA) connected to Thermo Scientific Hypersil GOLD columns (2.1 mm x 100 mm, 1.9 µm). Binary mobile phase was made by combining mobile phase A, which included 5 mmol l^{-1} ammonium acetate aqueous solution and 0.2 % (v/v) formic acid, with mobile phase B which contained methanol. The following mobile phase gradient program was applied: 10 % to 60 % in 15 min, 60 % to 95 % within the following 5 min and held for 5 min, then returned to 10 % in 1 min and held for 1 min. The flow rate was maintained at 0.3 ml min^{-1}, the column temperature was held at 40 °C, and the injection volume of samples was at 5 µl. Parameters for MS/MS conditions were summarized in **Appendix A**. All the analyses were carried out in duplicates.

3.7 Antibiotic Residue Risk Assessment

Ecological risk quotients (RQs) were calculated based on the European technical guidance document on risk assessment (European Commission, 2003) for the evaluation of potential ecological effects of antibiotic presence in the environment. RQs were calculated by the equation:

$$RQ = \frac{MEC}{PNEC} \tag{3.10}$$

where MEC was the "measured environmental concentration" and PNEC was the "predicted no-effect concentration" for the respective antibiotics. PNEC was the division of lethal concentration or effective concentration for 50 % of the exposed

population (EC50/LC50) against assessment factor (AF) based on toxicity data value on non-target aquatic organisms: algae, invertebrate *Daphnia magna*, and fish (**Appendix B**). RQs for all the above formulas were defined according to Xue et al. (2013): low risk ($0.01 < RQs < 0.1$), medium risk ($0.1 < RQs < 1$), and high risk ($RQs > 1$).

3.8 Isolation and Enumeration of Total Coliform and *E. coli*

Membrane filtration technique was used to isolate and enumerate total coliforms and *E. coli*. Briefly, 1 ml surface water sample was aliquoted into 9 ml of sterile 0.85 % saline water with three times serial dilution followed by filtration through a pre-sterilised 0.45 mm nitrocellulose filter in duplicates, whereby water sample was drawn through a sterile funnel assembly using a vacuum pump. The filters were then transferred onto CHROMagar™ ECC media (CHROMagar Inc., Paris, France). Incubation was performed at 37 ± 0.5 °C for 24 h.

Colonies were enumerated and confirmed visually with an unaided eye. Blue colonies were enumerated as *E. coli*, whereas mauve colonies were enumerated as total coliform. Presumptive *E. coli* isolates were further purified with Luria Bertani medium and preserved in stab and glycerol solution in a -80 °C freezer for further testing. The abundance of total coliform and *E. coli* were reported as colony forming unit per 100 ml (CFU 100 ml^{-1}).

3.9 *E. coli* Gram-stain

Gram stain was performed to further confirm the identity of isolates. First, a test bacterial colony was transferred into a drop of distilled water on a microscope slide. The bacterial suspension on the slide was dried by quickly passing it through the flame of a bunsen burner to create a thin smear. The smear was flooded with methyl violet followed by 2–3 drops of $NaHCO_3$ solution and stained for 1 min. Excess stains were rinsed with water. Iodine solution was applied for 1 min, and excess stains were rinsed with water. Next, the smear was decolourised with a decolourising agent for 2–3 s and rinsed with water. Lastly, the smear was flooded with safranin for 30–45 s and rinsed with water. The slide was blot dried and observed under a microscope. Gram-positive bacteria appear purple and Gram-negative bacteria appear red.

3.10 Deoxyribonucleic Acid (DNA) Template Preparation

Boiling lysis extraction method was applied to extract crude DNA from *E. coli* isolates. Briefly, pure presumptive *E. coli* isolates were sub-cultured on Nutrient Agar plates and incubated at 37 °C for 24 h. A single pure colony was picked and resuspended in 100 µl sterile water in a 1.5 ml microcentrifuge tube. The cell suspension was then heated up to 94 °C with a dry bath incubator (model: Major Science MD–01N) followed by rapid cooling at -20 °C for 10 min. Crude DNA produced from the bacterial suspension was diluted to an optical density of 0.1 at OD 600, vortexed for 1 min, and centrifuged at 5,000 rpm for 10 s. The crude DNA preparations were stored at 4 °C until used.

3.11 Gel Electrophoresis and Gel Imaging

PCR products were separated by horizontal gel electrophoresis in a 1.5 % agarose gel stained by nucleic acid stain (iNtRON Red SafeTM, #21141) while using tris/borate/EDTA (TBE) as a running buffer. Base pair size comparisons were made with the DNA size marker 100 bp DNA ladder (New England Biolabs, #N3231S) and 1 kb DNA ladder (New England Biolabs, #B7025), where appropriate. DNA was viewed under UV light and its image captured with a gel image documentation device (SASTEC, ST–GD1500).

3.12 *E. coli* Identification using *phoA* as a Housekeeping Gene

As described by Kong et al. (1999), the monoplex PCR assay that targets the *phoA* gene was adopted to further validate the presumptive *E. coli* isolates. The *phoA* gene used is an alkaline phosphatase precursor that is regarded as a housekeeping gene in *E. coli*. Briefly, the PCR reaction involves carrying out a total reaction volume of 25 µl. The reaction mixture consisted 5 µl DNA, 1× Green GoTaq buffer (pH 8.5), 0.5 U of Taq DNA polymerase (Promega, USA), 1.0 mM of $MgCl_2$, 140 µM dNTP, and 0.1 µM of each primer. Each PCR run includes a reaction mixture that has its DNA template replaced with sterile distilled water, which acts as a negative control. While positive control used was a reaction mixture that contained purified DNA extract from a known *E. coli* strain, ATCC *E. coli* 25922 (American Type Culture Collection, Virginia, USA). Primer sequences for the detection of target genes paired with PCR thermo-cycling conditions (Applied biosystems, 2720) and the master mixture concentration are all described in **Appendix C** and **Appendix D** respectively.

3.13 *E. coli* Phylogenetic Typing

The PCR assay used to assign *E. coli* isolates into phylogroups was developed and validated by Clermont et al. (2013), which enables an *E. coli* isolate to be rapidly assigned to either one of the seven phylogroups (A, B1, B2, C, D, E, and F) which belongs to *E. coli sensu stricto*, or to one of the five cryptic clades (I, II, III, IV, and V). The assay involves a multistep approach. Initially, a quadruplex PCR reaction was performed to assign phylogroup B1, B2, and F into confirmed groupings while phylogroup A, C, D, E, and cryptic clades (I–V) into temporary groupings. Generally, the combination of presence or absence of the genes *arpA*, *chuA*, *yjaA*, TspE4.C2 was used to determine the phylogenetic groups. Following that, ibeA gene was screened using monoplex PCR reaction as described by Johnson & Stell (2000) to confirm the identity of phylogroup B2. Then, phylogroup C-specific monoplex PCR reaction was performed in accordance with Lescat et al. (2009) to differentiate phylogroup C isolates from phylogroup A isolates. Next, phylogroup E-specific monoplex PCR reaction was also performed in accordance with Lescat et al. (2009) to differentiate isolates under phylogroup E from phylogroup D and cryptic clade I. Internal control was added for both C- and E-specific monoplex PCR named C-E-specific internal control. Finally, clade-specific multiplex PCR reactions from Clermont *et al.* (2011), clade (I–II), and clade (III–IV–V), were performed to determine the specific cryptic clade an isolate belongs to. Groupings not known to fall under any suggested groups by this iterative assay shall be identified using multi-locus sequence typing (MLST). The multistep procedure in assigning *E. coli* isolates to a phylogroup, or cryptic clade is summarised in **table 3.2.** For each quadruplex reaction, 20 µl reaction volume was carried out containing 3 µl DNA template, 2 µM each dNTP, 1x PCR buffer, 2 U Taq polymerase, 1 mM $MgCl_2$, 1 µM for primers except for Acek-F (2 µM), ArpA1-R (2 µM), trpBA-F (0.6 µM), and trpBA-R (0.6 µM).

Table 3.2: Quadruplex genotypes and steps required for assigning *E. coli* isolates to phylogroups

Initial step: Quadruplex genotype						
arpA (400 bp)	*chuA* (288 bp)	*yjaA* (211 bp)	TspE4.C2 (152 bp)	Temporary Phylogroup	**Next step**	Confirmed Phylogroup
+	-	-	-			A
+	-	-	+			B1
-	+	-	-			F
-	+	+	-			B2
-	+	+	+			B2
-	+	-	+	B2	Confirm with *ibeA* primers	If *ibeA*+ then B2, else perform MLST
+	-	+	-	A or C	Screen with C-specific primers	If C+ then C, else A
+	+	-	-	D or E	Screen with E-specific primers	If E+ then E, else D
+	+	-	+	D or E	Screen with E-specific primers	If E+ then E, else D
+	+	+	-	E or clade I	Screen with E-specific primers	If E- then clade I
-	-	+	-	Clade I or II	Confirm with cryptic clade primers	
Presence of single 476 bp product				Clade III, IV or V	Confirm with cryptic clade primers	
-	-	-	+	Unknown	Perform MLST	
-	-	+	+	Unknown	Perform MLST	
+	-	+	+	Unknown	Perform MLST	
+	+	+	+	Unknown	Perform MLST	
-	-	-	-	Unknown	Confirm with cryptic clade primers/ Perform MLST	

3.14 *E. coli* Antimicrobial Susceptibility Test (AST)

E. coli isolates were tested for antibiotic resistance by standard agar disc diffusion technique (Bauer et al., 1966) through commercial disc (Oxoid, UK) placement on Mueller Hinton agar (Difco, USA). Briefly, a sterile inoculation loop was used to isolate, transfer, and suspend *E. coli* colonies in a test tube containing 2 ml of sterile saline. The saline inoculum tube was vortexed until suspension appeared homogenised. Turbidity of the suspension was adjusted to 0.5 McFarland standard either by adding more *E. coli* colonies if suspensions had lower turbidity or diluting with sterile saline if the suspension had higher turbidity. Immediately, a sterile swab was dipped into the

inoculum tube, pressed against the side of the tube at above fluid level, and then to be inoculated onto a Mueller Hinton agar plate. To ensure even distribution of the inoculum, the swab was streaked multiple times while the plate was rotated about 60 degrees frequently. The swab was used to rim the plate before being discarded into a biohazard bag. A multidisc dispenser (Oxoid Antimicrobial Susceptibility disc Dispenser, ST6090) was used to dispense multiple antibiotic discs onto the surface of the inoculum plate at once. The antibiotic discs were applied to the inoculated plates and incubated at 37 ± 0.5 ˚C overnight. The following 11 antibiotic groups consisting of 20 antibiotics were used: sulphonamides (sulphafurazole 300 µg, SF; and sulphamethoxazole/trimethoprim 25 µg, SXT), macrolides (azithromycin 15 µg, AZM), quinolones (nalidixic acid 30 µg, NA; oxolinic acid 2 µg, OA; and ofloxacin 5 µg, OFX), fluoroquinolones (ciprofloxacin 5 µg, CIP; and enrofloxacin 5 µg, ENR), tetracyclines (tetracycline 30 µg, TE), penicillins (ampicillin 10 µg, AMP; amoxicillin/clavulanic acid 30 µg, AMC), cephalosporins (cephazolin 30 µg, KZ; and ceftriaxone 30 µg, CRO), Aminoglycoside (streptomycin 10 µg, S; gentamicin 10 µg, CN; and neomycin 30 µg, N) carbapenems (imipenem 10 µg, IPM), phenicols (chloramphenicol 30 µg, C; and florfenicol 30 µg, FFC), and nitrofurans (nitrofurantoin 100 µg, F). The zone of inhibition will be analysed according to the standards and interpretive criteria of the Clinical and Laboratory Standards Institute (CLSI, 2020). Briefly, *E. coli* plate was placed on a black, nonreflecting surface illuminated with reflected light. While viewing the plate directly above in a vertical line of sight, sizes of the zone of inhibitions for each antibiotic disc were measured and rounded to the nearest millimetre using a ruler. The diameter of the disc was used as a measurement; if the diameter could not be discerned, the radius will be measured and multiplied by 2 to determine an estimated diameter. *E. coli* ATCC 25922, which is a recommended reference strain for

antimicrobial susceptibility testing, was used as a control. The multiple antibiotic resistance (MAR) index was calculated by the equation:

$$\text{MAR} = \frac{\text{a}}{(b \text{ x } c)} \tag{3.11}$$

Where *a* is the aggregate resistance score of all isolates from a sample, *b* is the number of antibiotics tested, and *c* is the number of *E. coli* isolated per sample (Krumperman, 1983).

3.15 *E. coli* Virulence Gene

The first and second reaction of the three reactions 'three-sample multiplex PCR' assay designed and validated by Gómez-Duarte et al. (2009) was used. The assay could screen for nine virulent genes for the differentiation of six *E. coli* pathotypes, including enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E.coli* (ETEC), diffuse adherent *E. coli* (DAEC), and enteroinvasive *E. coli* (EIEC). *E. coli* isolate assignment to a specific pathotype is summarised in **table 3.3**. *E. coli* 2060-004, E2348/69, JM221, E9034A, C1845, and EC-12 were used as positive controls for EHEC, EPEC, EAEC, ETEC, DAEC, and EIEC respectively, whereas sterile water was used as the negative control.

Table 3.3: *E. coli* isolate assignment to pathotypes

Reaction	Target genes									Pathotype
	eae	VT	*bfpA*	*aggR*	*ipaH*	*virF*	*daaE*	LT	ST	
First	+	+	-	-						EHEC
	+	-	+	-						EPEC
	-	-	-	+						EAEC
Second					-	-	-	+	+	ETEC
					-	-	+	-	-	DAEC
					+	+	-	-	-	EIEC

The first reaction from the multiplex PCR assay that targets *eae,* VT, *bfpA*, and *aggR* genes was used to identify EHEC, EPEC, and EAEC. The second reaction in the multiplex PCR assay targeting *ipaH, virF*, *daaE*, LT, and ST genes was used to identify ETEC, DAEC, and EIEC. Both multiplex PCR assays had total reaction volumes of 25 µl.

