Whole genome sequencing and comparative genome analysis of a local multi-drug resistant uropathogenic *Escherichia coli* isolate.

Kainaat Khalil

Reg number: 00000330424

A thesis submitted in partial fulfillment of the requirements for the degree of

MS Industrial Biotechnology

Supervised by:
Dr. Amjad Ali

Industrial Biotechnology

Atta ur Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad, Pakistan.

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DECLARATION

I certify that this research work titled "**Whole genome sequencing and comparative genome analysis of a local multi-drug resistant uropathogenic *Escherichia coli* isolate**." is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources has been properly acknowledged/referred.

Signature of Student

Kainaat Khalil

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Dedicated to my Parents

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My first word of thanks to the One who taught words to Adam, Who blessed man with knowledge, Who is the sublime without Whose “Kun” nothing is possible. Secondly my humblest thanks from the core of my heart to my beloved Prophet HAZRAT MUHAMMAD (S.A.W.W) who is the eternal fountain of knowledge and guidance for the whole mankind.

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Kainaat Khalil

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List of Abbreviations

UTI	Urinary Tract Infections
ExPEC	Extra Intestinal Pathogenic *Escherichia coli*
NCBI	National Center for Biotechnology Information
LPS	Lipopolysaccharide
MDR	Multi Drug Resistance
UPEC	Uropathogenic *Escherichia coli*
VFDB	Virulence Factor Database
CGE	Center For Genomic Epidemiology
OMP	Outer Membrane Protein
GAR	Global Antimicrobial Resistance
RND	Resistance-Nodulation-Cell Division
SMR	Small Multidrug Resistance
MFS	Major Facilitator Superfamily
SNPs	Single Nucleotide Polymorphisms
MLST	Multi Locus Sequence Typing
ROS	Reactive Oxygen Species
CARD	Comprehensive Antibiotic Resistance Database
AMR	Antimicrobial Resistance
ESBL	Extended Spectrum Beta Lactamase
BLAST	Basic Logic Alignment Search Tool

Abstract

Urinary tract infections (UTIs) are one of the most common non-malignant extra-intestinal infection in urology with 300 million infections occurring annually worldwide. Uropathogenic *Escherichia coli* (UPEC), a gram-negative, multi drug resistant bacteria is the primary causative agent among the microbial spectrum involved in UTIs. Structural and secreted virulence factors of UPEC mainly contribute to the etiology of UTIs. Over a period of years, there is steady rise in number of UPEC infections in our setup and abuse of antibiotics have ensued in increased antibiotic resistance with subsequent increase in multi-drug resistant (MDR) *E.coli* strains. However, not much is known about the genetic attributes of different UPEC lineages in Pakistan. For a better understanding of genomic diversity, virulence and resistance determinants a local UPEC isolate was characterized and comparative genome analysis with global strains was carried out. The UPEC isolate U1 is a virulent multi-drug resistant strain with a genome size of 5.2 Mb containing 5376 coding sequences and a total of 389 subsystems. The strain U1 belongs to ST131 and have O25:H4 serogroup. It harbors several resistant determinants, four plasmids (designated as pU1_1, pU1_2, pU1_3 and pU1_4) harboring a TEM-1 resistant gene, 4 complete prophages, 3 miniature inverted repeats, 2 composite transposons, 15 insertion sequences and several virulence genes. Comparative analysis revealed that the strain U1 has a similar resistance and virulence profile reported earlier for strains from Sequence type 131. Moreover phylogenetic analysis revealed that the isolate U1 is evolutionary related to strain UPEC 4_0 and strain O25:bH4 reported from Sweden and Saudi Arabia respectively. These genetic insights will be helpful for effective control measures against this pathogen, for health care providence as well as research and development (R & D) organizations.

CHAPTER 1

INTRODUCTION

Escherichia coli belongs to a diverse group of bacterial species with the ability of colonizing ecological niches and animal hosts (Wiles et al., 2008). Uropathogenic *Escherichia coli* (UPEC) have highly diverse genomes and phylogenetic distribution. They are categorized as most commonly human-disease causing extraintestinal pathogenic *E. coli* (ExPEC) (Kaper et al., 2004). Urinary tract infections (UTIs) are considered to be the most common human infections and UPEC is the primary etiological agent in both community acquired and nosocomial urinary tract infections (Bower et al., 2005).

Several factor that influence the establishment of UTIs involves anatomical factors, defense mechanisms of the host, and the virulence factors of the causative agent (Nicolle, 2002). UPEC divert from their status as commensal strains in the intestinal flora, grow and persist in the urinary tract and exhibit a repository of virulence factors helping them to establish the disease (Shah et al., 2019). Inflammatory responses from the host are triggered due to the breach by UPEC in the sterile environment of the Urinary tract. This leads to the production of cytokines, neutrophil influx and bladder epithelial cells exfoliation (Bien et al., 2012).

Antibiotics are used for the treatment but are becoming ineffective because of its abuse causing an increase in antibiotic resistance and subsequent increase in the number of multi-drug resistant (MDR) *Escherichia coli* strains. UPEC has shown resistance to a broad spectrum of antibiotics (Kot, 2019) and due to high recurrence rate it is a serious public health concern in developing countries like Pakistan and has

contributed to increase morbidity, mortality, and the health care expenses.

Detailed studies regarding multi-drug resistant *Escherichia coli* strains involved in uropathogenic infections are being carried out throughout the world. And these scientific findings might be helpful in shifting the UTI treatment from routine antibiotics to pathogen specific therapeutics (Klein and Hultgren, 2020). However, not much is known about the genetic attributes of local UPEC lineages. Therefore an active surveillance program is required for monitoring prevalence and trends in antibiotic resistance, for implementation of effective control measures and optimal UTI management. Thus this study was designed to evaluate antibiotic resistance profile of local strains, and the factors that influence the severity of disease (virulence factors).

A draft genome of local UPEC strain was characterized and comparative genome analysis with the global strains was done for the said purpose. Using comparative genomics approaches we would be able to determine the phylogenetic relationship of local isolate with world isolates and identify the genetic diversity. We focused on UPEC pathogenicity, recent global and national resistance trends, treatment strategies, alternative antimicrobials and prophylactic approaches in the prevention of UTIs. These finding will ultimately help in rapid diagnosis and treatment of UPEC and to combat against antimicrobial resistance in Pakistan.

Research objectives:

1. Characterization of local Uropathogenic *E.coli* (UPEC) isolate.
2. To estimate the pangenome among all publicly available UPEC strains.
3. *Insilico* comparative genome analysis of UPEC strains to determine the genetic diversity, antibiotic resistance and virulence profiles.

CHAPTER 2

Literature Review

2.1 Urinary Tract Infections

Urinary tract infection (UTI) describes an infection that occur anywhere within the urinary system including bladder, kidneys, ureters and urethra (Tan and Chlebicki, 2016). The presence of bacteria generally $>10^5$/ml in the urine indicates an urinary tract infection (Smelov et al., 2016). UTIs are one of the most common non-malignant conditions in urology (Zhu et al., 2021). It is the most common extra-intestinal infection which occurs when the uropathogenic strains spread from the host intestine to the urinary tract where they acquire new virulence properties encoded by specific genes that allow them to colonize mucosal surfaces of the host and invade the normally sterile urinary tract (Lara et al., 2017, Xie et al., 2006, Khairy et al., 2019).

UTIs are categorized into community-acquired and nosocomial infections (Behzadi, 2018). A UTI can be caused by a wide range of pathogens that can successfully colonizes the urinary tract including Gram-negative and gram-positive bacteria and certain fungi (Foxman and Brown, 2003). Uropathogenic *Escherichia coli* (UPEC) is the primary etiological agent associated to the development of UTIs (Terlizzi et al., 2017).

If UTIs are left undiagnosed or untreated, it can lead to systemic bacteraemia infections, triggering sepsis and septic shock. UTIs are the leading cause of gram-negative bacteremia (Patton et al., 1991).

2.2 Epidemiology of UTIs

At least 300 million urinary tract infections (UTIs) occur annually worldwide (White, 2021). The severity of these infections range from mild to severe sepsis, with 20 - 40% of mortality rate (Zhu et al., 2021). UTIs are not only a global burden but are an economic burden too. Around 11 million cases are reported is united states each year that cost approximately $5 billion annually (Foxman and Brown, 2003). UTIs cause significant morbidity in infant boys, females of all age groups and older men. The frequency of these infections are affected by age and sex. Moreover UTIs are more prevalent among females as compared to males (Ejrnæs, 2011).

Epidemiological studies indicate that around 50-60% of women will experience at least one UTI episode during their lifetime (Medina and Castillo-Pino, 2019) and almost 12% men experience an episode of symptomatic urinary tract infections once in their lifetime and despite treatment with antibiotics almost 27-48% women will experience a recurrent UTIs within 3 to 4 months of the initial infection (Micali et al., 2014). About 75% of uncomplicated and 65% of complicated urinary tract infections are caused by Uropathogenic *Escherichia coli* worldwide. A recent study from south Punjab, Pakistan has also reported *E.coli* as most prevalent causative agent in UTIs in the region (Malik et al., 2020).

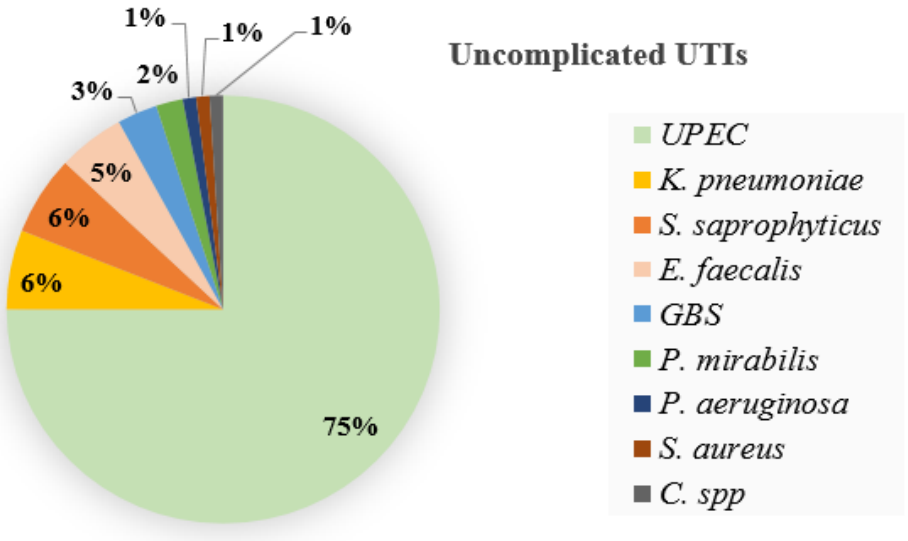

Figure 1(a) Epidemiology of urinary tract infections (Uncomplicated UTIs) (Flores-Mireles et al., 2015)

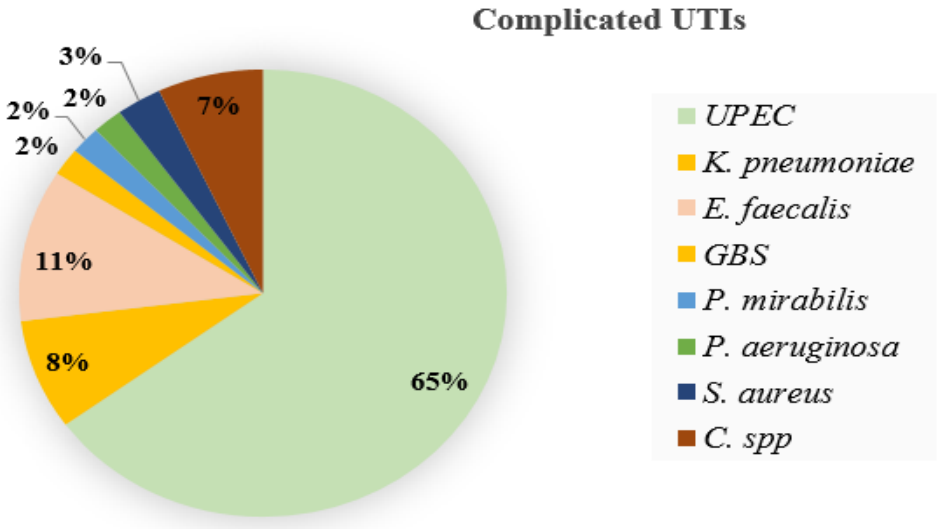

Figure 1(b) Epidemiology of urinary tract infections (Complicated UTIs) (Flores-Mireles et al., 2015)

2.3 Microbial Spectrum Involved In UTIs

A wide range of uropathogens are involved in the etiology of Urinary tract infections but uropathogenic *Escherichia coli* is the leading cause in both the complicated and uncomplicated UTIs (Medina and Castillo-Pino, 2019). UPEC is responsible for causing 70-90 % of community-acquired and 40-50% of nosocomial UTI among all age groups (Wiles et al., 2008a). Other uropathogens associated to the development of uncomplicated UTIs (in order of prevalence) are Klebsiella pneumoniae, Staphylococcus saprophyticus, Enterococcus faecalis, group B Streptococcus (GBS), Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus and Candida spp (Fig. 1) (Kline et al., 2011, Nielubowicz and Mobley, 2010, Ronald, 2002).

The most prevalent uropathogen causing complicated UTIs is Enterococcus spp., followed by K. pneumoniae, Candida spp., S. aureus, P. mirabilis, P. aeruginosa and group B Streptococcus (Chen et al., 2013b, Jacobsen et al., 2008, Fisher et al., 2011, Levison and Kaye, 2013). According to a worldwide phylogenetic analysis virulent extraintestinal *E. coli* strains belongs mainly to group B2 and D (to a lesser extent) among the eight Escherichia coli phylogroups (A, B1, B2, C, D, E, F, and Escherichia cryptic clade I) whereas the commensal strains are associated with group A or B1 (Kumar et al., 2017, Clermont et al., 2013, Basu et al., 2013, Ejrnæs, 2011, Moreno et al., 2008, Takahashi et al., 2006).

2.4 Classification of UTIs

Urinary tract infections are classified and characterized on the basis of different clinical manifestations, levels of severity, microbiological classification of pathogens, relevant complicating factors, location of infection in the host urinary tract and the presence or absence of symptoms (Bartoletti et al., 2016, Medina and Castillo-Pino, 2019).

2.4.1 Clinical Manifestation

The clinical situation of the urinary tract infection is classified as cystitis, pyelonephritis, urosepsis, urethritis and male adnexitis (Table 1) (Bartoletti et al., 2016, Smelov et al., 2016, Johansen et al., 2011). Cystitis can be defined as infection of the urinary bladder whereas symptomatic bacteremia of urinary tract origin is known as Urosepsis (Johnson, 1991) and the bacterial infection causing the inflation of kidneys is known as pyelonephritis. Pyelonephritis is an ascending UTI spreading from the bladder to the kidneys (Belyayeva and Jeong, 2022). Urethritis is an inflammation of the urethra (Young et al., 2022) whereas infections (chronic and acute) of the male urogenital tract is termed as male adnexitis. (Diemer and Gralla, 2008).

2.4.2 Levels of Severity

Severity grades for the clinical manifestation of UTIs include mild, moderate, severe and sepsis (Table 1). Urosepsis is always more severe than pyelonephritis and cystitis whereas pyelonephritis is always more severe than cystitis. The severity grading that can be adapted for urosepsis includes sepsis, severe sepsis and septic shock.

Furthermore pyelonephritis present as a mild and moderate infection that can be treated with antimicrobials whereas severe pyelonephritis infection requires therapy and hospitalization. (Bartoletti et al., 2016a, Johansen et al., 2011, Smelov et al., 2016).

2.4.3 Classification According To Risk Factors

Based on risk factors UTIs are classified as complicated and uncomplicated infections. Uncomplicated UTIs typically affect healthy individuals that have no urinary tract abnormalities (Hooton, 2012, Nielubowicz and Mobley, 2010, Kolman, 2019). Complicated UTIs results due to factors compromising host defense or the urinary tract. This may include renal transplantation, neurological disease resulting in urine retention, immunosuppression, renal failure, urinary obstruction, pregnancy and, indwelling catheters (Lichtenberger and Hooton, 2008, Levison and Kaye, 2013, Sabih and Leslie, 2022). These infections require prolonged therapy and patients have an increased risk of recurrent UTI (Mann et al., 2017).

