MOLECULAR DETECTION OF *qnrA* AND *qnrB* GENES IN UROPATHOGENIC *ESHCERICHIA COLI* (UPEC) ISOLATES FROM PERAK

By

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ABSTRACT

MOLECULAR DETECTION OF *qnrA* AND *qnrB* GENES IN UROPATHOGENIC *ESHCERICHIA COLI* (UPEC) ISOLATES FROM PERAK

AN HUI CHING

Uropathogenic *E. coli* (UPEC) is the causative agent in most urinary tract infection (UTI) cases in Malaysia. The overuse of fluoroquinolone antibiotics in treating UTI had been the major cause for the emergence of fluroquinolone antibiotic resistant strains. Several resistance genes had contributed the fluoroquinolone resistance in UPEC, including the *qnrA* and *qnrB* genes. Randomised samples of 75 UPEC clinical isolates had been collected from Hospital Permaisuri Bainun, Ipoh. In this study, the antibiotic resistance rate of the isolates were evaluated. The UPEC isolates were observed to be highly resistant to ampicillin (74.67%), followed by nalidixic acid (60%), levofloxacin (48%), ciprofloxacin (41.33%), and 36% for trimethoprim-sulfamethoxazole (SXT). None of the isolates were resistant to imipenem and tobramycin, while 20% and 2.67% of the isolates were resistant to gentamicin and amikacin respectively. Total DNA was extracted from each isolate and duplex PCR was carried out to detect for *qnrA* and *qnrB* genes. The *qnrB* gene was found to be more prevalent than *qnrA*, as 4.17% (2/48) of isolates resistant to the fluoroquinolone antibiotics (ciprofloxacin, nalidixic acid, levofloxacin) carried *qnrB*, while none of the isolates carried *qnrA* gene. No isolates sensitive to

fluoroquinolone harboured *qnrA* and *B* genes. No significant association had been found between the targeted *qnr* genes and fluoroquinolone resistance rate in the UPEC isolates.

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DECLARATION

I hereby declare that this final year project 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.

An Hui Ching

APPROVAL SHEET

This final year project report entitled "**MOLECULAR DETECTION OF *qnrA* AND *qnrB* GENES IN UROPATHOGENIC ESHCERICHIA COLI (UPEC) ISOLATES FROM PERAK**" was prepared by AN HUI CHING 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:

_____

Assoc. Prof Dr Chew Choy Hoong Date: 26th April 2023

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Date: 26th April 2023

PERMISSION SHEET

It is hereby certified that **AN HUI CHING** (ID No: **19ADB03144**) has completed this final year project thesis entitled "**MOLECULAR DETECTION OF *qnrA* AND *qnrB* GENES IN UROPATHOGENIC ESHCERICHIA COLI (UPEC) ISOLATES FROM PERAK**" under the supervision of Dr. Chew Choy Hoong (Supervisor) from Department of Allied Health Sciences, Faculty of 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,

Hui

(AN HUI CHING)

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LIST OF ABBREVIATIONS

A260	Absorbance at 260 nm
A280	Absorbance at 280 nm
A260/A280 ratio	Nucleic acid purity ratio
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended-spectrum beta lactamases
F	Female
M	Male
MDR	Multidrug Resistant
PCR	Polymerase chain reaction
R	Resistant
RNA	Ribonucleic acid
Rpm	Revolution per minute
S	Susceptible/Sensitive
SXT	Trimethoprim-sulfamethoxazole
Taq	*Thermus aquaticus*
TBE	Tris, boric acid, EDTA
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Urinary tract infection is the most common outpatient infection which mainly affect women. In Malaysia, 50%–80% of women are prone to UTI at least once per lifetime. The most prevalent causative agent for UTI in Malaysia is *Escherichia coli* which attributed to 80%–85% of cases, particularly uropathogenic *E. coli*. UTI is commonly treated with antibiotics. Nowadays, the overuse of antibiotics led to the emergence of antimicrobial resistant strains, including multidrug resistant strains (Manocha, 2012).

Fluoroquinolones are one of the antibiotics used in the empiric treatment for UTI. However, due to the high fluoroquinolone resistance rate in *E. coli*, the Ministry of Health Malaysia (2019) no longer recommends the use of fluoroquinolone to treat uncomplicated UTI. Nevertheless, fluoroquinolone remain as a good option in treating complicated UTI due to its wide spectrum of bacterial target and good pharmacokinetic properties (Maris et al., 2021).

Bacteria can resist to fluoroquinolone by harbouring fluoroquinolone resistance genes such as *qnr* genes. The prevalence of *qnr* genes in UPEC varies between different geographical areas such as China, Iran, Iraq, Pakistan, Egypt, and India (Zhao et al., 2014; Ali et al., 2016; Badamchi et al., 2019; Kammili et al., 2020; Koshki and Mozaffari, 2020; Esmaeel et al., 2022). Besides contributing to

fluoroquinolone resistance, *qnr* genes are also observed to commonly co-exist with beta lactam resistance genes in plasmids, which may give rise to multidrug resistance in bacteria (Salah et al., 2019). Thus, it is important to identify for the presence of *qnr* genes in UPEC before prescribing fluoroquinolone, to prevent treatment failure and further promote the emergence of fluoroquinolone resistant strains. Unfortunately, the prevalence of *qnr* genes in UPEC and their association with the fluoroquinolone resistance rate is still not well studied in Malaysia.

Therefore, the objectives for this project are:

a) To obtain antimicrobial susceptibility rate in UPEC isolates from Perak, Malaysia.
b) To perform duplex PCR detection for *qnr* genes in UPEC isolates from Perak, Malaysia.
c) To observe the distribution of *qnr* genes among UPEC isolates from Perak, Malaysia.
d) To evaluate the association between *qnr* genes carriage and antimicrobial susceptibility profile of UPEC isolates from Perak, Malaysia.

CHAPTER 2

LITERATURE REVIEW

2.1 Urinary tract infection (UTI)

2.1.1 Overview

Urinary tract infection is characterised as bacterial infection anywhere along the urinary tract, including kidneys, ureters, bladder, and urethra. In Malaysia, female is more prone to UTI, in which 50%–80% of them are found to acquire UTI at least once during their lifetime (Manocha, 2012). A study by Mustafa, Ramin and Balingi (2012) revealed the ratio of UTI in women to men was 2.3:1, based on Sabah, Malaysia.

Several signs and symptoms of UTI are dysuria, urgency and frequency to urinate, and cloudy urine (CDC, 2021). However, it is also possible for a patient with UTI to not experience any signs or symptoms. UTI could be diagnosed via urinalysis, in which patient's mid-stream urine sample is cultured, examined under microscope, or tested with dipstick test for the presence of leukocytes and nitrates (Manocha, 2012).

Several factors that lead to increased susceptibility to UTI are gender, age, urinary catheterisation, and frequent sexual intercourse (in female). Female have shorter urethra compared to male; thus, bacteria could easily access to urinary bladder after short distance traveling. Additionally, the postmenopausal changes of hormones

such as oestrogen among senior women weaken the urethra linings and its defence mechanism, allowing the bacteria to colonise and infect the urethra. Conversely, the benign prostatic hyperplasia (BPH), which is common among senior men, is the predisposing disease to UTI. Insertion of urinary catheter and frequent sexual intercourse may introduce bacteria from periurethral or vaginal areas into the urinary tract, therefore lead to UTI in female (Ho, 2019).

In Malaysia, the most prevalent causative agent for UTI is *Escherichia coli*, particularly uropathogenic *E. coli* (UPEC), it accounts for 80%–85% of cases; followed by *Staphylococcus saprophyticus* (5%–15%). The *Klebsiella* species, *Proteus* species, and *Pseudomonas aeruginosa* are more common in healthcare associated UTI. Most of the bacteria which lead to UTI are Gram-negative bacteria (Manocha, 2012).

2.1.2 Classification of UTI

The widely acceptable UTI classification system is developed by US Centers for Disease Control and Prevention (CDC), Infectious Diseases Society of America (IDSA), and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). This system classify UTI into complicated and uncomplicated based on the clinical symptoms, laboratory information and microbiological findings. However, this classification is found to be insufficient to reflect the complexity of the disease (Smelov, Naber and Johansen, 2016).

Therefore, according to Smelov, Naber and Johansen (2016), an improved UTI classification system was developed by the European Section of Infection in Urology (ESIU) together with EAU and International Consultation on Urological Diseases (ICUD) in 2012. From this system, the UTI is additionally classified into symptomatic and asymptomatic, based on the clinical manifestation and risk factors of patient. In addition, the ORENUC system is introduced to phenotype risk factors into groups (O, R, E, N, U, C) for the ease of UTI classification. Symptomatic UTI can be further classified into cystitis, pyelonephritis, and urosepsis based on clinical symptoms and severity (Smelov, Naber and Johansen, 2016).

2.1.3 Uropathogenic *Escherichia coli* (UPEC)

The major causative agent to community-acquired UTI is uropathogenic *Escherichia coli* (UPEC). UPEC is unique from other *E. coli* strains as UPEC carries genomic Pathogenicity Islands (PAI) which contains the genes encoding for UPEC virulence factors. The virulence proteins expressed by UPEC include lipopolysaccharide, flagella, pili, toxins and more (Figure 2.1). These virulence proteins are required for the adherence, colonisation, invasion, replication, and evasion of host immune system in host's urinary tract (Terlizzi, Gribaudo and Maffei, 2017).

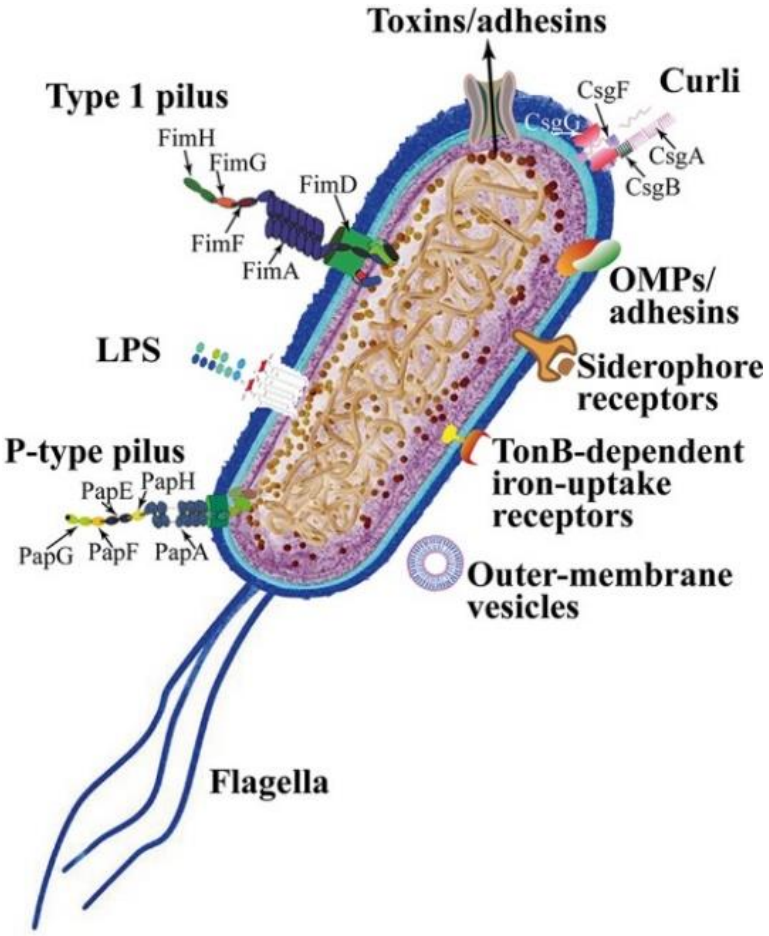

Figure 2.1: Examples of UPEC virulence proteins (Adapted from Terlizzi, Gribaudo and Maffei, 2017).

