Optimization and Screening of *CITM*, *A1*, *B1* and *ADK* genes in uropathogenic *Escherichia coli*

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In

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DEDICATION

To my Mommy, Daddy

DECLARATION

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere to any other degree.

BUSHRA NAZ AFRIDI

CERTIFICATE

This thesis submitted by the *Bushra Naz Afridi* is accepted in its present form by the Department of Microbiology, Quaid-I-Azam University, and Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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ABU	Asymptomatic bacteriuria
ACC	Acute complicated cystitis
AUP	Acute uncomplicated pyelonephritis
CFU	Colony forming unit
CLED	Cytosine lactose electrolyte deficient agar
CLSI	Clinical and laboratory standard institute
ESBL	Extended spectrum β-lactamases
ExPEC	Extraintestinal pathogenic *Escherichia coli*
ENR	Enrofloxacin
GNR	Gram-negative rod
IS	Intermediate sensitive
LS	Lactose fermenting
MIC	Minimum inhibitory concentration
MHB	Muller-Hinton broth
MLST	Multilocus sequence typing
PAIs	Pathogenicity island
Pap	Pathogenicity associated pili
RUTI	Recurrent urinary tract infection
RTX	Repeats in toxins
TBE	Tris-borate EDTA
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection
Vat	Vacuole associated toxins

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Bushra Naz Afridi

VI. ABSTRACT

Urinary tract infections (UTIs) are one of the most common bacterial infections worldwide. Recently, the World Health Organization (WHO) provided a list of ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp*.) The bacterium, *Escherichia coli*, is the major cause of urinary tract infections. The present study aimed to screen and optimize housekeeping, AmpC β-lactam and some novel genes among the clinical isolates of uropathogenic Escherichia coli involved in urinary tract infections. A total of 152 UPEC isolates were scrutinized for the AmpC β-lactamase gene (*CITM*). In addition, two other housekeeping genes *(Rec*A*, Adk),* and two uncharacterized genes *(A1, B1*) were also screened. The *A1*, *B1*, *RecA* and *Adk* genes were successfully detected. Overall plasmid-mediated β-lactamase AmpC genes were found in 23% (34/152) of the UPEC isolates including previously detected AmpC genes in this collection of isolates. In conclusion, this study successfully identified the *CITM* gene and confirmed the presence of novel genes (*A1, B1*) that provide insight for the further characterization of these genetic factors.

The MIC of fluoroquinolone antibiotic Enrofloxacin used for livestock (poultry, farms etc.) was performed which showed the high resistance against these human UTI patients isolated samples. The result of a current research study showed that 95% of isolates were resistant to this antibiotic (84% were highly resistant), and only 4% were sensitive to the antibiotic. The β-lactam positive gene samples showed 100% resistance against this fluoroquinolone antibiotic. Our finding showed that livestock antibiotics also indirectly affect humans which is an alarming situation because it is more difficult to treat infections. The use of antibiotics for livestock should be prohibited or have proper guidelines for their use. This research study showed that these UPEC strains were highly resistant to many antibiotics including livestock antibiotics. This research study proved that livestock antibiotics have a great adverse impact on humans and indirectly contribute to developing antibiotic resistance.

INTRODUCTION

Urinary tract infections (UTIs) are amongst the most prevalent medical health issues registered in different hospital settings. Respiratory tract infections are the most common pathological infections in humans while UTIs are the second most common type. Annual research shows that about 150 million individuals from around the world suffer from UTIs which leaves an economic burden in terms of medical expenses and cost of hospitalization. The anatomical or neurological urinary tract abnormalities suggest that UTIs could be clinically classified as either uncomplicated or complicated cases. (Zacché & Giarenis, *et al.,*2016). Statistical data suggest that around 40% of women are most likely to experience UTI at least once in their lifetime (Micali *et al*., 2014) and 11% of women (>18 years) experience relapsed episodes of urinary tract infection every year (Foxman & Brown,2003; Foxman *et al*., 2014). According to the US annual reports, 11 million cases of UTIs are reported each year with a total annual cost of $5 billion. The symptomatic UTIs are categorized into three groups as follows: pyelonephritis (or upper UTI, accompanied by the infection of the kidney), cystitis (or lower UTI, where bacteria invade the bladder) and urosepsis syndrome (Foxman, 2014; Smelov *et al*., 2016). Identification of UTI in the blood is characterized by the number of bacteria i.e., more than 105/ml in the urine sample. ORENUC, which is a name classification system, is an acronym. The O stands for NO known factors, the R stands for Risk for Recurrent UTI, the E stands for Extra urogenital risk factors, the N stands for Nephropathy, the U stands for Urological risk factors that can be resolved by therapy and lastly, the C stands for catheter and the risk factors that cannot be resolved by therapy. According to the current system of risk factors for urinary infections which is ORENUC, the risk factors have been divided into six groups (Smelov *et al*., 2016). Both men and women can have bacterial cystitis, often known as acute cystitis; some patients can get recurring urinary system infections (Fiore & Fox, *et al*., 2014). Recurrent urinary tract infections are those that occur three times in 12 months or twice in 6 months. Relapses are frequently caused by the same bacterial species as the initial illness. Up to 8% of children will experience at least one UTI between the ages of 1 month and 11 years and up to 30% of infants and children experience recurrent infections during the first six to 12 months after

initial UTI. (Silva, A. C. S., Oliveira, E. A., & Mak, R. H. *et al*., 2020) Around 50% of UTIs do not seek medical care, therefore these statistics are overstated. The same or different strains of UTI-causing pathogens can (re)inoculate the bladder leading to recurrent UTIs. In contrast, bacteria that live in the bladder epithelium could periodically reappear and induce a relapse of urinary tract infections (Silverman *et al*., 2013). In women aged 61 to 65, 29% of the cases of urinary incontinence and 50% of genital-urinary symptoms—all signs and symptoms of bacteriuria—were reported (Raz *et al*., 2001).

The natural microbiota of the vagina and gastrointestinal tract can be permanently altered by standard prophylaxis and treatment of UTIs with antibiotics, and the development of multidrug-resistant (MDR) bacteria can also occur. Recurrent UTIs (rUTIs) have the potential to negatively impact women's quality of life, global public health, and the economy (Larsson *et al*., 2022). Urinary tract infection is rUTI only if at least two of the symptoms reappear within 6 months or 12 months of the initial identification of infection. According to the theory of pathogenicity, rUTIs are caused by offending uropathogens, susceptible hosts, or any combination of both. *Escherichia coli* (*E. coli*) is held responsible for up to 80% of UTIs and is thought to be responsible for half of the world's estimated resistance to antibiotics (WHO: Geneva, Switzerland; Kang *et al*.,2018).

Escherich claimed in an 1885 report that it would seem pointless and dubious to examine and sort out the supposedly randomly acting bacteria in normal faeces and the digestive system because the problem appears to be handled by an array of coincidences. Despite obvious scepticism, Escherich isolated, cultured, and utilized microscopy to explain the microbes identified in samples of newborn stools taken at delivery and during the first several months of life. Escherich used the gram staining method of Christian Gram for the identification of rod-shaped, gram-negative bacteria which commonly occurs in newborns. Escherich described a Gram-negative, rod-shaped bacteria that is thought to be common among newborns using Christian Gram's gram staining method was invented in 1884. He named the bacterium coli commune, and it could develop "a massive luxurious deep growth" from just a few basic nutrition sources. To honour the memory of Theodor Escherich, the

Bacterium coli commune was renamed *Escherichia coli* in 1919. Surprisingly, maybe the first *E. coli* strain that Escherich isolated was just recently found and sequenced. The initial strain designated NCTC86 at the National Culture Type Collection (London, UK) was devoid of known pathogenicity and virulence genes and belonged to one of the four major phylogenetic lineages recognized for *E. coli* (phylogroup A). The strain belonged to multilocus sequence type 10, which is still often seen in healthy children and adults. *E. coli* has functioned as a model organism to comprehend biology at its most fundamental levels due to its significance to health and its ease of cultivation and manipulation in the lab. (Martinson, J. N., & Walk, S. T. *et al.*, 2020). It may be found in a variety of habitats, such as soil and water, and it can also be found in the gastrointestinal tracts of warm-blooded animals and people. Although *E. coli* is typically thought of as a benign commensal, some strains can cause extraintestinal (urinary tract infection, sepsis/meningitis, and gastroenteric disorders in humans and animals) and gastroenteric diseases.

E. coli is divided into commensal and pathogenic categories based on how well it interacts with the host. The human immune system, age, the morphology of the urinary tract, and the infectious organism's virulence profile each play major roles in the way UTIs develop. Sexual activity, the use of an interceptive device, and menopause can all enhance a woman's vulnerability to UTIs during pregnancy (Nicole *et al*., 2002).

The *E. coli* strains (termed UPEC) are found in the intestinal flora in the commensal state from where they move towards the urinary tract to inhabit this area and cause infection. The uropathogenic *Escherichia coli* (UPEC) in humans causes diseases like urosepsis and urinary tract infections (UTI). About 80-95% of community-acquired UTI cases occurred due to UPEC leaving a negative impact on the world's healthcare system (Bonten *et al*., 2021). These strains have many virulent factors and multiple mechanisms which infect the urinary tract and cause its diseases. The reason why these strains are termed UPEC is that they have a strong relationship with uropathogenic illness. The UPEC is considered as extraintestinal *E. coli* further divided into 8 serogroups because of the presence or absence of O antigen on their surface suggesting the significance of the repeating terminal polysaccharides adhered

to lipopolysaccharide surface. The eight serogroups are as follows: O1, O2, O4, O6, O7, O16, O18, and O75and phylogenetic grouping B2 & D depending upon phylogenetic analysis applying multilocus enzyme electrophoresis (Aubron *et al*.,2012; Kaur *et al*., 2022). Moreover, *E. coli* strains are grouped into five groups based on phylogenetic analysis and are as follows: A1, B1, B2, D and E. Group B2 includes major UPEC strains while group D has only minor strains. Whereas groups A1, B1 and E are directly associated with intestinal strains of *E. coli* (Spurbeck *et al*., 2011). The commensal *E. coli* strain which inhabits the human gastrointestinal system is referred to as the UPEC strain. Thus, the genes associated with extra-intestinal virulence are associated with UPEC located in the urinary tract system. Many genomic studies revealed that the genome of *E. coli* has a vast genomic pool and a core. However, the flexible gene pool could only be observed in ExPEC UPEC strains and pathogenic intestinal strains. However, the core genome is found in all different *E. coli* strains. The mobile genetic elements (like transposons, phages, plasmids, and pathogenicity islands) found in the flexible gene pool play an essential role in a cell's adaptation to its environment. The important genetic information required by a cell to perform vital activities is stored in the core genome (Pitout *et al*., 2012; Ulett et al., 2013). The genomic size of *E. coli* lies between 4.5 to 5.5 Mb while the size of intestinal commensal *E. coli* is around 4.5 Mb. Furthermore, the genomic content of ExPEC (such as UPEC) is 5Mb. The genome size of different strains varies as follows: UPEC antimicrobial resistant > UPEC antimicrobial sensitive>intestinal pathogen> commensal (Pitout *et al*.,2012; Agarwal *et al*., 2012).

The research shows that UPEC has a wide range of virulent genes and related factors which play an essential role in the development and occurrence of UTI symptoms. Many urinary tract infections primarily are regulated by the presence or absence of some virulence genes and their expression. The pathogenic process always starts with attachment with adhesins, and pathogens such as UPEC need a suitable condition that leads to colonization and biofilm formation. Previous research studies investigate a sophisticated balance between proteomic and genomic (Pitout *et al*.,2012; Spurbeck *et al*.,2011; Momtaz *et al*.,2013). Ten groups of virulence factors include afimbrial adhesins, capsule, motility, serum resistance, autotransporter adhesins, fimbrial adhesins, lipopolysaccharide (LPS), outer membrane

proteins, siderophores, and toxins. The gene pool of some UPECs also contains antibiotic-resistant genes. As previously mentioned, the virulence genes' presence and their expression level assess the bacterial attributes in collaboration with the clinical symptomizing of urinary tract infections which include pyelonephritis, asymptomatic bacteriuria, and cystitis in an individual. Some severe UPEC significant virulent genes include *pap, aer, cnf 1, hly, sfa, aer, afa, fim, foc, astA, iroN* etc. These virulent genes play an essential role in the pathogenicity of pathotypes. Most of these virulent genes are also recognized in plasmid for example *cnf, hly, pap, sfa* (Agarwal *et al*.,2012; Momtaz *et al*.,2013).

A recent study reported that 86% of isolates of UPEC are resistant to trimethoprim-sulfamethoxazole, 76.3% are resistant to co-amoxiclav and 42% showed resistance against fluoroquinolone (Wolff&Maclennan,2007). MDR *E. coli* acquired resistance to a wide range of antibiotics, and the emergence of MDR UPEC is challenging to treat the infections. The incidence of MDR strains of UPEC was investigated and it was regarded that 77.5% of all the screened isolates had been resistant to 3 or more than 3 of the tested antibiotics. For MDR strains of UPEC, similar trends had been found in Iran (77%), whereas, in India, they had been 92%, in Slovenia, they had been 42% and in USA., MDR charges had been 7.1% (Ali *et al*.,2014) The two important groups of *Escherichia coli* secreted virulence factors are bacterial cell surface and secreted virulence factor. Fimbriae, specifically type 1 and P fimbriae, are the maximum frequent bacterial cellular surface virulence elements. These fimbriae aid in the production of biofilms, cytokine induction, tissue invasion, adherence to the host mobile floor, and adhesion to the host cell surface. Moreover, flagellum, capsular lipopolysaccharide, and outer membrane proteins are bacterial cell surface virulence elements. The virulence factors hemolysin and siderophores are secreted. The virulence factors include variable adhesins that are necessary for the uroepithelial cells' irreversible adhesion and adherence (Klein& Hultgren, *et al.*, 2020). The most common virulence factor or toxin associated with pyelonephritis produced by UPEC is hemolysin followed by cytotoxic necrotizing factor 1 (CNF-1) and autotransporters. These agents perform the following functions: exfoliate epithelial cells of the bladder, induce cell death, diffuse through the mucosal membranes, and facilitate cells with iron and other essential nutrients

(Chen *et al*.,2006). Two types of autotransporters (1) vacuolating autotransporter toxin (VAT) and (2) secreted autotransporter toxin (SAT) cause cellular damage in the kidney and bladder cells along with other physiological functions like elongation of the cell, autophagy, degradation of coagulation factor V, and abnormal bladder epithelial barrier regulation (Díaz *et al*.,2020). Siderophores are methods for iron acquisition that guarantee sufficient intracellular iron levels to support bacterial life in the human urinary tract—enhancing the ascending character of UPEC by motility-mediating flagella (Klein & Hultgren, *et al*., 2020).

Numerous scientific surveys show that UPEC antibiotic-resistant strains such as extended resistance, plasmid-mediated AmpC β-lactamase, and spectrum β-lactamase (ESBL)have spread around the globe. Different antimicrobial-resistant genes are present in pathogens against various classes of antibiotic drugs. Previous studies investigated the following most frequent antibiotic-resistant genes *bla*$_{\text{CTX-M}}$*, bla*$_{\text{OXA-7}}$*, bla*$_{\text{PSE-4}}$*, bla*$_{\text{SHV}}$*, CITM against* β-lactams, *aph(3')-IA against Kanamycin against* Gentamicin, *dhfr1, dhfrVII against Trimethoprim, aadA1, ant(3'')-IIa against Streptomycin, cat1, cml* against Chloramphenicol, *qnr (qnrA, qnrBand qnrS) against Quinolones, sul1, sul2 against* Sulfonamides, and *tet(A), tet(B), tet(C), tet(D) against* Tetracyclines. (Pitout *et al*.,2012; Momtaz *et al*.,2013). The production of the extended-spectrum β-lactamases (resistance to broad-spectrum cephalosporins), carbapenemases (resistance to carbapenems), PMQR genes (plasmid-mediated quinolone resistance; resistance to fluoroquinolones), and mcr genes (resistance to polymyxins) in UPEC strains for antibiotic resistance mechanisms are the most difficult challenges (Poirel *et al*., 2018). The high antibiotic resistance and UTI prevalence and incapability of antibiotic therapies emphasize demanding new techniques for UTI treatment. Non-antibiotic treatments that target virulence factors responsible for UTI development are more appealing than antibiotic treatments (Sarshar *et al*., 2020).

Antimicrobial resistance genes are spread by mobile genetic elements such as integrons which are typically found in plasmids and transposons. Plasmids and transposons pose serious concerns regarding access to accurate diagnostic tools and effective antimicrobial therapeutics alternatives. For the diagnosis of UPEC strains and antibiotic-resistant UPEC

pathotypes the classical and preliminary diagnostic techniques are unreliable and inefficient. Therefore, the production of innovative molecular approaches with precise diagnosis and efficient treatment is a critical need. Polymerase chain reaction (PCR) is a diagnostic technique which efficiently identifies and diagnoses antimicrobial genes like UPEC virulence genes. Designing DNA microarray probes by using virulence genes is especially useful (Behzadi *et al*., 2015). The main causes of nosocomial and community-acquired infections up to 50% and 75% to 95% are caused by the bacterial pathogen UPEC. These pathotypes' negative outcomes include morbidity, higher healthcare expenses and fatal consequences (Leski *et al*., 2013). Different strains and the human immune system response cause variable UTI complications from symptomatic/asymptomatic, acute/chronic, complicated/uncomplicated, and lower/upper UTIs (Behzadi *et al.,* 2015). Distinct factors are associated with the progression of infections of the urinary tract like an individual's age, gender, genetics, personal hygiene, catheterization, hospitalization, and social interactions/relationships (Behzadi & Behzadi, *et al*., 2008).

A concerning rise in UPEC resistance has been noticed over the past two decades. Concern about the emergence of gentamicin-resistant UPEC in the community was raised in the UK and the rise in ciprofloxacin-resistant pathogens from the blood has also been reported in the UK. (Stone *et al*.,2009; Woodford *et al*.,2007). Numerous studies in Pakistan highlighted unanticipated trends in antibiotic resistance in UPEC and other etiological agents that cause UTIs. (Muhammad *et al*.,2011).

Fluoroquinolone is a highly effective class of antibiotics that are used against numerous pathogens including both gram-positive and gram-negative and highly prescribed antibiotics. Different UTI conditions such as pyelonephritis, uncomplicated cystitis, and catheter-associated UTIs require this type of first-line drug because of these useful properties like good tissue penetration, excellent bioavailability, and long half-life. Even so, frequent use of this drug leads to serious health problems and antibiotic resistance against UPEC and along with other pathogens causing UTIs (Warren *et al*.,1999; Gupta *et al*.,2011). This class of antibiotics follow two main discovered mechanisms of antibiotic resistance that are

further helpful for scientists to alter the chemical structure of antibiotics to increase the efficacy of drugs and lower the emergence of drug resistance. Regrettably, these efforts were relatively unsuccessful and resistance against fluoroquinolone antibiotics increased continuously. The recorded trend against fluoroquinolone resistance to *E. coli* in major parts of Europe rose to 50% increasing day by day (Kronvall *et al*.,2010)**.** Many antibiotics resistance biological mechanisms noted in the case of UPEC develop and contribute to resistance. The antimicrobial resistance genes among UPEC may take place via horizontal gene transfer mechanisms or DNA mutations. Two genes; (1) antimicrobial resistance genes and (2) virulence genes are intricately linked to each other and can influence each other through their degree of expression with them distinguish identification (Silva *et al*.,2012).

By having a thorough understanding of the processes and mechanisms governing the interactions among all the distinct factors involved in the development, transfer, and dissemination of antimicrobial resistance it is feasible to understand the emergence of mutant strains and the selection process of these mutant (resistant) strains. Furthermore, an increase in the evolution and UPEC's population structure can aid in the creation of innovative and efficient new strategies.

For phylogenetic analysis of UPEC, several types of typing techniques including biotyping, anti-biotyping, serotyping, phage typing, multilocus enzyme electrophoresis and polyacrylamide gel electrophoresis are utilized. For the identification of the product of gene expression, all these phenotypic methods were applied. All these typing methods vary in their sensitivity, precision, and availability but all of them have low discriminative power (Belkum *et al*., 2001). The gene structures can be found by using genotyping techniques. These techniques are ribotyping, restriction fragment length polymorphism (RFLP), Pulsed-field gel electrophoresis (PFGE), Random amplification of polymorphism DNA (RAPD), Enterobacterial repetitive intragenic consensus PCR (ERIC), and Multilocus sequence typing (MLST). Based on their reproductivity, cost, discriminative capacity, and accessibility, these strategies varied from one another. Of all these techniques, MLST is a newly developed approach with a high degree of discriminative power and broad

applicability for studying the phylogeny of pathogenic bacteria (Russo& Johnson, *et al.,*2020). Multilocus sequence typing (MLST) is the molecular typing technique used for the differentiation and classification of bacterial strains based on their genetic variability in many housekeeping genes. MLST is a useful method to investigate population structure, statistical analysis, and evolution of different bacterial strains like UPEC.

This study concentrated on the investigation of some housekeeping genes (*Rec*A, *Adk*), AmpC β-lactam genes (*CITM*) and two novel genes (*A1*, *B1*) screening and their optimization among UPEC isolates. The study aimed to highlight fluoroquinolone antibiotic-resistant AmpC β-lactam genes (*CITM*). It also aims to understand how excessive antibiotics used in farms and livestock are related to antibiotic resistance. The study also shows the relevancy of AmpC-resistant genes that were also resistant to the fluoroquinolone family of antibiotics.

AIMS AND OBJECTIVES

This study aimed to optimize and screen housekeeping, AmpC β-lactam and some novel genes among the clinical isolates of uropathogenic *Escherichia coli* involved in urinary tract infections, collected from the Islamabad Diagnostic Centre in Islamabad, Pakistan. The study investigates Enrofloxacin (Fluoroquinolone antibiotic) on human-isolated uropathogenic *Escherichia coli* to determine the link of MIC level with mutations responsible for fluoroquinolone resistance. While the major objectives set to achieve these aims were:

1. To optimize and screen the *CITM*, *RecA, Adk, A1* and *B1* genes.
2. To determine the minimum inhibitory concentration of fluoroquinolone antibiotic (Enrofloxacin) in these clinically isolated UPEC samples.
3. Relate the AmpC β-lactam gene (*CITM*) to fluoroquinolone antibiotic and determine the fluoroquinolone resistance in *CITM*-positive isolates.

LITERATURE REVIEW

Overview

The CDC describes UTI as an infection in any area of the urinary system, which includes the urethra, ureters, bladder, and kidneys. According to the Centers for Disease Control and Prevention (CDC, 2022), the condition is ranked as the sixth most prevalent healthcare-associated illness (HAI).

Multi-drug resistant (MDR) Uropathogenic *E. coli* (UPEC) strains are a major open well-being concern, as they can cause urinary tract contaminations that are troublesome to treat with anti-microbials. A few consider having explored the predominance and instruments of MDR in UPEC strains, with numerous detailing the far-reaching spread of resistance genes such as *CTX-M* and *TEM* beta-lactamases, fluoroquinolone resistance-conferring transformations within the *gyr*A and *par*C qualities, and aminoglycoside-modifying chemicals. In expansion, ponders have appeared that MDR UPEC strains are regularly related to expanded destructiveness, as proven by the nearness of harmful components such as fimbriae, hemolysins, and siderophores. Methodologies to combat MDR UPEC contaminations incorporate the improvement of modern anti-microbials, combination treatments, and elective treatment alternatives such as bacteriophages or immunotherapies. (Momtaz *et al*.,2013).