3.16 *tet* Antibiotic Resistance Gene Screening

Screening of fourteen *tet* resistance genes was modified from Ng et al. (2001), a total reaction volume of 25 µl where the genes were separated into four groupings, i.e. group I consisted of *tet*[(B), (C), and (D)], group II were *tet*[(A), (E), and (G)], group III covered *tet*[(K), (L), (M), (O), and (S)], and group IV included *tet*[P(A), (Q), and (X)].

3.17 *sul* Antibiotic Resistance Gene Screening

Screening of *sul* resistance genes was separated into a duplex PCR assay for *sul*1 and *sul*2 based on Kozak et al. (2009a), while a monoplex was performed for *sul*3 based on Pei et al. (2006). Both PCR had a total reaction volume of 25 µl.

3.18 Genetic Diversity by Repetitive Element Sequence-Based PCR (rep-PCR)

As described by Lim et al. (2009), rep-PCR is a DNA fingerprinting method that was utilised to amplify repetitive elements from purified *E. coli* isolates in the determination of genetic diversity among said isolates. Briefly, rep-PCR reaction involves performing a total reaction volume of 25 µl. While positive control used was a reaction mixture that contained purified DNA extract from a known *E. coli* strain (ATCC, *E. coli* 25922). The rep-PCR fingerprint patterns were managed and analysed with the bioinformatics software: BioNumerics Seven (APPLIED MATHS, Kortrijk, Belgium). DICE coefficient was performed to determine the similarity between profiles, while cluster analysis was performed with the unweighted pair group method using arithmetic averages (UPGMA). Shannon diversity index (*H*') was calculated using the following equation:

$$H' = -\Sigma\, Pi \log Pi \tag{3.12}$$

$$Pi = ni / \mathrm{N} \tag{3.13}$$

Whereby *ni* is the number of strains having each band pattern, N is the total number of isolate applied for rep-PCR.

3.19 Statistical Analysis

Statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) version 21.0 (IBM, Chicago, USA). The criterion for statistical significance for all the following analyses was at p-value ≤ 0.05. Pearson's chi-square (goodness of fit) test was performed to determine the significant difference among phylogenetic groups, *sul* resistance genes, and *tet* resistance mechanism types according to frequency data, whereas the chi-square test for independence was applied to determine any association between phylogroups and antibiotic resistance. Prevalence of antibiotic resistance was defined as the proportion of resistant *E. coli* isolates over total tested isolates. Correlation and linear regression analyses were performed to establish any association between water quality towards *E. coli* and total coliform abundance. Cluster analysis for sampling sites was performed based on the antibiotic susceptibility profile and antibiotic residue through the Bray-Curtis similarity index using PAST version 3.22 (Hammer et al., 2001). Besides that, canonical correlation analysis (CCA) was performed using PAST Statistics version 3.22 to analyse the following square root transformed variables: *E. coli* phylogenetic groups among sampling sites with respect to resistance genes *sul* and *tet*, water quality parameters, and antibiotic residue concentrations.

CHAPTER 4: RESULTS

4.1 Water Physicochemical Parameters

Among surface water from all sampling sites, the water physicochemical properties were summarised in **table 4.1**. The average water temperatures ranged from 22.7 °C to 31.8 °C, the pH ranged from 4.7 to 8.3, and the salinity increased significantly (ANOVA: $F = 3.315, p < 0.05$) from upstream to the estuary and are 0 ppt and 22.56 ppt respectively, while the DO ranged from 3.56 to 8.35 mg l^{-1} with the exception of the wastewater effluent from slaughterhouse that recorded values lower than the LQ.

Table 4.1: Water physicochemical parameters of Larut River compared to Sangga Besar

Sampling Stations	S1a	Zoo	Hospital	Slaughter house	S1b	S1c	S2a	S2b	S2c
Temp (°C)	28.66	26.75	28.93	26.99	22.70	31.18	31.56	31.78	31.46
Salinity (ppt)	0.07	0.00	0.06	0.73	9.07	18.72	16.44	22.56	12.42
pH	6.95	7.87	8.30	7.71	4.79	7.86	7.57	7.57	7.54
Dissolved Oxygen (mg l^{-1})	3.56	7.41	6.09	< LOD	6.31	7.17	8.31	8.31	8.35
Turbidity (NTU)	16.10	28.93	43.03	125.03	3.03	114.27	252.02	252.02	143.87

4.2 Inorganic Nutrient Parameters

For inorganic nitrogens, NH_4 was most abundant in Larut River followed by NO_3 and NO_2 (**Table 4.2**). NH_4 mean concentrations ranged from 0.004–0.513 mg L^{-1} with low concentrations at upstream S1a, and effluents from the three anthropogenic sites (0.003–0.067 mg l^{-1}), however higher concentration was observed at midstream S1b (0.513 mg l^{-1}) before decreasing at downstream S1c (0.213 mg l^{-1}). Both NO_3 and NO_2

had observed a similar trend, where each ranged from 0.0058–0.584 mg l^{-1} and 0.011–0.254 mg l^{-1} respectively, where both mean concentrations increased at midstream S1b at 0.493 mg l^{-1} and 0.213 mg l^{-1} before decreasing at downstream S1c at 0.271 mg l^{-1} and 0.086 mg l^{-1}. NH_4, NO_3, and NO_2 in Sangga Besar River have mean concentrations that were relatively higher than the effluent but lower than midstream S1b of Larut River, excluding NO_3 and NO_2 at S2a, which was higher than S1b. PO_4 concentrations were mostly ≤ 0.180 except for 2 sites, hospital and zoo, each recording 0.795 mg l^{-1} and 0.780 mg l^{-1} respectively.

Table 4.2: Mean inorganic nutrient concentrations (mg l^{-1}) of Larut River compared to Sangga Besar River

Sampling Stations	S1a	Zoo	Hospital	Slaughter house	S1b	S1c	S2a	S2b	S2c
Silicate (SiO_4)	2.190	2.797	1.713	3.753	2.820	1.840	1.713	1.310	1.040
Ammonium (NH_4)	0.013	0.007	0.067	0.003	0.517	0.217	0.110	0.143	0.230
Nitrite (NO_2)	0.037	0.030	0.013	0.037	0.213	0.087	0.250	0.103	0.087
Nitrate (NO_3)	0.370	0.203	0.057	0.093	0.493	0.270	0.587	0.307	0.320
Phosphate (PO_4)	0.030	0.793	0.793	0.000	0.100	0.183	0.147	0.067	0.057

4.3 Detection, Concentration and Ecological Risk of Antibiotic Residues

Of the 22 antibiotics screened, 16 types of antibiotic residues were positively detected among the sites except for S2b farm in Sangga Besar River. Overall, the total antibiotic concentration ranged from LQ to 1,262.30 ng l^{-1} and showed a mean of 13.05 ng l^{-1} (**Table 4.3**). The total antibiotic detection frequency was 88.89 %, whereby ETM-H_2O (77.78 %) and CIX (55.56 %) were the most prevalent antibiotics detected. The detection frequency of antibiotic residues in Larut River was higher in comparison to Sangga Besar, with concentrations ranging from LQ–18.28 ng l^{-1}. The upstream site

S1a of Larut River generally had a lower antibiotic detection frequency (9.09 %) compared to other positively detected sampling sites. Of the antibiotics examined, only ENRO (0.93 ng l^{-1}) and SMX (0.21 ng l^{-1}) were detected. Hospital, zoo, and slaughterhouse effluents from the midstream of Larut River were important contributors to antibiotic contamination along Larut River. Among the wastewater effluents, hospital wastewater effluent had the highest antibiotic detection frequency and the highest total concentration of antibiotics (54.55 %; 2,227.94 ng l^{-1}). For the downstream sites (S1b and S1c) fewer antibiotics were generally detected, with ETM-H_2O being detected for both sites with 6.42 ng l^{-1} and LQ–3.61 ng l^{-1} respectively. Similarly, at Sangga Besar River sites, low antibiotic residues were detected for S2a and S2c with a total concentration of 5.14 ng l^{-1} and 23.16 ng l^{-1} respectively.

Table 4.3: Summary of antibiotic concentrations detected in surface water of Larut River and Sangga Besar River by similarity

Anti-biotic class	Com-pound Abbr.	Total site (ng/L) Freq	Mean	Min	Max	Sampling site antibiotic concentration (ng/L) Hospital Min	Hospital Max	Zoo Min	Zoo Max	Slaughterhouse Min	Slaughterhouse Max	S1b Min	S1b Max	S1c Min	S1c Max	S2c Min	S2c Max	S1a Min	S1a Max	S2a Min	S2a Max	S2b Min	S2b Max
I	RTM	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
	CTM	22.22	0.99	0.14	13.15	3.28	14.45	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	0.14	LOD	LOD	LOD	LOD	LOD	LOD
	AZM	33.33	1.14	0.36	14.45	5.07	14.45	LOD	0.60	LOD	LOD	LOD	LOD	LOD	LOD	LOD	0.36	LOD	LOD	LOD	LOD	LOD	LOD
	ETM-H_2O	77.78	94.28	LQ	1 262.30	394.56	1 262.30	LOD	22.15	0.18	2.39	LOD	6.42	LQ	3.61	0.55	3.74	LOD	LOD	0.22	0.86	LOD	LOD
II	ENX	11.11	0.14	LQ	2.55	LOD	LQ	LQ	2.55	LOD	LQ	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
	ENRO	44.44	3.63	LQ	34.30	LOD	LQ	LOD	34.30	3.24	25.61	LOD	LOD	LOD	LOD	LQ	LQ	LOD	0.93	LOD	1.30	LOD	LOD
	NOX	33.33	0.37	LQ	2.55	2.45	2.55	LOD	LQ	LQ	1.55	LOD	LOD	LOD	0.18	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
	OFX	33.33	21.47	LQ	253.09	93.87	253.09	LOD	18.87	LOD	LOD	LOD	LOD	LOD	LOD	2.32	18.28	LOD	LOD	LOD	LQ	LOD	LOD
	CIX	55.56	56.41	LQ	577.97	387.65	577.97	LOD	23.18	0.68	5.38	LQ	0.54	LOD	LOD	7.36	12.62	LOD	LOD	LOD	LQ	LOD	LOD
III	CTC	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
	OTC	22.22	13.72	18.81	108.88	LOD	LOD	18.81	108.88	26.76	92.58	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LQ	LOD	LOD
	TC	11.11	64.41	66.93	1 092.49	66.93	1 092.49	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
IV	FF	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
	CAP	22.22	0.55	LQ	4.92	3.01	4.92	LQ	1.99	LOD	LOD	LOD	LQ	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LQ	LOD	LOD
V	TMP	33.33	9.73	1.03	93.15	75.58	93.15	LOD	5.39	LOD	1.03	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
VI	SAAM	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
	STZ	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
	SDM	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
	SMA	11.11	0.58	4.72	5.67	LOD	LOD	LOD	LOD	4.72	5.67	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
	SPD	11.11	0.13	0.00	2.35	LOD	2.35	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
	SDZ	33.33	0.91	0.19	12.59	LOD	2.64	LOD	LOD	1.02	12.59	LOD	0.19	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
	SMX	55.56	5.53	0.21	91.36	6.64	91.36	LOD	0.37	LOD	0.55	LOD	0.41	LOD	LOD	LOD	LOD	LOD	0.21	LOD	LOD	LOD	LOD
	Total	88.89	13.05	LOD	1 262.30	Freq:	54.55		45.46		40.91		18.18		9.09		22.73		9.09		9.09		LOD

I: Macrolide; II: Fluoroquinolone; III: Tetracycline; IV: Amphenicol; V: Trimethoprim; VI: Sulphonamide;
Roxithromycin: RTM; clarithromycin: CTM; azithromycin: AZM; erythromycin-H_2O: ETM-H_2O; enoxacin: ENX; enrofloxacin: ENRO; norfloxacin: NOX; ofloxacin: OFX; ciprofloxacin: CIX; chlortetracycline: CTC; oxytetracycline: OTC; tetracycline: TC; florfenicol: FF; chloramphenicol: CAP; trimethoprim: TMP; sulfacetamide: SAAM; sulfathiazole: STZ; sulfadimethoxine: SDM; sulfadimidine: SMA; sulfapyridine: SPD; sulfadiazine: SDZ; sulfamethoxazole: SMX;
LQ: Limit of quantification; LOD: Limit of detection, antibiotic concentrations equal and below the LOD were defined as "not detected or zero in their respective calculations".
FREQ: Frequency (%) of detection per sampling site; MEAN: Mean value of antibiotic concentrations (ng/L); MIN: Minimum concentration (ng/L); MAX: maximum concentration (ng/L).

In this study, PNEC of algae, invertebrate *Daphnia magna*, and fish were analyzed to assess the RQs. RQ for ENX was not calculated, as the toxicology data were unavailable for these aquatic organisms. The degree of sensitivity of the aquatic organisms towards antibiotics in surface waters of Larut and Sangga Besar Rivers was in the following descending order: Algae > *Daphnia magna* > fish. In **figure 4.1**, the risk assessment showed that among the macrolides detected, ETM-H_2O posed low to high ecological risk to algae among the study sites except for S1a and S2b, conversely, low risk was detected for invertebrates, whereas AZM posed a low to medium risk to algae. CTM detected in hospital was found to have a high risk for algae, contrastingly, algae in S2c of Sangga River was exposed to low ecological risk. For fluoroquinolones, OFX detected at zoo, hospital, and S2c posed a high ecological risk to algae, whereas at S2c only medium risk was found. CIX detected in hospital had a low risk for algae. Among tetracyclines, OTC detected in hospital, slaughterhouse and S2a posed low to medium risk for algae, whereas TC detected in hospital posed a medium risk for algae, medium risk for invertebrates, and low risk for fish. Among the sulphonamides compounds tested, only SMX and SMA detected in hospital and slaughterhouse posed medium and low risk respectively to algae.

