MOLECULAR DETECTION OF TETRACYCLINE RESISTANCE GENES IN ASSOCIATION WITH EFFLUX PUMP MECHANISM IN UROPATHOGENIC *Escherichia coli* (UPEC) ISOLATES

By

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ABSTRACT

MOLECULAR DETECTION OF TETRACYCLINE RESISTANCE GENES IN ASSOCIATION WITH EFFLUX PUMP MECHANISM IN UROPATHOGENIC *Escherichia coli* (UPEC) ISOLATES

Lee Shuet Yi

Uropathogenic *E. coli* (UPEC) is the most common causative agent of urinary tract infections (UTIs). In recent years, UPEC strains were reported to show increase rate in multidrug resistance (MDR), especially to tetracycline class. The rationale behind the continued emergence of antimicrobial resistance is the dissemination of tetracycline efflux genes among bacterial strains. Thus, the current study was conducted to detect the prevalence of *tetA* and *tetB* genes in correlation with efflux pump resistance mechanisms among the UPEC isolates through molecular screening. A total of 60 UPEC isolates retrieved from Hospital Raja Permaisuri Bainun, Ipoh were subjected to antimicrobial susceptibility testing, followed by molecular detection *tetA* and *tetB* genes through duplex PCR assay. UPEC isolates were predominantly collected from females (73.33%) and working age groups (53.33%). UPEC isolates were found to be most resistant to ampicillin (71.67%) and tetracycline (51.67%), but highly susceptible to imipenem (0.00%) and minocycline (1.67%). Among 60 UPEC isolates, *tetA* positive isolates (n = 26, 41.67%) were detected to be higher as compared to *tetB* positive isolates (n = 7, 11.67%), while only one isolate showed co-existence of both *tetA* and *tetB* genes (1.67%). Despite showing

negative *tet* gene association with demographic profiles of patients (p-values > 0.050), the *tet* efflux genes significantly correlate with the resistance profiles of tetracycline antibiotic class. The tetracycline resistance traits showed statistically significant positive association with both *tetA* ($p = 0.000$) and *tetB* ($p = 0.029$), whereas minocycline resistance profiles only associate positively with *tetB* ($p = 0.010$). All tetracycline-resistant isolates harboured at least one *tet* efflux gene. Meanwhile, only one out of 60 UPEC samples showed minocycline resistance, which was screened positive for *tetB* gene. In addition, *tetA* gene was discovered to positively associate with resistance profiles of nalidixic acid ($p = 0.020$), trimethoprim-sulfamethoxazole ($p = 0.001$) and ampicillin ($p = 0.000$).

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

LEE SHUET YI

APPROVAL SHEET

This project report entitled "**<u>MOLECULAR DETECTION OF TETRACYCLINE RESISTANCE GENES IN ASSOCIATION WITH EFFLUX PUMP MECHANISM IN UROPATHOGENIC *Escherichia coli* (UPEC) ISOLATES</u>**" was prepared by LEE SHUET YI and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

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Dr. Chew Choy Hoong Date: 30/5/2022.

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PERMISSION SHEET

It is hereby certified that **LEE SHUET YI** (ID No: **18ADB01539**) has completed this final year project entitled "**MOLECULAR DETECTION OF TETRACYCLINE RESISTANCE GENES IN ASSOCIATION WITH EFFLUX PUMP MECHANISM IN UROPATHOGENIC *Escherichia coli* (UPEC) ISOLATES**" under the supervision of Dr. Chew Choy Hoong from the Department of Allied Health Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(LEE SHUET YI)

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LIST OF ABBREVIATIONS

A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
AMR	Antimicrobial resistance
bp	Base pair
BLAST	Basic Local Alignment Search Tools
CDC	Centres for Disease Control and Prevention
cIAI	Complicated intraabdominal infections
CLSI	Clinical and Laboratory Standard Institute
cUTI	Complicated urinary tract infection
DHA	Drug/H^+ antiporters
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
EDTA	Ethylenediamine tetraacetic acid
ExPEC	Extraintestinal pathogenic *Escherichia coli*
FDA	Food and Drug Administration
H_2O	Water
HAI	Healthcare-associated infection
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionisation-Time of Flight
MDR	Multidrug resistance
MFS	Major facilitator superfamily
$MgCl_2$	Magnesium chloride
µl	Microlitre
µM	Micromolar

ml	Millilitre
mM	Millimolar
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometre
OM	Outer membrane
p-value	Probability value
PAI	Pathogenicity island
PCR	Polymerase chain reaction
QIDP	Qualified Infectious Disease Product
qPCR	Quantitative polymerase chain reaction
rpm	Revolutions per minute
Taq	*Thermus aquaticus*
TBE	Tris/Borate/EDTA
Tcr	Tetracycline resistance
TSA	Tryptic soy agar
UPEC	Uropathogenic *Escherichia coli*
U	Enzyme unit
UTI	Urinary tract infection
UV	Ultraviolet
V	Volt
w/v	Weight per volume

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

Urinary tract infection (UTI) is one of the most frequent infectious diseases that accounts for almost one-fourth of all clinical infections, contributing to significant morbidity and mortality worldwide (Al-Badr and Al-Shaikh, 2013). Various uropathogens may be responsible for UTIs, but uropathogenic *Escherichia coli* (UPEC) is the most prevalent causative agent accounting for nearly 90% of UTIs (Wiedemann et al., 2014; Flores-Mireles et al., 2015). Prescribing antimicrobials will be able to halt bacterial infections, but the widespread clinical use of these drugs in UTI treatment will lead to the emergence of antibiotic resistance. In recent years, UPEC strains have been discovered to have increased resistance to multiple drugs, and the resistance to tetracycline is the most prevalent (Bunduki et al., 2021).

Tetracycline resistance pattern is often associated with the acquisition of *tet* resistance genes through mobile genetic elements to confer resistance mechanisms including efflux pump, ribosomal protection and enzymatic deactivation (Grossman, 2016). Efflux pump is the most predominant tetracycline resistance mechanism found in Gram-negative bacteria (Grossman, 2016). Over the years, many studies had disclosed numerous tetracycline resistance genes that have attribution on conferring to efflux mediated resistance mechanism. Nonetheless, *tetA* and *tetB* genes were discovered as the two most common *tet* efflux genes, both of which responsible in encoding for major facilitator superfamily (MFS) efflux transporters (Warburton et al., 2012). The capability of MFS transporters in extruding multiple drugs had raised a major health concern.

Studying the association between the resistant strains and the efflux resistance mechanisms is crucial to formulate appropriate and effective drug therapies. To the best of our knowledge, the antibiotic resistance pattern among UPEC strains, typically tetracycline resistance mechanism and the clinical significance of *tet* efflux genes in UPEC clinical isolates has not been extensively studied in Malaysia.

Thus, the present study was undertaken to achieve the following aims:

1. To observe the occurrence of tetracycline resistance phenotypes by evaluating the antibiotic susceptibility profiles of UPEC clinical isolates.
2. To optimise the duplex PCR condition for *tet* genes screening.
3. To screen and identify the carriage of *tet* efflux genes (*tetA* and *tetB*) in UPEC clinical isolates via duplex PCR assay.
4. To analyse the distribution of *tetA* and *tetB* genes among all the UPEC isolates.
5. To evaluate the association of *tet* genes with demographic profiles of patients and antimicrobial susceptibility profiles.

CHAPTER 2

LITERATURE REVIEW

2.1 Urinary tract infections (UTIs)

2.1.1 Overview

According to Centres for Disease Control and Prevention (CDC), UTI is defined as an infection in any part of the urine system: urethra, ureters, bladder and all the way up to kidneys. The disease is described as the fifth most common healthcare-associated infection (HAI) (Centres for Disease Control and Prevention, 2022).

UTI can be caused by a broad range of pathogens, including bacteria, fungi, and viruses in rare cases. Some of the common uropathogens are *Escherichia coli, Enterococcus faecalis*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Staphylococcus saprophyticus*, however, uropathogenic *E. coli* is often reported as the most predominant cause of infection (Terlizzi et al., 2017). UTI is clinically categorised into uncomplicated and complicated. Uncomplicated cases refer to the infections affecting healthy individuals with no anatomical or functional anomalies, predominantly women due to anatomical factors. Meanwhile, complicated UTIs are associated with risk factors predisposing to the infection, such as structural urinary tract abnormalities, immunosuppression, compromised host defence, neurological bladder retention, renal failure, renal transplantation, and indwelling catheterisation (Flores-Mireles et al., 2015). Hence, the overall aetiological factors suggests that females and elderly are the common victims of UTIs.

2.1.2 Uropathogenic *Escherichia coli* (UPEC)

Uropathogenic *E. coli* (UPEC) is the major causative pathogen accounting for 90% of uncomplicated, community acquired UTI (Shah et al., 2019). UPEC are Gram-negative, facultative bacteria subclassified from extraintestinal pathogenic *E. coli* (ExPEC). These *E. coli* strains are originally live as commensal flora in human intestines, but later transition into pathogenic state after acquiring virulence factors and antimicrobial resistance (AMR). Aside from pathogenicity islands (PAI) conferring to virulency, AMR is another key factor that cannot be overlooked in UTI cases as it aids in facilitating the pathogenesis of UPEC by avoiding the antimicrobials action. This will consequently promote to the persist colonisation and establish bacterial reservoir to infect urinary tract. Throughout the years, an increase prevalence of multidrug resistance (MDR) is observed among UPEC strains, peculiarly to tetracycline (Bunduki et al., 2021). The emergence of MDR-UPEC strains resulting from indiscriminate and excessive use of broad-spectrum antibiotics has brought to global concern due to the limited available therapeutic options against the predominant pathogen.

2.2 Tetracycline

Tetracyclines were first discovered in the early 1940s, which were originally obtained and derived from the fermentation of a soil bacterium, *Streptomyces aureofaciens* (Das and Patra, 2017). Tetracycline constitutes wide spectrum of antimicrobial activity against pathogen, including both Gram-positive and Gram-negative bacteria by exerting bacteriostatic or even bactericidal effect in newer tetracycline generations via protein synthesis inhibition. Aside from that, tetracyclines also possess other criteria to be an ideal antimicrobial agent, including confirmed clinical safety, oral and intravenous bioavailability, and acceptable drug tolerability (Grossman, 2016). Thus, tetracyclines are said to be well-established as a drug treatment for various infections such as respiratory, gastrointestinal, urogenital, skin and other serious pathogenic infections (Grossman, 2016).