2.1. Urinary tract infection

About 150 million people worldwide develop urinary tract infections each year, with significant social costs of approximately US $6 billion. (Ahmed *et al*.,2016). Uropathogenic *E. coli* is the most common cause of UTI including 90% as compared to other bacterial species. (Flores-Mireles *et al*., 2015). Other bacteria, such as *Klebsiella pneumonia* and *Proteus mirabilis*, can also cause UTIs. Infection with different structures (pilus, pili, flexures, flagella) and bladder (toxins, iron sources) causes infections. Urinary tract infection (UTI) can occur in the community and the hospital. The most important bacteria of host urothelium were taught.

The UTI indicates the certain bacteria present in the urine (usually > 105/ ml) urosepsis syndrome, pyelonephritis (upper kidney infection) and cystitis (or lower urinary tract infections) (Foxman, 2014; Smelov *et al*.,2016). However, under normal circumstances, such as pain while urinating, and frequent urination followed by pus or blood in the urine, only 100 uropathogenic organisms per milliliter are considered significant. The urinary tract is normally sterile. Although UTI can affect both men and women, it is more common in women and also found in children except in premature babies. About 90% of UTIs in young women are caused by a Gram-negative rod-shaped *E. coli* that is a member of the normal microbiome (Johnson *et al*.,1991). Public washrooms, poor hygiene, washroom setups and toilet practices also may be one of the reasons for the spread of UTIs among people. Pathogen may remain inert or silent in the bladder, but the report also suggests that 8% of women may have asymptomatic bacteriuria and severe consequences may develop and lead to septicemia, shock and sometimes death of an individual (Maginni *et al*., 2009).

Symptoms of a UTI include frequent or painful urination, abdominal pain, and cloudy or foul-smelling urine. UTIs may or may not be complex, and complex UTIs occur in people with anatomical abnormalities, urinary catheters, or other conditions. Depending on the signs and symptoms UTIs can be symptomatic or asymptomatic. The infection of the normal genitourinary tract is considered an uncomplicated UTI and while identification of structural and functional abnormalities during diagnosis is referred to as a complicated UTI and is usually treated with antibiotics, usually trimethoprim-sulfamethoxazole, nitrofurantoin, and Fosfomycin. (Gupta *et al*., 2011). Complicated UTIs are mostly asymptomatic and include instrumentation e.g., urethral catheters. Isolation of significant bacteria from urine that can cause infection is termed asymptomatic bacteriuria (bacteria in urine) however, no local and systemic genitourinary symptoms are noticed. Recurrent urinary tract infections (rUTIs) were defined as two or more infections within six months or three or more infections per year. rUTIs are a serious medical condition that can be life-threatening and affects up to 40% of women that have faced urinary tract infections in the past. The main factors leading to rUTIs include the following urinary abnormalities, immunodeficiency, sexual activity, and immunity. Recurrent UTIs can be difficult to treat because using the same antibiotics can

cause resistance and affect the genital area, increasing the risk of infection. There are different methods for rUTIs treatment like estrogen therapy, behavior modification and the use of prophylactic antibiotics that are being assessed worldwide.

Antibiotics mitigate the risk of rUTIs but act only as a short-term solution because of the development of drug resistance. The risk of rUTIs in postmenopausal women is reduced by estrogen therapy due to the improvement of vaginal function. Also, behavioural modifications like consuming alcohol and vomiting after sex play a substantial role in controlling the risk of infection. However, there is still a need to explore these options in depth to evaluate their effectiveness and safety (Hooton *et al*., 2001).

In the past few years, a broad spectrum of antimicrobial-resistant UTIs has been discovered (Farshad *et al*., 2012). It is to be noted that pregnant females suffering from asymptomatic bacteriuria (ASB) need antimicrobial therapy (Flores-Mireles '*et al*., 2015; Pitout *et al*., 2016). Moreover, UPEC resistance emerged as a major limitation in the treatment of urinary tract infections, especially in pregnant females. Many types of UPEC have developed resistance against some common antibiotics like fluoroquinolones and trimethoprim-sulfamethoxazole. The emergence of the issue of multi-drug resistance has increased the chances of treatment failure (Lee *et al*., 2014).

Researchers around the world are striving to develop alternative antibiotic-resistant UTI treatments like the use of probiotics and phages.

In addition, studies are being conducted to develop new antibiotics and combinations of antibiotics to overcome resistance mechanisms in MDR UPEC strains.

2.2. Epidemiology and Prevalence of UTIs

The prevalence of UTI varies according to many factors such as age, gender, region of residence and underlying medical conditions.

Accurate analysis of incidents of UTIs is complicated on the basis that UTIs are not detailed very usually, nonetheless, it is known as the most familiar bacterial infection. The diagnosis pattern that followed is the positive urine culture as well as evident clinical symptoms but in

rehabilitant settings, uncomplicated UTIs are treated without using any culture technique. So, misconceptions arise during estimating the incidence rate of UTIs.

In the United States, about 7 million doctor appointments are linked with patients suffering from UTIs followed by 1 million emergency room visits, and 100,000 annual hospitalizations. Women are more susceptible to UTIs and have an estimated lifetime risk of at least one UTI of up to 50-60%. Men are less likely to develop a UTI, but the incidence increases with age, especially in those with an abnormality or device (Foxman *et al*.,2014).

Like all other regions, urinary tract infections (UTIs) are an especially important and real public health problem in Pakistan and the Asian region. In this region, the prevalence of UTI is influenced by many factors, including poor sanitation, inadequate water supply, and overuse and prevention of antibiotics. UTIs are a common cause of illness in Pakistan, especially among women. The reported incidence of UTIs varies depending on the study population and the diagnostic criteria used (Malik& Ahmed, 2005). A study conducted at a tertiary hospital in Lahore reported that about 50.7% of bacterial isolates were *E. coli* showing 39.5 % of its prevalence among patients. In another study conducted in Karachi, the prevalence of female outpatients admitted to gynaecology clinics was 20.5% (Jabeen *et al*.,2005). Females are more prone to UTIs as compared to males with an estimated ratio of 8:1 and the risk of UTIs increases with age. About 20-30% of females with previous cases of UTIs experience recurrent UTIs.

A case study administered in Pakistan by Amber *et al* declared that the frequency of UTI in females was 60.5% on the other hand the frequency of UTI in males is less significant at 39.5% (Amber *et al*.,2016; Mody & Juthani-Mehta, 2014).In children, the prevalence of UTI is highest in infancy, with a peak incidence occurring between the ages of 2-6 months, the increased prevalence rate in infants; 8.7% in boys & 7.5% in girls of less than 3 months and then decreases with age. UTIs are more prevalent in age groups of 21-30 years in women and 31-40 years in men, nevertheless, for infants and neonates it is 14% & 0.1-1% respectively (Birnie *et al*., 2017). The occurrence of UTI in pregnant women is highest in

the 21-25 age group at 41.02%, compared to 35.89% in the 26-30 age group (Amber *et al*.,2016).

Other countries in the Asian region also have rates of UTIs as high as 7.5% to 23%. A study in Nepal reported a prevalence of over 23%, with *E. coli* being the most isolated bacteria. High rates of antibiotic resistance have been reported in India, where UTIs account for approximately 25-30% of all outpatient visits (Bhatta *et al*.,2011; Kaur & Chaudhary, *et al.*, 2014).

Uropathogens that are resistant to multiple drugs hinder the treatment of UTIs especially in Asiatic regions like Pakistan. A recent study conducted at a hospital in Lahore suggested that 45.8% to 97.2% of uropathogenic patients are prescribed various antibiotics. Other similar studies in this region highlighted the need for the development of improved and safe antiretroviral services along with new treatment strategies. The rate of occurrence of UTIs varies according to the geographical region. Developing and poor countries are more likely to face this health issue due to the lack of clean drinking water and inadequate sanitary conditions.

Moreover, some individuals are more exposed to urinary tract infections than others due to factors like spinal cord injuries and compromised immune systems (Nicole LE *et al.,* 1991). About 40% of nosocomial infections occur due to urinary tract infections and leave clinical as well as economic burden on the country.

Research shows that approximately 75-90% of UTIs are caused by the pathogen *Escherichia coli*. Other bacteria such as Klebsiella pneumoniae, Proteus mirabilis, and Enterococcus faecalis are also frequently associated with UTIs.

The emergence of multi-drug resistance (MDR) has made it difficult to treat UTIs using antibiotics resulting in increased morbidity and mortality (Gajdács *et al*.,2019).

The prevalence of MDR uropathogens varies geographically and between patients, with higher prevalence in developing countries and patients with a history of recurrent infections or prior antibiotic use.

Published research shows that developing countries and privileged regions with less economic and social conditions are more prone to UTIs than developed countries. According to a study reported by Hussain *et al*. (2018), it was highlighted that UTI was an epidemic in Pakistan, especially with 74.53% of women and 25.46% of men (Hussain *et al*., 2018). Cases reported in India comprised 27.75% of the population group (n = 9,763) (Maji *et al*., 2016).

2.3. Etiological Agents and Major Risk Factors

The considerable risk factor that is liable towards UTIs progression involves gender, age, sexual activity, catheterization, kidney stones, enlarged prostate, use of calcium, pregnancy, anatomical abnormalities of the urinary tract, immune suppression, hormonal changes, ureteric stress, postmenopausal state, use of spermicides and underlying medical conditions such as diabetes, heart disease, obesity, cancer, and kidney disease (Gupta *et al*.,2011; Foxman,2010). The etiological agents of UTIs are mostly bacterial named *Escherichia coli, Staphylococcus saprophyticus, Enterococcus spp, Proteus mirabilis,* and *Klebsiella pneumoniae,* among these bacterial agents the gram-negative rods (GNRs) account for 80-85% infections. Out of GNRs uropathogenic *Escherichia coli* UPEC being the most usual causative agent causes 70-95% of infections and the remaining 15-20% infection responsible GNRs are *Proteus mirabilis*, *Staphylococcus saprophyticus* & *Klebsiella pneumoniae* (Flores-Mireles *et al*.,2015; Tandogdu & Wagenlehner, *et al.*, 2016). The type and number of bacteria that cause a UTI will vary depending on a person's health and vulnerability. For example, group B *Streptococci* and *Klebsiella* are more common in diabetic patients, while *Pseudomonas* bacteria are more common in catheterized patients.

Different age groups are vulnerable to UTIs but some subcategories such as pregnant females, infants, and patients with injuries and underlying medical conditions are at considerable risk factors. Based on gender, females are more sensitive to UTIs than males (Jackson *et al*.,2004). A reflective study reported an incidence of symptomatic UTI in 6-year-old children. Gender comparative studies indicate that girls (6.6%) are three times more susceptible to infection than boys (1.8%). It is estimated that 1 in 3 females experienced UTI at 24 years of age and need antimicrobial treatment. Overall, 40-50% of females have UTIs

once throughout life. Complicated UTIs demand hospitalization and lead to financial problems due to medical expenses and increased prevalence rate.

Recent studies investigate that women often seek medical procedures promptly, despite the presence of appropriate symptoms of UTI. Genetic and biological factors extensively involved can increase susceptibility to UTIs. The distinct genetic aspects involved non-sector status or ABO blood group antigens that can lead to an improved vulnerability against UTIs (Chita *et al*., 2017; Elikwu et *al*., 2017; Mittal *et al*., 2009). Biological diseases of the body include the occurrence of urinary tract obstructions and urinary tract infections in the past. Certain behavioural changes, such as the use of diaphragms, condoms, and spermicide, and the frequency of sexual intercourse in premenopausal women may increase the risk of UTI (Delcaru *et al*., 2016; Guglietta *et al*., 2017). Estrogen deficiency also increases susceptibility to UTIs, and women of all age groups are at risk for UTIs despite the use of antibiotics.

2.4. *Escherichia coli*

German bacteriologist Theodor Escherich first discovered bacteria in the human intestinal colon in 1885 that caused digestive problems such as diarrhoea and indigestion. This bacterium was originally called "bacterium coli" but was later renamed *Escherichia coli (E. coli*) by the scientist who first discovered it (Ali *et al*., 2016; Edberg *et al*., 2000). *Escherichia coli* is the most studied microorganism in the world in many aspects such as gene regulation mechanism in bacteria. *Escherichia coli* is part of the normal microflora of the human gastrointestinal tract (GIT) and colonizes in neonatal GIT at the time of delivery. However, it can cause illness in certain situations, such as in debilitated and immunocompromised patients (Johnson *et al*., 1989). Morphological characteristics specify that they are gram-ve, lactose fermenter, rod-shaped, facultative anaerobes, and non-spore-forming coliform bacteria. There are essentially two groups of *E. coli* bacteria: enteric and non-enteric. The most pervasive enteric bacteria is *E. coli* which is found in faecal flora. In the case of UTI, the disease is caused by extra enteric uropathogenic *E. coli* in 90% of reported cases. *E. coli* strains causing UTI to have no clonality; they are related to different

phenotypic groups (Phillips *et al*., 1988). Enteric pathogenic *E. coli* is categorized clinically as a virulent strain responsible for the many infections that cause diarrhoea, gram-positive bacteremia, UTIs, neonatal meningitis, and neonatal sepsis.

UPEC, a strain of *E. coli*, represents a variety of bacteria and strategies that alter the state of gut bacteria, allowing them to grow and remain in the urine, allowing infection and causing urinary tract infections. There are several different *E. coli* strains, but the ones associated with urinary tract pathogenicity are referred to as UPEC strains (Foxman *et al*., 2000). *E. coli* can be grouped into three distinct categories based on the type of infections they impose, for instance, Commensal bacteria are harmless, whereas intestinal pathogenic strains and pathogenic bacterial strains cause infections (Allocati *et al*., 2013).

Furthermore, the major *E. coli* strains are of two types: cell surface and secretory virulence factor. Most of the bacterial cells are made of either type P fimbriae or type 1 fimbriae playing an essential role in cell adhesion, tissue invasion, formation of biofilm and induction of cytokines. Bacterial cells are mainly composed of type 1 fimbriae and type P fimbriae. These fimbriae contribute to cell adhesion, tissue invasion (important in the pathogenesis of UPEC-induced UTI), biofilm formation, and cytokine induction. Bacterial cells also have flagella, capsular lipopolysaccharide, and outer membrane structures. Hemolysins and siderophores are secreted virulence factors (Emody *et al*.,2003).

UPEC has developed resistance, posing a threat to global health. Therefore, understanding UPEC prevalence and antibiotic resistance is important for the management of UPEC UTIs (Tabasi *et al*., 2015). Therefore, this study aimed to evaluate what types of different bacterial markers are found in UPEC isolates and assess their relationship with antibiotic resistance patterns.

2.5. Taxonomical classification of *E. coli*

Based on the characteristics, genetic makeup, and pathogenicity of *Escherichia coli* classified, here are some classifications of *Escherichia coli.*

According to taxonomical classification, *E. coli* belongs to the family Enterobacteriaceae and the genus Escherichia. The other species of this genus are *E. coli, E. albertii, E. fergusonii,* and *E. blattae.* (Table 2.5)

Table 2.5. Scientific Classification of *Escherichia coli*

Domain	Bacteria
Phylum	Pseudomonadota
Class	Gammaproteobacteria
Order	Enterobacterales
Family	Enterobacteriaceae
Genus	*Escherichia*
Species	***E. coli***

2.6. Phylogenetic classification of *Escherichia coli*

Escherichia coli (*E. coli*) strains are classified phylogenetically into several phylogroups based on their distinctive genomes and evolutionary relationships. A, *B1*, B2, C, D, E, F, and clade I are some of the phylogroups into which *E. coli* strains can be divided. Specific genetic markers or genes, such as *chuA*, *yjaA*, and *TspE4*.C2, have a significant role in determining the classification (Clermont *et al*.,2013). Pathogenic *E. coli* belongs to *B1*& D and non-pathogenic *E. coli* is related to A & B2 phylogroup (Picard *et al*.,1999).

We need to delve deeper into the bacteria's phylogenetic taxonomy to better comprehend the epidemiology and genetic variety of *E. coli* strains. This bacterium shows an interesting evolutionary history and has evolved with time to become more harmful. It has adapted to changing environments to spread among animals, humans, and various ecosystems (Clermont *et al*., 2013).

Pathogenic *E. coli* belongs to *B1*& D and non-pathogenic *E. coli* is related to A & B2 phylogroup.

2.7. Serotype of *E. coli*

E. coli is distributed into 700 different serotypes based on the serotyping system, which considers the antigens, namely antigen O (lipopolysaccharide) and antigen H (flagellar). *E. coli* strains are divided into the following phylogenetic groups (A, *B1*, B2, D, E, F) based on genetic markers (Tenaillon *et al*.,2010). Moreover, these strains could further be classified into various genomic groups based on the absence or presence of certain genes. For example, some strains may carry genes encoding virulence factors, antibiotic resistance, or metabolic pathways (Nowrouzian *et al*.,2005).

2.8. Pathotype of *E. coli*

Similarly, based on pathogenesis, they are divided into pathotypes; different strains of the same species have multiple virulence factors that enable them to cause disease. They are classified into two broad categories; (1) Enteric *E. coli* with 7 pathotypes and (2) Extraintestinal pathogenic *E. coli* (ExPEC) with three pathotypes and some details shown in the table.2.8(Allocati *et al*., 2013). Of ExPEC UTIs, approximately 90% are caused by uropathogenic *E. coli* (Ali *et al*., 2016).

Table 2.8. Pathotypes of *Escherichia coli* (Allocati *et al*., 2013)

Pathotype	Characteristics
Enteropathogenic *E. coli* (EPEC)	Causes infantile diarrhoea; adhesion and effacement lesion
Enterohemorrhagic *E. coli* (EHEC)	Causes hemorrhagic colitis and hemolytic uremic syndrome; Shiga toxin-producing
Enterotoxigenic *E. coli* (ETEC)	Causes traveller's diarrhoea; produces enterotoxins
Enteroaggregative *E. coli* (EAEC)	Causes persistent diarrhoea in children and adults; adheres to the intestinal mucosa
Diffusely Adherent *E. coli* (DAEC)	Causes diarrhoea; adheres to enterocytes
Uropathogenic *E. coli* (UPEC)	Causes urinary tract infections
Neonatal meningitis-associated *E. coli* (NMEC)	Causes meningitis in neonates
Extraintestinal pathogenic *E. coli* (ExPEC)	Causes sepsis, meningitis, and other extraintestinal infections

2.9. Evolution of uropathogenic *E. coli*

The specific extraintestinal pathogenic strain (ExPEC) of *E. coli* that causes UTI is UPEC. Recent studies reported that all UPEC strains do not inevitably share all the sets of virulence factors. O antigen serotype profiling classified UPEC into eight serogroups with 58% prevalence (O1, O2, O4, O6, O8, O9, O18 & O83). Most of the UPEC are in B2 and D phylogroups, while the commensal strains are related to A and B1 phylogroups. The existence of genomic Pathogenicity Islands (PAI) and the production of virulence variables such as adhesins, toxins, surface polysaccharides, flagella, and iron-acquisition systems have culminated in the identification of four primary UPEC phylogroups (A, B1, B2, and D) (Bien *et al*., 2012).

There are several UPEC strains recognized based on virulence factors and other characteristics. The common virulent strains are O1:K1:H7, O2:H6, O6: K2:H1, and O18:K1:H7. In newborns and young children, O1:K1:H7 is frequently linked to neonatal meningitis and UTIs (Pitout *et al*., 2016). O2:H6 is often related to recurrent UTIs in females

(Manges *et al*., 2001). O6: K2:H1 is often linked to pyelonephritis (kidney infection) and bacteremia (bloodstream bacterial infection) (Johnson *et al*., 2001). O18:K1:H7 has been associated with UTIs in aged individuals and those with underlying health problems (Totsika *et al*., 2012).

2.10. Pathogenesis of uropathogenic *E. coli*

A series of phases are included in the pathological process of UPEC during UTIs:

a) Colonization of the periurethral and vaginal regions with the colonization of the urethra
b) Ascent into the bladder lumen and growth as planktonic cells in the urine
c) Adhesion to the surface and interaction with the bladder epithelium defence system
d) Biofilm formation
e) Bladder formation's invasion and replication Quiescent intracellular reservoirs (QIRs) arise and reside in the underlying urothelium in intracellular bacterial communities (IBCs)
f) Kidney colonization and host tissue damage with a higher risk for bacteremia/septicemia.

Additionally, UPEC may establish intracellular bacterial communities, which are bacterial colonies that develop inside of host cells and have been linked to persistent UTIs. UPEC may also create biofilms on the surface of host tissues, which can help bacteria survive and develop antibiotic resistance (Mody & Juthani-Mehta, 2014).

Adhesins, chemicals, and iron uptake mechanisms are only a few of the virulence components involved in the complicated pathogenesis of UPEC. Bacterial adhesion and colonization require adhesins such as type 1 fimbriae, P fimbriae, and D fimbriae. Some toxins leave negative impacts on healthy tissues in attempts to fight germs, for instance, hemolysin and cytotoxic necrotizing factors. Bacteria can thrive and proliferate in the human urinary tract, but they need additional factors like heme absorption systems or other iron

acquisition mechanisms like siderophores to survive inside the host's urinary tract (Hultgren *et al*., 2018; Forster & Krieger, 2019).

Pathogenesis of UPEC and UTIs has been described in detail by many authors like Scott J. Hultgren and Catherine S. Forster & John N. Krieger in the respective books "Urinary Tract Infections: Molecular Pathogenesis and Clinical Management" and Urinary Tract Infections: Diagnosis and Management." Moreover, it is crucial for researchers to analyze and further understand the pathogenesis of UPEC and UTIs which would help develop novel strategies to overcome and treat these infections (Totsika *et al*., 2012; Pitout *et al*., 2016).

Molecular Pathogenesis and Clinical Management, a book explains the mechanism of pathogenesis of UPEC. According to the mentioned book, the first step in pathogenesis is adhesion in which bacteria gets attached to the surface of uroepithelial cells, followed by invasion and multiplication into the host's bladder epithelium. There it forms biofilms that actively develop resistance against antibiotics. The significance of virulence factors is also highlighted in this book stating that toxins and fimbriae are capable of colonizing and causing disease.

A similar mechanism of invasion is described by Catherine S. Forster and John N. Krieger enlightening the role of bacterial virulence factors in spreading the disease and suppressing the human immune response followed by the progression of urinary tract infections. To avoid challenges and the eventual development of antibiotic resistance, the book also stresses the need to properly recognize and treat UTIs.

Overall, both publications offer fascinating details about the aetiology of UPEC and UTIs and highlight how crucial it is to comprehend the molecular mechanisms behind these ailments to diagnose, treat, and prevent these illnesses effectively.

2.11. Treatment options and mode of action on UPEC

Antibiotics are either manufactured or natural agents that may eradicate or stop the development of bacteria. These are the sorts of drugs that are utilized for treating bacterial infections and can be taken orally, given intravenously, or applied topically. The kind of

bacterial illness being treated, and the patient's unique medical history determine the precise type and quantity of antibiotic that should be used (Flores-Mireles, 2014).

Bacteriostatic and bactericidal are the two basic classifications of antibacterials based on their method of action. The numerous kinds of antibiotics are based on the target location, such as a bacterial cell wall, cell membrane, nucleic acid, ribosomes (30s and 50s), or one of the many cellular enzymes present in bacteria. These include substances that block the formation of cell walls, cell membranes, DNA and RNA, protein, and other substances, as well as antimetabolites (Bbosa *et al*., 2014).