The pathogenesis of UPEC is described as follow. UPEC in the periurethral, or vaginal areas acquire access to urethra, and then travel to the host's urinary bladder with unknown mechanisms. In the urinary bladder, UPEC binds to facet cells, which is bladder superficial epithelial cells, using type 1 pili. This allows bacterial adherence and later, the internalisation of bacteria into the cells. UPEC will then colonise, and form biofilm-like masses known as intracellular bacterial communities (IBC) in the cell cytoplasm. In response to that, the IBC containing facet cells are

largely dissipated, releasing IBCs into urine. Furthermore, UPEC in IBC will also undergo structural changes to be released out of the cells and spread to the neighbouring cells (McLellan and Hunstad, 2016).

2.1.4 Treatments for UTI

Generally, the empirical treatment for UTI is varied based on its classification (complicated, uncomplicated, symptomatic, asymptomatic). Treatments for asymptomatic UTI is usually not required except for pregnant women or prior to performing urology surgical procedure. Conversely, symptomatic UTI is usually treated with antibiotics. Based on the treatment guideline by Ministry of Health Malaysia (2019), patient diagnosed with cystitis are treated with nitrofurantoin or cephalexin as first line drugs for 5-7 days. However, nitrofurantoin should be avoided if the patient's glomerular filtration rate (GFR) is below 30 ml/min, or the patient is in third trimester of pregnancy. Fosfomycin is recommended for pregnant patient with cystitis, who infected by multi-drug resistant (MDR) Gram-negative bacteria. On the other hand, the first line drugs for pyelonephritis are amoxicillin/clavulanate, or ampicillin/sulbactam for outpatients; and amoxicillin/clavulanate, ampicillin/sulbactam or cefuroxime for inpatient. Ultrasound of upper urinary tract should be performed prior to the treatment to eliminate obstructive pyelonephritis. Furthermore, oral therapy (amoxicillin/clavulanate, cephalexin) or parenteral therapy (amoxicillin/clavulanate, cefuroxime, ampicillin/sulbactam) with or without the addition of aminoglycoside are recommended for complicated UTI (Ministry of Health Malaysia, 2019). The

recommended clinical flow for treating UTI in Malaysian primary care settings is displayed in Figure 2.2.

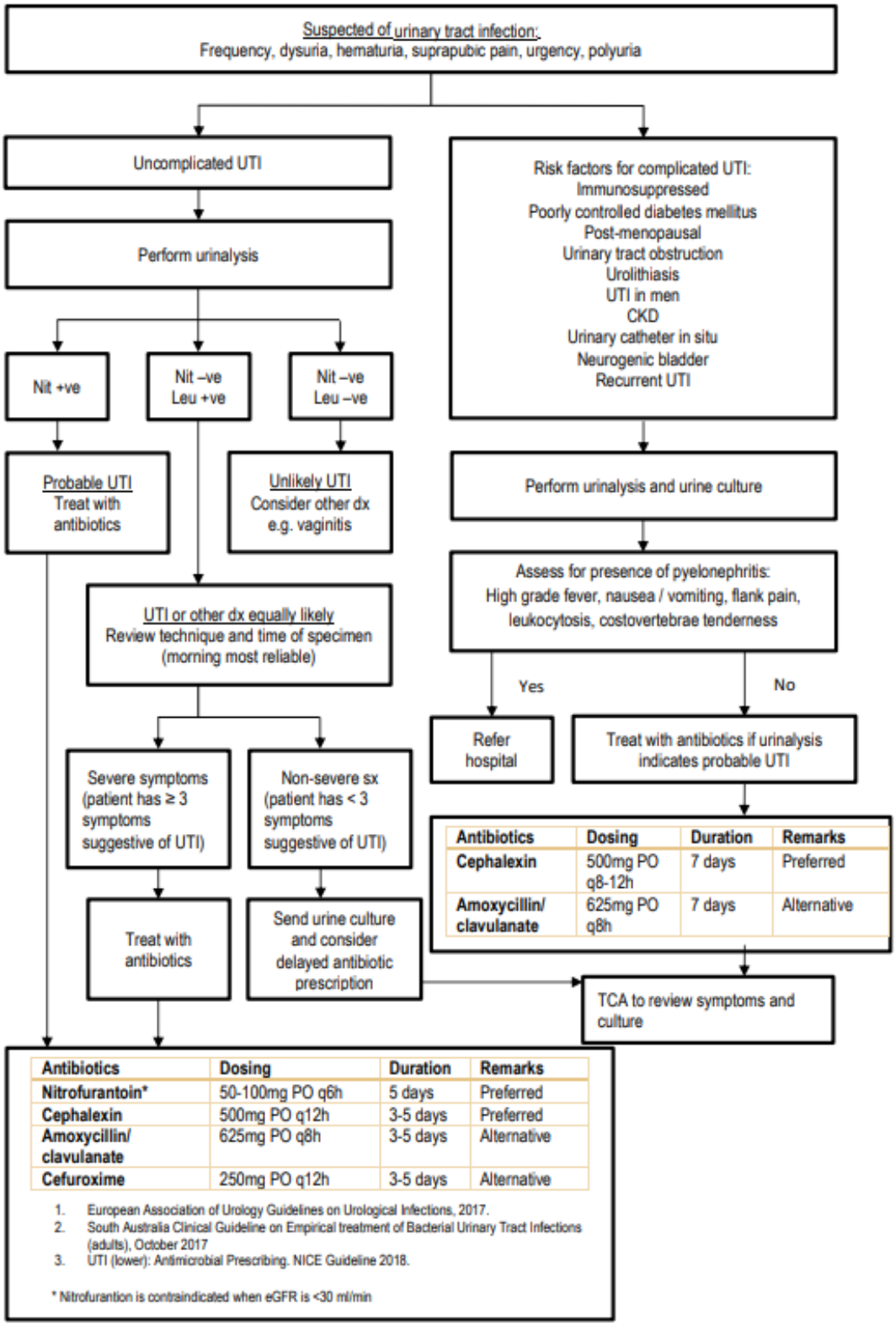

Figure 2.2: Clinical flow for treating UTI in Malaysian primary care settings (Adapted from Ministry of Health Malaysia, 2019).

Nevertheless, in Malaysian primary care settings, 70.3% patient diagnosed with UTI and 25.8% patient with UTI like symptoms are prescribed with antibiotics. The high usage of antibiotics for UTI in clinical settings has led to the emergence of antibiotic resistant strains. As a result, a relapse of UTI would occur due to the treatment failure in eradicating all the bacteria in the patient (Teng et al., 2011).

According to Terlizzi, Gribaudo and Maffei (2017), beside antibiotics, alternative cures are also available for UTI patient. Some of the cures are vaccines, probiotics, and oestrogens. The vaccines developed are based on bacterial (UPEC) virulence proteins such as adhesins, antimicrobial peptides and siderophores, which will increase the efficiency of bacteria removal by patient's immune system after injection. Vaccine is seen to be more efficient in treating upper urinary tract infection than lower urinary tract infection. However, the use of vaccines may lead to disruption of the proteobacteria population of *E. coli* in patient's gut. On the other hand, probiotics such as *Lactobacilli* species are suggested in treating recurrent UTI. Probiotics are observed to inhibit the growth of UPEC regardless of its sensitivity to antibiotics. Furthermore, vaginal oestrogen therapy with hyaluronic acid, chondroitin sulphate, curcumin and quercetin are suggested for post-menopausal women to prevent recurrent of UTI (Terlizzi, Gribaudo and Maffei, 2017).

2.2 Fluoroquinolones

2.2.1 Background

Fluoroquinolones are modified quinolones. The first quinolone, nalidixic acid, was discovered by George Lesher and his colleagues in 1962, when they were

investigating the by-product of the synthesis of anti-malarial chloroquine. Nalidixic acid was the first fluoroquinolone used in UTI treatment. Although nalidixic acid has great antibacterial activity against Gram-negative bacteria, it has short half-life in serum and fast renal elimination. Therefore, modification was done by substituting the sixth position of the core quinolone ring structure with fluorine and adding a piperazinyl derivative to the seventh position of the ring, which produce fluoroquinolone antibiotics. The fluoroquinolones are improved from generation to generation by further modifying its core quinolone structure to target wider range of bacteria and to improve its pharmacokinetic profiles (Figure 2.3), (Maris et al., 2021).

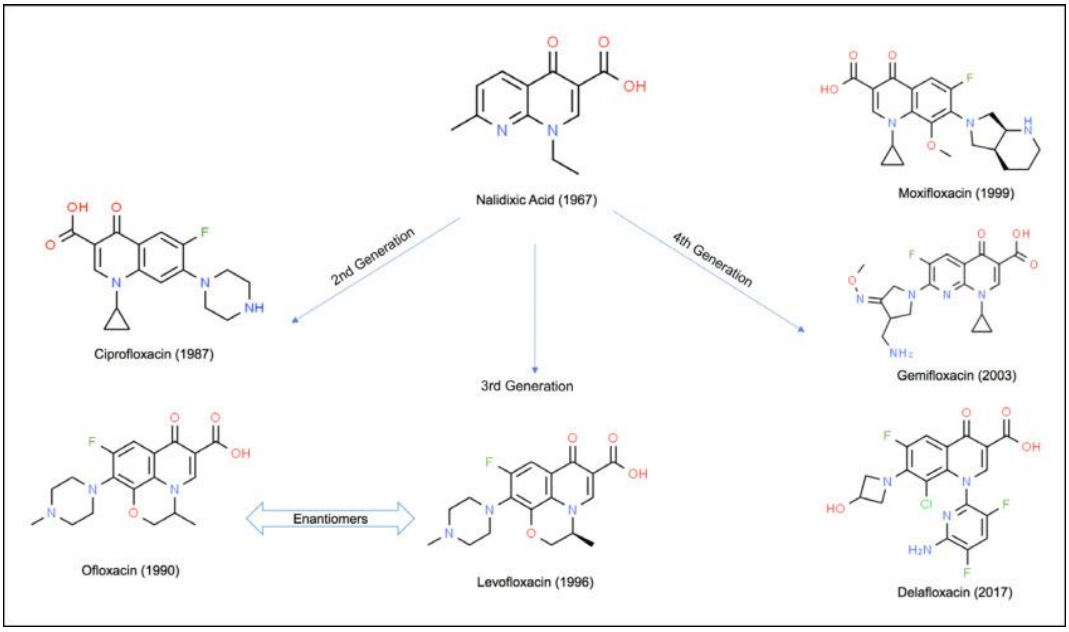

Figure 2.3: Structure of different generations of fluoroquinolone antibiotics by modifying their quinolone backbone (Adapted from Maris et al., 2021).

In early 2000s, the fluoroquinolone antibiotics are one of the most prescribed antibiotic classes in bacterial infection. Fluoroquinolone is favoured as it has potent activity against wide spectrum of bacteria, good oral bioavailability, applications in various fields, and beneficial pharmacokinetic properties. However, some rare and severe side effects, such as tendinopathy, arise with the wide use of fluoroquinolone. Since 2016, the Food and Drug Administration (FDA) advises to stop the prescription of fluoroquinolone in mild infections such as uncomplicated UTI, where the risks outweigh the benefits. However, fluoroquinolone is still a good therapeutic option for complicated UTI (Maris et al., 2019).

Nonetheless, the wide application of fluoroquinolone in clinical settings has led to the increased emergence of resistant bacterial strains. Therefore, fluoroquinolone is no longer recommended in Malaysian primary healthcare as empiric treatment for UTI. If the use of fluoroquinolone is necessary, combined prescription with other antibiotics are recommended (Ministry of Health Malaysia, 2019).