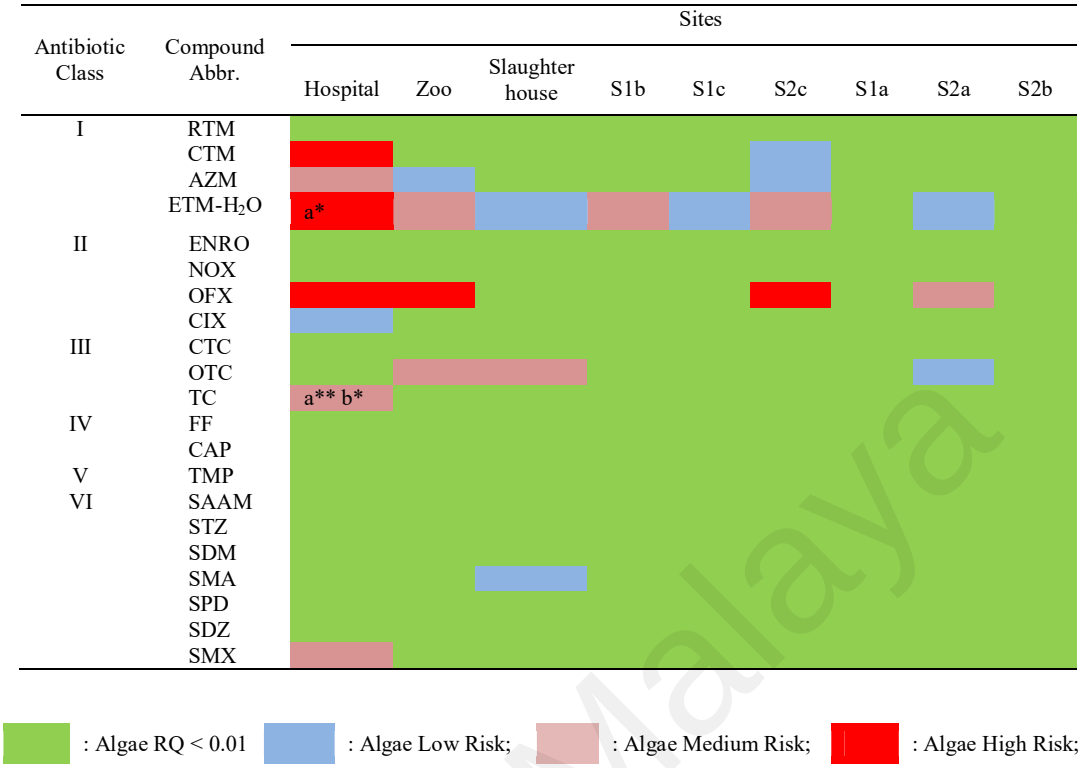

Figure 4.1: Calculated RQs for the 22 antibiotics in surface waters of 9 sampling sites in Larut River and Sangga Besar River towards algae, invertebrates, and fish

I: Macrolide; II: Fluoroquinolone; III: Tetracycline; IV: Amphenicol; V: Trimethoprim; VI: Sulphonamide; Roxithromycin: RTM; clarithromycin: CTM; azithromycin: AZM; erythromycin-H_2O: ETM-H_2O; enoxacin: ENX; enrofloxacin: ENRO; norfloxacin: NOX; ofloxacin: OFX; ciprofloxacin: CIX; chlortetracycline: CTC; oxytetracycline: OTC; tetracycline: TC; florfenicol: FF; chloramphenicol: CAP; trimethoprim: TMP; sulfacetamide: SAAM; sulfathiazole: STZ; sulfadimethoxine: SDM; sulfadimidine: SMA; sulfapyridine: SPD; sulfadiazine: SDZ; sulfamethoxazole: SMX; a*: Invertebrate low risk; a**: Invertebrate medium risk; b*: Fish low risk

4.4 Abundance of Coliform and *E. coli*

Coliform and *E. coli* were detected at all sampling sites, where both concentrations were higher at Larut River (up to 4.7×10^5 CFU 100 ml^{-1}; up to 4.1×10^5 CFU 100 ml^{-1}, respectively) than Sangga Besar River (up to 4.3×10^3 CFU 100 ml^{-1}; up to 2.5×10^3 CFU 100 ml^{-1}) (**Figure 4.2**). Overall, higher coliform and *E. coli* concentration (2–3 log CFU 100 ml^{-1} difference) were detected in the effluent sites compared to river waters ($p \leq 0.05$). According to site, the highest total coliform and *E. coli* concentration were observed in wastewater effluent from slaughterhouse. Mean total coliform in slaughterhouse, hospital, and zoo were approximately 2 log order higher than upstream (S1a) and 0.4 logs higher than downstream S1b while mean *E. coli* count were approximately 3 log order higher and about 1 log higher at S1b respectively.

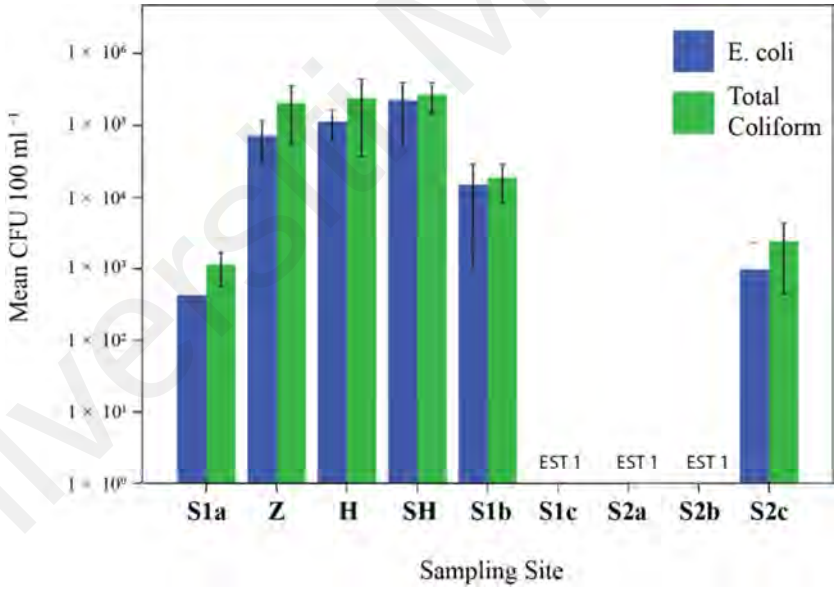

Figure 4.2: Total coliform and *E. coli* counts from each site

EST, estimation. Sampling sites: Z, Zoo; H, Hospital; SH, Slaughterhouse

4.5 Distribution of *E. coli* Phylogenetic Groups

Each phylogenetic group (A, B1, B2, C, D, E, and F) was assigned to the 503 *E. coli* isolates, whereas no cryptic clades were detected among them (**Figure 4.3**). Overall, phylogroup B1 (46.72 %) and phylogroup A (34.39 %) was the most prevalent phylogroup in this study. For the 6 sites in the Larut River along with 3 sites in the Sangga Besar River, the distribution of all phylogroups except phylogroup B1 was not homogenous according to Pearson's chi-square test. Nevertheless, phylogroup B1 was still higher at the effluents from zoo, slaughterhouse, and S1c from Larut River. Phylogroup A (34.39 %), the second most prevalent phylogroup in this study were found significantly higher ($\chi 2 = 55.314$, $df = 8$, $p = 0.000$) in effluents of hospital (58.57 %) and slaughterhouse (49.18 %) followed by S1b (51.67 %). In contrast, the remaining phylogroups only account for 18.89% of the total isolates, where phylogroup B2 and D was found to be significantly higher (B2: $\chi 2 = 134.94$, $df = 9$, $p = 0.00$; D: $\chi 2 = 32.87$, $df = 9$, $p = 0.00$) in upstream S1a. Remarkably, a distinct shift in phylogroup distribution was observed between the upstream S1a and anthropogenically impacted zoo, e.g. *E. coli* strains from zoo had phylogroup A and B1 as the predominant strains while phylogroup B2 representation is only at 2.63 %.

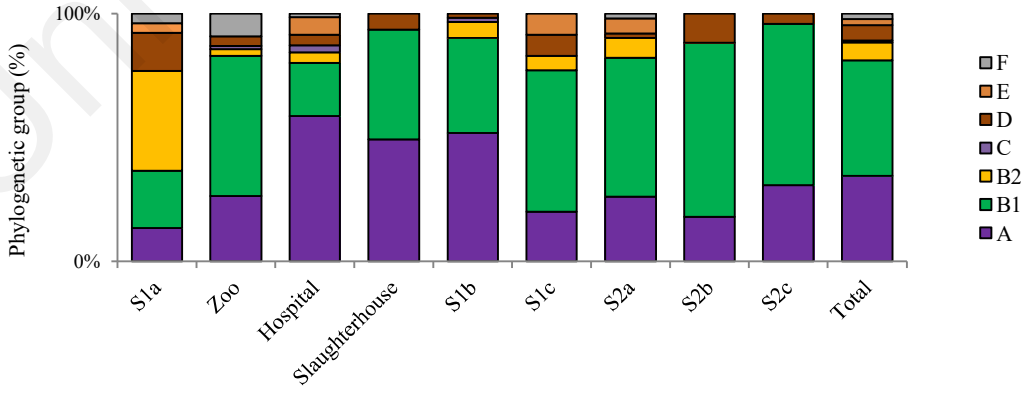

Figure 4.3: Distribution of *E. coli* phylogenetic groups by sites in Larut and Sangga Besar Rivers

4.6 Antimicrobial Susceptibility Profile

From the 11 antibiotic classes that covered 20 antibiotics, all except imipenem had positive resistance among the 503 *E. coli* isolates tested. According to antibiotic class, antibiotic resistance ranges were: tetracycline (10.34–86.84 %) > quinolones (3.45–80.33 %) > penicillins (0–75.00 %) > sulphonamides (0–65.79 %) > amphenicols (2.00–60.53 %) > fluoroquinolones (0–57.38 %) > aminoglycosides (0–47.37 %) > macrolides (0–19.74 %) > cephalosporins (0–18.57 %) > nitrofurans (0–3.45 %). From the total *E. coli* isolates, 339 (67.40 %) were resistant to ≥ 1 antibiotic whereby 53.08 % were MAR isolates (i.e. resistant to ≥ 3 different antibiotic classes). Among the MAR isolates, frequency was higher in wastewater effluents (zoo > slaughterhouse > hospital) than river waters (S1b > S2a > S1c > S2c > S1a > S2b) (**Figure 4.4**). Specifically, 24.45 % showed resistances to 3–5 classes, 27.24 % resisted against 6–8 classes, and seven isolates (1.39 %) were resistant to nine classes by which two MAR isolates carried 16 types of AR, each recovered from slaughterhouse and hospital effluent.

Between sites, cluster analysis showed the distribution of AR *E. coli* phenotype from zoo and slaughterhouse effluents was more similar than hospital effluent and S1b. The highest prevalence of antibiotic-resistant *E. coli* strains was observed in effluents from zoo (97.37 %) and slaughterhouse (90.16 %), along with S1b (75.00 %), with respective MAR isolate prevalence of 84.21 %, 72.13 %, and 56.67 %. In contrast, upstream S1a had the lowest prevalence of AR strains (44.23 %) and MAR strains (25.00 %). The MAR index of ≥ 0.20 was observed at S1a, zoo, hospital, slaughterhouse, and S1b. Greater risk of MAR *E. coli* detection was found in Larut River (MAR index: 0.28) where the prevalence of antibiotic-resistant and MAR isolates were 74.86 % and 59.61 % against Sangga Besar River (MAR index: 0.13) with 49.66 % and 37.58 % respectively.