By targeting the bacterial cell wall or cell membrane, antibiotics can stop pathogens from multiplying. The production of nucleic acids and proteins are additional targets produced by ribosomes. The nucleoprotein complexes in bacteria are composed of two components: small 30S and large 50S (as shown in the image).

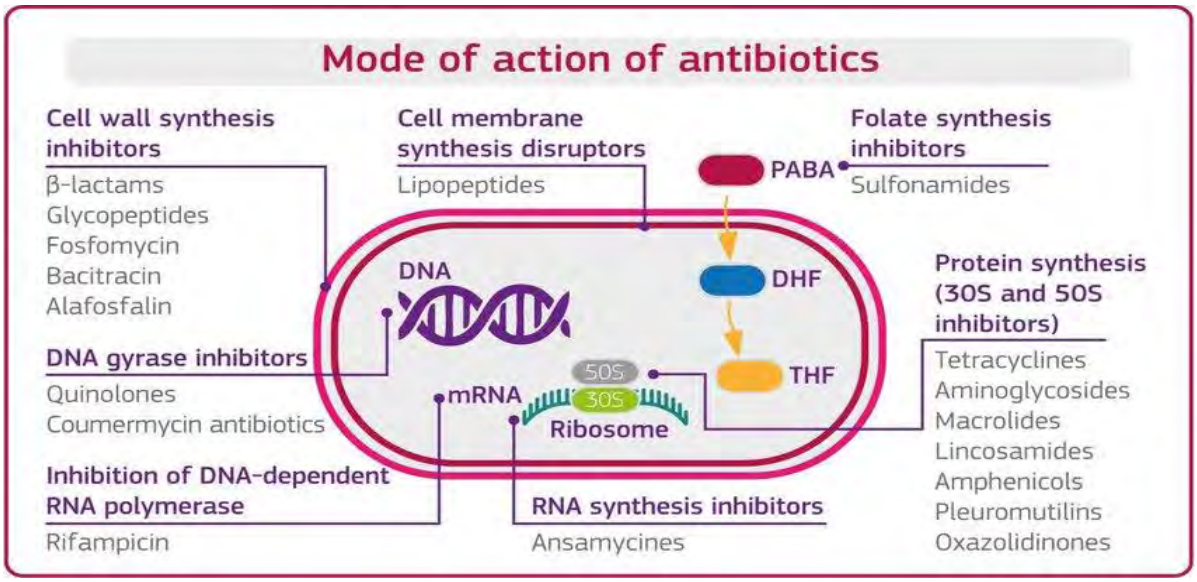


Figure 2.11 Diagrammatic representation of different classes of antibiotics and their mode of action (Sanseverino *et al*.,2018)

Most antibiotics in this case act as antimetabolites where they inhibit the DNA synthesis (following folate metabolism) through the series of changes using two folic acid precursors, dihydro folic acid (DHF) and tetrahydro folic acid (THF) and para-amino-benzoic acid

(PABA). Antibiotics physiologically disrupt the shape of the DNA by inhibiting the production of DNA gyrase which is associated with the process of DNA replication and transcription. Each mechanism's corresponding class of antibiotics is displayed (Sanseverino *et al*.,2018). Depending on the type of antibiotic and the bacterial target, different antibiotics have varying mechanisms of action against Uropathogenic *Escherichia coli* (UPEC).

For instance, by interfering with the metabolism of folic acid, antibiotics like sulfonamides, trimethoprim, and nitrofurantoin impede bacterial metabolism and DNA synthesis. These medications go against the enzymes responsible for producing folic acid, a vital component needed for bacterial development and growth. These antibiotics interfere with bacterial metabolism and stop bacterial growth by impeding the formation of folic acid (Gary *et al*., 2005).

On the contrary, antibiotics like fluoroquinolones (e.g., ciprofloxacin, levofloxacin) and aminoglycosides (e.g., gentamicin, amikacin) prevent bacterial DNA replication and transcription by specifically inhibiting bacterial topoisomerases and ribosomes, respectively. As bacterial topoisomerases, which are enzymes that control the configuration and molecular makeup of DNA during replication, are bound by fluoroquinolones, DNA replication is slowed down. Fluoroquinolones stop the transcription and replication processes of bacterial DNA by obstructing topoisomerases, which causes the bacteria to die. Similarly, aminoglycosides restrict the production of bacterial proteins by attaching to bacterial ribosomes and preventing messenger RNA (mRNA) from being translated into proteins. In the end, the bacterial cell collapses because of the synthesis of deactivated proteins (Toma& Deyno, 2015).

2.12. UTIs treatment

UTIs significantly lower people's quality of life and have a significant impact on the economy and public health (Kostakioti *et al*., 2012).

Antibiotics are suggested to treat many urinary tract infections especially the ones caused by Uropathogenic *Escherichia coli* (UPEC) and other virulent agents. The kind of pathogen

causing the infection and its severity determine the best antibiotic to use. The preliminary antibiotics used for uncomplicated UTIs include trimethoprim/sulfamethoxazole, nitrofurantoin, and Fosfomycin. These kinds of antibiotics have fewer adverse effects and are effective against the majority of UPEC strains. These medications may not always work, though, as antibiotic resistance is on the rise (Lucente *et al*.,2012).

Most patients with lower UTIs have been observed to get symptom relief in 25% of cases after 5-7 weeks, and in 80% of cases after waiting roughly 5 months without treatment Lower tract and upper tract infections, or complex and simple UTIs, are categorized as cystitis and pyelonephritis, respectively (Mandell *et al*., 2000). The selection of the best therapy and regimen is aided by this categorization. Short-course therapy lasting 3 to 7 days is used to treat uncomplicated UTIs, whereas extended therapy is used to treat complicated infections (Mandell *et al*.,2000).

Generally prescribed treatment based on signs & symptoms described by patients is the use of oral antimicrobials before any clinical diagnosis (Mishra *et al*., 2012).

The Infectious Disease Society of America has created and published evidence-based treatment recommendations for acute, uncomplicated UTIs (cystitis and pyelonephritis).

The recommended antibiotics for uncomplicated cystitis are Fosfomycin, nitrofurantoin, and trimethoprim/sulfamethoxazole (TMP-SMX). These medicines have fewer adverse effects and are effective against the majority of uropathogenic *E. coli* (UPEC) strains. Due to worries about rising resistance and potential adverse effects, fluoroquinolones, including ciprofloxacin and levofloxacin, are equally as effective but only administered for complex cystitis or as a second-line therapy. Uncomplicated cystitis normally requires 3-5 days of antibiotic therapy, but complicated cystitis may need 7–14 days. Some individuals, such as those with recurrent cystitis or risk factors for problems, could need an extended period of therapy (Gupta *et al*., 2011; Flores-Mireles *et al*., 2015).

Acute pyelonephritis is normally treated with antibiotics using intravenous infusions while the patient is hospitalized. Considering regional variations of antibiotic sensitivity and the

severity of the illness, the initial immediate therapy should be selected. Fluoroquinolones, cephalosporins, and aminoglycosides are the recommended antibiotics for treating acute pyelonephritis. Depending on how severe the illness is, therapy might last anywhere between 7 and 14 days. Following hospital release, patients may take oral antibiotics for a total of 14 days. Hoenigl *et al*. (2018) conducted a systemic review to highlight the effectiveness and safety of different antibiotic regimens for the treatment of acute pyelonephritis. According to the study, fluoroquinolones and cephalosporins were the specified initial intervention alternatives since they had comparable both safety and effectiveness aspects. Aminoglycosides should be used with caution, especially in individuals with pre-existing renal illness since they were discovered to have a greater risk of nephrotoxicity (Hoenigl *et al.,* 2018).

Antibiotics having a broader spectrum and strong penetrating power, such as fluoroquinolones, can be used to treat complicated UTIs in patients who have moderate to severe illnesses. However, there haven't been many controlled research trials in this area that could have addressed the best medication and length of treatment (Gupta K, *et al*.,2011).

Gupta *et al*. (2011) suggested that for acute uncomplicated cystitis (AUC) treatment with antibiotics. Usually taken at a dose of 100mg two times per day for 5-7 days, nitrofurantoin is a widely used antibiotic. Another antibiotic that is recommended for use is trimethoprim-sulfamethoxazole, which is taken twice daily for three days at a dosage of 160/800mg. Additionally, a single 3g dosage of Fosfomycin tromethamine is used to treat acute, simple cystitis. Acute, uncomplicated cystitis is treated with ciprofloxacin, another antibiotic. A dosage of 250-500mg administered twice a day for three days is recommended. Other suggested alternatives include fluoroquinolone for 3 days OR Amoxicillin-clavulanate or Cefdinir or Cefpodoxime-proxetil or Cefaclor for 3-7 days.

For the treatment of acute uncomplicated pyelonephritis, the prescribed antibiotics include Levofloxacin 750 mg, Ofloxacin 400 mg, Ciprofloxacin 500 mg, Cefuroxime axetil 500 mg, Cefpodoxime proxetil 200 mg, Trimethoprim/Sulfamethoxazole (160/800 mg), and Gentamicin 5-7 mg/kg with ampicillin 1 g every six hours.

It is essential to remember that the selection of antibiotics may change depending on local resistance patterns and patient-specific considerations like allergies or comorbidities. As a result, it is advised to speak with a healthcare professional to decide on the best course of therapy (Gupta *et al*.,2011).

The overall success rate of antibiotic treatment for simple cystitis was 73.2%, whereas the success rate for acute uncomplicated pyelonephritis was 86.4%, according to literature-based research on 27 randomized controlled trials (Flores-Mireles *et al*.,2015)

Trimethoprim-sulfamethoxazole, an antibiotic frequently used for treating UTIs, has an 18.3% overall risk of resistance, according to research published between 2000 and 2017. 1.4% of people had developed resistance to the antibiotic nitrofurantoin, which is also frequently used (Aminzadeh & Sadat, 2019).

2.13. Antibiotic resistance

According to a study carried out in Pakistan, ciprofloxacin, co-trimoxazole, and ampicillin, all have resistance rates of 46.8%, 49.4%, and 50.2% for uropathogenic *E. coli* (UPEC) (Shaikh *et al*.,2015).

Another research executed in Pakistan by Rehman *et al.* (2016) discovered that the UPEC had resistance rates to ciprofloxacin, nalidixic acid, and norfloxacin that were68.2%,71.6%, and 64.4%, respectively.

Antibiotic resistance has caused a change in how UTIs are treated recently, with a focus on minimizing needless antibiotic usage and encouraging the use of non-antibiotic therapy. As alternatives to antibiotics, methods including cautious waiting, delayed administration, and the use of probiotics have been investigated.

A wide range of antibiotics are being used to treat several types of urinary tract infections which have affected bacterial ecology enabling it to develop antibiotic resistance. The development of resistance is most observed during the treatment of recurrent microbial UTIs. The emergence of MDR UPEC and antimicrobial resistance in UPEC are health issues of clinal concern, especially affecting women suffering from recurrent UTIs. Fluoroquinolones,

cephalosporins, and aminoglycosides are only a few examples of the broad-spectrum antibiotics that are being overused because of the rising prevalence of MDR UPEC, especially in poor nations (Bartoletti *et al*., 2016; Sanchez *et al*., 2016).

Continuous and long-term use of antibiotics plays an essential role in the development of multi-drug resistance strains that are simultaneously resistant to three or more antibiotic classes. For example, Methicillin-resistant S. aureus (MRSA), Vancomycin-resistant Enterococci (VRE), and Vancomycin-resistant S. aureus (VRSA) are just a few examples of infectious individuals that now exhibit multi-drug resistance against antibiotics. In many countries, antimicrobial resistance is rising, demonstrating the heterogeneity associated with time and place (Fig.2.14).

Production of several -lactamase enzyme subtypes is associated with resistance to -lactams. Multiple forms of -lactamases (*bla* genes) are among the genes and are often found on plasmids (Adamus-Biaek *et al*., 2018). Penicillin, cephalosporin, monobactams, and carbapenems are examples of -lactam antibiotics. The amide bonds found in the four-membered-lactam ring are degraded by -lactamases (Noyal *et al*., 2009). In contrast to carbapenems, cephamycin, and beta-lactamase inhibitors, ESBL is an enzyme that gives resistance to all β-lactam antibiotics (including penicillin's, cephalosporins, and monobactams) (Baudry *et al*., 2009).

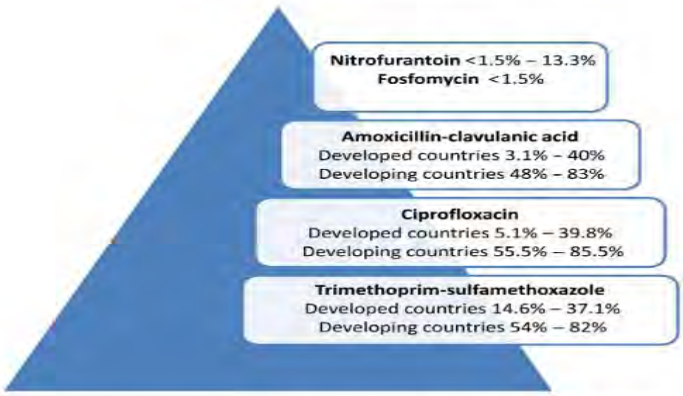


Fig. 2. The resistance of UPEC to antimicrobials using in the treatment of UTIs.
Amoxicillin-clavulanic acid; the developed countries (USA, 3.1–40%; Germany, 5.3%; Poland, 13.9%; England, 30%; France, 37.6%), developing countries (Nepal, 48%; Pakistan, 71%; Jordan, 83%). **Ciprofloxacin**; developed countries (USA, 5.1–12.1%; Belgium, 12.9%; Germany, 10.5–17.3%; Switzerland.17.4%; England, 20.4%; France, 24.8%; Spain, 39.8%), developing countries (Jordan, 55.5%, Mongolia, 58.1%; Pakistan, 60.8%; Nepal, 64.6%; Ethiopia, 85.5%). **Trimethoprim-sulfamethoxazole**; developed countries (Belgium, 14.6%; USA, 17.4%; Germany, 18.45%; Poland, 21.4%; Switzerland, 24.5%; Spain, 30.9%; France, 37.1%), developing countries (Iran, 54%; Mexico, 66%; Ethiopia, 68.5%; Mongolia, 70.9%; Jordan, 73,1%; Pakistan, 82%).

Figure 2.13 Bartoletti *et al*. (2016) and Sanchez *et al*. (2016) show the resistance of UPEC toward antibiotics.

2.14. Types of resistance

Several mechanisms are followed by bacteria for resistance that can be intrinsic or acquired. It is more challenging to cure infections when bacteria have developed multiple defence mechanisms against antibiotics. These defence mechanisms can be roughly divided into different types. Some bacteria produce beta-lactamases and other metabolic enzymes which cause inactivation of penicillin and cephalosporins, leading to the development of resistance. Another related phenomenon is observed by efflux pumps that actively expel drugs outside bacterial cell walls and store genetic information for resistance genes. By altering the process by which antimicrobial agents attach to bacterial target sites like DNA gyrase or ribosomal subunits, mutations in bacteria can also cause resistance. Furthermore, bacteria may alter or evade the metabolic pathways that antibiotics target to build resistance (Alekshun & Levy, *et al*., 2007).

2.15. Antibiotic resistance in uropathogenic *Escherichia coli*

Several types of antibiotic resistance may be present in UPEC (uropathogenic *Escherichia coli*), allowing growth and proliferation in the presence of antibiotics. Antibiotic resistance

observed in UPEC most commonly occurs due to Beta-lactamase synthesis. Beta-lactamase enzymes, which may render beta-lactam medicines like penicillin and cephalosporins inactive, may be produced by UPEC strains. These antibiotics lose their antibacterial effectiveness when the beta-lactam ring structure of the antibiotic is broken down by the beta-lactamase enzymes of UPEC.

Extended-Spectrum-β-lactamase (ESBL) are enzymes generated by bacteria that give resistance to several different beta-lactam antibiotics including penicillin and monobactams. Clinical settings now address a serious threat from UPEC strains that give rise to ESBLs (Pitout *et al*.,2012). AmpC β-Lactamase enzyme production by UPEC can hydrolyze a wide range of β-lactam medications causing resistance to them, including cephalosporins (Jacoby *et al*., 2009).

A frequent antibiotic class for treating UTIs is fluoroquinolones. UPEC bacteria acquire resistance against fluoroquinolones through a series of processes like transforming DNA gyrase and topoisomerase IV genes or efflux pump systems (Gupta *et al*., 2001).

Aminoglycosides are antibiotics that work well against a variety of Gram-negative bacteria. Through processes including enzymatic alteration or decreased absorption because of changes in the proteins that make up the bacterial outer membrane, UPEC strains can become resistant to aminoglycosides (Pitout *et al*., 2010).

For the treatment of UTIs, sulfamethoxazole and trimethoprim are routinely combined. Through mutations or the acquisition of resistance genes, such as those producing dihydrofolate reductase enzymes with decreased affinity for the medicine, UPEC strains can develop resistance to trimethoprim (Warren *et al*.,1999).

Another often employed antibiotic for UTIs is nitrofurantoin. UPEC strains can become resistant to nitrofurantoin in several ways, such as changes to the way the drug gets activated and transported through the body or mutations in the target enzymes.

2.16. Mechanism of resistance

Production of several β-lactamase enzyme subtypes is associated with resistance to β-lactams. Multiple forms of β-lactamases (*bla* genes) are among the genes frequently found on plasmids. Genetic analysis from a study conducted in hospital wards of Lodz, Poland showed that the UPEC strain i.e., the *TEM-1* gene is found in most patients (Adamus-Białek *et al.*, 2018). The enzymes, ESBLs provide resistance against different agents like penicillin, cephalosporins, and monobactam; however, some antibiotics like carbapenems, cephamycin's, and -lactamase inhibitors are efficient against this viral strain (Baudry *et al.*, 2009). About 20 years ago, scientists developed a novel type of ESBL called CTX-M (cefotaxime) which is a significant agent among ESBL generated by UPEC. Among the three classes of -lactamases most essential ones are TEM and SHV (Ojdana *et al*. 2014; Shahbazi *et al*. 2018). Developing countries like Iran, Nepal, Pakistan, and Jordan observe 37.1%, 38.9%, 40% and 50% cases of UPEC-producing ESBL (Ali *et al*., 2016; Parajuli *et al*., 2017; Shakhatreh *et al*., 2018).

Different regions of the world, and occasionally even different hospitals within the same nation, have varying rates of *E. coli* isolates that produce ESBLs. The *E. coli* isolates synthesizing ESBLs show resistance against many antimicrobial agents like β-lactam, tetracycline, aminoglycosides, and trimethoprim/sulfamethoxazole (Rezai *et al*. 2015). Shahbazi *et al*. (2018) reported that UPEC bacterial strains that produce ESBL are more resistant against aminoglycosides glycosides and quinolones when compared to the UPEC bacteria independent of ESBL. The administration of carbapenems (including, imipenem and meropenem) is highly effective in treating Urinary Tract Infections caused by bacterial strains producing ESBL (Idil *et al*., 2016). Lactamase inhibitors are-lactamase inhibitors are advised while using cephalosporins, penicillin, and monobactams (Bartoletti *et al*., 2016).

The two most prescribed antibiotics for UPEC are quinolones and fluoroquinolones. These antibiotics are considerably being consumed around the world contributing to the increased rate of antibiotic resistance in UPEC. Komp *et al*. (2003), reported that the fluoroquinolones bind to and inhibit the activity of DNA gyrase and topoisomerases II and IV. The encoding

of DNA gyrase is facilitated by *gyrA* and *gyrB* genes (Pourahmad & Mohiti, 2010). These genes catalyse the supercoiling of DNA, causing mutations in UPEC and ultimately causing quinolone resistance. The quinolone resistance-determining regions (QRDRs) lies between amino acids 67 (*Ala-67*) to 106 (*Gln-106*) at the N-terminal sequence of gyrA protein. Friedman *et al.* (2001) conducted thorough research to assess the phenotypic resistance against quinolones and fluoroquinolones and revealed that mutations in the gyrA gene's codons 83 and 106 in UPEC isolates cause antimicrobial resistance against the aforementioned antibiotics (Shenagari *et al*., 2018).

Some pathways of quinolone and fluoroquinolone resistance in *E. coli* include the existence of efflux pumps and reduced antibiotic absorption. It occurs due to alterations and disturbances in the outer membrane porin proteins (Asadi &Habibin, *et al*., 2019). Abdelhamid and Abozahra *et al*., 2017 highlighted that the increase in the expression rate of the efflux pump-coding genes *acrA* and *mdfA* is responsible for the development of levofloxacin resistance. This finding supports the idea that efflux pump systems are essential in the development of antibiotic resistance against fluoroquinolone in UPEC isolates.

Antibiotics are specific in their mode of action- for instance, Fosfomycin actively inhibits the cell wall production in bacteria leading to bacterial cell death (Dijkmans *et al*., 2017). Fosfomycin is similar to phosphoenolpyruvate (PEP) which binds and activates UDP-GlcNAc enol pyruvyl transferase for the synthesis of necessary peptidoglycans. Fosfomycin resistance is developed through three distinct pathways; (1) gene mutations affecting the production of glycerol-3-phosphate or glucose-6-phosphate transporter mitigating the rate of absorption of Fosfomycin, (2) point mutations at the binding sites of UDP-GlcNAc enol pyruvyl transferases, and (Kim *et al*., 1996) (3) enzymatic breakage of oxirane ring or phosphorylation in Fosfomycin causes its inactivation.

The rate of disruption of the oxirane ring is spread up by Glutathione transferase (*FosA*), Fosfomycin-specific epoxide hydrolase (*FosX*), and L-cysteine thiol transferase (*Fos B*) (Rigsby *et al*., 2005). For the time being, UPEC has very little nitrofurantoin resistance, making it an excellent choice for treating simple cystitis. As a result of the antimicrobial's

many targets in the bacterial cell, resistance to nitrofurantoin did not arise as quickly as resistance to other medications (Veeraraghavan & Shakti, *et al.,*2015). The mutation frequency in *E. coli* was shown to be roughly 107/cell where these mutations develop resistance to nitrofurantoin (Sandegren *et al*., 2008). Resistance to nitrofurantoin was brought about by mutations in the genes encoding oxygen-insensitive nitro reductases, *nfsA* and *nfsB*. Additionally, it proved that nitrofurantoin-resistant mutants had significantly lower rates of bacterial cell proliferation when the drug was present in therapeutic doses. It could imply that nitrofurantoin-resistant mutants were unable to initiate an infection when specific drugs were used (Sandegren *et al*., 2008).

2.16.1. Intrinsic resistance

The term "intrinsic resistance" describes a strain of Uropathogenic *Escherichia coli* (UPEC) that is naturally resistant to a particular antibiotic or antimicrobial treatment. UPEC has several mechanisms that add to its inherent resilience and enable it to live and persist in a variety of situations. The efficiency of antimicrobial medicines is constrained by these processes, which include both physical and metabolic barriers (Russo& Johnson,2020).

By nature, some bacteria inherently resist antibiotics due to their biological characteristics. For instance, Gram-negative bacteria like *E. coli* have a barrier-like outer layer that naturally protects them from several medications (Martínez,2008).