2.2.2 Mechanisms of fluoroquinolone

Fluoroquinolones exert its bactericidal effect by disrupting the bacterial DNA replication via inhibition of its DNA gyrase and topoisomerase VI. DNA gyrase is essential in bacteria as it functions to establish negative supercoiling in DNA ahead of the replication fork to release the tension of DNA created by helicase during DNA replication. On the other hand, the role of topoisomerase VI is decatenation of daughter chromosomes at the end of replication (Blondeau, 2004).

Fluoroquinolone bind to bacterial DNA, DNA gyrase and/or topoisomerase VI to form antimicrobial complex which result in the inhibition of the enzymes. Consequently, the positive supercoiling in DNA ahead of the replication fork prevents the unwinding activity of helicases, halting the movement of replication fork. Inhibition of topoisomerase VI also prevent the separation of nascent DNA. As a result, the DNA replication in bacteria is inhibited which eventually lead to cell death. In Gram-negative bacteria such as UPEC, fluroquinolone inhibit DNA gyrase while in Gram-positive bacteria topoisomerase VI is primarily inhibited. However, dual inhibition of both enzymes is also observed in Gram-positive bacteria. In addition, fluoroquinolone is specific towards DNA gyrase and topoisomerase VI which are not found in human (Blondeau, 2004).

2.3 Fluoroquinolone resistance genes

2.3.1 Overview

Fluoroquinolone resistance genes confer to survival of bacteria in the presence of fluoroquinolone antibiotics. In general, there are three types of resistance genes that contribute to fluoroquinolone resistance: target-mediated resistance genes, chromosomal-mediated resistance (CMR) genes and plasmid-mediated quinolone resistance (PMQR) genes. Examples of target mediated resistance genes are *gyr* gene and *par* gene, CMR genes are *omp* genes, and PMQR genes include *qnr* genes, *aac-(6')-Ib-cr* genes, *qep* genes (Aldred, Kerns and Osheroff, 2014). PMQR genes are usually carried in plasmid and thus transmitted horizontally among bacteria. In contrast, target-mediated resistance genes are transmitted vertically. Furthermore, co-existence of other resistance genes in plasmids, such as beta-lactam resistance

genes, with PMQR genes are common. Therefore, although bacteria that harbour PMQR genes confer to low level of resistance, it may lead to higher level resistance due to combination with other resistance mechanism or lead to selection of resistant mutant, which eventually increased the risk of failure in treatment (Jacoby, Strahilevitz and Hooper, 2014; Maris et al., 2021).

2.3.2 Fluoroquinolone resistance mechanisms

Generally, the fluoroquinolone resistance mechanisms can be grouped into three: target mediated resistance, PMQR mediated resistance, and chromosomal mediated quinolone resistance.

The resistance genes involve in target mediated resistance are *gyr* genes and *par* genes. these two genes are responsible for the synthesis of DNA gyrase subunits and topoisomerase VI subunits, and these two enzymes are the targets of fluoroquinolone. Thus, some fluoroquinolone resistant bacteria carry point mutation on *gyr* and/or *par* genes, which the mutations are localised to region of drug binding site on the enzymes. Consequently, fluoroquinolone unable to bind to the enzymes, therefore decrease their susceptibility to the antibiotic (Figure 2.4a). The *gyrA* or *parC* gene mutations are more common than *gyrB* or *parE* gene mutations. This may be explained by higher level fluoroquinolone resistance (higher MIC) conferred by *gyrA* gene mutation than *gyrB* gene. As for *par* genes, the reason for this phenomenon is yet to be known (Hooper, 2001; Hopkins, Davies and Threlfall, 2005; Correia et al., 2017).

The PMQR mediated resistance are due to *qnr*, *qep*, and/or *aac-(6')-Ib-cr* genes. The *qnr* genes encode for pentapeptide-repeat proteins which will bind to DNA gyrase and topoisomerase VI, to destabilise the antimicrobial complex formed. Therefore, the two enzymes are released and free to perform its function. On the other hand, the *aac-(6')-Ib-cr* genes encode for aminoglycoside acetyltransferase protein variant, which function to acetylate nitrogen of the C7 piperazine ring found on fluoroquinolone, thus reducing the fluoroquinolone's activity (Figure 2.4d). Lastly, the *qep* genes encode for efflux pump such as QepA and OqxAB. The QepA pump is from the major facilitator superfamily (MFS) transporter which was seen overexpressed in Gram-positive fluoroquinolone resistant bacteria. The OqxAB pump is from resistance nodulation division superfamily (RND) transporter which primarily observed with overexpression in Gram-negative fluoroquinolone resistant bacteria (Figure 2.4c), (Correia et al., 2017; Maris et al., 2021).

In addition, increased efflux of fluoroquinolone together with reduced influx contribute to fluoroquinolone resistance in Gram-negative bacteria. This is because under normal condition, the outer membrane of Gram-negative bacteria serve as extra permeability barrier for the fluoroquinolone to enter. Therefore, fluoroquinolone rely on the porin channels such as outer membrane protein (Omp) in bacterial outer membrane for entrance. These porin channels are encoded by *omp* genes. The influx of fluoroquinolone in Gram-negative bacteria is necessary for it to act on the cellular enzymes. In Gram-negative fluoroquinolone resistant bacteria, the genes encode for the Omp proteins are mutated, leading to its decrease or loss of expression, and reducing the influx of fluoroquinolone into the bacteria.

Moreover, the OmpX protein are found to be overexpressed in fluoroquinolone resistant bacteria. This protein acts as a down-regulator for the expression of other porin proteins (Figure 2.4b), (Hooper, 2001; Correia et al., 2017). Figure 2.4 shows the overview of fluoroquinolone resistance mechanisms.

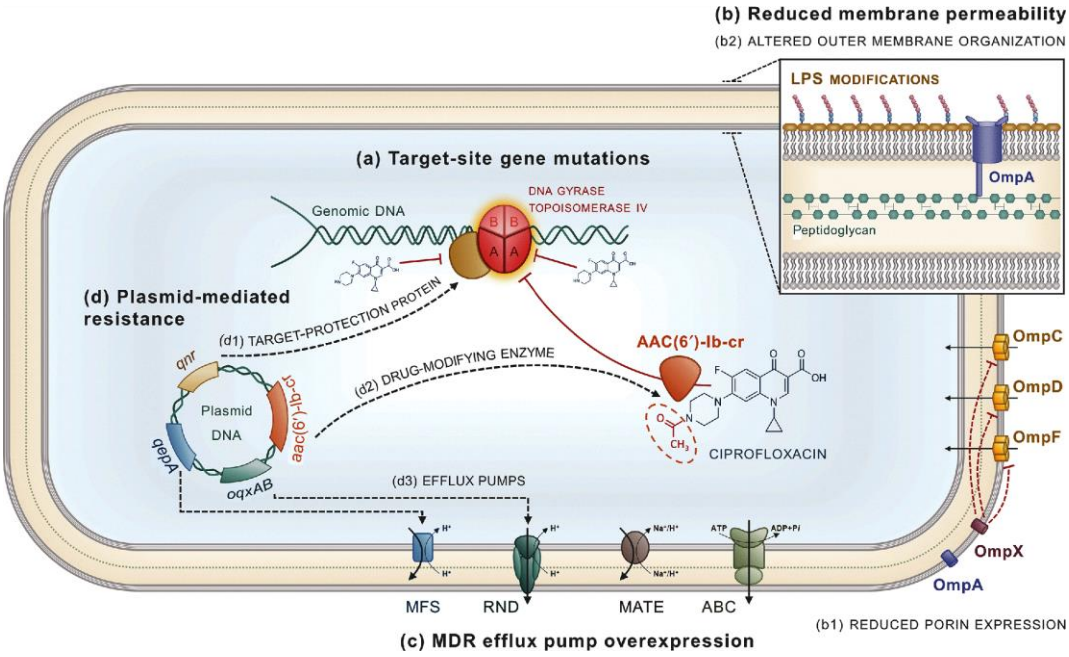

Figure 2.4: Overview of fluoroquinolone resistance mechanisms (Adapted from Correia et al., 2017).

2.4 The *qnr* genes

2.4.1 Overview

The *qnr* genes are one of the PMQR genes that confer to fluoroquinolone resistance in bacteria. The *qnrA* gene was the first discovered *qnr* genes in *K. pneumoniae*. Following its discovery, other plasmid-mediated *qnr* genes are discovered all around the world. The *qnr* genes is one family for various *qnr* variants. In general,

most of the variants are observed to vary ≥ 35% with *qnrA* or each other in sequence. Currently, the discovered *qnr* genes are *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *qnrVC*. Furthermore, allelic variants are also observed among each *qnr* genes respectively, which differ from each other by 10% or less. Examples of *qnr* allelic variants are *qnrA1*, *qnrB4* and *qnrS1* (Hooper and Jacoby, 2015).

The *qnr* family under PMQR genes are found in plasmids with different size and incompatibility specificity. They are also found consistently associated with transposable element particularly IS*CR1* and IS*26*. The IS*CR1* allow transposition of *qnr* genes and act as an active promoter for gene expression. However, the *qnrd1* and *qnrS2* are found located within mobile insertion cassettes, with flanking inverted repeats but with absence of transposase. On the other hand, *qnrVC* gene is currently the only one which located in cassette linked with *attC* site. Table 2.1 lists the *qnr* allelic variants and their associated mobilising elements, as well as their plasmid incompatibility (Inc) group. In addition, *qnr* genes found in plasmids are usually observed to co-exist with other resistance genes (Jacoby et al., 2014; Hooper and Jacoby, 2015).

Table 2.1: The *qnr* genes and their associated plasmids and mobilising elements.

***qnr* allelic variant**	**Plasmid Inc group(s)**	**Mobilising element(s)**
qnrA1	A/C2, F11, H12, I1, L/M, N	IS*CR1*
qnrA3	N	IS*CR1*, IS*26*
qnrA6	A/C	IS*CR1*
qnrB1	$F11_K$, H family, L/M	Orf1005, IS*26*
qnrB2	FIA, FII, L/M, N	IS*CR1*
qnrB4	FIA, FIIAs, L/M, R	IS*CR1*
qnrB6	FIIAs	IS*CR1*

Table 2.1 (continue):

qnrB10	UT	IS*CR1*
qnrB19	Cole, L/M, N	IS*Ecp1*, IS*26*
qnrB20	-	Orf1005, IS*26*
qnrS1	Cole, FI, HI1, HI2, I1, L/M, N, NT, R, UT, X1, X2	IS*2*, IS*26*, IS*Ecl2*
qnrS2	Q, U	Mic[b]
qnrC	-	IS*Pmi1*
qnrD1	UT[a]	Mic[b]
qnrVC1	-	*attC*
qnrVC4	-	IS*CR1*

Mic[b]: mobile insertion cassette
UT[a]: Untypable

(Adapted from Hooper and Jacoby, 2015).

2.4.2 Prevalence of *qnr* genes in UPEC

Compared to other *qnr* genes, *qnrA* (0%–18%), *qnrB* (0%–63.8%) and *qnrS* (3.53%–62.5%) are the prevalently detected by researchers from various geographical areas such as Iran, Iraq, India, Pakistan, China, and Egypt (Zhao et al., 2014; Ali et al., 2016; Badamchi et al., 2019; Kammili et al., 2020; Koshki and Mozaffari, 2020; Esmaeel et al., 2022).