Both pyelonephritis (Upper UTIs) and cystitis (lower UTIs) may occur as complicated and uncomplicated infections depending on underlying risk factors (Knottnerus et al., 2013). Risk factors associated with cystitis includes genetic susceptibility, a prior urinary tract infection, sexual activity, female gender, diabetes, obesity and vaginal infection (Hannan et al., 2012). Moreover, for phenotyping the risk factors the ORENUC system comprising of six groups was proposed (Table 2). For complicated UTI, risk factors belong to the N, U, and C categories of ORENUC system (Smelov et al., 2016, Johansen et al., 2011).

Table 1 Clinical manifestation of UTIs and grading of severity. Adapted from (Johansen et al., 2011)

Acronym	Clinical diagnosis	Clinical symptoms	Grade of severity
CY-1	Cystitis	Dysuria, frequency, urgency, suprapubic pain; sometimes unspecific symptoms	1
PN-2	Mild and moderate pyelonephritis	Fever, flank pain, CVA tenderness; sometimes unspecific symptoms	2
PN-3	Severe pyelonephritis	As PN-2, nausea and vomiting with or without symptoms of CY	3
US-4	Urosepsis (simple)*	Temperature >38 ˚C or 90 beats/min; respiratory rate >20 breaths/min or PaCO2 12 000 cells/mm3	4
US-5	Severe urosepsis*	As US-4, organ dysfunction, hypoperfusion, or hypotension. Hypoperfusion and perfusion abnormalities.	5
US-6	Uroseptic shock*	As US-4 or US-5, with hypotension despite adequate fluid resuscitation, perfusion abnormalities	6
CY = cystitis; PN = pyelonephritis; US = urosepsis; WBC = white blood cells.			

Table 2 Host risk factors in UTIs categorized according to the ORENUC system (Johansen et al., 2011)

Phenotype	Category of risk factor	Examples of risk factors
O	No known risk factor	– Otherwise healthy premenopausal women
R	Risk factors for recurrent urinary tract infection but no risk of more severe outcome	– Sexual behavior (frequency, spermicide) – Hormonal deficiency in post menopause – Secretor type of certain blood groups – Well-controlled diabetes mellitus
E	Extra urogenital risk factors with risk of more severe outcome	– Prematurity, new born – Pregnancy – Male gender – Badly controlled diabetes mellitus – Relevant immunosuppression (not well defined
N	Nephropathic diseases with risk of more severe outcome	– Relevant renal insufficiency (not well defined) – Polycystic nephropathy – Interstitial nephritis (e.g. due to analgesics)
U	Urologic risk factors with risk of more severe outcome, which can be resolved during therapy	– Ureteral obstruction due to a ureteral stone – Well-controlled neurogenic bladder disturbances – Transient short-term external urinary catheter – Asymptomatic bacteriuria*
C	Permanent urinary catheter and unresolvable urologic risk factors with risk of more severe outcome	– Long-term external urinary catheter – Unresolvable urinary obstruction – Badly controlled neurogenic bladder disturbances

2.4.4 Microbiological Characterization of Pathogens

E.coli and *S.saprophyticus* are essential uropathogens in 70-95% and 5-10% cases of uncomplicated UTIs respectively. Other causative agents includes *Proteus mirabilis* and *Klebsiella spp* that are isolated occasionally. In case of complicated UTIs microbiological spectrum includes *E.coli*, the most frequent pathogen and several gram-negative and gram-positive bacteria including *Enterobacter spp, P. aeruginosa, Citrobacter spp, Enterococcus spp* and *Staphylococcus spp* (Grabe et al., 2013).

2.4.5 Classification According To Source of Infection

UTIs are common pathological conditions occurring in either the health-care or community settings (Gupta et al., 2011, Lee et al., 2018). Among all hospital-acquired infections nosocomial UTIs comprises up to 40%. Patients with catheters or those undergoing urological treatments and long-stay elderly male patients have increased risk of developing nosocomial UTIs. The causative agent occasionally originate from a moist site in the hospital environment (Kalsi et al., 2003).

2.4.6 Classification Based On Presence and Absence of Symptoms

Cystitis (lower UTI) and pyelonephritis (upper UTI) are symptomatic UTIs where there are clinical symptoms indicative of UTI involving bladder and kidneys. In contrast infection without urinary symptoms whereby there is a growth of 10^5 bacteria or more in a single urine sample in men or two consecutive female urine specimens is termed as asymptomatic bacteriuria (Nicolle et al., 2005, Bartoletti et al., 2016).

2.5 Pathogenesis

The pathogenesis of mucosal surface infection encompasses a number of steps, including the bacterial attachment to the epithelium, colonization of urinary tract followed by tissue damage and in certain cases there involves invasion and dissemination (Sparling, 1983). Each stage depends on host factors as well as virulence properties of the bacteria (Beachey, 1981). Bacterial attachment and internalization are the essential steps (Connell et al., 1997). The adherence capacity of the bacteria depends on the infection site in the urinary tract as well as the presence and absence of the underlying functional and structural anomalies (Reid and Sobel, 1987).

Bacterial expression of fimbrial adhesions enables the attachment to urothelium and helps in colonizing the urinary tract (Connell et al., 1997). Uncomplicated UTIs begin with contamination of the periurethral region with gut residing pathogens followed by colonization of the urethra and migration to the bladder (Fig. 2) (McLellan and Hunstad, 2016, Chen et al., 2013, Hooton, 2001, Mak and Kuo, 2006). This is followed by colonization of the bladder which is mediated by pilli and adhesins.

Bacterial access to the bladder epithelial cell cytoplasm may results in the formation of biofilm like masses called as IBC (Intracellular bacterial communities) (Anderson et al., 2003, Schwartz et al., 2011). Inflammatory responses clear extracellular bacteria (Mulvey et al., 1998) while those evading the immune system undergo multiplication and form biofilms. These bacteria induce tissue damage through production of toxins and proteases and promote the survival of bacteria by the release of essential nutrients.

After colonizing the bladder, bacteria can ascend the ureters and colonize the kidneys resulting in upper urinary tract infection or pyelonephritis (Spaulding and Hultgren, 2016, Flores-Mireles et al., 2015). For a pathogen to cause complicated UTIs same steps will be followed except that the host bladder must be compromised for example in case of catheterization. Due to catheterization an immune response is induced resulting in the accumulation of fibrinogen on the catheter. UTIs occurs when the bacteria adhere to the catheter with subsequent formation of the biofilm (Werneburg et al., 2020).

2.6 Routes of Infection

Bacteria can cause UTI via three major routes of infection including lymphatic, haematogenous and ascending routes. In patients with established UTI, the most common route of infection is ascending route (Davis and Flood, 2011). In ascending route, the microbial pathogens originate from rectal flora and via urethra gain entry into the urinary tract to the bladder (Handley et al., 2002). Most cases of Pyelonephritis occurs due to ascension of the uropathogen from the bladder into the renal pelvis through host ureter (Busch and Huland, 1984). Kidney infection through the haematogenous route is uncommon in healthy individuals. (Smellie et al., 1975). In some cases a uropathogen utilizes the lymphatic route for penetration of the host urinary tract from the adjacent organs. (Davis and Flood, 2011).

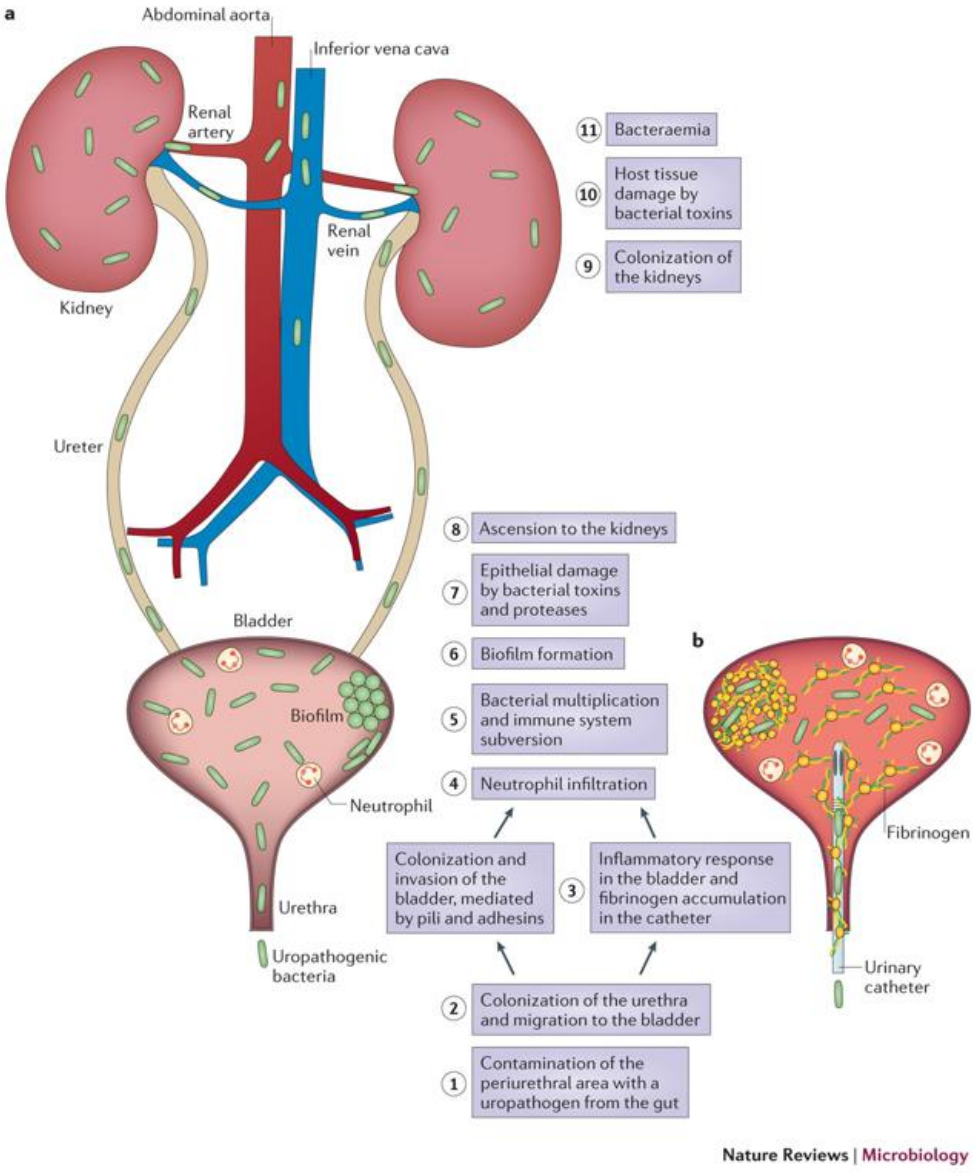

Figure 2 Pathogenesis of urinary tract infection (Flores-Mireles et al., 2015)

2.7 Virulence Factors (VF) of UPEC

UPEC express a repository of virulence factor genes that plays a significant role in the establishment of urinary tract infections. Those factors are broadly classified as cell surface and secreted virulence factors. These include fimbrial and afimbrial adhesins, flagellum, polysaccharide capsule, lipopolysaccharide (LPS), outer membrane proteins (OMPs), secreted toxins and iron uptake receptors such as siderophores (Fig. 3) (Johnson, 1991b, Emo et al., 2003, Vagarali et al., 2008, Shah et al., 2019, Terlizzi et al., 2017, Klemm et al., 2010b, O'Brien et al., 2016, Werneburg et al., 2015). These virulence factors plays vital role in bacterial adherence, colonization and persistence despite an effective defense mechanism of the host (Shah et al., 2019,Vagarali et al., 2008, Schlager et al., 2002, Yamamoto et al., 1997). Several studies had indicated that UPEC virulence genes are located on the pathogenicity islands (PAIs) transferred from other species through horizontal gene exchange (Behzadi, 2018).

2.7.1 Adhesins

Adhesins are categorized as fimbrial or afimbrial adhesins. This depends on whether these adhesins are presented as part of a surface glycoprotein called as fimbria. Fimbria/pilus act as ligands for glycoprotein and glycolipid receptors that are present on the uroepithelial cells (Davis and Flood, 2011). A number of adhesins are encoded by UPEC targeting a wide range of host receptors (Bower et al., 2005).

These adhesins can adhere onto both biotic (e.g. host cells) and abiotic (e.g. catheter) surfaces. Adhesins encoded by UPEC includes Curli adhesins, Dr adhesins, AFA adhesins, Type 1 and type 3 fimbriae, F1C fimbriae, S and P fimbriae, F9 fimbriae and Auf adhesins (Table 3) (Behzadi, 2018, Servin, 2014, Klemm et al., 2010a, Ulett et al., 2007, Spurbeck et al., 2011).

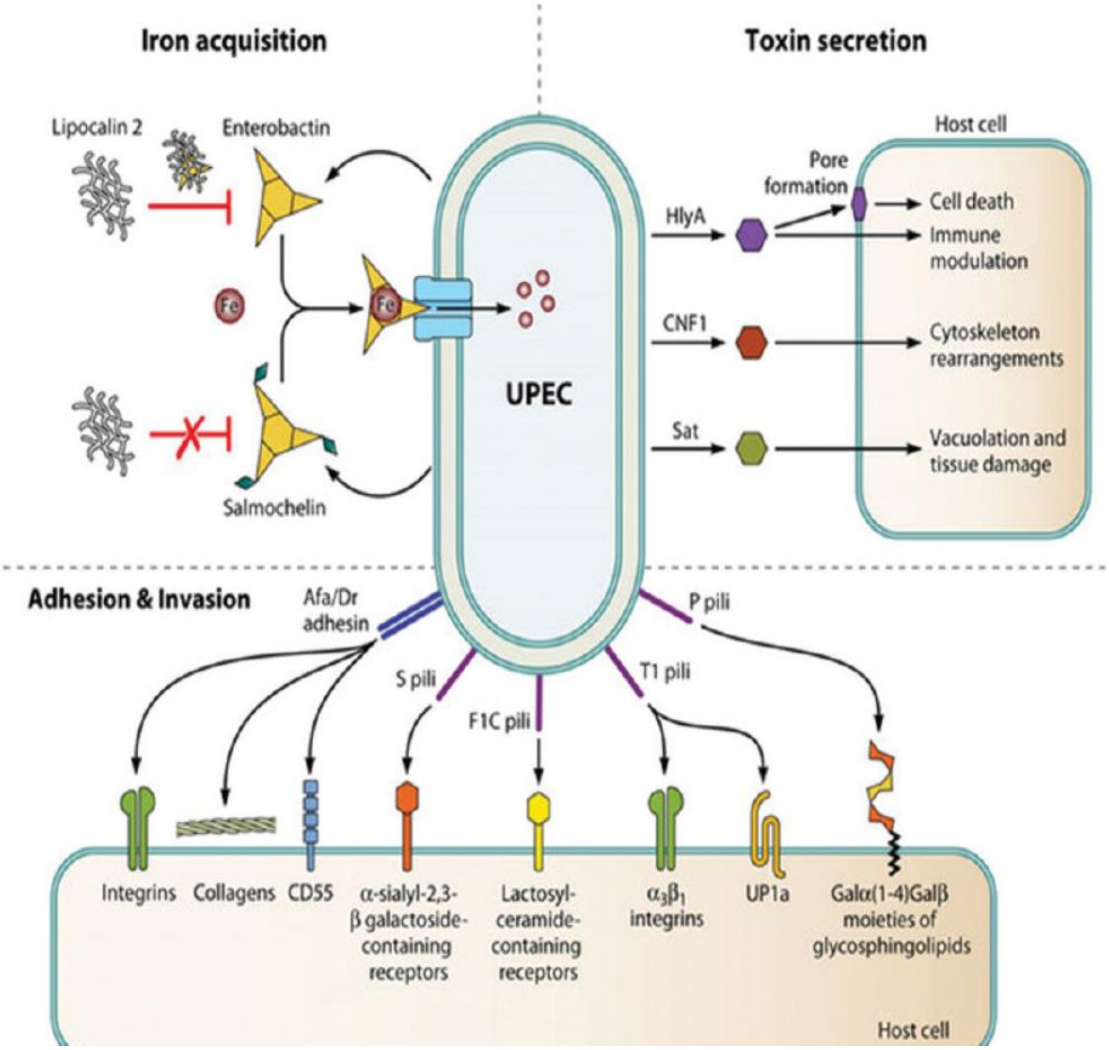

Figure 3 Virulence factors of Uropathogenic *Escherichia coli* (Barber et al., 2016)

