GENETIC DIVERSITY OF PATHOGENICITY ISLANDS (PAIS) OF UROPATHOGENIC *ESCHERICHIA COLI* (UPEC) STRAINS FROM PERAK, MALAYSIA

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By

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ABSTRACT

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Chin Jia Jin

Urinary tract infection (UTI) is a severe health issue worldwide that is primarily caused by uropathogenic *Escherichia coli* (UPEC). Hundreds of UPEC lineages with various phylogenetic backgrounds and virulence-associated traits have been reported worldwide, but the genetic profiles of UPEC strains remain largely unexplored in Malaysia. Therefore, this study aimed to examine the key molecular characteristics of the UPEC strains in Perak, Malaysia. A total of 105 UPEC strains were collected from a government-funded hospital in Perak, and their resistance profiles against 15 antimicrobials were determined through the disc diffusion method. Template DNAs were extracted using the fast-boil method. The distribution of the phylogroups, 32 virulence genes (VGs), eight pathogenicity island (PAI) markers and the genetic diversity of the UPEC isolates were determined through multiplex polymerase chain reactions. In this study, most UPEC strains were multidrug-resistant (MDR) (53/105; 50.5%) and showed the greatest resistance towards ampicillin (72/105; 68.6%), followed by levofloxacin (36/105; 34.3%). Common virulence-associated traits included *fimH* (102/105; 97.1%), PAI IV_{536} (91/105; 86.7%), *fyuA* (90/105; 85.7%) and *chuA* (88/105; 83.8%). Phylogroup B2 was the predominant phylogroup

(67/105; 63.8%) that exhibited consensus virulence-associated traits of *fimH*, *yfcV*, *kpsMT* II, *fyuA*, *chuA*, *malX*, PAI IV_{536} and PAI I_{CFT073} (63/67; 94.0%). Surprisingly, phylogroup B2 demonstrated significantly less MDR (p= 0.018) but exhibited the most virulence-associated traits. Among the eight clonal groups (CGs) (18/105; 17.1%), CG1 was dominant and displayed the highest number of virulence-associated traits. UPEC strains isolated from female patients (80/105; 76.2%) displayed a lower prevalence of MDR (p= 0.527) and virulence-associated traits, whereas those collected among the predominant 60-79 age group (42/105; 40.0%) were significantly more MDR (p= 0.002) and carried fewer virulence-associated traits than other age groups. In conclusion, this study elucidated multiple molecular characteristics of the UPEC strains in relation to the host age and gender in Perak.

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SUBMISSION OF DISSERTATION

It is hereby certified that **CHIN JIA JIN** (ID No: **20ADM06235**) has completed this dissertation entitled "**GENETIC DIVERSITY OF PATHOGENICITY ISLANDS (PAIS) OF UROPATHOGENIC *ESCHERICHIA COLI* (UPEC) STRAINS FROM PERAK, MALAYSIA**" under the supervision of Associate Professor Dr, Chew Choy Hoong (Supervisor) from the Department of Allied Health Sciences, Faculty of Science, and Assistant Professor Dr. Choo Quok Cheong (Co-Supervisor) from the Department of Biological Science, Faculty of Science.

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DECLARATION

I hereby declare that the dissertation 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.

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LIST OF ABBREVIATIONS

×*g*	Time gravity
°C	Degree Celsius
A/S	Ampicillin-sulbactam
AMC	Amoxicillin-clavulanic acid
AMP	Ampicillin
bp	Base pair
CAZ	Ceftazidime
CDT	Cytolethal distending toxin
CFU	Colony forming units
CG	Clonal group
CIP	Ciprofloxacin
cnf1	Cytotoxic necrotising factor 1
CTX	Cefotaxime
CXM	Cefuroxime
DAF	Human decay-accelerating factor
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
dNTP	Deoxynucleotide triphosphates
e.g.	For example
EDTA	Ethylenediaminetetraacetic acid
ERIC	Enterobacterial repetitive intergenic consensus
ESBL	Extended-spectrum beta-lactamase

ETP	Ertapenem
ExPEC	Extraintestinal *escherichia coli*
fim	Type 1 fimbria
GBD	Global burden of diseases, injuries, and risk factors study
GbO3	Globotriosylceramide
GbO4	Globotetraosylceramide
GbO5	Globopentaosylceramide
GEN	Gentamicin
hlyA	*α*-haemolysin
IBC	Intracellular bacterial communities
ICEs	Integrative and conjugative elements
IMP	Imipenem
iTOL	Interactive tree of life
kb	Kilobase
LB	Luria-Bertani
LE	Levofloxacin
LPS	Lipopolysaccharide
M	Molar
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MBL	Metallo-β-lactamase
MccV	Microcin V
MDR	Multidrug-resistant
MEM	Meropenem

$MgCl_2$	Magnesium chloride
MHA	Mueller Hinton agar
PAI	Pathogenicity island
pap	Pyelonephritis-associated pili
PCR	Polymerase chain reaction
pH	Potential of hydrogen
REP-PCR	Repetitive-element polymerase chain reaction
rpm	Revolution per minute
SPATEs	Serine protease autotransporter proteins
SPSS	Statistical package of the social sciences
ST	Sequence type
SXT	Ttrimethoprim-sulfamethoxazole
TBE	Tris-borate-EDTA
U	Unit
UPEC	Uropathogenic *Escherichia coli*
UPGMA	Unweighted pair group method with arithmetic mean
usp	uropathogenic specific protein
UTI	Urinary tract infection
UV	Ultraviolet light
V	Volt
vat	Vacuolating autotransporter toxins
VG	Virulence gene

CHAPTER 1

INTRODUCTION

Urinary tract infection (UTI) represents one of the most common causes of bacterial infections that annually affects 274 million people worldwide (Tabasi, et al., 2016; Global Burden of Diseases, Injuries, and Risk Factors Study 2017 (GBD 2017) Disease and Injury Incidence and Prevalence Collaborators, 2018; García, et al., 2021). It is often associated with high societal costs, including health care costs and economic loss due to the absence of work (Flores-Mireles, et al., 2015).

Epidemiological studies revealed that almost 80% of the UTI cases are predominated by uropathogenic *Escherichia coli* (UPEC) (Flores-Mireles, et al., 2015; Forde, et al., 2019). UPEC strains usually harbour a wide range of virulence genes (VGs) with diverse functional categories, such as adhesins, toxins, protectins, iron-uptake and miscellaneous (Johnson and Russo, 2018). Some of these VGs are encoded by the pathogenicity islands (PAIs), which can be readily transferred among the bacterial strains through horizontal gene transfer (Desvaux, et al., 2020). The PAIs are often involved in the genetic rearrangement, excision, transfer, and acquisition of additional DNA, therefore contributing to the evolution and the diversification of *E. coli* (Desvaux, et al., 2020).

Hundreds of UPEC lineages with diverse genetic backgrounds have been reported worldwide, suggesting that the UPEC strains may vary in their strategies for UTI pathogenesis in different geographical areas (Sintsova, et al., 2019; Biggel, et al., 2020). Nevertheless, most of the *E. coli* UTI cases are usually predominated by several pandemic UPEC lineages from a B2 or D phylogenetic background, for instance, sequence type (ST) 69, ST73, ST95 and ST131 (Riley, 2014; Biggel, et al., 2020). Factors that facilitate the global spread of these pandemic lineages remain to be clarified but are assumed to be driven by specific genetic determinants (Riley, 2014; Biggel, et al., 2020).

Antimicrobial therapy has been the first choice in treating and ameliorating the clinical symptoms of UTI (Wurpel, et al., 2015; Halaji, et al., 2022; Yang, et al., 2022). However, antimicrobials are often prescribed without prior identification of the antibiogram (Terlizzi, Gribaudo and Maffei, 2017; Gatya Al-Mayahie, et al., 2022). These practices have increased the risk of developing greater antimicrobial resistance (Gatya Al-Mayahie, et al., 2022). A higher failure rate in the empirical treatment may increase the need for hospitalisation, thus making the UTI treatment progressively more expensive and challenging (Kudinha, 2017; Sora, et al., 2021).

In Malaysia, the genetic profiles of the UPEC clinical strains are currently unexplored. To date, there is only one UPEC-related study carried out in the Sabah state of Malaysia, which reported on the prevalence of the uropathogenic specific protein (*usp*), α-haemolysin (*hlyA*) and cytotoxic necrotising factor 1 (*cnf1*) (Lai, et al., 2016). Hence, this study aims to examine

the molecular characteristics of the UPEC strains in relation to the patient demographics in the Perak state of Malaysia. A better understanding of the antimicrobial resistance profiles and the molecular characteristics of the UPEC isolates will aid in improving antimicrobial stewardship, monitoring high-risk clones and inventing new therapeutics for better infection control in Malaysia. The objectives of this study are:

1. To determine the antimicrobial susceptibility profiles of the UPEC isolates,
2. To identify the phylogroups of the UPEC isolates,
3. To examine the phylogenetic distribution of virulence-associated traits,
4. To investigate the relationship between phylogroups, virulence-associated traits and antimicrobial resistance,
5. To determine the genetic diversity of the UPEC isolates, and
6. To compare the characteristics of the UPEC isolates with the host age and gender.

CHAPTER 2

LITERATURE REVIEW

2.1 Urinary Tract Infection

UTI is indicated by the presence of infectious microorganisms in the urinary tract system that is not caused by contamination (Berard, et al., 2011). Virtually any microorganisms can cause UTIs in humans, but certain microbes with specific virulence and host susceptibility factors usually predominate most of the UTI cases (Berard, et al., 2011). UPEC is the predominant uropathogen that accounts for almost 80% of the UTI cases (Belete and Saravanan, 2020; Klein and Hultgren, 2020). Other microbes that are frequently associated with UTIs include *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Staphylococcus saprophyticus* (Terlizzi, Gribaudo and Maffei, 2017).

UTI represents a broad range of clinical spectrums that vary from asymptomatic bacteriuria (bacteria in the urine) to symptomatic infections, such as urethritis (inflammation of the urethra), cystitis (inflammation of the bladder), pyelonephritis (inflammation of the kidney) and urosepsis (bacteria in the blood) (Berard, et al., 2011; García, et al., 2021). Some patients may have clinical symptoms, including intense urge to urinate (urgency), difficulty to urinate (hesitancy), pain on urination (dysuria), fever, chills, and back pain (Bono, Leslie and Reygaert, 2022).

Although UTI is treatable, a severe case of UTI can be life-threatening. The delay and suppression in the host's proinflammatory response will result in permanent renal scarring, leading to the impairment of renal functions (Bien, Sokolova and Bozko, 2012). Besides, the kidney may be severely damaged due to the elicitation of intense immune responses against the invading pathogens (Bien, Sokolova and Bozko, 2012). The uropathogens may also access the bloodstream, causing sepsis or even death (Smith, et al., 2010; García, et al., 2021).

2.2 Phylogroups of UPEC Strains

Epidemiological studies reveal that most of the UTI cases reported worldwide are mainly caused by the UPEC strains belonging to the phylogroups B2 and D, including the Southeast Asia countries (e.g., Thailand and Vietnam), East Asia countries (e.g., Taiwan, Mongolia, and Korea) and European countries (e.g., Romania, Germany, and France) (Ramos, et al., 2011; Ramos, et al., 2012; Toval, et al., 2014; Lavigne, et al., 2016; Munkhdelger, et al., 2017; Cristea, et al., 2019; Tewawong, et al., 2020; Hyun, Lee and Kim, 2021; Lin, et al., 2021b). In contrast, phylogroup A is the most dominant phylogroup in Egypt (El-Mahdy, Mahmoud and Shrief, 2021). These findings suggest that although the phylogroups A and B1 are typically more associated with commensal strains, they can also outcompete the phylogroup B2 and D, which are assumed to be more virulent (Biggel, et al., 2020). Strikingly, the UPEC strains do not necessarily share the same phylogenetic distribution within the same country.

Phylogroup A is dominant in the Puebla state of Mexico, whereas phylogroup B2 is prevalent in the Sonora state of Mexico (Ballesteros-Monrreal, et al., 2020).

Characterising the phylogroups of the UPEC strains based on either phenotypic or genotypic features has been challenging due to the diverse genetic pools of the UPEC strains in different geographical areas, sites of infection, antibiotic resistance, ecological niches and the propensity to cause disease (Abdul-Razzaq, 2011). To meet the criteria of phylogenetic characterisation, the target gene must be obtained or deleted during the emergence of the new strain (Clermont, Bonacorsi and Bingen, 2000). Moreover, the target gene must be stable and not easily deleted nor exchanged through horizontal gene transfer among other phylogroups (Clermont, Bonacorsi and Bingen, 2000). Recombination should also rarely occur in the target gene (Clermont, Bonacorsi and Bingen, 2000).

In 2000, Clermont and colleagues developed a rapid and simple phylotyping method that targets three genes of the *E. coli* strains (e.g., *chuA* and *yjaA* gene and a DNA fragment known as TspE4.C2). *chuA* gene is present among the phylogroups B2 and D, which can be further discriminated with the sole presence of *yjaA* in the phylogroup B2 (Clermont, Bonacorsi and Bingen, 2000). On the contrary, the phylogroups B1 and A are absent of *chuA*, with the sole presence of TspE4.C2 DNA fragment in the phylogroup B1 (Clermont, Bonacorsi and Bingen, 2000). With this technique, the UPEC strains can be classified into four major phylogroups (A, B1, B2 and D) with an accuracy rate of more than 99% compared to the reference methods such as multilocus enzyme

electrophoresis and ribotyping (Clermont, Bonacorsi and Bingen, 2000). In 2013, this phylotyping method was revisited. With the introduction of additional target genes such as *arpA* and *trpA*, the UPEC strains can be assigned into eight phylogroups (e.g., A, B1, B2, C, D, E, F and clade I) (Clermont, et al., 2013). Phylogroup C is proposed to be more closely related to phylogroup B1, whereas phylogroup F is the sister group of phylogroup B2 (Clermont, et al., 2013).

While phylotyping can also be performed through other reference methods such as multilocus enzyme electrophoresis and ribotyping, these techniques are relatively more complex, time-consuming and often require a collection of typed strains as reference (Clermont, Bonacorsi and Bingen, 2000). In contrast, the Clermont PCR-phylotyping is fast, simple and does not require any reference strains, thereby has been widely used to determine the phylogroups of the UPEC strains (Clermont, Bonacorsi and Bingen, 2000; Clermont, et al., 2013).

2.3 Virulence Genes of UPEC Strains

In order to establish successful infection, the UPEC strains have to enter, adhere, absorb the host's nutrients, propagate and eventually disseminate by destroying the cells (Terlizzi, Gribaudo and Maffei, 2017). Therefore, each of the UPEC isolates typically harbours hundreds of VGs with diverse functional categories, for example, adhesins, siderophore systems, surface polysaccharides, toxins, serum resistance-associated traits and miscellaneous traits (Johnson and Russo, 2018; Biggel, et al., 2020).

The distribution of the VGs is assumed to be correlated with the severity of the infection. The whole-genome analysis demonstrated that invasive UPEC strains that cause more severe infection (e.g., pyelonephritis and urinary-source bacteraemia) possess significantly more VGs (e.g., iron-uptake and immune evasion or modulation functional categories) as compared to the non-invasive UPEC strains that cause less severe infection (e.g., cystitis and asymptomatic bacteriuria) (Biggel, et al., 2020). However, a VG should always refer to as a gene associates with rather than a gene contributes to the virulence of the UPEC strains because the epidemiological associations do not necessarily reflect causality (Johnson and Russo, 2018). Specific roles played by each VGs in UTI pathogenesis are reviewed in the following section.

2.3.1 Adhesion Genes

Adherence to host cells is critical in the early stage of infection so that the UPEC strains can contact, colonise and eventually establish the infection within the host (Svensson, et al., 2018; Sarshar, et al., 2020). Having multiple adhesins with different receptor binding preferences will ensure more successful bacterial attachments in different environmental niches (Sarshar, et al., 2020).

Among 38 different chaperone-usher fimbriae identified, type 1 fimbria is one of the most common chaperone-usher fimbriae among the UPEC strains (Sarshar, et al., 2020). Type 1 fimbria is a short filamentous organelle that is encoded by a highly conserved gene operon, *fimBEAICDFGH* (Behzadi, 2018). The adhesive tip of type 1 fimbria, known as *fimH*, binds to the uroplakins of the

host cells (Subashchandrabose and Mobley, 2015). The glycoprotein plaque uroplakin is one of the anatomical barriers of the urinary tract to protect against infection (Abraham and Miao, 2015; Poolman, 2017). A prior study demonstrated that mutants with the loss of *fimH* alone could not invade the urothelial cells successfully despite the presence of other functional VGs (Liu, et al., 2016). In contrast, having the *fimH* alone can successfully promote intracellular bacterial communities (IBCs) formation, which is critical for the persistence of the UPEC strains (Liu, et al., 2016). Hence, *fimH* has been considered one of the most important VGs in UTI pathogenesis (Ortega Martell, 2020).

Another well-characterised chaperone-usher fimbria among the UPEC strains is P fimbria. An estimation of 70% pyelonephritis isolates express the P fimbriae, whereas only 30% of the cystitis isolates express the *pap* gene clusters (Svensson, et al., 2018). Unlike type 1 fimbriae that are located at the genomic backbone, P fimbriae are encoded by 11 *pap* (pyelonephritis-associated pili) genes that reside within the pathogenicity islands, for example, *papB*, A, H, C, D, J, K, E, F, G and I (Marklund, et al., 1992; Bien, Sokolova and Bozko, 2012; Subashchandrabose and Mobley, 2015; Behzadi, 2020). *papA* encodes the major pilin subunits, *papH* encodes the terminator for pilus assembly, *papC* facilitates pilin polymerisation, *papE* encodes the major tip component, and *papF* encodes the adaptor that links *papG* and *papE* in correct stereochemical orientation (Marklund, et al., 1992; Durand, et al., 2009).

There are three major molecular variants for the tip adhesins of P fimbriae (*papG*) known as *papG* allele I, II and III (Behzadi, 2020). The *papG* allele II has been reported to cause pyelonephritis by binding to the globotriosylceramide (GbO3) and globotetraosylceramide (GbO4) in the kidneys (Lane and Mobley, 2007). On the contrary, *papG* allele III is associated with cystitis and binds to GloboA, an analogue of globopentaosylceramide (GbO5), which is expressed by those susceptible to lower UTIs (Johnson, et al., 2001a; Lane and Mobley, 2007; Desvaux, et al., 2020). *papG* allele I is a rare tip adhesion variant that preferably binds to GbO3 (Lane and Mobley, 2007; Lüthje and Brauner, 2014). It is seldom encountered in UTIs, so its clinical association remains unknown (Johnson, et al., 2001a; Lane and Mobley, 2007; Rezatofighi, Mirzarazi and Salehi, 2021).

Other chaperone-usher fimbriae, such as S and F1C fimbriae, are also observed among the UPEC strains (Munkhdelger, et al., 2017; Rezatofighi, Mirzarazi and Salehi, 2021). *focG* is the minor subunit of the F1C fimbriae that binds to the Gal-Nac-β-1,4-Gal-β molecules of the bladder and kidney endothelial cells (Behzadi, 2020). Thus, F1C fimbriae are often associated with cystitis and pyelonephritis (Behzadi, 2020). On the other hand, *sfaS* is the pilus tip adhesin of the S fimbriae that binds to the sialic acid receptors of the red blood cells, urinary bladder and kidney (Behzadi, 2018; 2020). As a result, S fimbriae are often associated with urosepsis and ascending UTIs (Bien, Sokolova and Bozko, 2012; Behzadi, 2020). Since the S and F1C fimbriae are highly homologous, primers that target the consensus region of the *sfa* and *foc* operons (*sfa/focDE*) were also included in the present study (Ott, et al., 1988; Le

Bouguenec, Archambaud and Labigne, 1992; Mitsumori, et al., 1998; Johnson and Stell, 2000).

yfcV is the major subunit of a putative chaperone usher fimbria (Spurbeck, et al., 2012). Although the precise role of *yfcV* in UTI pathogenesis remains unknown, *yfcV* is highly associated with the UPEC strains, especially those involved in upper UTIs such as acute uncomplicated pyelonephritis (Spurbeck, et al., 2012; Li, et al., 2021). *E. coli* isolates that carry *yfcV* also confer greater VGs than the negative isolates (Spurbeck, et al., 2012). While *yfcV* is also present among the non-pathogenic *E. coli* K-12, it only shares 70% amino acid identity with the UPEC CFT073 (Spurbeck, et al., 2012). Hence, the primers used for determining *yfcV* in the present study are specific to those present among the UPEC isolates (Spurbeck, et al., 2012).

Dr family or Afa/Dr fimbriae are chaperone usher fimbriae that comprise closely related operons, including *afa*, *dra*, *daa*, and *nfa* (Le Bouguénec and Servin, 2006). Afa/Dr fimbriae bind to the Dr blood group antigen on the human decay-accelerating factor (DAF) and facilitate the invasion of the bacteria into the intraepithelial cells to protect the bacteria against the host defence system and antimicrobial treatment (Sarowska, et al., 2019; Alvarez-Fraga, et al., 2022). Recently, the Afa/Dr fimbriae are frequently associated with pyelonephritis and recurrent cystitis in pregnant women (Alvarez-Fraga, et al., 2022).

Other adhesins that cause agglutination of the red blood cells (haemagglutinin) are G fimbriae and M agglutinin. *gafD* is the fimbrial lectin of the G fimbriae that causes the agglutination of the endo-β-galactosidase-treated red blood cells by binding to their *N*-acetyl-D-glucosamine receptors (Väisänen-Rhen, Korhonen and Finne, 1983; Saarela, et al., 1995; Saarela, et al., 1996; Tanskanen, et al., 2001). In contrast, *bmaE* encodes the precursor protein of the M agglutinin that recognises the human blood group M antigen (Rhen, et al., 1986; Rhen, Klemm and Korhonen, 1986). *gafD* and *bmaE* are more frequently detected among the pyelonephritis isolates than the cystitis and prostatitis isolates (Johnson, et al., 2005a). Besides, the UPEC strains that exhibit *bmaE* are often associated with greater biofilm formation potential (Zamani and Salehzadeh, 2018).

2.3.2 Toxic Genes

Despite having multiple adhesins for bacterial colonisation, the UPEC strains also secrete various toxins into their surroundings (Etefia, 2021). These toxins generally help to disrupt the host cell integrity for better nutrient absorption and protect the UPEC strains against the host immune system (Bunduki, et al., 2021; Etefia, 2021). Alpha-haemolysin (*hlyA*) is a repeat-in-toxin protein commonly found among gram-negative pathogens (Bien, Sokolova and Bozko, 2012; Gu, et al., 2021). Interestingly, *hlyA* acts differently depending on its concentration. Suppose the *hlyA* is expressed in high concentration, it will trigger the red blood cells and other nucleated host cells to burst, thereby allowing the UPEC strains to cross through the mucosal barrier easily to acquire the host nutrients (Bien,

Sokolova and Bozko, 2012; Etefia, 2021). If the *hlyA* is expressed in low concentration, it will trigger the neutrophils and T lymphocytes to undergo apoptosis (Bien, Sokolova and Bozko, 2012). Furthermore, it also induces the epithelial cells to undergo exfoliation so that the UPEC strains are disseminated into deeper layers of the urothelium (Bien, Sokolova and Bozko, 2012; Lüthje and Brauner, 2014).

Cytotoxic necrotising factor 1 (*cnf1*) is a member of Rho GTPase-activating toxins that was firstly discovered from the *E. coli* strains isolated from the stool specimens of paediatric patients suffering from acute gastroenteritis and severe diarrhoea (Chaoprasid and Dersch, 2021). *cnf1* is commonly expressed by 40% of the UPEC strains (Olson, Justice and Hunstad, 2015). It promotes the entry of the UPEC strains into the urothelial cells by facilitating the transient activation and degradation of Rac (Lüthje and Brauner, 2014; Chaoprasid and Dersch, 2021). It also induces severe inflammation in the murine ascending UTI models by mediating the downregulation of CD36 in macrophages to increase the UPEC and neutrophile titers in the infected tissues (Yang, et al., 2018).

Vacuolating autotransporter toxins (*vat*) are also widespread among the UPEC strains (Díaz, et al., 2020). *vat* was first described as a secreted protein encoded by the pathogenicity island of an avian *E. coli* strain Ec222 (Nichols, et al., 2016; Tapader, Basu and Pal, 2019). *vat* is classified under Class II serine protease autotransporter proteins of Enterobacteriaceae (SPATEs) and shares 78% homology with another autotransporter, known as *tsh* (Spurbeck, et al., 2012; Nichols, et al., 2016). *vat* cleaves the glycoproteins of the leukocytes and delays

the neutrophil infiltration (Ruiz-Perez, et al., 2011; Spurbeck, et al., 2012). In addition, it also induces cellular damage and vacuole formation in the bladder cells (Díaz, et al., 2020). Surprisingly, all urosepsis isolates express *vat* during infection (Nichols, et al., 2016; Díaz, et al., 2020).

Cytolethal distending toxin (CDT) is a bacterial genotoxin that possesses deoxyribonuclease I (DNase I) activity and breaks double-strand DNAs, leading to cellular distention or even death of the eukaryotic cells (Elwell and Dreyfus, 2000; Mirzarazi, et al., 2015). Production of an active CDT requires three polypeptides named *cdtA*, *cdtB* and *cdtC* (Scott and Kaper, 1994; Elwell and Dreyfus, 2000). *cdtB* mediates the DNase activity and promotes the cell cycle arrest, whereas *cdtA* and *cdtC* are involved in the binding and translocation of *cdtB* (Elwell and Dreyfus, 2000; Elwell, et al., 2001). Although *cdtB* has been isolated from UTI patients, the role of *cdtB* in UTI pathogenesis remains to be clarified (Abe, et al., 2008; Mostafavi, et al., 2019).

2.3.3 Iron-uptake Genes

Iron is essential for all living cells in biological metabolisms, such as oxygen transport, electron transport and DNA synthesis (Kudinha, 2017). The elemental iron usually binds to the glycoproteins or incorporates into haemoglobins and myoglobins within the hosts, thereby creating an iron-limited environment to reduce the survival of the UPEC strains (Subashchandrabose and Mobley, 2015). To ensure adequate levels of iron uptake, the UPEC strains harbour multiple iron acquisition mechanisms (Etefia, 2021). For instance, the UPEC strains can

secrete siderophores such as aerobactin (encoded by *iutA*) and yersiniabactin (encoded by *fyuA*) to chelate the ferric iron or express haem binding receptor (encoded by *chuA*) to acquire the iron released during the lysis of the red blood cells (Lüthje and Brauner, 2014; Subashchandrabose and Mobley, 2015; Etefia, 2021).

Yersiniabactin is one of the most prominent siderophores secreted by the UPEC strains for iron acquisition (Etefia, 2021). The yersiniabactin operon resides on the high pathogenicity island of the Enterobacteriaceae and comprises *fyuA*, which encodes for the outer membrane receptor protein (Kudinha, 2017). *In vivo* experiment demonstrates that the *fyuA* deficient mutants attenuate the UPEC in the cystitis, pyelonephritis and systemic infection of the murine models (Brumbaugh, et al., 2015). Besides, *fyuA* is also essential for efficient biofilm formation (Hancock and Klemm, 2007; Hancock, Ferrières and Klemm, 2008). The *fyuA* deficient mutant shows a 92% reduction in the biofilm formation as compared to the wild-type strains when growing in the urine flow-cell chambers (Hancock, Ferrières and Klemm, 2008).

On the other hand, the aerobactin operon comprises five genes: *iucABCD* and *iutA*. *iucABCD* gene clusters are involved in the biosynthesis of aerobactin, whereas *iutA* encodes the outer membrane receptor protein (Kudinha, 2017). Chromosomally encoded *iutA* is more frequently found among urosepsis patients, whereas the plasmid-borne *iutA* is often associated with antimicrobial resistance genes (Kudinha, 2017). Previous studies have demonstrated that the distribution of *iutA* differs in terms of clinical presentations. It is highly prevalent among the

pyelonephritis and urosepsis isolates as compared to the cystitis isolates (Johnson, et al., 2005a; Kudinha, 2017; Malekzadegan, et al., 2018; Massip, et al., 2020).

Apart from acquiring ferric iron through siderophores, the UPEC strains utilise outer membrane haem receptors such as *chuA* to acquire haem as a source of iron (Etefia, 2021). *In vivo* experiment demonstrated that the expression of *chuA* in the urine of the infected murine models increased by an average of 67-fold as compared to the *hma* haem receptor, which only increased by 24-fold, suggesting that *chuA* may contribute more to the haem uptake under an iron-limiting condition (Hagan and Mobley, 2009). Furthermore, the mutant strain deficient in *chuA* and *hma* is outcompeted by the wild-type strain in kidney colonisation, indicating the importance of haem as an essential source of iron in kidney infection (Hagan and Mobley, 2009). *chuA* deficient mutant also has smaller IBCs than the wild-type strain, which may result in reduced capabilities of the UPEC stains in bladder colonisation and persistence within the host (Lüthje and Brauner, 2014).