2.5 Multidrug resistance

Although *qnr* genes are resistance genes specific to fluoroquinolone antibiotics, the overuse of antibiotics in UTI treatment has contributed to the emergence of multidrug resistant (MDR) strains. For instance, the *qnr* genes are commonly observed to co-exist with beta lactam resistance genes in plasmids. Several resistance mechanisms might be utilised by MDR bacteria simultaneously. The mechanisms include enzymatic inactivation of antibiotics, reduce permeability of

antibiotics, and prevent binding of antibiotics to target sites. The emergence of MDR strains especially in UPEC had increased difficulty in treating UTI. Therefore, the prescription of antibiotics for UTI treatment should be regulated (Dehbanipour et al., 2016; Ochoa et al., 2016).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

Table 3.1 includes the list of chemicals and reagents used in this study, with their corresponding manufacturer and country.

Table 3.1: List of chemicals and reagents used with respective manufacturer and country.

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 (SXT), Ampicillin, Amikacin antibiotic discs	Oxoid Ltd., United Kingdom
Ciprofloxacin, Levofloxacin, Tobramycin antibiotic discs	Himedia Laboratories Pvt. Ltd., India

Table 3.1 (continue)

Gentamicin antibiotic discs	Becton Dickinson Company, United States
Nalidixic acid, Imipenem antibiotic discs	Liofilchem S.R.L., Italy
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 *qnrA*, Forward and reverse primers of *qnrB*	IDT, Singapore
100 bp DNA ladder RTU	Bio-Helix Co., Ltd., 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
QIAquick® PCR Purification Kit	QIAGEN®, Germany

3.2 General Plan of the Experimental Work

3.2.1 Sample collection

Clinical isolates of uropathogenic *Escherichia coli* (UPEC) were collected from Hospital Permaisuri Bainun, Ipoh, on 2020 to 2021. In this project, a total of 75 clinical isolates from urine samples were evaluated. The demographic data for each clinical isolates were acquired from the hospital (Appendix A). Prior to this project, ethical approval from Medical Research and Ethics Committee was obtained. The clinical isolates were identified through MALDTI-TOF mass spectrometry at Hospital Permaisuri Bainun in Ipoh and were classified as *Escherichia coli*. Only pure UPEC isolates with significant growth of more than 100,000 CFU/ml were collected from the hospital.

3.2.2 Sample preparation

The clinical isolates were inoculated from glycerol stock and streaked on tryptic soy agar (TSA) with aseptic techniques. After streaking, the samples were incubated overnight at 37ºC. The revived samples were stored at 4ºC until further testing.

3.2.3 Antimicrobial susceptibility test

The antimicrobial susceptibility test was carried out using Kirby-Bauer Disc Diffusion method (Hudzicki, 2009). The antibiotic discs used were trimethoprim-sulfamethoxazole (25 µg), ampicillin (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), tobramycin (10 µg), gentamicin (10 µg), and nalidixic acid (30 µg). To prepare colony suspension with turbidity of 0.5 McFarland standard, one or two colonies were taken from master culture plate with sterile inoculation loop and put into 5 ml

sterile saline water. A sterile cotton swab was dipped into the colony suspension, and excess saline water was removed by pressing the swab against the inner wall of centrifuge tube. Mueller Hinton agar was evenly streaked with suspension using the wet cotton swab and allowed to sit for 3–5 minutes. Antibiotic discs were placed on the swabbed Mueller Hinton agar with sterile forceps. The discs were gently pressed onto the agar. The agar plates were then incubated at 37ºC for 16 to 18 hours. Based on the standard of Clinical and Laboratory Standard Institute (CLSI) guidelines (2022), (Appendix B), the diameter of each inhibitory zones was measured in millimetres and classified as resistant (R), intermediate (I), or sensitive (S). *Escherichia coli* ATCC 25922 was used as negative control.

3.2.4 Total DNA extraction

The total DNA of each clinical isolate was extracted with fast-boil method (Kor, Choo and Chew, 2013). Prior to the DNA extraction, one colony of each clinical isolates was taken from respective master culture plate and inoculated into sterile nutrient broth. The colony suspensions were incubated for 20 hours at 37ºC in shaking incubator at 200 rpm. Subsequently, 1.2 ml inoculum was transferred into sterile 1.5 ml microcentrifuge tube. The inoculum was centrifuged at 11200 *g* for 5 minutes. The supernatant was removed, and the bacterial pellet was resuspended with 300 µl sterile distilled water. The suspension was boiled at 100ºC for 5 minutes using a heat block and immediately incubated on ice for another 2 minutes. The suspension was then centrifuged at 11200 *g* for 2 minutes. The supernatant which contained DNA was aspirated into new sterile 1.5 ml microcentrifuge tube. The DNA purity and concentration were measured via Thermo Scientific™ nanodrop™

2000/2000c Spectrophotometer. Any A260/A280 ratio within 1.8–2.0 for the DNA was interpreted as pure. The DNA was stored at -20ºC until subsequent assays.

3.2.5 Duplex PCR detection of fluoroquinolone resistance gene

Prior to the resistance gene detection, duplex PCR conditions from Salah et al. (2019) were used to ensure optimum amplification of the targeted gene, *qnrA* and *qnrB* genes. The PCR master mix was prepared as listed in Table 3.2. The DNA concentration of each isolate was standardised to 50 ng/µl. The primer sequences and their expected product sizes are included in Table 3.3. The cycling condition used for duplex PCR detection is shown in Table 3.4. All PCR reactions were performed using Bio-Rad T100 Thermal Cycler PCR machine.

Table 3.2: The components used to prepare PCR master mix for single reaction.

Components	**Initial Concentration**	**Final Concentration**	**Volume (µl)**
Sterile distilled water	-	-	11.00
Taq buffer	5x	1x	5.00
$MgCl_2$	25 mm	1.5 mm	1.50
qnrA primer (F)	10 µM	0.3 µM	0.75
qnrA primer (R)	10 µM	0.3 µM	0.75
qnrB primer (F)	10 µM	0.3 µM	0.75

Table 3.2 (continue):

qnrB primer (R)	10 µM	0.3 µM	0.75
dNTP	10 mm	100 µM	0.25
Taq DNA polymerase	5 U	1.25 U	0.25
DNA template	-	50 ng/µl	4.00
Total Volume	-	-	25.00

Table 3.3: Primer sequences and their expected product size.

Gene	Sequence (5'–3')	Size (bp)	Citation
qnrA	For.: ATTTCTCACGCCAGGATTTG	516	(Salah et al.,
	Rev.: GATCGGCAAAGGTTAGGTCA		2019).
qnrB	For.: GATCGTGAAAGCCAGAAAGG	469	
	Rev.: ACGATGCCTGGTAGTTGTCC		

Table 3.4: Cycling conditions of duplex PCR (Salah et al., 2019).

Steps	Temperature (ºC)	Duration (s)	Number of cycles
Initial denaturation	95	300	1
Denaturation	95	45	32
Annealing	53	45	
Extension	72	60	
Final extension	72	300	1

3.2.6 Agarose gel electrophoresis analysis

PCR products were analysed via agarose gel electrophoresis. 1x TBE buffer was prepared from the stock solution (10x TBE buffer) as running buffer. The reagents and quantity used for preparing 10x TBE buffer are listed in Table 3.5. The PCR products were analysed on 1.5% (w/v) agarose gel at 90V for 50 minutes. Two µl of GelRed was added into 50 ml agarose gel solution, to pre-stain the gel. The DNA ladder used was Bio-Helix 100 bp DNA ladder H3 RTU as molecular weight size marker for product size interpretation. After the run, the gel was viewed with Molecular Imager® Gel Doc^{TM} XR System.

Table 3.5: Reagents used for preparation of 10x TBE buffer (1L).

Reagents	Quantity (g)	Final Concentration	Citation
Tris base	121.1	1 M	(Cold Spring
Boric acid	61.8	1 M	Harbor
EDTA	7.4	0.02 M	Protocols, 2010)

3.2.7 PCR purification for DNA sequencing

After gel analysis, PCR product with matching band size displayed in Table 3.3 was purified with QIAquick® PCR Purification Kit. A total of 75 µl PCR product was purified following the protocol provided with the purification kit. The concentrated DNA template was sent to First Base Laboratories Sdn Bhd for Sanger sequencing to confirm the gene. The sequenced gene was identified via NCBI BLAST.

3.2.8 Statistical analysis

The data collected from this project was evaluated with IBM® SPSS® Statistics 26.0.0 software. The association between the prevalence of the *qnrA* and *qnrB* gene and antimicrobial susceptibility profile was analysed with *Chi*-square tests. All data were evaluated based on two-tailed tests of significance, with $p < 0.05$ considered as statistically significant for positive association.

CHAPTER 4

RESULTS

4.1 Overview

The antimicrobial susceptibility profile and *qnr* genes harbouring genotypic profile of 75 UPEC clinical isolates were collected via Kirby-Bauer disc diffusion method and duplex PCR gene detection, respectively. From the Kirby-Bauer disc diffusion test, most isolates showed resistance to ampicillin, followed by nalidixic acid and levofloxacin. Forty-eight (64%) isolates were resistant to antibiotics from the fluoroquinolone group, while 27 (36%) were sensitive to it. Among the fluoroquinolones resistant isolates, two out of 48 (4.17%) harboured *qnrB* gene, while none of the isolates harboured *qnrA* gene. The association between the phenotypic profile and genotypic profile of isolates was evaluated with two-tailed *Chi*-square test or Fisher's exact test. There was no statistical association between the fluoroquinolone resistance profile and *qnr* genes carriage in UPEC clinical isolates. The association between antimicrobial resistance profile and *qnr* genes carriage for tobramycin and imipenem could not be calculated as none of the isolates were found to be resistant to these two antibiotics.

4.2 Antimicrobial susceptibility profile

The clinical isolates' antimicrobial susceptibilities were tested against trimethoprim-sulfamethoxazole (SXT), ampicillin, amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin, nalidixic acid, and imipenem antibiotics.

The zone of inhibition for each antibiotic disc was measured, and its susceptibility was interpreted based on CLSI guidelines displayed in Appendix B. Isolates interpreted as ‘intermediate’ and ‘resistant’ were grouped as ‘resistant’ for ease of analysis, as shown in Appendix C. The representative image for the antimicrobial susceptibility test is presented in Figure 4.1.

From Appendix C, 64 out of 75 isolates (85.33%) were resistant to at least one antibiotic tested. Eight out of 75 isolates (10.67%) showed resistance to more than three antibiotic classes tested (fluoroquinolone, aminoglycoside, carbapenem, penicillin, sulphonamide), indicating that these isolates were multi-drug resistant strains.