Table 3 Adhesins encoded by uropathogenic *Escherichia coli* (Bower et al., 2005, Behzadi, 2018)

Adhesins	Type of adhesins	Genes	Role	Target structure
Curli	Afimbiral adhesin	*csgA, csgB, chg., csgD, csgE, csgF, csgG*	Adhesion for colonization (biofilm formation) & invasion	Matrix protein like fibronectin, laminin & plasminogen, mucosal cells
Dr	Fimbiral adhesin	*draA, draB, draC, draD, draE, draP*	Adhesion for colonization, Preparing invasion	A vast range of cells with Dr blood group antigens on their surface like urothelia, neutrophil cells, connective tissues in the upper part of urinary tract
AFA	Afimbiral adhesin	*afaI, afaII, afaIII, afaIV, drII*		
Type 1 fimbriae	Fimbiral adhesion (sensitive to mannose)	*fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH*	Adhesion for colonization (biofilm formation) & invasion	RBCs, mucosal membrane & Epithelium cells, uroplakin receptors in urine bladder
Type 3 fimbriae	Fimbiral adhesin	*mrkA, mrkB, march, mrkD, mrkE*		
F1C fimbriae		*focA, focC, focD, focf, focG, focH, focI*	Adhesion for colonization (biofilm formation)	Glycolipids of endothelial mucosal membrane & glomeruli
S fimbriae		*sfaA, sfaB, sfaC, sfaD, sfaE, sfaF, sfaG, sfaH, sfaS, sfaX, sfaY*	Adhesion and colonization	Sialic acid molecules on kidneys & glomeruli endothelial, epithelial and mucosal cells
P fimbriae	Fimbiral adhesion (resistant to mannose)	*papA, papB, papC, papD, papE, papF, papG, papH, papI*		Vascular epithelia, urothelia & mucosal cells
F9 fimbriae	Fimbiral adhesin	*c1931, c1932, 1933, 1934, c1935, c1936*	Adhesion for colonization (biofilm Formation)	Urothelial cells
Auf fimbriae		*aufA, aufB, aufC, aufD, aufE, aufF, aufG*		Unknown

2.7.2 Iron Acquisition Systems

There is a limited concentration of iron in the bladder environment during UTI. For uropathogens to grow in urine, they utilize an iron (Fe3+) scavenging system called as siderophore systems. These systems have three main components including assembly machinery for siderophore, an iron binding siderophore and a membrane receptor for internalization of siderophore bounded iron. Siderophore mediated iron utilization is significant for UPEC colonization of the urinary tract (Hagan and Mobley, 2009). UPEC produces several systems including yersiniabactin, aerobactin, enterobactin, and salmochelin of which aerobactin and yersiniabactin are essential in the urinary tract. (Neilands et al., 1985, Parvez and Rahman, 2018, Garcia et al., 2011). Aerobactin is a highly expressed system which is stable at low pH and exhibits higher levels of iron binding than enterobactin (Valdebenito et al., 2006, Watts et al., 2012).

Yersiniabactin plays a significant role in the formation of biofilm. It also has a protective role against copper stress by sequestering host-derived copper (Chaturvedi et al., 2012). The involvement of IroN (salmochelin siderophore receptor) in urothelial cells invasion has been reported. Thus it plays a dual role as iron uptake receptor and an internalization factor (Feldmann et al., 2007). Moreover Hemin uptake system uptakes free iron during urinary tract infection and includes ChuA and Hma. It has also been found to be involved in growth of uropathogens and formation of biofilm (Jahandeh et al., 2015, Reigstad et al., 2007, Garcia et al., 2011).

2.7.3 Capsule

Capsular virulence factors of UPEC includes K polysaccharides including K1,

K2, K3, K5, K12, K13, K20 and K51/KspMT. These polysaccharides play role in adhesion, formation of biofilm, resistance to antimicrobials, anti-phagocytosis, anti-serum and complement-mediated bactericidal effect. The K1 polysaccharide are involved in the development of IBC whereas K1 and K5 also plays a role in the prevention of host humoral immune response by a molecular mimicry to components of the host tissues and in several stages of pathogenesis (da Silva and Mendonça, 2012, Agarwal et al., 2012, Momtaz et al., 2013, Bekal et al., 2003, Jahandeh et al., 2015, Bien et al., 2012).

2.7.4 Lipopolysaccharides

Lipopolysaccharides have amphipathic properties comprising of an oligosaccharide core lined with fatty acids and the core is bounded to polysaccharide chain known as O antigen (Simpson et al., 2015). There are several aspects of the uropathogenic *Escherichia coli* life cycle that are mediated by the structural components of the lipopolysaccharides. This includes their ability to colonize bladders, formation of biofilm, and induce host adaptive and innate immune responses (Aguiniga et al., 2016). Lipopolysaccharides are also responsible for providing resistance to hydrophobic antibiotics. When the lipopolysaccharides concentration decreases at the cell surface hypersensitivity to hydrophobic toxic molecules occurs (Zhang et al., 2013). Lipopolysaccharides also induce the production of cytokines including IL-1, TNF-α and nitric oxide that are responsible for an increase in the inflammatory response (Bien et al., 2012b, Emody et al., 2003).

2.7.5 Toxins

UPEC secretes several toxic substances involved in its pathogenesis. Toxins have the ability of altering the cell signaling cascade of the host and mediate inflammatory responses. A number of studies have indicated that these toxins also play role in the stimulation of the host cell death and release of essential nutrients, providing the ability to reach deeper tissues in the urinary tract (Agarwal et al., 2012). These toxins can also cause the host cell lysis and functional and structural defects in host (Asadi Karam et al., 2019, Henderson et al., 2004, Wiles et al., 2008b). Cyclomodulins, a CDT toxin was the first virulent toxin reported in UPEC (Soltani et al., 2018). Other UPEC toxins reported includes α-hemolysin (HlyA), vacuolating autotransporter toxin (VAT), secreted autotransporter toxin (SAT), cytotoxic necrotizing factor 1 (CNF1), arginine succinyl-transferase (AST), cytolysin A, plasmid-encoded toxin (PET), Shigella enterotoxin-1 (ShET-1) etc. (Parvez and Rahman, 2018).

2.7.6 UPEC Flagella

A number of UPEC strains isolated from patients with asymptomatic, acute or recurrent UTIs has been observed to express a flagellum mediated motility. (Wright et al., 2005). It had been reported that flagella plays dynamic roles during biofilm formation such as adherence, maturation, and dispersal (Nakamura et al., 2016, Pratt and Kolter, 1998). Motility is also involved in the accession of the pathogen from the bladder to the kidneys (Lane et al., 2007). Flagellated UPEC cause about 70–90% of all UTIs (Bien et al., 2012b). Protein FliC forms flagella that is used by UPEC strains to evade host immune system (Honko and Mizel, 2005). It also enhances *E.coli* ability to attract and repel environmental stimulus (Emody et al., 2003).

2.7.7 Outer Membrane Proteins (OMPs)

Outer membrane proteins including OmpA, OmpC, OmpF, OmpT, OmpX, ompA, ompC, ompF, ompT ompX has been identified as UPEC virulence factors. Several OMPs act as porin and play role in transportation. Several proteins are also involved in the intratcellular virulence of the UPEC by acting as a facilitating factor (da Silva and Mendonça, 2012, Agarwal et al., 2012, Momtaz et al., 2013, Bekal et al., 2003, Jahandeh et al., 2015). A recent study suggested that OmpT plays a complex role in UTI pathogenesis including increased bacterial adhesion and invasion, IBCs formation and upregulated proinflammatory cytokines (He et al., 2015). Another recent study has indicated that ompF porin have some immuno-properties and also play role in adherence, invasion, and serum resistance in bacterial pathogenesis (Hejair et al., 2017, Su et al., 1990).

2.8 Mobile Genetic Elements

Mobile genetic elements are moveable segments of DNA that are transferred among bacterial genomes and have a significant role in the plasticity of bacterial genomes (Rankin et al., 2011). Mobile genetic elements are important drivers of the HGT and acquiring new MGEs may result into emergence of new *E.coli* pathotypes (AmbrožKičK et al., 1998, Szczepanowski et al., 2005). Mobilomes comprises of all mobile genetic elements that are responsible for bacterial evolution as well as antibiotic resistance and virulence. This includes transposable elements, plasmids, integrons, insertion sequences, bacteriophages, genomic islands (GI). The major players of spreading genes encoding antimicrobial resistance in UPEC pathotypes includes

conjugative integrons, plasmids and transposons (Carvalho et al., 2020). **Genomic islands** are large DNA regions frequently exchanged between bacterial isolates. GI encode for several properties including metabolic adaptation, resistance and bacterial virulence (Lloyd et al., 2007, Hacker et al., 1997). GIs that encodes for the virulence genes of bacteria are called pathogenicity islands (PAIs) (Hacker and Carniel, 2001).

PAIs carry often more than one virulence associated gene and they are linked to tRNA loci. The first PAIs have been identified in UPEC genomes. Most genes that code for the UPEC virulence factors are present on PAIs (Middendorf et al., 2001, Blum et al., 1995). **Plasmids** are also involved in carrying acquired virulence and resistance genes. They are classified into different Inc families according to the conjugative and incompatibility features (Noll et al., 2018, Michelacci et al., 2018, Velappan et al., 2007). IncF incompatibility group plasmids were found to encode both resistance and virulence genes (Rijavec et al., 2006a). **Bacteriophages** are also responsible for the dissemination of virulence and antimicrobial resistance genes among bacteria through phage-mediated transduction resulting in the emergence of new pathotypes (Bozcal, 2019, Barr et al., 2013). **Transposable elements** are DNA sequences transfer among different locations in the bacterial genome. Transposable elements harbors resistance genes that can be integrated and excised from the genome with the help of enzymes called as transposases. The simplest version of the transposable elements among uropathogens is the insertion sequence (IS) (Muñoz-López and García-Pérez, 2010, Brown-Jaque et al., 2015). Major features of prokaryotic IS families is given in table 4. Recent studies on UPEC has established a strong association between the presence of integrons and antimicrobial resistance in various regions of USA, Asia and Europe (Muhammad et al., 2011).

Table 4 Major features of prokaryotic IS families (Mahillon et al., 1998)

Family	Size range (bp)	Direct repeats (bp)	ENDS	Inverted repeats	No. of ORFs
IS1	770	9 (8–11)	GGT	Y	2
IS3	1200-1550	5	TGA	Y	2
IS5	800-1350	4	GG	Y	1 or 2
IS6	750–900	8	GG	Y	1
IS21	1,950–2,500	4 (5, 8)	TG	Y	2
IS30	1,000–1,250	2–3	-	Y	1
IS66	2,500–2,700	8	GTA	Y	>3
IS91	1,500–1,850	0	-	N	1
IS110	1,200–1,550	0	-	N	1
ISL3	1,300–1,550	8	GG	Y	1
ISAs1	200–1,350	8	C	Y	1

2.9 Antibiotic Treatment for UTI

UPEC affect significantly large proportion of individuals every year hence are a major target of antimicrobial therapy (Rijavec et al., 2006). Several factors including patient's age, history, gender, other underlying infections, clinical presentation and infection site are considered for the antibiotic therapy (Ejrnæs, 2011). Local resistance profiles of the causative agent is also considered while selecting the antimicrobials. Commonly used antimicrobial agents for the treatment of uncomplicated UTIs include trimethoprim, fluoroquinolones, β-lactams, fosfomycin tromethamine and nitrofurantoin. These antimicrobials are used due to their favorable pharmacokinetic profiles, their tolerability and the spectrum of activity (Jancel and Dudas, 2002, Neu, 1992). Fluoroquinolones including ciprofloxacin, prulifloxacin , levofloxacin may be considered as alternatives for treating uncomplicated cystitis (Bartoletti et al., 2016).

Several other antibiotics that are effective against UPEC strains includes Imipenem (100%), ertapenem (99.98%), amikacin (99.94%), and nitrofurantoin (99.91%). Imipenem is also most effective against ESBL producing UPEC strains (Idil et al., 2016). Nitrofurantoin is appropriate for empirical UTI therapy (Muhammad et al., 2011). For pyelonephritis or complicated urinary tract infections Amoxicillin-clavulanic acid is a recommended antibiotic. Also it can be used for empirical therapy for uncomplicated UTIs (Kot, 2019). Since the treatment of UTIs with antibiotics, the level of antibiotic resistances among community and nosocomial isolates has steadily increased and it is an alarming condition to overcome by the healthcare sector.

2.10 Global Antibiotic Resistance

Antibiotic resistance patterns among microbial spectrum involved in UTIs is changing (Rijavec et al., 2006) and has become a major concern for public health (Bunduki et al., 2021). Antibiotic abuse has indeed resulted in selection of strains that

have the ability of acquiring antimicrobial resistance genes via HGT (Davies and Davies, 2010, Leungtongkam et al., 2018). UPEC resistance to fluoroquinolones is significantly higher in developing countries (55.5–85.5%) than in developed countries (5.1–32.0%) (Kot, 2019) (Figure 4). UPEC resistance against Amoxicillin-clavulanic acid varied between infected population and geographical regions. Many studies have reported UPEC resistance to trimethoprim-sulfamethoxazole in many countries suggesting it not to be used as empiric UTI therapy (Kot, 2019).

Several studies have reported that biofilm producing UPEC show significant antimicrobial resistance in comparison with biofilm non-producers (Karam et al., 2016,Tadepalli et al., 2016, Tabasi et al., 2016). The prevalence of ESBL producing fluoroquinolones (FQ) resistant UPEC in the Asian-Pacific region is significantly higher (Ali et al., 2016, Dalhoff, 2012). UPEC isolates also shows resistance against cephalosporins and ampicillin (Moya-Dionisio et al., 2016).

In **Pakistan** resistance against quinolones has increased for Enterobacteriaceae in the last decade (Yasmin et al., 2013). A study had reported significantly higher antimicrobial resistance (for most tested antimicrobials) in UPEC strains from Pakistan (Muhammad et al., 2011). According to recent studies 82% of UPEC isolates are trimethoprim-sulfamethoxazole resistant (Ali et al., 2016) whereas 71% UPEC strains are amoxicillin-clavulanic acid resistant and 60.8% strains are ciprofloxacin resistant (Kot, 2019). UPEC strains isolated from Pakistani outpatients showed 29% and 4% resistance against gentamicin and amikacin (Ali et al., 2016). According to NIH-Pakistan antimicrobial resistance surveillance report 2017 - 2018 resistance profile of *E.coli* from urine samples shows a surge in resistance against ciprofloxacin, ceftriaxone, amoxicillin-clavulanic acid, nitrofurantoin and cefixime in year 2018 than 2017 (Figure 5).