Additionally, inherent resistance is shown towards certain antimicrobial peptides produced from the host immune system by UPEC. These peptides, like defensins and cathelicidins, have antibacterial characteristics and are especially important in the innate immune response. To combat the effects of these peptides, UPEC has developed defence mechanisms. These include altering the lipopolysaccharides in its outer membrane or producing certain proteins that either neutralize or break down the peptides (Russo & Johnson, 2020; Bonomo& Szabo, 2020; Torres *et al*.,2001).

2.16.2. Enzymatic inactivation

One aspect of intrinsic resistance in UPEC is enzyme inactivation. UPEC can create enzymes that chemically alter antibiotics, rendering them ineffective. This process is known as enzymatic inactivation. For example, beta-lactamase enzymes could destroy the beta-lactam ring structure of beta-lactam antibiotics rendering them useless (Martínez, 2008).

2.16.3. Target modification

UPEC may develop another aspect of intrinsic resistance genes or genetic changes that change the target areas for antibiotics. This modification may lower the antibiotics' propensity for attaching to their targets, decreasing their potency. Examples include changes to bacterial enzymes that fluoroquinolones target or adjustments to ribosomal subunits that aminoglycosides target (Martínez, 2008).

2.16.4. Efflux pumps

The existence of efflux pumps in UPEC is one facet of intrinsic resistance. UPEC Bacterial cells equipped with efflux pump systems aggressively expel antibiotics from the bacterial cell. To reduce the efficacy of antibiotics, these pumps assist in removing them before they reach their intended areas. Fluoroquinolones, tetracyclines, and macrolides are only a few of the antibiotics for which efflux pumps are responsible for resistance (Martínez,2008).

2.16.5. Reduced permeability

UPEC can alter the permeability of their outer membrane or cell wall to stop antibiotics from penetrating the cell. As a result, fewer antibiotics can reach the target locations, decreasing their efficacy (Martínez, 2008; Blair *et al*.,2015).

2.16.6. Biofilm formation

The intrinsic resistance in UPEC is strongly influenced by the presence of biofilms. Intricate bacterial colonies wrapped in a self-made extracellular matrix are known as biofilms and can be formed by UPEC. The human immune system is kept at bay and antimicrobial agent penetration is hindered by biofilms, which offer UPEC a haven. The biofilm matrix serves

as a physical barrier and can bind and sequester antibiotics, lowering their availability and efficacy (Russo & Johnson, 2020; Bonomo& Szabo, 2020; Torres *et al*.,2001).

Contrary to pathogens that are free-floating counterparts, bacteria discovered in biofilms have higher antibiotic resistance. The biofilm matrix functions as a physical barrier and may prevent antibiotics from penetrating fully. Furthermore, bacteria in biofilms can go into a latent condition, making the ability to go into a latent condition, which makes them less sensitive to antibiotics (Martínez,2008; Blair *et al*.,2015).

2.16.7. Acquired resistance.

In uropathogenic *Escherichia coli* (UPEC), acquired resistance describes the appearance of resistance to antibiotics because of genetic alterations the bacteria have acquired. The mobile genetic elements like plasmids or transposons as well as gene mutations, horizontal gene transfer, and other methods might result in these alterations.

The acquisition of genes encoding antimicrobial resistance determinants is one of the primary routes of acquired resistance in UPEC. The transmission of mobile genetic elements or from other bacteria in the environment are two ways that these genes might be acquired. For instance, UPEC can acquire genes for β-lactamases, enzymes that render inactive β-lactam drugs like penicillin and cephalosporins. Another typical method of acquired resistance in UPEC is the efflux pump development where cells actively expel antimicrobial drugs outside of the bacterial cell.

The discovery of plasmids or transposons containing numerous resistance genes is a crucial component of the acquired resistance mechanism in UPEC. These genetic components may contain genes that confer resistance to many antibiotic classes, allowing UPEC to concurrently withstand the effects of several antimicrobial drugs. Through horizontal gene transfer methods like conjugation or transduction, these genetic components can travel across different UPEC strains.

2.16.8. Multi-drug resistant UPEC

In vitro susceptibility analysis suggests that if one antibiotic shows resistance against more than three bacterial strains it is regarded as multi-drug resistant (Mahony *et al*., 2020). The trend of resistance towards multiple drugs in *E. coli* was discovered in the late 20th century. According to WHO, Enterobacterales that produce carbapenem-resistant, extended-spectrum - β-lactamase (ESBL) is one of the most concerning classes of bacteria that might produce multi-drug resistance. It is expected that by 2050 antibiotic-resistant Enterobacterales will spread disproportionality among different communities (Tacconelli *et al.*, 2018).

E. coli has shown a range of resistance to all kinds of antibiotics used to treat UPEC infections. The most prevalent class of antibiotics used to treat UPEC infections are β-lactams, which prevent vital penicillin-binding proteins from doing their necessary functions to prevent cell wall production. Usually, β-lactamases, which are enzymes able to hydrolyze this class of antibiotics and render them useless, mediate β-lactam resistance (Tooke *et al*.,2019).

2.17. Extended spectrum β-Lactamases

There are many variants of β-lactamases, but the most common ones include TEM, SHV, and CTX-M enzymes. These β-lactamases are further grouped into four different Ambler classes where TEM, SHV, and CTX-M belong to class A. The narrow spectrum β-lactamases like TEM-1 and SHV-1 cause the breakdown of penicillin and cephalosporins. Too much consumption of cephalosporins is most likely to cause mutations in TEM-1 and SHV-1 enzymes. Such unusual and unexpected mutations termed ESBLs cause resistance against different cephalosporin generations (2nd, 3^{rd,} and 4th) due to their wide action range (Heritage *et al*., 1999; Bush *et al*., 2013).

2.18. Plasmid-mediated AmpC-β-Lactamase

Ambler Class C is the classification for AmpC β-lactamases (Bush *et al*., 2013) except for rare mutant strains, *E. coli* only generates modest and mostly ineffective quantities of AmpC

β-lactamases, which are typically chromosomally encoded (Tamma *et al*.,2019). However, plasmids can also be used to transmit AmpC β-lactamases. All β-lactams, except for cefepime, cefpirome, and carbapenems, are resistant to AmpC β-lactamases (Meini e*t al*., 2019). According to multiple investigations, isolated *E. coli* (<7%) possess the AmpC β-lactamase enzyme, making it less common than ESBLs (Ribeiro *et al*., 2019).

2.19. Carbapenemases

Class A Carbapenemases are characterized by their chromosomal location but are often regulated by plasmids. All currently therapeutically available β-lactam antibiotics, except for monobactams, can be hydrolyzed by broad-spectrum-lactamases referred to as class B carbapenemases (Bader *et al*.,2020). In 2009, variations in New Delhi metallo-β-lactamase (NDM) strains were first discovered in *E. coli* (Wilson *et al*.,2018). Oxacillin-hydrolyzing enzymes are within the category of class D carbapenemases. Only a small number of the more than 200 enzymes are effective against carbapenems. Such enzymes found in *E. coli* are as follows: OXA-48, OXA-181, OXA-232, and OXA-484. Moreover, ESBLs cause third-generation carbapenem and cephalosporin resistance due to co-occurrence with some other mechanisms of resistance. However, co-occurrence with other resistance mechanisms, such as ESBLs, can result in 3rd-gen cephalosporin and carbapenem resistance. These carbapenemases have limited action on their own and frequently spare cephalosporins (Parh *et al*.,2021; Sommer *et al*.,2021).

2.20. Non-β-Lactam antibiotics resistance

In addition to β-lactam antibiotics, there are many other alternative medication options available for treating different UPEC infections. Some of these antibiotics are trimethoprim, Fosfomycin, fluoroquinolones, aminoglycosides, and sulfonamides (Allocati *et al*., 2013). Other resistance mechanisms exist that reduce the effectiveness of these medications and are frequently present in ESBL-producing bacteria (Bodendoerfer *et al*., 2020).

2.20.1. Fluoroquinolones

Fluoroquinolones have antibacterial activity against a variety of microorganisms. They function by obstructing DNA supercoiling. Resistance is frequently brought on by mutations in drug targets, but other factors, including increased efflux pumps, target structure protection, and decreased outer membrane permeability, are also likely to contribute to resistance (Poirel *et al*.,2018). A study conducted in Iran (2019) revealed that 45.2% of studied isolates showed resistance against fluoroquinolone. On the other hand, a study conducted in the US highlighted that 28.2% of strains under investigation were 28.2% (Begier *et al*.,2021). Among many other antibiotics, the most prescribed class of antibiotics to treat UTIs are fluoroquinolones. Statistics indicate that they are prescribed in over 50% of instances of simple UTIs (Adamus-Białek *et al*., 2017).

2.20.2. Aminoglycosides

When treating UPEC infections, aminoglycosides and β-lactams are frequently combined. They function by reversibly binding to the 50S subunit of the bacterial 70S ribosome and the 30S subunit of the 16S rRNA (Poirel *et al*.,2018). The 16S RNA and/or S5 and S12 ribosomal proteins in certain bacteria can mutate, leading to resistance. The presence of a large number of operons in *E. coli* limits the chance of extensive mutations. As a result, plasmid-mediated resistance to aminoglycosides is present in UPEC. Enzymatic inactivation of ribosomal methylases or their protection can also result in resistance to aminoglycosides (Poirel *et al*.,2018; Ojdana *et al*.,2018).

2.20.3. Fosfomycin

A versatile antibiotic, Fosfomycin is effective against both Gram-positive and Gram-negative bacteria. The antibiotic comes in three different forms that can either be administered orally or intravenously. Fosfomycin tromethamine and Fosfomycin calcium are given orally while Fosfomycin disodium is administered intravenously (Michalopoulos *et al*.,2011). While IV Fosfomycin has received FDA approval in several countries to be used as a treatment for severe infections caused by MDR Gram-negative bacteria, despite its

usage there, oral Fosfomycin is still widely used across the world. Fosfomycin, which is frequently used to treat cystitis, acts by obstructing the formation of peptidoglycan. There are two primary methods of resistance that Fosfomycin could acquire; the first one is the production of plasmids expressing Fosfomycin-modifying enzymes and the second one is mutations in proteins implicated in the uptake system (Dimopoulos *et al*.,2019).

2.20.4. Sulfonamides and Trimethoprim

Two bacteriostatic antibiotics (sulfonamides and trimethoprim in combined form) that prevent the production of folic acid by bacteria have beneficial effects. Resistance against sulfonamides or trimethoprim is brought on by either a change in the target enzyme or genes (Poirel *et al*.,2018).

2.21. Genotyping methods

The genetic variations and traits of organisms, including bacteria like Uropathogenic *Escherichia coli* (UPEC), are examined using genotyping techniques. Insightful information about several factors like genetic diversity, population makeup, and relatedness of various strains is acquired using this technique**.** Here are a few basic genotyping techniques.

2.21.1. Multilocus Sequence typing.

MLST method is one of the recently used genotyping methods. In MLST multiple genes nucleotide sequences change is compared, and their relatedness is determined. (Maiden *et al*.,1998; Spratt *et al*., 1999). Multilocus sequence typing (MLST)is used for sorting bacterial strains according to the sequence differences in several housekeeping genes. The genetic diversity, population structure, and epidemiology of UPEC have all been studied in detail using MLST. Tenaillon *et al*. (2010) carried out one of the important types of research on MLST in UPEC. They compared a collection of UPEC strains recovered from infections in the urinary tract to non-pathogenic strains using MLST analysis. The study found UPEC to have a complex population structure, which may indicate the existence of many clonal lineages. Additionally, they discovered that MLST sequence types were linked to clinical signs and patterns of antibiotic resistance.

To increase the method's discriminating strength, Clermont *et al*. (2013) modified the MLST scheme for UPEC by integrating more genes. Achtman MLST, an enhanced MLST method that added seven housekeeping genes, provided for more accurate subtyping and characterization of UPEC strains.

Additionally, MLST has been utilized to look at the transmission and dispersion of UPEC lineages around the world. For instance, Gordon *et al*. (2017) used MLST to analyse UPEC strains from several nations and found common sequence types throughout geographically distinct regions, indicating the spread of certain lineages across borders.

2.21.2. Pulsed-field gel electrophoresis (PFGE)

A popular technique for examining bacterial genomes, such as UPEC is pulsed-field gel electrophoresis (PFGE). Large DNA fragments may be separated and characterized by PFGE, revealing details on the clonal and genetic links between various strains (Hunter *et al*., 2005).

In PFGE, whole genomic DNA is embedded in an agarose gel and exposed to an electrical field in a sequence of pulses with different switch timings and angles. The size-dependent migration of the DNA fragments through the gel matrix is influenced by the alternating electric field. Therefore, a distinctive set of DNA bands—also referred to as a DNA fingerprint or profile—that accurately depicts the genetic makeup of the strain under study is produced (Ribot *et al*., 2006).

Researchers can analyse the epidemiological linkage between various UPEC strains and establish their relatedness by analyzing the PFGE patterns of those strains. Strains with identical or nearly identical PFGE patterns are likely to have experienced clonal growth or to have a common progenitor. In epidemic analyses, surveillance research, and strain surveillance for antibiotic resistance, PFGE has been extensively employed (Gautam *et al*.,1997).

While PFGE is an effective method for strain typing, it is crucial to be aware that it has several drawbacks. To maintain precision and interoperability across various laboratories,

the interpretation of PFGE patterns calls for expertise and standardized procedures. Additionally, PFGE may not be able to detect minute genetic variants since it primarily reveals information on large-scale genomic rearrangements (Gautam *et al*.,1997).

2.21.3. Whole-genome sequencing (WGS)

An organism's whole genome, including Uropathogenic *Escherichia coli* (UPEC), may be thoroughly analyzed using the genotyping technique known as whole-genome sequencing (WGS). Such sequencing helps in the determination of the complete DNA sequence of an individual's genome. It makes it possible and easy to detect genetic variants like single nucleotide polymorphisms (SNPs), deletions, insertions, and structural abnormalities (Köser *et al*., 2012). WGS involves extracting the UPEC strain's DNA, high throughput sequencing it, and then utilizing bioinformatics software to analyse it. The obtained genomic information can offer comprehensive details on the genetic makeup of the strain, including its main genome and accessory genome, virulence factors, antibiotic resistance genes, and phylogenetic relationships (Ingle *et al*.,2016).

Compared to conventional genotyping techniques, WGS has several benefits. Compared to techniques like pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST), it offers a better level of precision and accuracy in detecting genetic variants. Additionally, WGS enables the identification of novel strains or resistance mechanisms by capturing the fine-scale genomic diversity within a bacterial population (Grad *et al*.,2012).

Phylogenetic reconstruction, epidemic investigations, source tracing, and the discovery of putative virulence factors or resistance mechanisms are just a few of the downstream analyses that may be performed using WGS data. It enables strain comparison at the genomic level, assisting with the comprehension of the epidemiology and evolution of UPEC. (Grad *et al*.,2012)

The use of WGS for genotyping UPEC strains, however, necessitates complex laboratory setups, proficiency in bioinformatics analysis, and substantial computer resources. To

guarantee reliable and repeatable findings, standardized procedures and bioinformatics pipelines are crucial.

2.21.4. Polymerase chain reaction (PCR)-based techniques

Many genotyping approaches for bacteria, including Uropathogenic *Escherichia coli* (UPEC), are based on Polymerase Chain Reaction (PCR) technology. To identify and characterize diverse genetic components, such as virulence genes, antibiotic resistance genes, and other target areas, PCR enables the amplification of DNA sequences of interest. (Buberg *et al*., 2019)

For genotyping UPEC, a variety of PCR-based methods are employed:

- Multiplex PCR: This is an efficient method in which many targeted gene sequences are amplified simultaneously in a single reaction. Identification and classification of resistance genes or virulence factors is made possible using certain primer sets.
- Reverse Transcription PCR (RT-PCR): RT-PCR is used for the identification and measurement of gene expression by transforming RNA into complementary DNA (cDNA) with the help of the enzyme reverse transcriptase. This method could also be used to identify gene expression of virulence genes and other agents of UPEC pathogenesis.
- Real-time PCR: It measures the DNA and RNA amplification in real-time during the stage of amplification in the polymerase chain reaction. It also identifies several specific genes and genetic components, like quantifying gene expression and identifying bacterial load.

Nested PCR: It consists of two PCR amplification cycles where the result of the first PCR acts as a template for the following PCR cycle. It is efficient in enhancing the detection sensitivity of specific targets with lower abundance.

The significance of PCR-based genotyping technologies encompasses higher sensitivity, specificity, and decreased turnaround time. These methods help in the detection of UPEC

strains, identification of virulence factors, studying antibiotic resistance and understanding the epidemiology and genetic diversity of UPEC populations (Buberg *et al*., 2019).

2.21.5. DNA microarray

A DNA Microarray DNA chip or DNA microchip is a genotyping apparatus that simultaneously identifies the expression of thousands of genetic variants and genes in each biological sample. It provides researchers with an opportunity to study global gene expressions or identify the sequence of genes of interest (Bednar *et al*., 2000).

Probes are made specifically to complement certain genes or genetic variants. The DNA sample is marked with some marker-like fluorescent dye, which is then hybridized into microarray probes. The process of hybridization is responsible for the identification of the target sequence in the sample. Microarrays are also utilized to identify genetic elements like single nucleotide polymorphisms (SNPs) that are usually associated with the occurrence of some diseases or reactions to medications. Additionally, in microbial diagnostics, microarrays detect different bacterial strains based on their genetic profiles (Lockhart *et al*., 1996).

DNA microarray technology has been extensively used in the context of uropathogenic *Escherichia coli* (UPEC) to research the genetic profile and virulence characteristics of UPEC strains. Researchers can locate and characterize certain genes or genetic variants that contribute to the pathogenesis and virulence of UPEC using DNA microarrays. The expression of numerous genes having a relationship with virulency, including those involved in adhesion, invasion, iron acquisition, toxin synthesis, and biofilm formation, have been examined using DNA microarrays and UPEC. The gene expression patterns of several UPEC strains may be compared to help researchers pinpoint certain genetic elements linked to higher virulence or pathogenicity. DNA microarrays have furthermore been used to research the genetic variety and evolution of UPEC strains. Researchers can find strain-specific genetic markers and comprehend the genetic relatedness among UPEC strains from various geographic regions or patient groups by comparing the genetic profiles of various UPEC isolates (Totsika *et al*.,2011).

2.22. The resistance of UPEC lineages

Using the MLST technique helps in the exploration of ExPEC lineages. Many blood infections and clinical diseases like UTIs occur due to the pandemic ExPEC clones with sequence types ranging from 10, 69, 73, 95, 127 and 131 (Tartof *et al*., 2005; Adams-Sapper *et al*., 2013; Riley *et al*., 2014). More than 50% of ExPEC infections worldwide are caused by these genotypes. The isolates with sequence type 131 typically show multidrug resistance (Petty *et al*., 2014; Banerjee and Johnson, 2014). Adams-Sapper *et al*. (2013) conducted a study on patients in Northwest England and suggested that 92 % of multi-drug resistant isolates belonged to sequence type 131. In comparison to ST127 isolates, which were the most extensively responsive to antibiotics, ST131 showed increased levels of antibiotic resistance (Gibreel *et al*., 2012).

In 1999–2000 the reported 56% of UTI infections was caused by UPEC strains from ST 95, 127, 73, 69, 131, and 10. The same STs were the root of 64% of UTI infections from 2016 to 2017. According to Yamaji *et al*.'s (2018) study, four genotypes (ST 95, 127, 73, and 131) accounted for 46.4% of ampicillin-resistant isolates in 2016–2017 as opposed to just 21.8% in 1999–2000. Only in these genotypes was the rise in ampicillin resistance noted. According to Yamaji *et al*. (2018), TMP-SMZ isolates had the highest percentage of ST69 in both research periods. From 2016–2017 about 70% of the isolates with *CTX-M* were ST69, 127, and 131 while 58% of ciprofloxacin-resistant isolates belonged to ST131.

2.23. Virulence factors of UPEC

A virulence factor is defined as any part of a microbe or organism that could initiate infection or disease in an individual. UTIs and the establishment of infection inside the urinary tract are both possible outcomes of uropathogenic *Escherichia coli* (UPEC), which has several virulence characteristics. The four major categories of these virulence factors are adhesins, poisons, iron acquisition systems, and immune evasion strategies. The following are some of the main virulence characteristics of UPEC (Subashchandrabose *et al*.,2014; Mulvey *et al*.,2000).

- Adhesins: Different UPEC strains express adhesins that allow them to cling to and colonize the uroepithelium. Type 1 fimbriae, P fimbriae, S fimbriae, Dr fimbriae, and fimbria adhesins are a few examples of these adhesins. They facilitate bacterial colonization by mediating adhesion to certain host cell receptors.
- Toxins: The development of UTIs is aided by the toxins produced by UPEC, which harm host tissues. Hemolysin (HlyA), cytotoxic necrotizing factor (CNF), and -hemolysin (HlyA) are a few prominent toxins produced by UPEC. These toxins could harm cells, interfere with cell membranes, and bring on inflammation.
- Systems for Iron Acquisition: UPEC has developed diverse ways to take iron, a nutrient that is crucial for bacterial development, from the host. They create siderophores that scavenge iron from the host and make it easier for it to be absorbed, including enterobactin and yersiniabactin. Additionally, UPEC has iron-regulated surface determinants (IreA and IreB) that support iron uptake.
- Immune Evasion Mechanisms: UPEC uses some techniques to get around the host immune system. They can create biofilms, which are organized bacterial colonies encased in a barrier which resists the host's immune system and antimicrobial substances. UPEC also have antigenic variation and complement activation inhibition as defences against phagocytosis.
- Other Factors: UPEC strains may create extra virulence factors, such as capsule polysaccharides that help the bacteria evade host immune defences and proteases and autotransporters that help the bacteria spread and cause tissue damage.

Understanding the virulence factors of UPEC is crucial for comprehending the pathogenesis of UTIs and developing strategies for diagnosis, treatment, and prevention. By targeting these virulence factors, it may be possible to disrupt UPEC colonization and reduce the severity of UTIs (Subashchandrabose *et al*.,2014; Mulvey *et al*.,2000).

2.24. Significance of biofilm in UTIs

The pathogenesis and durability of Uropathogenic *Escherichia coli* (UPEC) infections are significantly influenced by biofilm development. A self-produced extracellular matrix that

attaches to surfaces and shields bacteria from environmental pressures including immunological reactions and antibiotic treatments is known as a biofilm. Biofilms are intricate colonies of bacteria. Numerous benefits result from UPEC-induced biofilm development in the urinary system. First, makes it possible for UPEC to stick to and colonize the uroepithelial cells, aiding in the spread of infection. The biofilm matrix shields the bacteria from phagocytosis, antibody-mediated killing, and others. Biofilm development leads to UPEC infections' resistance to antibiotic therapy. The biofilm matrix functions as a physical barrier that restricts the entry of antibiotics into the bacterial population, increasing the susceptibility of the microbial inhabitants of the biofilm to antimicrobial agents. Additionally, because of the biofilm's sluggish development rate, certain medicines that target rapidly dividing cells may be less effective against the bacteria there. Due to this, UPEC is better able to survive and avoid immune system clearance (Justice *et al*.,2006; Caza *et al*., 2015).

Research by Justice *et al*. (2006) found that UPEC strains that were isolated from female patients with recurrent UTIs had better biofilm-forming capacities than strains from patients with isolated episodes of infection. Similarly, Caza *et al*. (2015) examined the function of biofilms in catheter-related urinary tract infections and discovered that UPEC strains linked with catheter biofilms had higher antibiotic resistance compared to planktonic (free-floating) counterparts.