2.2.1 Mechanism of action of tetracycline

Tetracycline exerts its mode of action by binding to 16S part of the 30S ribosomal subunits to prevent the attachment of incoming aminoacyl-tRNA to the A site of the ribosome. This will arrest the translation process sterically and further hinder the protein synthesis that results in hampering bacterial cell growth (Das and Patra, 2017). Permeation of tetracycline across the bacterial cell membrane is required for the drug to exert its action as the translation machinery are enclosed within the cytoplasmic region. The uptake of tetracycline into Gram-negative bacteria, typically *E. coli* is most preferentially via passive diffusion through outer membrane (OM) porins like OmpF and OmpC, where the Donnan potential generated along the OM will drive the drug accumulation in the periplasm. In view that the porins are cation selective, tetracyclines are often chelated with metal ions like magnesium and calcium to allow the import of the cationic antimicrobial across OM by dissociating tetracycline from the metal ions. The uncharged, hydrophobic tetracycline released from metal ion dissociation will then diffuse across inner membrane into cytoplasm via active transport and target ribosome to exert its action (Grossman, 2016; Rusu and Buta, 2021).

Despite the broad-spectrum antimicrobial action, the Gram-negative UPEC strains were documented to have high tetracycline resistance over the years. According to an article review published in 2021, a meta-analysis involving 13 studies conducted from the year 2000 to 2019 had summarised the prevalence of virulence factors and antimicrobial resistance of UPEC isolates. In this review, a total of 1888 UPEC isolates were analysed and 69.10% of the isolates portrayed tetracycline resistance, which was the highest rate among all the other antibiotics (Bunduki et al., 2021). In view that MDR-UPEC resistant strains are poorly studied in Malaysia, the resistance mechanism serves as a good study focus in order to search for the most effective therapeutic options against UPEC-associated infections.

2.3 Tetracycline efflux pump resistance mechanism

Tetracycline resistance mechanisms are mainly described into three general types: efflux pumps, modification at the drug target site and ribosomal protection. Among the above, efflux pump is the most common tetracycline resistance mechanism that predominates in Gram-negative bacteria (Grossman, 2016). Efflux pumps are energy-dependent integral membrane proteins spanning across the lipid bilayer to export tetracycline out from bacterial cell. As a consequence, the intracellular concentration of tetracycline will be reduced to a point that is insufficient to exhibit antimicrobial effect, and thus protecting the pathogen from being a ribosomal target (Masci, 2020). In addition, the efflux system also promote time for pathogen to learn, adapt, and further emerge to acquire more resistance mechanisms such as upregulation of efflux pumps, and mutation or alteration at drug target site (Kumar and Varela, 2012). There are up to 26 classes of efflux pumps discovered attributing to tetracycline efflux system (Masci, 2020). The efflux pumps are categorised into five major groups, of which major facilitator superfamily (MFS) being one of the most common tetracycline-specific transporters (Kumar and Varela, 2012; Grossman, 2016). The members of MFS transport proteins are known to confer resistance to not only tetracycline, but also to wide range of antibiotic classes (Kumar and Varela, 2012).

2.3.1 Major facilitator superfamily (MFS)

The major facilitator superfamily (MFS) is discovered to be one of the biggest groups of secondary active transporters, which mainly attribute to the transport of substances across membrane (Choudhuri, 2014). The transport can be categorised into uniport (transport of single substrate), symport (co-transport of two substrates in one direction) and antiport (transport of two substrates in opposite directions), of which the process is driven by the existing electrochemical or ionic (H^+) gradient (Choudhuri, 2014; Kumar et al., 2020). The MFS transporters are often composed of around 400 to 600 amino acid residues that further assembled into 12 or 14 transmembrane α-helices (Brown et al., 2019; Kumar et al., 2020).

Initially, the transporters of this family are responsible in transferring wide diversity of molecules that serve for the bacterial cellular mechanisms, for instance monosaccharides, carbohydrates, solute and ions, Krebs cycle intermediates, metabolites, amino acid, nucleotides and so on. However, the specificity of MFS transporters is not only limited to these diverse range of substrates, but also to multiple drugs whereby the drug/H^+ antiporters (DHA) will be encoded to drive the extrusion of antimicrobials actively out from the intracellular compartment (Kumar et al., 2016; Kumar et al., 2020). Some of the well-acknowledged MFS multidrug efflux pumps are *MdfA*, *tetA*, *NorA*, and *EmrD*, and the last protein mentioned, *EmrD* protein, is the first MFS antimicrobial transporters discovered in *E. coli* (Kumar et al., 2020). In addition to that, the multidrug MFS transporter proteins were spotted to have conformational changes and structural amendment over the years to alter the binding specificity and improve hydrophobicity in order to drive the antimicrobial efflux and expel lipophilic drugs (Kumar et al., 2020). Hence, the specificity of MFS efflux proteins to a wide array of drugs and antimicrobial agents will confer to multidrug resistance (MDR), which in turn render the available therapeutic treatments ineffective.

2.3.2 Tetracycline resistance (Tcr) determinants

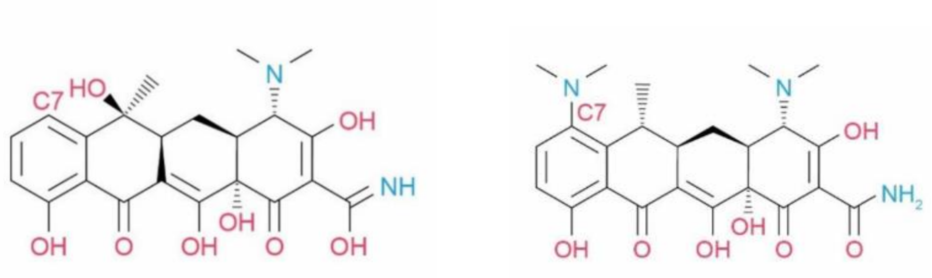
To date, more than 40 tetracycline resistance genes were discovered, majority of which are found to be associated with efflux pumps system (Sheykhsaran et al., 2019). The relevant efflux *tet* genes present in genus *Escherichia* are *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetI*, *tetY* (Chorpra and Robert, 2001). Among all the *tet* efflux genes, *tetA* and *tetB* were identified as the most prevalent *tet* resistance genes in *E. coli* strains (Jones et al., 2006; Koo and Woo, 2011). Both *tetA* and *tetB* genes are responsible to encode for MFS integral membrane protein to drive efflux via proton-motive force (Warburton et al., 2012). The Tcr determinants can be found in chromosomes or arise from mutation under antibiotic exposure, however, most of them are obtained through the acquisition of mobile genetic elements carrying Tcr genes, like transposons or mobile plasmids (Jahantigh et al., 2020). This will allow the transmission and dissemination of Tcr genes among bacterial species via horizontal gene transfer and further confers to the emergence of tetracycline resistance among UPEC strains. With the drastic rise in tetracycline resistance rate, the clinical application of tetracycline is compromised as the utility will be narrowed down to pathogens with confirmed susceptibility.

2.4 Generations of tetracycline

Considering the rapid bacterial evolution and widespread dissemination of Tcr genes, new approaches were carried out to optimise and create modern synthetic derivatives of tetracycline classes. The modern generations of tetracycline are derived to exhibit improved potency and efficacy to ensure the antimicrobial agents are capable to unleash full potential against emerging MDR pathogens (Grossman, 2016). To date, tetracyclines are classified into three generations. The drugs categorised in the first generation are tetracycline, oxytetracycline, chlortetracycline and demethylchlortetracycline (Janser, 2016). Meanwhile, the second generations of tetracyclines were further derived from the first generations to generate new antibiotics like doxycycline, minocycline, lymecycline and rolitetracyclin (Janser, 2016). Although the second generations exhibit a better antimicrobial action against microbes, the continued widespread dissemination of resistance determinants, especially efflux mediated resistance has rendered the first- and second-generation tetracycline ineffective. Thus, the development of subsequent third generation of tetracycline such as tigecycline, omadacycline, eravacycline and sarecycline was undertaken (Rusa and Buta, 2021).

2.4.1 Tetracycline and minocycline

The two antibiotics highlighted in the current study, tetracycline and minocycline belong to the same tetracycline class. Both drugs exert identical function in targeting 30S ribosomal subunit. However, minocycline is a second-generation derivative of the first-generation tetracycline, where the ring D at carbons 7 to 9 of original chemical structure was modified to give rise to a better membrane diffusion capability and ribosomal binding affinity as compared to tetracycline (Asadi et al., 2020; Roy et al., 2021). The chemical structural comparison between the two drugs is shown in Figure 2.1. Unlike the energy dependent process observed in the uptake of tetracycline, minocycline is able to enter bacterial cell via passive diffusion (Roy et al., 2021). Besides, minocycline also found to have a stronger binding affinity to ribosome, suggesting that minocycline may exhibit a better antimicrobial effect as compared to tetracycline. Despite the proven improved efficacy in clinical setting, bacterial strains that carry *tetB* gene were reported to have a certain resistance to minocycline. This is due to the fact where *tetB* gene is the only efflux gene that can encode for proteins to export both tetracycline and minocycline (Chorpra and Roberts, 2001).



(a) **(b)**

Figure 2.1: **Chemical structure of (a) tetracycline and (b) minocycline (Adapted from Bunick et al., 2021).**

2.4.2 Modern third-generation tetracycline

Undoubtedly, the emergence of antibiotic resistance has constrained the use of first and second generations of tetracyclines. Thus, the invention of the subsequent generation of tetracyclines are driven. The most recent novel tetracycline analogues reported are omadacycline and eravacycline, both of which are discovered to exhibit broad spectrum of activity against MDR pathogens. Eravacycline (Xerava) is a synthetic fluorocycline, that was specifically designated as a Qualified Infectious Disease Product (QIDP) by Food and Drug Administration (FDA) to treat complicated intraabdominal infections (cIAI) and complicated urinary tract infection (cUTI). Regrettably, eravacycline is only approved for cIAI treatment, but the potential shown in the newer generations of tetracycline may suggest for more medication approaches and brings a ray of hope for UTI treatment (Thakare et al., 2018).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Reagents

The list of chemicals and reagents used in this project with their respective manufacturer are displayed in Table 3.1.

Table 3.1: List of chemicals and reagents used and their corresponding manufacturers.