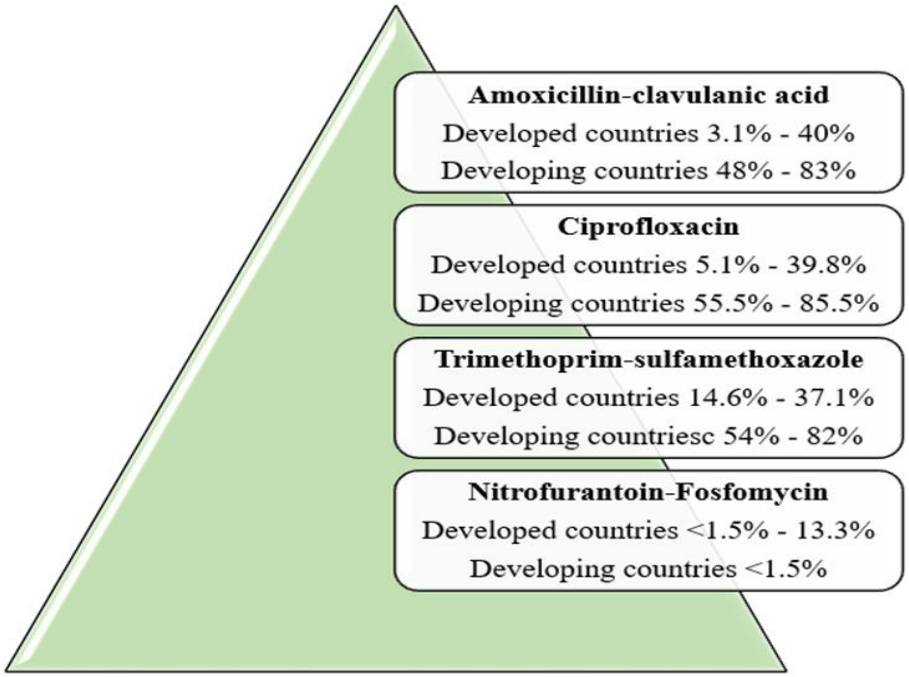

Figure 4 UPEC resistance to antimicrobials used in the treatment of UTIs (Kot, 2019)

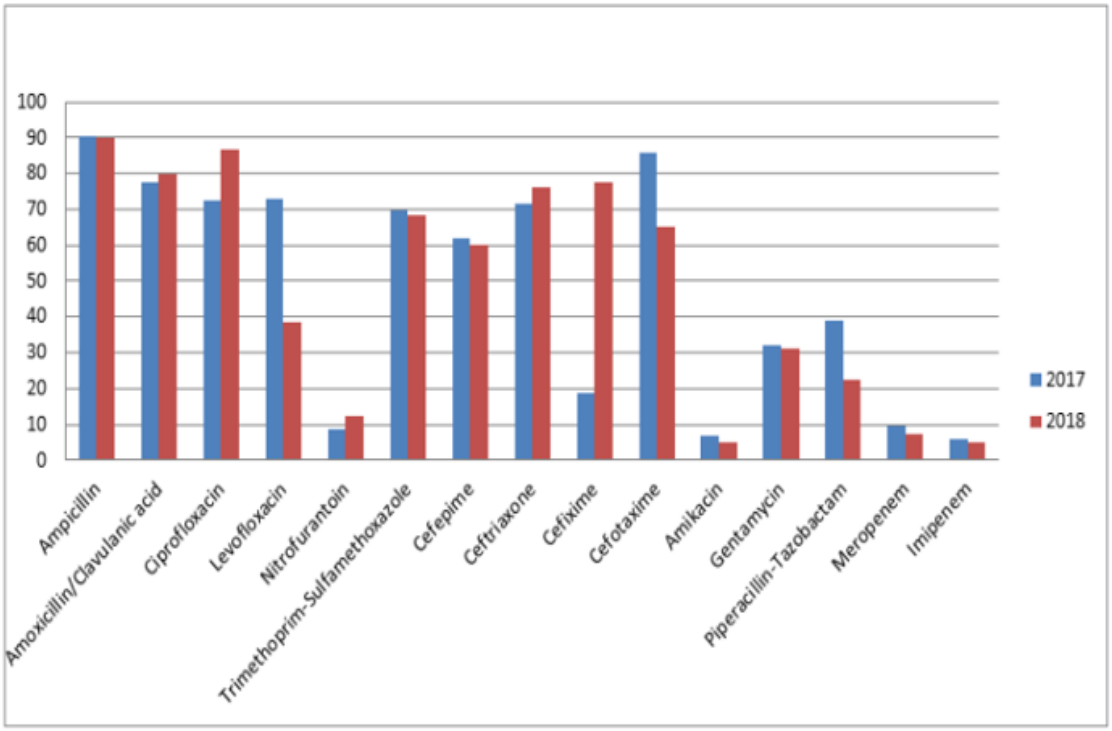

Figure 5 Graph representing resistance profile of Escherichia coli in Urine samples

2.11 Alternative Antimicrobial Strategies

Due to antibiotic abuse and long-term interference with microbiota of the intestine alternative strategies are required. Several alternative effective remedies reported against UPEC includes the following; development of **vaccines** using UPEC factors as vaccine antigens (McLellan and Hunstad, 2016). Adhesins, siderophores, and antimicrobial peptides of the pathogen are potential target among candidate antigens for vaccine development (Spaulding and Hultgren, 2016). **Probiotics** is another alternative used extensively for reducing the recurrent urinary tract infections (Zacchè and Giarenis, 2016). UPEC inhibitory activity have been shown by several Lactobacilli strains and their major role is their ability to remove reservoirs of UPEC preventing reoccurrence (Shim et al., 2016). Whereas use of **Estrogens** as an alternative modulates two epithelial defense mechanisms including AMPs induction and decrease in epithelial exfoliation (Lüthje et al., 2013).

Pillicides are interesting alternative as the necessity of pili for UPEC adhesion makes assembling chaperone-usher pathway (CUP) inhibitors a suitable target for the reduction of urinary tract infections (Aberg and Almqvist, 2007). Whereas the production of Type 1 pilus can be inhibited by **Curlicides**. They also inhibits the curli biogenesis thus inhibiting biofilm formation mediated by curli (Chapman et al., 2002). D-Mannose and D-Mannose-Derived **FimH antagonists** target bacterial adhesion by inhibiting FimH thereby reducing UPEC infection (Sauer et al., 2016). Phenols, polyphenols, Vitamin D, methaneamine, and several medicinal plants that exhibit antibacterial activity against UPEC are other alternative antimicrobial strategies (Terlizzi et al., 2017).

2.12 UPEC Antibiotic Resistance Mechanisms

β-lactamase enzymes hydrolyze amide bond of the beta lactam ring and thus are responsible for resistance against β-lactams (Adamus-Białek et al., 2018). Mutations in *blaSHV-1*, *blaTEM-1* and *blaTEM-2* genes results in the development of ESBL resulting in resistance to cephalosporins (Dashti et al., 2006). The frequency of ESBL producing UPEC is Pakistan is 38.9% (Ali et al., 2016). *gyrA* and *gyrB* genes mutations catalyze supercoiling of the DNA that results in resistance to quinolones (Friedman et al., 2001). Efflux pumps and variations in the porin proteins results in decrease antibiotic uptake causing resistance to fluoroquinolones and quinolones (Karam et al., 2019). Fosfomycin resistance is due to mutations in the G-3-P transporter or G-6-P transporter genes (Kadner and Winkler, 1973, Tsuruoka and Yamada, 1975). Also point mutation in the UDP-GlcNAc enolpyruvyl transferase binding site results in fosfomycin resistance (Kim et al. 1996). The phosphorylation of the phosphonate group or enzyme mediated cleavage of the oxirane ring results in the inactivation of Fosfomycin (Rigsby et al., 2005). Nitrofurantoin resistance is due to mutations in the *nsfA* and *nfsB* genes that code for nitroreductase that are oxygen insensitive resulting in resistance to nitrofurantoin (Sandegren et al., 2008).

2.13 Pan-genome Analysis

The concept of bacterial pan-genome was introduced in 2005 for the analysis of pathogenic bacterial species. Pan-genome encompasses the entire repertoire of genes that are accessible to a studied phylogenetic clade or a given species. The pangenome is classified into core genome and accessory genome including dispensable and unique genome. The core genome consists of conserved genes present among all strains

whereas dispensable genome consist of genes shared by some strains. Unique genes are strain specific genes (Tettelin et al., 2005, Chen et al., 2018). Thus far it is difficult to fully describe the bacterial species due to the addition of new gene sequences. This addition can be due to duplication or diversification of the existing genes resulting in high diversity in bacterial genomes (Venter et al., 2004).

Thus for the estimation of genetic diversity in bacterial strains analysis of a pan-genome is a suitable approach (Vernikos et al., 2015). It also provide information whether the pangenome of the respective specie is open or closed. The pace with which microbes are evolving there is a likelihood of increase in the microbial gene pool by several orders of magnitude. An in-house pipeline PanRV is available for pan-genome analysis.

CHAPTER 3

Materials and Methods

3.1 Study Approval and Sample Collection

The study was approved by the Industrial Biotechnology department of Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST, Islamabad to collect a UPEC isolate U1 from Fauji Foundation hospital, Rawalpindi, Pakistan and culturing of the isolate in ASAB labs. The strain U1 was isolated from a urine sample of a 65 years old female patient suffering from urinary tract infection. The isolate U1 was transported to Integrated Biology Lab, ASAB for preliminary identification (colony/cell morphology, biochemical and molecular characterization) and to perform antibiotic susceptibility assay.

3.2 Preliminary Identification

3.2.1 Colony Morphology

The Isolate U1 was streaked on MacConkey Agar plates; a selective media for *Escherichia coli* species and were incubated at 37 ˚C. After 24 hours of incubation, the shape, colony size, texture and margin were recorded and crosschecked with Bergey's Manual of Systematic Bacteriology (*Bergey's Manual® of systematic Bacteriology, 2005*).

3.2.2 Cell Morphology

To know about the cell morphology gram staining was performed. For Gram staining a smear of a pure culture was prepared on a glass slide. The glass slide was then stained with Crystal violet dye (primary dye) for two minutes. Then the slide was stained with iodine solution for two minutes.

Excessive stain in removed by flooding the slide with water. Purple stained were decolorized with 70% ethanol for about 1 minute. The slide was then stained with Secondary dye i-e. safranin for 45 seconds. Finally, the slide was washed, air dried and was examined under the microscope.

3.3 Biochemical Characterization

After preliminary identification, biochemical test were performed to phenotypically characterize the isolate U1.

3.3.1 Catalase Test

Bacteria uses catalase enzyme; an antioxidant enzyme to protect cell from oxidative damage by reactive oxygen species (ROS). Catalase enzyme has the ability to use hydrogen peroxide (H_2O_2), a non-radical ROS, as its substrate and catalyze it into O_2 and H_2O. Fresh colony of the isolate U1 was placed on a slide using sterilized loop and 3% of H_2O_2 was applied to observe the bubble formation.

3.3.2 Lactose Fermentation Test

Lactose Fermentation test is used to identify gram negative bacteria that can ferment lactose. *Escherichia coli* can ferment lactose producing acid end products which results in the decrease in the pH of the medium. A positive test consists of a color change from pink to red, indicating a pH change to acidic. The isolate U1 was streaked on MacConkey Agar plates; a differentiating agar that differentiate the gram-negative bacteria based on their lactose metabolism and were incubated at 37 ˚C for 24 hours.

3.4 Antibiotic Susceptibility Assay

The isolate U1 was tested against 15 antibiotics: Sulphamethoxazole-

Trimethoprim (SXT), Cefepime (FEP), Ciprofloxacin (CIP), Vancomycin (VA), Enoxacin (EN), Gentamicin (CN), Piperacillin (PRL), Kanamycin (K), Amoxycillin/ Clavulanic Acid 2:1 (AMC), Meropenem (MEM), Ampicillin (AMP), Amikacin (AK), Ceftriaxone (CRO), Imipenem (IPM) and Chloramphenicol (C) (Table 5) using the Kirby-Bauer method. Briefly, the bacterial culture at turbidity of 0.5 McFarland was prepared from an overnight culture by mixing the pure colony of isolate U1 in 1ml of normal saline. Then 50µl of bacterial suspension was then poured. Using the glass spreader the suspension is spread over Mueller-Hinton Agar (MHA) media plates to make a media lawn and 4-5 media plates were placed on each plate. The plates are then incubated at 37 ˚C for 24 hours. After incubation, zone of inhibition were recorded and examined according to the guideline of Clinical and Laboratory Standards Institute ('CLSI', 2019).

3.5 Glycerol Stock Preparation

An aqueous solution of Glycerol acts as a cryoprotectant and is used for preserving microbial culture at low temperature. For broth culture, the liquid LB broth medium was inoculated with fresh culture/fresh single colony of isolate U1 in a culture tube and incubated in a shaking incubator at 37 ˚C to ensure proper aeration and nutrient availability overnight. Shaking incubation also avoids bacterial clumping at the bottom of the culture tube. After incubation culture tube are checked for bacterial growth. The broth becomes cloudy from the bacterial growth. Then about 0.5ml bacterial culture from LB broth was mixed in 0.5ml of 80% sterile glycerol. The cryovials were then gently vortexed and freeze using liquid nitrogen before it was transferred to -70/80 ˚C freezer.

Table 5 Concentration of antibiotics used for antibiotic susceptibility assay.

No.	Class of Antibiotics	Antibiotics	Concentration (µg)
1.	Sulfonamides	Sulphamethoxazole/ Trimethoprim (SXT)	25
2.	Cephalosporin	Ceftriaxone (CRO)	30
3.		Cefepime (FEP)	30
4.	Fluoroquinolones.	Ciprofloxacin (CIP)	5
5.		Enoxacin (EN)	10
6.	Glycopeptides	Vancomycin (VA)	5
7.	Aminoglycosides	Gentamicin (CN)	10
8.		Amikacin (AK)	30
9.		Kanamycin (K)	30
10.	Penicillin	Piperacillin (PRL)	100
11.		Ampicillin (AMP)	25
12.		Amoxycillin/ Clavulanic Acid 2:1 (AMC)	30
13.	Carbapenem	Meropenem (MEM)	10
14.		Imipenem (IPM)	10
15.	Amphenicol	Chloramphenicol (C)	30

3.6 Molecular Identification

3.6.1 DNA Extraction

The genomic DNA of the isolate U1 was extracted by the GeneJet Genomic DNA Purification Kit (Thermo Fischer Scientific) using the self-optimized manufacturer's protocol. The pellet from the fresh broth culture was suspended in 180 µL of Digestion Solution followed by addition of 20 µL of Proteinase K Solution. The sample was incubated at 56 °C using a shaking water bath for about 30 minutes. Then 20 µL of RNase A Solution was added and incubated at room temperature followed by the addition of 200 µL of lysis Solution to the sample and about 400 µL of 50 % ethanol. The prepared lysate was transferred to a GeneJET Genomic DNA Purification Column inserted in a collection tube. The column was centrifuged for 1 min at 6,000 x g. The purification column was placed into a new 2 mL collection tube followed by the addition of 500 µL of Wash Buffer I. Then it was centrifuged for 1 minute at 8,000 x g and 500 µL of Wash Buffer II was added to the purification column and centrifuged for 3 minutes at maximum speed. The purification column was transferred to a sterile 1.5 mL micro centrifuge tube and about 100 µL of elution buffer was added to the center of the purification column membrane to elute genomic DNA. It was incubated for 2 minutes at room temperature and was centrifuge for 1 minute at 8,000 x g. The purification column was discarded and the purified DNA was stored at -20 °C.

3.6.2 DNA Quantification and Integrity

Extracted DNA of the isolate U1 was quantified using Qubit 2.0 fluorometer. For the quality and integrity analysis of extracted DNA, Agarose Gel Electrophoresis was performed using 1% agarose gel, with 1kb ladder.

3.7 Whole-Genome Sequencing, Assembly and Annotation

Whole genome of U1 isolate was sequenced using Illumina Hi seq 2500 platform. The genomic libraries were prepared via Nextera XT Library Prep Kit at MicrobesNG, Institute of Microbiology and Infection, University of Birmingham (Edgbaston, Birmingham). The quality of sequenced genomes was assessed using FASTQC (Andrews, 2010) and the raw reads were trimmed using Trimmomatic v0.30 tool followed by genome assembly using QIAGEN CLC Genomics Workbench V.20.0.4. The assembled genome was annonated using RAST annotation tool (Aziz et al., 2008) and cross checked by Prokka (Seemann, 2014).

3.8 *Insilico* Pathogen Identification

The generated contigs were used to confirm the *Escherichia coli* isolate using the KmerFinder 3.2 at Center of Genomic Epidemiology (Larsen et al., 2014).

3.9 Reference Based Assembly

For reference based assembly *E. coli* strain CFT073 reference genome (GenBank assembly accession GCA_000007445.1) was retrieved from NCBI database. The sequence files were aligned using QIAGEN CLC Genomics Workbench V.20.0.4 alignment tool. The newly generated multi-fasta .FNA file of uropathogenic *Escherichia coli* U1 contigs were in specified order as that of the reference *E.coli* CFT073 genome.

3.10 Submission of Draft Genome to NCBI

After assembly and alignment the draft genome sequence of the uropathogenic *Escherichia coli* isolate U1 was submitted to NCBI database. The raw reads of the

E.coli isolate U1 were submitted to the Sequence Read Archive (SRA).

3.11 Sequence Retrieval

A total of 701 *Escherichia coli* strains data was available on NCBI database that were screened for UPEC strains based on the host disease. The sequences of the respective uropathogenic *E.coli* whole genomes were retrieved from the NCBI database for comparative genome analysis and pangenome analysis in December 2021.