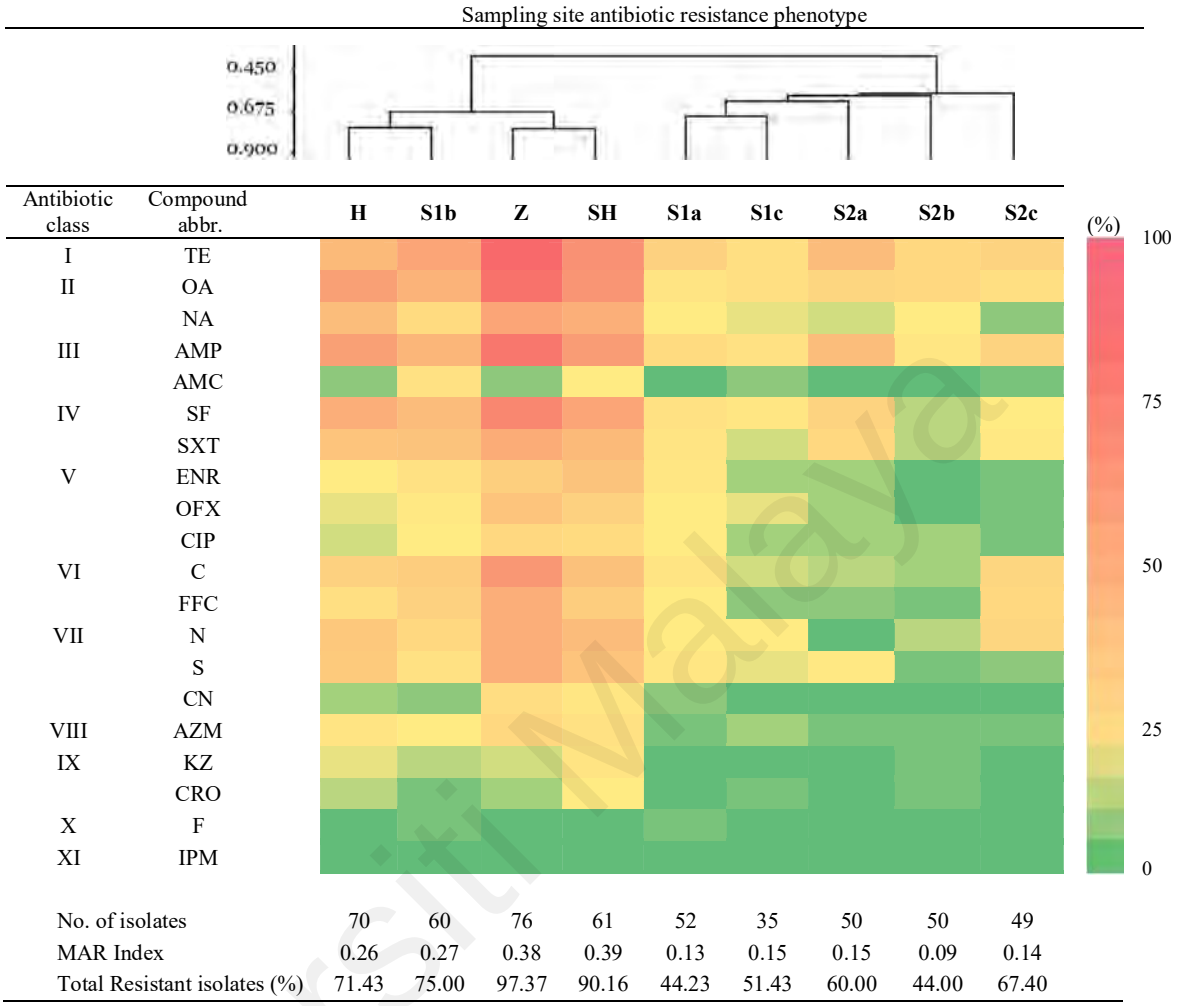

Figure 4.4: Antibiogram of antibiotic resistance phenotype detected in surface water among sampling sites in Larut River and Sangga Besar River organised by similarity

Abbr, abbreviation. Antibiotic class: I, Tetracycline; II, Quinolone; III, Penicillin; IV, Sulphonamide; V, Fluoroquinolone; VI, Amphenicol; VI, Aminoglycoside; VIII, Macrolide; XI, Cephalosporin; X, Nitrofuran; XI, Carbapenem. Antibiotic type: TE, Tetracycline; OA, Oxolinic acid; NA, Nalidixic acid; AMP, Ampicilin; AMC, Amoxycillin/Clavulanic acid; SF, Sulfafurazole; SXT, Sulfamethoxazole/trimethoprim; ENR, Enrofloxacin; OFX, Ofloxacin; CIP, Ciprofloxacin; C, Chloramphenicol; FFC, Florfenicol; N, Neomycin; S, Streptomycin; CN, Gentamicin; AZM; Azithromycin; KZ, Cephazolin; CRO, Ceftriaxone; F, Nitrofurantion; IPM, Imipenem. Sampling sites: Z, Zoo; H, Hospital; SH, Slaughterhouse

Among phylogroups, A showed the highest resistance prevalence of 81.50 % with 76.9 % MAR isolates. Moreover, phylogroup F and B1 had shown associated resistance towards tetracycline (TE, $\chi2 = 14.119$, $df = 6$, $p = 0.028$), quinolones (OA, $\chi2 = 67.221$, $df = 6$, $p = 0.000$; NA, $\chi2 = 48.444$, $df = 6$, $p = 0.000$), fluoroquinolones (CIP, $\chi2 = 19.858$, $df = 6$, $p = 0.001$), sulphonamides (SF, $\chi2 = 22.283$, $df = 6$, $p = 0.001$; SXT, $\chi2 = 26.879$, $df = 6$, $p = 0.000$), amphenicols (C, $\chi2 = 29.923$, $df = 6$, $p = 0.000$; FFC, $\chi2 = 26.073$, $df = 6$, $p = 0.000$), and macrolides (AZM, $\chi2 = 28.443$, $df = 6$, $p = 0.000$). Although phylogroup C had 100 % antibiotic resistance, it is important to note that only four isolates belonging to this group were isolated in this study. Notably, phylogroups F and B1 each carried a single isolate with 16 types of antibiotic resistance. Lower percentage of antibiotic resistance was observed in phylogenetic groups B2 and D. These phylogroups were detected more in upstream (S1a) which was associated with animal origin. Phylogroup B2 was found to have higher resistance against penicillin, quinolones/fluoroquinolones, sulphonamides and aminoglycosides whereas Phylogroup D was detected to have higher resistance against tetracycline, penicillin, and sulphonamides. In terms of MAR, phylogroups with a MAR index of greater than 2.0 were phylogroups A, B1, C, and F.

4.7 Pathotypes of *E. coli*

Eleven *E. coli* isolates (2.19 %) harboured ≥ 1 intestinal pathogenic *E. coli* (IPEC) associated virulence gene (**Table 4.4**). The virulence factor *aggR*, which indicates a positive EAEC pathotype was the most prevalent gene (n=5) and was detected in effluents of zoo and hospital. Importantly, these EAEC isolates collectively demonstrated MAR profiles of 5–16 antibiotics, with combinations of *sul2* and *sul3* genes, and *tet*(*B*), *tet*(*A*), *tet*(*L*), *tet*(*M*), and *tet*(*X*) genes. Additionally, *bfpA* (n=3),

ST (n=2), and *eae* (n=1) virulence genes were also detected along the river continuum with high susceptibility to antibiotics, except for a single phylogroup F isolate from zoo effluent. In terms of phylogroup, the phylogroup F strains (n=4/11] were found to carry virulent genes.

Table 4.4: Profiles of virulent gene carrying *E. coli* isolates

	Profile							
Virulence gene	Isolate	River	Site	Phylogroup	No. of antibiotic resistance phenotype	Antibiotic resistance	*sul* gene	*tet* gene
ST	1WF1	Larut	S1a	D	0	N/A	N/A	N/A
ST	1WF11	Larut	S1a	B2	0	N/A	N/A	N/A
*bfp*A	1Z1B5	Larut	Zoo	F	10	TE-OA-NA-AMP-SF-SXT-ENR-C-S-AZM	N/A	*tet*(A)
*bfp*A	2AQ7	Sangga Besar	S2b	B1	0	N/A	N/A	*tet*(E)(G)(L)
*bfp*A	2RM2	Sangga Besar	S2c	B1	1	AMP	N/A	*tet*(G)(L)
eae	2BP6	Larut	S1a	B2	0	N/A	N/A	*tet*(A)
*agg*R	1Z1B11	Larut	Zoo	F	5	TE-OA-NA-AMP-FFC	*sul*3	*tet*(B)(L)
*agg*R	1Z2 1	Larut	Zoo	B1	13	TE-OA-NA-AMP-SF-SXT-ENR-OFX-CIP-C-N-S-CN	*sul*3	*tet*(A)(L)(M)
*agg*R	1Z2 4	Larut	Zoo	F	12	TE-OA-NA-SF-SXT-ENR-OFX-CIP-C-N-S-CN	*sul*23	*tet*(A)(L)(M)
*agg*R	1HB11	Larut	Hospital	F	16	TE-OA-NA-SF-SXT-ENR-OFX-CIP-C-FFC-N-S-CN-KZ-CRO	*sul*3	*tet*(A)
*agg*R	1HN31	Larut	Hospital	B1	13	TE-OA-NA-AMP-AMC-SF-SXT-ENR-OFX-CIP-C-N-S	N/A	*tet*(X)

4.8 *tet* and *sul* Genes Distribution in *E. coli*

Overall, 394 (78.33 %) *E. coli* isolates harboured at least one of the tested *tet* genes except for *tet*(S*)* which codes for *tet* resistance mechanisms: efflux protein *tet* [(A), (B), (C), (D), (E), (G), (K), (L), and A(P)], ribosomal protection protein *tet* [(M), (O), and (Q)] and enzyme inactivation *tet*(X). The majority of the resistance genes were found in the zoo (93.42 %) effluents followed by slaughterhouse (85.25 %) effluents. There were 109 *E. coli* isolates that did not harbour any of the tested

resistance genes. The predominant *tet* genes carried by *E. coli* were *tet*(A) (46.32 %), *tet*(L) (31.01 %), *tet*(M) (9.54 %), and tet(X) (**Figure 4.5**). Among sites, *tet*(A) followed by *tet*(M) was the dominant gene in zoo effluent, *tet*(L) was abundant in S2b, and *tet*(X) was in hospital effluent. Efflux genes were prevalent among all sites, indicating that active efflux via membrane associated proteins was the main mechanism for resistance in *E. coli* that resided in Larut River and Sangga Besar River. Both *tet*(A) and *tet*(L) belonged to the active efflux resistant mechanism. Meanwhile, resistance mechanisms of active efflux along with ribosomal protection proteins (RPP) were found in zoo wastewater effluent, as evidenced by the dominance of *tet*(A) and *tet*(M) respectively. Certain *E. coli* (35.98 %) in this study were found to carry multiple *tet* resistance genes with a high variation of *tet* gene combination. Among the 57 different *tet* combinations harboured by *E. coli* isolates, the most prevalent multiple *tet* resistance genes *tet*(*A*)(*L*) (7.36%), *tet*(*A*)(*M*) (4.97 %), and *tet*(*A*)(*L*)(*M*) (3.18 %) were significantly dominant ($p \leq 0.05$) in wastewater effluents from hospital Notably, the presence of all three *tet* resistance mechanisms that includes enzyme inactivation, *tet*(X), in *E. coli* isolates isolated from hospital effluent were detected. In contrast, *E. coli* isolates that harboured significantly fewer resistance genes were observed at less polluted sites. Results indicated that tetracycline resistant *E. coli* acquired multiple mechanisms to confer resistance.

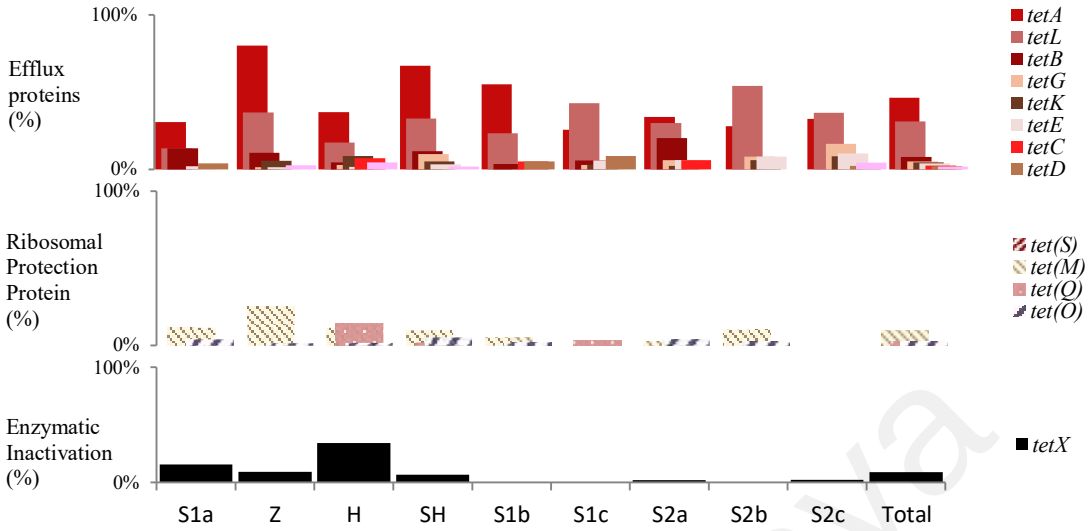

Figure 4.5: Prevalence of *tet* antibiotic resistance gene type categorised by resistance mechanism carried by *E. coli* isolated from sites in Larut River compared to Sangga Besar River

There were 149 (29.62 %) *E. coli* isolates which harboured ≥ 1 sulphonamide resistance gene, resulting in a gene-frequency distribution of *sul*3 (17.10 %) > *sul*2 (4.97 %) > *sul*1 (2.58 %) (**Figure 4.6**). Among sites, *sul* gene carrying *E. coli* was significantly higher with prevalence of 52.46 % in midstream slaughterhouse ($\chi 2 = 22.375$, $df = 5$, $p = 0.000$) and 46.05 % at zoo ($\chi 2 = 6.000$, $df = 4$, $p = 0.000$). In comparison, upstream S1a had the lowest detection frequency (3.45 %). Among rivers, greater prevalence was expectedly found in Larut River (35.31 %) compared to Sangga Besar River (16.11 %). The highest prevalence of *sul*3 was detected in wastewater effluent from zoo. Among the multiple *sul* gene combinations, the *sul*13 combination was most frequently detected. Furthermore, three isolates with combination *sul*123 were detected (0.60 %); specifically, two from slaughterhouse and one from hospital.