Chemicals and reagents	Manufacturer, Country
Mueller Hinton agar	HiMedia Laboratories Pvt. Ltd., India
Nutrient broth	HiMedia Laboratories Pvt. Ltd., India
Tryptic soy agar (TSA)	Merck KGaA, Germany
Agarose powder	1st BASE Laboratories, Singapore
Trimethoprim-sulfamethoxazole, Chloramphenicol, Ampicillin, Tetracycline, Minocycline antibiotic disks	Oxoid Ltd., United Kingdom
Nalidixic acid, Imipenem antibiotic disks	Liofilchem s.r.l., Italy
Ciprofloxacin, Levofloxacin antibiotic disks	HiMedia Laboratories Pvt. Ltd., India

Table 3.1 (continued)

Deoxynucleotide triphosphates (dNTP) mix	Promega Corporation, United States
Magnesium chloride ($MgCl_2$)	Promega Corporation, United States
5x GreenGoTaq® Flexi Buffer	Promega Corporation, United States
GoTaq® G2 Flexi DNA Polymerase	Promega Corporation, United States
Forward and reverse primers of *tetA*	Eurogentec Ait Pte Ltd., Singapore
Forward and reverse primers of *tetB*	Integrated DNA Technologies, United States
100 bp DNA ladder	SMOBIO Technology, Inc., Taiwan
GelRed stain	Yeastern Biotech Co., Ltd., Taiwan
Tris base	Thermo Fisher Scientific, United States
Boric acid	Merck KGaA, Germany
EDTA disodium salt	Grupo RNM, Portugal

3.2 Methodology

3.2.1 Sample collection

Clinical isolates of uropathogenic *E. coli* (UPEC) were collected from Hospital Raja Permaisuri Bainun, Ipoh. A total of 60 clinical isolates from urine samples were assessed in this current project. The data of each samples including the age and gender of patients were acquired. Prior to the project, ethical approval was obtained from Medical Research and Ethics Committee, and the isolates had undergone bacterial identification via MALDI-TOF mass spectrometry to be identified as *E. coli*. The samples were streaked on TSA, incubated overnight at 37°C and subsequently stored at 4°C for downstream assays.

3.2.2 Antimicrobial susceptibility test

The antimicrobial susceptibility profiles of UPEC isolates against trimethoprim-sulfamethoxazole (25 µg), nalidixic acid (30 µg), levofloxacin (5 µg), ciprofloxacin (5 µg), tetracycline (30 µg), minocycline (30 µg), imipenem (10 µg), ampicillin (10 µg) and chloramphenicol (30 µg) were conducted via the Kirby-Bauer disk diffusion method. Several colonies of each sample were transferred from TSA culture plate into a 5 ml sterile saline using a sterile inoculating loop to prepare for a colony suspension with the turbidity of 0.5 McFarland standard. The colony suspension was swabbed evenly on the surface of a Mueller Hinton agar plate via a sterile cotton swab. Antibiotic disks were gently placed on the swabbed plate with a sterile forceps and incubated at 37°C for 16 to 18 hours. The diameter of the inhibition zone was measured in millimetres and the results were interpreted as resistant (R), intermediate (I) and sensitive (S) according to the performance standard provided by Clinical and Laboratory Standard Institute (CLSI) guidelines (2021). The interpretive categories and zone diameter breakpoints for all antibiotics are listed in Appendix A. *E. coli* ATCC 25922 was used as the negative control for the susceptibility test.

3.2.3 Total DNA extraction

Total DNA extraction was conducted using fast-boil method (Kor et al., 2013). The isolates were first incubated aerobically into 7 ml of nutrient broth for 20 hours at 37°C with constant shaking (220 rpm). After incubation, 1.5 ml of inoculum was centrifuged at 12,000 rpm for 5 minutes. The pellet was resuspended with 300 µl of sterile deionised water. The samples were subjected to cell lysis via 5-minute boiling, and immediately followed by immersion on ice for 2 minutes. Subsequently, the tubes were centrifuged again at 12,000 rpm for 2 minutes. The supernatant was aspirated into new sterile microcentrifuge tubes and stored at -20ºC for the following PCR detection. The concentration and purity of DNA extracts were measured using Thermo Scientific Nanodrop^{TM} 1000 Spectrophotometer.

3.2.4 Optimisation of duplex PCR condition

Prior to the duplex PCR screening of *tet* genes among UPEC isolates, PCR conditions were optimised to ensure the optimal amplification of the two targeted *tetA* and *tetB* genes. Several PCR parameters including the concentration of magnesium chloride and *Taq* DNA polymerase, and primer dosage were optimised. Gradient PCR in a variation of temperatures ranging from 52°C to 58°C was carried out to optimise the annealing temperature of primers.

3.2.5 Duplex PCR detection of tetracycline resistance genes in samples

The optimised duplex PCR conditions were applied to the subsequent duplex PCR screening of DNA extracts to detect the carriage of *tetA* and *tetB* genes in UPEC isolates. The PCR reaction mixture with a total volume of 25 µl was prepared as listed in the Table 3.2 below. The primer sequences and the expected product size of respective *tet* resistance genes are displayed in Table 3.3, whereas the cycling condition used are shown in the subsequent Table 3.4. All PCR reactions were performed using Analytik JENA Model FlexCycler2 PCR Thermal Cycler.

Table 3.2: Components of duplex PCR reaction mixture in a final volume of 25 µl for the detection of *tetA* and *tetB* genes.

Component	Initial Concentration	Final Concentration	Volume (µl)
Deionised H_2O	-	-	14.0
Taq buffer	5×	1×	5.0
dNTP	10 mM	100 µM	0.25
$MgCl_2$	25 mM	1.5 mM	1.5
tetA primer (F)	10 µM	0.3 µM	0.75
(R)	10 µM	0.3 µM	0.75
tetB primer (F)	10 µM	0.3 µM	0.75
(R)	10 µM	0.3 µM	0.75
Taq DNA polymerase	5 U	1.25 U	0.25
DNA template	-	~ 180 ng/µl	1.0
Total volume			**25**

Table 3.3: Primer sequences and estimated amplicon size of *tetA* and *tetB* genes.

Primers	Sequence (5' – 3')	Expected amplicon size (bp)	References
tetA	F: 5'- GTGAAACCCAACATACCCC -3' R: 5'- GAAGGCAAGCAGGATGTAG -3'	888	(Maynard et al., 2003)
tetB	F: 5'- CCTCAGCTTCTCAACGCGTG -3' R: 5'- GCACCTTGCTGATGACTCTT -3'	634	(Dormanesh et al., 2014)

Table 3.4: Cycling conditions of duplex PCR.

Step	Temperature (°C)	Duration (seconds)	No. of cycle(s)
Initial denaturation	94	300	1
Denaturation	94	60	30
Annealing	56	30	
Extension	72	60	
Final extension	72	480	1

3.2.6 Agarose gel electrophoresis

Subsequent to PCR amplification, gel electrophoresis was carried out to detect the presence of the amplified *tet* genes. By using 1× TBE (1 M Tris base, 1 M Boric acid, 0.02 M EDTA) as the running buffer, the PCR products were electrophoresed on 1.5% (w/v) agarose gel at 90 V for 50 minutes. The gel was pre-stained with GelRed during gel preparation for gel visualisation under UV transilluminator. SMOBIO’s 100 bp DNA ladder was used as molecular-weight size marker to assess the product size of amplicons.

3.2.7 Statistical analysis

The data was computed and analysed by using IBM® SPSS® Statistics 25.0.0 software. The distribution of UPEC isolates and the prevalence of *tetA* and *tetB* genes in accordance with demographic profiles of patients were evaluated. Besides, the association of *tet* genes with demographic profiles, and the association between genotypic traits and antibiotic susceptibility profiles were analysed as well. The *p*-values < 0.05 was considered statistically significant for positive association.

CHAPTER 4

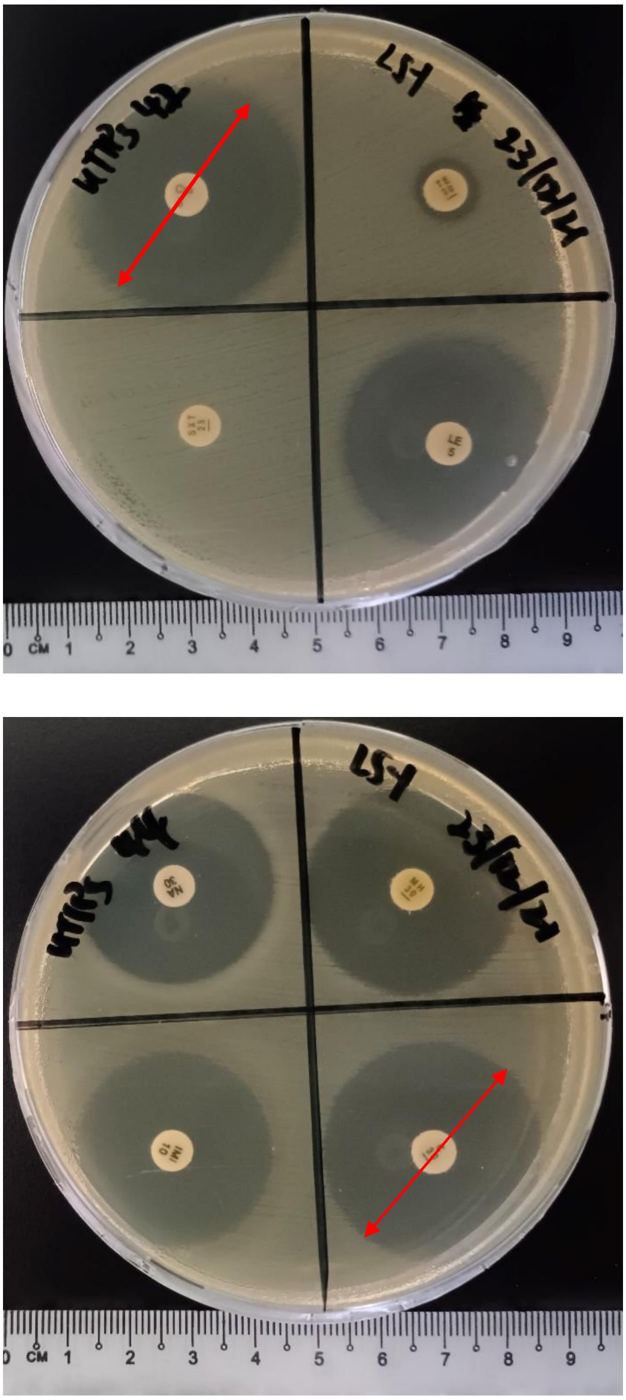
RESULTS

4.1 Overview

Sixty UPEC clinical isolates collected from Hospital Raja Permaisuri Bainun were screened for antimicrobial susceptibility testing and the carriage of tetracycline resistance genes were detected via duplex PCR to study the association between the phenotypic and genotypic profile of the isolates. The demographic data of each isolate are listed in Appendix B.

4.2 Antimicrobial susceptibility test

The antibiotic susceptibility profiles of UPEC isolates against different antibiotic classes including quinolone (nalidixic acid, ciprofloxacin and levofloxacin), tetracycline (tetracycline and minocycline), folate pathway antagonist (trimethoprim-sulfamethoxazole), β-lactam antibiotics (imipenem and ampicillin) and phenicol (chloramphenicol) are shown in Appendix C. Figure 4.1 shows the phenotypic susceptibility traits of isolates, whereby the inhibition zones were interpretated based on CLSI guidelines. For a better interpretation analysis, isolates with intermediate susceptibility were categorised as resistant. The distribution of resistant isolates to each antibiotic are summarised in Figure 4.2. The UPEC strains showed the highest level of resistance to ampicillin (71.67%), followed by tetracycline (51.67%), nalidixic acid (50.00%), trimethoprim-sulfamethoxazole (33.33%) and ciprofloxacin (31.67%). Meanwhile, the isolates show a certain degree of susceptibility to levofloxacin (26.67%) and chloramphenicol (16.67%). However, the strains were found to have least resistance towards imipenem (0.00%) followed by minocycline (1.67%), of which only one out of 60 samples are minocycline-resistant and none of the strains showed resistance to imipenem.