3.12 Pangenome Analysis

All UPEC genomes retrieved from the NCBI database were subjected to in-house pipeline PanRV to estimate the pan and core genome (Naz et al., 2019). PanRV consists of four modules. The first module; the pangenome estimation module (PGM) was used for pangenome analysis. PGM calculates pan, core, shell and cloud genes amongst UPEC genomes by making use of integrated rapid large-scale prokaryotic pangenome analysis pipeline, Roary (Page et al., 2015). PGM takes gff file as input therefore all the genome fasta files were annotated in gff format using Prokka. For the analysis, default parameters were used with 90% sequence identity threshold.

3.13 Multi-Locus Sequence Typing (MLST) of UPEC strains

Multilocus sequence typing (MLST) characterize the bacterial isolates using internal fragments of seven house-keeping genes sequences. The different sequences of each gene present within a bacterial species are assigned as distinct alleles and for each isolate the alleles at each of the seven loci define sequence type (ST). Multilocus sequence typing (MLST) profile of all complete genomes and UPEC strain U1 was obtained using multi locus sequence typing (MLST) tool version 2.0 at CGE using

default parameters (Larsen et al., 2012). For *Escherichia coli* scheme 1 of the MLST tool was used.

3.14 Serotyping of UPEC Strains

A combination of the two immunogenic structures is used for O:H serotyping including lipopolysaccharide and flagellar antigen. This prediction is based on O-antigen processing genes wzx, wzy, wzm, and wzt and the flagellin genes fliC, flkA, fllA, flmA, and flnA. For serotype prediction of all the UPEC genomes under study SerotypeFinder, a Web tool at CGE was used (Joensen et al., 2015).

3.15 Identification and Comparative Analysis of Antibiotic Resistance Genes

To know about the genetic basis of resistance and epidemiology of the resistance genes in the *Escherichia coli* isolate U1, the resistance genes in the genome were identified using Resistance gene identifier (RGI) tool at Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013) and ResFinder at CGE (Zankari et al., 2012). The selection criteria for CARD database was perfect and strict and 95% identity nudge was used. ResFinder threshold for %ID was set at 90% with 60% minimum length was used for the identification of the acquired antimicrobial resistance genes. The predicted acquired antimicrobial resistance genes in UPEC isolate U1 were compared with the UPEC complete genomes (n=71) retrieved from NCBI database to compare the resistance profiles of the strains and for the identification of novel resistance genes in local strain U1 if any.

3.16 Identification and Comparative Analysis of Virulence Genes

A pathogen’s ability to infect or damage its host tissues is determined by the

virulence factors it carries. Identification of these factors help in understanding how a pathogen invade the host cell, evade the immune system and establish an infection. The virulence factors of UPEC isolate U1 was identified using Virulence factor database (VFDB) (Chen et al., 2005) and VirulenceFinder (Joensen et al., 2014) 2.0 at CGE. The predicted virulence factors of uropathogenic *Escherichia coli* isolate U1 were then compared with the UPEC strains (n=71) retrieved from the NCBI database to identify any novel virulence factors in UPEC isolates.

3.17 Identification and Comparative Analysis of Plasmids

Plasmids are self-replicating extrachromosomal DNA segments that may contain genes encoding for the antimicrobial resistance and virulence factors. To identify the plasmid in the uropathogenic *Escherichia coli* isolate U1, Plasmidfinder v.2 (Carattoli et al., 2014) at CGE was utilized. The plasmids identified in the UPEC isolate U1 were also screened for the presence of virulence and antibiotic resistance genes. The identified plasmids were then compared with other global UPEC strains.

3.18 Identification and Comparative Analysis of Prophages

Prophages may also disseminate the resistance and virulence genes among bacteria. They can integrate and become part of the bacterial host genome. For the identification of prophages sequences in the UPEC isolate U1 PHASTER (Arndt et al., 2016) online tool available at https://phaster.ca/ was used. The identified prophage sequences were retrieved from the UPEC isolate U1 genome and further analyzed for the presence of antibiotic resistance and virulence genes using Resistance gene identifier (RGI) tool at CARD server (McArthur et al., 2013), ResFinder at CGE server (Zankari et al., 2012) and Virulence factor database (VFDB) (Chen et al., 2005). Then these sequences were compared with other UPEC strains retrieved from the NCBI

database.

3.19 Identification and Comparative Analysis of Insertion Sequences

Mobilome of bacteria also consist of transposable elements that may transfer among different locations in a bacterial genome and between bacterial genomes. The simplest version of the transposon is the insertion sequence that can mediate genome diversification. To identify these sequences MobileElementFinder (Johansson et al., 2020) was used with default quality parameters.

3.20 SNP based phylogeny

To infer the SNP based phylogenetic relationship of UPEC isolate U1 with UPEC strains reported from diverse geographical locations CSI phylogeny 1.4 (Kaas et al., 2014) at CGE was used with default parameters. CSI Phylogeny calls SNPs against the reference genome *Escherichia coli* CFT073, filters the SNPs, does site validation and infers a phylogeny based on the concatenated alignment of the high quality SNPs. Circularized SNP tree of 72 (test strain n = 1, and global strains n =71) UPEC genomes is visualized by using interactive tree of life (iTOL) v.2 (Letunic and Bork, 2021).

CHAPTER 4

Results

4.1 Characterization of Local Isolate U1

The isolate U1 was able to grow on MacConkey Agar plates and visible colonies of the isolate U1 were observed after 24 hours of incubation at 37 ˚C. The isolated colonies were further analyzed for morphological and biochemical characteristics. Preliminary identification revealed pink, flat, dry and rough U1 colonies. The colonies were Gram–negative. The biochemical test showed that the colonies were catalase positive. Lactose positive colonies were seen on MacConkey Agar after 24 H incubation at 37°C. Hence based on morphological and biochemical characterization the isolate U1 was identified as an uropathogenic *Escherichia coli*.

4.2 Phenotypic Resistance Profile

The antibiotic resistance assay provide the phenotypic resistance profile of the UPEC isolate U1. The isolate U1 showed resistance to 11 antibiotics whereas it was susceptible to 4 antibiotics. The UPEC isolate U1 was resistant against Sulphamethoxazole/ Trimethoprim (25µg), Cefepime (30 µg), Ciprofloxacin (5µg), Vancomycin (5µg), Enoxacin (10µg), Piperacillin (100µg), Kanamycin (30µg), Amoxycillin/ Clavulanic Acid 2:1 (30µg), Meropenem (10µg), Ampicillin (25µg), Ceftriaxone (30µg). Whereas the isolate was susceptible to Amikacin (30µg), Imipenem (10µg), Chloramphenicol (30µg). The zone of diameter is given in table 2 and 3.

Table 6 Antibiotics against the isolate U1 was resistant

No.	Class of Antibiotics	Antibiotics	Concentration (µg)	Zone Diameter (mm)
1.	Sulfonamides	Sulphamethoxazole/ Trimethoprim (SXT)	25	0
2.	Cephalosporin	Cefepime (FEP)	30	0
3.		Ceftriaxone (CRO)	30	0
4.	Fluoroquinolones	Ciprofloxacin (CIP)	5	0
5.		Enoxacin (EN)	10	0
6.	Glycopeptides	Vancomycin (VA)	5	0
7.	Penicillin	Piperacillin (PRL)	100	0
8.		Ampicillin (AMP)	25	0
9.		Amoxycillin/ Clavulanic Acid 2:1 (AMC)	30	0
10	Aminoglycosides	Kanamycin (K)	30	8
11	Carbapenem	Meropenem (MEM)	10	9

Table 7 Antibiotics against the Isolate U1 was susceptible

No.	Class of Antibiotics	Antibiotics	Zone Diameter (mm)
1	Aminoglycosides	Gentamicin CN (10µg)	16mm
2		Amikacin AK (30µg)	20mm
3	Carbapenem	Imipenem IPM (10µg)	33mm
4	Amphenicol	Chloramphenicol C (30µg)	28mm

4.3 DNA Extraction and Quantification

The genomic DNA of the isolate U1 was extracted by the GeneJet Genomic DNA Purification Kit (Thermo Fischer Scientific) and extracted DNA of the isolate U1 was quantified to be 19.7ng/µl.

4.4 Whole Genome Sequencing, Assembly and Annotation

The illumina sequencing platform 2500 yielded 1112641 paired end reads. The generated pair end reads were assembled into 118 contigs. The sequence size was 5,272,959 bp. The N50 value was 151471 bp and L50 value was 13 and the GC content was 50.7% (Table 8). The genome sequence of isolate U1 was then aligned with uropathogenic *E. coli* CFT073 (5.2Mb) reference genome (GenBank assembly accession GCA_000007445.1) via QIAGEN CLC Genomics Workbench V.20.0.4. Reference based genome assembly identifies several insertions and deletions within the U1 genome at various positions. Genome annotation yielded a total of 5376 coding sequences and 79 RNA encoding genes (Table 8). *Insilico* pathogen identification also confirmed the strain U1 as uropathogenic *Escherichia coli*.

4.5 Sequence Submission to NCBI

After assembly and alignment the draft genome sequence of the uropathogenic *Escherichia coli* isolate U1 was submitted to NCBI database available under accession number JAJAWD000000000. The raw reads of the *E.coli* isolate U1 were submitted to the Sequence Read Archive (SRA). Sequence read archive accession was assigned to the raw reads (SRX12734653).

Table 8 Genomic characteristics of UPEC isolate U1

Genomic Characteristics	
Size	5,272,959 bp
Number of Contigs	118
GC content	50.7%.
N50	151471 bp
L50	13
Number of coding sequences	5376
Longest contig size	314483 bp
Smallest contig size	507 bp.
Mean sequence size	44686.1 bp
Number of subsystems	389
tRNA	71
rRNA	7
tmRNA	1

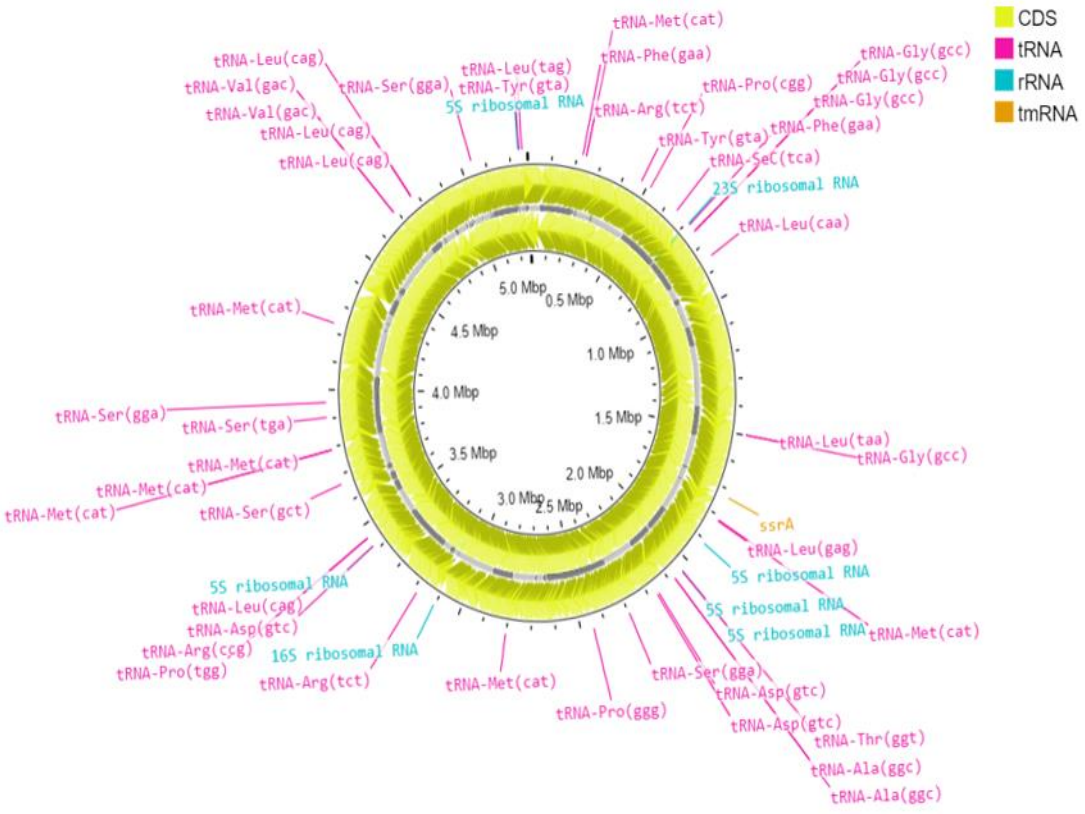

Figure 6 Circularized view of UPEC U1 genome

4.6 UPEC Sequence Retrieval

A total of 71 complete genome sequences of *Escherichia coli* causing urinary tract infections were retrieved out of total 701 complete uropathogenic *Escherichia coli* genomes reported from around the world from NCBI database on 14th of November 2021. The *Escherichia coli* strains included in the study were all reported of causing urinary tract infections in human host. Similarly, 71 proteomes of completely sequenced UPEC genomes were retrieved from NCBI database on 15th of November 2021.

4.7 Multi Locus Sequence Typing of UPEC Strains

The MLST analysis of all the UPEC strains (n=72) were conducted using the scheme that comprises of seven housekeeping genes including *adk, fumC, gyrB, icd, mdh, purA, recA*. MLST analysis revealed 37 different sequence types among 72 strains that shows high genetic diversity between UPEC genomes. The most frequent sequence type was ST131 (n=14) and UPEC isolate U1 also belong to sequence type 131. Among other STs shared by UPEC isolates, the most frequently encountered were ST127 (6 isolates), ST95 (6 isolates), ST1193 (4 isolates), and ST73 (4 isolates) (Table 9).

Table 9 Multi Locus Sequence Type of the studied UPEC Strains

Housekeeping genes	**Sequence types (STs)**	**No. of isolates**
adk, fumC, gyrB, icd, mdh, purA, recA	ST131	15
	ST127	6
	ST95	6
	ST73, ST1193, ST648	4, 4, 3 respectively
	ST219, ST69, ST101, ST167, ST410	2, 2, 2, 2, 2 respectively
	ST10522, ST10, ST537, ST617, ST2487, ST393, ST429, ST2279, ST1485 ST117, ST448, ST602, ST538, ST58, ST1434, ST394, ST453, ST10754, ST48, ST773, ST3902, ST216, ST46, ST2840	1 isolate from each sequence type

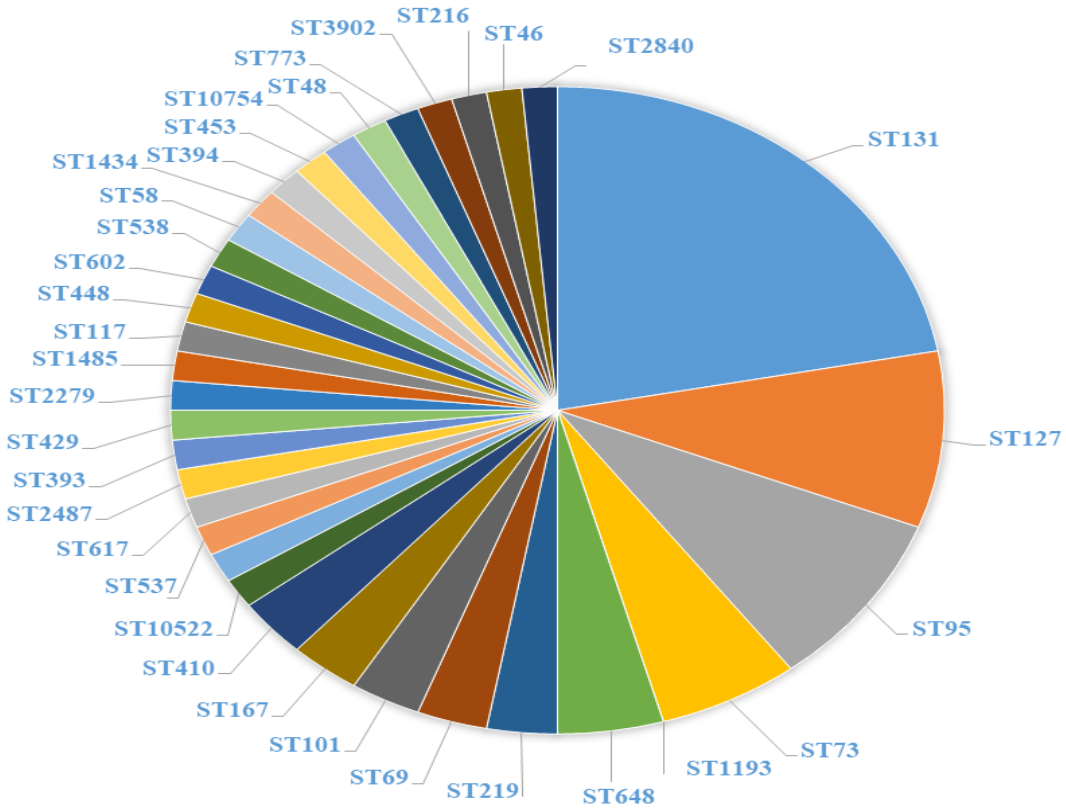

Figure 7 Pie chart representing sequence type diversity among studied UPEC strains

4.8 Serotyping of UPEC Strains

22 distinct O-serogroups were identified and the most frequently occurring were O25. Six O-nontypeable UPEC strains were also identified that fall into H31, H28, H8, H9, H21, H11 group. There were several O serotypes which were similar to each other on both wzx and wzy variants including O13/O129 & O13/O129/0135 (where H type is H4), O17/O77 & O17/O44 (H type is H18) and O2/O50 (H type is H2) respectively. 23 different H types were identified among the studied UPEC strains and the most frequently occurring H type was H4. Considering the O:H serotypes, 30 different O:H serogroups were identified among 72 UPEC genomes and O25:H4 was the most frequent and detected in (15/72) of the UPEC isolates. All UPEC strains that have sequence type 131 have O25:H4 serotype. Hence an extensive diversity of O:H serotypes is present among the UPEC genomes. The other serotypes that were identified among the strains are mentioned in Table 10.