2.3.4 Protectin Genes

To protect against phagocytosis and bactericidal actions of the host's complement system, the UPEC strains generally produce diverse types of capsular polysaccharides (Sarowska, et al., 2019). To date, more than 80 distinct types of capsular polysaccharides have been identified in the *E. coli* isolates (Sarkar, et al., 2014; Azurmendi, et al., 2020). These capsular polysaccharides

are classified into four major groups (groups 1 to 4) based on their genetic assembly, biochemistry and physical attributes (Johnson, 1991; Sarkar, et al., 2014). Among the four major groups of capsular polysaccharides, group II capsular polysaccharide is the most closely related to the UPEC strains (Sarkar, et al., 2014).

Group II capsular polysaccharide operon comprises three clusters of genes located near the *serA* locus of the chromosomal DNA (Johnson, 1991; Goller, et al., 2014). Region 1, 2 and 3 gene clusters are involved in the polymerisation of the carbohydrate subunits, postpolymerisation and the exportation of the polysaccharides, respectively (Johnson, 1991). Both region 1 and region 3 gene clusters are conserved among all the group II capsules (Owaif, 2016). However, the region 2 gene cluster is serotype-specific. It encodes different allelic types of group II capsules (e.g., Kl, K2, K5, K6, K12, K13, K14, K15, K20, K23, K51, K52 and K54) by adding different phosphatidic acid groups in the postpolymerisation process (Johnson, 1991; Owaif, 2016).

Epidemiological studies reveal that K1 is one of the leading capsule subtypes among the UPEC strains (Anderson, et al., 2010; Olson, Justice and Hunstad, 2015). It protects the UPEC strains from innate immunity by impeding the phagocytosis of the neutrophils (Anderson, et al., 2010). In addition, it is also crucial in the IBC formation, which helps the UPEC strains to persist within the uroepithelium and develop cystitis (Anderson, et al., 2010; Olson, Justice and Hunstad, 2015). Apart from K1 capsule, K5 capsule is also commonly found among the UPEC strains (Hyun, Lee and Kim, 2021; Rezatofighi, Mirzarazi and

Salehi, 2021). K5 capsule protects the bacteria against phagocytosis by preventing the initial attachment and the internalisation of the polymorphonuclear leukocytes (Burns and Hull, 1999). As the group II capsules rely on the conversed ATP-binding cassette transporters for exporting the capsular polysaccharides, primers that target *kpsM* and *kpsT* genes were used to detect the presence of group II capsules in the present study (Bliss and Silver, 1996). Besides, primers specific to the region 2 gene cluster of the K1 and K5 group II capsules were also included (Johnson and Stell, 2000).

Similarly, group III capsules of the *E. coli* strains also confer a serotype-specific region 2 gene cluster that encodes different allelic types of group III capsule (e.g., K2, K3, K10, K11 and K19), resembling the group II capsules (Russo, et al., 1998; Owaif, 2016). Nevertheless, the genetic organisations of the region 1 and 3 gene clusters of the group III capsules differ from the group II capsules (Owaif, 2016). Region 1 gene cluster of the group III capsule consists of four genes that encode KpsE, KpsD, KpsM, and KpsT proteins, whereas region 3 gene cluster encodes KspS and KpsC proteins (Clarke, Pearce and Roberts, 1999; Owaif, 2016). *In vivo*, the mutants deficient in both group III K54 capsular polysaccharide and O4-specific antigen showed lower inflammatory scores and significantly reduced the ability to cause bladder and kidney infection in the murine UTI models (Russo, et al., 1996). Nonetheless, the group III capsule is rarely found among the UPEC strains as compared to the group II capsule (Johnson, et al., 2005a; Hyun, Lee and Kim, 2021; Rezatofighi, Mirzarazi and Salehi, 2021).

Lipopolysaccharide (LPS) is one of the major structural components of the outer membrane that serves as the primary permeability barrier of the *E. coli* strains against environmental threats such as antibiotics and bile salts (Lukomski, Hull and Hull, 1996; Bertani and Ruiz, 2018; Etefia, 2021). LPS comprises three major domains, including the lipid A, core oligosaccharide, and O antigen (Lukomski, Hull and Hull, 1996). O antigen, an extended polysaccharide on the cell surface, protects the cell against the serum complement and phagocytosis of the polymorphonuclear leukocytes (Lukomski, Hull and Hull, 1996; Bertani and Ruiz, 2018). While the overall structure of the LPS is conserved, the O antigens are highly diverse within the strain levels (Bertani and Ruiz, 2018). Among the 200 different O antigens identified, the O4 serogroup is frequently found among the UPEC strains and acute pyelonephritis patients (Lukomski, Hull and Hull, 1996; Sarkar, et al., 2014; Bertani and Ruiz, 2018). In addition, the UPEC strains with the O4 serogroups have the highest antimicrobial resistance (Mohammed, et al., 2021). *rfc* locus, which is involved in the biosynthesis of O4 LPS, was targeted in the present study (Lukomski, Hull and Hull, 1996; Johnson and Stell, 2000).

2.3.5 Miscellaneous Genes

In this study, VGs such as *cvaC*, *traT*, *malX* and *ibeA*, which do not fall into any functional categories of adhesins, toxins, protectins and iron-uptake, were categorised as miscellaneous genes.

Microcin V (MccV), formerly colicin V, is a peptide antibiotic that kills the bacteria within the genera *Escherichia*, *Klebsiella*, *Salmonella*, and *Shigella* by disrupting their membrane potential (Gérard, Pradel and Wu, 2005; Baquero, et al., 2019). MccV operon consists of four plasmid-borne genes, including *cvaA*, *cvaB*, *cvaC* and *cvi* (Gérard, Pradel and Wu, 2005; Johnson, et al., 2006). *cvaC* is involved in the synthesis of MccV, whereas *cvaA* and *cvaB* are involved in the secretion of MccV (Baquero, et al., 2019). On the other hand, *cvi* encodes the immunity protein that protects the microcin-producing strains from bactericidal activity (Gérard, Pradel and Wu, 2005). A high prevalence of MccV observed among the pyelonephritis isolates suggests that MccV may play a role in the pathogenesis of pyelonephritis (Johnson, et al., 2005a). MccV plasmids also carry iron-uptake genes, which may provide a competitive advantage over non microcin-producing strains to survive in an iron-limited environment, such as the urinary tract (Da Cruz Campos, et al., 2019).

traT is one of the most common miscellaneous genes among the UPEC strains (Rezatofighi, Mirzarazi and Salehi, 2021; Tanabe, et al., 2022). It encodes for an outer membrane protein that mediates surface exclusion through inhibiting the formation of stable mating aggregate (Montenegro, et al., 1985; Gago-Córdoba, et al., 2019). *traT* is always carried on large IncF plasmids. Therefore,

bacteria that carry the F plasmids typically have a 100- to 300-fold reduction in their ability to act as recipients in conjugation as compared to those that do not carry the F plasmids (Johnson, 1991; Sora, et al., 2021). *traT* also protects the cells from the complement-mediated killing of the serum and phagocytosis (Montenegro, et al., 1985; Johnson, 1991; Derakhshandeh, et al., 2015).

malX is a common miscellaneous gene present in the pathogenicity island of the UPEC prototype CFT073 (Campos, et al., 2018; Hyun, Lee and Kim, 2021; Zeng, et al., 2021). It encodes for the phosphotransferase system enzyme II, which recognises maltose and glucose as substrates (Reidl and Boos, 1991; Johnson and Stell, 2000). A previous study suggests that *malX* may be essential for the intestinal persistence of *E. coli* strains (Ostblom, et al., 2011). Furthermore, a high prevalence of *malX* is frequently observed among the UPEC isolates involved in recurrent or relapse UTIs (Ejrnæs, et al., 2011; Agarwal, et al., 2014; Pompilio, et al., 2018).

ibeA was first identified in the RS218 *E. coli* strain isolated from the cerebrospinal fluid of a neonatal meningitis patient (Cortes, et al., 2008; Wijetunge, et al., 2015). It facilitates the invasion of *E. coli* strains into the brain endothelial cells by interacting with the Caspr1 host receptor proteins (Xu, et al., 2020). It is also involved in the invasion of the Caco-2 and M-like cells, macrophage survival and the inflammatory response of the adherent-invasive *E. coli* strains (Cieza, et al., 2015). Recently, *ibeA* has been detected among the extraintestinal *E. coli* (ExPEC) sequence type 131 and is correlated with low *in vivo* virulence in the mouse sepsis model (Mora, et al., 2014; Duprilot, et al.,

2020). Nevertheless, a high occurrence of *ibeA* is usually observed among the *E. coli* strains isolated from dogs instead of humans (Henriques, et al., 2014; Naziri, et al., 2020; Valat, et al., 2020).

2.4 Pathogenicity Islands of UPEC Strains

Pathogenicity island (PAI) refers to a large VGs containing DNA fragment that is readily exchanged among the bacterial strains through horizontal gene transfer (Hacker, et al., 1997; Maniam, et al., 2022). It usually has a fragment size of more than 10 kb and harbours an atypical GC content that distinguishes it from the rest of the bacterial host genome (Desvaux, et al., 2020). It is often flanked by direct repeats and mobile elements, such as insertion sequence elements, plasmids and bacteriophages (Desvaux, et al., 2020).

PAI is non-replicative and unable to mobilise by itself (Desvaux, et al., 2020). Thus, it encodes integrases and recombination directionality factors to mediate the integration and excision processes, which lead to different variations of PAIs (Desvaux, et al., 2020). Alternative mechanisms include the horizontal transfer of PAIs through conjugative plasmids, integrative and conjugative elements (ICEs), and *att* sites of the phages (Desvaux, et al., 2020). Besides, homologous DNA recombination also aids in disseminating the PAIs if the donor bacteria share the same sequence with the recipient bacteria in their plasmids or PAIs (Desvaux, et al., 2020).

Whole-genome phylogenetic studies suggest that the UPEC strains may have evolved from commensal *E. coli* strains through the horizontal transfer of these PAIs, thereby demonstrating the crucial roles of the PAIs in the emergence of pathogenic variants and the development of diseases (Desvaux, et al., 2020). Among the UPEC strains, PAI I-IV of UPEC 536, PAI I and II of UPEC CFT073 and PAI I and II of UPEC J96 are the most studied of the PAIs (Sabaté, et al., 2006). Common VGs encoded by these PAIs are summarised in Table 2.1 (Ostblom, et al., 2011; Samei, Haghi and Zeighami, 2016). Notably, certain VGs present redundantly in these PAIs. For example, α-haemolysin is encoded by the PAI I and II of UPEC 536, whereas P fimbria is encoded by the PAI I and II of UPEC CFT073 (Desvaux, et al., 2020). Epidemiological studies regarding the PAIs of the UPEC prototypes strains (e.g., UTI89, CFT073, J96 and 536) suggest that these PAIs may act synergically in UTI pathogenesis (Desvaux, et al., 2020).

Table 2.1: Common virulence genes encoded by the pathogenicity islands of the UPEC 536, CFT073 and J96 (adapted from Ostblom, et al., 2011; Samei, Haghi and Zeighami, 2016).

Pathogenicity Islands	Virulence Genes
PAI I_{536}	α-Haemolysin, F17-like fimbriae, CS12-like fimbriae
PAI II_{536}	Hek adhesin, P fimbriae, α-Haemolysin, hemagglutinin-like adhesin
PAI III_{536}	S fimbriae, salmochelin, HmuR-like haem receptor, Sat toxin, Tsh-like haemoglobin protease, antigen 43
PAI IV_{536}	Yersiniabactin siderophore system
PAI I_{CFT073}	α-Haemolysin, P fimbriae and aerobactin
PAI II_{CFT073}	P fimbriae, iron-regulated proteins
PAI I_{J96}	α-Haemolysin and P fimbriae
PAI II_{J96}	α-Haemolysin, Prs fimbriae, cytotoxic necrotising factor

The acquisition of PAIs in the UPEC strains has been hypothesised to be driven by a programmed and sequential mechanism rather than a random event (Sabaté, et al., 2006). PAI IV_{536} is the most prevalent PAI in Enterobacteriaceae, whereas PAI II_{536} and PAI III_{536} are rare and exclusively detected in more virulent UPEC isolates (Sabaté, et al., 2006; Samei, Haghi and Zeighami, 2016). The high frequency of PAI IV_{536} in the UPEC strains may be driven by its relatively more stable properties, thereby allowing it to be acquired first in the bacterial genome as compared to the PAI II_{536} and PAI III_{536} (Sabaté, et al., 2006; Samei, Haghi and Zeighami, 2016).

The distribution of the PAIs may correlate with the phylogenetic background of the UPEC strains. Previous studies have demonstrated a higher prevalence of PAI markers in phylogroup B2 (Sabaté, et al., 2006; Navidinia, et al., 2013). Interestingly, the insertion sequences IS1 and IS2 are found exclusively in this phylogroup (Sabaté, et al., 2006). As most of the PAI-related integrases are located adjacent to the tDNAs, the tDNAs targeted for the integration in the phylogroup B2 may differ in the phylogroups A and B1, thereby affecting the acquisition or excision of PAIs (Desvaux, et al., 2020).

2.5 Antimicrobial Resistance of UPEC Strains

In recent years, the UPEC strains have gained greater resistance towards first-line antimicrobials. For instance, trimethoprim-sulfamethoxazole, which serves as the mainstay for uncomplicated cystitis treatment, is less effective in countries, including Pakistan (82%), Mexico (72.7%), and Mongolia (70.9%) due to the

high resistance rates (Ramírez-Castillo, et al., 2018; Kot, 2019). Furthermore, ciprofloxacin, the empirical oral prescription for uncomplicated pyelonephritis, also shows a profound level of resistance in Ethiopia (85.5%), Taiwan (79.5%), and Thailand (65.4%) (Kot, 2019; Tewawong, et al., 2020; Lin, et al., 2021a). Moreover, amoxicillin-clavulanic acid, which is recommended for treating mild and moderate pyelonephritis, has a high level of resistance in countries such as Jordan (83%), Turkey (50.9%), and France (37.6%) (Lavigne, et al., 2016; Kot, 2019; Yılmaz and Aslantaş, 2020). In contrast, the resistance rates of the UPEC strains for trimethoprim-sulfamethoxazole (34.1%), ciprofloxacin (27.0%) and amoxicillin-clavulanic acid (13.4%) are relatively lower in Malaysia, as reported in the latest National Antibiotic Resistance Surveillance Report 2020 Malaysia (Institute for Medical Research, 2020). However, the resistance rates of amoxicillin-clavulanic acid and ciprofloxacin have increased from 13.2% and 26.3% in 2019 to 13.4% and 27.0% in 2020, respectively (Institute for Medical Research, 2020).

There is some controversy regarding the relationships between the antimicrobial resistance of the UPEC strains and the number of VGs carriage. Some prior studies have revealed the inverse correlation between the antimicrobial resistance of the UPEC strains and the number of VGs carriage (Takahashi, et al., 2009; Johnson and Russo, 2018; Zeng, et al., 2021). For instance, the fluoroquinolone-resistant isolates harbour a significantly lower prevalence of VGs such as *papA*, *papC*, *sfa/foc*, *cnf1*, *hly*, *kpsMT* II and *ibeA* compared to the susceptible isolates (Takahashi, et al., 2009; Zeng, et al., 2021). Nonetheless, the current global spread of ST131 UPEC strains is extensively

drug-resistant and confers a broad range of VG profiles (Johnson and Russo, 2018). Further studies are needed to elucidate the relationship between antimicrobial resistance and the virulence of the UPEC strains.

2.6 Repetitive-element Polymerase Chain Reaction

Repetitive-element Polymerase Chain Reaction (REP-PCR) is a PCR-based genomic profiling method commonly used to assess the clonal variability of bacterial strains (Johnson and Russo, 2018). As opposed to the Clermont phylotyping that only targets several DNA markers, REP-PCR amplifies the sequences between the highly conserved repetitive DNA regions (e.g., BOX and Enterobacterial Repetitive Intergenic Consensus (ERIC) elements) interspersed throughout the entire bacterial genome (Versalovic and Lupski, 2002; Pitout, et al., 2009). Therefore, REP-PCR has a greater discrimination power for differentiating closely related bacterial strains (Ardakani and Ranjbar, 2016; Bilung, et al., 2018; Mahmoud, et al., 2020).

REP-PCR has been considered a useful genotyping tool for exploring the clonality and genetic variability among *E. coli* isolates (Da Moreira Silva, et al., 2017; Kaushik, et al., 2019; Mahmoud, et al., 2020). Simple "same-versus-different" comparisons of the DNA fingerprints enable researchers to have an overview of the genetic diversity of the bacterial isolates faster and more affordable (Ardakani and Ranjbar, 2016; Bilung, et al., 2018; Mahmoud, et al., 2020). Nevertheless, the REP-PCR is unreliable for large-scale comparisons. The comparisons of different UPEC strains are made merely based on the

amplicon size, so the data may be less reliable and consistent as compared to the other sequence-based typing method, such as multilocus sequence typing.

Epidemiological studies show that most of the UPEC strains belong to specific phylogenetic groups (B2 or D) or clonal groups (Riley, 2014). For instance, major pandemic clonal lineages such as ST131, ST95, ST69, ST393 and ST73 have been reported across the continents and account for most of the extraintestinal infections, including UTIs (Manges, et al., 2008; Peirano, et al., 2012; Johnson, et al., 2017b; Denamur, et al., 2021). The clonal dissemination of these pandemic lineages has been proposed to be driven by either biological factors, such as enhanced virulence due to the carriage of consensus virulence profiles, or competitive advantage against antimicrobial selective pressure due to the acquired antimicrobial resistance genes, such as the extended-spectrum beta-lactamases (Riley, 2014; Bogema, et al., 2020). None of these hypotheses is validated to explain the global dissemination of these pandemic clonal lineages (Riley, 2014; Bogema, et al., 2020).

2.7 Risk Factors of UTI

In general, almost half of the females will have at least one UTI in their lifetime (Griebling, 2005). Females are prone to community-acquired UTIs due to anatomical differences such as shorter urethral length and closer distance between the urethra and anus (Tewawong, et al., 2020). Although UTIs are typically less common in males, males are often at a higher risk of infection by the MDR UPEC strains (Tamadonfar, et al., 2019; Raphael, Glymour and

Chambers, 2021). Extensive research has revealed a significant correlation between the male gender and the MDR phenotype (Almomani, et al., 2018; Benaissa, et al., 2021; Raphael, Glymour and Chambers, 2021; Shakya, et al., 2021). In the DNA microarray detection of 98 antimicrobial resistance genes, the UPEC strains isolated from male patients in Mexico harboured more antimicrobial genes than female patients (mean of 7.9 ± 5.5 versus 4 ± 3.0) (Ramírez-Castillo, et al., 2018).

On the other hand, the incidence of UTIs also increases with age (Schmiemann, et al., 2010; Ahmed, et al., 2018; Palacios-Ceña, et al., 2021). A large retrospective observational study in the United Kingdom demonstrated an increasing prevalence of UTI cases with the host age, peaking at 85 years and above in both genders (Ahmed, et al., 2018). Of note, hospital admissions due to UTIs are more prevalent in males, especially those over 85 years (Redondo-Sánchez, et al., 2021). This phenomenon may be due to incomplete bladder emptying, which is often associated with prostatic enlargement in older male patients (Woodford and George, 2011). Other factors include higher catheter usage and immunosenescence (decline of the immune system due to ageing) also increases the risk of UTIs in the elderly (Woodford and George, 2011; Cristina, et al., 2021).

Furthermore, elders might be at a higher risk of infection by the low-virulent UPEC strains. In Taiwan, the UPEC strains isolated from elderly patients (above 80 years) exhibited fewer VGs than the younger generation (3 years and below) (Lin, et al., 2021a; Lin, et al., 2021b). This phenomenon may

be related to the decline of the immune system due to ageing (Lin, et al., 2021a). Besides, elders might be more prone to MDR UPEC infections. For example, a retrospective study in China demonstrated an increasing prevalence of the MDR UPEC strains with the host age, having the highest rate of 68.8% in geriatric patients (above 65 years) (Huang, et al., 2021). Similar studies in Taiwan and Germany also showed that the UPEC strains collected from elderly patients exhibited greater antimicrobial resistance to most of the commonly used antimicrobials (Lin, et al., 2021a; Lin, et al., 2021b; Manseck, et al., 2022).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The materials used in the present study are listed in Table 3.1. On the other hand, the chemical reagents, agars, and medium were prepared as described in Table 3.2 and Table 3.3.

Table 3.1: Materials used and their manufacturers.

Material	Manufacturer, country
GoTaq®G2 DNA polymerase (with reaction buffer, $MgCl_2$ and dNTPs)	Promega
Oligonucleotides	Integrated DNA Technologies
Agarose gel	Invitrogen
EtB"Out" nucleic acid stain	Yeastern Biotech
100 bp DNA ladder (100 bp − 1500 bp)	Smobio, Taiwan
100 bp DNA ladder (100 bp − 3000 bp)	Cleaver Scientific, United Kingdom
1 kb DNA ladder (250 bp − 10 kb)	Vivantis, Malaysia
Tris base	Fisher bioreagents
Boric acid	Merck
Ethylenediaminetetraacetic acid (EDTA) di-sodium salt	Chemiz
Sodium chloride	Merck
Barium chloride	Merck
Sulfuric acid	Merck
Glycerol	Fisher Scientific
Luria-Bertani (LB) agar (Lennox)	Pronadisa
LB broth (Lennox)	Pronadisa
Mueller Hinton Agar (MHA)	Himedia
Antibiotic discs	BD BBL

Table 3.2: Preparation of the chemical reagents.

Chemical reagent	Formulation
10X Tris-Borate-EDTA (TBE) buffer	To make 1 L 121.1 g of Tris base 61.8 g of boric acid 7.4 g of EDTA di-sodium salt 1 L of distilled water
50% (v/v) glycerol	To make 1 L 0.5 L of 100% (w/v) glycerol 0.5 L of distilled water
0.85% (w/v) saline	To make 1 L 8.5 g of sodium chloride 1 L of distilled water
0.5 McFarland standard	To make 10 mL 0.05 mL of 1% (w/v) of barium chloride 9.95 mL of 1% (v/v) of sulfuric acid

Table 3.3: Preparation of the agars and medium.

Agar/broth	Formulation
LB agar	To make 1 L 40 g of LB agar powder 1 L of distilled water
LB broth	To make 1 L 20 g of LB broth powder 1 L of distilled water
MHA	To make 1 L 38 g of MHA powder 1 L of distilled water

3.2 Ethical Clearance and Consent

This research study has been approved by the Medical Research and Ethics Committee of the Ministry of Health Malaysia (reference number of KKM/NIHSEC/P21-31(4)) and the Scientific and Ethical Review Committee of the Universiti Tunku Abdul Rahman (reference number: U/SERC/111/2020). Moreover, the consent for collecting the UPEC samples and patient demographics was also obtained, with the reference number: IPSR/RMC/UTARRF/2020-C1/C02 (HRPB-UTIPS).

3.3 UPEC Sample Collection

As the incidence of UTI remains unknown in Malaysia, Solvin's formula was applied for calculating the sample size of the present study using the online software (SurveyMonkey; https://www.surveymonkey.com/mp/sample-size-calculator/). In 2020, the Perak population was estimated to be 2.51 million (Department of Statistics Malaysia, 2021). Therefore, a minimum of 97 samples were required to reach the confidence level of 95% and the margin of error of 10%.

In this study, a total of 105 UPEC strains were consecutively collected from the Raja Permaisuri Bainun Hospital from August 2020 to January 2021. All samples harvested from the urine specimens were cultured on the Cystine Lactose Electrolyte-Deficient and MacConkey agar (Oxoid, United Kingdom) using MAST BACTERURITEST strips (Abtek, United Kingdom) and incubated overnight at 37°C. The suspected *E. coli* bacterial isolates were further verified

through the Microflex® LT/SH MALDI-TOF biotyper (Bruker, Germany). Only pure UPEC isolates with significant bacterial growth of more than 100,000 CFU/mL were subcultured on the slant LB agar. All bacterial samples were suspended in 15% (v/v) glycerol and stored at -80°C. Besides, the demographic data of the study population (e.g., age, gender, hospitalisation (inpatient or outpatient) and geographical areas) were retrieved from the patient database of the microbiological laboratory. All the bacterial identification and isolation procedures mentioned earlier were carried out by the laboratory technicians of the Raja Permaisuri Bainun Hospital.

3.4 Antimicrobial Susceptibility Testing

A total of fifteen antimicrobials representing ten antimicrobial categories were tested in this study (Table 3.4). The antimicrobial susceptibility profiles of the UPEC isolates were determined through the disc diffusion method as described in the Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute, 2021). *E. coli* ATCC 25922 served as the quality control strain.

First, a single UPEC colony was streaked on the MHA and incubated at 37°C for 16 to 18 hours. Then, several bacterial colonies were inoculated into 0.85% sterilised saline solution until the turbidity was equivalent to 0.5 McFarland standard. A sterilised cotton swab was then dipped into the bacterial suspension and pressed firmly against the wall of the glass test tube several times before streaking on the MHA. This procedure was repeated twice, where the

plate was rotated approximately 60° each time. Then, the rim of the agar was also swabbed to ensure homogenous inoculum density throughout the agar plate. After a few minutes, antimicrobial discs were placed onto the agar, approximately 24 mm centre to centre apart from each other. Eventually, the zone of inhibition was measured using a ruler after incubating at 37°C for 16 to 18 hours.

For analysis, results that were interpreted as intermediate using the Clinical and Laboratory Standards Institute criteria were also treated as resistant. The multidrug-resistant (MDR) isolates referred to the UPEC isolates that showed resistance to at least one antimicrobial agent in three or more antimicrobial categories (Magiorakos, et al., 2012).

Table 3.4: β-lactam and non β-lactam antimicrobials for antimicrobial susceptibility testing.

Antimicrobial category	**Antimicrobial (disk content)**
Penicillin	Ampicillin[1] (10 µg)
Penicillin with beta-lactamase inhibitor	Ampicillin-sulbactam[1] (10/10 µg) and amoxicillin-clavulanate[1] (20/10 µg)
Non-extended spectrum cephalosporin	Cefuroxime[1] (30 µg)
Extended-spectrum cephalosporin	Cefotaxime[1] (30 µg) and ceftazidime[1] (30 µg)
Carbapenem	Ertapenem[1] (10 µg), meropenem[1] (10 µg) and imipenem[1] (10 µg)
Aminoglycoside	Gentamicin[2] (10 µg)
Nitrofuran	Nitrofurantoin[2] (300 µg)
Folate pathway inhibitor	Trimethoprim-sulfamethoxazole[2] (1.25/23.75 µg)
Fluoroquinolone	Levofloxacin[2] (5 µg) and ciprofloxacin[2] (5 µg)
Phosphonic acid	Fosfomycin[2] (200 µg)

[1]β-lactams
[2]Non-β-lactams

3.5 DNA Extraction

In this study, all the template DNAs were extracted through the fast-boil method as described by Kor, Choo and Chew (2013). A single bacterial colony was inoculated into 5 mL of LB medium and cultured overnight at 37°C with agitation at 220 rpm. Negative control consisting of only 5 mL of LB medium was also included. Then, 1.5 mL of the overnight culture was aliquoted into a 1.5 mL microcentrifuge tube and centrifuged at 11,200 *g* for five minutes. The supernatant was discarded and the pellet was washed with 300 µL of sterile distilled water. The bacterial suspension was then boiled for five minutes and placed immediately on ice for two minutes. Eventually, the supernatant was harvested after centrifuging at 11,200 *g* for two minutes. The purity of all template DNAs was checked through the NanoDrop™ 1000 spectrophotometer (Thermo Scientific, United States) before storing at -20°C.

3.6 Clermont Phylotyping Polymerase Chain Reaction

The phylogroup identification of the UPEC isolates was carried out according to the established protocol by Clermont and colleagues (2013). The Clermont phylotyping method consists of seven sets of primers that target *arpA*, *chuA*, *trpA*, *yjaA* and DNA fragment TspE4.C2, as listed in Table 3.5 and Table 3.6. This method comprises two major parts, a quadruplex PCR and additional E or C-specific PCRs. The UPEC isolates that could not be assigned directly based on the quadruplex genotypes underwent additional E or C-specific PCRs. All the UPEC isolates would have at least one PCR product in the quadruplex PCR

assays, which helped to assess the quality of the template DNAs and reduced the false-negative results (Clermont, et al., 2013).

All the PCR assays were performed in a total volume of 25 µL reaction mixture, containing a final concentration of 1X buffer, 1.5 mM of magnesium chloride ($MgCl_2$), 0.2 mM each deoxynucleotide triphosphate (dNTP), 1.25 U of *Taq* polymerase, 100 ng of template DNA and primers listed in Table 3.5 and Table 3.6. The conditions for the Clermont phylotyping PCRs are listed in Table 3.7.

Table 3.5: Primer sequences and amplicon sizes for quadruplex PCR (adapted from Clermont, et al., 2013).