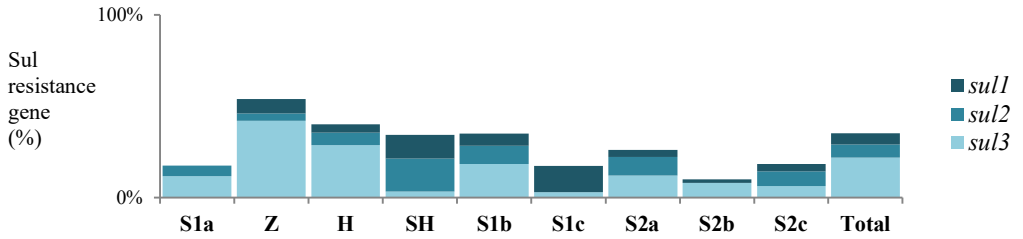

Figure 4.6: Cumulative *sul* resistance gene distribution of *E. coli* isolates by site

4.9 Relationship Between Physicochemical and Antibiotic Residues on *E. coli* Phylogenetic Distribution

CCA analysis was used to discern the possible relationships between *E. coli* phylogenetic groups and the variables for physicochemical and antibiotic residues in the water samples among sites. Variables were square root transformed within the CCA. A total of 78.41 % of the variance was explained from the CCA, where the horizontal axis (CCA 1) and the vertical axis (CCA 2) respectively explained 52.60 % and 25.81 % of the explanatory variables.

CCA analysis revealed that the *E. coli* phylogroups A and C were greater in deteriorating water quality with high NH_4 and PO_4 (**Figure 4.7 A**). In comparison, phylogroup B1 seemed to grow well in turbid water, with NO_2, and have better tolerance to salinity, and pH. While the other lower frequency phylogroups (B2, D, E, F) were associated with SiO_4, temperature, and NO_3. Results also showed that the distribution of different phylogroups and antibiotic resistance genes were also affected by antibiotic use. The antibiotics detected in hospital, slaughterhouse, and S1b exhibited a significant correlation with phylogenetic groups A, C, *sul*, and *tet* genes, while oxytetracycline (OTC), fluoroquinolones (ENRO and ENX) detected in zoo showed positive associations with phylogroup B1 (**Figure 4.7 B**). However, no correlation was observed in the lower frequency phylogroups (B2, D, E, and F).

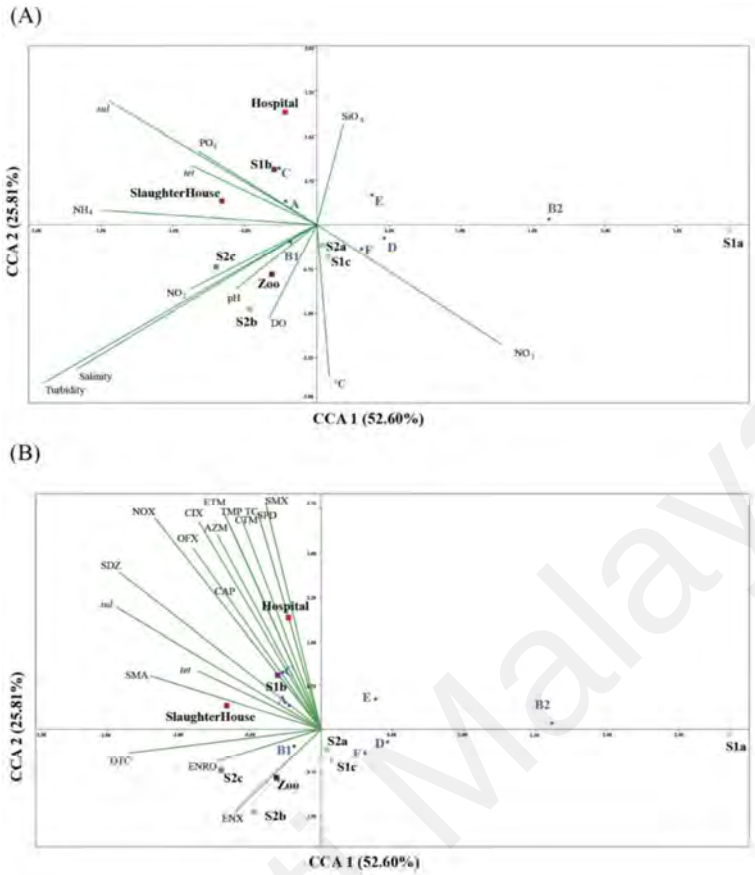

FIGURE 4.7: Square root normalised CCA ordination showing the distribution of *E. coli* phylogenetic groups by sampling sites in Larut River and Sangga Besar River with respect to their phylogenetic group and resistance genes *tet* and *sul*

(A) Relationship between *E. coli* and water physicochemical parameters of Larut River and Sangga Besar River collected. **(B)** Relationship between *E. coli* and water antibiotic residue detected in Larut River and Sangga Besar River

Water physicochemical factors abbreviation: Temp (°C), salinity (ppt), pH, dissolved Oxygen (DO), turbidity and concentrations (µM) of Silicate (SiO_4), Ammonium (NH_4), Nitrite (NO_2), Nitrite (NO_2), Nitrate (NO_3), Phosphate (PO_4) Antibiotic resistance gene: *sul*, sulphonamide resistance gene; *tet*, tetracycline resistance gene Antibiotic residue: RTM, Roxithromycin; CTM, clarithromycin, AZM, azithromycin; ETM-H_2O, erythromycin-H_2O; ENX, enoxacin; ENRO, enrofloxacin; NOX, norfloxacin; OFX, ofloxacin, CIX, ciprofloxacin; CTC, chlortetracycline; OTC, oxytetracycline; TC, tetracycline; FF, florfenicol; CAP, chloramphenicol; TMP, trimethoprim; SAAM, sulfacetamide; STZ, sulfathiazole; SDM, sulfadimethoxine; SMA, sulfadimidine; SPD, sulfapyridine; SDZ, sulfadiazine; SMX, sulfamethoxazole

4.10 Genetic Diversity of *E. coli* Through DNA Fingerprinting Method

A total of 503 band patterns of variable band numbers with amplicon sizes ranging from 100 bp–2000 bp were generated by rep-PCR from all the *E. coli* isolates identified from the nine sites. Among sites, the highest diversity was observed in slaughterhouse (*H'*, 3.53), followed by zoo (*H'*, 3.38), whereas the lowest *H'* was detected in S2c (*H'*, 2.60) (**Table 4.5**). In contrast, hospital effluent had a low *E. coli* genetic diversity (*H'*: 2.91) among effluent sites. Among rivers, Larut River showed higher diversity (*H'*, 4.58%) in comparison with Sangga Besar River (*H'*, 3.79).

Due to the large population size (n = 503), only non-repeating genotypes (< 85% similarity, n = 90) were selected to have the genetic relationships of *E. coli* in this study analysed. By calculating the DNA band patterns using band-based DICE coefficient of similarity and generated by unweighted pair group method with arithmetic mean (UPGMA), a dendrogram representing seven major clusters at 55 % cut-off value was constructed (**Figure 4.8**). The highest number of isolates were in Cluster I (n = 40), with isolates found with nearly equal distribution at all sites along Larut River. The only exception is hospital effluent, which contained isolates (85.00 %) characterised under the generalist phylogroups A and B1 with phenotypic resistance (67.50 %). Similar to cluster I, cluster VI (n = 13) were phylogroup A and B1 strains with high antibiotic resistance but differed spatially. In contrast, low antibiotic-resistant phylogroup B2 isolates were distinctively associated with clusters IV, V, and VII. The aforementioned clusters collectively contained isolates (n = 15) that were isolated at non-anthropogenic sites except for two strains from zoo and hospital separately. The remaining clusters II and III had a diverse phylogeny similar to that of cluster I but instead had intermediate antibiotic resistance.

Table 4.5: Non-repeating genotypes and genetic diversity of *E. coli* isolates

River	Site	No. of Isolates	No. of non-repeating genotype (< 85 % similarity)	Diversity index (*H'*)
Larut River	S1a	52	19	3.30
	Zoo	76	10	3.38
	Hospital	70	7	2.91
	Slaughterhouse	61	8	3.53
	S1b	60	16	3.22
	S1c	35	7	2.66
	Sum	354	67	4.58
Sangga Besar River	S2a	50	7	3.06
	S2b	50	11	2.86
	S2c	49	5	2.60
	Sum	149	24	3.79
	Total	503	90	4.76

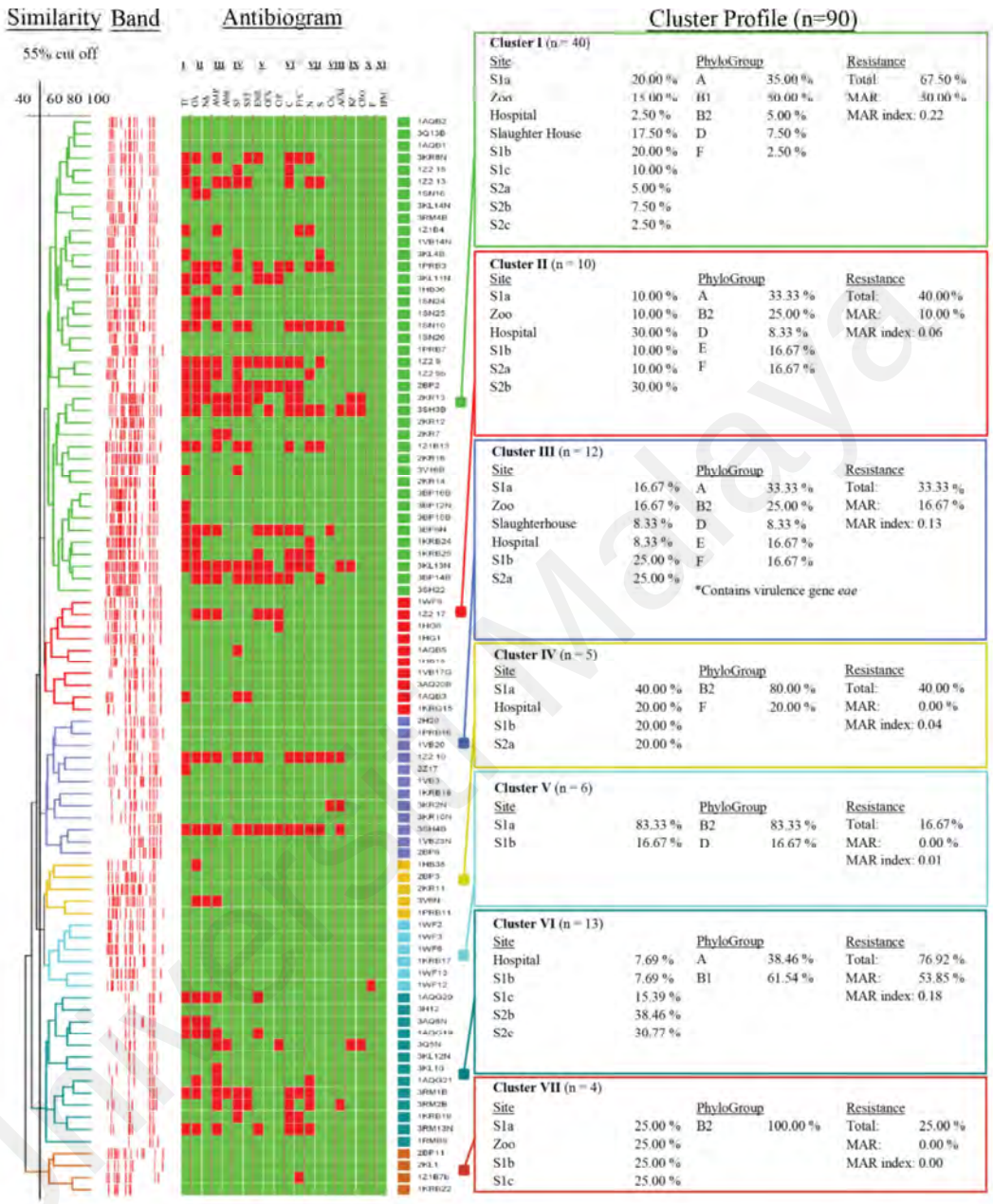

Figure 4.8: Dendrogram showing similarity of *E. coli* strains isolated from different sampling sites as determined by rep-PCR fingerprint analysis using REP primer

I, Tetracycline; II, Quinolone; III, Penicillin; IV, Sulphonamide; V, Fluoroquinolone; VI, Amphenicol; VII, Aminoglycoside; VIII, Macrolide; IX, Cephalosporin; X, Nitrofuran; XI, Carbapenem

CHAPTER 5: DISCUSSION

5.1 Occurrence of Antibiotics in Water

Sites from Sangga Besar River generally had low antibiotic detections and concentrations likely attributed to the ten times lower population density of Sangga Besar compared to Larut River (Ghaderpour et al., 2015). Studies have shown significant correlations between population density and antibiotic compounds in the surface waters of a river (Osorio et al., 2016). However, no correlation between antibiotic concentrations and water parameters measured was found (Lye et al. 2019), even though some studies have shown that physicochemical properties of antibiotics were affected by the local environment parameters (e.g. temperature, pH, salinity, moisture, oxygen level, etc) (Luo et al., 2011; Lu et al., 2015; Yang et al., 2015). A possible explanation is hydrodynamics and microbiological activity of community bacteria instead played a larger role in the degradation and persistence of antibiotics in this environment (Gauthier et al., 2010; García-Galán et al., 2011; Tappe et al., 2013; Topp et al., 2013).