2.25. Antibiotic utilization in farming and its Effect on human

Since the late 1940s, it has been known to administer antibiotics in subtherapeutic concentrations to cattle making them grow faster and cheaper (Sneeringer *et al*., 2015).

Farm animals act as hosts for the inoculation and growth of antibiotic-resistant bacteria which transfer to humans following several different routes. These bacterial strains pollute soil and groundwater indirectly posing a threat to farmers. They then spread these bacterial strains to human communities either through direct contact or consumption of contaminated meat. Analysis of retail meat including chicken, beef, pork, and turkey showed elevated levels of Enterobacteriaceae (Elliott, 2015; Johnson *et al*., 2006). It shows a substantial

number of these bacteria are indigestible and antibiotic-resistant - people can fall sick if this meat is not cooked properly. The CDC reported that bacterial disease transmission through the consumption of contaminated meat products contributes to 20% of antibiotic-resistant infections in humans (CDC, 2013,). There is a chance of biases in this data and actual figures may be even higher, which shows the need for further exploration.

Dr Levy in 1976 observed that the administration of fluoroquinolones to farm animals develops fluoroquinolone-resistant Enterobacteriaceae infections in humans, this study was later supported by Silbergeld *et al*. (2008). Similarly, fluoroquinolone-resistant Enterobacteriaceae infections were observed in the United States when they started using fluoroquinolones to treat bacterial infections in their livestock (Gupta *et al*., 2004).

MATERIALS AND METHODS

3.1. Specimen collection

From IDC (Islamabad Diagnostic Center) thoroughly about 250 urine samples were taken from non-hospitalized and hospitalized patients with complaints of UTI. They were collected with the patient's ethical consent. A sterile bottle and screw-capped container were given to the patient for midstream urine collection and all samples were labelled with patient ID, gender & date. Then for further analysis collected samples were brought to the microbiology lab within 30 minutes. Out of 250 collected samples for the current study,152 UPEC isolates were taken from UTI-confirmed patients.

3.2. Microbiological Testing and Analysis

3.2.1. Bacterial isolation

According to the available protocol calibrated sterile wire loop of 01µl (0.001ml) was used for the inoculation of the specimen on Cystin Lactose Electrolytes Deficient Agar (CLED Agar). The agar plates were incubated at 37°C for 24 hours under aerobic conditions. Growth appears at a concentration of > 100 colonies forming unit/ml was considered significant (Rijavee *et al.*, 2006). The collected primary culture was grown using MacConkey agar. The pure cultures preserved for a shorter period were put either on MacConkey plates or nutrient agar slants. On the other hand, the samples that needed to be stored for longer periods were preserved using 15% glycerol which was later stored at -20°C. Similarly, the nutrient agar, TSA, LB and EMB agar medium also streaked and observed their growth on all media.

3.2.2. Bacterial Diagnostic Identification

For bacterial identification, morphological and biochemical tests were conducted routinely. These practical methods were used to distinguish organisms from one another to verify the authenticity or utility of the strain. Two levels of identification, primary and secondary, were done by following the available different testing protocols. Based on gram staining and colony morphology, primary identification was made, and then the second level of

identification includes biochemical tests and molecular techniques known as the secondary level.

3.3. Identification of isolates

Widely used techniques for bacterial identification were Gram staining and biochemical testing to identify pure isolates. PCR was used for further identification and confirmation.

3.3.1. Phenotypic and Cultural Identification

Selective media in the case of UPEC such as CLED, and MacConkey agar were used to screen the isolates by checking their growth on selected agar. Morpho-cultural characteristics of Gram-negative bacteria especially *E. coli* on agar plates size (small, moderate, and large), colour (yellow, pink), shape (round, irregular colonies), border and prominence (convex, concave, raised) were considered while picking up colonies for further analysis such as Gram staining and biochemical testing.

3.3.2. Gram-staining

For gram-staining and further analysis, the selected colonies from MacConkey agar or CLED were picked carefully. The following steps were followed for gram staining.

Smear preparation

In the first step take a neat glass slide and place one drop of sterile normal saline on it. Then, with a hap of a sterile wire loop pick a single colony from afore mentioned agar and transfer it on a glass slide in normal saline. Make a thin uniform smear by mixing colony in normal saline in a circular motion in an area of 25-30mm across. The smear was left to air dry for a while.

Smear fixation

The air-dried prepared smear was passed two to three times through the flame for proper fixation. After passing through the flame the fixed smear slide was then allowed to cool for 30-50 seconds.

Staining methodology

- According to the protocol of Gephardt *et al*, 1981, the primary dye crystal violet was used to cover the fixed smear for one minute.
- After 1 minute wash off the stain gently with an indirect stream of tape water or distilled water then all the water was tipped off from the slide within a couple of seconds (s).
- Flood the slide with the drops of mordent (Gram iodine) for 60 seconds, and gently wash the slide under tap water or distilled water for 2 s.
- Now Flood the slide with a decolorizing agent, leave it for 15 seconds or add single drops until the decolourizer runs from the slide.
- After that cover, the smear with counter-dye safranine for 30 s to 1 minute.
- Finally, the smear was washed with an indirect stream of tap water until no colour appeared in the effluent and then allowed to air dry or blot dry with absorbent paper.
- Now take a microscope with a 100x lens and observe results under oil immersion.

3.3.3. Biochemical and molecular identification

Many biochemical tests, mainly seven tests were conducted Indole ring test, Urea hydrolysis test, Triple sugar iron (TSI), Citrate utilization, Catalase test, Lactose fermentation, and Methyl-red test were performed for further identification.

1. Indole test

It is used as a part of the IMViC procedure, for the confirmation and distinction among members of the Enterobacteriaceae family of bacteria, this test is used.

Objective

To determine the ability of organisms (bacterial samples) to produce the enzyme tryptophanase.

Principle

This principle states that the bacterial enzyme tryptophanase accumulates indole from tryptophan (an amino acid). Tryptophan shows deamination and hydrolysis by certain

bacteria producing tryptophanase enzyme and converting tryptophan to indole. The test was performed to check the ability of certain bacteria to decompose tryptophan to Indole, which gives a Colour reaction with para-dimethylaminobenzaldehyde (DMAB) (Kovac's reagent).

When indole is combined with Kovac's reagent a cherry red to brown colour ring is formed in peptone water at the top of the broth. Cherry red ring forms on the top of the broth because amyl alcohol is not water-soluble, and it is an oily layer. The lack of ring formation indicates that no indole was utilized. The following reaction will occur during the test.

Tryptophan + H2O ———— tryptophanase enzyme ——> Indole + Pyruvic acid + Ammonia

Material required.

Glass test tubes, sterile loop, Peptone broth or SIM media (a nutrient enriched with Amino acid tryptophan), 18-24hrs culture plates bacterial sample (*Escherichia coli and Klebsiella sp.*), Kovac's reagent (p-Dimethylaminobenzaldehyde, Hydrochloric Acid-37%, Amyl Alcohol), incubator.

Procedure

- Prepare tryptophan broth (peptone broth) autoclave broth and put 4ml broth in each glass test tube.
- Take test tubes with broth, cover them with air-tight lids and sterilize them in an autoclave.
- The colonies were isolated from the 18-24hrs fresh culture plate by using a sterile loop, inoculated into the 4ml peptone broth, and grown at 37°C for 24-28hrs.
- After incubation time 0.5ml of the Kovac's reagent was added to the tube drop by drop touching the wall of the glass tubes.
- Roll the glass tubes between palms to mix the reagent through the culture or shake properly for mixing.
- Let stand for a while and the result was observed.

- Note the development of a cherry red ring on the top of the peptone broth in the case of UPEC. The two possible results were as given.
- Indole test positive: cherry red colour can be used as a control if testing for negative isolates. *(E. coli*)
- Indole test negative: no red colour can be used as a control if testing for positive isolates. *(Klebsiella pneumonia*)

2. Urea hydrolysis test

The urea hydrolysis test efficiently differentiates between two bacterial strains: (1) *E. coli* and (2) *Klebsiella pneumonia*.

Objective

To determine the organism's (bacteria sample) capability of producing urease enzyme that is required for the breakdown of urea.

Principle

The Urea that is most toxic to living organisms is the common metabolic waste product of protein digestion. Urease, an enzyme helps in the degradation of urea into simple and less toxic products that as ammonia and carbon dioxide. The violated bacteria are inoculated in a slant containing urea and indicator phenol road. If this strain is urease-producing the culture medium will be degraded into the desired product. The hydrolysis of this strain is possible which yields CO2 and ammonia. The ammonia will make the medium alkaline shown by the colour change. This process of hydrolysis raises the pH of agar causing a change of colour from orange to reddish pink. Bacteria produce Urease and ensure that hydrolyze urea into ammonia and CO2. Organisms that don't have this enzyme will show no change in the colour of the media. The hydrolysis of urea in the positive test is given as.

Urea + H2O————urease enzyme———>Ammonia + CO2

Material required.

Christensen's Urea broth or MIU (mortality indole urea) medium, Burner, Incubator, Glass test tubes, sterile loop, 18-24hrs fresh Bacterial culture plates; *Proteus sp, klebsiella pneumonia* and *E. coli.*

Procedure

- Prepare a urea Christensen's Urea broth or MIU (mortality indole urea) medium by dissolving 2.9g of powder in 150 ml of distilled water.
- Autoclave the media and then add urea to avoid its breakdown.
- The freshly grown and isolated colonies will be picked carefully using a sterile inoculating needle.
- The organisms under study will be inoculated using Christensen's urea agar slants. Then it will be incubated for 18-24 hours at 37°C under aerobic conditions.
- The colour change will be observed and noted on the following day.
- Urease test Positive: Red-pink collar (*Proteus vulgaris, klebsiella sp*)
- Urease test Negative: No pink colour (*E. coli*)

3. Triple sugar iron test (TSI)

This test helps to differentiate Enterobacteriaceae from non-Enterobacteriaceae.

Objective

To assess the capacity of an organism for producing H2S gas and its ability for fermenting sugar (lactose, sucrose).

Principle

The principle which governs TSI is associated with the use of carbohydrates, ferrous ions and amino acids found in the medium. TSIA contains three sugars lactose, sucrose, and glucose as a source of carbon. For the identification of gram-negative enteric bacteria, this test is used. These types of enteric bacteria produce enzymes for glucose utilization and the other two sugars must first convert into glucose through different pathways. The phenol red in the medium changes its colour to yellow because of the acid. The inoculation of

microorganisms into the TSI agar slant makes it essential to observe colour changes in the slant and butt. Similarly, protein also degraded in aerobic conditions into alkaline products and changed the medium colour to pink-red. Gas H2S is also released and detected when holes are formed, and the medium is broken. The colour change that is observed due to H2S is black. The following type of colour change is observed in the TSI test.

The yellow colour shows fermentation with acid accumulation.

A pink or red colour shows protein catabolism with alkaline products.

Black precipitate indicates the production of Hydrogen Sulfide (H2S).

$FeSO4 + H2S \longrightarrow H2SO4 + FeS$ (Black ppt)

Material required.

TSIA slant, Glass test tubes, sterile loop, 18-24hrs fresh bacterial cultures (*E. coli and Klebsiella*), sterile Inoculating wire, Burner, Incubator, weighing balance.

Procedure

- Prepare 15-20ml TSI agar with distilled water and agar powder and autoclave them with test tubes.
- Take 5-7ml TSI agar in each test tube and tilt the test tubes to make a slant till the media solidifies.
- A flamed inoculating needle will be used to collect the well-isolated colony from a bunch of freshly grown colonies.
- To inoculate the organism in agar, the slant will be stabbed from the bottom within 3-5 mm depth.
- After stabbing the needle will be withdrawn and then with the same loop streak the entire slant surface of the agar medium.
- The test tube will be capped loosely to incubate for 18-24 hours at 37°C under aerobic conditions.

- After overnight colour changes in the slant and butt will be observed and results will be noted.

4. Citrate utilization test

After TSI, the citrate test will be used for the identification of the difference between Enterobacteriaceae and non-Enterobacteriaceae. This method is specifically utilized to differentiate among different members of Enterobacteriaceae.

Objective

To find out the organism that can produce citrase enzyme.

Principle

The principle aims to utilize citrate as the only carbon source found in the medium. The citrase enzymes produced by bacteria degrade citrate without the intervention of coenzymes to oxaloacetic acid, acetic acid, and carbon dioxide. This carbon dioxide combines with the sodium that is present in the medium and forms sodium carbon carbonate which is responsible for raising pH. Citrate results in the degradation of ammonium salts into ammonia and causes alkalinity. Citrate utilizes bacteria as a sole source of carbon and utilizes ammonium salt as the sole source of nitrogen present in the medium. This shift in pH to alkaline as above 7.5 results in a colour change of the bromothymol indicator which is green as neutral pH in the medium changes into Prussian blue. In the case of zero utilization of citrate, there will be no change in colour observed. The reaction is as follows.

Citrate ——— citrate enzyme —-> Oxaloacetic acid + acetic acid

Oxaloacetic acid ————-> pyruvic acid + CO_2

$Na + CO_2$ ————>Na_2CO_3

Ammonium salts ⟶ NH_3

Material required.

Simmon's citrate agar slant, 18-24hrs fresh culture bacterial samples (*E. coli* and *Klebsiella* sp.), sterile Inoculating loop, glass test tubes, sterile Inoculating wire, Burner, Incubator, weighing balance.

Procedure

- Prepare Simmon citrate agar or Koser's citrate medium and put 5ml in each test tube.
- Tilt the test tubes to prepare the slant and butt that contain the agar medium in melted form.
- For the collection of inoculums from an isolated colony a sterile inoculating needle will be used.
- Then the citrate agar slant will be used for back-and-forth streaking with the help of a loop followed by labelling.
- The test tubes will be capped loosely.
- Then, the test tubes will be incubated for 18-24 hours at 37°C under aerobic conditions.
- After overnight incubation, colour changes will be observed and noted.
- Citrate test Positive: the colour of media changes to blue (*Klebsiella sp.*)
- Citrate test Negative: No change in colour of media (*E. coli*)

5. Catalase test

The test is used to identify the catalase-producing organism that detoxifies and degrades hydrogen peroxide into water and oxygen gas.

Objective

This test is used to check whether the microorganism is aerobic or anaerobic.

Principle

The basic principle is the breakdown of hydrogen peroxide with the catalase enzyme that bacteria produce. Aerobic and facultative anaerobic bacteria convert toxic hydrogen peroxide to non-toxic products because H_2O_2 is highly reactive and damages the cell components.

H_2O_2 ———— catalase ——>H_2O + O_2

Material required.

18-24hrs fresh culture of *Escherichia coli*, 3% Hydrogen peroxide, A glass slide, test tubes, and sterile Glass rod.

Procedure

- With the help of a sterile glass rod pick the fresh culture colonies of *Escherichia coli* and spread them on the glass slide.
- Prepare a fresh 3% hydrogen peroxide solution.
- With the help of a dropper, a drop of 3% hydrogen peroxide was placed on the bacterial colonies on a glass slide.
- Observe the immediate bubbling effervescence of O2.

Lactose fermentation test

This type of test is used to differentiate between differentiating coliforms from non-coliforms.

Objective

To check the ability of bacteria to ferment the carbohydrate (lactose) as a carbon source.

Principle

The principle of this test states the capacity of some bacterial groups to assimilate necessary carbohydrates found in media like CLED agar and MacConkey and added lactose with indicator. A colour change in the indicator is observed.

Material required.

Petri plates, permanent marker, sanitizer, flame, MacConkey agar, CLED agar, incubator, autoclave, toothpicks, fresh inoculum, lactose, indicator.

Procedure

- Clean the hood, burn the flame, and place autoclaved material including petri plates, toothpicks and other the in hood with ethanol spirit or hand sanitizer.
- The tested organism *Escherichia coli* is streaked upon MacConkey agar plates under controlled conditions.
- Label them with isolated numbers that are streaked upon them.
- These streaked agar plates were incubated for 18-24 hours at 37° under aerobic conditions.
- A change in the colour of the medium was observed the next day.

6. Methyl-red test:

All Enterobacteriaceae family convert glucose into pyruvic acid and further enter two other pathways. During the fermentation of glucose, this test is used to find enough acid production by bacterial species. This is the quantitative measuring of acid production.

Objective

To check the ability of bacteria to ferment glucose and convert it into acids.

Principle

This test is based on the ability of certain bacterial groups to utilize glucose in media such as CLED agar and MacConkey and convert it into acetic acid or formic acid and lactic acid with indicator. A colour change in the methyl indicator is observed. The species-specific stable acid is formed by a mixed acid pathway at the end of the metabolization of glucose and the intermediate product is pyruvic acid. When the bacteria utilize glucose present in the medium the methyl red changes from yellow to red during the addition of methyl red indicator in the broth medium with bacterial growth. The pH of the medium also changes because of the acid accumulation and converts to acidic pH with time.

Material required.

Petri plates, permanent marker, sanitizer, flame, MacConkey agar, CLED agar, incubator, autoclave, toothpicks, fresh inoculum, glucose, indicator, MRVP broth (pH 6.9), Methyl red solution, 0.02%.

Procedure

- Clean the hood, burning flame, and place autoclaved material that includes petri plates, toothpicks, and others in the hood with ethanol spirit or hand sanitizer.
- The organism from 18 to 24 hours pure culture will be taken to lightly inoculate the medium.
- The sample will be incubated for 24 hours aerobically at 37°C.
- After 24 hours of incubation, aliquot 1ml of the broth to a clean test tube.
- The remaining broth will be reintubated in an additional 24 hours.
- About 2 to 3 drops of methyl red indicator will be added to the aliquot.
- Observe for red colour immediately.

7. API (Analytical Profile Index) 20E/10S Test

The Kits include strips to perform 20 or 10 miniature biochemical tests and provide accurate identification of gram-negative and gram-positive bacteria to the species level. It is based on an extensive database and is standardized. This API 20E/10S is a biochemical panel for the identification and differentiation of members of the Enterobacteriaceae family. It is an easy-to-use Test system.

Objective

To identify and differentiate members of the family of Enterobacteriaceae.

Principle

The API series provides an established, scaled-down version of the earlier identification methods, which were difficult to read and complicated to apply. The plastic strip of API 20E has twenty miniature test chambers and 10S has ten miniature test chambers with the dehydrated medium that have specific chemical compositions for each test. Enzymatic

activity, particularly involving the inoculated organisms' catabolism of proteins or amino acids or fermentation of carbohydrates, is often detected by them.

The strips are incubated after each well has been rehydrated with a bacterial solution. During incubation, metabolism results in colour changes that can occur naturally or are brought on by the addition of chemicals. A profile number is generated from the sum of all positive and negative test results, which is then compared to profile numbers in a commercial codebook (or online) to identify the bacterial species.

Material required.

API kit, bacterial isolate, sterile distilled water, sterile oil, and other necessary equipment.

Procedure

- Verify the culture is Enterobacteriaceae. A fast oxidase test for cytochrome c oxidase may be carried out to verify this.
- Select one isolated colony (from a pure culture) and prepare a suspension of it in sterile distilled water.
- Consider the API 20E/10S Biochemical Test Strip, which has 20 or 10 distinct compartments for dehydrated bacterial media/biochemical reagents.
- Fill the compartments with the bacterial suspension using a Pasteur pipette.
- The ADH, LDC, ODC, H2S, and URE compartments should all be filled with sterile oil.
- Add a few water droplets to the tray, insert the API Test strip, and shut it.
- Incubate the tray at 37oC for 18 to 24 hours.

3.4. Genomic characterization and analysis

The techniques for molecular identification will be used after the implementation of phenotypic and biochemical identifications. For this purpose, several steps were followed, like DNA extraction using PCR technique to gel electrophoresis. Three basic methods are used in our lab for DNA extraction that we used named Phenol-chloroform method, the boiling method and the CTAB method. I followed the CTAB method for DNA extraction and followed the following protocol that is below.

3.4.1. CTAB (cetyltrimethylammonium bromide) Method for DNA extraction

One of the most useful and best methods for DNA extraction. It will help to separate polysaccharides and rescue nucleic acid. EDTA is present in the CTAB that prevents DNA degradation.

Principle

CTAB is an ionic detergent that disrupts membranes and a chloroform-isoamyl alcohol mixture that separates contaminants from the organic phase and nucleic acid into the aqueous phase.

STEP 1: Streaking of *E. coli* isolates on MacConkey agar

Clinical isolates of *E. coli* identified by microbiological and biochemical techniques were put on MacConkey agar plates by streaking from the preserved glycerol stock, followed by an incubation period of 18-24 hours at 37°C under aerobic conditions.

STEP 2: Refresh culture for DNA extraction

Material required.

Working hood, UV, Nutrient broth, blue tips, yellow tips, Eppendorf, autoclave, permanent marker, shaker incubator, a pipette of different ranges, discarder, weighing balance, flask, burner etc.

Protocol

- Prepare nutrient broth as per protocol with distilled water and nutrient powder and autoclaved.
- Take autoclaved Eppendorf and label them. Put 1 ml of nutrient broth in each Eppendorf.
- Label Eppendorf with respective bacterial isolate numbers.
- Isolated colonies of *E. coli* from the fresh culture of MacConkey were picked up very carefully with an aseptic wire loop and suspended into an Eppendorf-containing nutrient broth.

- Place the Eppendorf in a shaker incubator for 18-24 hours at 37°C under aerobic conditions.
- We can also use a stock of glycerol to refresh bacterial isolates.
- Preserved stock is present in glycerol, but to prevent any sort of contamination, we don't use it regularly. Hence, make new fresh stock.
- Use 1ml nutrient broth in Eppendorf that is prepared and take a 50µl culture from preserved stock and add it in media.
- Incubate it in a shaker incubator for 24 hours or more hours at 37°C under aerobic conditions.
- After incubation, if the media was turbid, it means that there has been growth and then we move toward plating.
- The platting method is used to refresh and preserve stock.

STEP 3: DNA extraction by using CTAB.

Material required.

TE buffer, 5M NaCl, CTAB, Chloroform, phenol-chloroform, Na-acetate, ice-cold ethanol, Eppendorf, and other equipment.

CTAB Methodology

- In this method, 1 ml of an overnight culture of *E. coli* as aforementioned was taken and centrifuged at 3000rpm to pellet cells.
- Then the supernatant was discarded.
- Now 80µl TE buffer and 20µl NaC1 (5M) are added to the pellet and the pellet is re-suspended by vertexing.
- After that 10µl CTAB was added, and tubes were incubated at 60°C for 20 minutes.
- After incubating, 100µl chloroform was added and tubes were vortexed followed by incubation on ice for 30 minutes.
- The tubes were centrifuged in 21 microfuges at 10,000rpm for 10 minutes. The supernatant was then transferred to newly labelled Eppendorf tubes.

- Now 100µl phenol-chloroform was added and the tubes were vortexed to get a milky solution followed by centrifugation at top speed for 5 minutes.
- Supernatant was transferred to a new labelled tube.
- Now 100µl of chloroform was added and tubes were vortexed followed by centrifugation for 5 min.
- The supernatant was finally transmitted to a new labelled tube to which 10µl of Na-acetate and 200µl of ice-cold ethanol were added to facilitate DNA precipitation.
- After that, the tubes were incubated at -20°C for speed for 10 minutes.
- Ethanol is drained and the pellet is about 1 hour and spins at the top are retained.
- The pellet is re-suspended by adding 100µl of 70% ethanol and centrifuged to obtain the DNA pellet.
- After that ethanol is dried from the tubes by keeping the lids open.
- Finally, the pellet is resuspended in 20µl TE buffer and stored at -20°C to -80°C. (William, Feil, & Copeland, *et al*., 2012).