Primer	Primer sequence (5'-3')	Target site	Amplicon size (bp)	Reference
chuA.1b[a]	ATGGTACCGGACGAACCAAC	*chuA*	288	Clermont, et al., 2013
chuA.2[a]	TGCCGCCAGTACCAAAGACA			Clermont, Bonacorsi and Bingen, 2000
yjaA.2b[a]	CAAACGTGAAGTGTCAGGAG	*yjaA*	211	Clermont, et al., 2013
yjaA.2b[a]	AATGCGTTCCTCAACCTGTG			Clermont, et al., 2013
TspE4C2.1b[a]	CACTATTCGTAAGGTCATCC	TspE4.C2	152	Clermont, et al., 2013
TspE4C2.2b[a]	AGTTTATCGCTGCGGGTCGC			Clermont, et al., 2013
AceK.f[b]	AACGCTATTCGCCAGCTTGC	*arpA*	400	Clermont, et al., 2013
ArpA1.r[b]	TCTCCCCATACCGTACGCTA			Clermont, Bonacorsi and Bingen, 2004

[a]The final concentration of the primer was 1 µM.
[b]The final concentration of the primer was 2 µM.

Table 3.6: Primer sequences and amplicon sizes of C- and E-specific PCRs (adapted from Clermont, et al., 2013).

Reaction	Primer	Primer sequence (5'-3')	Target site	Amplicon size (bp)	Reference
Group C	trpAgpC.1[b]	AGTTTTATGCC CAGTGCGAG	*trpA*	219	Lescat, et al., 2013
	trpAgpC.2[b]	TCTGCGCCGGT CACGCCC			Lescat, et al., 2013
Group E	ArpAgpE.f[b]	GATTCCATCTT GTCAAAATAT GCC	*arpA*	301	Lescat, et al., 2013
	ArpAgpE.r[b]	GAAAAGAAAA AGAATTCCCA AGAG			Lescat, et al., 2013
Internal control for group C and E	trpBA.f[a]	CGGCGATAAA GACATCTTCAC	*trpA*	489	Clermont, et al., 2008
	trpBA.r[a]	GCAACGCGGC CTGGCGGAAG			Clermont, et al., 2008

[a]The final concentration of the primer was 0.6 µM.
[b]The final concentration of the primer was 1 µM.

Table 3.7: Conditions for the Clermont phylotyping PCRs (adapted from Clermont, et al., 2013).

Step	Temperature	Duration
Initial denaturation	94°C	4 minutes
Denaturation	94°C	5 seconds
Annealing	57°C for (E-specific PCR) or 59°C (quadruplex and C-specific PCRs)	20 seconds
Final extension	72°C	5 minutes

3.7 Virulence Genes Multiplex Polymerase Chain Reaction

Thirty-two VGs representing five functional categories (e.g., adhesins, toxins, protectins, iron-uptake and miscellaneous) were identified through six multiplex PCR assays as shown in Table 3.8 (Johnson and Stell, 2000; Spurbeck, et al., 2012). All the multiplex PCR assays were carried out in a total volume of 25 µL reaction mixture, containing a final concentration of 1X buffer, 4 mM of $MgCl_2$ (except for Pool 2; 3.5 mM of $MgCl_2$ and Pool 5; 3.2 mM of $MgCl_2$), 0.8 mM each dNTP, 2.5 U of *Taq* polymerase, 100 ng of template DNA and the primers listed in Table 3.9 to Table 3.14. Six UPEC strains (e.g., J96, 2H16, 2H25, V27, PM9 and L31) served as the positive controls to validate the presence of all the VGs tested (Table 3.15 and Table 3.16). Sterile distilled water served as the negative control. The multiplex PCR assays started with the initial denaturation at 94°C for five minutes, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 63°C for 30 seconds and extension at 68°C for three minutes, with a final extension at 72°C for 10 minutes.

Table 3.8: Virulence genes tested in the virulence gene PCR assays (adapted from Johnson and Stell, 2000; Spurbeck, et al., 2012).

Functional category	Virulence gene	Comment
Adhesin	*papAH*	subunit of P fimbriae
	papC	assembly subunit of P fimbriae
	papEF	subunit of P fimbriae
	papG I	class 1 adhesin tip of P fimbriae (*papG*)
	papG II_III	conserved region of class II and III *papG* adhesin
	papG allele I	*papG* variants associated to J96 ($papG_{J96}$)
	papG allele II	*papG* variants associated to pyelonephritis ($papG_{1A2}$)
	papG allele III	*papG* variants associated with cystitis ($prsG_{J96}$)
	sfa/focDE	central regions of S and F1C fimbriae operons
	sfaS	pilus tip adhesin of S fimbriae
	focG	minor subunit of F1C fimbriae
	afa/draBC	central regions of Dr antigen-specific fimbrial and afimbiral adhesion operons
	bmaE	blood group M-specific adhesion
	gafD	N-acetyl-D-glucosamine-specific fimbriae
	fimH	adhesive tip of type 1 fimbriae
	yfcV	chaperone-usher fimbriae
Toxins	*hlyA*	α-Haemolysin A
	cnf1	cytototoxic necrotising factor 1
	cdtB	cytolethal distending toxin
	vat	vacuolating autotransporter toxin
Protectins	*kpsMT* II	group II capsule synthesis
	K1	specific to K1 group II capsule
	K5	specific to K5 group II capsule
	kpsMT III	group III capsule synthesis
	rfc	O4 lipopolysaccharide synthesis
Iron-uptake	*fyuA*	Yersiniabactin receptor
	chuA	haem binding receptor
	iutA	ferric aerobactin receptor
Miscellaneous	*cvaC*	Microcin V, formerly colicin V synthesis
	ibeA	brain endothelium invasion
	traT	serum resistance-associated protein
	malX	phosphotransferase system enzyme II (also known as PAI I_{CFT073})

Table 3.9: Primer sequences and amplicon sizes of the pool 1 virulence gene PCR assay (adapted from Johnson and Stell, 2000).

Primer	Primer sequence (5'-to 3')	Target site	Amplicon size (bp)	Reference
RPAi f	GGACATCCTGTTACAGCG CGCA	*malX*	925	Johnson and Stell, 2000
RPAi r	TCGCCACCAATCACAGCC GAAC			
PapA f	ATGGCAGTGGTGTCTTTT GGTG	*papAH*	717	Johnson and Stell, 2000
PapA r	CGTCCCACCATACGTGCT CTTC			
FimH f	TGCAGAACGGATAAGCC GTGG	*fimH*	508	Johnson and Stell, 2000
FimH r	GCAGTCACCTGCCCTCCG GTA			
KpsIII f	TCCTCTTGCTACTATTCCC CCT	*kpsMT* III	392	Johnson and Stell, 2000
KpsIII r	AGGCGTATCCATCCCTCC TAAC			
PapEF f	GCAACAGCAACGCTGGTT GCATCAT	*papEF*	326	Yamamoto, et al., 1995
PapEF r	AGAGAGAGCCACTCTTAT ACGGACA			
ibe10 f	AGGCAGGTGTGCGCCGC GTAC	*ibeA*	171	Huang, et al., 1995
ibe10 r	TGGTGCTCCGGCAAACCA TGC			Johnson and Stell, 2000

The final concentrations for all the primers were 0.6 µM.

Table 3.10: Primer sequences and amplicon sizes of the pool 2 virulence gene PCR assay (adapted from Johnson and Stell, 2000).

Primer	Primer sequence (5'-to 3')	Target site	Amplicon size (bp)	Reference
FyuA f[a]	TGATTAACCCCGCGACG GGAA	*fyuA*	787	Johnson and Stell, 2000
FyuA r[a]	CGCAGTAGGCACGATG TTGTA			Schubert, et al., 1998
bmaE-f[c]	ATGGCGCTAACTTGCCA TGCTG	*bmaE*	507	Johnson and Stell, 2000
bmaE-r[c]	AGGGGGACATATAGCC CCCTTC			
sfa1[a]	CTCCGGAGAACTGGGT GCATCTTAC	*sfa/focDE*	410	Le Bouguenec, Archambaud
sfa2[a]	CGGAGGAGTAATTACA AACCTGGCA			and Labigne, 1992
AerJ f[a]	GGCTGGACATCATGGG AACTGG	*iutA*	302	Johnson, et al., 1998
AerJ r[a]	CGTCGGGAACGGGTAG AATCG			
AlleleIII-f[a]	GGCCTGCAATGGATTTA CCTGG	*papG* allele III	258	Johnson and Brown, 1996
AlleleIII-r[a]	CCACCAAATGACCATGC CAGAC			
K1-fc[b]	TAGCAAACGTTCTATTG GTGC	*kpsMT* K1	153	Johnson and Stell, 2000
kpsII r[b]	CATCCAGACGATAAGC ATGAGCA			

[a]The final concentration of the primer was 0.3 µM.
[b]The final concentration of the primer was 0.6 µM.
[c]The final concentration of the primer was 1.2 µM.

Table 3.11: Primer sequences and amplicon sizes of the pool 3 virulence gene PCR assay (adapted from Johnson and Stell, 2000).

Primer	Primer sequence (5'-to 3')	Target site	Amplicon size (bp)	Reference
hly f[b]	AACAAGGATAAGCACT GTTCTGGCT	*hlyA*	1177	Yamamoto, et al., 1995
hly r[b]	ACCATATAAGCGGTCAT TCCCGTCA			
rfc-f[b]	ATCCATCAGGAGGGGA CTGGA	*rfc*	788	Johnson and Stell, 2000
rfc-r[b]	AACCATACCAACCAAT GCGAG			
AlleleI-f[a]	TCGTGCTCAGGTCCGGA ATTT	*papG* allele I	464	Mitsumori, et al., 1998
AlleleI-r[a]	TGGCATCCCCCAACATT ATCG			
kpsII f[a]	GCGCATTTGCTGATACT GTTG	*kpsMT* II	272	Johnson and Stell, 2000
kpsII r[a]	CATCCAGACGATAAGC ATGAGCA			
PapC f[a]	GTGGCAGTATGAGTAAT GACCGTTA	*papC*	205	Johnson and Stell, 2000
PapC r[a]	ATATCCTTTCTGCAGGG ATGCAATA			Le Bouguenec, Archambaud and Labigne, 1992

[a]The final concentration of the primer was 0.3 µM.
[b]The final concentration of the primer was 0.6 µM.

Table 3.12: Primer sequences and amplicon sizes of the pool 4 virulence gene PCR assay (adapted from Johnson and Stell, 2000).

Primer	Primer sequence (5'-to 3')	Target site	Amplicon size (bp)	Reference
gafD-f[c]	TGTTGGACCGTCTCAGGGCTC	*gafD*	952	Johnson and Stell, 2000
gafD-r[c]	CTCCCGGAACTCGCTGTTACT			
ColV-Cf[c]	CACACACAAACGGGAGCTGTT	*cvaC*	697	Johnson and Stell, 2000
ColV-Cr[c]	CTTCCCGCAGCATAGTTCCAT			
FocG f[a]	CAGCACAGGCAGTGGATACGA	*focG*	364	Johnson and Stell, 2000
FocG r[a]	GAATGTCGCCTGCCCATTGCT			
TraT f[a]	GGTGTGGTGCGATGAGCACAG	*traT*	290	Johnson and Stell, 2000
TraT r[a]	CACGGTTCAGCCATCCCTGAG			
AlleleII-f[b]	GGGATGAGCGGGCCTTTGAT	*papG* allele II	190	Johnson and Brown, 1996
AlleleII-r[b]	CGGGCCCCCAAGTAACTCG			

[a]The final concentration of the primer was 0.6 µM.
[b]The final concentration of the primer was 0.84 µM.
[c]The final concentration of the primer was 0.9 µM.

Table 3.13: Primer sequences and amplicon sizes of the pool 5 virulence gene PCR assay (adapted from Johnson and Stell, 2000).

Primer	Primer sequence (5'-to 3')	Target site	Amplicon size (bp)	Reference
pGf[g]	CTGTAATTACGGAAGTGATTTCTG	*papG* I	1190	Marklund, et al., 1992
pGr[f]	ACTATCCGGCTCCGGATAAACCAT			
pGf[g]	CTGTAATTACGGAAGTGATTTCTG	*papG* II_III	1070	Marklund, et al., 1992
pG1"r*[d]	TCCAGAAATAGCTCATGTAACCCG			Johnson and Stell, 2000
Afa f[a]	GGCAGAGGGCCGGCAACAGGC	*afa/draBC*	594	Johnson and Stell, 2000
Afa r[a]	CCCGTAACGCGCCAGCATCTC			
cnf1[c]	AAGATGGAGTTTCCTATGCAGGAG	*cnf1*	498	Yamamoto, et al., 1995
cnf2[c]	CATTCAGAGTCCTGCCCTCATTATT			
SfaS f[b]	GTGGATACGACGATTACTGTG	*sfaS*	244	Johnson and Stell, 2000
SfaS r[b]	CCGCCAGCATTCCCTGTATTC			
K5-fc[e]	CAGTATCAGCAATCGTTCTGTA	*kpsMT* K5	159	Johnson and Stell, 2000
kpsII r[c]	CATCCAGACGATAAGCATGAGCA			

[a]The final concentration of the primer was 0.09 µM.
[b]The final concentration of the primer was 0.54 µM.
[c]The final concentration of the primer was 0.6 µM.
[d]The final concentration of the primer was 0.72 µM.
[e]The final concentration of the primer was 0.9 µM.
[f]The final concentration of the primer was 1.38 µM.
[g]The final concentration of the primer was 2.1 µM.

Table 3.14: Primer sequences and amplicon sizes of the pool 6 virulence gene PCR assay (adapted from Johnson and Stell, 2000; Spurbeck, et al., 2012).

Primer	Primer sequence (5'-to 3')	Target site	Amplicon size (bp)	Reference
vat-f[a]	TCAGGACACGTTCAGGC ATTCAGT	*vat*	1100	Vigil, et al., 2011
vat-r[a]	GGCCAGAACATTTGCTC CCTTGTT			
fyuA-f[a]	GTAAACAATCTTCCCGC TCGGCAT	*fyuA*	850	Vigil, et al., 2011
fyuA-r[a]	TGACGATTAACGAACC GGAAGGGA			
chuA-f[c]	CTGAAACCATGACCGTT ACG	*chuA*	652	Spurbeck, et al., 2012
chuA-r[c]	TTGTAGTAACGCACTAA ACC			
cdt-s2[c]	GAAAATAAATGGAACA CACATGTCCG	*cdtB*	466	Johnson and Stell, 2000
cdt-a2[c]	AAATCTCCTGCAATCAT CCAGTTTA			
cdt-s1[c]	GAAAGTAAATGGAATA TAAATGTCCG		466	Johnson and Stell, 2000
cdt-a1[c]	AAATCACCAAGAATCA TCCAGTTA			
yfcV-f[b]	ACATGGAGACCACGTTC ACC	*yfcV*	292	Spurbeck, et al., 2011
yfcV-r[b]	GTAATCTGGAATGTGGT CAGG			

[a]The final concentration of the primer was 0.15 µM.
[b]The final concentration of the primer was 0.6 µM.
[c]The final concentration of the primer was 0.9 µM.
[d]The final concentration of the primer was 0.72 µM.

Table 3.15: Positive control strains for the virulence gene multiplex PCR assays.

UPEC strain	Description	Reference
J96	Pyelonephritis isolate collected in mid-1980s in Seattle	Johnson, et al., 1997
2H16, 2H25, V27 and PM9	Urosepsis adult patients isolates collected in the mid-1980s in Seattle Washington	Johnson and Stell, 2000
L31	Canine UTI isolate	Johnson, et al., 2001b

Table 3.16: Formulation of the positive controls for the virulence gene multiplex PCR assays.

Pool	Template DNA (Ratio)	Concentration (ng/µL)
1	2H25:J96 (1:1)	100
2	2H25:PM9 (1:1)	100
3	J96:L31 (1:1)	100
4	PM9:V27 (1:1)	100
5	J96: Mix (4:1) Mix=2H25:V27:2H16 (1:1:1)	100
6	V27	100

3.8 Pathogenicity Island Marker Multiplex Polymerase Chain Reaction

A total of eight PAI markers in three UPEC prototype strains (CFT073, J96 and 536) were determined through the multiplex PCR assays (Sabaté, et al., 2006). PAI III_{536}, IV_{536} and II_{CFT073} were detected in the multiplex PCR A assay, while the remaining PAI markers (e.g., PAI I_{J96}, II_{J96}, I_{536}, II_{536} and PAI I_{CFT073}) were detected in the multiplex PCR B assay, as shown in Table 3.17 and Table 3.18.

The PAI marker multiplex PCR A assays were carried out in a total volume of 25 µL reaction mixture, containing a final concentration of 1X buffer, 2 mM of $MgCl_2$, 0.5 mM each dNTP, 1.25 U of *Taq* polymerase, 100 ng of template DNA and the primers listed in Table 3.17. On the other hand, the PAI marker multiplex PCR B assays were carried out in a total volume of 25 µL reaction mixture, containing a final concentration of 1X buffer, 2.5 mM of $MgCl_2$, 0.5 mM each dNTP, 2.5 U of *Taq* polymerase, 100 ng of template DNA and the primers listed in Table 3.18. *E. coli* 536 and J96 served as the positive control for the multiplex PCR A and B assays, respectively.

The multiplex PCR assays started with the initial denaturation at 94°C for five minutes, followed by 30 cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute and extension at 72°C for one minute, with a final extension at 72°C for 10 minutes.

Table 3.17: Primer sequences and amplicon sizes of the PAI marker multiplex PCR A assay (adapted from Sabaté, et al., 2006).

Primer	Primer sequence (5'-to 3')	Target site	Amplicon size (bp)	Reference
sfaAI.1[a]	CGGGCATGCATCAATTATCTTTG	PAI III_{536}	161	Dobrindt, et al., 2001
sfaAI.2[a]	TGTGTAGATGCAGTCACTCCG			
IRP2FP[a]	AAGGATTCGCTGTTACCGGAC	PAI IV_{536}	280	Karch, et al., 1999
IRP2RP[a]	TCGTCGGGCAGCGTTTCTTCT			
cft073.2Ent1[b]	ATGGATGTTGTATCGCGC	PAI II_{CFT073}	400	Sabaté, et al., 2006
cft073.2Ent2[b]	ACGAGCATGTGGATCTGC			

[a]The final concentration of the primer was 0.5 µM.
[b]The final concentration of the primer was 0.7 µM.

Table 3.18: Primer sequences and amplicon sizes of the PAI marker multiplex PCR B assay (adapted from Sabaté, et al., 2006).

Primer	Primer sequence (5'-to 3')	Target site	Amplicon size (bp)	Reference
papGIf[b]	TCGTGCTCAGGTCCGGAATTT	PAI I_{J96}	461	Johnson and Brown, 1996
papGIr[b]	TGGCATCCCACATTATCG			
RPAi[a]	GGACATCCTGTTACAGCGCGCA	PAI I_{CFT073}	930	Johnson and Stell, 2000
RPAf[a]	TCGCCACCAATCACAGCGAAC			
orf1up[b]	CATGTCCAAAGCTCGAGCC	PAI II_{536}	1000	Sabaté, et al., 2006
orf1down[b]	CTACGTCAGGCTGGCTTTG			
I.9[c]	TAATGCCGGAGATTCATTGTC	PAI I_{536}	1800	Sabaté, et al., 2006
I.10[c]	AGGATTTGTCTCAGGGCTTT			
hlyd[d]	GGATCCATGAAAACATGGTTAATGGG	PAI II_{J96}	2300	Landraud, et al., 2003
cnf[d]	GATATTTTTGTTGCCATTGGTTACC			

[a]The final concentration of the primer was 0.12 µM.
[b]The final concentration of the primer was 0.2 µM.
[c]The final concentration of the primer was 0.8 µM.
[d]The final concentration of the primer was 1.2 µM.

3.9 Repetitive-element Polymerase Chain Reaction Assay

The genetic diversity of the UPEC isolates was assessed through the REP-PCR assays using BOXA1R (5-CTACGGCAAGGCGACGCTGACG-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') primers (Johnson and O'Bryan, 2000).

The REP-PCR assays were carried out in a total volume of 25 µL reaction mixture, containing a final concentration of 1X buffer, 1.5 mM of $MgCl_2$, 0.2 mM each dNTP, 1.25 U of *Taq* polymerase, 100 ng of template DNA and 2 µM of BOXA1R or ERIC2 primer. The REP-PCR assays started with the initial denaturation at 94°C for seven minutes, followed by 30 cycles of denaturation at 90°C for 30 seconds, annealing at 52°C for one minute and extension at 65°C for eight minutes, with a final extension at 65°C for 16 minutes.

All BOXA1R and ERIC2 DNA fingerprints were computed into binary data, in which the presence or absence of each band was indicated by 1 or 0, respectively. Then, the binary data of the BOXA1R and ERIC2 DNA fingerprints of each sample was combined from head-to-tail to form a composite DNA fingerprint. Dice similarity coefficient was used to compare the binary datasets. The dendrogram was constructed based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method using the Interactive Tree of Life (iTOL) version 6 online tool. The UPEC isolates that exhibited identical composite DNA fingerprints were classified under the same clonal group.

3.10 Agarose Gel Electrophoresis

PCR products of all the multiplex PCR assays were loaded on 2.5% (w/v) agarose gel prestained with EtB“Out” nucleic acid stain (Yeastern Biotech, Taiwan), except for the PAI marker multiplex PCR B assay, which was loaded on 1.5% (w/v) agarose gel for the better separation of the larger amplified DNA fragments. After electrophoresis at 1X TBE buffer at 80 V for approximately 40 minutes, the agarose gel was visualised under UV light using the Molecular Imager® ChemiDoc™ XRS+ (Bio-Rad, United States). The gel image was captured through the ChemiDoc™ XRS+ with Image Lab™ software (Bio-Rad, United States).

3.11 Statistical Analysis

The statistical analysis was performed using the Statistical Package of the Social Sciences (SPSS) version 26 software (IBM, United States). The analysis of categorical variables was conducted through the Pearson’s *Chi*-square test or Fisher’s exact test. Fisher’s exact test was used if an expected cell count of less than five was detected in at least one of the contingency table cells. On the other hand, the Mann-Whitney *U* test was applied for analysing the continuous variables. All the statistical tests were two-tailed, and a *p*-value of less than 0.05 was considered statistically significant throughout the study.

CHAPTER 4

RESULTS

4.1 Patient Demographics

The study population consisted of 76.2% (80/105) female and 23.8% (25/105) male patients, most of whom were inpatients (56/105; 53.3%). The median age of the patients was 57 years (ranging from 0 to 88 years). For analysis, the age of the patients was divided into five groups: 19 years and below, 20-39 years, 40-59 years, 60-79 years, and 80 years and above. Most of the UPEC samples (42/105; 40.0%) were isolated from the 60-79 age group. In contrast, only 7.6% (8/105) of the isolates were collected from patients aged 80 years and above. Figure 4.1 shows that the ratio of male and female patients in the 19 years and below and the 80 years and above age groups were similar. Nevertheless, a higher prevalence of female patients was observed in other age groups. Interestingly, no male patient was observed in the 20-39 age group.

The UPEC samples were collected from different regions of the Perak state in Malaysia. Out of the 16 regions, the majority of the UPEC samples were collected from Ipoh (74/105; 70.5%), followed by Kampar (8/105; 7.6%), Batu Gajah (5/105; 4.8%), Sungai Siput (5/105; 4.8%) and other areas (13/105; 12.3%).

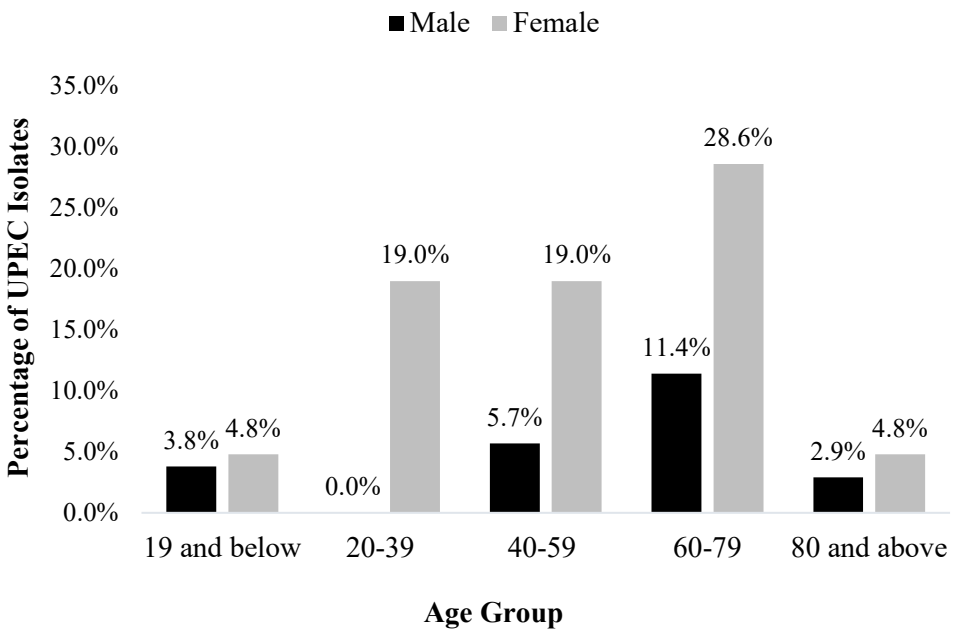


Figure 4.1: Prevalence among age groups and gender of patients

4.2 Antimicrobial Resistance Profiles of the UPEC Isolates

Out of the 15 antimicrobials tested, the UPEC isolates showed the highest resistance rates towards ampicillin (72/105; 68.6%), as shown in Table 4.1. Besides, high resistance rates were also observed in antimicrobials such as levofloxacin (36/105; 34.3%), amoxicillin-clavulanic acid (35/105; 33.3%), trimethoprim-sulfamethoxazole (35/105; 33.3%) and ciprofloxacin (32/105; 30.5%) (Table 4.1). For the extended and non-extended spectrum cephalosporins, the resistance rates ranged from 21.0% to 27.6%. Most of the UPEC isolates were susceptible to ampicillin-sulbactam (7/105; 6.7%), fosfomycin (2/105; 1.9%) and nitrofurantoin (1/105; 1.0%). Meanwhile, none of the UPEC isolates was resistant to carbapenems, including ertapenem, imipenem and meropenem (Table 4.1).

Table 4.1: Prevalence of antimicrobial resistance among the UPEC isolates.

Antimicrobial	Total (n= 105) No. of isolates (%)
Penicillin	
Ampicillin	72(68.6)
Penicillin with β-lactamase inhibitor	
Amoxicillin-clavulanic acid	35(33.3)
Ampicillin-sulbactam	7(6.7)
Non-extended spectrum cephalosporin	
Cefuroxime	29(27.6)
Extended spectrum cephalosporin	
Cefotaxime	28(26.7)
Ceftazidime	22(21.0)
Fluoroquinolone	
Ciprofloxacin	32(30.5)
Levofloxacin	36(34.3)
Aminoglycoside	
Gentamicin	19(18.1)
Nitrofuran	
Nitrofurantoin	1(1.0)
Folate pathway inhibitor	
Trimethoprim-sulfamethoxazole	35(33.3)
Phosphonic acid	
Fosfomycin	2(1.9)
Carbapenem	
Ertapenem	0(0.0)
Imipenem	0(0.0)
Meropenem	0(0.0)

4.3 Phylogroups of UPEC Isolates

In this study, the Clermont phylotyping PCRs were carried out to classify the phylogroups of the UPEC isolates, as described in Section 3.6. The representative gel images of the quadruplex, C-specific and E-specific PCRs are shown in Figure 4.2, Figure 4.3 and Figure 4.4, respectively. All UPEC isolates yielded at least one PCR product in the quadruplex PCRs, demonstrating that the template DNA quality was satisfactory for the PCR reactions (Clermont, et al., 2013).

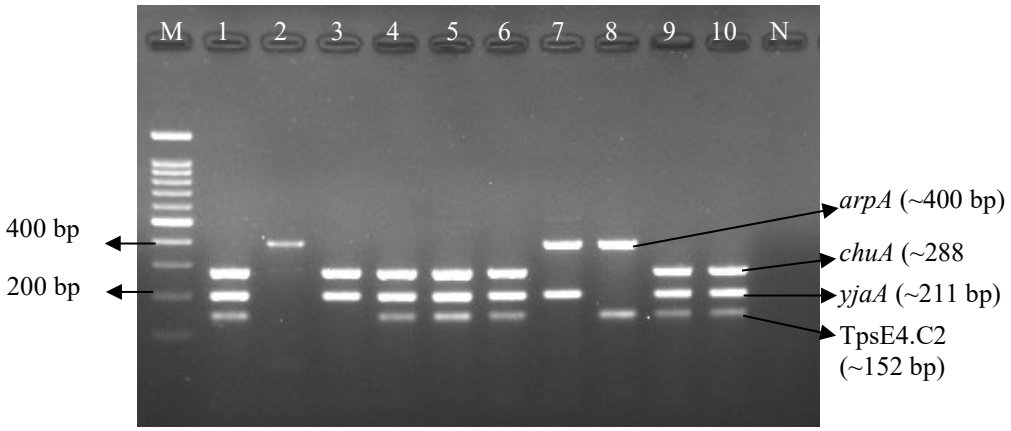


Figure 4.2: Representative gel image of the quadruplex multiplex PCR assay. M indicates the 100 bp DNA ladder (SMOBIO, Taiwan), lanes 1 to 10 indicate the representative UPEC samples and N indicates the negative control.