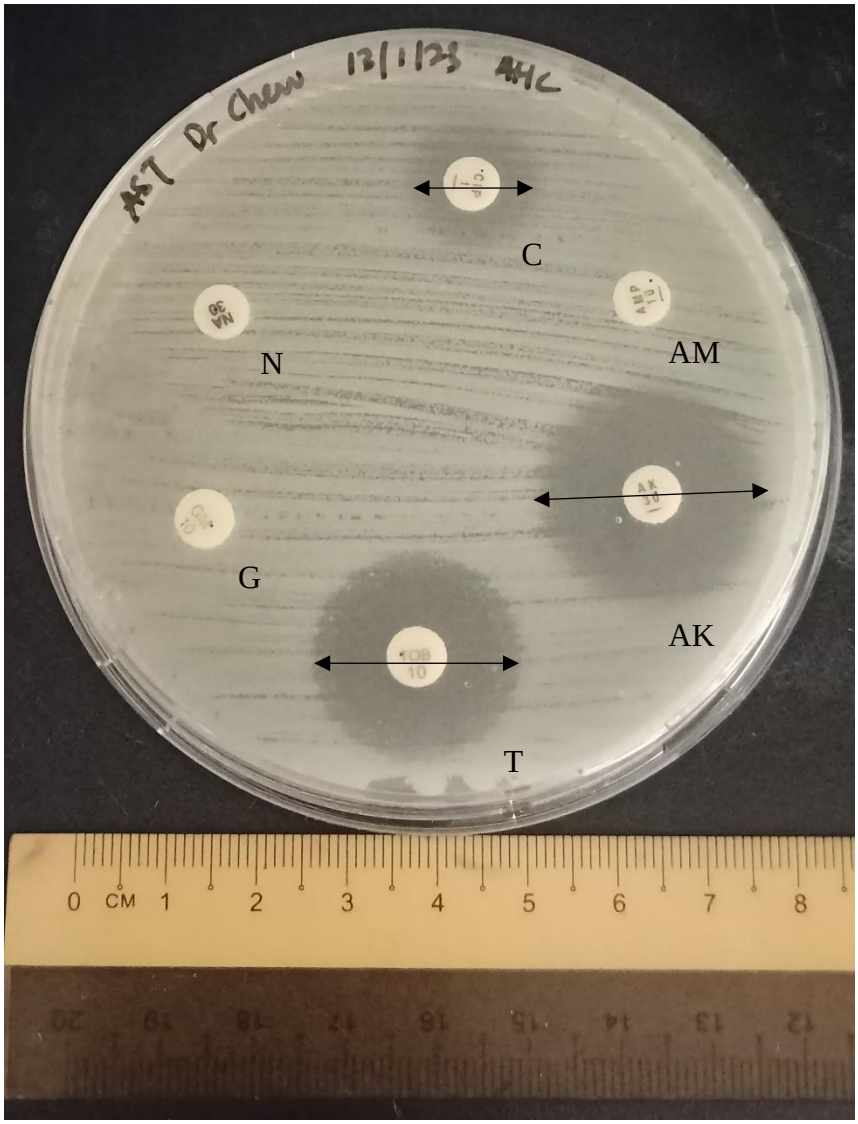

Figure 4.1: Representative image for antimicrobial susceptibility test.

AK: Amikacin (30 µg); **T:** Tobramycin (10 µg); **G:** Gentamicin (10 µg); **N:** Nalidixic Acid (30 µg); **C:** Ciprofloxacin (5 µg); **AM:** Ampicillin (10 µg). The zone of inhibition was indicated by black arrows. UPEC32 were fully resistant to gentamicin (0 mm), nalidixic acid (0 mm), ciprofloxacin (14 mm) and ampicillin (0 mm), while sensitive to tobramycin (23 mm) and amikacin (26 mm).

The distribution of antimicrobial susceptibility profile for the isolates is presented in Figure 4.2. The antibiotic with the highest number of isolates resistant to, was ampicillin (74.67%), followed by the fluoroquinolones: nalidixic acid, levofloxacin,

and ciprofloxacin with the frequency of 60%, 48%, and 41.33%, respectively. None of the isolates were resistant to imipenem and tobramycin. Among the aminoglycoside group, tobramycin had zero isolates resistant to it, followed by amikacin (2.67%) and gentamicin being the highest in the group (20%). On the other hand, 27 out of 75 isolates (36%) showed resistance to SXT.

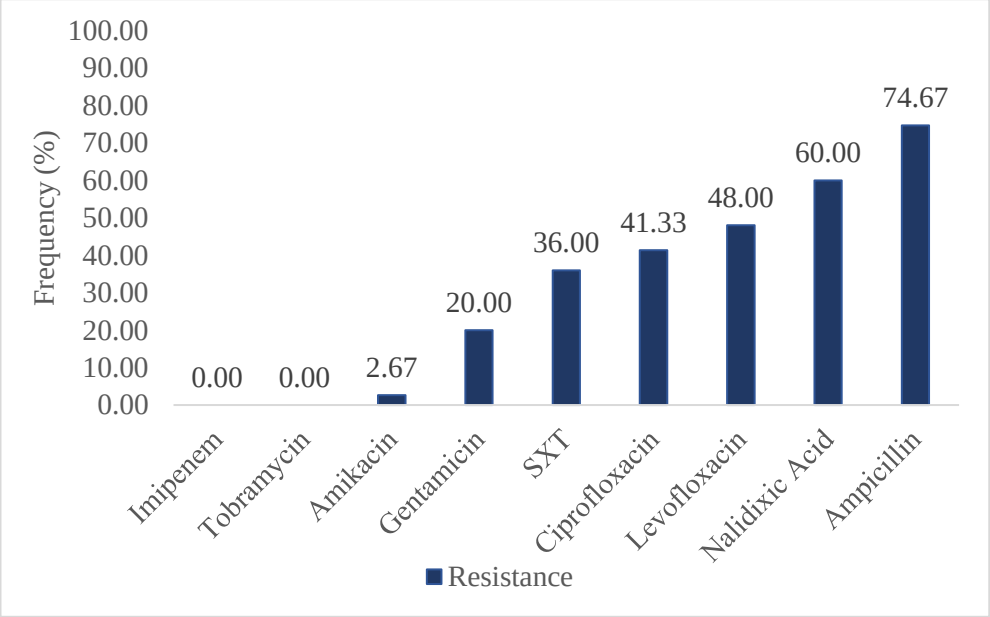

Figure 4.2: Distribution of antimicrobial susceptibility profile in 75 UPEC isolates.

4.3 DNA concentration and purity

The purity and concentration of each extracted DNA were measured. The purity of DNA was interpreted based on nucleic acid 260/280 ratios (Blue-Ray Biotech, 2019). The purity and concentration of the extracted DNA were included in Appendix D. The DNA concentration of samples were ranged from 59.7 ng/µL to

406.7 ng/µL. The A260/A280 ratio of most of the extracted DNA were over 2.0, which indicated RNA contamination. However, this was expected as fast boil method was isolating whole nucleic acids from cell, including RNA.

4.4 Detection of fluoroquinolone resistance genes (*qnr*) in isolates

Seventy-five UPEC clinical isolates were screened for two fluoroquinolone resistance genes, *qnrA* (469 bp) and *qnrB* (519 bp) using duplex PCR. Only two (2.7%) UPEC strains screened positive for *qnrB* gene, while none screened positive for *qnrA* gene. Thus, the *qnrB* gene has a higher prevalence in UPEC strains compared to *qnrA* gene. Seventy-three (97.33%) UPEC strains screened did not carry either *qnrA* or *qnrB* genes, and the coexistence of *qnrA* and *qnrB* genes was not found in any UPEC strains. Figure 4.3 shows the representative gel electrophoresis image of duplex PCR screening for *qnrA* and *qnrB* genes, and Figure 4.4 shows the distribution of *qnr* genes among the samples. Band size (469 bp) was seen in lane 2 to 5 which corresponded to the expected amplicon size of *qnrB* amplification.

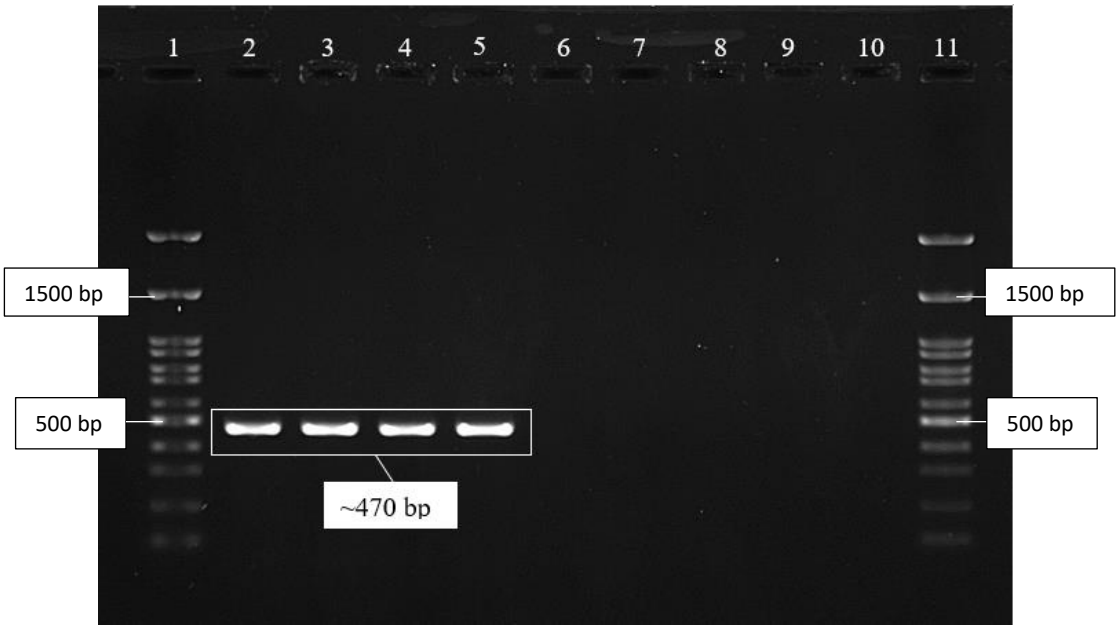

Figure 4.3: Representative gel electrophoresis image of duplex PCR screening for *qnr* genes.

Lane one and lane 11 were loaded with 100 bp DNA ladder. Two *qnrB* (469 bp) positive UPEC strains (UPEC32 and UPEC35) were shown in the image (lane two, three and lane four, five respectively). Lane three was an experimental duplicate of UPEC32 (in lane two), and lane four was an experimental duplicate of UPEC35 (in lane three). Lane six to nine were loaded with *qnr* genes negative UPEC strains. Lane 10 was loaded with sterile distilled water as negative control.

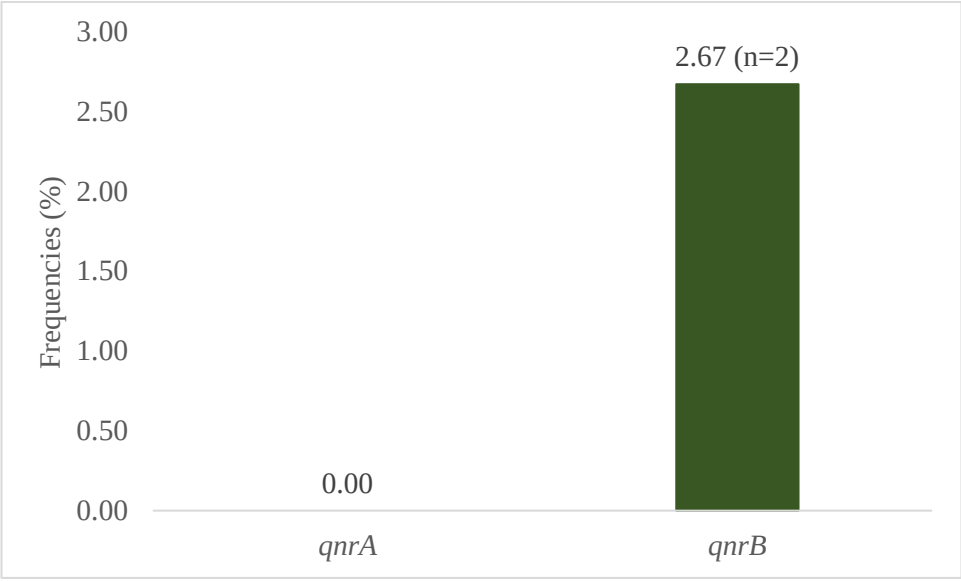

Figure 4.4: Distribution of *qnr* genes in 75 UPEC clinical isolates.