3.5. Quantification of DNA and its Evaluation

Nucleic acid solutions can be quantified by many methods. If the solution is pure one can use Spectrophotometric quantitation. Contrary to this small DNA amount has a higher risk of contamination; therefore, it's better to choose ultraviolet (UV)-induced emission of fluorescence. In this study, DNA quantitation was done by both aforementioned techniques.

3.5.1. DNA Quantification Methods.

1. Ethidium bromide fluorescence

Principle

The principle of gel-based ethidium bromide quantitation of DNA is based on the fact of embolism of nucleotides by ethidium bromide and then the release of its fluorescence under ultraviolet light.

Procedure

- 1% agarose gel (Life Technologies) gel was prepared in IX TBE buffer.
  For 1% add 0.4g agar powder in 36ml distilled water 4ml TBE buffer,
  For 1.5% add 0.6g agar powder in 36ml distilled water+ 4ml TBE buffer,
  For 2% add 0.8g agar powder in 36ml distilled water 4ml TBE buffer.
- Place the above mixture in a beaker on a hot plate. Do not allow much boiling remove at intervals, and boil until transparency appears in the solution.
- Remove from the hot plate and allow it to cool.
- The ethidium bromide solution in quantity 2-4 µl will be added.
- After the gel was solidified, approximately 2-3µl of extracted DNA was mixed with 2µl of 6X loading dye (Therma Scientific) and was loaded into wells, next to a DNA marker (Therma Scientific 1 kb ladder).
- The gel was then run for 35-40 minutes at a voltage of 75 Volts in IX TBE buffer (BIO-RAD gel electrophoresis tank).
- After the adjusted period, the gel was observed by a UV trans-illuminator to visualize DNA bands.
- The BIO-RAD Gel Documentation system was able to capture band photos.

2. Spectrophotometric quantitation

Principle

The principle of Spectrophotometric analysis is based on the Lambert-Beer equation and on the fact that nucleic acids have a maximum absorption at 260 mm.

Procedure

- For Spectrophotometric quantitation genomic DNA was suspended in IX TE buffer or distilled H_2O to prepare different dilutions of DNA.
- DNA concentration was calculated by using the formula:

  dsDNA concentration µg/ml = abs260 × 50µg/ml.

- For diluted samples, the formula used was:
  dsDNA concentration µg/ml - abs260 x 50ug/mI x Dilution factor

3.6. PCR optimization for molecular detection of *E. coli*

For molecular detection of *E. coli* isolates the PCR conditions will be adjusted accordingly to facilitate the amplification of the gene of interest. Thus, the primers with 450bp proximal and distal conserved flaking sites previously used by Khan *et al*. (2007) will be used. Primers were provided by Montreal Quebec.

Table 3.6 The table shows the designed primer sequence, length, and source.

***E. coli* 16s rRNA gene primer**	**Length**	**Source**
5′-CAATTTTCGTGTCCCCTTCG-3′	450bp	LUH. Khan *et al*, 2007
3′- GTTAATGATAGTGTGTCGAA-5′		

3.6.1. PCR reaction mixture

For the PCR reaction mixture, SOLIS BIODYNE 5X FIREPOL® Master Mix was used. It encompasses all the reagents required for PCR, buffer 7.5mM. $MgCI_2$., 200µM each dNTP, Tag polymerase, except template, primers, and water. The total reaction volume was kept at 20µl. The scheme of volumes used in this experiment while making the PCR reaction mixture is presented in the table below.

Table 3.6.1. Scheme of volumes used in PCR reaction mixtures.

Reaction components	Volume	Final Concentration
5X FIREPOL® Master Mix	4µl	1X
Forward primer(10pmol/µl) Reverse primer(10pmol/µl)	0.6µl 0.6µl	0.3µM 0.3µM
Template DNA	2µl	___

PCR water	12.8 µl	___
Total Reaction Mixture	20 µl	___

3.7. PCR amplification of Uropathogenic *Escherichia coli* genes

Primers of the specific genes were provided by Montreal Quebec and PCR conditions were optimized based on the research group and previous study.

3.7.1. PCR reaction mixture

For the PCR reaction mixture, SOLIS BIODYNE 5X FIREPOL® Master Mix was prepared. It encompasses all the reagents required for PCR, Taq buffer 10X, $MgCI_2$ 25mM, 10µM each dNTPs, and Taq polymerase. The total reaction volume was kept at 45µl for 5 samples and 90µl for 10 samples. The following method of volumes was employed while preparing the PC reaction mixture.

PCR reagents were provided by Solis Bio Dyne which contains all reagents of Master Mix. With the help of these reaction components, the master mix was prepared by using the following volume of reagents given in the table.

Table.3.7.1a. The table shows the scheme of volume used to prepare a master mix for PCR.

Reaction components	**Volume For 5 samples**	**Volume For 10 samples**
PCR water	31.5 µl	63 µl
Taq buffer (10X)	5 µl	10 µl
$MgCl_2$ (25mM)	3 µl	6 µl
dNTPs (10mM)	1 µl	2 µl
Forward Primer (10pM/ µl)	2 µl	4 µl
Reverse Primer (10pM/ µl)	2 µl	4 µl
Taq. Polymerase	0.5 µl	1 µl
Total Volume	45 µl	90 µl

The other protocol used for Master mix preparation includes the following reagents and concentrations.

Table 3.7.1b. The table shows the scheme of volume used to prepare a master mix for PCR with Taq buffer with $MgCl_2$.

Reaction components	Volume for 5 samples	Volume for 10 samples
PCR water	34.5 µl	69 µl
Taq buffer with $MgCl_2$	5 µl	10 µl
dNTPs (25mM)	1 µl	2 µl
Forward Primer (10pM/ µl)	2 µl	4 µl
Reverse Primer (10pM/ µl)	2 µl	4 µl
Taq. Polymerase	0.5 µl	1 µl
Total volume	45 µl	90 µl

- Add this reaction mixture of 9 µl in each PCR tube.
- Label them with an isolated number. Then add 1.5 µl of DNA in each labelled PCR tube.
- For negative control add 2 µl of DNA and 2 µl of PCR water to the respective PCR tube and mix it.

3.7.2. Screening and Optimization of the Housekeeping Gene

The two primers of housekeeping genes of UPEC were used for identification and the sequence of the primer used is shown in Table 3.7.2a.

Table.3.7.2a. The Forward and Reverse sequence with details of screened housekeeping genes (*Adk, RecA*).

Gene Name	Primer Sequence (5´ → 3´)		Annealing Temperature (°C)	Amplicon Size (bp)	Reference
ADK	F	ATTCTGCTTGGCGCTCCGGG			

(Adenylate kinase)	R	CCGTCAACTTTCGCGTATTT	66 °C	583bp	Tartof *et al*., 2005
*Rec*A (Recombinase A ATP/GTP Binding motif)	F	CGCATTCGCTTTACCCTGACC	67 °C	780bp	Tartof *et al*., 2005
	R	TCGTCGAAATCTACGGACCGGA			

Screening and Optimization of bla*CITM* Gene of UPEC

The primer used for the screening and optimization of the gene for β- lactam Amp C of UPEC is named *CITM* and the detail about the used primer is shown in the table.3.7.2b. below.

Table 3.7.2b. The table shows the Forward and Reverse sequence with details of the screened bla*CITM* Gene.

Gene Name	**Primer Sequence (5´⟶ 3´)**		**Annealing Temperature (°C)**	**Amplicon Size (bp)**	**Reference**
CITM (AmpC class resistant gene)	F	TGGCCAGAACTGACAGGCAAA	66.5°C	462bp	(Van *et al*2008)
	R	TTTCTCCTGAACGTGGCTGGC			

Screening and Optimization of Noval Genes UPEC

The sequence of the primer that was used to screen and optimize the gene in the uropathogenic strains of *E. coli* is shown in the given Table 3.7.2c.

Table.3.7.2c. The table shows the Forward and Reverse sequence with details of screened *A1, and B1* genes.

Gene Name	Primer Sequence (5′ ⟶ 3′)		Annealing Temperature (°C)	Amplicon Size (bp)	Nucleotide position	Reference
A1	F	AAAGGGTGGTC CTGG	54°C	223bp	72559 to 72781	Totsika *et al*., 2011
	R	ACGTCAGTTGC TGGC				
B1	F	CGAAGTTCAGC CCGCTATGT	58°C	397bp	23259 to 23655	Totsika *et al*., 2011
	R	GCTTTCCCAAG ATGCCTCAG				

3.8. PCR Parameters and Optimization Techniques

The PCR amplification technique will be used for *E. coli* identification. The optimizing conditions will include a pre-heating temperature of 95°C for 5 min and 35 cycles of amplification followed by denaturation at 95°C. the biosystem thermocycler will operate at the following conditions 2700 °C for 45 s (annealing), 72 °C for 01 minutes (extension), 72 °C for 10 min (final extension) and finally ∞ at 4 °C.

3.8.1. Agarose gel electrophoresis

Prepare 2% agarose in IX TBE buffer by adding 3-4µl Ethidium Bromide. After amplification 3-5µl of PCR product was added to the loading dye (2µl) (Thermo Scientific) which was then loaded in the specific wells of the gel prepared. The Thermo scientific kb ladder (a DNA marker) was also loaded in one of the wells. The gel was allowed to run for

45-55 min at 100 Volt in a gel electrophoresis tank (in IX TBE buffer). Then, the gel was studied under a UV trans-illuminator to identify and visualize necessary bands. The BIO-RAD Gel Documentation system was then used to take images of these bands.

3.9. Minimum inhibitory concentration

The Minimum Inhibitory Concentration (MIC) is the lowest ability of the antibiotic to inhibit the visible growth of the organism being studied under certain conditions. The MIC values are utilized to determine the susceptibility of bacteria to drugs and to evaluate the activity of new antibiotics. MIC is considered the gold standard for knowing the antimicrobial susceptibility patterns of bacteria. They also provide valuable information about pathogenic bacteria, unlike diffusion methods that only determine the quality of the antibiotic. The most common methods used for MIC determination are agar dilution and broth dilution. However, other techniques such as the E-strip method can also be used. Agar dilutions of antibiotics contain varying concentrations of nutrient agar, while in liquid dilutions it is known that the volume of juice containing the inoculum is mixed with a sample of bacterial cells containing copious amounts of antibiotics. Liquid mixing was included in our study and followed the guidelines recommended by CLSI and EUCAST.

Broth dilution

Principle

The technique works on the principle in which different concentrations of antibiotics are prepared in a liquid medium. After this, the known volume of inoculum is added to the standard number of cells.

Quality control

- The medium used must be properly autoclaved and its conditions must be adjusted according to the requirements of a particular antibiotic group.
- Bacteria to be tested should be identified up to genus and species level.
- Isolated and pure colonies of test organisms should be used.

- Standardization of bacterial suspension to be added is of crucial importance in susceptibility.
- Controls should be added along, to check the fidelity of protocol.

McFarland standards

Principle

McFarland standards are used to standardize the approximate number of bacterial cells in a suspension by comparing the turbidity of test suspension to McFarland standards.

Material required.

Antibiotic Enrofloxacin, 96-well Mic plate, Pipette, DMSO, McFarland standard, UPEC isolates, MHA broth, test tubes, Eppendorf's, Flame, weighing balance and other required material that used in all protocols.

Preparation

In clinical microbiology practices, for antimicrobial susceptibility testing, usually, 0.5 McFarland standard is used, containing approximately 0.5 X 10^8 CFU/ml.

The standard is prepared by mixing Barium Chloride ($BaCI_2$) and sulfuric acid (H_2SO4); as a result, Barium sulfate ($BaSO_4$) precipitates are formed resulting in turbidity.

To prepare 0.5 McFarland standard 0.5ml of 1.175% (w/v) $BaCI_2$.$2H_2O$ was mixed with 9.95ml of 1% H_2SO_4 to give a total volume of 10ml.

Antibiotic stock solution preparation

The antibiotic used was Enrofloxacin provided by Zhejiang Longhua Pharmaceutical Company Limited. Their stock solutions were prepared as per the manufacturer's instructions. First, the antibiotic stock would be prepared in the appropriate solvent and dilutant that is required. The drug would be weighed and dissolved properly to yield the required concentration.

The following formula was used to determine the amount of powder and dilutant for the preparation of a standard stock solution of Enrofloxacin.

Weight (mg) = Volume (ml)•Concentration (µg/ml)/Potency (µg/mg)

Volume (ml) = Weight (mg)•Potency (µg/mg)/ Concentration (µg/ml)

To find out the weight of powder for stock preparation the following formula was used.

W (mg) = [V (ml) x C (µg/ml)]/P (µg/mg)

W=weight of antibiotic (mg), C=final concentration of solution (5120µg/ml), V=volume required (ml)

P=potency of the drug (µg/mg) (given by the manufacturer or can be found by using molecular formula)

Potency = Anhydrous molecular formula of antibiotic x 100/Hydrous molecular formula of antibiotic.

Enrofloxacin stock

A stock solution of 5120µg/ml of Enrofloxacin' was prepared in DMSO (Dimethyl sulfoxide) solvent, or we can also use ethanol as a solvent and add PBS (Phosphate buffer saline) at room temperature.

0.56g of Enrofloxacin powder was properly weighed and added up to 10ml of DMSO and used within 3 days.

The final volume was adjusted to 10ml to give a stock concentration of 5120ug/ml. The stock was stored at room temperature till use.

Mueller-Hinton broth preparation

Mueller Hinton broth provided by (OXOIDE ENGLAND) was prepared according to the manufacturer's instructions in one litre of distilled water, boiled to completely dissolve it and then autoclaved at 121°C for 15 minutes.

Antibiotic dilution preparation for MIC

Dilutions of antibiotics were prepared according to CLSI 2013 guidelines; the dilution range was kept between 512µg/ml to 0.25µg/ml, using sterile, autoclaved MHB. Each dilution was kept 2X of the dilution to be tested. Prepare a working solution of the drug in 1 test tube for one microtiter plate. Add 1480µl MHB and 180µl stock solution in a test tube. To the microtiter plate 100µl of Mueller Hinton Broth (MHB) would be added from column second to column twelve. 200µl of the working solution was added to all the wells of the first column in the microtiter plate and then diluted by taking 100µl from the first to the second column using the multichannel pipette and mixing properly and taking 100µl from the diluted second well to the third and mixed and so on till the last well of last column wells diluted and discard 100µl at the end.

Microbial suspension preparation

As per CLSI recommendations, samples were streaked on MacConkey agar plates and incubated overnight to get pure and isolated colonies. After overnight incubation 3- get pure 3-5 isolated colonies were touched using a sterile wire loop and were suspended in 1ml MHB in Eppendorf. To get a uniform suspension, mix it properly and place the prepared primary suspension in a shaker incubator for 15-20min. Check the turbidity after 15-20 minutes. Then using a micro-pipette 20µl of this suspension was added into 1980µl of MHB to get a final cell count of 5 $x10^5$ to $1x10^5$ CFU/ml and this is the secondary suspension.

Controls

As per CLSI recommendations, two controls were also added, in one well of the microtiter plate 100 µl autoclaved sterile media without antibiotics was added to check the sterility of the media. In the second control 100µl of working solution with antibiotic was added and 100µl of inoculum / secondary suspension of each isolate without antibiotic in wells.

MIC determination

The results were noted after overnight incubation of test tubes where they ranged from slightly turbid to non-turbid. The decision of turbidity depends on the visualization of the

solution in the tubes. The minimum inhibitory concertation will be determined when the lowest possible amount inhibits bacterial growth.

RESULTS

A total of 250 isolates were collected from IDC (Islamabad Diagnostic Center) and out of these 250 isolates 152 were confirmed as uropathogenic *E. coli* by using different techniques including Morpho-cultural, biochemical, and molecular techniques.

4.1. Bacterial Isolation:

The bacterial morpho-cultural characteristics and gram staining results appeared as:

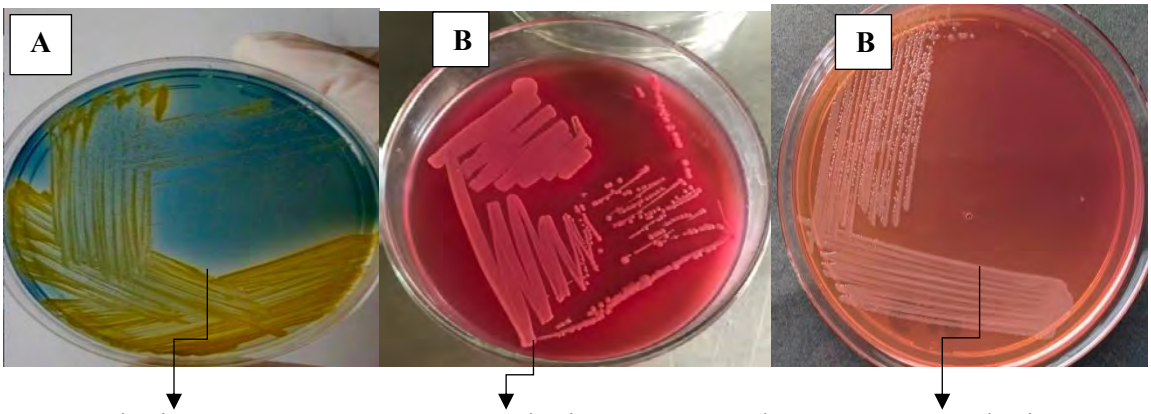
The given table 4.1.1 and Figure 4.1.2 below show the appearance and morphology of the selected UPEC isolates.

Table. 4.1.1 Primary Level of Identification of Uropathogenic *E. coli* and Interpretation

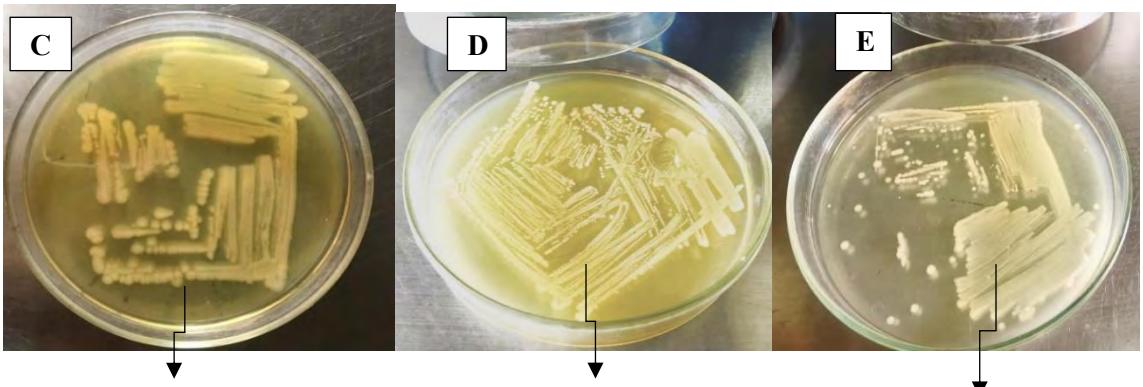
Techniques	**Results**
1. Phenotypic characteristics	-----
CLED agar **(A)**	Yellow, moderate, round, and convex colonies.
MacConkey agar LF **(B)**	Pink, moderate, large, elevated and doughnut-shaped colonies.
MacConkey agar NLF**(B)**	flat, dry, pale, non-mucoid colonies with a darker pink area
LB agar **(C)**	Large, thick, greyish-white, moist, smooth, opaque colonies.
TSA agar **(D)**	Large, thick, greyish-white, moist, smooth, opaque colonies.
Nutrient agar **(E)**	Large, thick, greyish-white, moist, smooth, opaque colonies.
EMB agar **(F)**	Green metallic sheen colonies
2. Gram staining (G)	Gram-negative (pink), short rods.

The figure here shows (A to G) the colonies on different selected media in the form of images. The lactose fermenter and non-lactose fermenters can be seen clearly on the agar in petri plates. The microscopic image of the isolate's gram staining showed pink and rod-shaped colonies of gram-positive *E. coli.*

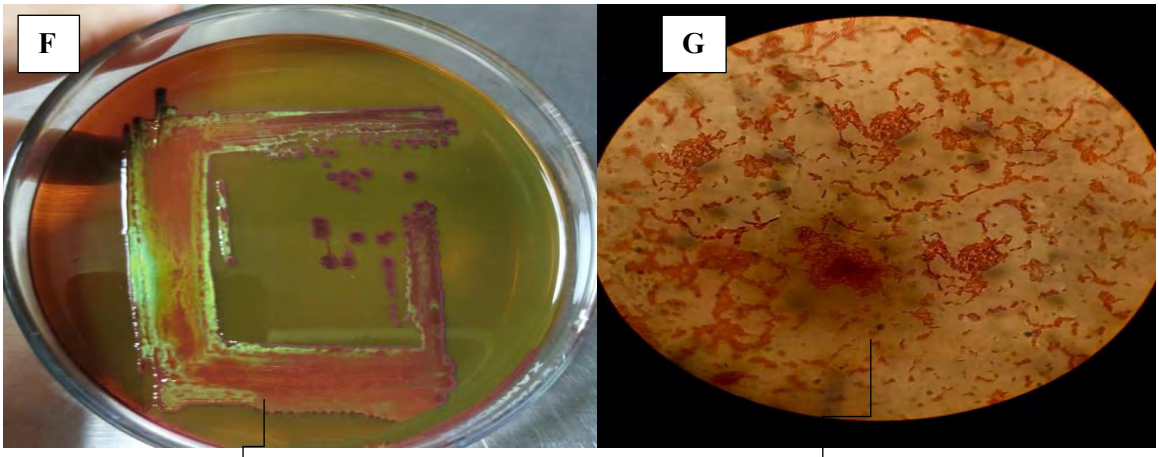
Figure 4.1.2 The figure shows the results of the morphology of *E. coli* on different agar mediums and Gram staining.



A. LF colonies on CLED agar **B.** LF colonies on MacConkey agar, NLF colonies on MA



C. *E. coli* colonies on LB agar **D**. *E. coli* colonies on TSA **E**. *E. coli* colonies on NA



F. *E. coli* metallic sheen colonies on EMB agar. **G.***E. coli* Gram -ive, pink, short rods

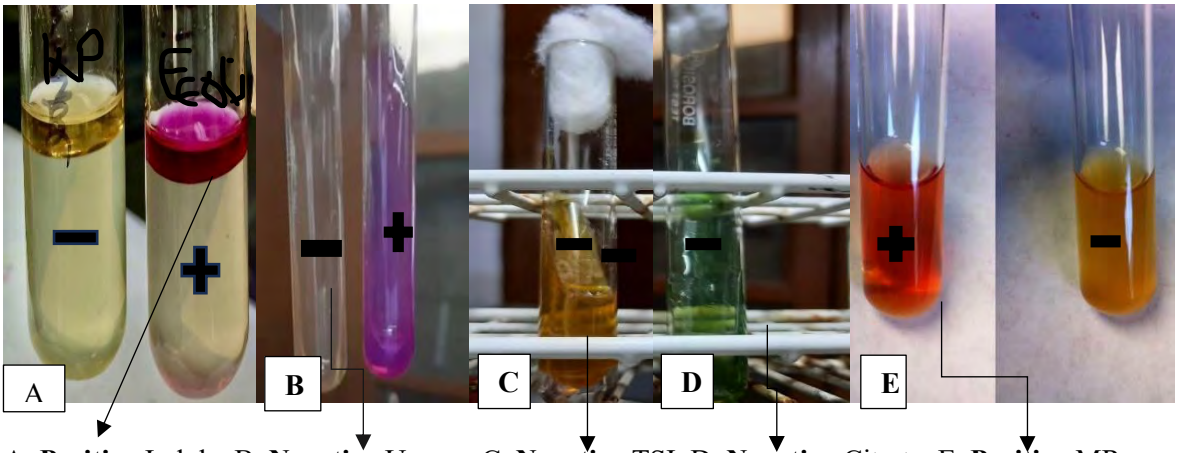
4.2. Biochemical Identification

Biochemical and enzymatic characteristics of microorganisms used for the secondary level of identification. Seven biochemical tests were conducted including the Indole ring test, Urea hydrolysis test, Triple sugar iron (TSI), Citrate utilization, Catalase test, Lactose fermentation, and Methyl-red test. The 160 isolates were confirmed as Uropathogenic *E. coli* based on these biochemical tests. The characteristics possessed by *E. coli* on behalf of these biochemical tests are the following.