(a)

(b)

Figure 4.1: Representative images of Kirby-Bauer disk diffusion antibiotic susceptibility test on UPEC clinical isolates.

(a) UTIPS 42 showed resistance to tetracycline and trimethoprim-sulfamethoxazole, the isolates were fully resistant to trimethoprim-sulfamethoxazole, (b) UTIPS 44 was susceptible to all four antibiotics including imipenem, chloramphenicol, minocycline and nalidixic acid.

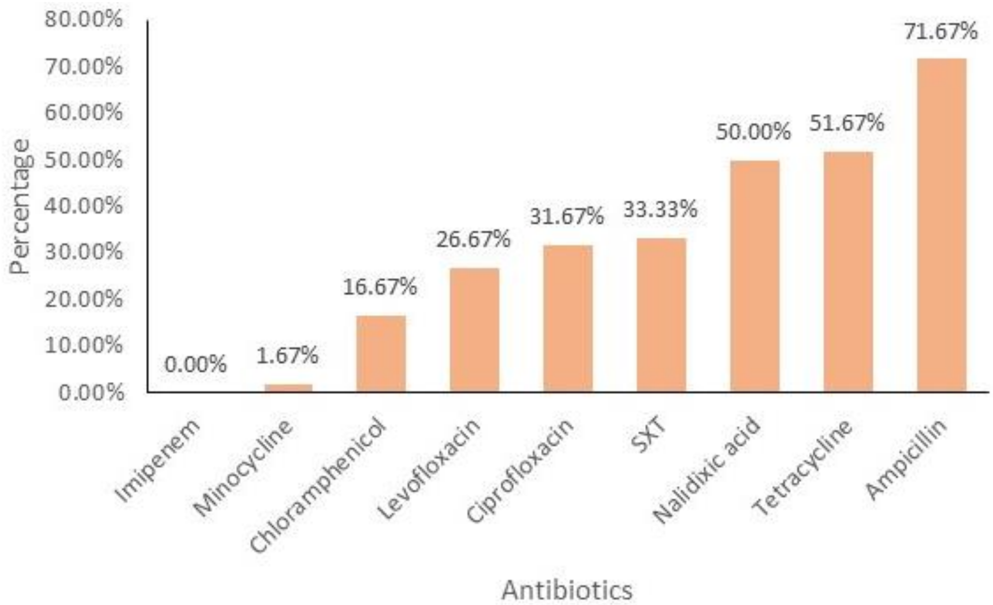


Figure 4.2: Distribution of resistant isolates according to antibiotics.

4.3 Concentration and purity of DNA extracts

The ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280} ratio) of DNA extracts were measured to assess the purity of extracted samples. The extracts were regarded to be relatively pure with minimal or no contamination as the A_{260}/A_{280} ratio of samples fell within the range of 1.8 and 2.0. The concentration and purity were recorded in Appendix D.

4.4 Optimisation of duplex PCR condition

The outcome of gel image for gradient PCR is shown in Figure 4.3. Annealing temperatures at 51.9°C and 53.8°C were opted out as these temperatures yielded off-target amplifications at around 150 bp. Meanwhile, the band intensity of *tetA* was slightly lower at 58.1°C as compared to 56.2°C, suggesting that the annealing temperature at 56.2°C was the most optimum temperature for the duplex PCR. The optimum duplex PCR was finalised to be performed in a final volume of 25 µl, which contains 1× buffer, 100 µM of dNTPs, 1.5 mM of magnesium chloride, 0.3 µM of each primer, 1.25 U of *Taq* DNA polymerase and 180 ng/µl of DNA template. The optimised cycling condition of duplex PCR was as follows: 5 minutes at 94°C for initial denaturation, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 30 seconds and extension at 72°C for 60 seconds, while the final extension step was at 72°C for 8 minutes.

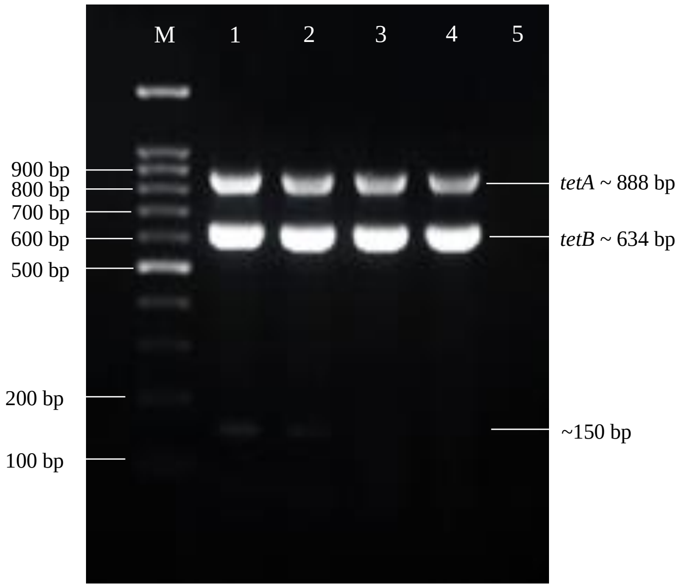


Figure 4.3: Optimisation of PCR condition based on annealing temperature gradient.

Lane M represents 100 bp DNA ladder. Lane 1 to 4 were loaded with UTIPS 14 and subjected to gradient PCR with the annealing temperatures of 51.9°C, 53.8°C, 56.2°C and 58.1°C, respectively. Lane 1 and 2 showed non-specific amplifications at around 150 bp. Lane 5 is the negative control for the PCR condition.

4.5 Duplex PCR detection of *tetA* and *tetB* genes

All UPEC clinical isolates were subjected to duplex PCR detection of *tetA* and *tetB* genes. Out of 60 screened isolates, 25 (41.67%) samples showed positive for *tetA* gene, and 7 (11.67%) showed the carriage of *tetB* gene. Surprisingly, only one sample (1.67%) showed co-carriage of *tetA* and *tetB* genes. In general, the UPEC isolates illustrated a higher prevalence of *tetA* gene as compared to *tetB* gene. The prevalence of *tetA* and *tetB* genes are summarised in Table 4.1. Figure 4.4 displays the representative duplex PCR gel image outcome to assess the presence of *tetA* and *tetB* genes in UPEC isolates. The genotypic profile of each sample is listed in Appendix E.

Figure 4.4: Representative image of duplex PCR screening of *tetA* and *tetB* genes.

Lane M represents 100 bp DNA ladder. Lane 1 to 9 were loaded with UTIPS 44, UTIPS 49, UTIPS 34, UTIPS 42, UTIPS 47, UTIPS 54, UTIPS 39, UTIPS 50, UTIPS 56, respectively. Lane 10 was loaded with UTIPS 14 to serve as positive control. Positive strains of *E. coli* K21 and H47 with confirmed carriage of corresponding genes were loaded into lane 11 and lane 12, respectively. Lane 13 is the negative control for the duplex PCR screening.

Table 4.1: Number of UPEC isolates screened positive for *tet* genes.

***tet* gene(s)**	**Number of Positive Isolate(s)**
tetA	25/60 (41.67%)
tetB	7/60 (11.67%)
tetA and *tetB*	1/60 (1.67%)

4.6 Distribution of UPEC clinical isolates in relation to gender and age groups of patients

Figures 4.5 and 4.6 display the distribution of UPEC clinical isolates based on gender and age groups, respectively. In a total of 60 samples, UPEC strains were predominantly found in females (n = 44, 73.33%) as compared to males (n = 16, 26.67%). In terms of age groups, the age of patients was classified into young age group, working age group and old age group according to the Department of Statistics Malaysia. Among the 60 isolates, the highest frequency of clinical isolates was discovered to be collected from patients in working age group (n = 32, 53.33%), followed by the old age group (n = 24, 40.00%) and the young age group (n = 4, 6.67%).

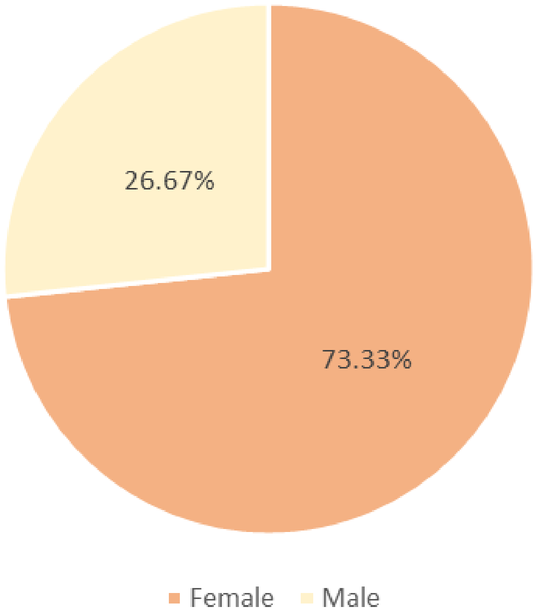


Figure 4.5: **Distribution of UPEC clinical isolates based on gender.**

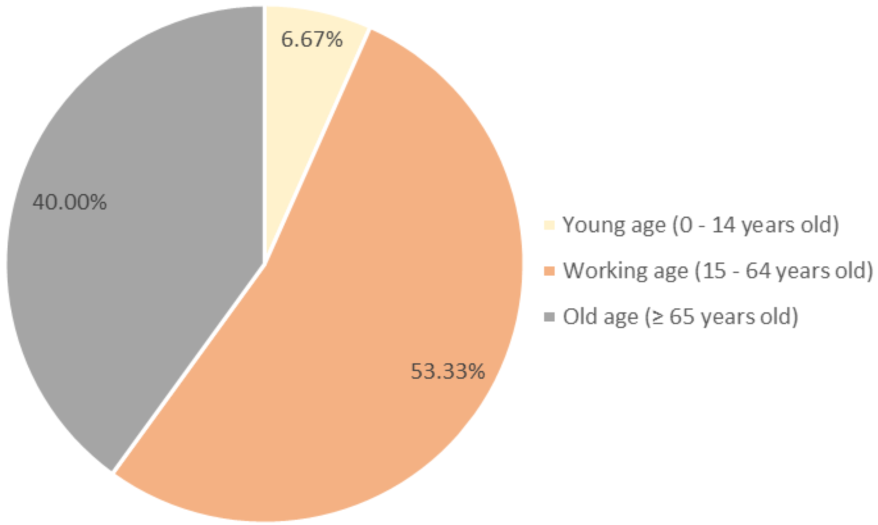


Figure 4.6: **Distribution of UPEC clinical isolates based on age groups.**

4.7 Prevalence of tetracycline resistance genes in accordance with gender and age groups of patients

The overall gene prevalence in accordance with demographic profiles is summarised in Table 4.2. The representative data of the association between *tet* genes and the demographic profiles are listed in Appendix F. Both *tetA* and *tetB* genes show no association to neither gender nor age groups as the *p*-values are greater than 0.05. The distribution of *tetA* and *tetB* genes based on gender is shown in Figure 4.7. The prevalence of *tetA* genes shows not much difference in both males (43.75%) and females (45.45%), however *tetB* genes in females (15.90%) were slightly outnumbered than that of males (6.25%). The gene prevalence in both genders show resemblance whereby the frequency of *tetA* gene is higher than that of *tetB* gene.