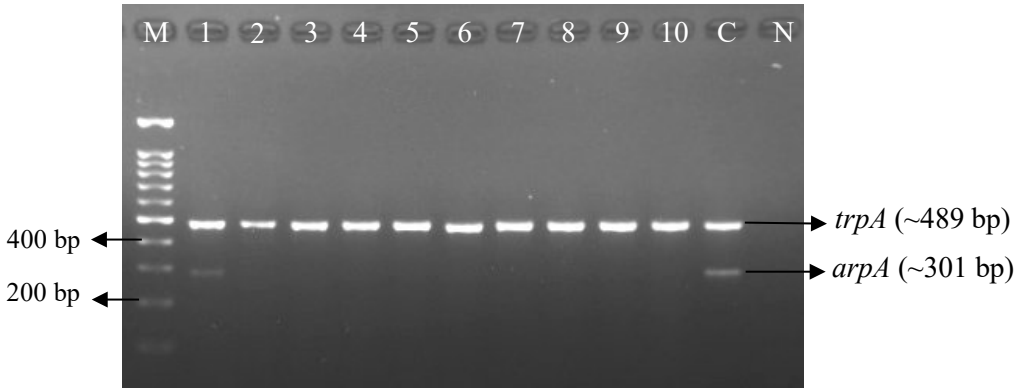


Figure 4.3: Representative gel image of the E-specific multiplex PCR assay. M indicates the 100 bp DNA ladder (SMOBIO, Taiwan), lanes 1 to 10 indicate the representative UPEC samples, C indicates the positive control and N indicates the negative control.

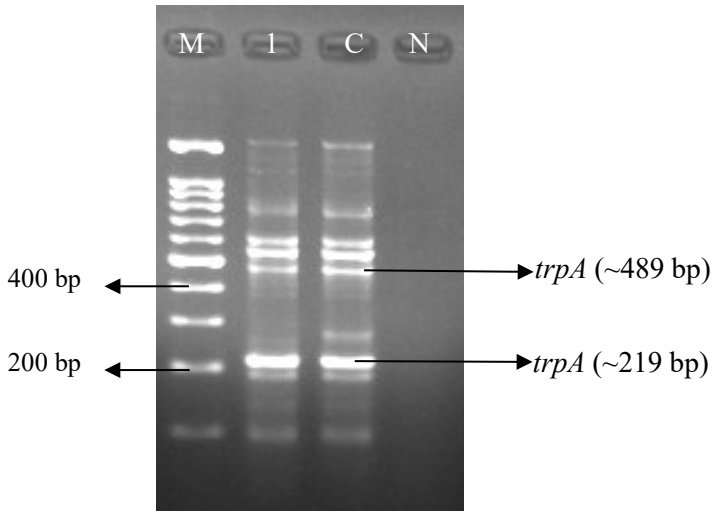


Figure 4.4: Representative gel image of the C-specific multiplex PCR assay. M indicates the 100 bp DNA ladder (SMOBIO, Taiwan), lane 1 indicates the representative UPEC sample, C indicates the positive control and N indicates the negative control.

One hundred and five UPEC isolates were classified into seven different phylogroups, where most of the isolates belonged to the phylogroup B2 (67/105; 63.8%), followed by phylogroup B1 (13/105; 12.4%) and phylogroup D (13/105; 12.4%). The remaining UPEC isolates were assigned to the phylogroups F (7/105; 6.7%), A (3/105; 2.9%), C (1/105; 0.9%) and E (1/105; 0.9%).

4.4 Prevalence of the Virulence Genes and Pathogenicity Island Markers

A total of 32 VGs representing five major functional categories were identified through the multiplex PCR assays as described in Section 3.7. The representative gel images of the VG multiplex PCR assays are shown in Figure 4.5 to Figure 4.10. On the other hand, eight PAI markers were tested as described in Section 3.8. The representative gel images of the PAI marker multiplex PCR assays are shown in Figure 4.11 to Figure 4.12.

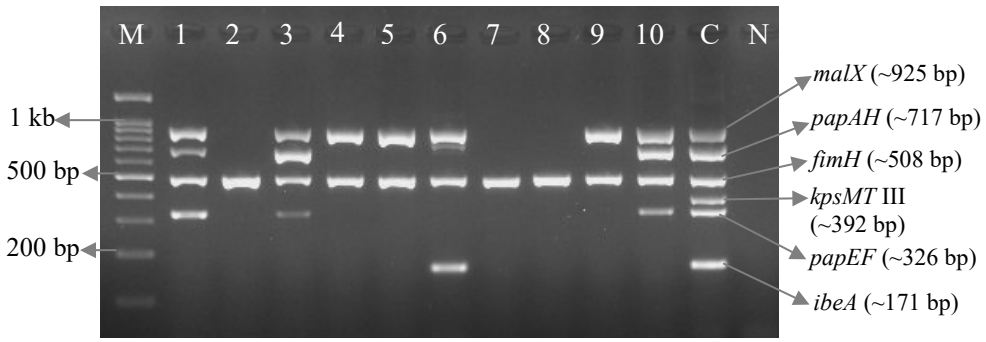


Figure 4.5: Representative gel image of the pool 1 virulence gene multiplex PCR assay. M indicates the 100 bp DNA ladder (SMOBIO, Taiwan), lanes 1 to 10 indicate the representative UPEC samples, C indicates the positive control and N indicates the negative control.

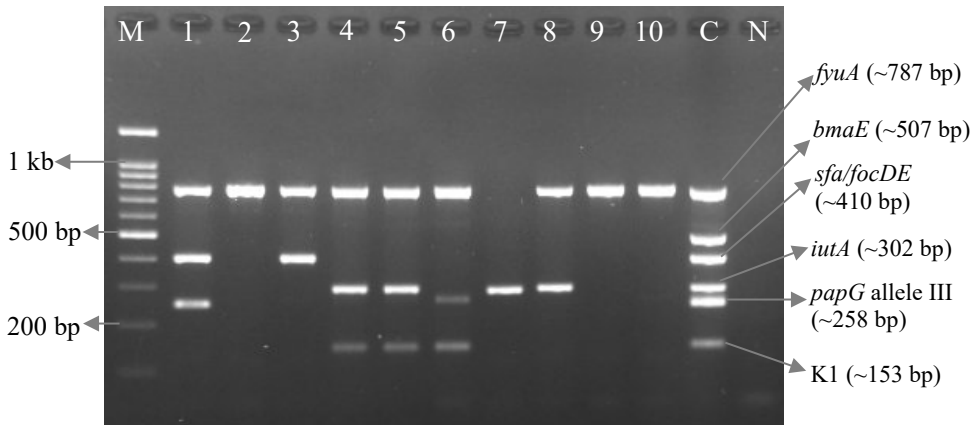


Figure 4.6: Representative gel image of the pool 2 virulence gene multiplex PCR assay. M indicates the 100 bp DNA ladder (SMOBIO, Taiwan), lanes 1 to 10 indicate the representative UPEC samples, C indicates the positive control and N indicates the negative control.

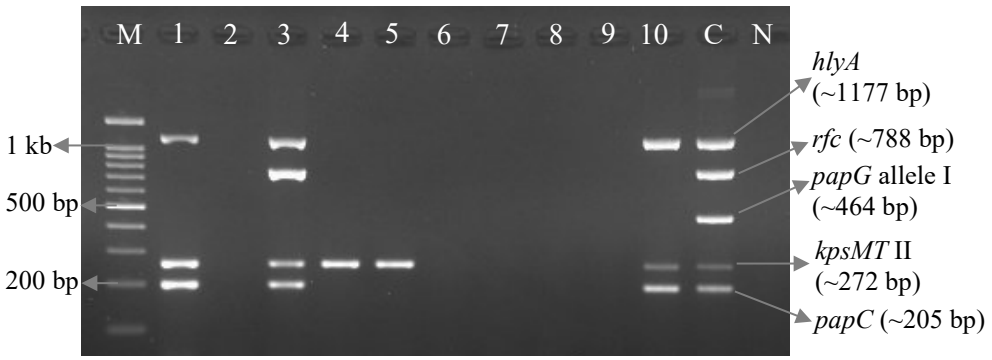


Figure 4.7: Representative gel image of the pool 3 virulence gene multiplex PCR assay. M indicates the 100 bp DNA ladder (SMOBIO, Taiwan), lanes 1 to 10 indicate the representative UPEC samples, C indicates the positive control and N indicates the negative control.

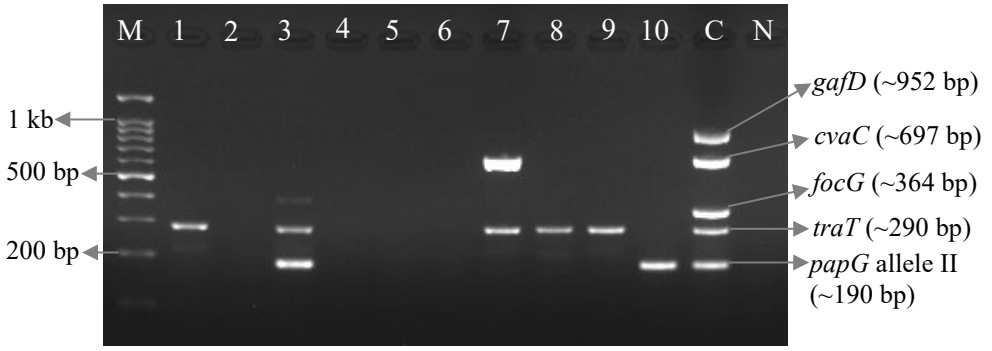


Figure 4.8: Representative gel image of the pool 4 virulence gene multiplex PCR assay. M indicates the 100 bp DNA ladder (SMOBIO, Taiwan), lanes 1 to 10 indicate the representative UPEC samples, C indicates the positive control and N indicates the negative control.

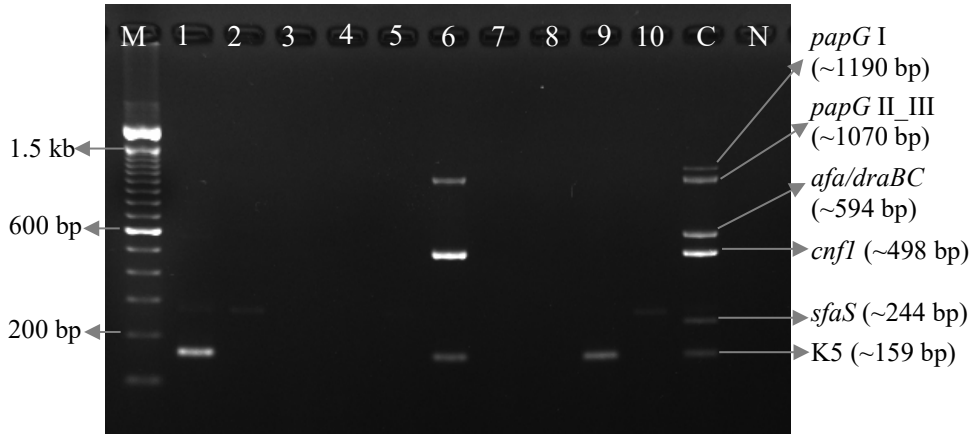


Figure 4.9: Representative gel image of the pool 5 virulence gene multiplex PCR assay. M indicates the 100 bp DNA ladder (Invitrogen, United States), lanes 1 to 10 indicate the representative UPEC samples, C indicates the positive control and N indicates the negative control.

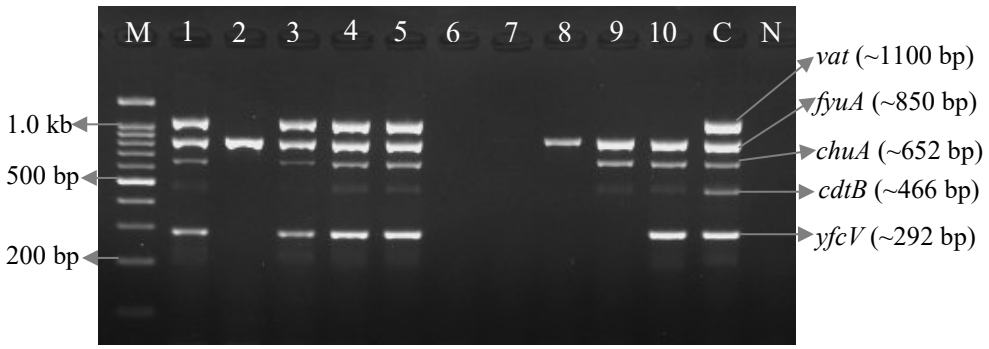


Figure 4.10: Representative gel image of the pool 6 virulence gene multiplex PCR assay. M indicates the 100 bp DNA ladder (SMOBIO, Taiwan), lanes 1 to 10 indicate the representative UPEC samples, C indicates the positive control and N indicates the negative control.

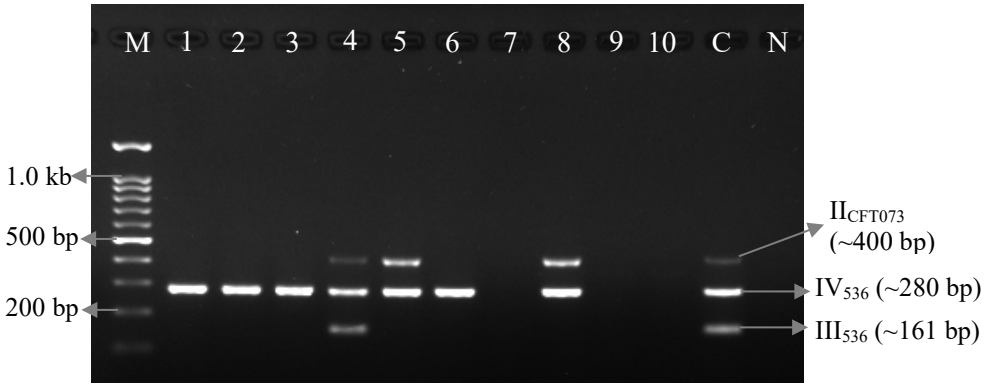


Figure 4.11: Representative gel image of the PAI marker multiplex PCR A assay. M indicates the 100 bp DNA ladder (SMOBIO, Taiwan), lanes 1 to 10 indicate the representative UPEC samples, C indicates the positive control and N indicates the negative control.

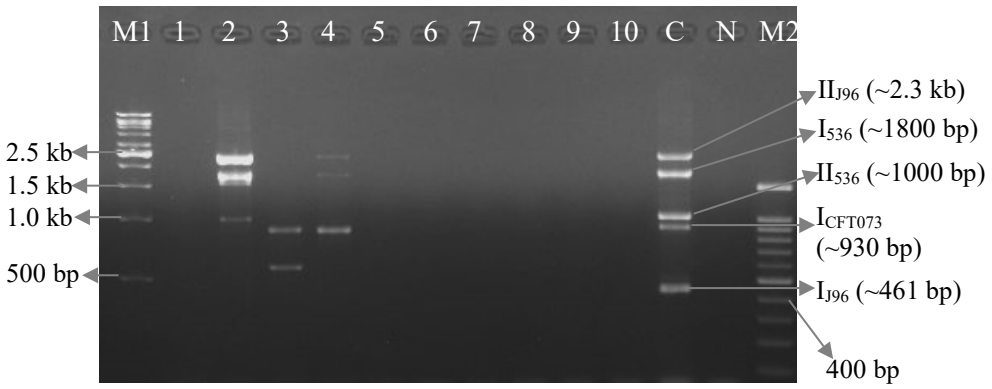


Figure 4.12: Representative gel image of the PAI marker multiplex PCR B assay. M1 and M2 indicate the 1.0 kb DNA ladder (Vivantis, Malaysia) and 100 bp DNA ladder (SMOBIO, Taiwan), respectively. Lanes 1 to 10 indicate the representative UPEC samples, C indicates the positive control and N indicates the negative control.

Out of the 32 VGs studied, *fimH* (102/105; 97.1%) was the most prevalent gene among the UPEC strains, followed by *fyuA* (90/105; 85.7%) and *chuA* (88/105; 83.8%), as shown in Table 4.2. For the adhesion genes, *yfcV* (70/105; 66.7%), *papEF* (49/105; 46.7%), *papAH* (48/105; 45.7%), *papC* (45/105; 42.9%) and *papG* II_III (44/105; 41.9%) were more commonly found. In contrast, *papG* allele II (26/105; 24.8%), *papG* allele III (24/105; 22.9%), *sfa/focDE* (24/105; 22.9%), *sfaS* (13/105; 12.4%), *afa/draBC* (8/105; 7.6%) and *focG* (7/105; 6.7%) were less common (Table 4.2). None of the UPEC isolates harboured *papG* I, *papG* allele I, *bmaE* and *gafD*, as shown in Table 4.2.

Of the studied toxic genes, *vat* (54/105; 51.4%) was more frequently found than *cnf1* (26/105; 24.8%), *hlyA* (25/105; 23.8%) and *cdtB* (1/105; 1.0%) (Table 4.2). *kpsMT* II (83/105; 79.0%) was the most prominent protectins among the UPEC isolates, followed by K5 (43/105; 41.0%), K1 (33/105; 31.4%), *rfc* (3/105; 2.9%) and *kpsMT* III (1/105; 1.0%). On the other hand, *iutA* (54/105; 51.4%) was the least prevalent iron-uptake gene among the UPEC isolates. For the miscellaneous genes, *malX* (71/105; 67.6%) and *traT* (68/105; 64.8%) were more commonly found compared to *cvaC* (12/105; 11.4%) and *ibeA* (5/105; 4.8%).

Table 4.2: Prevalence of the VGs among the UPEC isolates.

VG	Total (n= 105) No. of isolates (%)
Adhesin	
papAH	48(45.7)
papC	45(42.9)
papEF	49(46.7)
papG I	0(0.0)
papG II_III	44(41.9)
papG allele I	0(0.0)
papG allele II	26(24.8)
papG allele III	24(22.9)
sfa/focDE	24(22.9)
sfaS	13(12.4)
focG	7(6.7)
afa/draBC	8(7.6)
fimH	102(97.1)
yfcV	70(66.7)
gafD	0(0.0)
bmaE	0(0.0)
Toxin	
hlyA	25(23.8)
cnf1	26(24.8)
cdtB	1(1.0)
vat	54(51.4)
Protectin	
kpsMT II	83(79.0)
K1	33(31.4)
K5	43(41.0)
kpsMT III	1(1.0)
rfc	3(2.9)
Iron-uptake	
fyuA	90(85.7)
chuA	88(83.8)
iutA	54(51.4)
Miscellaneous	
cvaC	12(11.4)
ibeA	5(4.8)
traT	68(64.8)
malX	71(67.6)

Abbreviations: VG, virulence gene.

Out of the eight PAI markers tested, PAI IV_{536} (91/105; 86.7%) was the most prominent PAI marker, followed by the PAI I_{CFT073} (71/105; 67.6%) and PAI II_{CFT073} (55/105; 52.4%) (Table 4.3). PAI I_{536} (25/105; 23.8%), II_{J96} (25/105; 23.8%), II_{536} (23/105; 21.9%) were more commonly found as compared to the PAI III_{536} (13/105; 12.4%). Meanwhile, none of the UPEC isolates harboured the PAI I_{J96} (Table 4.3).

Table 4.3: Prevalence of the PAI markers among the UPEC isolates.

PAI markers	Total (n= 105) No. of isolates (%)
I_{536}	25(23.8)
II_{536}	23(21.9)
III_{536}	13(12.4)
IV_{536}	91(86.7)
I_{CFT073}	71(67.6)
II_{CFT073}	55(52.4)
I_{J96}	0(0.0)
II_{J96}	25(23.8)

Both primer sets used for detecting the *malX* and PAI I_{CFT073} were from the same reference. Abbreviations: PAI, pathogenicity island.

4.5 Phylogenetic Distribution of Virulence-associated Traits

To investigate the relationship between phylogroups and virulence-associated traits, the phylogenetic distribution of the VGs and PAI markers were compared and analysed using Pearson's *Chi*-square test or Fisher's exact test, as described in Section 3.11. On the other hand, the Mann-Whitney *U* test was used to compare the median of VGs and PAI markers among different phylogroups. A *p*-value of less than 0.05 was considered statistically significant.

Table 4.4 and Table 4.5 show that 17 VGs (e.g., *papAH*, *papC*, *papEF*, *papG* II_III, *papG* allele III, *sfa/focDE*, *sfaS*, *fimH*, *yfcV*, *hlyA*, *cnf1*, *vat*, *kpsMT* II, K1, *fyuA*, *chuA*, *malX*) and seven PAI markers (e.g., PAI I_{536}, PAI II_{536}, PAI III_{536}, PAI IV_{536}, PAI I_{CFT073}, PAI II_{CFT073} and PAI II_{J96}) were more frequently detected among the phylogroup B2 (all $p< 0.05$). In contrast, no virulence-associated trait was significantly more prevalent among other phylogroups (Table 4.4 and Table 4.5). Besides, six virulence-associated traits (e.g., *sfaS*, *cnf1*, *cdtB*, *ibeA*, PAI II_{536} and PAI III_{536}) were exclusively detected among the phylogroup B2, whereas *kpsMT* III was only observed in the phylogroup D (Table 4.4 and Table 4.5).

Notably, a distinct combination of virulence-associated traits that included *fimH*, *yfcV*, *kpsMT* II, *fyuA*, *chuA*, *malX*, PAI IV_{536} and PAI I_{CFT073} were detected in over 94.0% (63/67) of the phylogroup B2 isolates (all $p< 0.05$). In contrast, no virulence-associated trait was shared among the non-phylogroup B2 isolates, except for *fimH* (35/38; 92.1%) (Table 4.4 and Table 4.5). Overall, the phylogroup B2 exhibited the most VGs (median of 14 versus five) and PAI markers (median of three versus one) as compared to the non-phylogroup B2 (Table 4.6). As shown in Table 4.6, the phylogroups B1 and D had a lower prevalence of virulence-associated traits.

Table 4.4: Phylogenetic distribution of the VGs.

VG	No. (%) of UPEC isolates carrying VGs							
	A (n= 3)	B1 (n= 13)	B2 (n= 67)	C (n= 1)	D (n= 13)	E (n= 1)	F (n= 7)	Total (n= 105)
papAH	0(0.0)	1(7.7)[a]	41(61.2)[a]	0(0.0)	2(15.4)[a]	0(0.0)	4(57.1)	48(45.7)
papC	0(0.0)	1(7.7)[a]	38(56.7)[a]	0(0.0)	2(15.4)[a]	0(0.0)	4(57.1)	45(42.9)
papEF	0(0.0)	1(7.7)[a]	38(56.7)[a]	0(0.0)	6(46.2)	0(0.0)	4(57.1)	49(46.7)
papG II_III	0(0.0)	1(7.7)[a]	38(56.7)[a]	0(0.0)	2(15.4)[a]	0(0.0)	3(42.9)	44(41.9)
papG allele II	0(0.0)	1(7.7)	20(29.9)	0(0.0)	2(15.4)	0(0.0)	3(42.9)	26(24.8)
papG allele III	0(0.0)	1(7.7)	23(34.3)[a]	0(0.0)	0(0.0)[a]	0(0.0)	0(0.0)	24(22.9)
sfa/focDE	0(0.0)	1(7.7)	23(34.3)[a]	0(0.0)	0(0.0)[a]	0(0.0)	0(0.0)	24(22.9)
sfaS	0(0.0)	0(0.0)	13(19.4)[a]	0(0.0)	0(0.0)	0(0.0)	0(0.0)	13(12.4)
focG	0(0.0)	1(7.7)	6(9.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	7(6.7)
afa/draBC	0(0.0)	0(0.0)	7(10.4)	0(0.0)	1(7.7)	0(0.0)	0(0.0)	8(7.6)
fimH	2(66.7)	13(100.0)	67(100.0)[a]	1(100.0)	13(100.0)	0(0.0)[a]	6(85.7)	102(97.1)
yfcV	0(0.0)[a]	0(0.0)[a]	65(97.0)[a]	0(0.0)	0(0.0)[a]	0(0.0)	5(71.4)	70(66.7)
hlyA	0(0.0)	0(0.0)[a]	24(35.8)[a]	0(0.0)	1(7.7)	0(0.0)	0(0.0)	25(23.8)
cnf1	0(0.0)	0(0.0)[a]	26(38.8)[a]	0(0.0)	0(0.0)[a]	0(0.0)	0(0.0)	26(24.8)
cdtB	0(0.0)	0(0.0)	1(1.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(1.0)
vat	0(0.0)	2(15.4)[a]	49(73.1)[a]	1(100.0)	1(7.7)[a]	0(0.0)	1(14.3)	54(51.4)
kpsMT II	0(0.0)[a]	1(7.7)[a]	64(95.5)[a]	0(0.0)	12(92.3)	1(100.0)	5(71.4)	83(79.0)
K1	0(0.0)	0(0.0)[a]	26(38.8)[a]	0(0.0)	3(23.1)	1(100.0)	3(42.9)	33(31.4)
K5	0(0.0)	1(7.7)[a]	32(47.8)	0(0.0)	8(61.5)	0(0.0)	2(28.6)	43(41.0)
kpsMT III	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(7.7)	0(0.0)	0(0.0)	1(1.0)
rfc	0(0.0)	1(7.7)	2(3.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	3(2.9)

Table 4.4 (Cont.): Phylogenetic distribution of the VGs.

VG	No. (%) of UPEC isolates carrying virulence genes							
	A	B1	B2	C	D	E	F	Total
	(n= 3)	(n= 13)	(n= 67)	(n= 1)	(n= 13)	(n= 1)	(n= 7)	(n= 105)
fyuA	2(66.7)	5(38.5)[a]	67(100.0)[a]	0(0.0)	11(84.6)	1(100.0)	4(57.1)	90(85.7)
chuA	0(0.0)[a]	0(0.0)[a]	67(100.0)[a]	0(0.0)	13(100.0)	1(100.0)	7(100.0)	88(83.8)
iutA	1(33.3)	5(38.5)	33(49.3)	1(100.0)	9(69.2)	0(0.0)	5(71.4)	54(51.4)
cvaC	1(33.3)	3(23.1)	5(7.5)	1(100.0)	2(15.4)	0(0.0)	0(0.0)	12(11.4)
ibeA	0(0.0)	0(0.0)	5(7.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	5(4.8)
traT	2(66.7)	10(76.9)	42(62.7)	1(100.0)	9(69.2)	0(0.0)	4(57.1)	68(64.8)
malX	1(33.3)	0(0.0)[a]	66(98.5)[a]	0(0.0)	1(7.7)[a]	0(0.0)	3(42.9)	71(67.6)

[a]$p < 0.05$ was considered statistically significant. *papG* I, *papG* allele I, *gafD* and *bmaE* were not detected among the UPEC isolates. Abbreviations: UPEC, uropathogenic *Escherichia coli*.

Table 4.5: Phylogenetic distribution of the PAI markers.

PAI marker	No. (%) of UPEC isolates carrying PAI marker							
	A (n= 3)	B1 (n= 13)	B2 (n= 67)	C (n= 1)	D (n= 13)	E (n= 1)	F (n= 7)	Total (n= 105)
I_{536}	1(33.3)	1(7.7)	23(34.3)[a]	0(0.0)	0(0.0)[a]	0(0.0)	0(0.0)	25(23.8)
II_{536}	0(0.0)	0(0.0)	23(34.3)[a]	0(0.0)	0(0.0)	0(0.0)	0(0.0)	23(21.9)
III_{536}	0(0.0)	0(0.0)	13(19.4)[a]	0(0.0)	0(0.0)	0(0.0)	0(0.0)	13(12.4)
IV_{536}	2(66.7)	6(46.2)[a]	67(100.0)[a]	0(0.0)	11(84.6)	1(100.0)	4(57.1)[a]	91(86.7)
I_{CFT073}	1(33.3)	0(0.0)[a]	66(98.5)[a]	0(0.0)	1(7.7)[a]	0(0.0)	3(42.9)	71(67.6)
II_{CFT073}	1(33.3)	0(0.0)[a]	53(79.1)[a]	0(0.0)	0(0.0)[a]	0(0.0)	1(14.3)	55(52.4)
II_{J96}	1(33.3)	0(0.0)[a]	24(35.8)[a]	0(0.0)	0(0.0)[a]	0(0.0)	0(0.0)	25(23.8)

[a]$p< 0.05$ was considered statistically significant. PAI I_{J96} was not detected among the UPEC isolates. Abbreviations: PAI, pathogenicity island; UPEC, uropathogenic *Escherichia coli*.

Table 4.6: Comparison of the virulence-associated traits among different phylogroups.

Phylogroups (no. of isolates)	Median of VGs (range)		*p*-value	Median of PAI markers (range)		*p*-value
	Within phylogroup	Other isolates		Within phylogroup	Other isolates	
A (n= 3)	3(2-4)	10(1-21)	0.010	1(0-5)	3(0-7)	0.363
B1 (n= 13)	4(1-13)	10.5(2-21)	<0.0001	0(0-2)	3(0-7)	<0.0001
B2 (n= 67)	14(5-21)	5(1-13)	<0.0001	3(1-7)	1(0-5)	<0.0001
D (n= 13)	8(4-13)	10(1-21)	0.012	1(0-2)	3(0-7)	<0.0001
F (n= 7)	8(6-13)	9.5(1-21)	0.370	1(0-2)	3(0-7)	0.016

$p< 0.05$ was considered statistically significant. Abbreviations: VG, virulence gene; PAI, pathogenicity island.