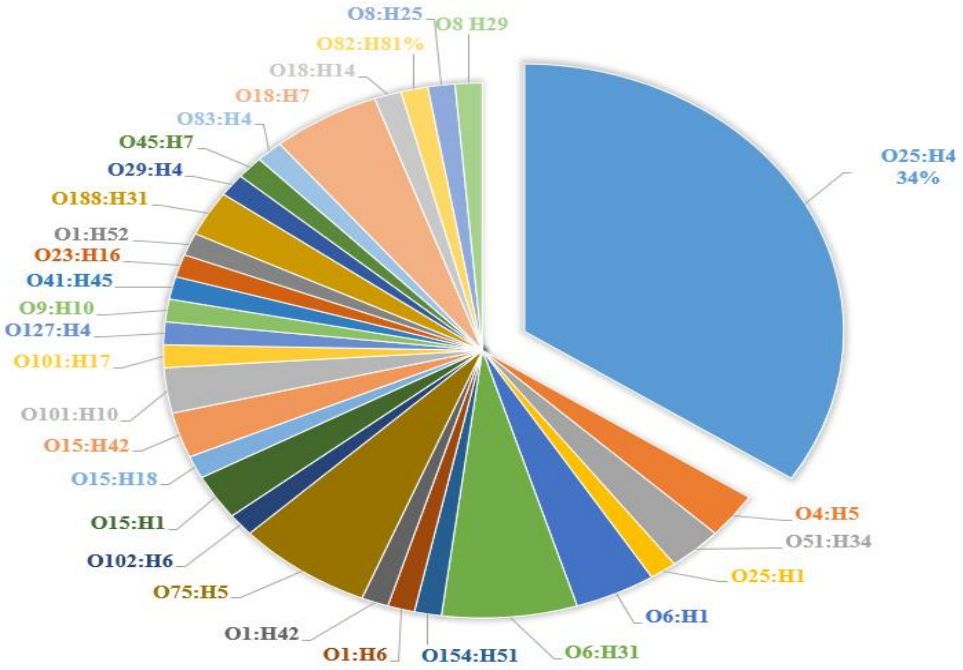

Figure 8 Pie chart representing serogroups diversity among the studied UPEC strains

Table 10 Serogroups identified among 72 UPEC strains

O Serogroup	H Type	No. of isolates	O Serogroup	H Type	No of isolates
O4	H5	2	O127	H4	1
O51	H34	2	O9	H10	1
O25	H4	15	O41	H45	1
	H1	1	O23	H16	1
O6	H1	3	O11	H52	1
	H31	5	O188	H31	2
O154	H51	1	O29	H4	1
O1	H6	1	O45	H7	1
	H42	1	O83	H4	1
O75	H5	5	O18	H7	4
O102	H6	1		H14	1
O15	H1	2	O82	H8	1
	H18	1			
	H42	2			
O101	H10	2	O8	H25	1
	H17	1		H9	1

4.9 Pangenome of Uropathogenic *Escherichia coli*

PanRV estimated a total of 17408 genes in 72 uropathogenic *Escherichia coli* genomes, out of which 2559 were core genes that were conserved among 90-100% strains, 661 soft core genes present among 89-90% strains, 2835 shell or accessory genes present among 15-89% of the strains and 11,353 cloud or unique genes (Fig. 9). Figure 10 shows a phylogenetic tree based on the presence and absence of core and accessory genes among UPEC genomes. Our analysis also revealed that UPEC has an open pangenome; there is an increase in number of unique genes (Figure 11) and total genes (Figure12) with an increase in the number of total genomes.

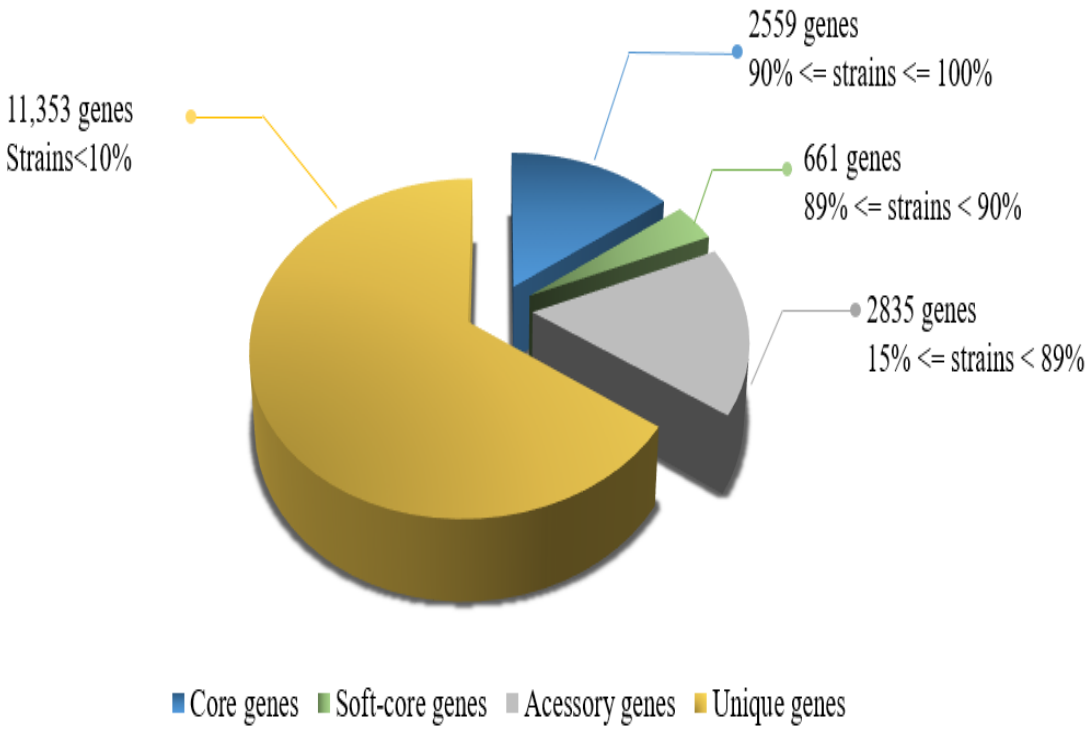

Figure 9 Pie chart of the breakdown of genes and the number of UPEC isolate they are present in.

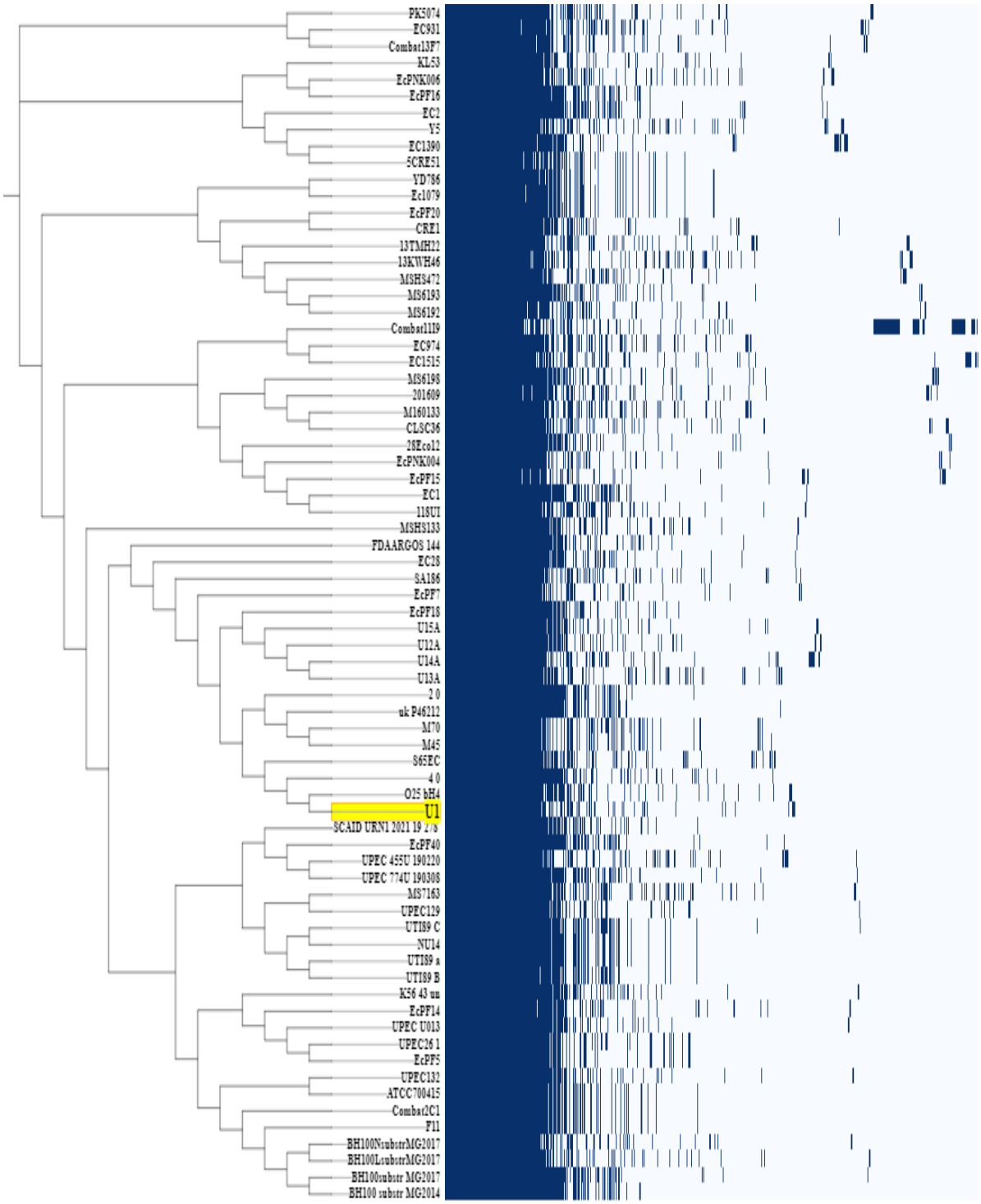

Figure 10 Tree compared to a matrix with the presence and absence of core and accessory genes among UPEC genomes

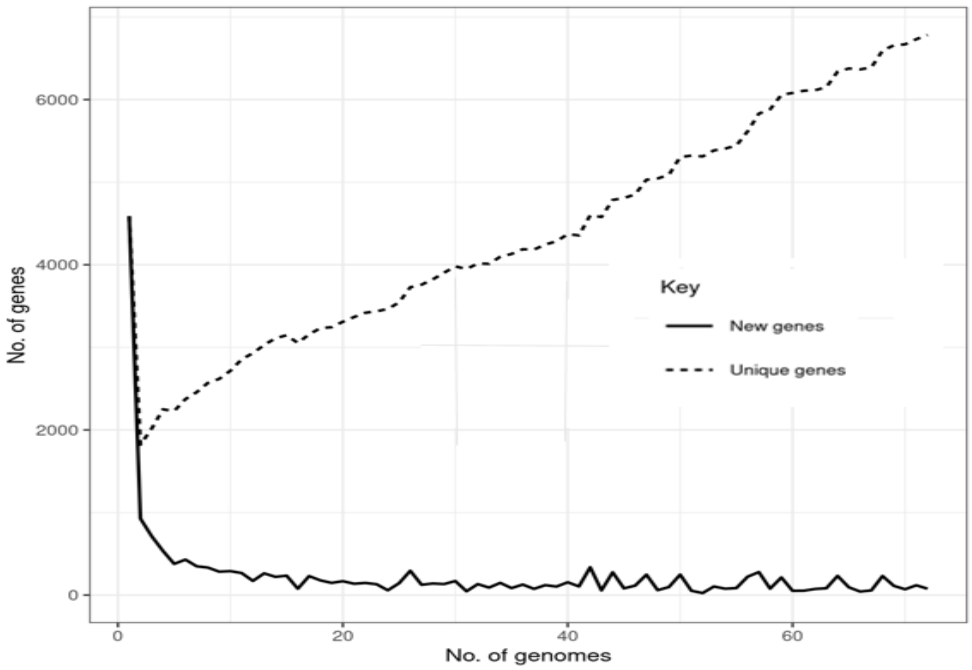

Figure 11 Graph showing an increase in the number of unique genes with the increase in the number of UPEC genomes and then consistency in number of new genes with the increase in number of genomes.

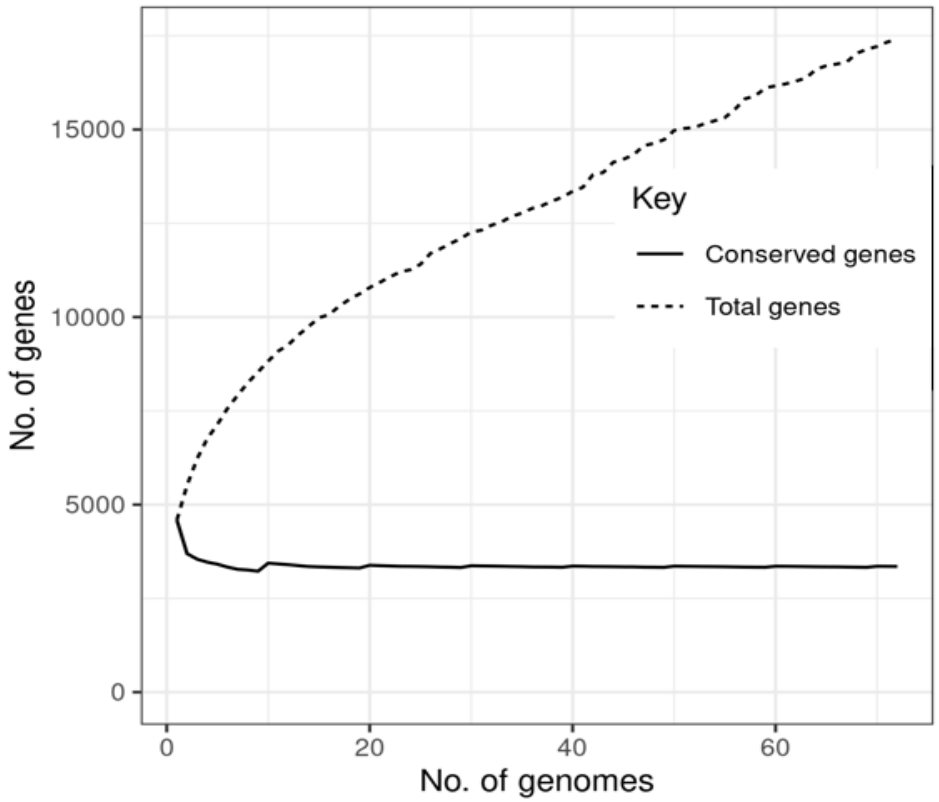

Figure 12 Graph showing an increase in the number of total genes with the increase in the number of genomes while after some point the number of conserved genes become consistent

4.10 Identification and Comparative Analysis of Plasmids

PlasmidFinder identify 4 plasmid in the local UPEC isolate U1 designated as pU1_1, pU1_2, pU1_3 and pU1_4 (Table 11). Plasmids were further analyzed for the presence of antibiotic resistance genes and virulence genes. pU1_4 was harboring gene TEM-1 from TEM beta-lactamase family providing resistance against cefazolin, amoxicillin, piperacillin, ampicillin and cefalotin. No virulence gene was identified in any of the plasmids identified in the isolate U1. Comparative analysis revealed that plasmids identified in UPEC isolate U1 are common between UPEC strains (with 100% and 99% sequence similarity) whereas the most frequent replicons among the UPEC strains were FIA, FIB, FII, and FIC which are associated with the IncF group.