Figure 4.8 shows the prevalence of *tetA* and *tetB* genes according to age groups. The percentage of isolates with *tetA* gene carriage was discovered to be identical in young age and working age group (50.00%), which is higher than that of old age group (37.50%). Meanwhile, *tetB* genes were found to be the most predominant in old age group (25.00%), followed by working age group (6.25%), while none of the young age patients harbour *tetB* gene. Comparable to Figure 4.7, the prevalence of *tetA* gene is higher than *tetB* gene across all age groups.

Table 4.2. Distribution of *tet* genes in accordance with gender and age of patients.

Demographic profile		Duplex PCR detection of genes					
		***tetA*+**	***tetA*-**	***p*-value***	***tetB*+**	***tetB*-**	***p*-value***
Gender	**Female (n= 44)**	20 (45.5%)	24 (54.5%)	0.907	7 (15.9%)	37 (84.1%)	0.330
	Male (n= 16)	7 (43.8%)	9 (56.3%)		1 (6.3%)	15 (93.8%)	
Age group	**Young age (n= 4)**	2 (50.0%)	2 (50.0%)	0.635	0 (0.0%)	4 (100.0%)	0.089
	Working age (n= 32)	16 (50.0%)	16 (50.0%)		2 (6.3%)	30 (93.8%)	
	Old age (n= 24)	9 (37.5%)	15 (62.5%)		6 (25.0%)	18 (75.0%)	

* Positive association is considered to be statistically significant with *p*-value < 0.05.

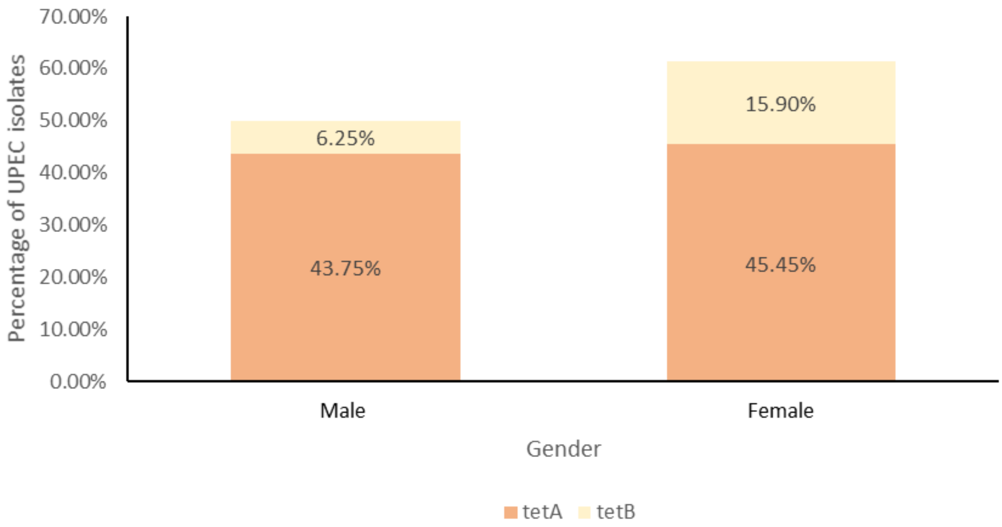


Figure 4.7: Distribution of *tetA* and *tetB* genes according to gender of patients.

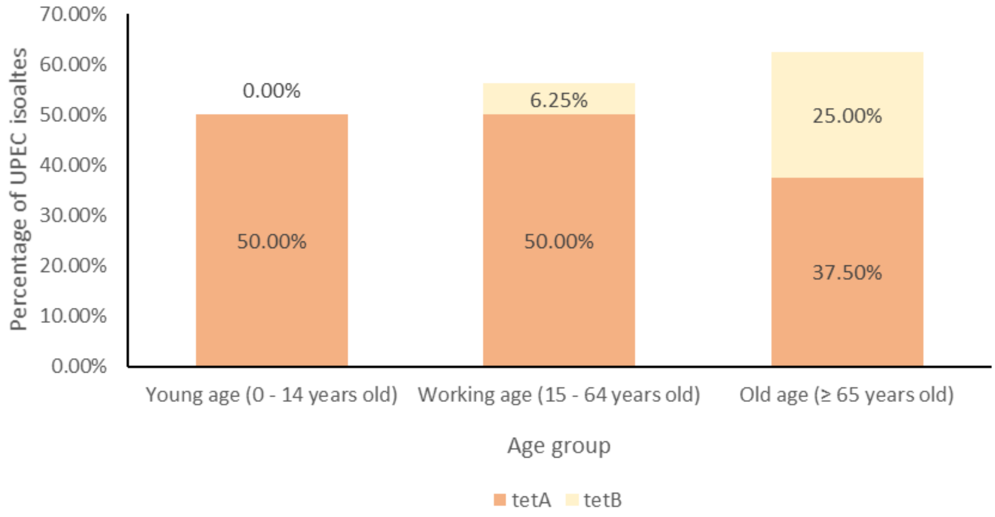


Figure 4.8: Distribution of *tetA* and *tetB* genes according to age groups of patients.

4.8 Association between phenotypic antimicrobial resistance traits and genotypic profile of clinical isolates

Table 4.3 summarises the association between the genotypic profile and the resistance phenotype of UPEC isolates, and the statistical analysis are shown in Appendix G. In a total of 60 UPEC isolates, 31 (51.67%) exhibited resistance to tetracycline, and all the resistant isolates harboured at least one of the *tet* genes. Among the 31 tetracycline-resistant isolates, 25 (80.65%) carried *tetA* genes, while 7 (22.58%) isolates screened positive for *tetB* genes as shown in Figure 4.9. The high *tet* genes prevalence observed in tetracycline-resistant isolates suggests that the genotypic profile may correspond with the phenotypic resistance profile. The positive association between the tetracycline resistance traits and the gene prevalence of *tetA* and *tetB* were further confirmed to be statistically significant (p = 0.000; p = 0.029).

Contrary to tetracycline resistance profile, the UPEC clinical isolates displayed a high susceptibility to minocycline as only one out of 60 samples was resistant to minocycline, and was detected to harbour *tetB* gene (100.00%). Despite majority (59 out of 60) of the UPEC isolates were minocycline-susceptible, the duplex PCR screening revealed resistance genes carriage in 34 strains (57.63%). Among the 59 susceptible isolates, 27 (45.76%) were *tetA* positive and 7 (11.86%) were *tetB* positive as displayed in Figure 4.10. In terms of association between phenotypic and genotypic variables, the minocycline susceptibility profile shows positive correlation with *tetB* ($p = 0.010$), but negative association with *tetA* ($p = 0.362$).

Apart from tetracycline antibiotic class, *tetA* gene was also found to be positively associated with other antimicrobials including nalidixic acid ($p = 0.020$), trimethoprim-sulfamethoxazole ($p = 0.001$) and ampicillin ($p = 0.000$). Meanwhile, considering that all UPEC isolates were susceptible to imipenem, the association between *tet* genes and imipenem susceptibility profile was unable to be computed as the variable of imipenem susceptibility profile is a constant. Anyhow, the negative association of *tet* genes with antimicrobial susceptibility profiles against the other antibiotics including ciprofloxacin, levofloxacin, chloramphenicol, were also observed as the p-values > 0.050.

Table 4.3: Association of *tet* genes with antimicrobial susceptibility profiles of 60 UPEC isolates.

Antimicrobial Susceptibility		Duplex PCR detection of genes					
		tetA+	***tetA-***	***p*-values**	***tetB+***	***tetB-***	***p*-values**
Tetracycline	**R** (n=31)	25 (80.6%)	6 (19.4%)	0.000*	7 (22.6%)	24 (77.4%)	0.029*
	S (n=29)	2 (6.9%)	27 (93.1%)		1 (3.4%)	28 (96.6%)	
Minocycline	**R** (n=1)	0 (0.0%)	1 (100%)	0.362	1 (100%)	0 (0.0%)	0.010*
	S (n=59)	27 (45.8%)	32 (54.2%)		7 (11.9%)	52 (88.1%)	
Nalidixic acid	**R** (n=30)	18 (60.0%)	12 (40.0%)	0.020*	5 (16.7%)	25 (83.3%)	0.448
	S (n=30)	9 (30.0%)	21 (70.0%)		3 (10.0%)	27 (90.0%)	
Ciprofloxacin	**R** (n=19)	12 (63.2%)	7 (36.8%)	0.054	3 (15.8%)	16 (84.2%)	0.703
	S (n=41)	15 (36.6%)	26 (63.4%)		5 (12.2%)	36 (87.8%)	
Levofloxacin	**R** (n=16)	10 (62.5%)	6 (37.5%)	0.100	2 (12.5%)	14 (87.5%)	0.909
	S (n=44)	17 (38.6%)	27 (61.4%)		6 (13.6%)	38 (86.4%)	
Trimethoprim-sulfamethoxazole	**R** (n=20)	15 (75.0%)	5 (25.0%)	0.001*	4 (20.0%)	16 (80.0%)	0.283
	S (n=40)	12 (30.0%)	28 (70.0%)		4 (10.0%)	36 (90.0%)	
Imipenem	**R** (n=0)	–	–	–**	–	–	–**
	S (n=60)	27 (45.0%)	33 (55.5%)		8 (13.3%)	52 (86.7%)	
Ampicillin	**R** (n=43)	26 (60.5%)	17 (39.5%)	0.000*	7 (16.3%)	36 (83.7%)	0.286
	S (n=17)	1 (5.9%)	16 (94.1%)		1 (5.9%)	16 (94.1%)	
Chloramphenicol	**R** (n=10)	5 (50.0%)	5 (50.0%)	0.728	2 (20.0%)	8 (80.0%)	0.497
	S (n=50)	22 (44.0%)	28 (56.0%)		6 (12.0%)	44 (88.0%)	

'R' stands for resistant, and 'S' stands for susceptible.

* Positive association is statistically significant with p-value < 0.05.