This study identified hospital, zoo, and slaughterhouse effluents from Larut River as likely sources of antibiotic contamination along Larut River, which was consistent with Lye et al. (2019). Cluster analysis showed that antibiotic contamination in hospital effluent was unlike other effluent sites, i.e. zoo and slaughterhouse were more similar to one another (69.00 %) than hospital. The high prevalence of antibiotic residues in hospital concurred with other studies (Verlicchi et al., 2012; Ory et al., 2016). Among antibiotics detected, ETM-H_2O was found to be the most prevalent, verifying a more recent report that identified it as the second most commonly prescribed antibiotic in Malaysia (22.4 %, Ministry of Health, 2017b) because of its use in bacterial infection

treatment and/or as a motility agent in critically ill patients in clinical (Ministry of Health, 2014b; Shamsuddin et al., 2016). This is followed by fluoroquinolones, where CIX was the second-most common expenditure in Malaysian public hospitals between 2009 and 2010 and are commonly used as systemic medication and to treat bacterial eye infection with utilisation of 0.365 defined daily dose (DDD) 1,000 inhabitants^{-1} day^{-1} and 0.022 DDD 1,000 inhabitants^{-1} day^{-1}, respectively whereas the usage in livestock reached 4,615 kg year^{-1} (Ministry of Health, 2014b; Marzuki, 2017). The third most prevalent was tetracycline, where its usage was the second-most commonly used veterinary antibiotic in Malaysia and had reportedly reached 73,910 kg year^{-1} (Marzuki, 2017). Together, the use of erythromycin, ciprofloxacin, and tetracycline was reportedly common in Malaysian hospitals back in 2007 (Ministry of Health, 2008), and evidence of antibiotic contamination in hospital effluent from the current environmental study could still corroborate this earlier report from more than a decade ago.

Antibiotic practices and doses applied in both humans and animals vary between regions and countries, which may greatly influence the type, distribution, and variation of antibiotic residues in aquatic environments (Managaki et al., 2007; Shimizu et al., 2013). Among the three major antibiotic residues in this study, Tan et al. (2017) revealed that the macrolide ETM-ethylsuccinate was one of the antibiotics that were inappropriately prescribed in a Malaysia hospital for upper respiratory tract infections, as most of the prescribers were unaware of the removal of this drug from Malaysia's National Antibiotic Guideline due to the resistance developed by Streptococci. Besides, ETM usage is legally permitted in Malaysia for poultry and cattle farming (Hassali et al., 2018), the total amount used in 2015 was 218,290 kg year^{-1} (Marzuki. 2017). Furthermore, ETM has good stability in the aquatic environment (Li et al., 2018). The detected concentrations in this study were

comparable to the levels from Pearl River (not detected–1,540 ng l^{-1}, Li et al., 2018) but higher than Tamagawa River, Japan (21.0–120.0 ng l^{-1}, Managaki et al., 2007), Lake Taihu (not detected–624.80 ng l^{-1}, Xu et al., 2014), and South Yellow Sea (not detected–138.90 nd l^{-1}, Du et al., 2017). Yet, lower than WWTPs in Brazil (not detected –1,586.0 ng l^{-1}, Jank et al., 2014) and hospital effluents from Romania Hospital (not detected–7,520.00 ng l^{-1}, Szekeres et al., 2017).

The use of fluoroquinolones has increased substantially globally due to their broad-spectrum antibiotic properties against Gram-negative pathogens, especially those resistant to other antibiotic classes (Pham et al., 2019). Among the fluoroquinolone compounds examined, second-generation CIX and OFX were the most prevalent. OFX is for systemic use and to treat outer ear infections. According to Malaysian Statistic Medicines 2011–2014, an increasing trend was observed for the usage of anti-infective OFX ear drop from 0.015 DDD 1,000 inhabitants^{-1} day^{-1} in 2011 to 0.018 DDD 1,000 inhabitants^{-1} day^{-1} in 2014 (Ministry of Health, 2017a). Generally, NOX is less potent than CIX, therefore, their usage rate as systemic medication (0.024 DDD 1,000 inhabitants^{-1} day^{-1}) and for bacterial eye infection treatment (0.001 DDD 1,000 inhabitants^{-1} day^{-1}) was much lower. For ENRO, it was detected in S1a, zoo, slaughterhouse, and S1a, whereas ENX was only detected in the zoo. The usage of these two antibiotics was legally permitted in animal feed for preventive measures in Malaysia (Marzuki, 2017). Overall, the fluoroquinolone concentrations detected in this work were below the global mean. However, CIX showed an increasing systemic utilisation and was the drug with the second highest expenditure in Malaysian public hospitals between 2009 and 2010 (Ministry of Health, 2014b). CIX levels in this study (not detected–577.97 ng l^{-1}) were higher if not similar to rivers and basins from Lui, Gombak, and Selangor Rivers, Malaysia (52.50 -299.88 ng l^{-1}, Praveena et al. 2018), DongJiang River, China (not detected–442.10 ng l^{-1},

Chen et al., 2018), ChongQing, China (not detected–458.00 ng l^{-1}, Chang et al., 2010), and WWTP near a hospital in Lake Victoria Basin, Kenya (not detected–420.00 ng l^{-1}, Kimosop et al., 2016), but were several order lower compared to WWTPs from Ter River, Spain (4.7–13,779.70 ng l^{-1}, Rodriguez-Mozaz et al., 2015), New York, USA (not detected–5,600.00 ng l^{-1}, Batt et al., 2006), and sewage treatment plants (STP)s from Okhla, India (2,900.00–45,400.00 ng l^{-1}, Mutiyar and Mittal, 2014), hospitals from Ujjain, India (not detected–236,600 ng l^{-1}, Diwan et al., 2010).

Tetracyclines were one of the considerably cheaper classes of antibiotics available, making it attractive to be used for human and veterinary drugs in developing countries like Malaysia (Michalova et al., 2004; Roberts et al., 2011; Ministry of Health, 2014b). The concentrations of tetracyclines detected in this work (LOD –1,092.49 ng l^{-1}, **Figure 5.1**) were higher than the global mean concentrations for surface waters (1.01–254,820 ng l^{-1}), rivers in HuangPu, China (not detected –135.50 ng l^{-1}, Jiang et al., 2011) and Cache La Poudre River, USA (not detected–1,210.00 ng l^{-1}, Kim and Carlson, 2006), lakes in Taihu, China (not detected–142.50 ng l^{-1}, Xu et al., 2014), and Choptank Basin, USA (not detected–388.00 ng l^{-1}, Arikan et al., 2008) but lower than hospital effluents from XinXiang, China (1,147.83–1,727.05 ng l^{-1}, Wang et al., 2018) and Romania (not detected–1,340.00 ng l^{-1}, Szekeres et al., 2017).

Apart from the three major antibiotic residues in this study, TMP was detected in locations including hospital, zoo, and slaughterhouse. Due to TMP being used in combination with sulphonamides for their synergistic antibacterial properties (Ministry of Health, 2014b, 2017a), sulphonamide detection was a near match with TMP. For sulphonamides, SMX was the most abundant and frequently detected residue type in this work, which is in agreement with Lye et al. (2019). Sulphonamides were

reported as ubiquitous in tropical Asian countries (Shimizu et al., 2013), yet the concentrations in this study were below the mean antibiotic concentration in surface waters for the Asia region. Sulphonamides in this study (not detected–93.15 ng l^{-1}) had low levels comparable to the Choptank watershed (not detected–9.00 ng l^{-1}, Arikan et al., 2008) and Bohai Sea (not detected–96.00 ng l^{-1}, Zhang et al., 2013). Specifically, SMX in this study had levels similar to Taihu lake (not detected–114.70 ng l^{-1}, Xu et al., 2014). In contrast, CAP was the only amphenicol compound detected in hospital and zoo. CAP was used as a systemic medicine and a topical ear drop to treat ear infections in Malaysia (Mohamad et al., 2014) while commonly used in veterinary for horses (McElligott et al., 2017). Malaysia has banned the usage of CAP for food use in food-producing animals since 1998 (Malaysia Food Act 1983 (Act 281) and Regulations) (Ministry of Health, 2014a). The concentration levels of CAP (LOD–4.92 ng l^{-1}) in the current work were lower than HuangPu River, China (4.18–2.36 ng l^{-1}, Jiang et al., 2011), Taff and Ely River, South Wales (not detected–40.00 ng l^{-1}, Kasprzyk-Hordern et al., 2008), Owo River, Nigeria (not detected–360.00 ng l^{-1}, Olarinmoye et al., 2016), WWTP in Lake Victoria Basin, Nigeria (not detected–60.00 ng l^{-1}, Kimosop et al., 2016), South Yellow Sea (not detected –73.20 ng l^{-1}, Du et al., 2017) and hospitals in Lake Victoria Basin, Nigeria (70.00–80.00 ng l^{-1}, Kimosop et al., 2016).

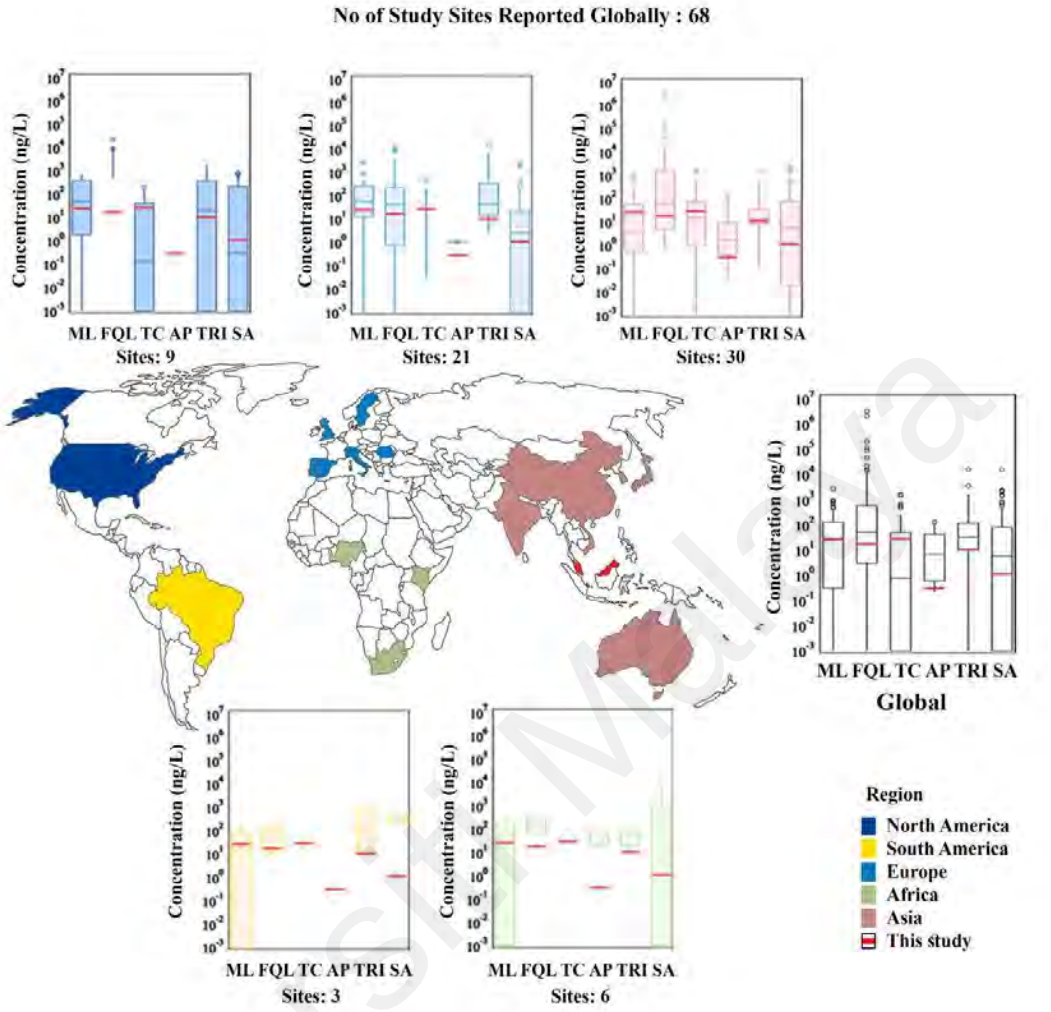

Figure 5.1: Countries with antibiotic residue detected in surface waters over this work and reported literature totaling 68 sites

Box plots show the concentrations (ng l^{-1}) of six antibiotic classes: Macrolides (ML); Fluoroquinolones (FQL); Tetracylines (TC); Amphenicols (AP); Trimethoprim (TRI); Sulphonamides (SA). Red lines show concentrations detected in Larut River and SanggaBesar River, Malaysia in this study

5.2 Risks Posed by Antibiotic Residues in Surface Waters

Individual antibiotic residue could be a risk to aquatic organisms according to the calculated RQs. Hospital and zoo had been categorised as risk sites in this study. ETM-H_2O, CTM, and OFX specifically posed high risks to algae ecology. Although

algae's sensitivity towards antibiotics in the aquatic environment had been verified by other studies (Halling-Sørensen, 2000; Ando et al., 2007; Magdaleno et al., 2015; Li et al., 2018), it affected 78 % of the studied sites, and this suggests ecological risks posed is still important. Environmental toxicology data for concentrations of antibiotics in river waters indicates that most antibiotics except ETM-H_2O, TC, CTM, and OFX were not high enough to cause acute effects on more complex aquatic organisms. To support this, similar high risks for OFX were present in sites from Laizhou Bay, China (Zhang et al., 2012), Korean aquatic environment (Lee et al., 2008), and Hong Kong sewage (Deng et al., 2016).

The assessment indicates that the risk of promoting antibiotic resistance selection for ETM-H_2O, CTM, and OFX from hospital effluent is significant in the environment (Kemper, 2008). Antibiotics in the environment could increase the prevalence of resistance after a period by selecting resistant phenotypes via inhibition of sensitive strains (Ågerstrand et al., 2015). In the absence of selective pressure from sulphonamide, sulphonamide-resistant bacteria still remained stable in the environment for at least 5 to 10 years longer than sulphonamide itself (Gao et al., 2012). Therefore, high-risk antibiotics should be used minimally and monitored to control any detrimental effects.