4.6 Antimicrobial Resistance of Different Phylogroups

To investigate the relationship between phylogroups and antimicrobial resistance, the resistance rates of the phylogroups were compared and analysed through the Pearson's *Chi*-square test or Fisher's exact test, as described in Section 3.11.

All phylogroup A isolates (3/3; 100.0%) were resistant to ciprofloxacin (p= 0.026), levofloxacin (p= 0.038) and trimethoprim-sulfamethoxazole (p= 0.035), as shown in Table 4.7. On the other hand, the majority of the phylogroup B1 and phylogroup B2 isolates were resistant to amoxicillin-clavulanic acid (8/13; 61.5%; p= 0.029) and ampicillin (41/67; 61.2%; p= 0.031), respectively. In contrast, phylogroup E was susceptible to all the antimicrobials tested (Table 4.7).

Table 4.7 shows that 50.5% (53/105) of the UPEC isolates were MDR. Although the MDR isolates were predominated by the phylogroup B2 (28/53; 52.8%), a greater proportion of the phylogroup B2 isolates were not MDR (39/67; 58.2%; p= 0.018). In contrast, the majority of the non-phylogroup B2 isolates were MDR (25/38; 65.8%; p= 0.018), especially for the phylogroup B1 (10/13; 76.9%; p= 0.042).

Table 4.7: Prevalence of antimicrobial resistance among different phylogroups.

Antimicrobial	No. (%) of the resistant UPEC isolates							
	A (n= 3)	B1 (n= 13)	B2 (n= 67)	C (n= 1)	D (n= 13)	E (n= 1)	F (n= 7)	Total (n= 105)
Penicillin								
Ampicillin	3(100.0)	11(84.6)	41(61.2)[a]	1(100.0)	11(84.6)	0(0.0)	5(71.4)	72(68.6)
Penicillin with β-lactamase inhibitor								
Amoxicillin-clavulanic acid	1(33.3)	8(61.5)[a]	19(28.4)	0(0.0)	6(46.2)	0(0.0)	1(14.3)	35(33.3)
Ampicillin-sulbactam	1(33.3)	1(7.7)	4(6.0)	0(0.0)	1(7.7)	0(0.0)	0(0.0)	7(6.7)
Non-extended spectrum cephalosporin								
Cefuroxime	0(0.0)	5(38.5)	16(23.9)	1(100.0)	4(30.8)	0(0.0)	3(42.9)	29(27.6)
Extended spectrum cephalosporin								
Cefotaxime	0(0.0)	5(38.5)	16(23.9)	1(100.0)	3(23.1)	0(0.0)	3(42.9)	28(26.7)
Ceftazidime	0(0.0)	5(38.5)	11(16.4)	1(100.0)	2(15.4)	0(0.0)	3(42.9)	22(21.0)
Fluoroquinolone								
Ciprofloxacin	3(100.0)[a]	6(46.2)	20(29.9)	1(100.0)	1(7.7)	0(0.0)	1(14.3)	32(30.5)
Levofloxacin	3(100.0)[a]	5(38.5)	22(32.8)	1(100.0)	4(30.8)	0(0.0)	1(14.3)	36(34.3)
Aminoglycoside								
Gentamicin	1(33.3)	4(30.8)	11(16.4)	0(0.0)	2(15.4)	0(0.0)	1(14.3)	19(18.1)
Nitrofuran								
Nitrofurantoin	0(0.0)	0(0.0)	1(1.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(1.0)
Folate pathway inhibitor								
Trimethoprim-sulfamethoxazole	3(100.0)[a]	6(46.2)	19(28.4)	1(100.0)	3(23.1)	0(0.0)	3(42.9)	35(33.3)
Phosphonic acid								
Fosfomycin	0(0.0)	1(7.7)	1(1.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	2(1.9)
MDR	3(100.0)	10(76.9)[a]	28(41.8)[a]	1(100.0)	7(53.8)	0(0.0)	4(57.1)	53(50.5)

[a]$p < 0.05$ was considered statistically significant. All isolates were susceptible to ertapenem, imipenem and meropenem. Abbreviation: MDR, multidrug-resistant; UPEC, uropathogenic *Escherichia coli*.

4.7 Association between Virulence-associated Traits and Antimicrobial Resistance

To investigate the relationship between virulence-associated traits and MDR, the distribution of the VGs and PAI markers among the MDR and non-MDR isolates were compared and analysed using the Pearson's *Chi*-square test or Fisher's exact test, as described in Section 3.11. On the other hand, the Mann-Whitney *U* test was used to compare the median of VGs and PAI markers among the MDR and non-MDR isolates.

Out of the 40 virulence-associated traits tested, 17 virulence-associated traits were significantly more prevalent among the non-MDR isolates, including *papAH*, *papC*, *papEF*, *papG* II_III, *papG* allele III, *sfa/focDE*, *sfaS*, *yfcV*, *hlyA*, *cnf1*, *vat*, *kpsMT* II, *chuA*, PAI I_{536}, PAI II_{536}, PAI III_{536} and PAI II_{J96} (Table 4.8). In contrast, none of the virulence-associated traits was significantly prevalent among the MDR isolates, except for *iutA* (35/53; 66.0%; p= 0.002). Table 4.8 shows that *sfaS* (13/52; 25.0%), *cdtB* (1/52; 1.9%), *kpsMT* III (1/52; 1.9%) and PAI III_{536} (13/52; 25.0%) were exclusively detected among the non-MDR isolates. Overall, the MDR isolates exhibited significantly fewer VGs (median of nine versus 15; p< 0.0001) and PAI markers (median of two versus three, p= 0.008) than the non-MDR isolates (Table 4.8).

Table 4.8: Distribution of virulence-associated traits among MDR and non-MDR isolates.

Virulence-associated trait	MDR (n= 53)	Non-MDR (n= 52)	Total (n= 105)	*p*-value
VG				
papAH	16(30.2)	32(61.5)	48(45.7)	0.001
papC	14(26.4)	31(59.6)	45(42.9)	0.001
papEF	17(32.1)	32(61.5)	49(46.7)	0.002
papG II_III	13(24.5)	31(59.6)	44(41.9)	<0.0001
papG allele II	13(24.5)	13(25.0)	26(24.8)	0.955
papG allele III	3(5.7)	21(40.4)	24(22.9)	<0.0001
sfa/focDE	5(9.4)	19(36.5)	24(22.9)	0.001
sfaS	0(0.0)	13(25.0)	13(12.4)	<0.0001
focG	4(7.5)	3(5.8)	7(6.7)	1.000
afa/draBC	5(9.4)	3(5.8)	8(7.6)	0.716
fimH	52(98.1)	50(96.2)	102(97.1)	0.618
yfcV	29(54.7)	41(78.8)	70(66.7)	0.009
hlyA	5(9.4)	20(38.5)	25(23.8)	<0.0001
cnf1	5(9.4)	21(40.4)	26(24.8)	<0.0001
cdtB	0(0.0)	1(1.9)	1(1.0)	0.495
vat	19(35.8)	35(67.3)	54(51.4)	0.001
kpsMT II	37(69.8)	46(88.5)	83(79.0)	0.019
K1	14(26.4)	19(36.5)	33(31.4)	0.264
K5	17(32.1)	26(50.0)	43(41.0)	0.062
kpsMT III	0(0.0)	1(1.9)	1(1.0)	0.495
rfc	2(3.8)	1(1.9)	3(2.9)	1.000
fyuA	42(79.2)	48(92.3)	90(85.7)	0.056
chuA	39(73.6)	49(94.2)	88(83.8)	0.004
iutA	35(66.0)	19(36.5)	54(51.4)	0.002
cvaC	6(11.3)	6(11.5)	12(11.4)	0.972
ibeA	1(1.9)	4(7.7)	5(4.8)	0.205
traT	35(66.0)	33(63.5)	68(64.8)	0.782
malX	32(60.4)	39(75.0)	71(67.6)	0.109
Median (range)	9(1-18)	15(1-21)		<0.0001
PAI marker				
I_{536}	7(13.2)	18(34.6)	25(23.8)	0.010
II_{536}	4(7.5)	19(36.5)	23(21.9)	<0.0001
III_{536}	0(0.0)	13(25.0)	13(12.4)	<0.0001
IV_{536}	43(81.1)	48(92.3)	91(86.7)	0.092
I_{CFT073}	32(60.4)	39(75.0)	71(67.6)	0.109
II_{CFT073}	25(47.2)	30(57.7)	55(52.4)	0.280
II_{J96}	6(11.3)	19(36.5)	25(23.8)	0.002
Median (range)	2(0-6)	3(0-7)		0.008

$p < 0.05$ was considered statistically significant. *papG* I, *papG* allele I, *gafD*, *bmaE* and PAI I_{J96} were not detected among the UPEC isolates. Abbreviations: VG, virulence gene; PAI, pathogenicity island; MDR, multidrug-resistant.

4.8 Characteristics of the UPEC Strains in Relation to the Patient Demographics

To determine the risk factors of UTIs, the antimicrobial resistance and genetic profiles of the UPEC isolates were compared against the patient demographics, including age and gender.

4.8.1 Comparisons of Phylogroups with Host Age and Gender

Table 4.9 shows that the phylogroup B2 isolates were more frequently detected among all the five age groups. In contrast, phylogroups A, C and E were only detected among the 60-79 age group, 20-39 age group and 40-59 age group, respectively (Table 4.9). In this study, none of the phylogroups was significantly correlated with host age (all $p > 0.05$) (Table 4.9). However, the phylogroup B1 and phylogroup B2 were significantly associated with the host gender (Table 4.10). Most of the UPEC isolates collected from male patients belonged to the phylogroup B2 (21/25; 84.0%; $p = 0.016$), but the phylogroup B1 was only observed among female patients (Table 4.10).

Table 4.9: Prevalence of the phylogroups among different age groups.

Phylogroup	Host age group (years old) (No. (%) of UPEC isolates)					Total	*p*-value
	19 and below (n= 9)	20-39 (n= 20)	40-59 (n= 26)	60-79 (n= 42)	80 and above (n= 8)	(n= 105)	
A	0(0.0)	0(0.0)	0(0.0)	3(7.1)	0(0.0)	3(2.9)	0.327
B1	0(0.0)	1(5.0)	4(15.4)	7(16.7)	1(12.5)	13(12.4)	0.524
B2	6(66.7)	12(60.0)	19(73.1)	26(61.9)	4(50.0)	67(63.8)	0.763
C	0(0.0)	1(5.0)	0(0.0)	0(0.0)	0(0.0)	1(1.0)	0.368
D	3(33.3)	4(20.0)	1(3.8)	3(7.1)	2(25.0)	13(12.4)	0.069
E	0(0.0)	0(0.0)	1(3.8)	0(0.0)	0(0.0)	1(1.0)	0.547
F	0(0.0)	2(10.0)	1(3.8)	3(7.1)	1(12.5)	7(6.7)	0.775

$p < 0.05$ was considered statistically significant. Abbreviation: UPEC, uropathogenic *Escherichia coli*.

Table 4.10: Prevalence of the phylogroups in male and female patients.

Phylogroup	Host gender (No. (%) of UPEC isolates)		Total	*p*-value
	Female (n= 80)	Male (n= 25)	(n= 105)	
A	3(3.8)	0(0.0)	3(2.9)	1.000
B1	13(16.3)	0(0.0)	13(12.4)	0.035
B2	46(57.5)	21(84.0)	67(63.8)	0.016
C	1(1.3)	0(0.0)	1(1.0)	1.000
D	10(12.5)	3(12.0)	13(12.4)	1.000
E	1(1.3)	0(0.0)	1(1.0)	1.000
F	6(7.5)	1(4.0)	7(6.7)	1.000

$p < 0.05$ was considered statistically significant. Abbreviation: UPEC, uropathogenic *Escherichia coli*.

4.8.2 Comparisons of Virulence-associated Traits with Host Age and Gender

As shown in Table 4.11, the UPEC isolates collected from the 40-59 age group had significantly more *papC*, *papG* II_III, *papG* allele III, *sfa/focDE*, *hlyA*, *cnf1*, PAI I_{536}, PAI II_{536} and PAI II_{J96}. Nonetheless, nine virulence-associated traits, including *papAH*, *papC*, *papEF*, *papG* II_III, *papG* allele III, *sfa/focDE*, *cnf1*, *kpsMT* II and PAI II_{536} were significantly less prevalent among the 60-79 age group. Furthermore, the UPEC isolates collected from the 20-39 age group carried fewer PAI II_{CFT073} (6/20; 30.0%; p= 0.026) (Table 4.11). Overall, the UPEC isolates collected from the 40-59 age group exhibited the most VGs (median of 14.5 versus nine; p= 0.028) and PAI markers (median of three versus two; p= 0.034) when compared to the other age groups (Table 4.12). On the contrary, a lower prevalence of VGs was observed among the UPEC isolates collected from the 60-79 age group (median of nine versus 12; p= 0.014).

Table 4.11: Distribution of the virulence-associated traits among different age groups.

Virulence-associated trait	Host age group (years old) (No. (%) of UPEC isolates)					Total
	19 and below (n= 9)	20-39 (n= 20)	40-59 (n= 26)	60-79 (n= 42)	80 and above (n= 8)	(n= 105)
VG						
papAH	3(33.3)	11(55.0)	16(61.5)	13(31.0)[a]	5(62.5)	48(45.7)
papC	3(33.3)	10(50.0)	16(61.5)[a]	11(26.2)[a]	5(62.5)	45(42.9)
papEF	3(33.3)	11(55.0)	16(61.5)	13(31.0)[a]	6(75.0)	49(46.7)
papG II_III	3(33.3)	10(50.0)	16(61.5)[a]	10(23.8)[a]	5(62.5)	44(41.9)
papG allele II	2(22.2)	6(30.0)	7(26.9)	7(16.7)	4(50.0)	26(24.8)
papG allele III	1(11.1)	5(25.0)	12(46.2)[a]	5(11.9)[a]	1(12.5)	24(22.9)
sfa/focDE	1(11.1)	4(20.0)	12(46.2)[a]	5(11.9)[a]	2(25.0)	24(22.9)
sfaS	1(11.1)	3(15.0)	6(23.1)	2(4.8)	1(12.5)	13(12.4)
focG	0(0.0)	1(5.0)	3(11.5)	2(4.8)	1(12.5)	7(6.7)
afa/draBC	2(22.2)	0(0.0)	1(3.8)	5(11.9)	0(0.0)	8(7.6)
fimH	9(100.0)	20(100.0)	25(96.2)	41(97.6)	7(87.5)	102(97.1)
yfcV	6(66.7)	13(65.0)	20(76.9)	26(61.9)	5(62.5)	70(66.7)
hlyA	1(11.1)	5(25.0)	10(38.5)[a]	6(14.3)	3(37.5)	25(23.8)
cnf1	1(11.1)	4(20.0)	12(46.2)[a]	6(14.3)[a]	3(37.5)	26(24.8)
cdtB	0(0.0)	0(0.0)	1(3.8)	0(0.0)	0(0.0)	1(1.0)
vat	6(66.7)	11(55.0)	17(65.4)	18(42.9)	2(25.0)	54(51.4)
kpsMT II	8(88.9)	18(90.0)	23(88.5)	28(66.7)[a]	6(75.0)	83(79.0)
K1	5(55.6)	9(45.0)	8(30.8)	10(23.8)	1(12.5)	33(31.4)
K5	3(33.3)	8(40.0)	14(53.8)	14(33.3)	4(50.0)	43(41.0)
kpsMT III	1(11.1)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(1.0)
rfc	0(0.0)	0(0.0)	1(3.8)	2(4.8)	0(0.0)	3(2.9)

Table 4.11 (Cont.): Distribution of the virulence-associated traits among different age groups.

Virulence-associated trait	Host age group (years old) (No. (%) of UPEC isolates)					Total
	19 and below (n= 9)	20-39 (n= 20)	40-59 (n= 26)	60-79 (n= 42)	80 and above (n= 8)	(n= 105)
VG						
fyuA	8(88.9)	16(80.0)	23(88.5)	35(83.3)	8(100.0)	90(85.7)
chuA	9(100.0)	18(90.0)	22(84.6)	32(76.2)	7(87.5)	88(83.8)
iutA	7(77.8)	7(35.0)	10(38.5)	24(57.1)	6(75.0)	54(51.4)
cvaC	2(22.2)	3(15.0)	2(7.7)	4(9.5)	1(12.5)	12(11.4)
ibeA	0(0.0)	2(10.0)	1(3.8)	2(4.8)	0(0.0)	5(4.8)
traT	6(66.7)	13(65.0)	15(57.7)	29(69.0)	5(62.5)	68(64.8)
malX	6(66.7)	13(65.0)	19(73.1)	28(66.7)	5(62.5)	71(67.6)
PAI marker						
I_{536}	1(11.1)	4(20.0)	10(38.5)[a]	8(19.0)	2(25.0)	25(23.8)
II_{536}	1(11.1)	4(20.0)	11(42.3)[a]	5(11.9)[a]	2(25.0)	23(21.9)
III_{536}	1(11.1)	3(15.0)	6(23.1)	2(4.8)	1(12.5)	13(12.4)
IV_{536}	8(88.9)	16(80.0)	24(92.3)	35(83.3)	8(100.0)	91(86.7)
I_{CFT073}	6(66.7)	13(65.0)	19(73.1)	28(66.7)	5(62.5)	71(67.6)
II_{CFT073}	4(44.4)	6(30.0)[a]	17(65.4)	24(57.1)	4(50.0)	55(52.4)
II_{J96}	1(11.1)	4(20.0)	11(42.3)[a]	7(16.7)	2(25.0)	25(23.8)

[a]$p < 0.05$ was considered statistically significant. *papG* I, *papG* allele I, *gafD*, *bmaE* and PAI I_{J96} were not detected among the UPEC isolates. Abbreviations: VG, virulence gene; PAI, pathogenicity island; UPEC, uropathogenic *Escherichia coli*.

Table 4.12: Comparison of the virulence-associated traits carried by different age groups.

Age group	Median of VGs (range)		*p*-value	Median of PAI markers (range)		*p*-value
(no. of isolates)	Within age group	Other isolates		Within age group	Other isolates	
19 and below (n= 9)	9(4-18)	9.5(1-21)	0.991	2(0-7)	3(0-7)	0.617
20-39 (n= 20)	11.5(2-18)	9(1-21)	0.796	2(0-7)	3(0-7)	0.221
40-59 (n= 26)	14.5(1-21)	9(1-18)	0.028	3(0-7)	2(0-7)	0.034
60-79 (n= 42)	9(1-18)	12(1-21)	0.014	3(0-7)	2(0-7)	0.512
80 and above (n= 8)	11.5(5-18)	9(1-21)	0.581	2.5(1-7)	3(0-7)	0.922

$p < 0.05$ was considered statistically significant. Abbreviations: VG, virulence gene; PAI, pathogenicity island; UPEC, uropathogenic *Escherichia coli*.

Table 4.13 shows that most of the UPEC isolates collected from male patients harboured *yfcV*, *chuA*, *malX*, PAI I_{CFT073} and PAI II_{CFT073}, ranging from 72.0% to 100.0% (all $p< 0.05$). Even though more VGs were detected among male patients than female patients (median of 11 versus nine), the difference was not statistically significant ($p= 0.077$). On the other hand, the UPEC strains isolated from male patients had significantly more PAI markers than female patients (median of three versus two; $p= 0.045$). As shown in Table 4.13, no virulence-associated trait was significantly more prevalent among female patients.

Table 4.13: Distribution of the virulence-associated traits in male and female patients.

Virulence-associated traits	Host gender (No. (%) of UPEC isolates)		Total	*p*-value
	Female (n= 80)	Male (n= 25)	(n= 105)	
VG				
papAH	34(42.5)	14(56.0)	48(45.7)	0.237
papC	32(40.0)	13(52.0)	45(42.9)	0.290
papEF	35(43.8)	14(56.0)	49(46.7)	0.284
papG II_III	31(38.8)	13(52.0)	44(41.9)	0.241
papG allele II	17(21.3)	9(36.0)	26(24.8)	0.136
papG allele III	18(22.5)	6(24.0)	24(22.9)	0.876
sfa/focDE	17(21.3)	7(28.0)	24(22.9)	0.483
sfaS	10(12.5)	3(12.0)	13(12.4)	1.000
focG	4(5.0)	3(12.0)	7(6.7)	0.353
afa/draBC	5(6.3)	3(12.0)	8(7.6)	0.392
fimH	78(97.5)	24(96.0)	102(97.1)	0.562
yfcV	49(61.3)	21(84.0)	70(66.7)	0.035
hlyA	18(22.5)	7(28.0)	25(23.8)	0.573
cnf1	18(22.5)	8(32.0)	26(24.8)	0.337
cdtB	0(0.0)	1(4.0)	1(1.0)	0.238
vat	42(52.5)	12(48.0)	54(51.4)	0.694
kpsMT II	60(75.0)	23(92.0)	83(79.0)	0.068
K1	26(32.5)	7(28.0)	33(31.4)	0.672
K5	30(37.5)	13(52.0)	43(41.0)	0.198
kpsMT III	1(1.3)	0(0.0)	1(1.0)	1.000
rfc	2(2.5)	1(4.0)	3(2.9)	0.562
fyuA	66(82.5)	24(96.0)	90(85.7)	0.112
chuA	63(78.8)	25(100.0)	88(83.8)	0.011
iutA	40(50.0)	14(56.0)	54(51.4)	0.600
cvaC	10(12.5)	2(8.0)	12(11.4)	0.727
ibeA	4(5.0)	1(4.0)	5(4.8)	1.000
traT	51(63.7)	17(68.0)	68(64.8)	0.698
malX	49(61.3)	22(88.0)	71(67.6)	0.013
Median (range)	9(1-19)	11(4-21)		0.077
PAI marker				
I_{536}	19(23.8)	6(24.0)	25(23.8)	0.980
II_{536}	17(21.3)	6(24.0)	23(21.9)	0.772
III_{536}	10(12.5)	3(12.0)	13(12.4)	1.000
IV_{536}	67(83.8)	24(96.0)	91(86.7)	0.179
I_{CFT073}	49(61.3)	22(88.0)	71(67.6)	0.013
II_{CFT073}	37(46.3)	18(72.0)	55(52.4)	0.024
II_{J96}	19(23.8)	6(24.0)	25(23.8)	0.980
Median (range)	2(0-7)	3(0-7)		0.045

$p< 0.05$ was considered statistically significant. *papG* I, *papG* allele I, *gafD*, *bmaE* and PAI I_{J96} were not detected among the UPEC isolates. Abbreviations: VG, virulence gene; PAI, pathogenicity island; UPEC, uropathogenic *Escherichia coli*.

4.8.3 Comparison of the Antimicrobial Resistance with Host Age and Gender

The UPEC isolates collected from the 60-79 age group were significantly more resistant towards antimicrobials, including cefuroxime (18/42; 42.9%), cefotaxime (18/42; 42.9%), ceftazidime (15/42; 35.7%), levofloxacin (21/42; 50.0%) and gentamicin (12/42; 28.6%), as shown in Table 4.14. On the contrary, the UPEC isolates collected from the 40-59 age group were significantly more susceptible to cefuroxime (3/26; 11.5%; p= 0.034) and cefotaxime (3/26; 11.5%; p= 0.044).

In the present study, the MDR phenotype was correlated with the host age. Table 4.14 shows that a higher prevalence of MDR was observed among the 60-79 age group (29/42; 69.0%; p= 0.002). Overall, the prevalence of the MDR isolates fluctuated across five age groups, reaching the first peak at the 20-39 age group (9/20; 45.0%) and the second peak at the 60-79 years age group (29/42; 69.0%) (Table 4.14).

Besides, the UPEC isolates collected from male patients were more MDR (14/25; 56.0%; p= 0.527) and exhibited higher resistance rates towards most of the antimicrobials tested, especially for cefuroxime (11/25; 44.0%; p= 0.036), cefotaxime (11/25; 44.0%; p= 0.025) and ceftazidime (9/25; 36.0%; p= 0.034) (Table 4.15). The only exception was fosfomycin, where the resistant isolates were only detected among female patients (2/80; 2.5%; p= 1.000).

Table 4.14: Antimicrobial resistance profiles of different age groups.

Antimicrobial	Host age group (years old) (No. (%) of resistant isolates)					Total
	19 and below (n= 9)	20-39 (n= 20)	40-59 (n= 26)	60-79 (n= 42)	80 and above (n= 8)	(n= 105)
Ampicillin	7(77.8)	15(75.0)	14(53.8)	32(76.2)	4(50.0)	72(68.6)
Amoxicillin-clavulanic acid	2(22.2)	7(35.0)	8(30.8)	17(40.5)	1(12.5)	35(33.3)
Ampicillin-sulbactam	0(0.0)	2(10.0)	0(0.0)	5(11.9)	0(0.0)	7(6.7)
Cefuroxime	1(11.1)	5(25.0)	3(11.5)[a]	18(42.9)[a]	2(25.0)	29(27.6)
Cefotaxime	1(11.1)	5(25.0)	3(11.5)[a]	18(42.9)[a]	1(12.5)	28(26.7)
Ceftazidime	1(11.1)	4(20.0)	2(7.7)	15(35.7)[a]	0(0.0)	22(21.0)
Ciprofloxacin	2(22.2)	4(20.0)	7(26.9)	17(40.5)	2(25.0)	32(30.5)
Levofloxacin	3(33.3)	4(20.0)	6(23.1)	21(50.0)[a]	2(25.0)	36(34.3)
Gentamicin	3(33.3)	1(5.0)	3(11.5)	12(28.6)[a]	0(0.0)	19(18.1)
Nitrofurantoin	0(0.0)	0(0.0)	0(0.0)	1(2.4)	0(0.0)	1(1.0)
Trimethoprim-sulfamethoxazole	3(33.3)	8(40.0)	9(34.6)	14(33.3)	1(12.5)	35(33.3)
Fosfomycin	0(0.0)	0(0.0)	0(0.0)	2(4.8)	0(0.0)	2(1.9)
MDR	3(33.3)	9(45.0)	10(38.5)	29(69.0)[a]	2(25.0)	53(50.5)

[a]$p < 0.05$ was considered statistically significant. Abbreviations: MDR, multidrug-resistant.

Table 4.15: Antimicrobial resistance profiles of the UPEC strains isolated from male and female patients.

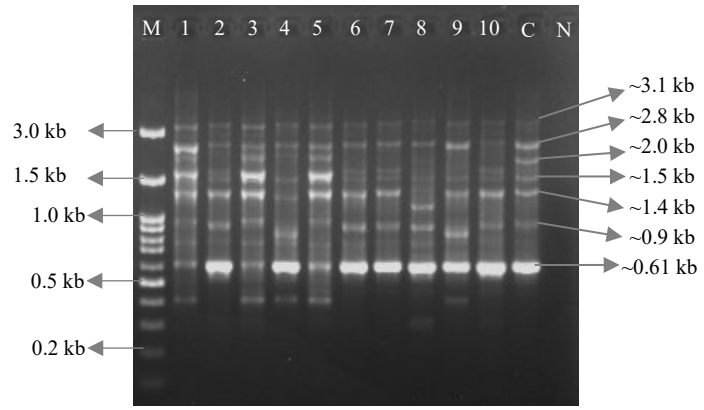
Antimicrobial	Host gender (No. (%) of resistant isolates)		Total	*p*-value
	Female (n= 80)	Male (n= 25)	(n= 105)	
Ampicillin	54(67.5)	18(72.0)	72(68.6)	0.672
Amoxicillin-clavulanic acid	26(32.5)	9(36.0)	35(33.3)	0.746
Ampicillin-sulbactam	4(5.0)	3(12.0)	7(6.7)	0.353
Cefuroxime	18(22.5)	11(44.0)	29(27.6)	0.036
Cefotaxime	17(21.3)	11(44.0)	28(26.7)	0.025
Ceftazidime	13(16.3)	9(36.0)	22(21.0)	0.034
Ciprofloxacin	22(27.5)	10(40.0)	32(30.5)	0.236
Levofloxacin	24(30.0)	12(48.0)	36(34.3)	0.098
Gentamicin	13(16.3)	6(24.0)	19(18.1)	0.384
Nitrofurantoin	0(0.0)	1(4.0)	1(1.0)	0.238
Trimethoprim-sulfamethoxazole	26(32.5)	9(36.0)	35(33.3)	0.746
Fosfomycin	2(2.5)	0(0.0)	2(1.9)	1.000
MDR	39(48.8)	14(56.0)	53(50.5)	0.527

$p < 0.05$ was considered statistically significant. Abbreviations: MDR, multidrug-resistant.

4.9 Genetic Diversity of the UPEC Isolates

To assess the clonal relatedness of the UPEC isolates, the REP-PCR assays were carried out as described in Section 3.9. The representative gel images of the REP-PCR assays are shown in Figure 4.13.