** p-value is not computed because the variable of imipenem susceptibility profile is a constant.

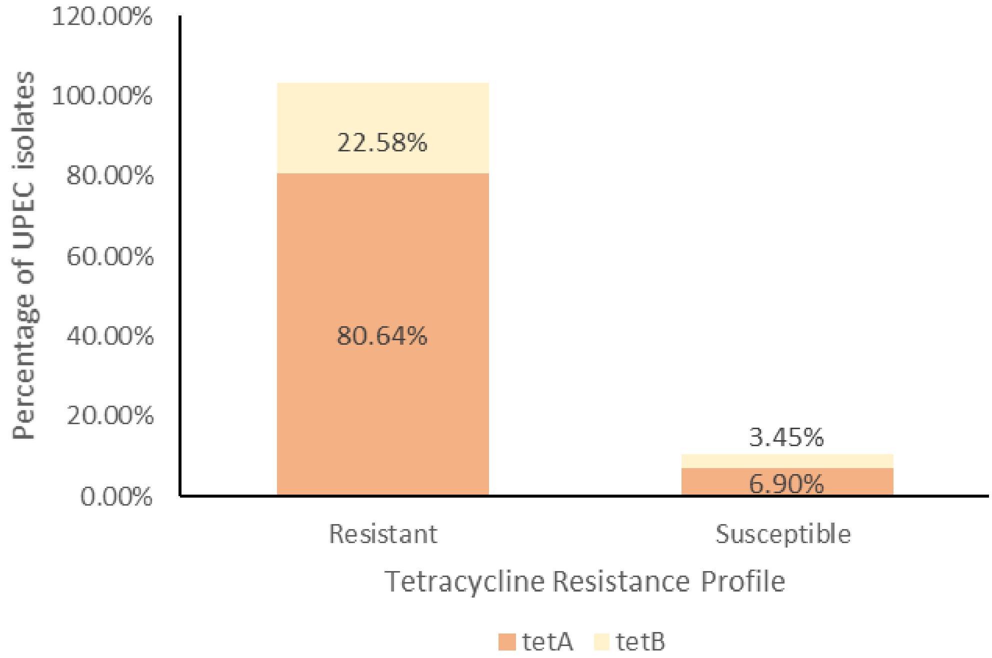


Figure 4.9: Distribution of *tetA* and *tetB* genes in UPEC isolates based on the tetracycline resistance profile.

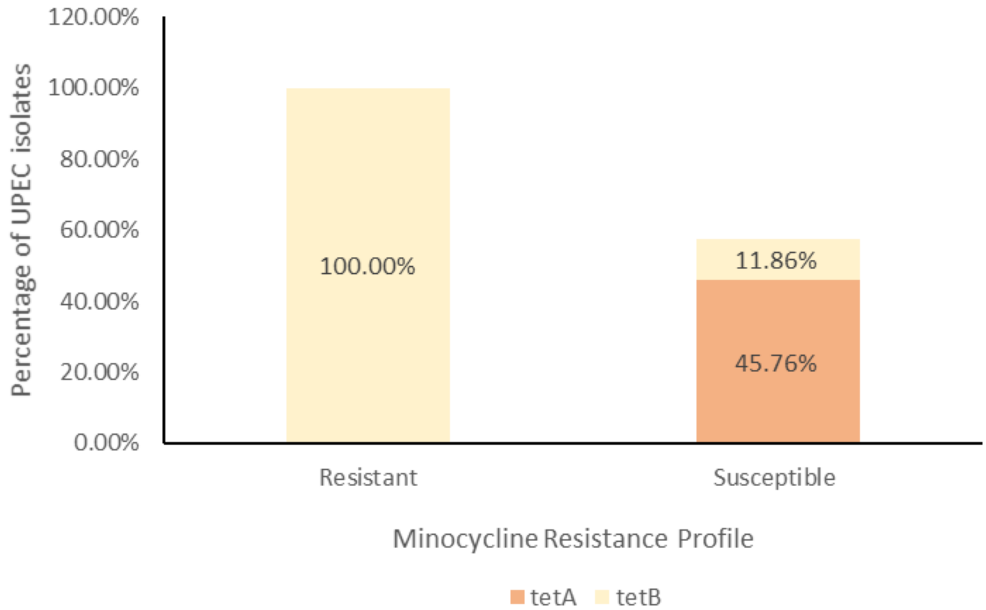


Figure 4.10: Distribution of *tetA* and *tetB* genes in UPEC isolates based on the minocycline resistance profile.

CHAPTER 5

DISCUSSION

5.1 Overview

The project was conducted to attain the following objectives: to assess the phenotypic antimicrobial susceptibility profiles of UPEC clinical isolates, to screen for the carriage of tetracycline resistance genes, *tetA* and *tetB* genes among UPEC isolates via duplex PCR detection, to evaluate the distribution of tetracycline resistance genes in UPEC isolates and to analyse the association of *tet* genes with demographic profiles of patients and resistance traits.

5.2 Antimicrobial susceptibility test

The UPEC clinical isolates retrieved in this study exhibited multidrug resistance to different antimicrobial agents, particularly to ampicillin (71.67%), tetracycline (51.67%) and nalidixic acid (50.00%). This outcome is in line with the research conducted by Olowe et al. (2013) who showed the rise in resistance rate against ampicillin (96.10%), tetracycline (77.80%), and nalidixic acid (38.40%). The high resistance pattern observed is likely to correlate with the widespread and indiscriminate use of antimicrobial agents in clinical use, which promotes the spreading of antimicrobial resistance determinants among the pathogenic *E. coli* strains and results in the emergence of multidrug strains to counteract actions of different antibiotics (Olowe et al., 2013). In particular, ampicillin, a semi-synthetic β-lactam antibiotics is one of the commonly prescribed agents for *E. coli* infection treatment in human (Chen et al., 2019).

Although imipenem serves as β-lactam antibiotic just like ampicillin, all UPEC isolates showed susceptibility to imipenem in this study. This result conforms with the findings obtained by Dehkordi et al. (2020), where the UPEC samples showed least resistance rate of 9.09% to imipenem. The high susceptibility against imipenem may be because imipenem is often prescribed in combination with other drugs such as cilastatin and relebactam to supress multidrug resistance development as the drug combination can act as a β-lactamase inhibitor (Chen et al., 2020). Besides, the combination therapy of imipenem/cilastatin/relebactam was also approved to treat cUTIs and cIAIs (Chen et al., 2020). Despite the ideal antimicrobial action may suggest imipenem could be a possible long term drug treatment for UTIs, imipenem, which is a carbapenem, often serve as the last resort agent for MDR strains due to the increasing resistance rate against β-lactam antibiotics (Sekyere, 2016; Chen et al., 2020).

Meanwhile, the UPEC isolates showed a relatively higher susceptibility to minocycline (1.70%) as compared to tetracycline (51.7%), suggesting that minocycline is able to exhibit a better antimicrobial efficacy. The higher efficacy and potency of minocycline is reasonable as the antibiotic is a second-generation tetracycline, which is the derivatives of the first-generation tetracycline that were developed to exhibits a higher diffusion capability and stronger ribosomal binding affinity as compared to tetracycline. Thus, minocycline is capable to demonstrate a better efficacy in eradicating the Gram-negative pathogen (Asadi et al., 2020).

5.3 Prevalence of *tetA* and *tetB* genes in UPEC clinical isolates

Both *tetA* and *tetB* genes, which account for the energy-dependent efflux pump mechanism, have been commonly reported to be the most common tetracycline resistance genes in *E. coli* isolates from human and animal origin (Koo and Woo, 2011). The current study revealed a predominant distribution of *tetA* gene (41.67%) in comparison with *tetB* gene (11.67%), which agrees with the findings by Olowe et al. (2013) whereby the *E. coli* isolates from human samples harbouring *tetA* gene showed the highest percentage rate (43.80%), followed by the isolates that carry *tetB* gene (32.00%). Despite *tetB* gene is frequently identified as the most prevalent tetracycline resistance determinant in previous clinical studies, some researchers believed that *tetA* gene has a greater transferability than *tetB* gene, especially among animal stocks, which consequently give rise to the predominancy of *tetA* gene (Koo and Woo, 2011; Olowe et al., 2013). Besides, findings reported by Skočková et al. (2012) also aligned with the abovementioned statement as distribution shift from *tetB* to *tetA* gene in *E. coli* isolates was spotted across the years.

Aside from the predominancy of *tetA* gene, the current finding also showed low co-carriage of *tetA* and *tetB* genes (1.67%). In accordance with this study, Olowe's research also revealed a low coexistence incidence of resistance genes with only 4.40% of isolates carrying both *tetA* and *tetB* genes (Olowe et al., 2013). Considering that *tet* resistance determinants are often embedded in mobile genetic elements, plasmid incompatibility may be one of the possible reasons resulting in the negative coexistence of *tetA* and *tetB* genes (Maynard et al, 2003).

5.4 Prevalence of UPEC isolates based on demographic profiles of patients

A higher prevalence of UPEC isolates was observed among the female patients (73.33%) across all age groups in this study. This observation is in agreement with the findings obtained by Dormanesh et al. (2014), who stated that the primary rationale behind the predominancy of the pathogenic *E. coli* strains is the difference in urogenital system and structure between males and females. Females have a relatively short and wide urethra, which may promote a greater chance for *E. coli* to gain access into urinary tract and cause infection, typically those who are sexually active and those who practice poor hygiene. Besides, imbalance in vaginal flora may also put the women at risk (Dormanesh et al., 2014). All these factors will predispose women to *E. coli* colonisation in urinary systems, which explains the high distribution of UPEC isolates in female patients.

In terms of age groups, highest frequency of UPEC isolates were collected from working age (53.33%), which is explicable due to its wide age range. However, second to the working age, the isolates also predominate in old age group (40.00%). The UPEC isolates prevalence shows conformity to the findings reported by Vargová et al. (2017) that revealed the increased incidence of urinary tract infection in patients over 60 years of age. Some of the risk factors in aging population are rendered immune system, nosocomial pathogen exposure and prolonged catheterisation. Besides, postmenopausal women are also found to be more prone to infection as the decline in oestrogen level will result in vagina flora alteration (Rowe and Mehta, 2013).

5.5 Distribution of *tet* genes based on demographic profiles of patients

In this study, the gene prevalence of *tetA* was more or less the same in both genders, whereas the gene prevalence of *tetB* was higher in females. In comparison to other age groups, old age group showed lower gene prevalence of *tetA*, but a higher gene prevalence of *tetB*. Despite UPEC isolates were predominantly recovered from females and working age groups, the *tet* genes were found to have no significant correlation with the demographic profiles. To date, there are insufficient research or findings evaluate on the association between *tet* genes and the demographic profiles of patients. The *tet* gene distribution pattern based on age and gender, as well as the negative association of *tet* genes with demographic profiles observed in this study are inexplicable.