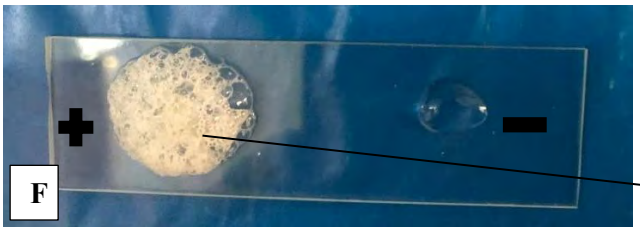
Table 4.2.1 Secondary level of identification (Biochemical Tests) and their interpretation

#	**Name of Biochemical Test**	**Result**	**Visual confirmation**
1	Indole Test	+ive	The reddish-brown ring formed on top.
2	Urea hydrolysis Test	-ive	No colour changes.
3	Triple sugar Iron Test	-ive	Yellow butt and yellow slant.
4	Citrate Utilization Test	-ive	No colour changes.
5	Catalase Test	+ive	Bubbles formation.
6	Lactose Fermentation Test	+ive	Pink colonies on MA & yellow colonies on CLED
7	Methyl-Red Test	+ive	MR VP broth colour changes and turns red.

Figure 4.2.2 The figure shows the results of biochemical tests.



A. **Positive** Indole, B. **Negative** Urease, C. **Negative** TSI, D. **Negative** Citrate, E. **Positive** MR,



F. **Positive** Catalase.

4.3. API (Analytical Profile Index) 10s Test Results

After individual biochemical testing, the 10 miniature tests were performed in a 10S test strip of the API kit, and the result is shown in the table and figure.

Table 4.3.1. The result of these 10 tests of the API kit is given in the table here.

Tests	Result	Visual confirmation
ONPG	+ive	Yellow colour
GLU	+ive	Yellow grey colour
ARA	+ive	Yellow colour
LDC	+ive	Red-orange colour
ODC	+ive	Red-orange colour
CIT	-ive	Pale green/ yellow colour
H2S	-ive	Colourless/greyish colour
URE	-ive	Yellow colour
TDA	-ive	Yellow colour
IND	+ive	Pink colour

Figure 4.3.2 The result of the API Test is shown as

4.4. Refresh culture for DNA extraction

Before DNA extraction refreshes bacterial isolates in Nutrient broth. Turbid Eppendorf contains grown cultures whereas the transparent Eppendorf represents sterility or Media control having only media.

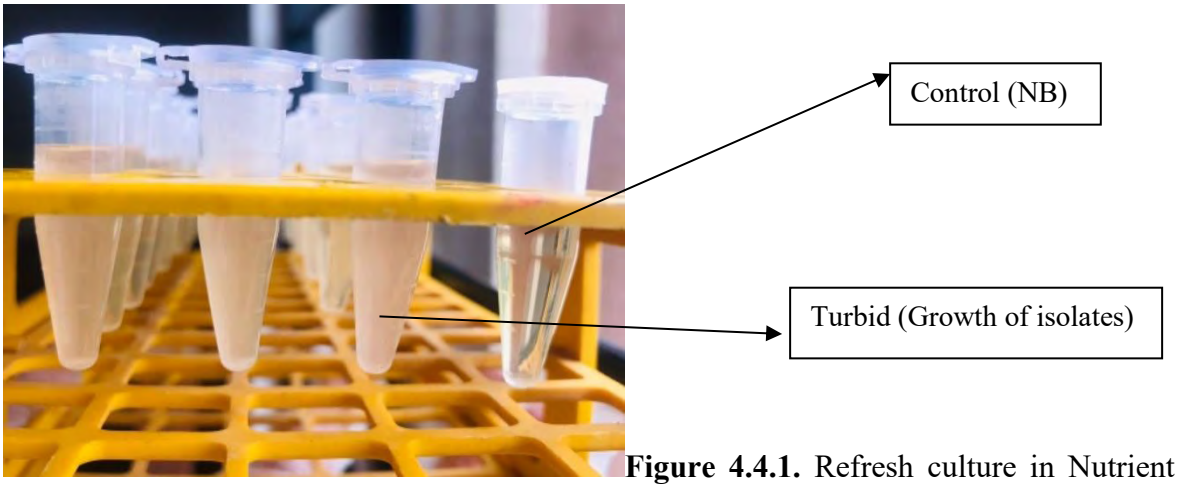


Figure 4.4.1. Refresh culture in Nutrient broth

4.5. PCR optimization for molecular detection of *E. coli.*

After biochemical identification, the DNA was extracted by the CTAB method and confirmed through an agarose gel, and molecular identification of *E. coli* strains was carried out. For further confirmation of *E. coli* isolates the PCR technique with a known pair of primers of 450bp of proximal and distal conserved flanking end of 16s rRNA was used, previously designed, and explained by Khan *et al*., 2007. Out of the 160 confirmed by biochemical testing, out of them, 152 were confirmed as *E. coli* by PCR.

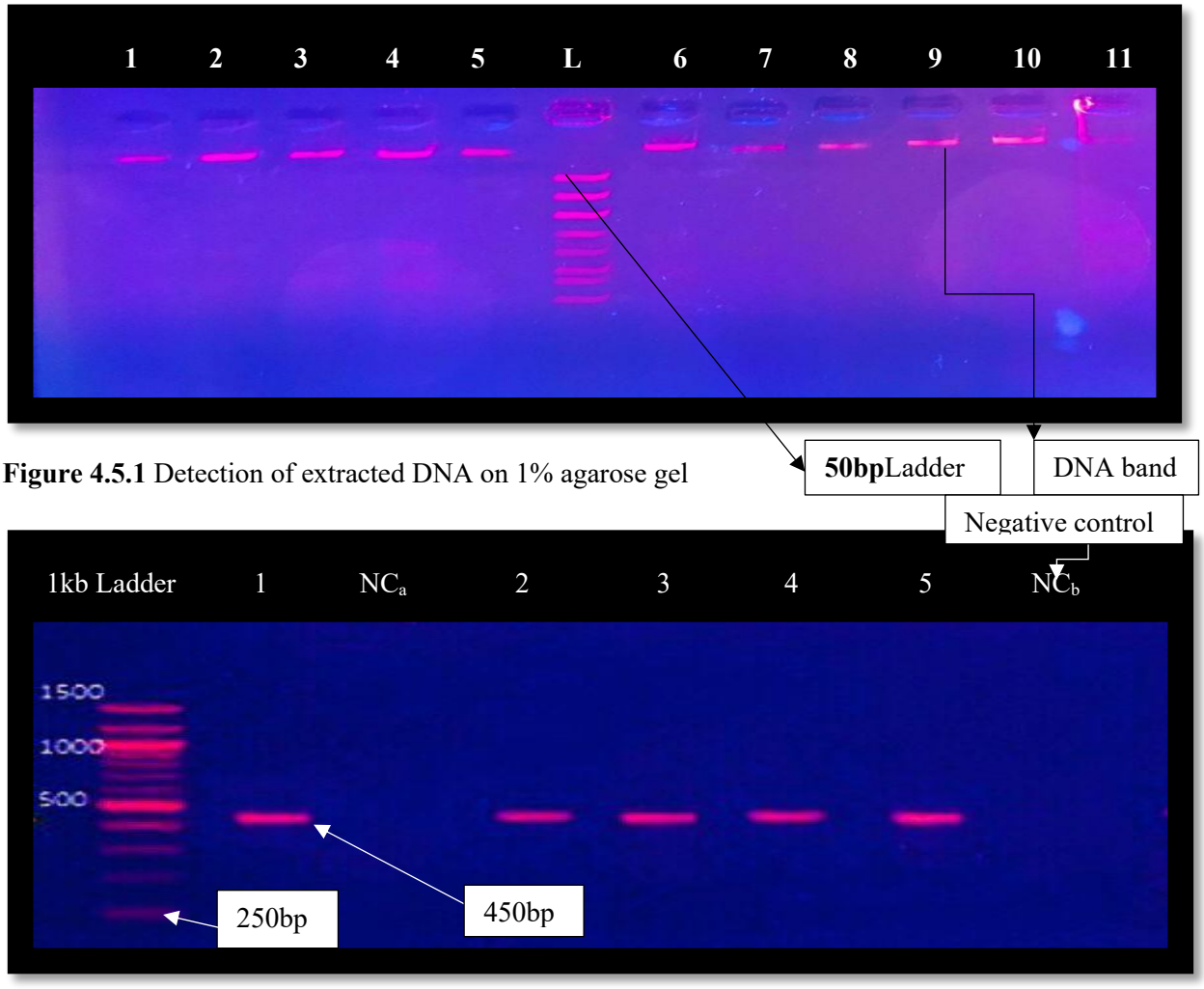


Figure 4.5.1 Detection of extracted DNA on 1% agarose gel

Figure 4.5.2. Molecular detection of *E. coli* (450bp 16s rRNA) on 1% agarose gel.

4.6. Molecular detection of housekeeping genes in Uropathogenic *Escherichia coli.*

Housekeeping genes of UPEC named *ADK* and Rec A were amplified. The fragment size of UPEC amplified housekeeping genes was compared with the marker of the 100bp DNA ladder. The size of the amplified UPEC housekeeping genes of *ADK* was (583bp) and Rec A (780bp).

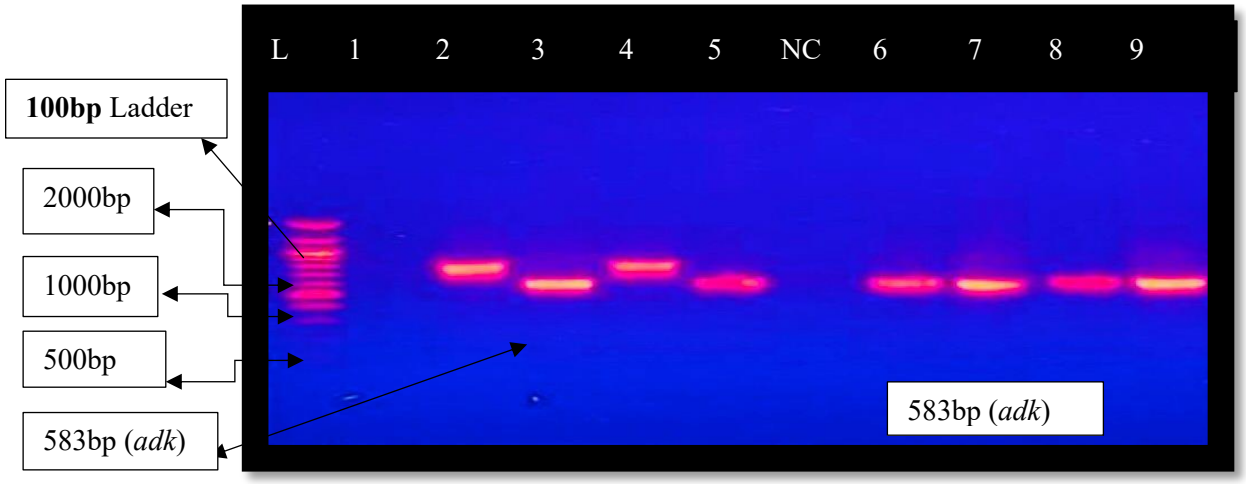


Figure 4.6.1 Amplified product of *Adk* gene (583bp) in 3,5,6,7,8,9 well along with 100bp ladder (L-Ladder) and negative control (NC)

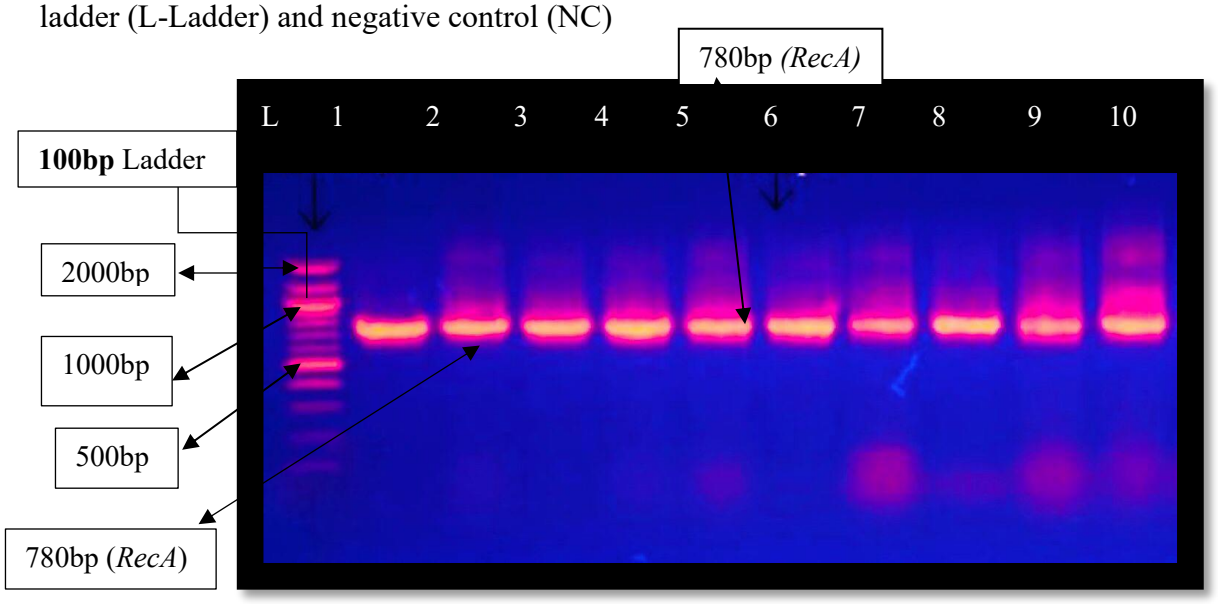


Figure 4.6.2 Amplified product of RecA (780bp) from 1-10 well along with 100bp ladder (L-Ladder)

4.7. Molecular detection of bla$_{CITM}$ gene in Uropathogenic *Escherichia coli*

The amplified bla$_{CITM}$ gene fragments were compared with the marker of the 50bp DNA Ladder. The size of the amplified β-lactam gene *CITM* was detected as 462bp.

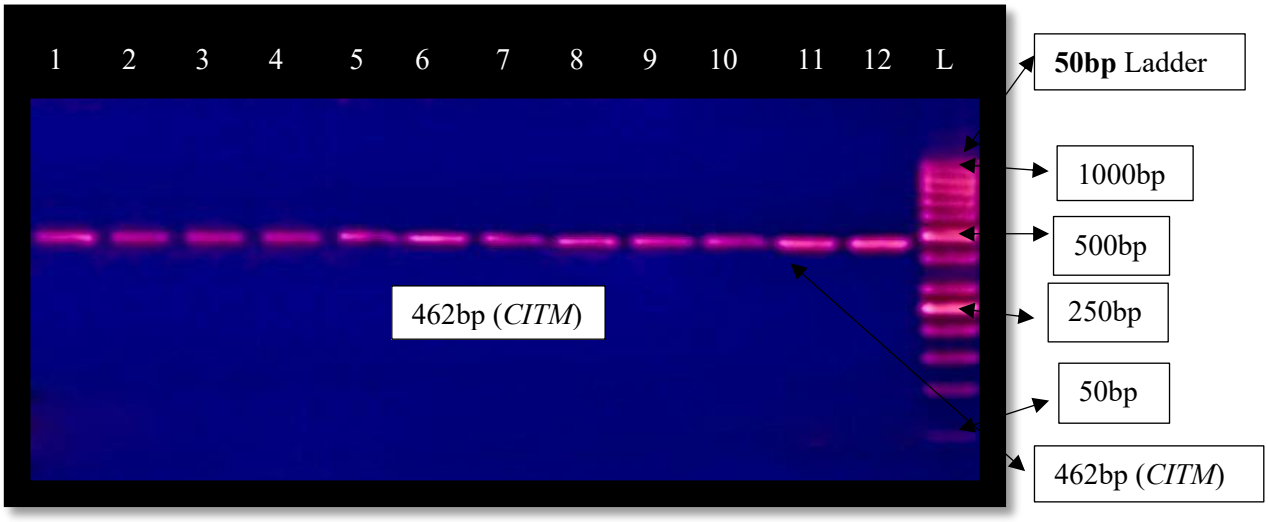


Figure 4.7.1 Molecular detection of *CITM* (462bp) on 2% Agarose gel runs against a 50bp ladder (L-Ladder).

4.8. Molecular detection of *A1* and *B1* gene in Uropathogenic *Escherichia coli*

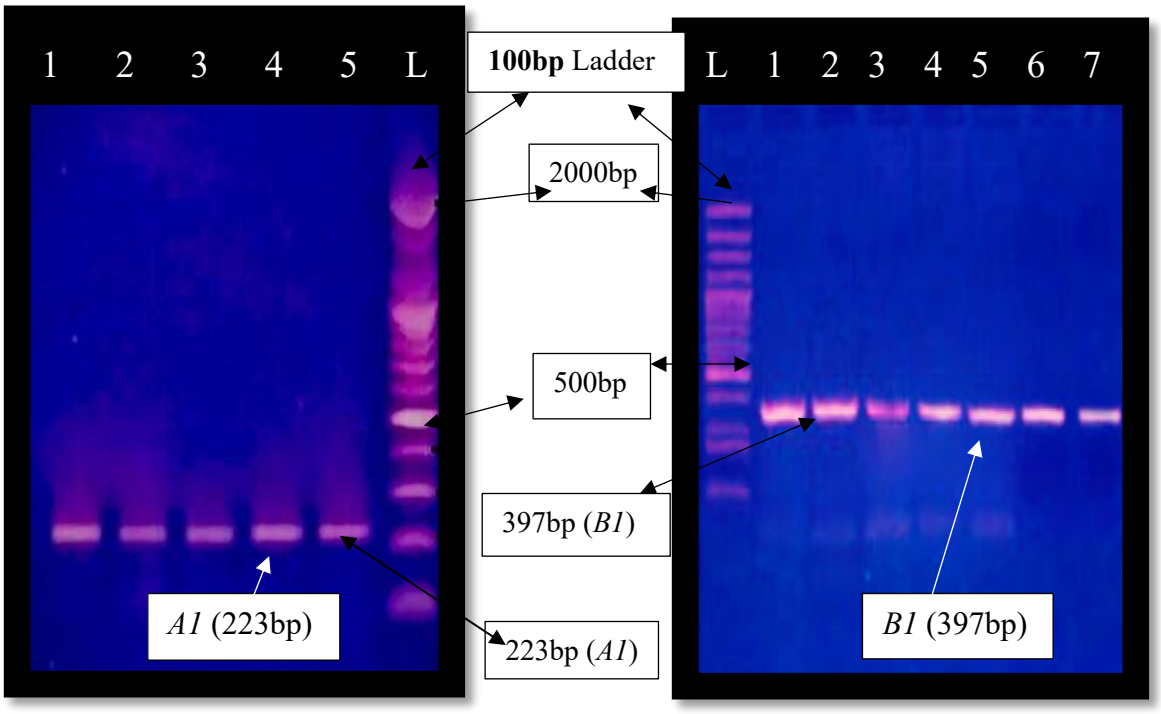


Figure 4.8.1 Molecular detection of *A1* (223bp) and *B1* (397bp) genes on 2% Agarose gel runs against a 100bp ladder (L-Ladder).

4.9. Results of Amplified Genes in Uropathogenic *Escherichia coli*

Prevalence of UTI in genders due to UPEC

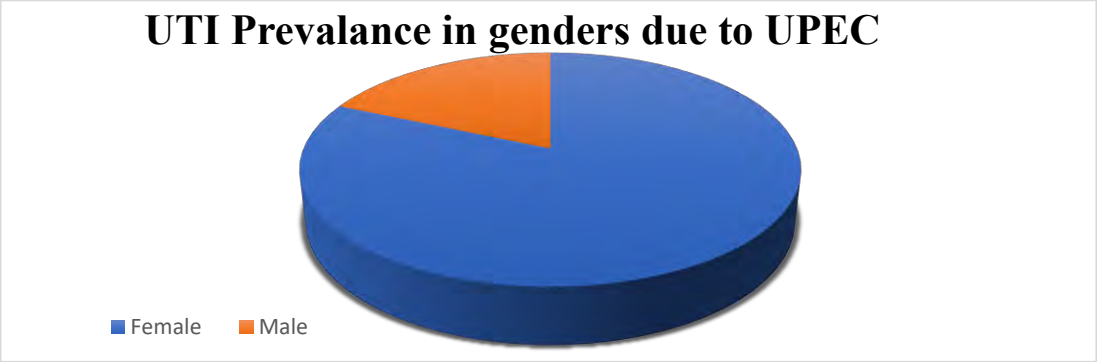


Figure 4.9.1. The pie chart shows the percentage of the male-female ratio of UTI due to UPEC Total number of isolates was 152. The ratio of female to male were 124:28, F(female)=124, M(male)=28.

Occurrence of screened housekeeping genes in Uropathogenic *E. coli*

The Pie-chart represents the percentage of *RecA* genes in Uropathogenic E.*coli*

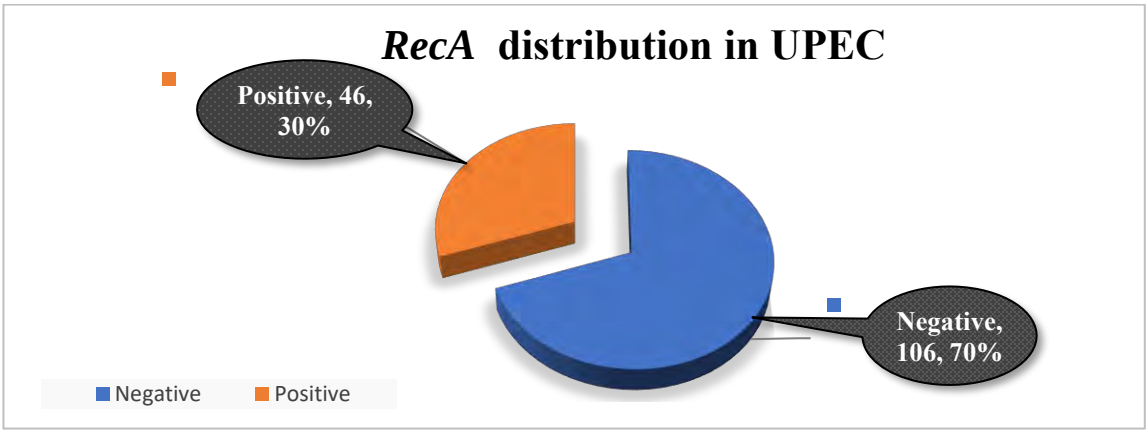


Figure 4.9.2: Showing percentage of *RecA* (housekeeping gene) in Uropathogenic *E. coli* strains, n=152, P=n=45(30%) and N=n=107(70%).