4.5 DNA purification and Sanger sequencing

The PCR product with band size corresponding to *qnrB* expected amplicon size (469 bp) was purified and sent for Sanger sequencing. The DNA sequence of obtained is displayed in Figure 4.5. The DNA sequence was analysed using NCBI Nucleotide Blast, and the result obtained is shown in Table 4.1.

```
>1st_BASE_4712026_32_qnrB_qnrB_primer_F
NCTAGCTGAAGNNGCCATTCGTCAAAGCTGGGACCTCTCCATGGCTGATTCCAGGAATATCCATGCGCTGGGA
ATCGAAATTCGCCACTGCCGGGCACAAGGGTCAGATTTTCGCGGCGCAAGTTTTATGACTATGATCCCCACCC
CCACCTGGTTTTGTATCGCCTATATCACCAATACCAACTTAAGCTACGCCAACTTTTCAAAAGTCGTACTGGA
AAAGTGCGAGCTGTGGGAAAACCGCTGGATGGGTACTCAGGTGCTGGGCGCAACGTTCAGTGGATCAGACCTC
TCTGGCGGCGAGTTTTCATCCTTCGACTGGCGAGCAGCAAACGTTACGCACTGTGATTTGACCAATTCGGAAC
TGGGCGATTTAGATATCCGCGGGGTTGATTTGCAAGGCGTCAAACTGGACAACTACCAGGCATCGTAA
```

Figure 4.5: DNA sequence of *qnrB* amplicon, via Sanger sequencing.

Table 4.1: Top three sequences with highest similarity to the analysed sequence.

No.	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Length	Accession
1	Escherichia coli quinolone resistance pentapeptide repeat protein (*qnrB*) gene, partial cds	*Escherichia coli*	706	706	94%	0.0	97.80%	415	OM791372.1
2	Klebsiella pneumoniae strain BA13643 plasmid plncR, complete sequence	*Klebsiella pneumoniae*	701	701	96%	0.0	96.90%	158043	CP102491.1
3	Klebsiella pneumoniae strain P1-1 plasmid pP1-1, complete sequence	*Klebsiella pneumoniae*	701	701	96%	0.0	96.90%	241435	CP092078.1

Based on Table 4.1, the sequence with the highest similarity (97.80%) to the analysed sequence was 'Escherichia coli quinolone resistance pentapeptide repeat protein (*qnrB*) gene, partial CDS'. This indicated that the gene purified from the PCR product was indeed qnrB gene from E. coli. The pairwise alignment output of the analysed sequence and database (OM791372.1) is included in Appendix E.

4.6 Association between antimicrobial susceptibility profile and genotypic profile of 75 UPEC isolates

The overall distribution of *qnr* genes based on fluoroquinolone resistance profile is presented in Figure 4.6. The resistance profile included all fluoroquinolone tested (ciprofloxacin, nalidixic acid and levofloxacin). Based on Figure 4.6, two out of 48 (4.17%) isolates resistant to fluoroquinolone carried *qnr* genes, while 46 out of 48 (95.83%) fluoroquinolone resistant isolates were screened negative for *qnrA* and *B* genes. None of the isolates which sensitive to fluroquinolone harboured the *qnrA* and *B* genes. Figure 4.7 illustrates the distribution of *qnrA* and *qnrB* genes based on fluoroquinolone resistance profile. Based on Figure 4.7, none of the isolates resistant nor sensitive to fluoroquinolone harboured *qnrA* gene, while two out of 48 isolates (4.17%) resistant to fluoroquinolone carried *qnrB* gene. None of the fluoroquinolone susceptible isolates carried *qnrB* gene.

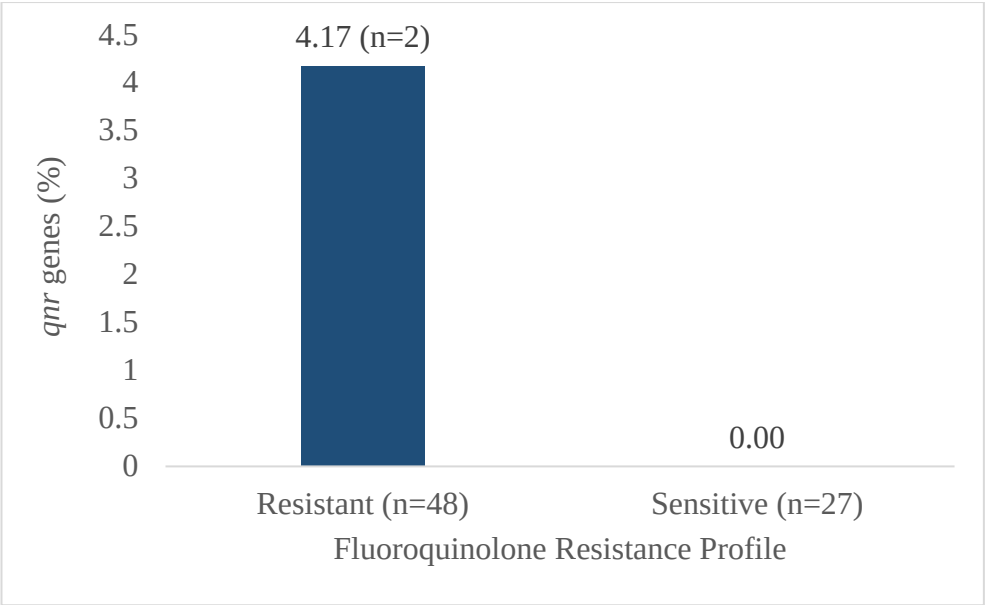

Figure 4.6: Overall distribution of *qnr* genes in isolates based on fluoroquinolone resistance profile.

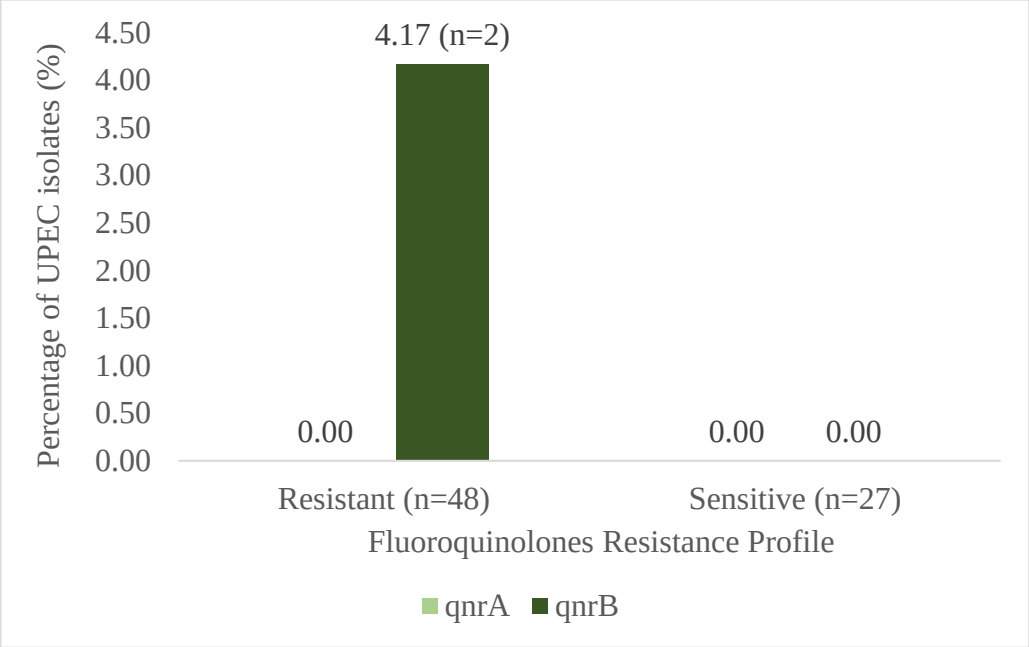

Figure 4.7: Percentage of UPEC isolates harboured *qnrA* and *qnrB* genes based on the fluoroquinolones resistance profile.

The association between phenotypic and genotypic profiles of isolates were analysed and presented in Table 4.2. The *p* value was calculated with Pearson's *Chi*-square test when the expected frequency value was more than five or Fisher's exact test when the expected frequency value was less than five. From Table 4.2, the association for imipenem and tobramycin resistance profile with respective genotypic profile could not be calculated as none of the isolates were resistant to imipenem nor tobramycin. No statistical association was found between the carriage of *qnr* genes and the fluoroquinolone antibiotics (ciprofloxacin, levofloxacin and nalidixic acid) or any other antibiotics. Thus, there was no positive association between the variables of fluoroquinolones resistance and *qnr* genes carriage.

Table 4.2: Association between antimicrobial susceptibility profiles of 75 isolates and *qnr* genes carriage.

Antibiotics		*qnrA*		*P* value	*qnrB*		*P* value
		Present	Absent		Present	Absent	
Ciprofloxacin	R	0 (0.0%)	31 (100.0%)	-	0 (0.0%)	31 (100.0%)	0.508
	S	0 (0.0%)	44 (100.0%)		2 (4.5%)	42 (95.5%)	
Levofloxacin	R	0 (0.0%)	36 (100.0%)	-	1 (2.8%)	35 (97.2%)	1.000
	S	0 (0.0%)	39 (100.0%)		1 (2.6%)	38 (97.4%)	
Nalidixic Acid	R	0 (0.0%)	45 (100.0%)	-	2 (4.4%)	43 (95.6%)	0.514
	S	0 (0.0%)	30 (100.0%)		0 (0.0%)	30 (100.0%)	
Tobramycin	R	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	0 (0.0%)	-[a]
	S	0 (0.0%)	75 (100.0%)		2 (2.7%)	73 (97.3%)	
Amikacin	R	0 (0.0%)	2 (100.0%)	-	0 (0.0%)	2 (100.0%)	1.000
	S	0 (0.0%)	73 (100.0%)		2 (2.7%)	71 (97.3%)	
Gentamicin	R	0 (0.0%)	15 (100.0%)	-	1 (6.7%)	14 (93.3%)	0.362
	S	0 (0.0%)	60 (100.0%)		1 (1.7%)	59 (98.3%)	
Ampicillin	R	0 (0.0%)	56 (100.0%)	-	1 (1.8%)	55 (98.2%)	0.416
	S	0 (0.0%)	19 (100.0%)		1 (5.3%)	18 (94.7%)	
Imipenem	R	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	0 (0.0%)	-[b]
	S	0 (0.0%)	75 (100.0%)		2 (2.7%)	73 (97.3%)	
SXT	R	0 (0.0%)	27 (100.0%)	-	0 (0.0%)	27 (100.0%)	0.533
	S	0 (0.0%)	48 (100.0%)		2 (4.2%)	46 (95.8%)	

'R': UPEC strains that resistant against tested antibiotics. 'S': UPEC strains that sensitive against tested antibiotics.

'[a]': No *p* value is generated as the antimicrobial susceptibility profile of tobramycin is constant.

'[b]': No *p* value is generated as the antimicrobial susceptibility profile of imipenem is constant.

**P* value was calculated with Pearson's χ^2 test for analysis of antimicrobial susceptibility profile in *qnr* positive and *qnr* negative isolates. *P* value less than 0.05 was interpreted as statistically significant.

***Fisher's exact test was used when at least one cell of the contingency table had an expected cell count of <5.

CHAPTER 5

DISCUSSION

5.1 Overview

For this study, antimicrobial susceptibility tests and duplex PCR were carried out to collect the resistance profile and to detect *qnrA* and *qnrB* genes from UPEC isolates. The association between genotypic and phenotypic profile of the isolates were also evaluated, via Fisher's exact test or *Chi* square test.