5.6 Association between phenotypic antimicrobial susceptible profiles and the tetracycline resistance genes prevalence

In the present study, all the tetracycline-resistant isolates were screened positive for the carriage of at least one *tet* efflux gene. The presence of *tetA* and *tetB* genes among UPEC isolates is proven to be positively associated (p-values < 0.030) with phenotypic resistance traits. Previously conducted research showed the similar correlation pattern where all the tetracycline-resistant UPEC isolates were found to harbour at least one tetracycline resistance determinants (Gharajalar and Sofiani, 2017). The most conceivable hypothesis for the occurrence of tetracycline resistance pattern among UPEC isolates is likely to be the successful expression of *tetA* and *tetB* genes in UPEC isolates that contributes to the efflux pump resistance mechanism and counteract with tetracycline.

On the contrary, the minocycline resistance pattern only associates positively with *tetB* (*p*-value = 0.010), but not *tetA* (*p*-value = 0.362). Among all the 60 isolates, the one and only sample that showed minocycline resistance was detected to carry *tetB* gene. This is because *tetB* gene is the only efflux gene that is capable to encode major facilitator superfamily transporters that are effective towards minocycline (Chorpra and Roberts, 2001; Asadi et al., 2020). This statement conforms with the research reported by Huys et al. (2005) whereby an increase of minimum inhibitory concentrations (MICs) of minocycline was spotted in tetracycline-resistant multidrug resistant (MDR) Gram-negative strains that was screened positive for *tetB* genes. Hence, the carriage of *tetB* gene may explain the resistance phenotype of UPEC strains towards minocycline. Nonetheless, Wang et al. (2017) proposed that the absence of *tetB* gene can be a possible reliable marker for minocycline susceptibility as the findings revealed that the *tetB* positive isolates had 100% sensitivity to minocycline resistance. However, this finding contradicts to the current study as seven out of 59 susceptible isolates were detected to harbour *tetB* gene. Anyhow, a study reported by Tuckman et al. (2007) showed conformity to the current findings, where 16 minocycline-susceptible isolates (MICs ≤ 64 µg/ml) were screened positive for *tetB* gene. The possible explanation behind the *tetB* gene carriage in susceptible isolates is that the *tetB* genes were not expressed or poorly expressed (Tuckman et al., 2007). This also suggests that the carriage of resistance genes in UPEC strains does not necessarily reflecting the phenotypic expression of resistance, and one of the reasons may be due to the gene regulation mechanism in the bacteria (Møller et al., 2016).

Aside from tetracycline antibiotic class, *tetA* gene also discovered to have significant positive association with the resistance profiles against nalidixic acid, ampicillin and trimethoprim-sulfamethoxazole. This suggest that the MFS efflux pump encoded by *tetA* is not selective to tetracycline class solely, but also able to expel other drugs, which further confers to multidrug resistance (Warburton et al., 2012).

On the other hand, there are resistant isolates that were screened negative for both *tetA* and *tetB* genes, which possibly suggests that the UPEC isolates harbour different *tet* efflux genes other than *tetA* and *tetB* genes, for instance *tetC, MdfA,* and *NorA* genes, to exhibit similar drug efflux mechanism. Besides, the UPEC strains may also carries other genes accounting for different resistance mechanisms, including ribosomal protection, enzymatic inactivation and drug target site modification, resulting in the appearance of resistance traits shown in the current study (Chhetri et al., 2015; Grossman, 2016). For instance, *tetO*, *tetM* and *tetW* are some of the commonly reported genes which encode for ribosomal protection proteins (RPPs), while *tetX* is responsible in tetracycline inactivation by adding an extra hydroxyl group to the C-11a position of the tetracycline core (Grossman, 2016).

Meanwhile, several susceptible isolates to different antimicrobials were found to be positive for *tet* genes carriage. Notably, a high prevalence of minocycline-susceptible isolates was screened positive for *tet* genes carriage. This suggest the acquisition of resistance genes among the susceptible isolates, which also implies that the UPEC strains had progressed to be a possible significant reservoir of *tet* efflux gene and may further emerge to become antimicrobial resistant in the future (Boerlin et al., 2005).

5.7 Limitations and future studies

Taking note of the phenotypic resistance pattern is highly dependent on the expressing ability of resistance determinants, nonetheless, the current study comes with limitations as the molecular detection only screen for the gene carriage without studying in-depth on the expression level of the resistance genes. To obtain a more thorough insight on the tetracycline resistance mechanism, it is considerable to further analyse the regulation of resistance genes through qPCR to quantify the expression level of determinants. In addition, studying on the two most predominant *tet* efflux genes is inadequate to understand the thorough efflux resistance mechanisms. Thus, more resistance determinants can be included in the future studies. Moreover, DNA sequencing was not included in the current study to verify the PCR assay. This issue should be underscored in the upcoming studies to ensure the gene sequences are aligned with the existing NCBI BLASTN databases. Besides, larger sample size is also preferable as 60 clinical isolates collected from one localised hospital is insufficient to reflect the UPEC isolates distribution in Malaysia population.

CHAPTER 6

CONCLUSION

Overall, UPEC isolates are predominantly recovered from female patients. The highest resistance rate of UPEC isolates was found to be against ampicillin, followed by tetracycline and nalidixic acid, while the isolates showed the highest susceptibility to imipenem and minocycline. The current study revealed a high prevalence of *tetA* genes as compared to *tetB* genes, which may propose the higher occurrence of widespread and dissemination of *tetA* efflux genes among the UPEC strains in hospital settings. Both *tet* genes showed negative association to age and gender of patients, however, there is a significant positive association between genotypic profile and phenotypic profile of tetracycline antibiotic class. All tetracycline-resistant isolates harboured at least one *tet* efflux gene, while one minocycline-resistant isolate harboured *tetB* gene. Although minocycline phenotypically showed a better efficacy in comparison to tetracycline, the high prevalence of *tet* genes carriage in minocycline-susceptible isolates revealed that the reservoir for *tet* genes has expanded in UPEC strains despite the low level or no expression of *tet* genes, which promotes gene dissemination among species. Meanwhile, *tetA* genes also correlate with the resistance profiles of other drugs, which is in line with the fact where *tet* genes are capable in encoding multidrug MFS transporters.

In a nutshell, the continued emergence of efflux mediated multidrug resistance among UPEC strains will be detrimental to the current therapeutic paradigm for UTIs. To impede the emergence of antimicrobial resistance, therapeutic strategy shift from broad-spectrum antimicrobials into antibiotics that target selectively to core resistance determinants has to be highlighted. Since the findings obtained in this study only focus on the basic molecular standpoints of efflux gene carriage in conferring to corresponding resistance traits, further in-depth studies are preferable to expand the understanding of specific important determinants in order to improvise and idealise the therapeutic strategy for UTI treatment.

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APPENDICES

Appendix A

Table 1: Interpretive categories and zone diameter breakpoints (in millimetres) of each antibiotic based on CLSI guidelines.

	Tetracycline (30 µg)	Minocycline (30 µg)	Trimethoprim-sulfamethoxazole (25 µg)	Ciprofloxacin (5 µg)	Nalidixic acid (30 µg)	Levofloxacin (5 µg)	Imipenem (10 µg)	Ampicillin (10 µg)	Chloramphenicol (30 µg)
Susceptible	≥15	≥16	≥16	≥26	≥19	≥21	≥23	≥17	≥18
Intermediate	12-14	13-15	11-15	22-25	14-18	17-20	20-22	14-16	13-17
Resistant	≤11	≤12	≤10	≤21	≤13	≤16	≤19	≤13	≤12

(Adapted from Clinical and Laboratory Standard Institute, 2021)

Appendix B

Table 2: Demographic data of each UPEC clinical isolates.

Sample	Gender	Age
UTIPS 1	M	2
UTIPS 2	F	78
UTIPS 3	F	48
UTIPS 4	F	26
UTIPS 5	F	67
UTIPS 6	F	36
UTIPS 7	F	77
UTIPS 8	F	76
UTIPS 9	M	69
UTIPS 10	M	52
UTIPS 12	F	66
UTIPS 13	F	24
UTIPS 14	F	74
UTIPS 15	M	40
UTIPS 16	F	41
UTIPS 17	F	30
UTIPS 18	F	51
UTIPS 19	M	63
UTIPS 20	F	88
UTIPS 21	F	53
UTIPS 22	F	63
UTIPS 23	F	63
UTIPS 24	M	77
UTIPS 25	F	79
UTIPS 26	F	61
UTIPS 27	F	76
UTIPS 28	F	66
UTIPS 29	F	38

Table 2 (continued)

UTIPS 30	F	73
UTIPS 31	M	63
UTIPS 32	M	68
UTIPS 33	F	68
UTIPS 34	F	64
UTIPS 35	F	56
UTIPS 36	F	64
UTIPS 37	F	59
UTIPS 38	F	79
UTIPS 39	F	86
UTIPS 40	F	35
UTIPS 41	F	70
UTIPS 42	F	17
UTIPS 43	F	17
UTIPS 44	M	57
UTIPS 45	F	30
UTIPS 46	F	48
UTIPS 47	F	26
UTIPS 48	M	82
UTIPS 49	M	87
UTIPS 50	F	53
UTIPS 51	F	75
UTIPS 52	M	13
UTIPS 53	F	5
UTIPS 54	F	39
UTIPS 55	M	53
UTIPS 56	M	49
UTIPS 57	F	67
UTIPS 58	F	44
UTIPS 59	M	82
UTIPS 60	F	3
UTIPS 61	M	68

‘M’ stands for male and ‘F’ stands for female.

Appendix C

Table 3: Antimicrobial susceptibility profile of UPEC isolates.