5.3 Abundance of Coliform and *E. coli*

The abundance of coliform and *E. coli* serves as indicators of water quality. Similar to past studies on polluted rivers from Malaysia and other locations around the globe, coliform and *E. coli* concentrations in this study were within the categorical range of poor water quality (Chatterjee et al., 2010; Lyautey et al., 2010; Pereira et al., 2013; Al-Badaii & Shuhaimi-Othman, 2015; Bong et al., 2020). To demonstrate, the standard

maximum 100 CFU 100 ml^{-1} limit was exceeded for this study according to the National Water Quality Standards (NWQS) class II for rivers set by the DOE Malaysia and the Malaysia Interim Marine Water Quality Standards (Department of Environment, 2019b). Of which, mean total coliform and *E. coli* count in sites receiving effluents from zoo, hospital, and slaughterhouse were reportedly higher than riverine sites S1a, S1b, and S2c. Thus, reaffirming a difference among the aforementioned sites.

Apart from anthropogenic factors, natural factors such as salinity ($R^2 = 0.26$, $df = 16$, n = 18) negatively impacted coliform and *E. coli* concentration in this study. Where the inhibitory effect of salinity (> 16 ppt) likely affected bacterial survival and growth rates for sites S1c, S2a, and S2b with low colony counts (Rozen & Belkin, 2001; Lye et al., 2019).

5.4 Distribution of *E. coli* Phylogenetic Groups

The phylogenetic distribution of *E. coli* isolates was not homogenous between the nine sites. In aquatic environments, phylogroups A and B1 are broadly more predominant than B2 and D (Figueira et al., 2011; Ghaderpour et al., 2015). Compared to phylogroup A, B1 was frequently the more prevalent phylogroup in environmental waters (Garcia-Aljaro et al., 2009; Hu et al., 2013; Pereira et al., 2013; Ghaderpour et al., 2015). Past studies have shown an association between the site of contamination and the predominance of a certain phylogroup in the aquatic environment (Hamelin et al., 2007; Sabaté et al., 2008; Mokracka et al., 2011). The varied survivability of *E. coli* and its ability to overcome stresses in water contribute to community distribution and diversity (Berthe et al., 2013). Land use, sources of pollution, availability of nutrients, *in situ* physicochemical parameters

(dissolved oxygen, pH, salinity, etc.), protozoan and bacterial predators, and hydrological conditions constitute the multiple factors influencing distribution (Lyautey et al., 2010; van Elsas et al., 2011). Interestingly, human commensal *E. coli* population structure had been observed to differ significantly from wildlife, even if certain dominant strains could be shared between hosts in contact (Mercat et al. 2016). Among the two studied rivers, Larut River, which was consistent with a higher human population, had recorded a greater abundance in phylogroup A (Massot et al., 2016; Stoppe et al., 2017). In contrast, Sangga Besar River contained predominantly phylogroup B1, where each sampling site was closer to the sea, suggesting possible natural influence such as a salinity gradient towards these isolates (Bong et al., 2020). To support this, B1 expressed longer persistency in saline estuarine and coastal waters compared to other phylogroups (Ratajczak et al., 2010; Berthe et al., 2013; Bong et al., 2020). Likewise, B1 was also found abundantly in wastewaters of zoo and slaughterhouse. As B1 tends to be isolated from animal faeces, particularly herbivorous animals, this may indicate that the effluents discharged in these sites contained elevated animal waste (Carlos et al., 2010). In contrast, phylogroups B2, C, D, E, and F were less abundant and rarely detected in Larut River and Sangga Besar River (Sen et al., 2019; Giacometti et al., 2021). Higher detection frequencies of B2 ($\chi2 = 134.94$, $df = 9$, $p = 0.00$) and D ($\chi2 = 32.87$, $df = 9$, $p = 0.00$) were observed at upstream S1a, probably resulting from commensal isolates from birds (Nakhaee et al., 2015) and wild mammals (Lu et al., 2016; Alonso et al., 2017) from Larut Hill Forest Reserve that shelters a rich biodiversity of 227 bird and 27 mammal species (World Wildlife Fund Malaysia, 2001). A shift in phylogroup predominance along the riverine continuum, from phylogroup B2 and D to phylogroup A and B1, was observed along with the anthropogenically impacted sites just downstream from S1a. Demonstrating a shift from natural *E. coli* population towards an anthropogenically

influenced population. This finding is in agreement with Petit et al. (2017) and Ghaderpour et al. (2015) where they too similarly found a significantly higher abundance of B2 and D at a less-populated upstream site located near a forest.

Among MAR *E. coli*, results were consistent with other studies that found phylogroups A and B1 to be dominant (Ghaderpour et al., 2015; Vignaroli et al., 2016) and, to a certain extent, phylogroup C. Despite phylogroup C being rare in this study (n = 4), the isolates were characterised by a high prevalence of AR. In support of this, phylogroup C strains in commensal *E. coli* from human stool samples were also reportedly higher than those of the A, B1, B2, E, and F strains (Massot et al., 2016). Whilst phylogroup A was often described to resist antibiotics, the same strain had also been observed to undergo a trade-off between AR (e.g. ciprofloxacin) and virulence (Anssour et al., 2016). In contrast, phylogroup B2 and D isolates showed relatively lower resistance (Picard & Goullet, 1989; Johnson et al., 1991; Garcia-Aljaro et al., 2009; Ghaderpour et al., 2015). Both phylogroups were more prevalent in upstream S1a, a site associated with animal origin. Phylogroup B2 has greater resistance toward penicillin, quinolones/fluoroquinolones, and sulphonamides, while Phylogroup D has greater resistance towards tetracycline, penicillin, and sulphonamides. Studies support the occurrence of both phylogroups in wild, companion, and food animals, suggesting diet may be the main factor introducing animals to MAR bacteria (Vredenburg et al., 2014; Manges et al., 2015; Hertz et al., 2016; Alonso et al., 2017; Borges et al., 2017). The emergence of MAR bacteria in animals through the food chain is likely a result of rapid urbanisation that led to habitat loss and concurrent exposure of animals to various environmental pollutants (Literak et al., 2010; Smith et al., 2014; Borges et al., 2017). Thus, the genotype of *E. coli* population could be used as an indicator, as there appears to be a relationship

between sites and the predominance of a certain *E. coli* phylogenetic group in the aquatic environment (Hamelin et al., 2007; Sabaté et al., 2008; Mokracka et al., 2011).

5.5 Antimicrobial Susceptibility Profile

The development of AR in the environment is a global problem, with growing evidence pointing at water bodies becoming potential reservoirs for AR microorganisms and ARGs (Hoa et al., 2011; Zhang et al., 2013). This study demonstrated that about two in three *E. coli* isolates were resistant to ≥ 1 antibiotic tested, while approximately half were MAR strains. Among which, older antibiotic classes that were widely used in human and veterinary medicine were observed to be widely resisted by *E. coli* strains in aquatic environments (Watkinson et al., 2007; Agwu & Oluwagunke, 2014; Alves et al., 2014; Ghaderpour et al., 2015; Divya & Hatha, 2019), including tetracyclines introduced in 1948 and sulphonamides introduced in 1936. In this study, significant association were found among the resistance phenotypes TE against C ($\chi2 = 117.937$, $df = 1$, $p < 0.000$) or CIP ($\chi2 = 35.307$, $df = 1$, $p < 0.000$) as supported by other studies (Chen et al., 2017), indicating co-selection for resistance among environmental *E. coli* (Dang et al., 2006). When compared to similar aquatic systems, the prevalence of environmental AR *E. coli* in this study was similar to Kat River in South Africa (Nontongana et al., 2014) and Cochin Estuary in India (Sukumaran et al., 2012) while higher than Matang Estuary in Malaysia (Ghaderpour et al., 2015), Tagus Estuary in Spain (Pereira et al., 2013), Kshipra River in India (Diwan et al., 2018), San Pedro River in Mexico (Ramírez Castillo et al., 2013), and generally lower than DongJiang River in China (88.00 %) (Su et al., 2012).

It was expected that the more populated Larut River was observed to possess a higher prevalence of AR isolates compared to the less populated Sangga Besar River.

Among sites, the high prevalence of MAR *E. coli* isolates was mainly attributed to surface waters of anthropogenic sites in Larut River with the order of descending prevalence: zoo, slaughterhouse, and hospital, along with S1b. Of which zoo peaked at the highest MAR prevalence of 84.21 %, while S1b isolates were remarkably resistant to 19/20 of the tested antibiotics. Whilst not designated an anthropogenic site, AR *E. coli* phenotype from S1b do cluster closely with effluent from hospital, zoo, and slaughterhouse, hence indicating possible pollution from non-point source(s). For MAR index, all three anthropogenic sites along with S1b had predictably exceeded an index of 0.2 (Krumperman, 1983). This suggests that these sites received various inputs that contained contaminants capable of influencing the prevalence of MAR *E. coli*. Other studies had likewise found AR *E. coli* to be detected in aquatic environments near anthropogenic sources such as hospital (Korzeniewska et al., 2013), wastewater treatment plant (Mokracka et al., 2011), municipal sewage (Osińska et al., 2017), agricultural farm (Araújo et al., 2017), livestock pen (Gao et al., 2015), recreational water body (De Luca Rebello & Regua-Mangia, 2014) and aquaculture farm (Ng et al., 2014). Elevated resistance towards common clinical drugs such as tetracycline, quinolones, penicillins, sulphonamides, and fluoroquinolones was prevalent in all anthropogenic sites and S1b. Besides, they were also characterised to have high coliform and *E. coli* count, high *E. coli* genetic diversity, and high *sul* and *tet* resistance gene prevalence. Conversely, a majority of *E. coli* isolates that had susceptibility towards all 20 antibiotics tested were likely isolated from sites such as S1a, S2b, and S2c instead. This is in agreement with Berto et al. (2009), where numbers of AR bacteria isolated in polluted waters were higher than unpolluted waters. For instance, lower antibiotic resistance was recorded from upstream S1a for all tested antibiotic groups, whereby *E. coli* isolates were suspected to be of wildlife origin (Sayah et al., 2005; Edge & Hill, 2007).

This is then followed by downstream sites such as S1c, S2b, and S2c with a slightly lower prevalence of MAR *E. coli* isolates, likely from water dilution effect and/or being inactivated by high salinity levels (Gao et al., 2013). MAR prevalence pattern in this study was similarly observed by Petit et al. (2017) where they had similarly reported a significant increase in MAR *E. coli* moving from upstream sites to downstream sites in Seine River, France. Results here indicate that sites under anthropogenic pressure contribute to the spatial change of AR *E. coli* population along Larut River.

5.6 Pathotypes of *E. coli*

Faecal pollution could pose pathogenic *E. coli* infection risks. Certain regions along the Larut River were used for recreational activities, it may constitute a source of bacterial contamination that could infect humans or animals through direct contact, aerosol, or incidental consumption. Approximating the major contributors of faecal pollution in environmental waters could support pollution control and sustainable water quality management. Hence, virulence factors were evaluated as risk indicators in the environment, due to their association with pathogenicity among *E. coli* isolates (Pereira et al., 2007). Results revealed an overall scarcity of virulence genes present where only 11 virulence gene carrying *E. coli* was detected in this study, which concurred with other aquatic environments with non-point source contamination (Hamelin et al., 2007; Ghaderpour et al., 2015; Bong et al., 2020) but contrasted with waters impacted by a sewage treatment plant (Anastasi et al., 2012). Discharge from sewage overflows likely transported these *E. coli* isolates with virulent factors from faeces of animals and humans into environmental waters. Among which, 6 isolates showed MAR characteristics that carry varying *tet* and *sul* resistance genotypes. Antibiotic resistance is common in pathogenic *E. coli* due to the presence of virulence

and antibiotic resistance determinants being encoded on plasmids, bacteriophages, and/or pathogenicity islands (Djordjevic et al., 2013; Koczura et al., 2013; Pereira et al., 2013). Among virulent *E.* coli isolated in this study, the highest resistance frequency of 54.55 % was shared by tetracycline, oxolinic acid, nalidixic acid, and ampicillin. Although these 11 *E. coli* isolates each carried virulence genes, it is noteworthy that six of them do not correspond to a specific pathotype and are thus not considered as pathotypes. Namely, the observation of virulence factors itself does not necessarily lead to pathogenicity (Rehman et al., 2017). They consisted of virulence genes *bfpA* (n = 3), ST (n = 2), and *eae* (n = 1). Nevertheless, these detected virulence genes still contributed to lesion attachment, adherence, ion outflow induction, etc. (Osińska et al., 2017). Detection of single virulence gene patterns in *E. coli* isolates could be explained by HGT between cells (Sidhu et al., 2013). Specifically, the diarrhoeagenic enteroaggregative *E. coli* (EAEC) was the only pathotype detected in this study and was isolated from midstream surface waters of hospital and zoo effluents. As emerging diarrhoeal pathogens, EAEC causes acute diarrhoea in developing countries (Savarino, 1993). This study reaffirmed the observation made by Ghaderpour et al. (2015), whereby EAEC isolates in their study had similarly originated from anthropogenic sources from a village. The pathogenic isolates were characterised as belonging to phylogroups B1 and F, possessed MAR profiles of 5–16 antibiotics, with combinations of *sul2* and *sul3* genes, and *tet*(*B*), *tet*(*A*), *tet*(*L*), *tet*(*M*), and *tet*(*X*) genes. Studies have revealed similar findings on EAEC strains to be associated with water surfaces (Müller et al., 2016) and MAR EAEC belonging to phylogroup F from a stream with poor water quality (Furlan et al., 2020). Results suggest that the dissemination of both virulence and resistance determinants could occur in the same anthropogenic site and therefore pose a health risk. Through HGT, either pathogenic or resistant isolates, when in contact with autochthonous bacteria, could then disseminate

resistance and virulence determinants among natural ecosystems (Calhau et al., 2015). A study between environmental and clinical *E. coli* strains found that environmental isolates which harboured virulence genes were located, interestingly, in B1 groups while the newly described phylogroups such as phylogroup F were also not inferior to B2 and D phylogroups in their virulence potential (El-shaer et al., 2018). In this work, one such isolate (1HB11) belonging to phylogroup F is detected in hospital effluent water with resistance to 16 types of antibiotics. Interestingly, 4/11 (36.36 %) phylogroup F strains are found to carry virulent genes in this study. Arguably, this was not always the case, as an Australian study found that only a minority of phylogroup F strains possess virulence traits, albeit with extra-intestinal infection (Vangchhia et al., 2016). Likely, a relationship between the genetic background of a strain and its virulence factors could be present in this study (Escobar-Páramo et al., 2004a). Perhaps given sufficient sampling efforts, distribution and prevalence data of a certain phylogroup may gain predictive power in risk identification for pathogenic *E. coli* contamination events.