(A)



(B)

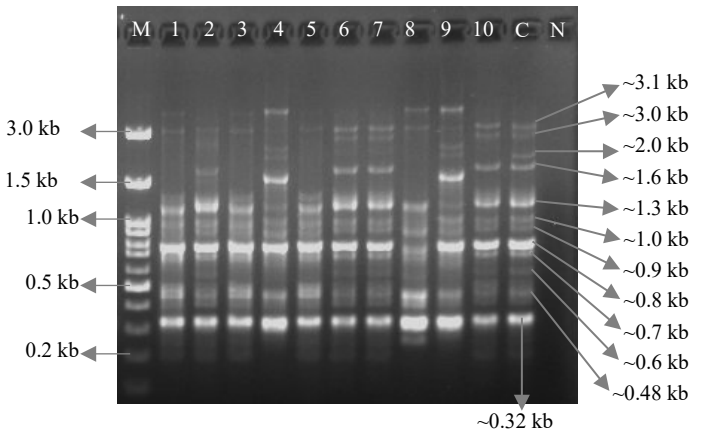


Figure 4.13: Representative gel image of the REP-PCR assays using the (A) BOXA1R and (B) ERIC2 primers. M indicates the 100 bp DNA ladder (Cleaver Scientific, United Kingdom) whereas lanes 1 to 10 indicate the representative UPEC samples. C indicates the positive control, whereas N indicates the negative control.

The REP-PCR assays revealed that the majority of the UPEC strains (87/105; 82.9%) were genetically diverse. Among the eight different clonal groups (CGs) identified (18/105; 17.1%), CG1 was the most prevalent clonal group, which comprised four isolates, including UTIPS 1, UTIPS 77, UTIPS 80 and UTIPS 101 (Figure 4.14). Each of the remaining clonal groups (e.g., CG2 to CG8) only contained two isolates (Figure 4.14).

While most of the UPEC isolates within the same phylogroups were clustered closely in the dendrogram, some of the isolates were spread over different clades, as shown in Figure 4.14. For instance, a phylogroup B1 isolate (UTIPS 30) was clustered into the same clade as a phylogroup A isolate (UTIPS 2). In addition, phylogroup F isolates (UTIPS 13 and UTIPS 48) were clustered together with phylogroup D isolates (UTIPS 90 and UTIPS 47), respectively.

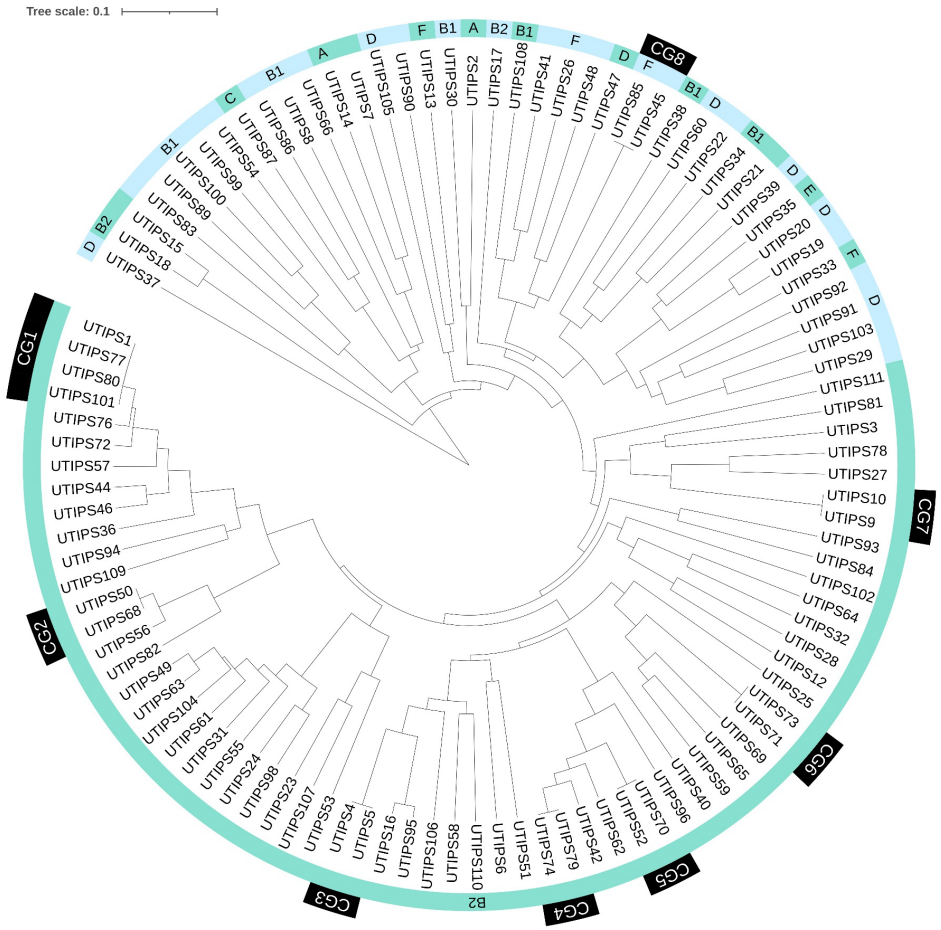


Figure 4.14: The UPGMA dendrogram based on the Dice similarity coefficient among 105 UPEC isolates evaluated using the BOXA1R and ERIC2 primers. The dendrogram was visualised using the Interactive Tree of Life (iTOL). UPEC isolates with identical BOXA1R and ERIC2 DNA fingerprints were classified as a single clonal group. Abbreviations: CG, clonal group.

4.10 Clonal Dissemination of the UPEC Isolates

To determine if the closely related UPEC isolates would share similar molecular characteristics, the phylogenetic background, virulence-associated traits and antimicrobial resistance profiles of the clonal group isolates were compared, as shown in Table 4.16.

Overall, the clonal UPEC isolates were highly homogeneous in their phylogeny, VG and PAI marker background but differed in their antimicrobial resistance phenotypes, except for CG7 isolates (Table 4.16). For instance, CG2, CG4, and CG8 isolates had similar virulence-associated traits, but their antimicrobial resistance varied substantially from non-MDR to MDR. Moreover, the UPEC isolates from different clonal groups also conferred identical virulence-associated traits. For instance, CG3 and CG6 isolates shared identical virulence-associated trait profiles of *fimH*, *yfcV*, *vat*, *kpsMT* II, K1, *fyuA*, *chuA*, *iutA*, *malX*, PAI IV_{536}, PAI I_{CFT073} and PAI II_{CFT073} (Table 4.16).

As shown in Table 4.16, the dominant CG1 isolates carried the highest number of 25 virulence-associated traits but were susceptible to all the antimicrobials tested. On the contrary, some of the CG isolates (e.g., CG2, CG4 and CG7) were both MDR and harboured 18 to 22 virulence-associated traits.

Table 4.16: Antimicrobial resistance and genetic profiles of the closely related UPEC isolates.

Clonal group	Phylogroup	Virulence-associated Trait	Antimicrobial resistance
CG1	B2	*papAH*, *papC*, *papEF*, *papG* II_III, *papG* allele III, *sfa/focDE*, *sfaS*, *fimH*, *yfcV*, *hlyA*, *cnf1*, *vat*, *kpsMT* II, K5, *fyuA*, *chuA*, *traT*, *malX*, I_{536}, II_{536}, III_{536}, IV_{536}, I_{CFT073}, II_{CFT073}, II_{J96}	All susceptible
CG2	B2	*papAH*, *papC*, *papEF*, *papG* II_III, *papG* allele III, *sfa/focDE*, *focG*, *fimH*, *yfcV*, *cnf1*, *vat*, *kpsMT* II, K5, *fyuA*, *chuA*, *malX*, I_{536}, II_{536}, IV_{536}, I_{CFT073}, II_{CFT073}, II_{J96}	AMP, AMC, SXT
		papAH, *papC*, *papEF*, *papG* II_III, *papG* allele III, *sfa/focDE*, *sfaS*, *fimH*, *yfcV*, *hlyA*, *cnf1*, *vat*, *kpsMT* II, *K5*, *fyuA*, *chuA*, *malX*, I_{536}, II_{536}, III_{536}, IV_{536}, I_{CFT073}, II_{CFT073}, II_{J96}	All susceptible
CG3	B2	*fimH*, *yfcV*, *vat*, *kpsMT* II, K1, *fyuA*, *chuA*, *iutA*, *malX*, IV_{536}, I_{CFT073}, II_{CFT073}	AMP, CIP, LE, GEN, SXT
		fimH, *yfcV*, *vat*, *kpsMT* II, K1, *fyuA*, *chuA*, *iutA*, *malX*, IV_{536}, I_{CFT073}, II_{CFT073}	AMP, LE, GEN
CG4	B2	*papAH*, *papC*, *papEF*, *papG* II_III, *papG* allele II, *fimH*, *yfcV*, *vat*, *kpsMT* II, K1, *fyuA*, *chuA*, *iutA*, *cvaC*, *traT*, *malX*, IV_{536}, I_{CFT073}	AMP
		papAH, *papC*, *papEF*, *papG* II_III, *papG* allele II, *fimH*, *yfcV*, *vat*, *kpsMT* II, K1, *fyuA*, *chuA*, *iutA*, *traT*, *malX*, IV_{536}, I_{CFT073}, II_{CFT073}	AMP, AMC, GEN, SXT
CG5	B2	*papAH*, *papC*, *papEF*, *papG* II_III, *papG* allele II, *fimH*, *yfcV*, *vat*, *kpsMT* II, K1, *fyuA*, *chuA*, *iutA*, *cvaC*, *traT*, *malX*, IV_{536}, I_{CFT073}	AMP
		papAH, *papC*, *papEF*, *papG* II_III, *papG* allele II, *fimH*, *yfcV*, *vat*, *kpsMT* II, K1, *fyuA*, *chuA*, *iutA*, *cvaC*, *traT*, *malX*, IV_{536}, I_{CFT073}	All susceptible
CG6	B2	*fimH*, *yfcV*, *vat*, *kpsMT* II, K1, *fyuA*, *chuA*, *iutA*, *malX*, IV_{536}, I_{CFT073}, II_{CFT073}	AMP, CIP, LE
		fimH, *yfcV*, *vat*, *kpsMT* II, K1, *fyuA*, *chuA*, *iutA*, *malX*, IV_{536}, I_{CFT073}	LE
CG7	B2	*fimH*, *fyuA*, *chuA*, *traT*, *malX*, IV_{536}, I_{CFT073}, II_{CFT073}	AMP, AMC, A/S, CXM, CAZ, CTX, CIP, LE, GEN, SXT
		papAH, *papC*, *papEF*, *papG* II_III, *papG* allele II, *fimH*, *yfcV*, *hlyA*, *cnf1*, *kpsMT* II, *fyuA*, *chuA*, *malX*, II_{536}, IV_{536}, I_{CFT073}, II_{CFT073}, II_{J96}	AMP, AMC, CTX, CAZ, CXM, SXT

Table 4.16 (Cont.): Antimicrobial resistance and genetic profiles of the closely related UPEC isolates.

Clonal group	Phylogroup	Virulence-associated Trait	Antimicrobial resistance
CG8	F	*papAH*, *papC*, *papEF*, *papG* II_III, *papG* allele II, *fimH*, *yfcV*, *kpsMT* II, K1, *fyuA*, *chuA*, *iutA*, IV_{536}	SXT
		papAH, *papC*, *papEF*, *papG* II_III, *papG* allele II, *fimH*, *yfcV*, *kpsMT* II, K1, *fyuA*, *chuA*, *iutA*, *traT*, IV_{536}	AMP, AMC, SXT

Abbreviations: CG, clonal group; AMP, ampicillin; AMC, amoxicillin-clavulanic acid, A/S, ampicillin-sulbactam, CXM, cefuroxime, CAZ, ceftazidime; CTX, cefotaxime; CIP, ciprofloxacin; LE, levofloxacin; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole.

CHAPTER 5

DISCUSSION

5.1 Female and Elderly Patients are at Higher Risks for UPEC Infection

In accordance with global trends, the female patients (80/105; 76.2%) in the present study are more prevalent than male patients (25/105; 23.8%), with a ratio of 3.2:1 (Tabasi, et al., 2016; Ramírez-Castillo, et al., 2018; Huang, et al., 2021; Lin, et al., 2021a; Yuan, et al., 2021; Mohapatra, et al., 2022). This phenomenon may be due to the relatively shorter urethra or closer distance between the urethra and anus in females, which increases the risk of infection (Tewawong, et al., 2020). Besides, the urethral openings of females are closer to the bladder, so bacteria can ascend into the bladder more easily (Foxman, 2014). Moreover, the periurethral of females also provide more moist areas for bacterial growth than males (Foxman, 2014).

Ramírez-Castillo, et al. (2018) reported that young children, especially female children between two to ten years old, were the most prevalent age group for UTIs. However, Yuan, et al. (2021) reported that the highest incidence of UTI for male and female patients in China was between 50-54 years and 55-59 years, respectively. In this study, the UPEC isolates (42/105; 40%) were mainly collected from the 60-79 age group, as shown in Figure 4.1. This finding is similar to the previous work in Taiwan, in which the 61-80 age group was the most prominent (Lin, et al., 2021a). The high incidence of infection in elderly

female patients may be due to the lower oestrogen level after menopause. Oestrogen is one of the greatest host defences against UTIs owing to its crucial roles in promoting the acidic condition in the vagina and the proliferation of lactobacillus (Dielubanza and Schaeffer, 2011; Aydin, et al., 2015). A low level of oestrogen may result in physiological changes, including vaginal atrophy, higher vaginal potential of hydrogen (pH), and alteration in vaginal flora (Raz and Stamm, 1993; Gunardi, et al., 2021). As a result, uropathogens can colonise the urogenital tract more easily (Gunardi, et al., 2021).

Surprisingly, this study had a significantly lower frequency of the UPEC strains among the 80 years and above age group (8/105; 7.6%) compared to those reported by Lin, et al. (2021a) (146/907; 16.1%). Elderly patients are more physically challenged to follow the procedures in collecting midstream urine samples, thereby more likely resulting in contaminated urine samples (Manseck, et al., 2022). Since only pure *E. coli* isolates harvested from the urine specimens were included in this study, this may result in fewer UPEC samples collected from the 80 years and above age group.

5.2 Antimicrobial Resistance of the UPEC Isolates in Comparison with the National Surveillance Report of Malaysia and International Studies

In this study, low resistance rates (0.0% to 6.7%) were observed towards antimicrobials, including ampicillin-sulbactam, nitrofurantoin, fosfomycin, ertapenem, imipenem and meropenem, indicating that these antimicrobials remained largely efficacious in the local scene (Table 4.1). In contrast, other antimicrobial agents included gentamicin, cefuroxime, cefotaxime, ceftazidime, trimethoprim-sulfamethoxazole, amoxicillin-clavulanic acid, levofloxacin, ciprofloxacin and ampicillin should be used judiciously due to their high antimicrobial resistance (18.1% to 68.6%) (Table 4.1).

In the present study, the UPEC isolates showed greater antimicrobial resistance towards six antimicrobials, including ampicillin, amoxicillin-clavulanic acid, cefotaxime, ceftazidime, cefuroxime and ciprofloxacin when compared to the national antimicrobial resistance trends of the UPEC strains as revealed in the National Antibiotic Resistance Surveillance Report 2020 Malaysia (Institute for Medical Research, 2020) (Table 5.1). Of note, the amoxicillin-clavulanic acid resistance rate was incredibly higher than those reported by the surveillance report (33.3% vs 13.4%) (Table 5.1). In contrast, the resistance rates of ampicillin-sulbactam, trimethoprim-sulfamethoxazole, imipenem and meropenem in the present study were lower than in the surveillance report, as shown in Table 5.1. The data of the national surveillance report was analysed based on a vast number of *E. coli* clinical samples collected from various states of Malaysia (Institute for Medical Research, 2020). However, the present study only involved a small sample size from the Perak

state, which could explain the observed discrepancies in the antimicrobial resistance of the UPEC strains.

Table 5.1: Comparison of the antimicrobial resistance of the present study and the national antibiotic resistance surveillance report 2020 Malaysia.

Antimicrobial	Antimicrobial resistance rate (%)	
	Present study	National antibiotic resistance surveillance report 2020 Malaysia
Ampicillin	68.6	62.4
Amoxicillin-clavulanic acid	33.3	13.4
Cefotaxime	26.7	21.6
Ceftazidime	21.0	13.2
Cefuroxime	27.6	23.1
Ciprofloxacin	30.5	27.0
Ampicillin-sulbactam	6.7	17.0
Trimethoprim-sulfamethoxazole	33.3	34.1
Imipenem	0.0	0.4
Meropenem	0.0	0.4

There is some controversy regarding the antimicrobial resistance of UPEC strains across different countries. For instance, the resistance rates of trimethoprim-sulfamethoxazole (33.3%) and ciprofloxacin (30.5%) were lower compared to the previous studies in Taiwan, Mexico and Turkey (45.1% to 72.7% and 35.9% to 79.5%, respectively) (Ramírez-Castillo, et al., 2018; Yılmaz and Aslantaş, 2020; Lin, et al., 2021a). The gentamicin resistance rate in the present study (18.1%) was also lower compared to the recent study in Taiwan (28.7%) (Lin, et al., 2021a). In addition, the ampicillin-sulbactam resistance rate in the present study (6.7%) was lower than those reported in Taiwan (15.0%), Libya (30.8%) and Mexico (55.5%) (Abujnah, et al., 2015; Ramírez-Castillo, et al., 2018; Lin, et al., 2021b).

On the contrary, the resistance rates of ampicillin (68.6%) and gentamicin (18.1%) were similar to the study in Turkey (67.3% and 20.9%, respectively) (Yılmaz and Aslantaş, 2020). Earlier studies also reported low resistance rates towards carbapenems, including ertapenem (0.0% to 1.4%), meropenem (0.0% to 1.4%) and imipenem (0.0% to 1.4%) (Abujnah, et al., 2015; Ramírez-Castillo, et al., 2018; Cristea, et al., 2019; Tewawong, et al., 2020; Yılmaz and Aslantaş, 2020; Lin, et al., 2021b).

The observed discrepancies in the antimicrobial resistance of the UPEC strains may be due to the differential usage of antimicrobials in different countries. Trimethoprim-sulfamethoxazole and ciprofloxacin are the more preferred antibiotics for treating UTIs in countries such as Taiwan, Mexico and Turkey (Arredondo-García, et al., 2007; Yürüyen, et al., 2017; Yi-Te, et al., 2020). In contrast, ciprofloxacin and other quinolones are no longer recommended as the empirical treatment for UTIs in Malaysia due to the selection of resistance (Ministry of Health Malaysia, 2019). Furthermore, gentamicin is commonly used for treating UTIs in Taiwan (Yi-Te, et al., 2020), but this antibiotic is mostly used to treat paediatric UTIs in Malaysia (Ministry of Health Malaysia, 2019).

The prevalence of MDR isolates in this study (50.5%) was lower compared to other studies in Taiwan (55.0%), Brazil (61.7%), Thailand (62.0%), Mexico (63%) and Mongolia (93.9%) (Munkhdelger, et al., 2017; Campos, et al., 2018; Ramírez-Castillo, et al., 2018; Tewawong, et al., 2020; Lin, et al., 2021b). Nonetheless, the rate of MDR in the present study (50.5%) was higher

than those reported in Libya (33.2%), Turkey (34.6%) and Iran (49.4%) (Abujnah, et al., 2015; Hadifar, et al., 2017; Yılmaz and Aslantaş, 2020).

Factors that might have contributed to the high antimicrobial resistance in the present study include the misuse of antimicrobials by healthcare professionals. In Malaysia, the antimicrobial prescription rate remains high in primary care settings (21.1%) (Ab Rahman, Teng and Sivasampu, 2016). Notably, the antimicrobial prescribing rate in the private clinics (30.8%) was at least four times higher than in the public clinics (6.8%) (Ab Rahman, Teng and Sivasampu, 2016). Careless usage of antimicrobials without prior routine antimicrobial susceptibility testing in private clinics may also facilitate the emergence of MDR strains (Gatya Al-Mayahie, et al., 2022).

5.3 Predominance of Phylogroup B2

In this study, the UPEC strains mainly belonged to the phylogroup B2 (67/105; 63.8%). This predominance of phylogroup B2 is also observed in other countries such as the United States, Iran, Taiwan, Mongolia and Thailand (Munkhdelger, et al., 2017; Schreiber, et al., 2017; Tewawong, et al., 2020; Lin, et al., 2021b; Halaji, et al., 2022). Phylogroup B1, which is more frequently associated with the commensal and intestinal pathogenic *E. coli*, had the same prevalence as the phylogroup D in the present study (Section 4.3). Similarly, phylogroups B1 and D are the third most common phylogroups among the UPEC isolates in the Brazilian community (Campos, et al., 2018). In contrast, phylogroup D is predominated in China (42.4%) and Mexico (23.6%) (Wang,

et al., 2014; Ramírez-Castillo, et al., 2018). Besides, the predominance of phylogroup A has also been reported in Egypt (48.9%) (El-Mahdy, Mahmoud and Shrief, 2021). These observed discrepancies may be due to the variations in the sample sizes, geographical areas, research designs and health status of the host (Javed, Mirani and Pirzada, 2021).

5.4 Distribution of Virulence Genes among the UPEC Isolates

Of the 32 studied VGs, *fimH* (102/105; 97.1%) was the most frequently detected VG in the present study (Table 4.2). The high occurrence of *fimH* among the UPEC isolates was also observed in other countries such as Mongolia, Brazil, Mexico, Taiwan and Iran (Munkhdelger, et al., 2017; Campos, et al., 2018; Ramírez-Castillo, et al., 2018; Lin, et al., 2021a; Rezatofighi, Mirzarazi and Salehi, 2021). In UTI pathogenesis, *fimH* (adhesive tip of the type 1 fimbriae) is vital in the early stage of bacterial colonisation by binding to the uroplakins of the host cells (Subashchandrabose and Mobley, 2015; Sarshar, et al., 2020). Martinez, et al. (2000) demonstrated that *fimH* alone was sufficient to mediate the adherence and the invasion of the UPEC isolates into the human bladder epithelial cells. Moreover, type 1 fimbriae operons are located at the genomic backbone of the UPEC isolates, which could explain the high prevalence of *fimH* in the present study (Subashchandrabose and Mobley, 2015).

yfcV (70/105; 66.7%) was the second most common adhesion gene in the present study (Table 4.2). This finding contradicted those reported in Mexico, where *yfcV* was only present among 20.0% of the UPEC isolates (Ramírez-Castillo, et al., 2018). *E. coli* isolates that exhibit *yfcV*, *vat*, *fyuA* and *chuA* have a significant advantage in murine bladder colonisation, with an approximately 1000-fold increase in median colonisation than the negative isolates without these VGs (Spurbeck, et al., 2012). The high occurrence of *yfcV* among the UPEC isolates in the present study suggests that this VG may be vital in the early stage of bacterial colonisation.

On the other hand, *pap* genes that encode for the P fimbriae, including *papAH* (48/105; 45.7%), *papC* (45/105; 42.9%), *papEF* (49/105; 46.7%), *papG* II_III (44/105; 41.9%), *papG* allele II (26/105; 24.8%) and *papG* allele III (24/105; 22.9%), ranged from 22.9% to 46.7% in the present study (Table 4.2). The distribution of *papC* (42.9%) and *papAH* (45.7%) were similar to those reported in Mexico (42.7%) and Brazil (44.9%) (Campos, et al., 2018; Ramírez-Castillo, et al., 2018). Nevertheless, a similar study in Iran reported a lower prevalence of the *pap* genes among the UPEC isolates, including *papG* allele II (6.5%), *papG* allele III (11.5%), *papAH* (19.5%), *papEF* (21.0%) and *papC* (21.7%) (Rezatofighi, Mirzarazi and Salehi, 2021). The UPEC strains isolated from the patients in Mongolia also had a lower distribution of *papG* allele III (1.4%), *papG* allele II (17.6%) and *papC* (20.3%) (Munkhdelger, et al., 2017). The observed discrepancies in the distribution of *pap* genes among the UPEC isolates may be due to the variations in the geographical areas and sample sizes. The sample size of the UPEC collected in the present study was 105 as

compared to the other aforementioned studies conducted in different countries, such as Iran and Mongolia, in which their sample sizes ranged from 138 to 148 (Munkhdelger, et al., 2017; Rezatofighi, Mirzarazi and Salehi, 2021).

Furthermore, *sfa/focDE* (24/105; 22.9%) was more frequently detected in the present study compared to other studies (8.1% to 14.4%) (Wang, et al., 2014; Calhau, et al., 2015; Munkhdelger, et al., 2017; Rezatofighi, Mirzarazi and Salehi, 2021). In particular, *sfaS* (13/105; 12.4%) was more frequently detected among the UPEC isolates than *focG* (7/105; 6.7%), as shown in Table 4.2. This finding was in accordance with the recent study carried out in Iran, in which the distribution of the *sfaS* and *focG* were 12.3% and 0.7%, respectively (Rezatofighi, Mirzarazi and Salehi, 2021). As the S fimbriae are positioned within the pathogenicity island III of the UPEC 536 strain, this may explain the higher prevalence of *sfaS* than *focG* in this study.

On the other hand, the prevalence of *afa/draBC* (8/105; 7.6%) in this study was similar to those reported in Mexico (8.2%) but was relatively lower than those reported in Iran (15.5%) and Mongolia (15.9%) (Munkhdelger, et al., 2017; Ramírez-Castillo, et al., 2018; Rezatofighi, Mirzarazi and Salehi, 2021). *afa/draBC* is the most prominent adhesion gene among the UPEC strains isolated from cats (91.9%) (Liu, Thungrat and Boothe, 2015). Therefore, the low prevalence of *afa/draBC* in the present study suggests that this adhesion gene may be less important in causing UTIs in humans. Other adhesion genes, such as *bmaE* (0.0%) and *gafD* (0.0%), were not detected in this study, suggesting that these adhesion genes may not be necessary for UTI pathogenesis.

Of the studied toxic genes, *vat* (54/105; 51.4%) was more frequently found than *cnf1* (26/105; 24.8%), *hlyA* (25/105; 23.8%) and *cdtB* (1/105; 1.0%) as shown in Table 4.2. Similar findings were reported by Ramírez-Castillo, et al. (2018), in which *vat* (20.0%) was more frequently detected than *hlyA* (9.1%) and *cnf1* (2.7%). In addition, the distribution of *hlyA* (23.8%) and *cnf1* (24.8%) in this study were similar to those reported in Taiwan (23.0% and 20.2%, respectively), but were relatively higher than those reported in Mexico, China and Turkey (1.3% to 11.6% and 2.7% to 7.8%, respectively) (Wang, et al., 2014; Ramírez-Castillo, et al., 2018; Yılmaz and Aslantaş, 2020; Lin, et al., 2021a). The low incidence of *cdtB* (1.0%) in the present study was consistent with similar studies performed by Tabasi, et al. (2016) and Rezatofighi, Mirzarazi and Salehi (2021) (both were 0.0%). *vat* was first described as a secreted protein encoded by the pathogenicity island of an avian *E. coli* (Tapader, Basu and Pal, 2019), whereas *hlyA* and *cnf1* are often carried by the PAI II of UPEC J96 (Lüthje and Brauner, 2014). These VGs could be transferred and acquired more easily among the UPEC strains than *cdtB* (Smith, et al., 2008; Tapader, Basu and Pal, 2019). Therefore, the prevalence of *vat*, *hlyA* and *cnf1* were higher than *cdtB* in the present study.

Of the studied protectins, *kpsMT* II (83/105; 79.0%) was the most prominent protectin, followed by K5 (43/105; 41.0%), K1 (33/105; 31.4%), *rfc* (3/105; 2.9%) and *kpsMT* III (1/105; 1.0%) (Table 4.2). The distribution of *kpsMT* II in this study (79.0%) was relatively higher than those reported in other countries (32.6% to 58.8%) (Calhau, et al., 2015; Munkhdelger, et al., 2017; Ramírez-Castillo, et al., 2018; Rezatofighi, Mirzarazi and Salehi, 2021).

Likewise, the K1 (31.4%), K5 (41.0%) and *kpsMT* III (1.0%) capsules were relatively higher than the similar study carried out in Iran (7.9%, 13.0% and 14.4% respectively) (Rezatofighi, Mirzarazi and Salehi, 2021). A low prevalence of *rfc* (0.7%) was also observed among the UPEC isolates collected in Iran (Rezatofighi, Mirzarazi and Salehi, 2021). The high occurrence of *kpsMT* II, including K1 and K5 in the present study, suggests that the group II capsules may serve as the primary protections against the phagocytosis of the leukocytes (Burns and Hull, 1999; Anderson, et al., 2010).

For the iron-uptake genes, *fyuA* (90/105; 85.7%) and *chuA* (88/105; 83.8%) were more commonly found among the UPEC isolates than *iutA* (54/105; 51.4%), as shown in Table 4.2. In Mexico, the UPEC strains isolated from the UTI patients also exhibited more *fyuA* (68.2%) as compared to *chuA* (49.1%) (Ramírez-Castillo, et al., 2018). Similar scenarios were also observed in the other studies in which the *fyuA* was more prevalent than *iutA* (Munkhdelger, et al., 2017; Campos, et al., 2018).

fyuA is widespread among the UPEC strains and is highly expressed during UTIs (Brumbaugh, Smith and Mobley, 2013). The *fyuA* deficient mutants attenuated the UPEC in cystitis, pyelonephritis and systemic infection of the murine models, indicating the importance of *fyuA* in UTI pathogenesis (Brumbaugh, et al., 2015). Moreover, *fyuA* is carried on the PAI IV of the UPEC 536, this may explain the higher occurrence of *fyuA* than *chuA* and *iutA* in this study. Epidemiological studies revealed that *chuA*, which encodes for the haem transporter, is only present among *E. coli* strains derived from the phylogroups

B2, D, E, F and clade I (Clermont, et al., 2013). Since the majority of the UPEC isolates in the present study belonged to these phylogroups (88/105; 83.8%), a higher prevalence of *chuA* was observed compared to *iutA*. Previous work by Landgraf, et al. (2012) also suggested that *iutA* might be advantageous but not essential for UPEC infection, which could explain its low frequency in this study.