Sample	NAL	CIP	LEX	TET	MIN	SXT	AMP	CHL	IPM
UTIPS 1	S	S	S	S	S	S	S	R	S
UTIPS 2	R	R	S	R	S	S	R	S	S
UTIPS 3	S	S	S	S	S	S	S	S	S
UTIPS 4	R	R	R	R	S	R	R	S	S
UTIPS 5	R	R	R	R	S	S	R	S	S
UTIPS 6	S	S	S	S	S	S	R	S	S
UTIPS 7	R	R	R	R	S	R	R	R	S
UTIPS 8	S	S	S	R	S	S	R	S	S
UTIPS 9	R	R	R	R	S	R	R	S	S
UTIPS 10	R	S	S	R	S	R	R	R	S
UTIPS 12	S	S	S	S	S	S	R	R	S
UTIPS 13	S	S	S	R	S	S	R	S	S
UTIPS 14	R	R	R	R	S	R	R	S	S
UTIPS 15	R	R	R	R	S	R	R	S	S
UTIPS 16	R	R	R	R	S	R	R	S	S
UTIPS 17	S	S	S	R	S	R	R	S	S
UTIPS 18	R	R	R	R	S	R	R	S	S
UTIPS 19	R	S	S	R	S	R	R	R	S

Table 3 (continued)

UTIPS 20	S	S	S	R	S	S	R	R	S
UTIPS 21	R	R	R	S	S	S	R	S	S
UTIPS 22	S	S	S	R	S	R	S	S	S
UTIPS 23	S	S	S	S	S	S	S	S	S
UTIPS 24	R	R	R	S	S	S	R	S	S
UTIPS 25	S	S	S	S	S	S	R	S	S
UTIPS 26	S	S	S	S	S	S	R	S	S
UTIPS 27	R	S	S	S	S	S	R	S	S
UTIPS 28	S	S	S	S	S	S	S	S	S
UTIPS 29	S	S	S	R	S	S	R	R	S
UTIPS 30	R	S	S	S	S	S	R	S	S
UTIPS 31	R	R	R	S	S	S	R	S	S
UTIPS 32	R	R	R	R	S	S	R	R	S
UTIPS 33	R	S	S	S	S	S	R	S	S
UTIPS 34	R	R	R	R	S	R	R	S	S
UTIPS 35	R	S	S	S	S	S	S	S	S
UTIPS 36	S	S	S	S	S	S	S	S	S
UTIPS 37	S	S	S	S	S	S	S	R	S
UTIPS 38	S	S	S	R	S	S	R	S	S
UTIPS 39	R	S	S	R	S	S	R	S	S
UTIPS 40	S	S	S	S	S	R	R	S	S
UTIPS 41	R	R	S	R	S	R	R	S	S

Table 3 (continued)

UTIPS 42	R	S	S	R	S	R	R	S	S
UTIPS 43	R	S	S	R	S	R	R	S	S
UTIPS 44	S	S	S	S	S	S	R	S	S
UTIPS 45	R	S	S	S	S	R	S	S	S
UTIPS 46	S	S	S	S	S	S	S	S	S
UTIPS 47	R	R	S	R	S	S	R	S	S
UTIPS 48	S	S	S	S	S	S	S	S	S
UTIPS 49	R	R	R	S	S	S	S	S	S
UTIPS 50	S	S	S	R	S	R	R	S	S
UTIPS 51	S	S	S	S	S	S	S	S	S
UTIPS 52	S	S	S	R	S	S	R	S	S
UTIPS 53	S	S	S	S	S	S	R	R	S
UTIPS 54	R	S	S	R	S	R	R	S	S
UTIPS 55	R	R	R	R	S	R	R	S	S
UTIPS 56	S	S	S	R	R	S	S	S	S
UTIPS 57	S	S	S	S	S	S	S	S	S
UTIPS 58	S	S	S	S	S	S	S	S	S
UTIPS 59	S	S	S	S	S	S	S	S	S
UTIPS 60	S	S	S	R	S	S	R	S	S
UTIPS 61	R	R	R	S	S	S	R	S	S

‘R’ stands for resistant, and ‘S’ stands for susceptible.

‘NA’ denotes nalidixic acid, ‘CIP’ denotes ciprofloxacin, ‘LEX’ denotes levofloxacin, ‘TET’ denotes tetracycline, ‘MIN’ denotes minocycline, ‘SXT’ denotes trimethoprim-sulfamethoxazole, ‘AMP’ denotes ampicillin, ‘CHL’ denotes chloramphenicol, ‘IMP’ denotes imipenem.

Appendix D

Table 4: The concentration and purity of DNA extracts from UPEC clinical isolates.

Sample	DNA concentration (ng/µl)	A_{260}/A_{280} ratio
UTIPS 1	182.52	1.84
UTIPS 2	195.10	1.90
UTIPS 3	211.00	1.88
UTIPS 4	223.60	1.76
UTIPS 5	186.97	1.84
UTIPS 6	196.85	1.90
UTIPS 7	244.06	1.82
UTIPS 8	189.48	1.86
UTIPS 9	222.32	1.93
UTIPS 10	149.31	1.84
UTIPS 12	222.47	1.77
UTIPS 13	247.61	1.88
UTIPS 14	265.59	1.75
UTIPS 15	228.27	1.81
UTIPS 16	209.98	1.89
UTIPS 17	268.06	1.84
UTIPS 18	228.72	1.85
UTIPS 19	151.45	1.93
UTIPS 20	169.07	1.83
UTIPS 21	199.92	1.97
UTIPS 22	175.11	1.75
UTIPS 23	167.75	1.92
UTIPS 24	237.89	1.90
UTIPS 25	187.85	1.78
UTIPS 26	134.36	1.92
UTIPS 27	195.65	1.81
UTIPS 28	147.94	1.93
UTIPS 29	173.10	1.75
UTIPS 30	150.39	1.76
UTIPS 31	186.12	1.92
UTIPS 32	210.41	1.97
UTIPS 33	186.08	1.93
UTIPS 34	179.50	1.81
UTIPS 35	167.08	1.81

Table 4 (continued)

UTIPS 36	188.58	1.98
UTIPS 37	206.35	1.78
UTIPS 38	131.62	1.91
UTIPS 39	192.39	1.91
UTIPS 40	224.02	1.97
UTIPS 41	158.59	1.88
UTIPS 42	160.28	1.82
UTIPS 43	156.76	1.82
UTIPS 44	159.63	1.83
UTIPS 45	184.43	1.96
UTIPS 46	122.98	1.99
UTIPS 47	198.54	1.91
UTIPS 48	223.63	1.85
UTIPS 49	164.18	1.92
UTIPS 50	156.13	1.94
UTIPS 51	207.73	1.92
UTIPS 52	150.46	1.83
UTIPS 53	134.55	1.80
UTIPS 54	197.75	1.87
UTIPS 55	170.40	2.00
UTIPS 56	211.77	1.95
UTIPS 57	153.31	1.81
UTIPS 58	208.89	1.90
UTIPS 59	190.67	1.95
UTIPS 60	182.55	1.92
UTIPS 61	225.89	1.88

Appendix E

Table 5: PCR screening of clinical isolates and the antimicrobial susceptibility profiles towards tetracycline and minocycline.

Sample	TET	MIN	*tetA*	*tetB*
UTIPS 1	S	S	-	-
UTIPS 2	R	S	+	-
UTIPS 3	S	S	-	-
UTIPS 4	R	S	+	-
UTIPS 5	R	S	+	-
UTIPS 6	S	S	-	-
UTIPS 7	R	S	-	+
UTIPS 8	R	S	+	-
UTIPS 9	R	S	+	-
UTIPS 10	R	S	+	-
UTIPS 12	S	S	+	-
UTIPS 13	R	S	+	-
UTIPS 14	R	S	+	+
UTIPS 15	R	S	+	-
UTIPS 16	R	S	+	-
UTIPS 17	R	S	+	-
UTIPS 18	R	S	+	-
UTIPS 19	R	S	+	-
UTIPS 20	R	S	-	+
UTIPS 21	S	S	-	-
UTIPS 22	R	S	+	-
UTIPS 23	S	S	-	-
UTIPS 24	S	S	-	-
UTIPS 25	S	S	-	-
UTIPS 26	S	S	-	-
UTIPS 27	S	S	-	-
UTIPS 28	S	S	-	-
UTIPS 29	R	S	+	-
UTIPS 30	S	S	+	-
UTIPS 31	S	S	-	-
UTIPS 32	R	S	+	-
UTIPS 33	S	S	-	+

Table 5 (continued)

UTIPS 34	R	S	+	-
UTIPS 35	S	S	-	-
UTIPS 36	S	S	-	-
UTIPS 37	S	S	-	-
UTIPS 38	R	S	+	-
UTIPS 39	R	S	-	+
UTIPS 40	S	S	-	-
UTIPS 41	R	S	-	+
UTIPS 42	R	S	+	-
UTIPS 43	R	S	+	-
UTIPS 44	S	S	-	-
UTIPS 45	S	S	-	-
UTIPS 46	S	S	-	-
UTIPS 47	R	S	+	-
UTIPS 48	S	S	-	-
UTIPS 49	S	S	-	-
UTIPS 50	R	S	-	+
UTIPS 51	S	S	-	-
UTIPS 52	R	S	+	-
UTIPS 53	S	S	-	-
UTIPS 54	R	S	+	-
UTIPS 55	R	S	+	-
UTIPS 56	R	R	-	+
UTIPS 57	S	S	-	-
UTIPS 58	S	S	-	-
UTIPS 59	S	S	-	-
UTIPS 60	R	S	+	-
UTIPS 61	S	S	-	-

‘R’ stands for resistant, and ‘S’ stands for susceptible.
‘+’ signifies positive for the gene screened while ‘-’ signifies negative for the gene screened.

Appendix F

Table 6: Representative data of statistical analysis on the negative association between *tetA* gene and gender of patients.

			tetA absent	*tetA* present	Total
sex	Female	Count	24	20	44
		% within sex	54.5%	45.5%	100.0%
		% within *tetA*	72.7%	74.1%	73.3%
		% of Total	40.0%	33.3%	73.3%
	Male	Count	9	7	16
		% within sex	56.3%	43.8%	100.0%
		% within *tetA*	27.3%	25.9%	26.7%
		% of Total	15.0%	11.7%	26.7%
Total		Count	33	27	60
		% within sex	55.0%	45.0%	100.0%
		% within *tetA*	100.0%	100.0%	100.0%
		% of Total	55.0%	45.0%	100.0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.014[a]	1	.907		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.014	1	.907		
Fisher's Exact Test				1.000	.571
N of Valid Cases	60				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 7.20.

b. Computed only for a 2x2 table

Symmetric Measures

		Value	Approximate Significance
Nominal by Nominal	Phi	-.015	.907
	Cramer's V	.015	.907
N of Valid Cases		60	

Appendix G

Table 7: **Representative data of statistical analysis on the positive association between *tetA* gene and tetracycline resistance profile.**

			tetA		
			absent	present	Total
tetracycline	resistant	Count	6	25	31
		% within tetracycline	19.4%	80.6%	100.0%
		% within *tetA*	18.2%	92.6%	51.7%
		% of Total	10.0%	41.7%	51.7%
	susceptible	Count	27	2	29
		% within tetracycline	93.1%	6.9%	100.0%
		% within *tetA*	81.8%	7.4%	48.3%
		% of Total	45.0%	3.3%	48.3%
Total		Count	33	27	60
		% within tetracycline	55.0%	45.0%	100.0%
		% within *tetA*	100.0%	100.0%	100.0%
		% of Total	55.0%	45.0%	100.0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	32.926[a]	1	.000		
Continuity Correction[b]	30.014	1	.000		
Likelihood Ratio	37.559	1	.000		
Fisher's Exact Test				.000	.000
N of Valid Cases	60				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 13.05.

b. Computed only for a 2x2 table

Symmetric Measures

		Value	Approximate Significance
Nominal by Nominal	Phi	-.741	.000
	Cramer's V	.741	.000
N of Valid Cases		60	

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