5.7 *tet* and *sul* Genes Distribution in *E. coli*

For tetracycline, 394 (78.33 %) *E. coli* isolates harboured ≥ 1 *tet* resistance genes. Generally, findings concurred with past studies that *tet* resistant genes are ubiquitous in aquatic environments (Jia et al., 2014; Chen et al., 2015). Among a diverse collection of 11 sole *tet* gene types and 57 *tet* gene combinations detected among *E. coli* isolates in this study, *tet*(A) was the most predominant (46.32 %), which was also reported in aquatic waters from other aquatic environments (Hu et al., 2008; Tacão et al., 2012; Pereira et al., 2013; Divya & Hatha, 2019). Many *E. coli* studies generally screened *tet* resistance genes under efflux pump (Tao et al., 2010; Adefisoye & Okoh, 2016;

Cho et al., 2019), in which this study found *tet*(A) and *tet*(L) active efflux resistant mechanism abundant. The predominance of *tet*(A) and *tet*(L) could be caused by the low concentrations of tetracycline (mean: 64.4 ng l^{-1}) in the surrounding, as the expression of these genes were mainly induced at low tetracycline level (Wang et al., 2019). Conversely, the expression of ribosomal protection protein *tet*(M) is mainly induced at high tetracycline levels (Ammor et al., 2008), which possibly explained the relatively low abundance of *tet*(M) in this study. Certain isolates (35.98 %) contained combinations of two or more tet genes, of which 15.51 % could be explained by *tet*(A)(L), *tet*(L)(M), and *tet*(A)(L)(M). *E. coli* isolates that harboured significantly fewer resistance genes were observed at less polluted sites. In contrast, hospital effluent in this study carried a significantly higher ($\chi 2 = 26.966$, $df = 6$, $p < 0.001$) number of *E. coli* isolates (22.86 %) that carried all three *tet* resistance mechanisms, inclusive of *tet*(X), an ARG commonly isolated from environmental sources (Roberts & Schwarz, 2016) which possessed the ability to resist the last-resort antibiotic tigecycline (Volkers et al., 2011). Additionally, high levels of tetracycline resistant strains with co-resistance towards erythromycin and ciprofloxacin were reported in *Staphylococcus aureus* strains from a tertiary hospital in Malaysia from 2003 to 2008 (Lim et al., 2012). Again, HGT from other bacteria communities probably explains the high prevalence of *tet* genes (Chopra & Roberts, 2001). The difference in ARG prevalence among various geospatial locations may reflect different antibiotic usage activities and different compositions of microflora in various environmental compartments. To demonstrate, Osińska et al. (2017) found *E. coli* isolates from wastewater samples, whether treated or untreated, had harboured greater diversity of *tet* ARG compared to isolates from both upstream and downstream portions of the river.

For sulphonamides, the principal resistance mechanism is via acquisition of an alternative Deoxyhypusine Synthase (DHPS) gene, consisting of three identified *sul*

genes (*sul*1, *sul*2, and *sul*3) (Perreten & Boerlin, 2003; Wu et al., 2010; Ben et al., 2017). From 149 (29.62 %) *E. coli* isolates that harboured ≥ 1 *sul* resistance gene, a gene-frequency distribution of *sul*3 > *sul*2 > *sul*1 were constructed, which incidentally was not consistent with literature that commonly depicted *sul*2 dominance (Su et al., 2012; Wang et al., 2013; Zhang *et al*., 2015). This unexpected result instead concurred with a recent study on urban freshwater aquatic recipients of Sweden (Lai et al., 2021). The origin of *sul*3 was initially suspected to be human (Grape et al., 2003), however numerous studies have since reported their prevalence in *E. coli* isolated from wildlife and livestock (Guerra et al., 2003; Perreten & Boerlin, 2003; Hammerum et al., 2006). Perhaps the trend in the emergence of *sul*3 in animals indicated potential selective pressure for sulphonamide resistance due to the presence of trimethoprim/sulphamethoxazole and/or other veterinary antibiotics (Liu et al., 2009). Thus, the widespread distribution of *sul*3 among *E. coli* isolates in this work was potentially due to the consumption of sulphonamide by a combination of humans and veterinary use (Lye et al., 2019; Thiang et al., 2021). To support this, sulphonamides were indeed widely used in Asian countries. For Malaysia, the usage in veterinary medicine was approximately 18,000 kg year^{-1} (Marzuki, 2017), whereas for healthcare, the usage ranged from 0.0982-5.9900 DDD 1,000 population^{-1} day^{-1} (Ministry of Health, 2017a). Among sites, *sul* gene carrying *E. coli* was significantly higher with prevalence of 52.46 % in midstream slaughterhouse ($\chi2 = 22.375$, $df = 5$, $p < 0.001$) and 46.05 % at zoo ($\chi2 = 6.000$, $df = 4$, $p < 0.001$). In comparison, upstream S1a had the lowest detection frequency (3.45 %). Again, findings from zoo effluent were consistent with pollution from animal sources, this is supported by past studies on commensal *E. coli*, which found food animals such as porcine (Perreten & Boerlin, 2003; Hammerum et al., 2006), poultry (Kozak et al., 2009b), and cattle (Guerra et al., 2003) to possess a higher prevalence of *sul*3 genes. Furthermore, a

minority of the isolates simultaneously harboured multiple *sul* combinations, of which the *sul*13 combination was predominant (2.58 %). Three isolates with a *sul*123 combination were detected (0.60 %); specifically, two from slaughterhouse and one from hospital. Past studies had also isolated *E. coli* isolates carrying *sul*123 within poultry (Kozak et al., 2009b) and porcine (Hammerum et al., 2006; Fazel et al., 2019). Sixty-seven *E.coli* isolates (32.52 %) expressed sulphonamide phenotype resistance yet carried none of the tested *sul* gene. The ability to acquire other resistance mechanisms (e.g., mutations in the chromosomal DHPS gene *flop*) more frequently among environmental *E. coli* may contribute to the sulphonamide resistant phenotypes that carried no resistant determinants (Changkaew et al., 2014; Ogura et al., 2020). Alternatively, the putative sulphonamide resistance gene *sul*4 could potentially fill the gap in this study as it was also found widespread across Asia and Europe (Razavi et al., 2017).

5.8 Relationship Between Physicochemical and Antibiotic Residues on *E. coli* Phylogenetic Distribution

The relationship between physicochemical and antibiotic residues in *E. coli* phylogenetic distribution studies has shown that *E. coli* phylogenetic groups are adaptable and genotypically affected by environmental changes (Jang et al., 2014). In this study, the correlation coefficient has shown that salinity explained 26.00 % of the variation in coliform abundance, specifically in Larut River. Other factors in combination may also contribute to the distribution of *E. coli* and their activity in the environment. Phylogroups A, B1, and C generally correlate better in deteriorating water quality in Larut River. Findings were consistent with Jang et al. (2014) and Bong et al. (2020), who reported that the occurrence and distribution of *E. coli*

phylogenetic distribution can be affected by environmental variables. Collectively, better adaptation to environmental drivers paired with a high turnover of gene repertoires made phylogroup B1 exquisitely versatile in the environment compared to other phylogenetic groups (Touchon et al., 2020). Findings were in line with previous studies that nutrient concentrations (C, N, P) are one of the important factors influencing the growth and survival of *E. coli* in the environment (Jang et al., 2017; Taabodi et al., 2019). The addition of nutrient concentrations could also enhance the horizontal transfer of genetic resistance materials (Blanco et al., 2009), which further enhances the adaptive ability and plasticity of *E. coli* in a variety of environments. Lye et al. (2019) detected a positive correlation between PO_4 with sulfonamide resistant heterotrophic bacteria and sulfonamide enteric bacteria in Larut River. They had found that on four occasions, exceptionally high PO_4 concentration but low nitrogen concentration was observed in wastewater effluents from both zoo and hospital. However, further studies are still required to understand the nature of these anthropogenic stressors and the exact mechanisms in shaping the *E. coli* prevalence, diversity, and dissemination of antibiotic resistance in this river.

No correlation was observed in the lower frequency phylogroups (B2, D, E, and F). This observation of correlations concurred with Varela et al. (2014), Lye et al. (2019), and Low et al. (2021), who have revealed that the wastewater effluents from zoo, slaughterhouse, and hospital are important antibiotic pollutant sources to the Larut River. Thus, the antibiotic residues in these effluents are expected to have a strong impact of selective pressure on antibiotic resistance in environmental *E. coli* compared to river waters. The variation in the response of different phylogenetic groups of *E. coli*, including resistant genes to different antibiotic residues, might be attributed to types of antibiotics detected, physiochemical properties and persistence of antibiotics in water (Diwan et al., 2018), water quality (Berthe et al., 2013), acquired resistance mechanisms

(Kawamura-Sato et al., 2010; Smet et al., 2010), environmental fitness of *E. coli* and indigenous microflora (van Elsas et al., 2011).

5.9 Genetic Diversity of *E. coli* Through Rep-PCR Fingerprint

The relationship of Larut River with the less impacted Sangga Besar River concurred with Jang et al. (2011), where diversity of *E. coli* genotypes tends to be greater with increasing proximity to anthropogenic urban sites. However, genetic heterogeneity between isolates from the natural environment may be caused by differences between sampling sites (e.g., sampling sites were probably subjected to high pollution from various sources) (Dos Anjos Borges et al., 2003). In contrast, hospital effluent had a low *E. coli* genetic diversity (*H'*: 2.91) among effluent sites even though Low et al. (2021) observed elevated antibiotic concentrations. A similar observation was found by McLellan (2004), who reported lower diversity of *E. coli* in contaminated surface waters in which environmental survival may be the factor that influence the recovery of the composition of strains from contaminated waters.

From the 90 non-repeating *E. coli* genotypes, isolates from cluster I were described as phenotypically (67.50 %) resistant strains and were found in roughly equal distribution at all sites at this study. This cluster constituted the prevalence of background antibiotic resistance in this study (Davies and Davies, 2010; D'Costa et al., 2011; Tamminen et al., 2011). Similar to cluster I, cluster VI (n = 13) included phylogroup A and B1 strains with high AR, but differed in terms of site isolated. Conversely, the remaining clusters were generally susceptible to antibiotics except cluster III, affirming a genetic link in antibiotic resistance for *E. coli* in this study. Cluster III (n = 13) had phenotypically resistant B1 strains (76.92 %) where most strains from downstream sites S1c, S2b, and S2c co-clustered with one isolate from

hospital, thus suggesting a potential contamination source. Similar to Liang et al. (2016), there was no clear pattern of *E. coli* clustering according to sites in this study. It is important to note that the main aim of this study is not to trace the exact host sources of commensal *E. coli* in a complex aquatic environment. *E. coli* genotypic and phenotypic diversity is very large. According to literature, a collection of more than 20,000 isolates had only captured 27.00 % of the predicted genotypes as estimated by rarefaction analysis (Lyautey et al., 2010). This was evidenced by hospital and zoo isolates consisting of a diverse population with five and four different clades, respectively, which were also observed by Ghaderpour et al. (2015). Given the limitation, this study did, however, demonstrate that AR *E. coli* population diversity in riverine estuarine water was instead related to the proximity of source contamination, stream order, and the land use as observed by other studies (Hamelin et al., 2007; Lyautey et al., 2010).

CHAPTER 6: CONCLUSION

The present study affirmed the prevalence and diversification of antibiotic resistant *E. coli* in Larut River were intensified by wastewater effluent from zoo, hospital, and slaughterhouse as sources of antibiotic residues. Sixteen antibiotic residues with concentrations ranging from LOD to 1,262.3 ng l^{-1} were detected in Larut River and Sangga Besar River. Results showed a wide prevalence of antibiotics in the sampling site, where fluoroquinolones and macrolides were detected frequently in the water samples. RQs identified ETM-H_2O, CTM, and OFX detected from hospital and zoo had posed a high risk to algae, while TC had low to medium ecological risk towards all tested aquatic organisms: algae, invertebrate *Daphnia magna*, and fish. The phylogroups B1 and A were predominant with the presence of resistance genes. The cluster analysis revealed that antibiotic resistance phenotype distribution of *E. coli* isolates from the zoo and the slaughterhouse effluents were more similar than hospital effluent and downstream site (S1b). The *tet* efflux genes were detected in the majority of the *E. coli* isolates, suggesting *E. coli* may be an important carrier and/or reservoir of tetracycline resistance genes conferring resistance. The prevalence of *sul3* gene in *E. coli* isolates might be attributed to the consumption of sulfonamide for humans and veterinary use. CCA analysis revealed a significant association between phylogroup and resistance genes with physicochemical properties and antibiotic residues on the environmental persistence of antibiotic resistant *E. coli*. All these findings are important to provide information on the global comparison of the persistence of antibiotic resistant *E. coli* in different aquatic ecosystems and the need to have surveillance and monitoring of virulence and antibiotic resistance in fresh river water to mitigate emerging resistance and dissemination through water and environment. The wastewater effluents from

hospital, zoo, and slaughterhouse introduced into the Larut River should be closely monitored.

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