Table 11 Plasmids identified in UPEC strain U1

Plasmids	Plasmid replicon	Length	GC content %
pU1_1	IncFIB (AP001918)	20115bp	46.3%
pU1_2	Col156	8900bp	46%
pU1_3	Col(BS512)	2101bp	47.2%
pU1_4	IncFII	7555bp	47%

4.11 SNP Based Phylogenetic Analysis

Single nucleotide polymorphism based phylogenetic analysis provided an evolutionary relationship between different UPEC genomes. The SNP based tree with the reference genome of the uropathogenic *Escherichia coli* strain CFT073 divided the strains into 12 clades. The UPEC isolate U1 was grouped in a clade with strain UPEC 4_0 reported from Sweden. The local isolate was also found to be related to the strains with similar MLST profile (ST-131) and serotype (O25:H4) emerging from same common ancestral strain. These results were in concordant with the phylogenetic tree based on accessory genome that grouped UPEC isolate U1 in a clade closely to strains with similar MLST and serotype profiles. Circularized SNP tree of 72 UPEC genomes is given in Figure 13.

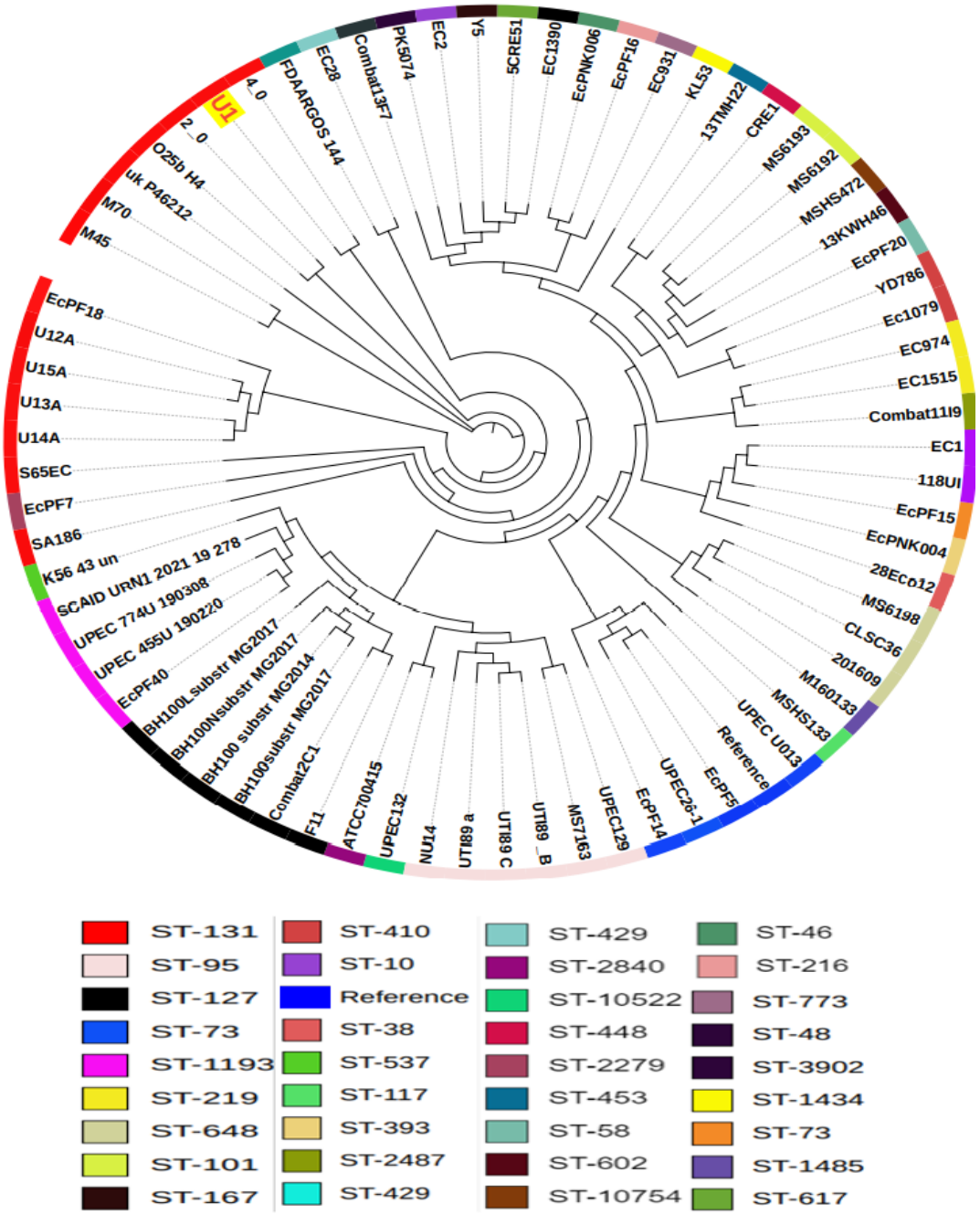

Figure 13 Circularized SNP tree of 72 (test strains n = 1, and global strains n = 71) UPEC genomes. Color keys indicating ST are provided alongside the circularized tree.

4.12 Identification and Comparative Analysis of Insertion sequences

A total of 15 insertion sequences (IS) had been identified in UPEC strain U1. These include IS640, ISEc52, ISEc1, ISEc38, IS26, IS30, ISEc10, IS629, IS682, ISKpn37 and ISEc53 belonging to 8 different IS families including IS21, IS3, ISAs1, ISL3, IS66, IS6, IS30 and IS1380. Out of all the mobile genetic elements predicted in isolate U1 the majority were insertion sequences. The comparative analysis of the insertion sequences revealed that the majority of the insertion sequences identified in UPEC strains from ST131 belong to IS3 family. The number of insertion sequences per isolate varied between UPEC strains. The insertion rate of insertion sequences vary between different UPEC genomes but predicted insertion sequences suggest high level of transposition within the UPEC genomes. Various insertion sequences identified in UPEC strains from ST131 is given in Table 12.

Table 12 Insertion sequences identified among UPEC strains from ST131

UPEC Strains	IS21	IS3	ISL3	ISAs1	IS66	IS4
4_0	ISEc10	ISSfl10 ISKpn37 IS629	ISEc53 ISEc38 ISEc52	ISEc1	IS682	-
M70	ISEc10	ISEc52 ISSfl10 IS629	ISEc53 ISEc38	ISEc1	IS682	-
U1	**IS640 ISEc10**	**ISEc52 IS629 ISKpn37**	**ISEc38 ISEc53**	**ISEc1**	**IS682**	-
SA186	IS100	ISSen4 IS629	ISEc38	ISEc1	IS682	IS421
U12A	IS100	ISKpn8 ISEc38 IS3 ISEc52 IS629	ISPst2 ISEc53	ISEc1	IS682	-
M45	ISEc10 IS640	ISEc52 ISSfl10	ISEc53 ISEc38 IS629	ISEc1	IS682	-
uk_P46212	ISEc10 IS640	ISEc52 ISSfl10 IS629	ISEc53 ISEc38	ISEc1	IS682	-
EcPF18	IS100	ISKpn8 ISSfl10 IS629	ISEc53 ISEc38 ISEc52	ISEc1	IS682	-
S65EC	ISEc10	ISEc52 ISSfl10 ISKpn37 IS629	ISEc53 ISEc38	ISEc1	IS682	IS4
U13A	IS100	ISKpn8 IS3 ISEc52 ISSfl10 IS629	ISEc53 ISEc38	ISEc1	IS682	-
2_0	ISEc10 IS640	ISKpn8 ISEc52 ISSen4 ISSfl10 IS629	ISEc53 ISEc38	ISEc1	IS6100 ISKpn24 IS682	IS4

4.13 Identification and Comparative Analysis of Prophage Sequences

PHASTER identified 13 prophage regions, out of which 4 regions were intact, 8 regions were incomplete and 1 region was questionable. The genome size of the four complete prophages was 43.4kb, 23.3kb, 24.4kb and 30.2kb (Fig. 14). Characteristics of complete prophages present in UPEC isolate U1 is given in Table 13. The prophages were further screened for the presence of antibiotic resistance genes and virulence genes.

No resistance gene has been carried by prophages identified in isolate U1 however an acquired virulence factor *iss* has been identified on the prophage region 5 encoding for protein involved in increase serum survival. Identified complete prophages were not unique to UPEC strain U1 and were also present in other UPEC strains reported from other geographical regions.

Table 13 Characteristics of complete prophages present in UPEC strain U1

Region	Region Length	Total Proteins	Phage Hit Proteins	GC %	Specific Keywords	Most Common Phage
3	43.4kb	60	42	48.07 %	Tail, plate, head, capsid, terminase, integrase	PHAGE_Pectob_ZF40_NC_019522
4	23.3kb	23	21	54.36 %	Tail, protease, portal, terminase, lysis	PHAGE_Entero_mEp460_NC_019716
5	24.4kb	29	26	51.65 %	Tail, transposase, head, capsid, portal, terminase, lysin	PHAGE_Entero_cdtI_NC_009514
12	30.2kb	41	36	51.74 %	Tail, plate, lysin, head, terminase, capsid, portal, recombinase, integrase	PHAGE_Entero_P88_NC_026014

Figure 14 Detailed linear genome display of 4 complete prophages in UPEC isolate U1

4.14 Identification and Comparative Analysis of Antibiotic Resistance Genes

Several antibiotic resistance genes were predicted by ResFinder and CARD in UPEC isolate U1 against multiple classes of antibiotics including fluoroquinolone antibiotic (*evgA, gadW, emrR, mdtH, emrA, AAC(6')-Ib-cr6, acrB, AcrS*), cephalosporin (*H-NS, TEM-1, CTX-M-15, OXA-1, marA*), phosphonic acid antibiotic (*mdtG, E.coli UhpT, GlpT, PtsI* with mutation conferring resistance to fosfomycin), aminoglycoside antibiotic (*cpxA, AAC(6')-Ib-cr6, baeR, APH(3'')-Ib, TolC*), tetracycline antibiotic (*acrB, AcrS, emrY, tet(A), Escherichia coli AcrAB-TolC* with *AcrR* mutation), phosphoethanolamine transferase, cephalosporin, carbapenem, glycopeptide antibiotic, macrolide antibiotic (*gadW, mphA, CRP, gadX*). Several antimicrobial gene families encoding for MFS, RND and SMR antibiotic efflux pump are also identified providing resistance against macrolide, fluoroquinolone, cephalosporin, cephamycin, penam and tetracycline antibiotics (Fig. 15 & 16). Majority of these resistance determinants are harbored on genomic islands within U1 genome. Resistance mechanism adopted by UPEC against antibiotics includes antibiotic efflux, antibiotic target replacement, antibiotic inactivation and reduced permeability to antibiotics. No novel resistance gene has been identified in the local strain U1. Some of the resistance genes common between UPEC strains are given in Table 14.

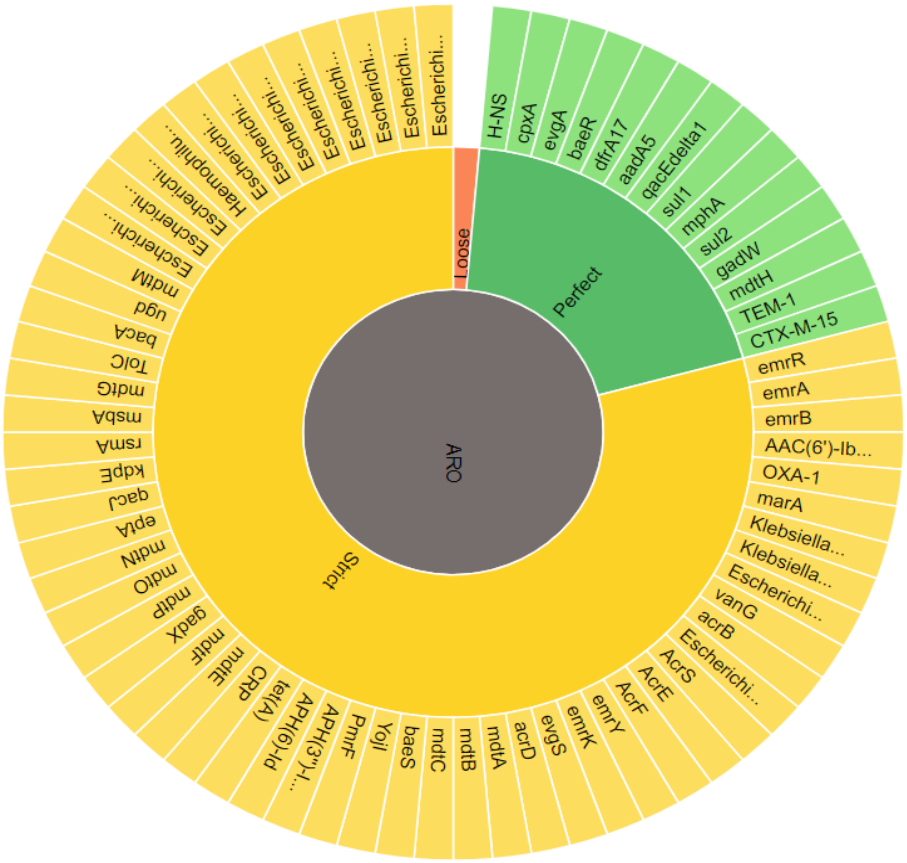

Figure 15 Resistance genes identified in UPEC isolate U1

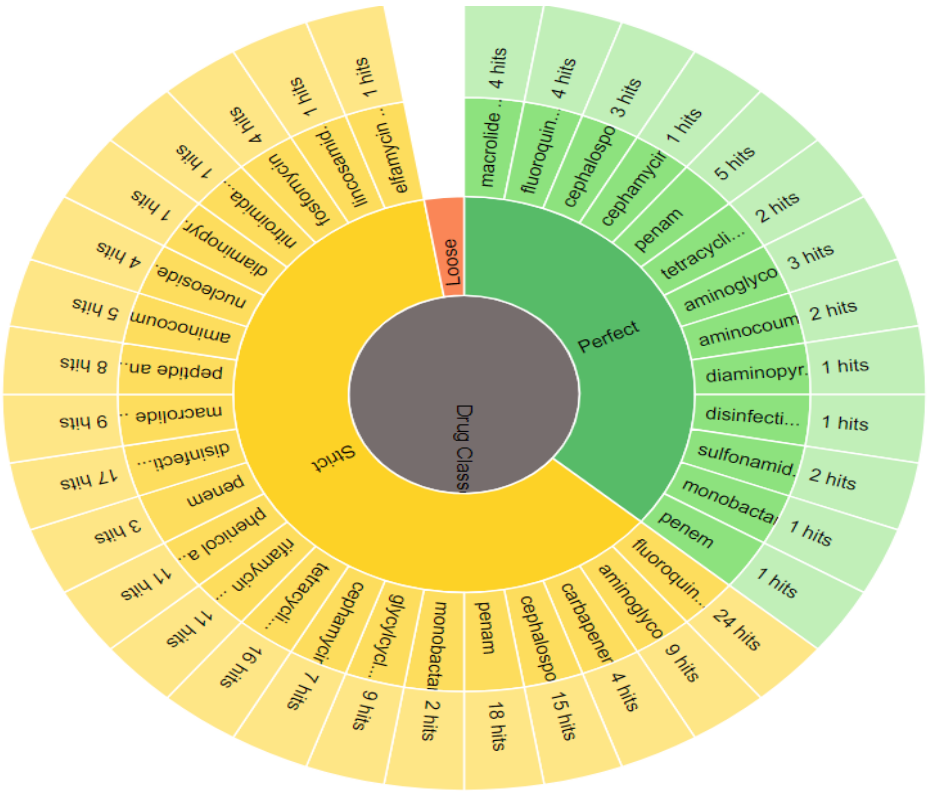

Figure 16 Drug classes against which UPEC strain U1 shows resistance

Table 14 Resistance genes common between UPEC strains

ARO Term	AMR Gene Family	Drug Class	Resistance Mechanism
kdpE	*kdpDE*	Aminoglycoside antibiotic	Antibiotic efflux
msbA	ABC antibiotic efflux pump	Nitroimidazole antibiotic	Antibiotic efflux
ugd	phosphoethanolamine transferase	Peptide antibiotic	Antibiotic target alteration
pmrF	phosphoethanolamine transferase	Peptide antibiotic	Antibiotic target alteration
E.coli emrE	*SMR antibiotic efflux pump	Macrolide antibiotic	Antibiotic efflux
sul2	sulfonamide resistant *sul*	Sulfonamide antibiotic	Antibiotic target replacement
TEM-1	*TEM* beta-lactamase	Monobactam, cephalosporin, penam, penem	Antibiotic inactivation
E. coli soxR with mutation	*ABC, *MFS and *RND antibiotic efflux pump	Fluoroquinolone cephalosporin, glycylcycline, tetracycline antibiotic	Antibiotic target alteration, antibiotic efflux
APH(3'')-Ib	*APH(6)*	Aminoglycoside antibiotic	Antibiotic inactivation
E.coli UhpT with mutation	antibiotic-resistant *UhpT*	Fosfomycin	Antibiotic target alteration
dfrA17	trimethoprim resistant dihydrofolate reductase *dfr*	Diaminopyrimidine antibiotic	Antibiotic target replacement