5.2 Antimicrobial susceptibility profile of UPEC clinical isolates

The UPEC isolates in this study showed high resistance to ciprofloxacin (41.33%). The results were comparable to the findings in Iraq (40%) but slightly lower than those reported in Iran (48.4%) and Mexico (47.3%), (Ramírez-Castillo, 2018; Alfuraiji et al., 2022; Malekzadegan et al., 2019). Nevertheless, the ciprofloxacin resistance rate was higher than those reported in Brazil, which was 20% (Souza da-Silva et al., 2020). Additionally, the resistance rate to levofloxacin (48%) resembled to the findings in Iran (47.9%) but was higher than the reported prevalence in Mexico (43.6%) and India (38%) (Mishra, Sarangi and Padhy, 2016; Ramírez-Castillo, 2018; Malekzadegan et al., 2018). In this study, 60% UPEC isolates were resistant to nalidixic acid. This rate was much lower than the one reported in two studies in Iran, which showed much higher nalidixic acid resistance rates of 71% and 71.9% (Abbasi and Ranjbar, 2018; Malekzadegan et al., 2019). Regardless, our

finding was similar to Malekzadegan et al. (2019), which showed higher resistant to nalidixic acid among the three fluoroquinolones tested.

From our findings, the resistance rate of ampicillin was the highest (74.67%) among UPEC. This result resembled the one reported in Mexico (70.9%) but was much lower than the prevalence reported in Iran (88.9%), (Malekzadegan et al., 2018; Souza da Silva et al., 2020). The high resistance rate may be due to the long-term usage of ampicillin in different fields such as farming and clinical practice since the first use of ampicillin in 1961. Since then, transmissible ampicillin resistance gene was detected in Europe in 1963, due to the extensive usage of ampicillin in farming. Additionally, the extensive usage of ampicillin in clinical settings, especially in UTI, further caused the emergence of ampicillin resistant UPEC strains (Tran-Dien et al., 2017).

In this study, the UPEC isolates were fully susceptible to imipenem and tobramycin. The low prevalence of imipenem- and tobramycin- resistance in isolates could be due to combined administration with other drugs, which could lead to better potency and prevention of resistance development. Imipenem is normally administered with cilastatin, while tobramycin is commonly administered with beta-lactam antibiotics for empiric therapy in serious infection (Scholar, 2007; Serio et al., 2018). Furthermore, tobramycin is not recommended to use in uncomplicated urinary tract infections unless the causative agent is resistant to antibiotics with lower toxicity. This would reduce the exposure of UPEC to tobramycin (FDA, 2013).

Overall, the antimicrobial susceptibility rates of UPEC isolates varied by regions and countries. In our study, high resistance seen toward ampicillin (74.67%), nalidixic acid (60%), levofloxacin (48%), and ciprofloxacin (41.33%) suggest that these antibiotics are not suitable for UTI empiric treatment, as the application of these antibiotics may cause selection of resistant strains and increases the risk of treatment failure (Leekha, Terell and Edson, 2011; Bischoff et al., 2018; O'Grady et al., 2018).

5.3 Prevalence of targeted fluoroquinolone resistance genes (*qnr*) in isolates

In this study, none of the UPEC isolates harboured *qnrA* gene while low prevalence of isolates carrying *qnrB* gene was detected (2.7%). The coexistence of *qnr* genes were not found in any isolates. These results were identical to Al-Agamy et al. (2018) which showed low prevalence for *qnrA* gene (0%) and *qnrB* gene (3.2%). Abbasi and Ranjbar (2018) also reported the absence of *qnrA* gene, however the prevalence observed in their isolates with *qnrB* gene was higher (30.9%). Likewise, the result is also comparable to findings by Zhao et al. (2014) which observed a prevalence of 1.5% for *qnrB* and 0% for *qnrA*, in China. Conversely, these reported prevalence were lower than those reported by Badamchi et al. (2019) from Iran, which reported a prevalence of 18% for *qnrA* and 30.9% for *qnrB* in ESBL-producing UPEC. Another study which focused on the detection of *qnr* genes in ESBL-producing UPEC revealed that 10% of the isolates contained *qnrA* and 21% of isolates contained *qnrB*. From the study, coexistence of *qnrA* and *qnrB* genes were also observed, but it was merely 1% (Farajzadehsheikh et al., 2019).

Most of the studies discovered that *qnrS* is the most prevalence *qnr* gene in UPEC compared to *qnrA* and *qnrB*. Thus, this could justify the low prevalence of *qnrA* and *qnrB* in our result. In addition, the result may be falsely negative due to the lack of positive control in the PCR runs. In conclusion, low prevalence of *qnrA* and *qnrB* genes were observed among our UPEC samples.

Additionally, ESBL-producing UPEC were frequently observed to carry *qnr* genes. ESBL-producing isolates were characterized as those able to produce β-lactamase, hydrolyse third generation cephalosporins and aztreonam, and was inhibited by clavulanic acid (Rawat and Nair, 2010). Association between *qnr* genes and ESBL isolates were reported by various studies (Lavilla et al., 2007; Bouchakour et al., 2010; Silva-Sanchez et al., 2011). However, the UPEC isolates evaluated in this study were not ESBL-producing strains. Nevertheless, more experiments would have to be performed to validate this statement.

5.4 Association between antimicrobial resistance profile and *qnr* genes profile

In this study, the presence of *qnrA* and *qnrB* genes were not associated to the fluoroquinolone resistance profile in UPEC isolates. Thus, the resistance may be conferred by other fluoroquinolone resistance genes such as *gyr* or *par* genes, which were not evaluated in this study. The high prevalence of mutated *gyrA* gene (95.74%), *parC* (90.43%) and *parE* genes (75.53%) in fluoroquinolone resistant isolates were reported by Zeng et al. (2021). The *gyr* genes were responsible for encoding DNA gyrase, while *par* genes encode for topoisomerase IV. Together they

form antimicrobial complex with fluoroquinolone and eventually kill the bacteria. Therefore, mutation in either *gyr* or *par* genes would lead to fluoroquinolone resistance in the bacteria (Rodríguez-Martínez, 2005).

Furthermore, as mentioned before, different studies revealed *qnrS* as the most prevalent *qnr* gene in UPEC isolates. Although the high prevalence of *qnrS* was observed, a study by Malekzadegan et al. (2019) reported no significant association between *qnr* genes (*qnrA*, *qnrB*, *qnrS*) and fluoroquinolone resistance profile. This suggested *qnr* family does not seem to be involved in the fluoroquinolone resistance mechanism in UPEC. Nonetheless, these statistics are varied in different regions.

Although *qnr* genes were not associated with fluroquinolone resistance in UPEC, the co-existence of *qnr* genes with other resistance determinants such as beta lactamase in plasmids are commonly observed in clinical isolates (Salah et al., 2019). Thus, although confer to low level of fluoroquinolone resistance, *qnr* were suspected to promote higher level of resistance in the bacteria (Yang, Nam and Lee, 2014).

5.5 Limitations and future studies

The current study encountered several limitations such as lack of investigation on other *qnr* gene family, as this study did not investigate on more prominent *qnrS* gene. Thus, it may not be sufficient to draw a conclusion that *qnr* genes were not involved in the fluroquinolone resistance mechanism in isolates. Furthermore, small sample size collected from hospital may also be the reason for the low prevalence of *qnr* genes in isolates. Thus, bigger sample size could be included to draw a more solid

conclusion. Moreover, the lack of positive control in this study may lead to invalid conclusion. Lastly, other fluoroquinolone resistance genes such as *gyr* or *par* gene were not evaluated, therefore the result may not be adequate to reflect all the fluoroquinolone resistant isolates.

In the future, the association between fluoroquinolone resistance genes and fluoroquinolone resistant isolates could be further studied. Molecular typing could be done to identify the genetic relatedness between the fluoroquinolone resistant strains. Moreover, the association between the ESBL-producing isolates and prevalence of *qnr* genes could also be evaluated. Additionally, the prevalence of other *qnr* determinants in multi drug resistant strains could be evaluated to assess its clinical significance.

CHAPTER 6

CONCLUSION

The objective of this study was to detect the carriage of fluoroquinolone resistance genes (*qnrA* and *qnrB*) in UPEC using duplex PCR, and to evaluate the association between the genotypic and phenotypic variables in UPEC isolates from patients. A total of 75 UPEC samples were collected from the Hospital Raja Permaisuri Bainun, Ipoh. The prevalence of isolates carried *qnrA* was 0%, and 2.67% for *qnrB* gene.

Merely 4.17% (2/48) of fluoroquinolones resistant UPEC isolates harboured *qnrB* gene, while none of the isolates harboured *qnrA* gene. Negative association was found between the carriage of *qnrA* and *B* genes and the fluoroquinolone resistance profile in isolates. This statistical analysis suggested that the fluoroquinolone resistance in UPEC was not due to *qnrA* and *B*, but other fluoroquinolone resistance genes which were not evaluated in this study. Furthermore, the *qnr* genes studied were also not associated with resistance profile of other antibiotics.

The findings of current research have given researchers a better insight on the prevalence and association of the targeted *qnr* genes in UPEC isolates collected from Ipoh, Perak. As presented, the investigated *qnr* determinants are not suitable to be used to predict the probability of the antibiotic resistance development in UPEC isolates from the respective hospital. However, the high fluoroquinolone resistance rate in the UPEC samples (ciprofloxacin, 41.33%; levofloxacin, 48%; and

nalidixic acid, 60%) suggested that fluoroquinolones should not be prescribed as primary treatment for UTI patients.

Nevertheless, this study encountered limitations such as lack of evaluation of other *qnr* genes and fluroquinolone resistance genes, small sample size and lack of positive control. Further studies should be conducted to determine the suitable antibiotics in treating UTI in Malaysia. Furthermore, the prescription of antibiotics should refer to the local occurrence of causative uropathogenic agents and their respective antimicrobial sensitivities, rather than to global guidelines, to reduce the development of antibiotic resistant strains in Malaysia.

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APPENDICES

Appendix A

Table 1: The demographic data of each UPEC clinical isolates collected from Hospital Permaisuri Bainun, Ipoh.

Samples	Age	Gender
UPEC 1	2	M
UPEC 2	78	F
UPEC 3	48	F
UPEC 4	26	F
UPEC 5	67	F
UPEC 6	36	F
UPEC 7	77	F
UPEC 8	76	F
UPEC 9	69	M
UPEC 10	52	M
UPEC 12	66	F
UPEC 13	24	F
UPEC 14	74	F
UPEC 15	40	M
UPEC 16	41	F
UPEC 17	30	F
UPEC 18	51	F
UPEC 19	63	M
UPEC 20	88	F
UPEC 21	53	F
UPEC 22	63	F
UPEC 23	63	F
UPEC 24	77	M
UPEC 25	79	F
UPEC 26	61	F
UPEC 27	76	F
UPEC 28	66	F
UPEC 29	38	F
UPEC 30	73	F
UPEC 31	63	M
UPEC 32	68	M
UPEC 33	68	F

Table 1 (continue):

UPEC 34	64	F
UPEC 35	56	F
UPEC 36	64	F
UPEC 37	59	F
UPEC 38	79	F
UPEC 39	86	F
UPEC 40	35	F
UPEC 41	70	F
UPEC 42	17	F
UPEC 44	57	M
UPEC 45	30	F
UPEC 46	48	F
UPEC 47	26	F
UPEC 48	82	M
UPEC 49	87	M
UPEC 50	53	F
UPEC 51	75	F
UPEC 52	13	M
UPEC 53	5	F
UPEC 54	39	F
UPEC 55	53	M
UPEC 56	49	M
UPEC 57	67	F
UPEC 58	44	F
UPEC 59	82	M
UPEC 60	3	F
UPEC 61	68	M
UPEC 62	25	F
UPEC 63	70	F
UPEC 71	7	F
UPEC 73	68	F
UPEC 87	22	F
UPEC 89	54	F
UPEC 90	0.17	M
UPEC 95	64	F
UPEC 96	23	F
UPEC 98	87	F
UPEC 99	66	F
UPEC 104	61	M
UPEC 105	77	F
UPEC 106	19	F
UPEC 110	75	M
UPEC 111	67	F

F: Female
M: Male

Appendix B

Table 2: Antimicrobial susceptibility test interpretive categories and zone diameter breakpoints (mm) based on CLSI (2022).