As shown in Table 4.2, the UPEC isolates exhibited more *malX* (71/105; 67.6%) and *traT* (68/105; 64.8%) compared to *cvaC* (12/105; 11.4%) and *ibeA* (5/105; 4.8%). The occurrence of *malX* gene in the present study (67.6%) was relatively lower than those reported in Brazil (96.3%) but was higher than those reported in Iran (32.6%) (Campos, et al., 2018; Rezatofighi, Mirzarazi and Salehi, 2021). Besides, the distribution of *traT* gene in this study (64.8%) was consistent with those reported in Mongolia (66.2%) but was relatively higher than those reported in Iran (42.0%) (Munkhdelger, et al., 2017; Rezatofighi, Mirzarazi and Salehi, 2021). The low prevalence of *cvaC* (11.4%) and *ibeA* (4.8%) were also observed in other studies, which ranged from 0.7% to 4.3% and 4.7% to 5.7%, respectively (Munkhdelger, et al., 2017; Rezatofighi, Mirzarazi and Salehi, 2021). *malX* is located within the pathogenicity island of UPEC prototype CFT073, whereas *traT* is always carried on large IncF plasmids (Johnson, 1991; Johnson and Stell, 2000). Thus, *malX* and *traT* may be easier to be transferred and acquired by other UPEC strains as compared to *cvaC* and *ibeA*, resulting in a higher frequency of *malX* and *traT* in the present study.

5.5 Distribution of PAI Markers among the UPEC Isolates

Out of the eight PAI markers tested, PAI IV_{536} was the most prominent PAI marker, followed by PAI I_{CFT073}, PAI II_{CFT073}, PAI II_{J96}, PAI I_{536}, PAI II_{536}, PAI III_{536} and PAI I_{J96} as shown in Table 4.3. The prevalence of these eight PAI markers was consistent with the hypothesis proposed by Sabaté, et al. (2006), in which the acquisitions of the PAI markers occur sequentially, starting with the PAI IV_{536}, followed by PAI I_{CFT073}, PAI II_{CFT073}, PAI II_{J96}, PAI I_{536}, PAI II_{536} and PAI III_{536}.

The predominance of the PAI IV_{536} (86.7%) among the UPEC isolates in the present study was concordant with recent studies in Iran (Samei, Haghi and Zeighami, 2016; Najafi, et al., 2018). PAI IV_{536} is the most ubiquitous expressed PAI marker in Enterobacteriaceae and is relatively stable in the UPEC 536 (Sabaté, et al., 2006; Rasoulinasab, et al., 2021), which could explain its high frequency in the present study.

The distribution of the PAI I_{CFT073} (67.6%), PAI II_{CFT073} (52.4%) and PAI II_{J96} (23.8%) in the present study were relatively higher compared to other studies (40.7% to 61.3%, 40.7% to 43.3% and 8.0% to 12.9%, respectively) (Calhau, et al., 2015; Samei, Haghi and Zeighami, 2016; Najafi, et al., 2018). These PAIs carry VGs of three major functions, including adhesins (P and Prs fimbriae), iron-uptake (aerobactin and iron-regulated proteins) and toxins (α-haemolysin and cytotoxic necrotising factor), suggesting their important roles in UTI pathogenesis (Sabaté, et al., 2006).

In this study, PAI II_{536} (21.9%) was more frequently detected than the PAI III_{536} (12.4%) and PAI I_{J96} (0.0%). On the contrary, similar studies in Iran revealed that the prevalence of PAI III_{536} (13.6% to 21.3%) among the UPEC strains was higher than the PAI II_{536} (12.0% to 12.1%), PAI II_{J96} (8.0% to 12.9%) and PAI I_{J96} (0% to 0.7%) (Samei, Haghi and Zeighami, 2016; Najafi, et al., 2018). While the PAI I_{J96} is known to encode for the α-haemolysin and P fimbriae, this PAI marker is rarely found among the UPEC isolates (Samei, Haghi and Zeighami, 2016; Najafi, et al., 2018). The absence of the PAI I_{J96} suggests that the UPEC isolates may rely more on the other PAI markers for encoding the α-haemolysin and P fimbriae (e.g., PAI I_{536}, PAI II_{536}, PAI I_{CFT073}, PAI II_{CFT073} and PAI II_{J96}).

5.6 Inverse Correlation between the MDR Phenotype and the Distribution of the Virulence-associated Traits

Of the 40 virulence-associated traits tested, 17 virulence-associated traits (e.g., *papAH*, *papC*, *papEF*, *papG* II_III, *papG* allele III, *sfa/focDE*, *sfaS*, *yfcV*, *hlyA*, *cnf1*, *vat*, *kpsMT* II, *chuA*, PAI I_{536}, PAI II_{536}, PAI III_{536} and PAI II_{J96}) were significantly more prevalent among the non-MDR isolates, as described in Section 4.7. These findings further reinforced the previous observations of the inverse correlation between MDR phenotype and the distribution of VGs among the UPEC strains. For instance, *sfa/focDE*, *hlyA* and *cnf1* were significantly more prevalent among the non-MDR isolates in China (Wang, et al., 2014). Similarly, Er, et al. (2015) also reported that the MDR isolates exhibited a significantly lower prevalence of *sfa/focDE* (24.7%) and *focG* (14.1%). Liu,

Thungrat and Boothe (2015) also demonstrated an inverse correlation between the distribution of VGs and antimicrobial resistance phenotypes, wherein the highest number of VGs were detected among the susceptible isolates, followed by non-MDR isolates and MDR isolates. The low prevalence of virulence-associated traits among the MDR isolates in the present study may be due to the fitness cost concomitant with the acquisition of antimicrobial resistance as the antimicrobials interfere with the crucial cellular processes of the bacterial strains, including the supercoiling of the chromosomes, synthesis of cell walls and RNA transcription (Andersson and Hughes, 2010; Bunduki, et al., 2021). However, this hypothesis needs to be validated, and there is still a lack of studies which elucidates the impact of antimicrobial resistance on the carriage of VGs among the UPEC strains. A recent study conducted by Rajer and Sandegren (2022) demonstrated that the introduction of MDR pUUH239.2 plasmid into the *E. coli* K-12 MG1655 stains resulted in a reduced fitness of 2.9% per generation. While 11 of 13 antimicrobial genes did not constitute any fitness cost individually, the *tetAR* and $bla_{\text{CTX-M-15}}$ resistance gene clusters were accountable for the entire fitness loss (Rajer and Sandegren, 2022). These findings highlighted that not all the acquisition of resistance genes would increase the fitness burden of the *E. coli* strains (Rajer and Sandegren, 2022).

This study also showed that *iutA* was significantly more prevalent among the MDR isolates (35/53; 66.0%; p= 0.002) (Table 4.8). This finding contradicted the report by Er, et al. (2015), who demonstrated a significant correlation between *afa/draBC* and the MDR phenotype. Although the aerobactin system (*iutA*) can be encoded either by the pathogenicity islands

(PAI I_{CFT073}) or plasmids (Kudinha, 2017), the observed positive correlation between *iutA* and the MDR isolates in the present study suggests that *iutA* may be carried by transmissible MDR plasmids that contain multiple resistance genes. Indeed, a recent study has revealed the presence of *iutA* on a MDR plasmid through the whole-genome sequencing approach (Zurfluh, et al., 2018).

5.7 Association between Phylogroup B2, Virulence-associated Traits and Antimicrobial Resistance

In the present study, the phylogroup B2 exhibited the highest number of virulence-associated traits compared to other non-phylogroup B2 (median of 14 VGs and three PAI markers versus five VGs and one PAI marker) (Table 4.6). Similar scenarios were observed in other studies, in which the phylogroup B2 harboured more virulence-associated traits than other phylogroups (Johnson, et al., 2005a; Er, et al., 2015; Najafi, et al., 2018; Rezatofighi, Mirzarazi and Salehi, 2021).

Furthermore, 17 VGs and seven PAI markers were widespread among the phylogroup B2 (all $p < 0.05$), as described in Section 4.5. Similarly, Najafi, et al. (2018) reported that the phylogroup B2 had a higher frequency of PAI I_{536}, PAI II_{536}, PAI IV_{536}, PAI I_{CFT073} and PAI II_{CFT073} (all $p< 0.05$). Although Rezatofighi, Mirzarazi and Salehi (2021) also showed that phylogroup B2 exhibited significantly more *papC*, *fimH*, *fyuA*, *iutA*, *kpsMT* II, *kpsMT* K5 and *malX* (all $p< 0.05$), the significant correlations between phylogroup B2 and VGs

such as *papG* allele III, *sfa/focDE*, *sfaS* and K1 were only observed in this study (Table 4.4).

A vast number of virulence-associated traits presumably offer various pathogenic strategies for more successful infection. For instance, the phylogroup B2 displayed a high prevalence of *fyuA*, *chuA*, *iutA* iron-uptake genes and PAI IV_{536}, indicating the presence of various iron acquisition systems within this phylogroup (Table 4.4). In addition, 70% of the phylogroup B2 isolates in the present study acquired *fyuA*, *chuA*, *yfcV* and *vat* (Table 4.4). These VGs have been demonstrated to improve the capabilities of *E. coli* strains in urinary tract colonisation (Spurbeck, et al., 2012). On the other hand, this phylogroup also harboured a higher prevalence of VGs (e.g., *papC*, *papG* allele II, *iutA*, K1, K5, *hlyA* and *malX*) and PAI markers (e.g., PAI I_{536}, PAI II_{536}, PAI III_{536}, PAI IV_{536}, PAI II_{CFT073} and PAI II_{J96}) that are involved in the intestinal colonisation (Table 4.4 and Table 4.5) (Nowrouzian, Adlerberth and Wold, 2001; Nowrouzian, Wold and Adlerberth, 2001; Nowrouzian, et al., 2003; Ostblom, et al., 2011). The accumulation of extensive virulence-associated traits in the phylogroup B2 is assumed to be driven by the subsequent vertical inheritance of virulence-associated traits through its ancestors or the pre-existing features of its genome, which increase the compatibility between these virulence-associated traits and phylogroup B2 (Escobar-Páramo, et al., 2004).

Despite heterogeneous genetic background, 94.0% of the phylogroup B2 exhibited *fimH*, *yfcV*, *kpsMT* II, *fyuA*, *chuA*, *malX*, PAI IV_{536} and PAI I_{CFT073} (Section 4.5). While VGs such as *fimH*, *kpsMT* II, *fyuA*, and *malX* are also present among other UPEC lineages worldwide (Riley, 2014), the co-occurrence of these eight virulence-associated traits has not been documented. This distinct characteristic of the phylogroup B2 isolates suggests that these virulence-associated traits may promote the colonisation or the infection-causing ability of the UPEC strains in the local scene.

Despite having extensive virulence-associated traits, most of the phylogroup B2 isolates in the present study were not MDR (39/67; 58.2%; p= 0.018). This inverse correlation between antimicrobial resistance and the virulence-associated traits among phylogroup B2 was similar to those reported by Wang, et al. (2014). The phylogroup B2 was correlated with a higher prevalence of VGs (e.g., *fimH*, *papEF*, *sfa/focDE*, *cnf1*, *hlyA* and *kpsMT* II), but lower rates of antimicrobial resistance (e.g., ampicillin, ampicillin-sulbactam, ciprofloxacin, trimethoprim-sulfamethoxazole, cefuroxime and cefotaxime) compared to phylogroups A, B1 and D (Wang, et al., 2014). The mechanisms behind these incidents remain to be clarified but have been proposed to be driven by the loss of incompatible PAIs among the antimicrobial-resistant strains, presumably contributing to a lower prevalence of VGs (Wang, et al., 2014).

Similarly, a recent study in China revealed that the antimicrobial susceptible isolates mainly belonged to the phylogroup B2 and typically harboured more VGs than the antimicrobial-resistant isolates (Zeng, et al., 2021). Nevertheless, the current predominance of phylogroup B2 ST131-H30 pandemic lineage is extensively MDR and enriches with a wide range of VGs (Johnson, et al., 2017a; Johnson and Russo, 2018). Therefore, future studies are needed to clarify the relationships between phylogeny, virulence-associated traits and antimicrobial resistance.

5.8 Association between Non-phylogroup B2, Virulence-associated Traits and Antimicrobial Resistance

Non-phylogroup B2 isolates (e.g., phylogroups A, B1, C, D, E and F) exhibited significantly fewer virulence-associated traits than the phylogroup B2 isolates (median of five VGs and one PAI marker versus 14 VGs and three PAI markers), as described in Section 4.5. Similar findings were also reported by Rezatofighi, Mirzarazi and Salehi (2021), in which no VG tested was significantly more prevalent among the non-phylogroup B2. The low prevalence of virulence-associated traits among the non-phylogroup B2 isolates in the present study suggests that these isolates may be less virulent and cause less severe UTI symptoms (Johnson, et al., 2005a; Biggel, et al., 2020).

Besides, non-phylogroup B2 isolates also displayed a higher prevalence of MDR than phylogroup B2 (65.8% versus 41.8%; p= 0.018), as described in Section 4.6. Similarly, Lin, et al. (2021b) observed a higher prevalence of MDR among the phylogroups C (83.3%), F (73.3%), A (70.6%) and D (58.6%) as

compared to the phylogroup B2 (50.4%). While Ramírez-Castillo, et al. (2018) has revealed a significant association between the phylogroup D and the MDR phenotype, this relationship was not observed in the present study (Section 4.6). The observed discrepancies may be due to the small sample size, which hinders the detection of the significant correlations between the MDR phenotype and non-phylogroup B2.

5.9 Host Age Related Difference in Relation to the Bacterial Traits

There is some controversy regarding the relationship between the phylogroups of the UPEC isolates and host age. In the present study, the phylogroup B2 was widespread among all the five age groups, but the relationship was not statistically significant ($p > 0.05$) (Section 4.8.1 and Table 4.9). This result contradicted the previous finding in Taiwan, where the phylogroups of the UPEC strains were significantly associated with host age ($p = 0.001$) (Lin, et al., 2021a).

Prior research has documented the inverse correlation between VGs and host age (Lin, et al., 2021b; Lin, et al., 2021a). For instance, Lin, et al. (2021b) revealed that the prevalence of VGs such as *papG* allele II, *papG* allele III, *cnf1*, *hlyA*, and *foc* was lower among the elderly age group (above 80 years) than in the younger age group (3 years and below). In the current investigation, *papG* allele II, *papG* allele III, *cnf1*, *hlyA* and *focG* were more frequently detected in the 80 years and above age group than in the 19 years and below age group (Table 4.11).

Recently, a retrospective study in China demonstrated an increasing prevalence of MDR with the host age, having the lowest rate of 26.0% in newborn babies (28 days and below) and the highest rate of 68.8% in geriatric patients (above 65 years) (Huang, et al., 2021). In contrast, the rate of MDR fluctuated across the age groups in the present study, with the lowest rate of 25.0% in the 80 years and above age group and the highest rate of 69.0% in the 60-79 years age group (Section 4.8.3).

In addition, an earlier study showed that the antimicrobial resistance of the UPEC strains increased significantly with age (Lin, et al., 2021a). For example, the UPEC isolates collected from elderly patients above 80 years displayed greater resistance to most of the commonly used antimicrobials, including amoxicillin-clavulanic acid, cefuroxime, ciprofloxacin and levofloxacin (Lin, et al., 2021a). On the contrary, the highest rates of antimicrobial resistance were observed among the 60-79 age group instead of the 80 years and above age group (Table 4.14). These observed discrepancies in the antimicrobial resistance may be due to the significant differences in the sample sizes between these age groups, in which the number of UPEC isolates in the 60-79 age group (n= 42) was five times higher than those collected in 80 years and above age group (n= 8) (Table 4.14).

5.10 Host Gender Related Difference in Relation to the Bacterial Traits

In this study, the phylogroups B1 and B2 were significantly more prevalent among female (13/80; 16.3%; p= 0.035) and male (21/25; 84.0%; p= 0.016) patients, respectively (Section 4.8.1 and Table 4.10). Likewise, Lin, et al. (2021a) revealed that the phylogroups B1 and B2 were more frequently detected among female (11.2%) and male patients (65.7%), but these relationships were not statistically significant (p= 0.084).

The UPEC isolates collected from female patients in the present study exhibited fewer virulence-associated traits than those collected from male patients (median of nine VGs and two PAI markers versus 11 VGs and three PAI markers). Furthermore, no virulence-associated trait was significantly more prevalent among female patients (Section 4.8.2). In contrast, Ramírez-Castillo, et al. (2018) showed that the UPEC isolates collected from female patients in Mexico harboured more VGs than male patients (mean of 36.3 ± 17.0 versus 31.7 ± 13.0). Besides, Lin, et al. (2021a) also reported a higher prevalence of K1 antigen among female patients.

The UPEC isolates collected from male patients in the present study harboured a significantly higher prevalence of *yfcV*, *chuA*, *malX*, PAI I_{CFT073} and PAI II_{CFT073}, ranging from 72.0% to 100.0% (Table 4.13). However, Lin, et al. (2021a) reported a higher prevalence of *cnf1* and *hlyA* among male patients. The high occurrence of virulence-associated traits among male patients in the present study suggests that males are more likely to suffer from UTIs caused by high virulence UPEC strains (Kudinha, et al., 2013). Indeed, the UPEC isolates

collected from male patients with febrile UTI exhibited a higher prevalence of VGs, including *papG* allele II, *papG* allele III, *sfa/focDE*, *focG*, *cnf1*, *fyuA*, *iutA*, *kpsMT* II, *rfc* and *malX* despite the presence of compromising conditions such as urinary tract instrumentation, diabetes mellitus and renal cortical scarring that predispose the hosts to UTI (Johnson, et al., 2005b; Kudinha, et al., 2013).

In this study, the UPEC isolates collected from male patients displayed greater resistance to most of the antimicrobials tested and were more MDR (14/25; 56.0%) than those collected from female patients (Section 4.8.3 and Table 4.15). Nonetheless, the *Chi*-square test analysis demonstrated no significant correlation between the male gender and the MDR phenotype (p= 0.527) (Table 4.15). In contrast, prior studies have documented the significant associations between the male gender and the MDR or extended-spectrum beta-lactamase (ESBL)-producing UTI (Almomani, et al., 2018; Benaissa, et al., 2021; Raphael, Glymour and Chambers, 2021; Shakya, et al., 2021). This discrepancy may be due to different sample sizes or the geographical origins of the study population.

5.11 Potential Pandemic UPEC Lineages

The majority of the UPEC isolates in the present study were genetically diverse, with only 17.1% (18/105) being classified into eight clonal groups (Section 4.9 and Figure 4.14). These data suggest no large-scale clonal dissemination of a specific UPEC lineage. Nevertheless, the REP-PCRs revealed several clonal groups that may be threatened to emerge as pandemic lineages. The

antimicrobial resistance advantage, combined with extensive virulence-associated traits among some of the CG2, CG4 and CG7 isolates, suggesting that they may be easier to be transmitted and adapt in various hosts (Cummins, et al., 2021).

Of the eight clonal groups, CG1 was the dominant clonal group that exhibited the most virulence-associated traits (Table 4.16). Although these isolates were susceptible to all the antimicrobials tested, carriage of a vast number of virulence-associated traits suggests that they may be more virulent and cause more severe UTI symptoms as compared to other clonal group isolates (Biggel, et al., 2020).

5.12 Potential Targets for Anti-Virulence Therapeutics

Antimicrobial resistance has become a major health concern worldwide, with approximate 0.7 million deaths caused by MDR pathogens (Spaulding, et al., 2018; Fleitas Martínez, et al., 2019; Cummins, et al., 2021; Gatya Al-Mayahie, et al., 2022). The current predominance of ST131 UPEC lineage that carries ESBLs and metallo-β-lactamases (MBLs) has also greatly reduced the treatment options of antimicrobial therapy (McNally, et al., 2019). To circumvent the spread of MDR UPEC isolates, anti-virulence therapy has emerged as a promising alternative therapeutic strategy for UTI treatment (Clatworthy, Pierson and Hung, 2007; Fleitas Martínez, et al., 2019).

A wide range of VGs has been investigated for anti-virulence therapy. These potential candidates usually play vital roles in UTI pathogenesis, including adhesins, toxins and iron-uptake genes (Clatworthy, Pierson and Hung, 2007; Kot, 2017; Fleitas Martínez, et al., 2019). In the present study, the high prevalence of *fimH* and *fyuA* among the UPEC isolates may serve as attractive targets for the anti-virulence therapeutics in the local scene.

In UTI pathogenesis, *fimH* is vital in the early stage of bacterial colonisation and sustaining the infection due to its crucial role in mediating the adhesion, invasion and formation of intracellular bacterial communities (Sarshar, et al., 2020). In the phase 1a/1b clinical trial, the vaccination of *fimCH* to the patient cohorts with 24-month recurrent UTI histories resulted in an almost 70% reduction in total UTI (Tamadonfar, et al., 2019). Most importantly, the United States Food and Drug Administration approved the compassionate use of this experimental vaccine for patients suffering from UTIs caused by multidrug-resistant UPEC strains (Tamadonfar, et al., 2019; Sarshar, et al., 2020).

Likewise, the *fyuA* gene is also an ideal vaccine candidate. The Yersiniabactin siderophore system encoded by *fyuA* plays a crucial role in iron uptake and the survival of UPEC (Sora, et al., 2021). *In vivo*, the *fyuA* deficient mutants had significantly lower bacterial colonisation in the bladder (2.6-fold) and kidney (6.4-fold) of the murine models compared with the wild-type strains (Brumbaugh et al., 2015). In addition, the intranasal vaccination of *fyuA* also

elicits a long-lived humoral immune response (Brumbaugh, Smith and Mobley, 2013).

5.13 Strengths and Limitations of the Study

One of the biggest strengths of the present study is the examination of the multiple bacterial traits of the UPEC strains among different affected hosts, including antimicrobial resistance, phylogroups, virulence-associated traits and clonal relatedness. Furthermore, this study also examined an extensive array of 40 virulence-associated traits that are crucial in UTI pathogenesis. Besides, the study population comprised all the community patients (e.g., male and female patients of all ages with any clinical conditions) from various regions in Perak within a specific time frame, better reflecting the incidence of UTIs among the Perak population.

Nonetheless, there were several limitations in this study. If a mutation occurs at the primer sites, some of the virulence-associated traits may not be detected through PCR (Rezatofighi, Mirzarazi and Salehi, 2021). Therefore, negative PCR results do not necessarily equivalent to the absence of the corresponding virulence-associated traits (Rezatofighi, Mirzarazi and Salehi, 2021). Type II error may also occur owing to the uneven distribution of the UPEC isolates and relatively sample size in the phylogroups A (3/105), C (1/105) and E (1/105). This study did not assess other risk factors of UTIs, such as urinary catheters, previous UTIs and blockages in the urinary tract.

5.14 Future Studies

To increase the statistical power and to determine if the distinct set of virulence-associated traits among the phylogroup B2 observed in this study is truly specific to the Perak population, more UPEC samples should be collected from the other states of Malaysia. Additional UPEC strains should also be collected from patients with different clinical syndromes (e.g., asymptomatic bacteriuria, cystitis, pyelonephritis and bacteraemia) or different predisposing conditions (e.g., pregnancy, diabetes, cancer) to better understand the relationship between the bacterial traits and the host factors.

Moreover, whole-genome sequencing could be performed to reveal other important genetic information (e.g., protein-coding genes, RNA genes, genomic islands and antimicrobial resistance genes). Comparative genome analysis can also be performed to determine the genomic variation between the local UPEC strains and other globally disseminated UPEC strains in the NCBI database. This analysis may provide valuable insights into new putative VGs or PAIs that contribute to their local predominance in UTIs.

To better reveal the relationship between the antimicrobial resistance and virulence of the UPEC strains, resistance genes that confer specific antimicrobial resistance phenotypes should also be tested, for example, beta-lactamases (e.g., *bla*TEM, *bla*SHV and *bla*OXA). While this study has revealed various virulence-associated traits, their expressions were not evaluated. Of note, the presence of a specific VG does not imply its functional expression. A recent study has demonstrated that the expression of VGs varies from patient to patient,

suggesting different requirements of a specific VG during the course of infection (Sintsova, et al., 2019).

CHAPTER 6

CONCLUSION

This study revealed the antimicrobial resistance profiles and the molecular characteristics of the UPEC strains in relation to the host age and gender. The majority of the UPEC strains were MDR (53/105; 50.5%) and showed higher resistance towards ampicillin, levofloxacin, amoxicillin-clavulanic acid, trimethoprim-sulfamethoxazole and ciprofloxacin. In contrast, ampicillin-sulbactam, fosfomycin, nitrofurantoin, ertapenem, imipenem and meropenem remained highly effective against the UPEC strains.

Out of the 40 virulence-associated studied, *fimH* (102/105; 97.1%), PAI IV_{536} (91/105; 86.7%), *fyuA* (90/105; 85.7%) and *chuA* (88/105; 83.8%) were more commonly found among the UPEC isolates. Most of the virulence-associated traits were significantly prevalent among the predominant phylogroup B2 (67/105; 63.8%) and non-MDR isolates. These results reinforced the previous findings wherein the antimicrobial resistance was inversely correlated to the virulence-associated traits or a B2 phylogenetic background. Notably, 94.0% (63/67) of the phylogroup B2 isolates shared a distinct set of virulence-associated traits, including *fimH*, *yfcV*, *kpsMT* II, *fyuA*, *chuA*, *malX*, PAI IV_{536} and PAI I_{CFT073}.

The UPEC isolates displayed different characteristics in relation to the host age and gender. The UPEC isolates collected from male patients primarily belonged to the phylogroup B2 (21/25; 84.0%; p= 0.016), had higher rates of antimicrobial resistance and exhibited more virulence-associated traits as compared to female patients. In contrast, host age showed no significant relationship with the phylogenetic background. The UPEC isolates collected from elderly patients in the 60-79 age group (42/105; 40.0%) were significantly more resistant towards antimicrobial agents but carried fewer virulence-associated traits compared with other age groups. Cephalosporins such as cefuroxime, cefotaxime and ceftazidime are not recommended for male and elderly patients aged 60-79 years due to the higher rates of antimicrobial resistance observed.

The REP-PCR assays only revealed the presence of eight clonal groups (18/105; 17.1%) among the UPEC isolates, indicating no large-scale clonal spread of a single UPEC lineage. Nevertheless, several clonal group isolates (e.g., CG2, CG4 and CG7) were MDR and enriched with extensive virulence-associated traits, threatening to emerge as pandemic lineages.

In conclusion, this study examined multiple bacterial traits of the UPEC strains collected in the Perak state of Malaysia. Unravelling the virulence-associated traits profiles of the UPEC isolates provides novel insights into their key pathogenic strategies during infection. These findings are critically important for the development of new therapeutics. The present study also discovered several high-risk clonal groups that might spread in the local scene.

Lastly, comparing antimicrobial resistance profiles among different affected hosts also provides valuable information for the physicians in prescribing the best medications.