*ABC; ATP-binding cassette antibiotic efflux pump

*SMR; Small multidrug resistance antibiotic efflux pump

*MFS; Major facilitator superfamily antibiotic efflux pump

*RND; Resistance-nodulation-cell division antibiotic efflux pump

4.15 Identification and Comparative Analysis of Virulence genes

Both structural and secreted virulence factors are predicted in UPEC strain U1 including genes encoding for UPEC pilus, fimbriae, iron acquisition systems, outer membrane proteins, autotransporter proteins and toxins (Figure 17). Acquired virulence gene *iss* encoding for protein providing increase serum survival has also been identified. Strain U1 virulence profile was further compared with other ST131 strains. All strains from ST131 harbors similar virulence factors except *galE* gene (encoding for exopolysaccaride) that was missing from majority of the strains. Whereas strains from other sequence type were also encoding for additional virulence factors including afimrial adhesin (*afa*), S fimbriae (*sfa*), curli (*csg*) and salmochelin siderophore (*iroN*) that were absent in most of ST131 strains.

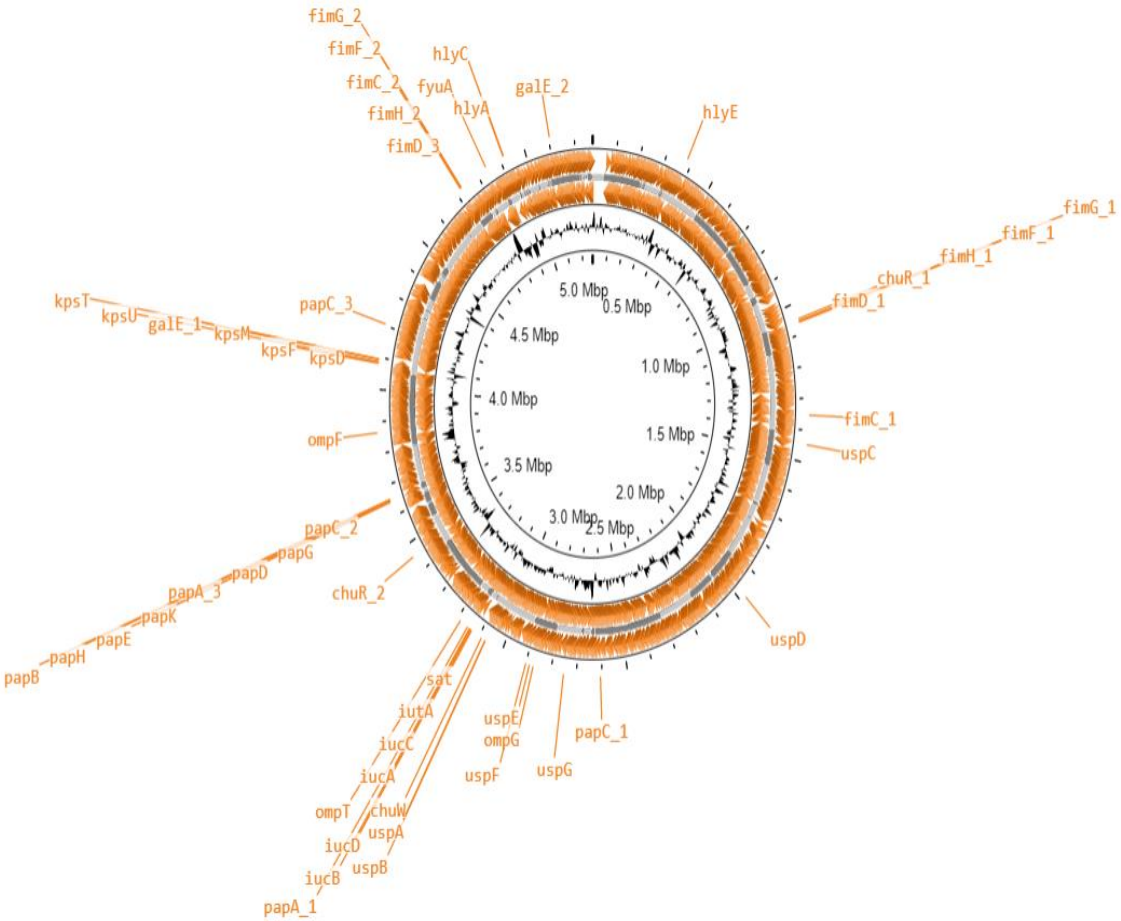

Figure 17 Circularized view of virulence genes identified in UPEC strain U1

CHAPTER 5

Discussion

Urinary tract infections ranked as second most frequently occurring bacterial infections in humans (Klumpp et al., 2006) predominantly caused be uropathogenic *Escherichia coli* (UPEC). Although UPEC strains can cause disease in the urinary tract, they can also exist within the human intestinal tract as part of the normal microbiota. The severity of urinary tract infections range from mild to severe sepsis, with 20 - 40% of mortality rate (Zhu et al., 2021). UTIs are categorized as community-acquired and nosocomial infections (Behzadi, 2018). These infections can be symptomatic or asymptomatic, complicated or uncomplicated, and recurrent UTIs also occur frequently among females despite the antibiotic therapy and most of the time these infections are caused by the same causative agent that causes the initial infection (Cusumano et al., 2010).

The recurrence rates of urinary tract infections are increasing and so does the antimicrobial resistance among the UPEC strains. This leads to an increase in the economic burden of these infections throughout the world (Patton et al., 1991). UPEC strains harbors extragenetic material (on PAIs) coding for genes products contributing to UPEC pathogenesis that makes them different from the commensal strains of the *E.coli* (Mobley et al., 2009). The transferable plasmid is responsible for the wide spread of the virulent MDR genes of *Escherichia coli* serotype O25:H4. The emergence of these strains carrying ESBL genes has been well documented worldwide (Peirano and Pitout, 2010, Alghoribi et al., 2015). Detailed work has been carried out on different UPEC lineages around the world but not much known about UPEC lineages in Pakistan including their genetic attributes, their resistance and virulence profiles, the genetic diversity of UPEC strains in Pakistan and the recent antimicrobial resistance trends.

Therefore in this study we have reported the WGS of a multidrug resistant uropathogenic *Escherichia coli* isolate U1 and conducted a detail comparative genome analysis with complete genome sequences of the global UPEC strains (n=71) with the purpose to get insightful information regarding the genomic divergence and correlate virulence factors and antibiotic resistance profiles. Our analysis identified local UPEC strain U1 as a virulent multidrug resistant strain. The strain U1 possess mobile genetic elements harboring the resistance gene as well as virulence gene responsible for disease severity and resistance against multiple drug classes.

Serotype analysis of the UPEC strains reveal that they belong to diverse serotypes similar to as reported by Rijavec et al., 2006. The local UPEC isolate U1 belong to ST131 which is currently the most prevalent extraintestinal E. coli lineage (Johnson et al., 2010). Our results were in concordant with the study conducted by Peirano and Pitout et al., whereby all of the ST131 UPEC strains were associated with serogroup O25:H4 among other serogroups identified for the UPEC strains. O25 serogroup was most prevalent among the studied strains. This serogroup has been reported to be highly prevalent among UTI strains (Dehkordi et al., 2020).

UPEC strains responsible for urinary tract infections fall under B2 and D phylogroups (kumar et al., 2017). Phylogenetic analysis of UPEC strains using distinct phylogenetic markers including pangenome, single nucleotide polymorphism (SNP) provided consistent branching patterns suggesting similar phylogeny for UPEC strains. The strain U1 is evolutionary related to UPEC strain 4_0 and O25:bH4 reported from Sweden and Saudi Arabia respectively which suggest a possible cross continent dissemination. These strains have similar virulence and resistance profile suggesting a common ancestral UPEC strain. Pangenome analysis estimated a total of 17408 genes and 11,353 unique genes in the UPEC pangenome depicting high genomic diversity

among the studied UPEC strains and the strains are evolving through acquisition of the genes and diversification. Our analysis also indicated that with the increase in the number of genomes there is an increase in the number of total genes while conserved genes become less and addition of new genomes will further increase its size suggesting an open UPEC pangenome as reported earlier by Rasko et al., 2008.

The prevalence of antibiotic resistance among UPEC strains has been significantly increasing globally and has become a serious problem for health care providers (kahlmeter et al., 2003). The local UPEC isolate U1 is identified as a multidrug resistant strain responsible for resistance against multiple antibiotic classes used commonly for the treatment of urinary tract infections in Pakistan. The identified genotypic resistance determinants support our observed phenotypic resistance profile of strain U1. Majority of these resistance determinants are harbored on genomic islands and plasmids that suggest the possible acquisition of resistance in the strain U1. Urinary tract infections caused by multidrug resistant strains is responsible for serious implication for the empiric therapy against microbial spectrum involved in causing UTIs and for the co-selection of pathogens with resistance properties (Mukherjee and Mukherjee, 2019). In concordant with the study conducted by Ali et al., most of the ST-131 strains were multi drug resistant. Resistance mechanisms identified in isolate U1 are similar to those reported for the global isolates. However, for determination of the population structure, spread of AMR clones, availability of more genomes sequences of UPEC is required.

Plasmid analysis showed UPEC isolate U1 possess 4 plasmids designated as pU1_1 (IncFIB (AP001918), pU1_2 (Col156), pU1_3 (Col (BS512) and pU1_4 (IncFII) with high sequence identity to previously reported pDA33135-139 (GenBank: CP029577.1), plasmid p1 (GenBank: CP054233.1), plasmid unnamed3 (GenBank:

CP077284.1) and pF16EC0211-1 (GenBank: CP088428.1) plasmids respectively. pU1_4 is identified as a resistance plasmid harboring a resistance gene TEM-1 from TEM beta-lactamase family providing resistance against cefazolin, amoxicillin, piperacillin, ampicillin and cefalotin. TEM-1 β-lactamase, a plasmid encoded enzyme is the most common beta lactamase among Enterobacteriaceae throughout the world. It is responsible for transferable resistance against ampicillin (Bush, 2010). Several factors are involved in resistance genes dissemination among causative pathogens whereby the plasmid mediated HGT of MDR is the most significant mechanism (Akingbade et al., 2014).

The strain U1 possessed a reservoir of virulence genes similar to strains from MLST group 131 and serogroup O25:H4 that must be responsible for the severity of the disease caused by the UPEC strains. Isolate U1 contains *fim* operon coding for Type I fimbriae and *pap* gene clusters encoding for P Fimbriae responsible for adhesion and invasion of UPEC to the urinary epithelium cells and initiation of the infection which is the significant step in the establishment of the infection (Connell et al., 1997). Another important virulence factor identified in isolate U1 is outer membrane protein T (*ompT). ompT* plays a multifaceted role in UPEC pathogenesis; it is involved in UPEC adhesion and invasion as well as IBC formation and unregulated pro - inflammatory cytokines (He et al., 2015). *iss* gene is also identified in strain U1. The role of the increased serum survival gene (*iss*) in virulence of the ExPEC strains has been recognized (Johnson et al., 2008).

Other predicted common virulence factors among global strain and local strain includes type 6 secretion system; a widely distributed secretion system in gram negative bacteria, responsible for the transport and delivery of effector toxins (Douzi et al., 2016), autotransporter proteins, iron acquisition systems, capsular polysaccharides,

exopolysaccharides and toxins. A wide range of insertion sequences has been identified among the studied UPEC genomes. Insertion rate of these insertion sequences vary between different UPEC genomes but predicted insertion sequences suggest high level of transposition within the UPEC genomes.

Urinary tract infections are commonly encountered infections in Pakistan predominantly among females of all age groups whereby antibiotic resistance is rapidly increasing and has become the most urgent threat to public health worldwide. The emergence of new resistant bacteria is on the rise, endangering the efficacy of existing antibiotics and the resistance patterns are spreading continuously. Uropathogenic *E.coli* is one of the most common multi drug-resistant infectious bacteria and are mostly resistant to beta-lactam antibiotics. Over a period of years, there has been a steady rise in number of UPEC isolates in Pakistani populations. Therefore, there is an urgent need to control this fast growing antimicrobial resistant pathogen through approaches alternative to antibiotics.

CHAPTER 6

Conclusion and Future Prospects

Our study concluded that uropathogenic *Escherichia coli* strain U1 is a virulent, multidrug resistant strain with ST131 and belong to serogroup 025:H4. Evolutionary strain U1 is closely related to strain 4_0 and strain O25:bH4 from Sweden and Saudi Arabia respectively. Comparative genome analysis revealed high genetic diversity among UPEC strains that are evolving through acquisition of the genes and diversification. All studied UPEC strains are multi-drug resistant hence there is a need for alternative antimicrobial approaches for appropriate UTI management. Appropriate surveillance studies are needed to determine UTI prevalence in our region, routes of infection & local antibiotic resistance trends. More whole genome sequences of this species should be made available in order to accurately estimate the size of pangenome, to study diversity and population structure that may help the healthcare professionals in controlling and preventing the further spread of UPEC and urinary tract infections. Also urinary tract infections must be deliberated as a social problem that effects an individual's quality of life and affects one's occupational and relational activities.

CHAPTER 7

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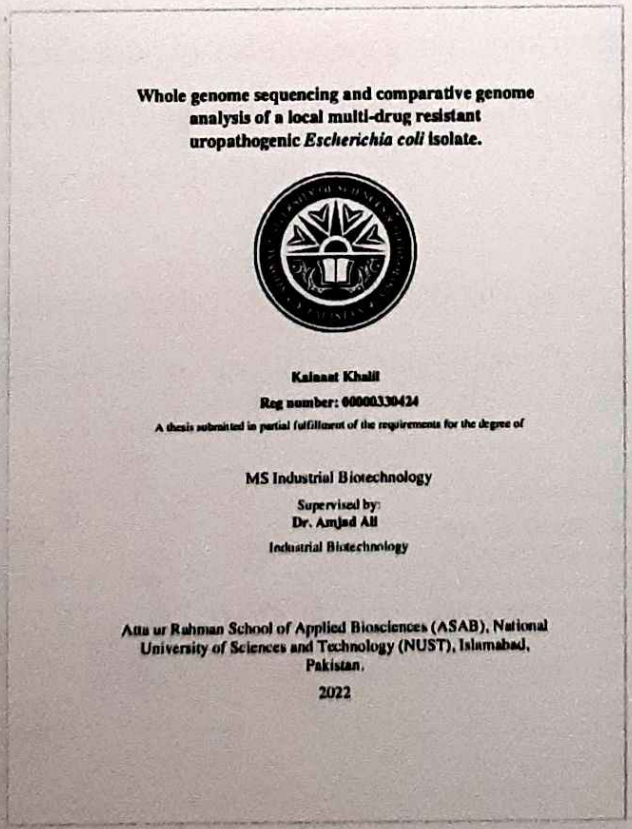

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