Categories	Ampicillin (10 µg), mm	Ciprofloxacin (5 µg), mm	Levofloxacin (5 µg), mm	Imipenem (10 µg), mm	Gentamicin (10 µg), mm
Susceptible	≥ 17	≥ 21	⩾ 17	⩾ 23	⩾ 15
Intermediate	-	16–20	14–16	20–22	13–14
Resistant	≤ 16	≤ 15	⩽ 13	⩽19	⩽ 12

Table 2 (continue):

Categories	Nalidixic acid (30 µg), mm	Trimethoprim-sulfamethoxazole (SXT) (25 µg), mm	Tobramycin (10 µg), mm	Amikacin (30 µg), mm
Susceptible	≥ 19	≥ 16	≥ 15	≥ 17
Intermediate	14–18	11–15	13–14	15–16
Resistant	≤ 13	≤ 10	≤ 12	≤ 14

Appendix C

Table 3: UPEC isolates antimicrobial susceptibility test collected data interpreted based on CLSI (2022).

Sample	Ampicillin	Ciprofloxacin	Levofloxacin	Imipenem	Gentamicin	Trimethoprim-sulfamethoxazole (SXT)	Nalidixic Acid	Amikacin	Tobramycin
UPEC 1	S	S	S	S	S	S	S	S	S
UPEC 2	R	R	R	S	S	R	R	S	S
UPEC 3	S	S	S	S	S	S	S	S	S
UPEC 4	R	R	R	S	R	R	R	S	S
UPEC 5	R	S	R	S	R	S	R	S	S
UPEC 6	R	S	S	S	S	S	S	S	S
UPEC 7	R	R	R	S	S	R	R	S	S
UPEC 8	R	S	S	S	R	R	S	S	S
UPEC 9	R	R	R	S	R	R	R	S	S
UPEC 10	R	S	S	S	S	R	R	S	S

Table 3 (continue):

UPEC 12	R	S	S	S	S	S	S	S	S
UPEC 13	R	S	S	S	S	S	S	S	S
UPEC 14	R	R	R	S	R	R	R	S	S
UPEC 15	R	R	R	S	R	R	R	S	S
UPEC 16	R	R	R	S	R	R	R	S	S
UPEC 17	R	S	S	S	S	R	S	S	S
UPEC 18	R	R	R	S	S	R	R	S	S
UPEC 19	R	S	R	S	R	R	R	S	S
UPEC 20	R	S	S	S	S	S	S	S	S
UPEC 21	R	R	R	S	S	S	R	S	S
UPEC 22	S	S	S	S	S	S	S	S	S
UPEC 23	S	S	S	S	S	S	S	S	S
UPEC 24	R	R	R	S	S	S	R	S	S
UPEC 25	R	S	S	S	S	S	S	S	S
UPEC 26	R	S	S	S	S	S	S	S	S

Table 3 (continue):

UPEC 27	R	S	S	S	S	S	R	S	S
UPEC 28	S	S	S	S	S	S	S	S	S
UPEC 29	R	S	S	S	S	S	S	S	S
UPEC 30	R	R	R	S	S	S	R	S	S
UPEC 31	R	R	R	S	S	R	R	S	S
UPEC 32	R	S	R	S	R	S	R	S	S
UTIPS 33	R	S	S	S	R	S	R	S	S
UPEC 34	R	R	R	S	S	R	R	S	S
UPEC 35	S	S	S	S	S	S	R	S	S
UPEC 36	S	S	S	S	S	S	S	S	S
UPEC 37	S	S	S	S	S	S	S	S	S
UPEC 38	R	S	S	S	S	S	S	S	S
UPEC 39	R	S	S	S	S	S	R	S	S
UPEC 40	R	S	S	S	S	R	S	R	S
UPEC 41	R	R	R	S	S	R	R	S	S

Table 3 (continue):

UPEC 42	R	S	S	S	R	R	R	S	S
UPEC 44	R	S	S	S	S	S	R	S	S
UPEC 45	S	S	S	S	S	R	S	S	S
UPEC 46	S	S	S	S	S	S	R	R	S
UPEC 47	R	R	R	S	S	S	S	S	S
UPEC 48	S	S	S	S	S	S	R	S	S
UPEC 49	S	R	R	S	S	S	S	S	S
UPEC 50	R	S	S	S	S	R	R	S	S
UPEC 51	S	S	S	S	S	S	S	S	S
UPEC 52	R	S	S	S	S	S	S	S	S
UPEC 53	R	S	S	S	R	R	S	S	S
UPEC 54	R	S	S	S	S	R	S	S	S
UPEC 55	R	R	R	S	S	R	R	S	S
UPEC 56	S	R	S	S	S	R	R	S	S
UPEC 57	S	S	S	S	S	S	S	S	S

Table 3 (continue):

UPEC 58	S	S	S	S	S	S	S	S	S
UPEC 59	S	S	S	S	S	S	S	S	S
UPEC 60	R	S	S	S	S	S	S	S	S
UPEC 61	R	R	R	S	S	S	S	S	S
UPEC 62	R	S	S	S	S	S	R	S	S
UPEC 63	R	R	R	S	S	R	R	S	S
UPEC 71	R	R	R	S	S	S	R	S	S
UPEC 73	S	S	R	S	S	S	R	S	S
UPEC 87	R	R	R	S	S	R	R	S	S
UPEC 89	R	R	R	S	R	S	R	S	S
UPEC 90	R	S	R	S	S	S	R	S	S
UPEC 95	R	R	R	S	R	R	R	S	S
UPEC 96	R	R	R	S	S	R	R	S	S
UPEC 98	R	R	R	S	S	S	R	S	S
UPEC 99	R	R	R	S	R	S	R	S	S

Table 3 (continue):

UPEC 104	R	R	R	S	S	S	R	S	S
UPEC 105	R	S	R	S	S	S	R	S	S
UPEC 106	S	R	R	S	S	S	R	S	S
UPEC 110	R	R	R	S	S	S	R	S	S
UPEC 111	R	R	R	S	S	S	R	S	S

R: Resistant
S: Sensitive

Appendix D

Table 4: Nucleic acid purification and concentration of extracted DNA.

Sample	Nucleic acid concentration (ng/µL)	A260/A280 ratio
UPEC 1	201.3	1.97
UPEC 2	168.1	2.15
UPEC 3	236.4	2.12
UPEC 4	157.4	2.15
UPEC 5	150.4	2.21
UPEC 6	139.6	2.23
UPEC 7	233.1	1.76
UPEC 8	406.7	2.11
UPEC 9	122.1	2.09
UPEC 10	134.3	2.23
UPEC 12	72.9	2.19
UPEC 13	62.7	2.01
UPEC 14	55.6	1.97
UPEC 15	86.0	2.01
UPEC 16	74.3	2.16
UPEC 17	97.4	2.21
UPEC 18	93.0	2.18
UPEC 19	127.4	2.01
UPEC 20	93.1	2.23
UPEC 21	150.8	2.20
UPEC 22	194.0	2.16
UPEC 23	218.9	2.19
UPEC 24	228.5	2.30
UPEC 25	118.9	2.12
UPEC 26	128.8	2.24
UPEC 27	151.6	2.21
UPEC 28	193.1	2.19
UPEC 29	154.6	2.23
UPEC 30	141.5	2.24
UPEC 31	200.4	2.25
UPEC 32	93.1	2.09
UPEC 33	137.1	2.05
UPEC 34	203.5	2.14
UPEC 35	149.5	2.03

Table 4 (continue):

UPEC 36	150.8	2.04
UPEC 37	101.7	2.11
UPEC 38	118.5	2.16
UPEC 39	145.7	2.25
UPEC 40	82.5	2.38
UPEC 41	160.6	2.20
UPEC 42	105.1	2.26
UPEC 44	229.5	2.28
UPEC 45	181.9	2.52
UPEC 46	198.5	2.07
UPEC 47	84.9	2.21
UPEC 48	64.8	2.28
UPEC 49	120.9	2.25
UPEC 50	163.5	1.80
UPEC 51	88.0	2.28
UPEC 52	59.7	2.33
UPEC 53	176.5	2.34
UPEC 54	270.6	2.23
UPEC 55	291.4	2.33
UPEC 56	432.8	2.16
UPEC 57	216.7	2.26
UPEC 58	253.1	2.19
UPEC 59	261.1	2.28
UPEC 60	286.8	2.25
UPEC 61	355.5	2.29
UPEC 62	236.4	2.09
UPEC 63	242.8	2.19
UPEC 71	69.0	2.01
UPEC 73	63.0	2.04
UPEC 87	91.1	2.05
UPEC 89	98.1	2.01
UPEC 90	88.3	1.95
UPEC 95	114.3	2.04
UPEC 96	91.2	2.10
UPEC 98	104.8	2.00
UPEC 99	84.2	2.06
UPEC 104	70.6	2.06
UPEC 105	77.5	2.13
UPEC 106	73.8	1.90
UPEC 110	111.1	1.99
UPEC 111	89.8	1.96

Appendix E

Escherichia coli quinolone resistance pentapeptide repeat protein (qnrB) gene, partial cds

Sequence ID: OM791372.1 **Length:** 415 **Number of Matches:** 1

Range 1: 7 to 415 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
706 bits(382)	0.0	400/409(98%)	0/409(0%)	Plus/Plus

```
Query  24   AAAGCTGGGACCTCTCCATGGCTGATTCCAGGAATATCCATGCGCTGGGAATCGAAATTC  83
            ||||  ||  || ||||||||||||||| |||||||||| ||||||||||||||||||||
Sbjct  7    AAAGTTGTGATCTCTCCATGGCTGATTTCAGGAATATCAATGCGCTGGGAATCGAAATTC  66

Query  84   GCCACTGCCGGGCACAAGGGTCAGATTTTCGCGGCGCAAGTTTTATGACTATGATCCCCA  143
            ||||||||||||||||||||||||||||||||||||||||||||||| ||||||| |||
Sbjct  67   GCCACTGCCGGGCACAAGGGTCAGATTTTCGCGGCGCAAGTTTTATGAATATGATCACCA  126

Query  144  CCCCCACCTGGTTTTGTATCGCCTATATCACCAATACCAACTTAAGCTACGCCAACTTTT  203
            ||| ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  127  CCCGCACCTGGTTTTGTAGCGCCTATATCACCAATACCAACTTAAGCTACGCCAACTTTT  186

Query  204  CAAAAGTCGTACTGGAAAAGTGCGAGCTGTGGGAAAACCGCTGGATGGGTACTCAGGTGC  263
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187  CAAAAGTCGTACTGGAAAAGTGCGAGCTGTGGGAAAACCGCTGGATGGGTACTCAGGTGC  246

Query  264  TGGGCGCAACGTTCAGTGGATCAGACCTCTCTGGCGGCGAGTTTTCATCCTTCGACTGGC  323
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  247  TGGGCGCAACGTTCAGTGGATCAGACCTCTCTGGCGGCGAGTTTTCATCCTTCGACTGGC  306

Query  324  GAGCAGCAAACGTTACGCACTGTGATTTGACCAATTCGGAACTGGGCGATTTAGATATCC  383
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  307  GAGCAGCAAACGTTACGCACTGTGATTTGACCAATTCGGAACTGGGCGATTTAGATATCC  366

Query  384  GCGGGGTTGATTTGCAAGGCGTCAAACTGGACAACTACCAGGCATCGTA  432
            |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  367  GCGGGGTTGATTTGCAAGGCGTCAAACTGGACAACTACCAGGCATCGTA  415
```

Figure 1: The pairwise alignment output of analysed sequence and database (OM791372.1).