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APPENDICES

Appendix A

Patient's data for one-hundred and five UPEC Samples

Sample ID	Sex	Age	Geographical Area	Inpatient
UTIPS 1	M	2	Ipoh	No
UTIPS 2	F	78	Kampar	Yes
UTIPS 3	F	48	Seri Iskandar	No
UTIPS 4	F	26	Ipoh	No
UTIPS 5	F	67	Ipoh	Yes
UTIPS 6	F	36	Ipoh	No
UTIPS 7	F	77	Tanah rata (Cameron Highland)	No
UTIPS 8	F	76	Ipoh	No
UTIPS 9	M	69	Sungai Siput	No
UTIPS 10	M	52	Batu Gajah	Yes
UTIPS 12	F	66	Kampar	Yes
UTIPS 13	F	24	Ipoh	Yes
UTIPS 14	F	74	Ipoh	No
UTIPS 15	M	40	Batu Gajah	No
UTIPS 16	F	41	Ulu Kinta	Yes
UTIPS 17	F	30	KK Monjoi	No
UTIPS 18	F	51	KK Pasir Pinji	No
UTIPS 19	M	63	Ipoh	Yes
UTIPS 20	F	88	Ipoh	Yes
UTIPS 21	F	53	Ipoh	No
UTIPS 22	F	63	Ipoh	No
UTIPS 23	F	63	Ipoh	No
UTIPS 24	M	77	Ipoh	No
UTIPS 25	F	79	Ipoh	No
UTIPS 26	F	61	Ipoh	Yes
UTIPS 27	F	76	Ipoh	Yes
UTIPS 28	F	66	Ipoh	No
UTIPS 29	F	38	Chemor	Yes
UTIPS 30	F	73	Ipoh	Yes
UTIPS 31	M	63	Sungai Siput	No
UTIPS 32	M	68	Ipoh	Yes
UTIPS 33	F	68	Batu Gajah	Yes
UTIPS 34	F	64	Ipoh	Yes
UTIPS 35	F	56	Ipoh	Yes
UTIPS 36	F	64	Chemor	No
UTIPS 37	F	59	Ipoh	No
UTIPS 38	F	79	Ipoh	No
UTIPS 39	F	86	Ipoh	No
UTIPS 40	F	35	Kampar	Yes

UTIPS 41	F	70	Ipoh	Yes
UTIPS 42	F	17	Ipoh	No
UTIPS 44	M	57	Ipoh	Yes
UTIPS 45	F	30	Chemor	No
UTIPS 46	F	48	Ipoh	No
UTIPS 47	F	26	Karai	No
UTIPS 48	M	82	Kampar	Yes
UTIPS 49	M	87	Ipoh	No
UTIPS 50	F	53	Ipoh	Yes
UTIPS 51	F	75	Sungai Siput	Yes
UTIPS 52	M	13	Ipoh	Yes
UTIPS 53	F	5	KK Menglembu	No
UTIPS 54	F	39	Ipoh	Yes
UTIPS 55	M	53	Ipoh	No
UTIPS 56	M	49	Ipoh	Yes
UTIPS 57	F	67	Ipoh	Yes
UTIPS 58	F	44	Ipoh	Yes
UTIPS 59	M	82	Ipoh	No
UTIPS 60	F	3	Ipoh	Yes
UTIPS 61	M	68	Ipoh	Yes
UTIPS 62	F	25	Ipoh	No
UTIPS 63	F	70	Ipoh	Yes
UTIPS 64	M	61	Ipoh	No
UTIPS 65	M	41	Ipoh	No
UTIPS 66	F	66	Ipoh	Yes
UTIPS 68	F	33	Batu Gajah	Yes
UTIPS 69	F	22	Ipoh	Yes
UTIPS 70	M	74	Ipoh	Yes
UTIPS 71	F	7	Ipoh	Yes
UTIPS 72	F	51	Ipoh	Yes
UTIPS 73	F	68	Ipoh	No
UTIPS 74	F	42	Sg Siput	Yes
UTIPS 76	M	68	Ipoh	No
UTIPS 77	F	30	Kampar	No
UTIPS 78	F	64	Ipoh	Yes
UTIPS 79	F	64	Changkat Melintang	Yes
UTIPS 80	F	88	Ipoh	Yes
UTIPS 81	F	57	Ipoh	Yes
UTIPS 82	F	53	Ipoh	Yes
UTIPS 83	F	44	Ipoh	Yes
UTIPS 84	F	34	Ipoh	Yes
UTIPS 85	F	55	Sungai Siput	Yes
UTIPS 86	F	85	Kampar	Yes
UTIPS 87	F	22	Ipoh	Yes
UTIPS 89	F	54	Ipoh	No
UTIPS 90	M	0.17	Kampar	Yes
UTIPS 91	F	27	Batu gajah	Yes
UTIPS 92	F	26	Ipoh	Yes

UTIPS 93	F	33	Ipoh	No
UTIPS 94	F	54	Ipoh	No
UTIPS 95	F	64	Ipoh	No
UTIPS 96	F	23	Lambor kiri	No
UTIPS 98	F	87	Ipoh	No
UTIPS 99	F	66	Ipoh	Yes
UTIPS 100	F	43	Ipoh	Yes
UTIPS 101	F	29	Chemor	No
UTIPS 102	M	67	Ipoh	No
UTIPS 103	M	7	Kampar	Yes
UTIPS 104	M	61	Ipoh	No
UTIPS 105	F	77	Ipoh	Yes
UTIPS 106	F	19	Ipoh	Yes
UTIPS 107	F	50	Ipoh	No
UTIPS 108	F	68	Ipoh	Yes
UTIPS 109	F	49	Ipoh	No
UTIPS 110	M	75	Ipoh	No
UTIPS 111	F	67	Ipoh	No

Appendix B

Antimicrobial resistant profiles of one-hundred and five UPEC samples

Sample ID	AMP	AMC	A/S	CTX	CAZ	CXM	CIP	LE	ETP	IMP	MEM	GEN	NIT	SXT	FOS
UTIPS 1	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 2	R	R	S	S	S	S	R	R	S	S	S	S	S	R	S
UTIPS 3	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 4	R	S	S	S	S	S	R	R	S	S	S	R	S	R	S
UTIPS 5	R	S	S	S	S	S	S	R	S	S	S	R	S	S	S
UTIPS 6	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 7	R	S	R	S	S	S	R	R	S	S	S	S	S	R	S
UTIPS 8	R	S	S	R	R	R	S	S	S	S	S	R	S	R	S
UTIPS 9	R	R	R	R	R	R	R	R	S	S	S	R	S	R	S
UTIPS 10	R	R	S	R	R	R	S	S	S	S	S	S	S	R	S
UTIPS 12	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 13	R	S	S	R	R	R	S	S	S	S	S	S	S	S	S
UTIPS 14	R	S	S	S	S	S	R	R	S	S	S	R	S	R	S
UTIPS 15	R	R	S	S	S	S	R	R	S	S	S	R	S	R	S
UTIPS 16	R	S	S	S	S	S	R	R	S	S	S	R	S	R	S
UTIPS 17	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S
UTIPS 18	R	R	S	R	R	R	R	R	S	S	S	S	S	R	S
UTIPS 19	R	S	S	S	S	S	S	R	S	S	S	R	S	R	S

UTIPS 20	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 21	R	R	S	S	S	S	R	R	S	S	S	S	S	S	S
UTIPS 22	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 23	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 24	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S
UTIPS 25	R	R	S	R	R	R	S	S	S	S	S	S	S	S	S
UTIPS 26	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 27	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 28	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 29	R	S	S	R	R	R	S	S	S	S	S	S	S	S	S
UTIPS 30	R	R	S	R	R	R	R	R	S	S	S	S	S	S	R
UTIPS 31	R	S	S	R	S	R	R	R	S	S	S	S	S	R	S
UTIPS 32	R	R	S	S	S	S	S	R	S	S	S	R	S	S	S
UTIPS 33	R	S	S	R	R	R	S	S	S	S	S	R	S	S	S
UTIPS 34	R	R	S	S	S	S	R	R	S	S	S	S	S	R	S
UTIPS 35	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 36	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 37	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 38	R	R	S	R	R	R	S	S	S	S	S	S	S	S	S
UTIPS 39	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S
UTIPS 40	R	S	R	S	S	S	S	S	S	S	S	S	S	R	S
UTIPS 41	R	S	S	R	R	R	R	R	S	S	S	S	S	R	S
UTIPS 42	R	S	S	S	S	S	S	S	S	S	S	R	S	R	S
UTIPS 44	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 45	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
UTIPS 46	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

UTIPS 47	R	R	S	R	S	R	R	R	S	S	S	S	S	S	S
UTIPS 48	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 49	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S
UTIPS 50	R	R	S	S	S	S	S	S	S	S	S	S	S	R	S
UTIPS 51	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 52	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 53	R	R	S	S	S	S	S	S	S	S	S	R	S	R	S
UTIPS 54	R	R	R	R	R	R	S	S	S	S	S	S	S	R	S
UTIPS 55	R	S	S	R	S	R	R	R	S	S	S	S	S	R	S
UTIPS 56	S	S	S	S	S	S	R	S	S	S	S	S	S	R	S
UTIPS 57	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 58	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 59	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 60	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 61	R	R	R	R	R	R	R	R	S	S	S	S	R	S	S
UTIPS 62	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 63	R	S	S	R	S	R	R	R	S	S	S	S	S	R	S
UTIPS 64	R	R	S	R	R	R	S	S	S	S	S	S	S	S	S
UTIPS 65	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 66	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 68	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 69	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 70	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 71	R	S	S	S	S	S	R	R	S	S	S	S	S	S	S
UTIPS 72	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 73	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S

UTIPS 74	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 76	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 77	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 78	R	S	S	R	S	R	S	S	S	S	S	S	S	S	S
UTIPS 79	R	R	S	S	S	S	S	S	S	S	S	R	S	R	S
UTIPS 80	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 81	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 82	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 83	R	R	S	S	S	S	S	S	S	S	S	S	S	R	S
UTIPS 84	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 85	R	R	S	S	S	S	S	S	S	S	S	S	S	R	S
UTIPS 86	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S
UTIPS 87	R	S	S	R	R	R	R	R	S	S	S	S	S	R	S
UTIPS 89	R	S	S	S	S	S	R	R	S	S	S	R	S	S	S
UTIPS 90	R	S	S	S	S	S	S	R	S	S	S	S	S	S	S
UTIPS 91	R	R	S	S	S	S	S	S	S	S	S	S	S	R	S
UTIPS 92	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 93	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 94	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 95	R	S	S	S	S	S	R	R	S	S	S	R	S	R	S
UTIPS 96	R	R	S	S	S	S	R	R	S	S	S	S	S	R	S
UTIPS 98	R	S	S	R	S	R	R	R	S	S	S	S	S	S	S
UTIPS 99	R	R	S	S	S	S	R	R	S	S	S	R	S	S	S
UTIPS 100	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 101	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 102	R	R	S	R	R	R	S	S	S	S	S	R	S	R	S

UTIPS 103	R	S	S	R	R	R	S	S	S	S	S	R	S	R	S
UTIPS 104	R	S	S	R	R	R	R	R	S	S	S	S	S	S	S
UTIPS 105	R	R	R	S	S	S	S	R	S	S	S	S	S	S	S
UTIPS 106	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S
UTIPS 107	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 108	R	R	S	R	R	R	R	S	S	S	S	R	S	R	S
UTIPS 109	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
UTIPS 110	R	R	S	R	R	R	R	R	S	S	S	S	S	S	S
UTIPS 111	R	R	S	R	R	R	R	R	S	S	S	S	S	S	R

Abbreviations: CG, clonal group; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; A/S, ampicillin-sulbactam; CTX, cefotaxime; CAZ, ceftazidime; CXM, cefuroxime; CIP, ciprofloxacin; LE, levofloxacin; ETP, ertapenem; IMP, imipenem; MEM, meropenem; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole.

Appendix C

Adhesion gene profiles of one-hundred and five UPEC samples

Sample ID	*papA*	*papC*	*papEF*	*papG* I	*papG* II_III	*papG* allele I	*papG* allele II	*papG* allele III	*sfa/focDE*	*sfaS*	*focG*	*afa/draBC*	*fim H*	*yfcV*	*gafD*	*bmaE*
UTIPS 1	+	+	+	-	+	-	-	+	+	+	-	-	+	+	-	-
UTIPS 2	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 3	+	+	+	-	+	-	+	-	+	-	-	-	+	+	-	-
UTIPS 4	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 5	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 6	+	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-
UTIPS 7	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 8	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 9	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 10	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
UTIPS 12	+	+	+	-	+	-	-	+	-	-	-	-	+	+	-	-
UTIPS 13	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UTIPS 15	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 16	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 17	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 18	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-

UTIPS 19	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 20	+	+	+	-	+	-	+	-	-	-	-	-	+	-	-	-
UTIPS 21	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 22	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 23	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
UTIPS 24	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
UTIPS 25	+	+	+	-	+	-	+	-	+	-	+	-	+	+	-	-
UTIPS 26	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 27	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
UTIPS 28	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 29	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 30	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 31	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 32	+	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-
UTIPS 33	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 34	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UTIPS 36	+	+	+	-	+	-	-	+	-	-	-	-	+	+	-	-
UTIPS 37	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 38	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 39	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 40	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 41	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 42	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
UTIPS 44	+	+	+	-	+	-	-	+	+	+	-	-	+	+	-	-
UTIPS 45	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-

UTIPS 46	+	+	+	-	+	-	-	+	+	+	-	-	+	+	-	-
UTIPS 47	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 48	+	+	+	-	+	-	+	-	-	-	-	-	-	+	-	-
UTIPS 49	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 50	+	+	+	-	+	-	-	+	+	-	+	-	+	+	-	-
UTIPS 51	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 52	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
UTIPS 53	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
UTIPS 54	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 55	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 56	+	+	+	-	+	-	-	+	-	-	-	-	+	+	-	-
UTIPS 57	+	+	+	-	+	-	-	+	+	+	-	-	+	+	-	-
UTIPS 58	+	+	+	-	+	-	-	+	+	-	-	-	+	+	-	-
UTIPS 59	+	+	+	-	+	-	+	-	+	-	+	-	+	+	-	-
UTIPS 60	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 61	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
UTIPS 62	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
UTIPS 63	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 64	+	+	+	-	+	-	+	+	+	-	+	-	+	+	-	-
UTIPS 65	+	+	+	-	+	-	+	+	+	-	+	-	+	+	-	-
UTIPS 66	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 68	+	+	+	-	+	-	-	+	+	+	-	-	+	+	-	-
UTIPS 69	+	+	+	-	+	-	-	+	+	-	+	-	+	+	-	-
UTIPS 70	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
UTIPS 71	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 72	+	+	+	-	+	-	-	+	+	+	-	-	+	+	-	-

UTIPS 73	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 74	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
UTIPS 76	+	+	+	-	+	-	-	+	+	+	-	-	+	+	-	-
UTIPS 77	+	+	+	-	+	-	-	+	+	+	-	-	+	+	-	-
UTIPS 78	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
UTIPS 79	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
UTIPS 80	+	+	+	-	+	-	-	+	+	+	-	-	+	+	-	-
UTIPS 81	+	+	+	-	+	-	-	+	+	-	-	-	+	+	-	-
UTIPS 82	+	+	+	-	+	-	+	+	+	+	-	-	+	+	-	-
UTIPS 83	+	+	+	-	+	-	+	+	+	-	+	-	+	-	-	-
UTIPS 84	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
UTIPS 85	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
UTIPS 86	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 87	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 89	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 90	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 91	+	+	+	-	+	-	+	-	-	-	-	-	+	-	-	-
UTIPS 92	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 93	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
UTIPS 94	+	+	+	-	+	-	-	+	+	+	-	-	+	+	-	-
UTIPS 95	+	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-
UTIPS 96	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
UTIPS 98	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
UTIPS 99	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 100	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 101	+	+	+	-	+	-	-	+	+	+	-	-	+	+	-	-

UTIPS 102	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
UTIPS 103	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
UTIPS 104	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
UTIPS 105	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 106	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 107	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
UTIPS 108	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UTIPS 109	+	+	+	-	+	-	-	+	+	+	-	-	+	+	-	-
UTIPS 110	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
UTIPS 111	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-

Appendix D

Toxic, protectin, iron-uptake and miscellaneous genes profiles of one-hundred and five UPEC samples

Sample ID	*hlyA*	*cnf1*	*cdtB*	*vat*	*kpsMT* II	K1	K5	*kpsMT* III	*rfc*	*fyuA*	*chuA*	*iutA*	*cvaC*	*ibeA*	*traT*	*malX*
UTIPS 1	+	+	-	+	+	-	+	-	-	+	+	-	-	-	+	+
UTIPS 2	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
UTIPS 3	+	-	-	+	+	-	+	-	+	+	+	-	-	-	+	+
UTIPS 4	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+
UTIPS 5	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+
UTIPS 6	-	-	-	+	+	+	-	-	-	+	+	-	-	+	-	+
UTIPS 7	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-
UTIPS 8	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-
UTIPS 9	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+
UTIPS 10	+	+	-	-	+	-	-	-	-	+	+	-	-	-	-	+
UTIPS 12	+	+	-	+	+	-	+	-	-	+	+	-	-	-	+	+
UTIPS 13	-	-	-	-	+	-	+	-	-	-	+	+	-	-	+	+
UTIPS 14	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+
UTIPS 15	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+
UTIPS 16	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+
UTIPS 17	-	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-
UTIPS 18	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+
UTIPS 19	-	-	-	-	+	-	+	-	-	+	+	+	-	-	+	-
UTIPS 20	-	-	-	-	+	-	+	-	-	+	+	+	-	-	+	-

UTIPS 21	-	-	-	-	+	-	+	-	-	+	-	-	-	-	+	-
UTIPS 22	-	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-
UTIPS 23	-	-	-	-	+	-	+	-	-	+	+	+	-	-	+	+
UTIPS 24	-	-	-	-	+	-	+	-	-	+	+	-	-	-	+	+
UTIPS 25	+	+	-	+	+	-	+	-	-	+	+	+	-	-	-	+
UTIPS 26	-	-	-	-	+	-	+	-	-	-	+	+	-	-	+	-
UTIPS 27	-	-	-	-	+	-	-	-	-	+	+	+	-	-	+	+
UTIPS 28	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	+
UTIPS 29	-	-	-	-	+	-	+	-	-	-	+	-	-	-	-	-
UTIPS 30	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-
UTIPS 31	-	-	-	-	+	-	+	-	-	+	+	+	-	-	+	+
UTIPS 32	-	-	-	+	+	+	-	-	-	+	+	-	-	+	-	+
UTIPS 33	-	-	-	+	-	-	-	-	-	+	+	+	-	-	+	-
UTIPS 34	-	-	-	+	-	-	-	-	-	-	-	+	+	-	+	-
UTIPS 35	-	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-
UTIPS 36	+	+	-	+	+	-	+	-	-	+	+	-	-	-	+	+
UTIPS 37	-	-	-	-	+	+	-	-	-	+	+	+	-	-	-	-
UTIPS 38	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	-
UTIPS 39	-	-	-	-	+	-	+	-	-	+	+	+	-	-	+	-
UTIPS 40	-	-	-	-	+	+	-	-	-	+	+	-	+	-	-	+
UTIPS 41	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
UTIPS 42	-	-	-	+	+	+	-	-	-	+	+	+	-	-	+	+
UTIPS 44	-	+	-	+	+	-	+	-	-	+	+	-	-	-	+	+
UTIPS 45	-	-	-	-	+	+	-	-	-	+	+	+	-	-	-	-
UTIPS 46	-	+	-	+	+	-	+	-	-	+	+	-	-	-	+	+
UTIPS 47	-	-	-	-	+	-	-	-	-	+	+	-	-	-	+	+

UTIPS 48	-	-	-	-	+	+	-	-	-	+	+	-	-	-	-	+
UTIPS 49	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	+
UTIPS 50	-	+	-	+	+	-	+	-	-	+	+	-	-	-	-	+
UTIPS 51	-	-	-	+	+	+	-	-	-	+	+	-	-	+	+	+
UTIPS 52	-	-	-	+	+	+	-	-	-	+	+	+	+	-	+	+
UTIPS 53	-	-	-	-	+	-	+	-	-	+	+	+	-	-	+	+
UTIPS 54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
UTIPS 55	-	-	-	-	+	-	+	-	-	+	+	+	-	-	+	+
UTIPS 56	+	+	-	+	+	-	+	-	-	+	+	-	-	-	+	+
UTIPS 57	+	+	-	+	+	-	+	-	-	+	+	-	-	-	+	+
UTIPS 58	+	+	-	+	+	+	-	-	-	+	+	-	-	-	-	+
UTIPS 59	+	+	-	+	+	-	+	-	-	+	+	+	-	-	-	+
UTIPS 60	-	-	-	+	-	-	-	+	-	+	+	+	+	-	+	-
UTIPS 61	+	+	-	-	+	-	-	-	-	+	+	+	-	-	-	+
UTIPS 62	-	-	-	+	+	+	-	-	-	+	+	-	+	-	+	+
UTIPS 63	-	-	-	-	+	-	+	-	-	+	+	+	-	-	+	+
UTIPS 64	-	-	-	+	+	-	+	-	+	+	+	-	-	-	+	+
UTIPS 65	+	+	+	+	+	-	+	-	-	+	+	+	-	-	+	+
UTIPS 66	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UTIPS 68	+	+	-	+	+	-	+	-	-	+	+	-	-	-	-	+
UTIPS 69	+	+	-	+	+	-	+	-	-	+	+	-	-	-	+	+
UTIPS 70	-	-	-	+	+	+	-	-	-	+	+	+	+	-	+	+
UTIPS 71	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+
UTIPS 72	+	+	-	+	+	-	+	-	-	+	+	-	-	-	+	+
UTIPS 73	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+
UTIPS 74	-	-	-	+	+	+	-	-	-	+	+	+	+	-	+	+

UTIPS 76	+	+	-	+	+		-	+	-		-	+	+	-	-	-	+	+
UTIPS 77	+	+	-	+	+		-	+	-		-	+	+	-	-	-	+	+
UTIPS 78	-	-	-	-	+		-	-	-		-	+	+	+	-	-	+	+
UTIPS 79	-	-	-	+	+		+	-	-		-	+	+	+	-	-	+	+
UTIPS 80	+	+	-	+	+		-	+	-		-	+	+	-	-	-	+	+
UTIPS 81	+	+	-	+	+		-	+	-		-	+	+	-	-	+	-	+
UTIPS 82	+	+	-	+	+		-	+	-		-	+	+	-	-	-	+	+
UTIPS 83	-	-	-	+	-		-	-	-		-	-	-	+	+	-	+	-
UTIPS 84	-	-	-	+	+		+	-	-		-	+	+	-	-	+	+	+
UTIPS 85	-	-	-	-	+		+	-	-		-	+	+	+	-	-	+	-
UTIPS 86	-	-	-	-	-		-	-	-		-	+	-	+	+	-	+	-
UTIPS 87	-	-	-	+	-		-	-	-		-	-	-	+	+	-	+	-
UTIPS 89	-	-	-	-	-		-	-	-		-	-	-	-	-	-	+	-
UTIPS 90	-	-	-	-	+		+	-	-		-	-	+	-	-	-	-	-
UTIPS 91	+	-	-	-	+		-	+	-		-	+	+	+	-	-	+	-
UTIPS 92	-	-	-	-	+		-	+	-		-	+	+	+	-	-	+	-
UTIPS 93	-	-	-	+	+		+	-	-		-	+	+	-	-	-	+	+
UTIPS 94	+	+	-	+	+		-	+	-		-	+	+	-	-	-	-	+
UTIPS 95	-	-	-	+	+		+	-	-		-	+	+	+	-	-	+	+
UTIPS 96	-	-	-	+	+		+	-	-		-	+	+	+	-	-	+	+
UTIPS 98	+	+	-	-	+		-	-	-		-	+	+	+	-	-	-	+
UTIPS 99	-	-	-	-	-		-	-	-		-	-	-	-	-	-	-	-
UTIPS 100	-	-	-	-	-		-	-	-		-	-	-	-	-	-	-	-
UTIPS 101	+	+	-	+	+		-	+	-		-	+	+	-	-	-	+	+
UTIPS 102	-	-	-	-	+		-	-	-		-	+	+	+	-	-	+	+
UTIPS 103	-	-	-	-	+		-	+	-		-	+	+	+	-	-	+	-

UTIPS 104	-	-	-	-	+	-	+	-	-	+	+	+	-	-	+	+
UTIPS 105	-	-	-	-	+	-	+	-	-	+	+	+	+	-	+	-
UTIPS 106	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+
UTIPS 107	-	-	-	-	+	-	+	-	-	+	+	+	-	-	+	+
UTIPS 108	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
UTIPS 109	+	+	-	+	+	-	+	-	-	+	+	-	-	-	+	+
UTIPS 110	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+
UTIPS 111	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+

Appendix E

PAI marker profiles of one-hundred and five UPEC samples

Sample ID	PAI I_{536}	PAI II_{536}	PAI III_{536}	PAI IV_{536}	PAI I_{CFT073}	PAI II_{CFT073}	PAI I_{J96}	PAI II_{J96}
UTIPS 1	+	+	+	+	+	+	-	+
UTIPS 2	-	-	-	+	-	-	-	-
UTIPS 3	-	-	-	+	+	+	-	-
UTIPS 4	-	-	-	+	+	+	-	-
UTIPS 5	-	-	-	+	+	+	-	-
UTIPS 6	-	-	-	+	+	+	-	-
UTIPS 7	-	-	-	-	-	-	-	-
UTIPS 8	-	-	-	+	-	-	-	-
UTIPS 9	-	-	-	+	+	+	-	-
UTIPS 10	-	+	-	+	+	+	-	+
UTIPS 12	+	+	-	+	+	+	-	+
UTIPS 13	-	-	-	-	+	-	-	-
UTIPS 14	+	-	-	+	+	+	-	+
UTIPS 15	-	-	-	+	+	+	-	-
UTIPS 16	-	-	-	+	+	-	-	-
UTIPS 17	-	-	-	+	-	-	-	-
UTIPS 18	-	-	-	+	+	+	-	-
UTIPS 19	-	-	-	+	-	-	-	-
UTIPS 20	-	-	-	+	-	-	-	-

UTIPS 21	-	-	-	+	-	-	-	-
UTIPS 22	-	-	-	+	-	-	-	-
UTIPS 23	-	-	-	+	+	+	-	-
UTIPS 24	+	-	-	+	+	+	-	-
UTIPS 25	+	-	-	+	+	+	-	+
UTIPS 26	-	-	-	-	-	-	-	-
UTIPS 27	-	-	-	+	+	+	-	-
UTIPS 28	-	-	-	+	+	+	-	-
UTIPS 29	-	-	-	-	-	-	-	-
UTIPS 30	-	-	-	+	-	-	-	-
UTIPS 31	-	-	-	+	+	+	-	-
UTIPS 32	-	-	-	+	+	-	-	-
UTIPS 33	-	-	-	+	-	+	-	-
UTIPS 34	-	-	-	-	-	-	-	-
UTIPS 35	-	-	-	+	-	-	-	-
UTIPS 36	+	+	-	+	+	+	-	+
UTIPS 37	-	-	-	+	-	-	-	-
UTIPS 38	-	-	-	+	-	-	-	-
UTIPS 39	-	-	-	+	-	-	-	-
UTIPS 40	-	-	-	+	+	-	-	-
UTIPS 41	-	-	-	-	+	-	-	-
UTIPS 42	-	-	-	+	+	+	-	-
UTIPS 44	+	+	+	+	+	+	-	+
UTIPS 45	-	-	-	+	-	-	-	-
UTIPS 46	+	+	+	+	+	+	-	+
UTIPS 47	-	-	-	+	+	-	-	-

UTIPS 48	-	-	-	+	+	-	-	-
UTIPS 49	-	-	-	+	+	+	-	-
UTIPS 50	+	+	-	+	+	+	-	+
UTIPS 51	-	-	-	+	+	+	-	-
UTIPS 52	-	-	-	+	+	-	-	-
UTIPS 53	-	-	-	+	+	+	-	-
UTIPS 54	-	-	-	-	-	-	-	-
UTIPS 55	-	-	-	+	+	+	-	-
UTIPS 56	-	+	-	+	+	+	-	+
UTIPS 57	+	+	+	+	+	+	-	+
UTIPS 58	+	+	-	+	+	+	-	+
UTIPS 59	-	-	-	+	+	+	-	-
UTIPS 60	-	-	-	+	-	-	-	-
UTIPS 61	+	+	-	+	+	+	-	+
UTIPS 62	-	-	-	+	+	-	-	-
UTIPS 63	-	-	-	+	+	+	-	-
UTIPS 64	-	-	-	+	+	+	-	-
UTIPS 65	+	-	-	+	+	+	-	-
UTIPS 66	-	-	-	-	-	-	-	-
UTIPS 68	+	+	+	+	+	+	-	+
UTIPS 69	+	+	-	+	+	+	-	+
UTIPS 70	-	-	-	+	+	-	-	-
UTIPS 71	-	-	-	+	+	+	-	-
UTIPS 72	+	+	+	+	+	+	-	+
UTIPS 73	-	-	-	+	+	-	-	-
UTIPS 74	-	-	-	+	+	-	-	-

UTIPS 76	+	+	+	+	+	+	-	+
UTIPS 77	+	+	+	+	+	+	-	+
UTIPS 78	-	-	-	+	+	+	-	-
UTIPS 79	-	-	-	+	+	+	-	-
UTIPS 80	+	+	+	+	+	+	-	+
UTIPS 81	+	+	-	+	+	+	-	+
UTIPS 82	-	+	+	+	+	+	-	+
UTIPS 83	+	-	-	+	-	-	-	-
UTIPS 84	-	-	-	+	+	-	-	-
UTIPS 85	-	-	-	+	-	-	-	-
UTIPS 86	-	-	-	+	-	-	-	-
UTIPS 87	-	-	-	-	-	-	-	-
UTIPS 89	-	-	-	-	-	-	-	-
UTIPS 90	-	-	-	-	-	-	-	-
UTIPS 91	-	-	-	+	-	-	-	-
UTIPS 92	-	-	-	+	-	-	-	-
UTIPS 93	-	-	-	+	+	-	-	-
UTIPS 94	+	+	+	+	+	+	-	+
UTIPS 95	-	-	-	+	+	+	-	-
UTIPS 96	-	-	-	+	+	-	-	-
UTIPS 98	+	+	-	+	+	+	-	+
UTIPS 99	-	-	-	-	-	-	-	-
UTIPS 100	-	-	-	-	-	-	-	-
UTIPS 101	+	+	+	+	+	+	-	+
UTIPS 102	-	-	-	+	+	+	-	-
UTIPS 103	-	-	-	+	-	-	-	-

UTIPS 104	-	-	-	+	+	+	-	-
UTIPS 105	-	-	-	+	-	-	-	-
UTIPS 106	-	-	-	+	+	-	-	-
UTIPS 107	-	-	-	+	+	+	-	-
UTIPS 108	-	-	-	-	-	-	-	-
UTIPS 109	+	+	+	+	+	+	-	+
UTIPS 110	-	-	-	+	+	+	-	-
UTIPS 111	-	-	-	+	+	-	-	-