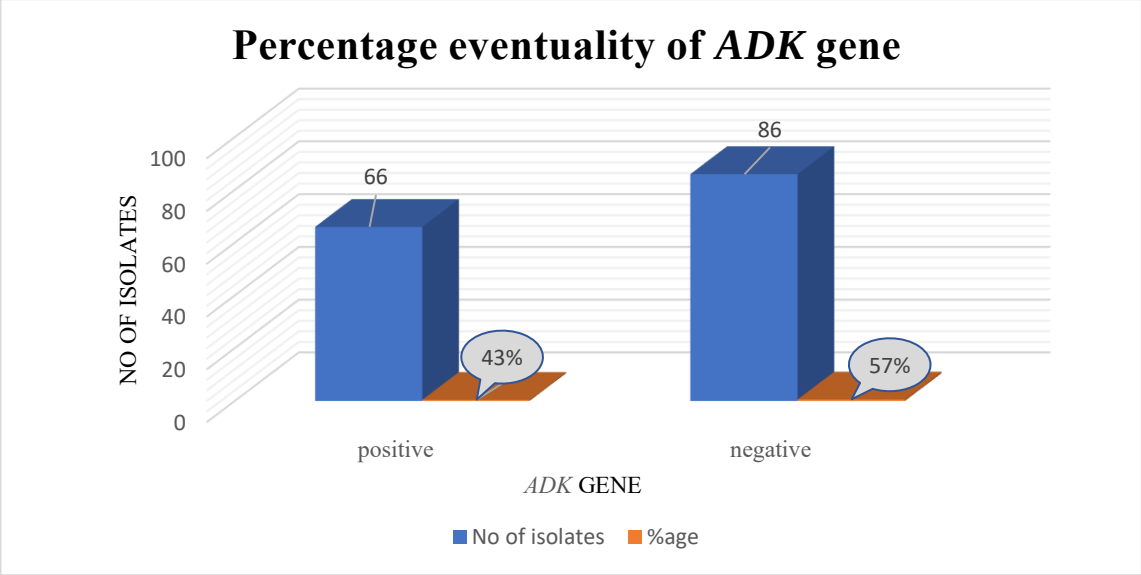


Figure 4.9.3: Depicting the percentage of *ADK* (housekeeping genes) in uropathogenic *E. coli* strains isolated from urine samples of UTI patients.

Detection of Amp-C genes among uropathogenic *Escherichia coli.*

The other Amp-C resistant gene families *FOX* and *DHA* were already amplified separately by using specific primer sequences. This *CITM* gene also belongs to the Amp-C family and was amplified by using specific primer sequences and find out their occurrence in UTI patients which is shown in the bar chart. Out of 152 UPEC isolates 4 were *CITM* positive.

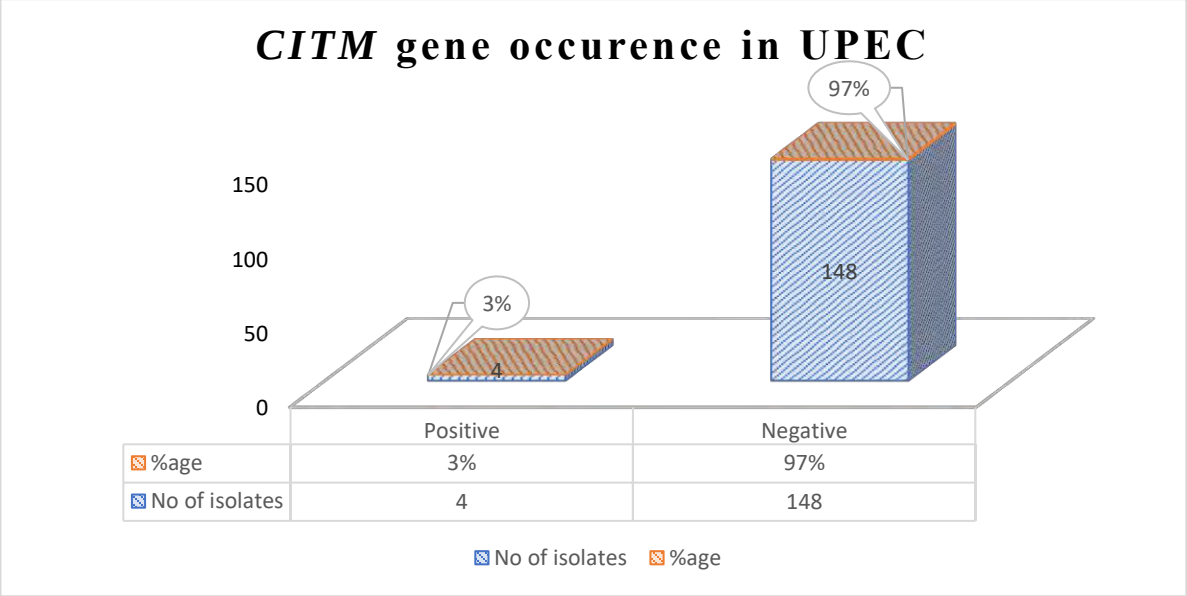


Figure 4.9.4. The following figure shows the Ampicillin C β- lactam gene percentage in *E. coli* strains of uropathogenic *E. coli* strains. (Total no. of isolates=n=152, Positive=P=4, Negative=N=148).

ESBLs and PABLs producers among these isolates

ESBLs detection was done by Ihsan *et al*., 2014 and PABLs detection was also done by another colleague with the same isolates. They reported a 42% prevalence of ESBLs and a 23% prevalence of PABLs producers among these isolates. Of the total isolates under study, 42% of isolates were ESBL formers from these isolates 1.5% contained the *CITM* gene.

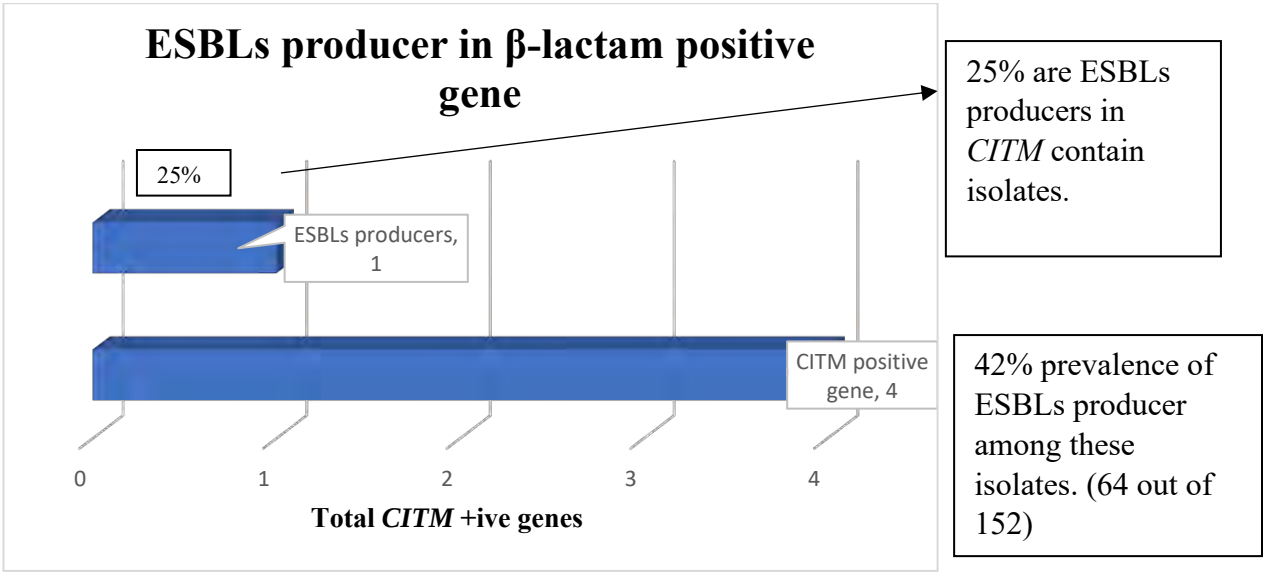


Figure 4.9.5 The bar graph shows the ESBL (Extended-spectrum β- lactam) producer in positive *CITM* isolates.

Distribution of amplified *A1* and *B1 genes* among Uropathogenic *E. coli.*

The percentage frequency of these novel genes was determined. Among all 152 samples, 37 isolates were *A1 positive*, and 51 isolates were *B1* positive shown in the figure below.

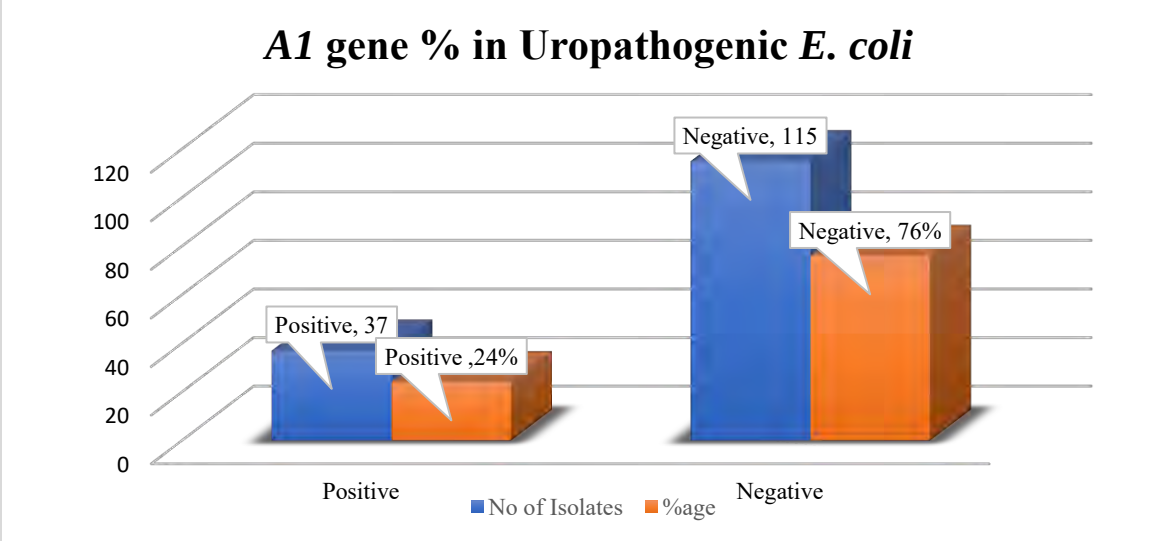


Figure 4.9.6: This graph shows the percentage of the *A1* gene in hospital-isolated uropathogenic *E. coli* strains. Total number of isolates were 152, N=115, P=37.

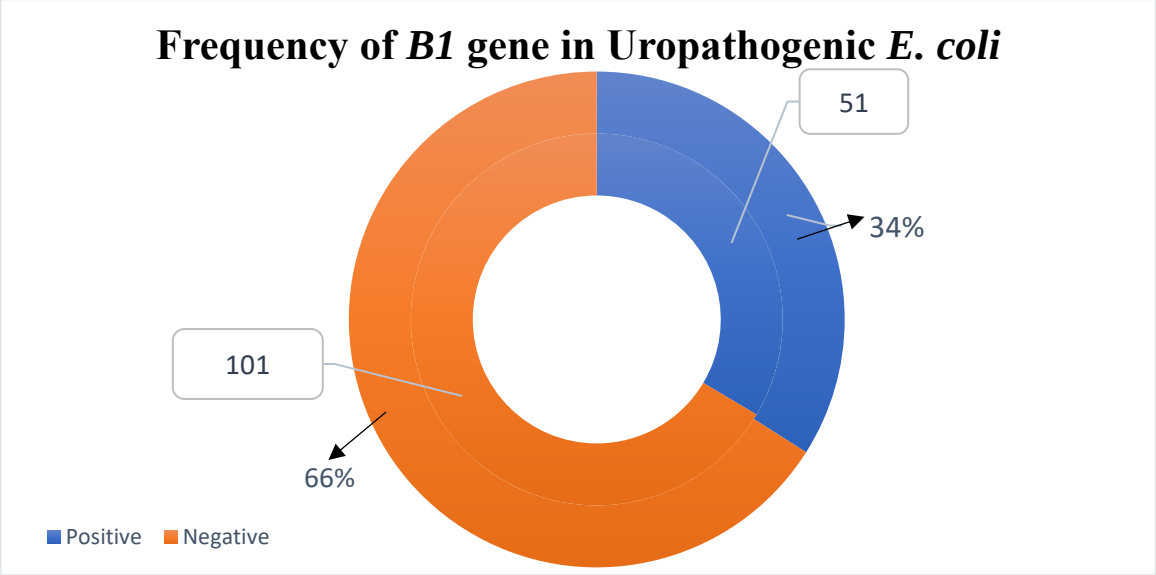


Figure 4.9.7: This pie chart shows the %age of the *B2* gene in UPEC. Total number of isolates were 152(n), P=51, N=101

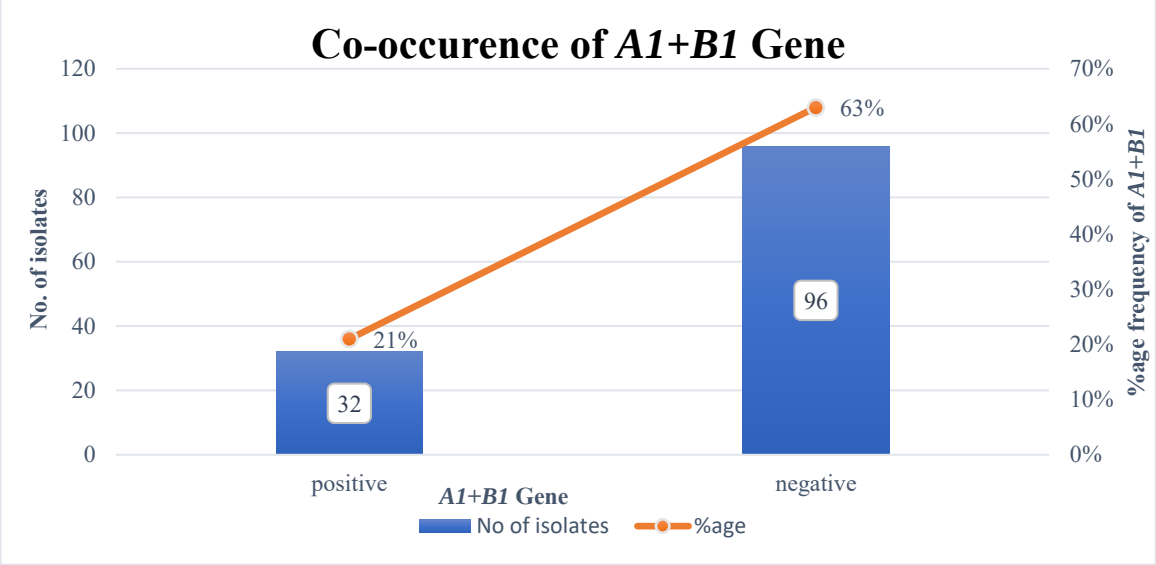


Figure 4.9.8: (*A1+B1*) Gene pair %age ubiquity in Uropathogenic *E. coli*N=96, P=32

Co-occurrence of these screened genes along UPEC

The various screened genes under study among all samples were also studied in pairs and combinations. The co-occurrence of the *A1+B1* gene was significant and showed their importance among isolates. The results are shown below in graphical form.

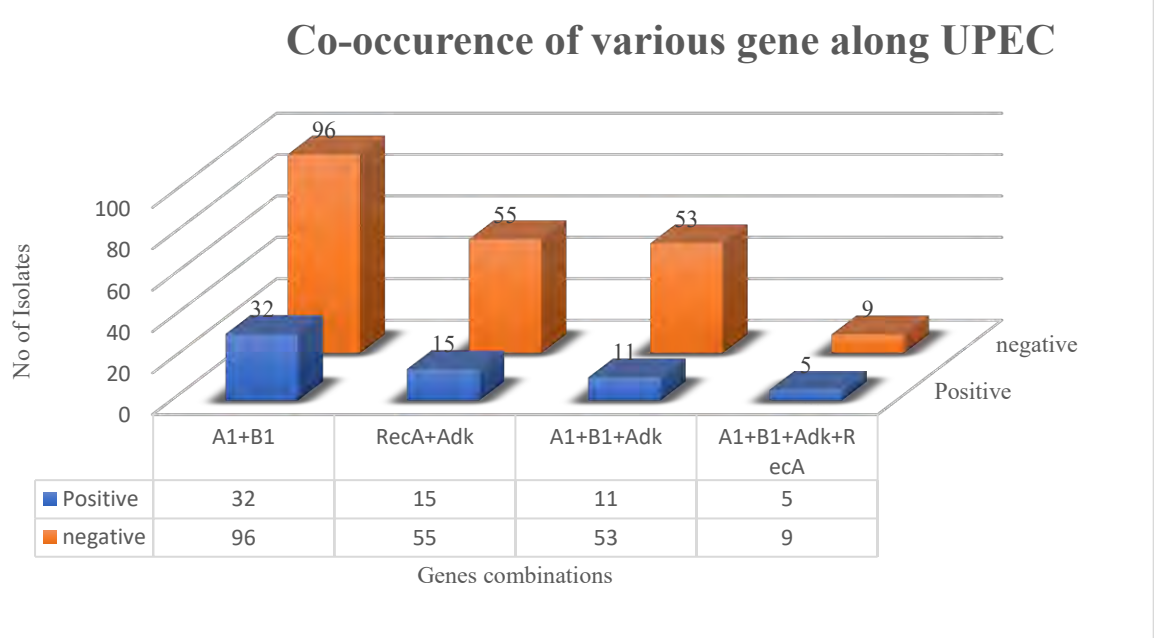


Figure 4.9.9. The following bar graph depicts the co-occurrence of screened genes among UPEC isolates.

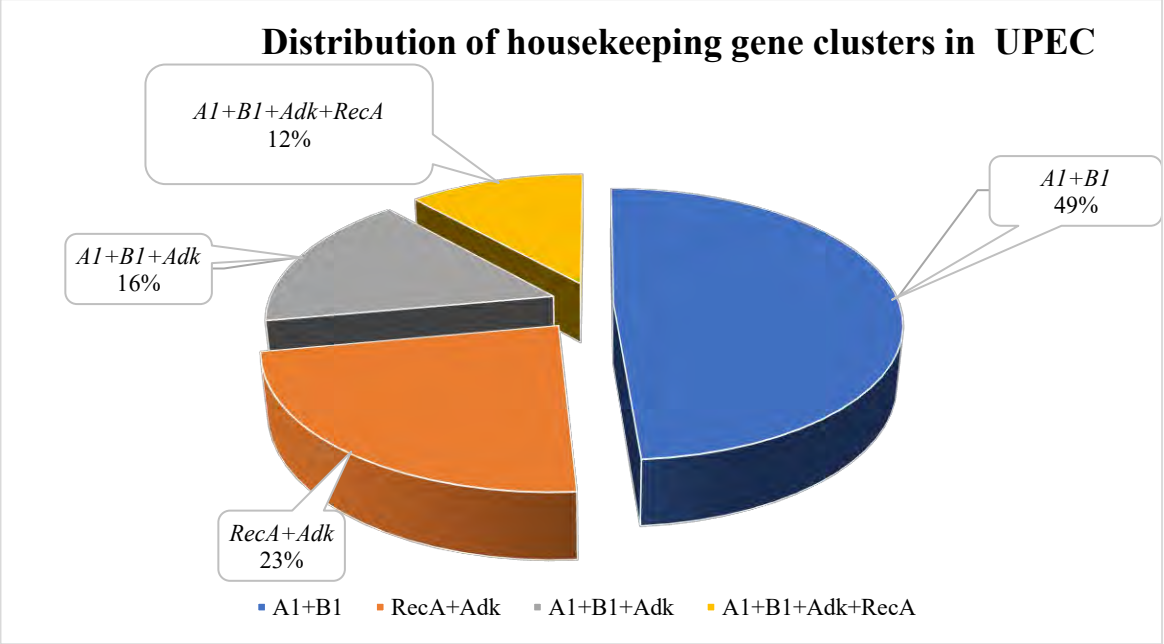


Figure 4.9.10. Different genes combination percentage frequencies distribution in UPEC.

The total UPEC. isolates were 152 the different combinations of screened genes were *A1+B1*=32, *RecA+Adk*=15, *A1+B1+Adk*=11, *A1+B1+Adk+RecA*=5

The screened genes were amplified in all ST131 strains. The housekeeping genes *RecA* were found in 27% and *Adk* were found in 54%. The percentage frequency of *A1* was 27% and *B1* was 66%. Comparison of the genes under study among these isolates showed the significant %age of *A1* and *B1 genes* related to housekeeping genes *RecA* (27%) and *Adk* (54%). The β-lactam gene *CITM* was only found in 2% of isolates of UPEC.

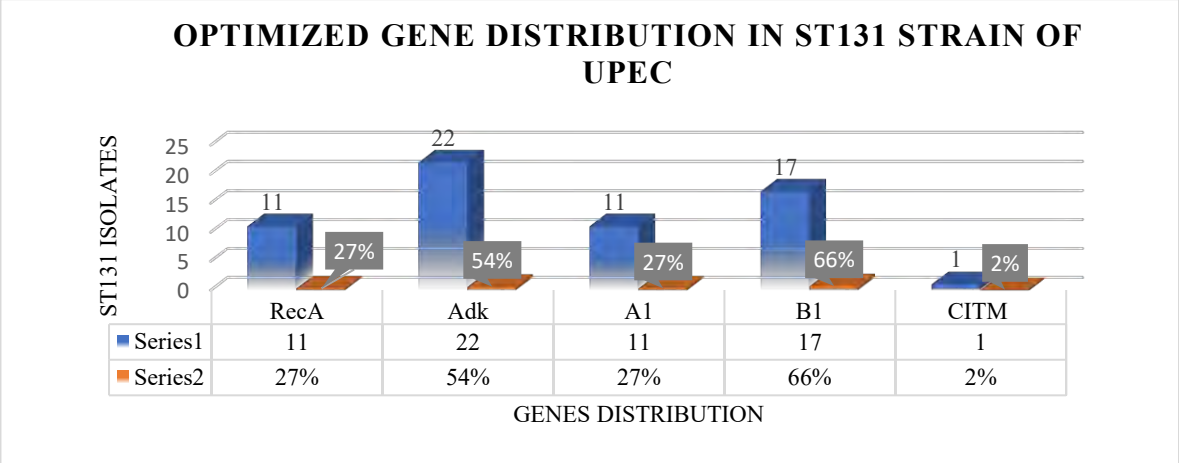


Figure 4.9.11. The following graph shows the percentage of *RecA*, *Adk*, *A1*, *B1* and *CITM* genes in the ST131 strain of uropathogenic *E. coli*.

4.10. Minimum Inhibitory concentration

Out of 100 samples of uropathogenic *Escherichia coli* the MIC results of Enrofloxacin showed that 95% of isolates (95/100) were resistant to ENR, 1% (1/100) were intermediate and 4% (4/100) were sensitive to selected antibiotics. All these UPEC strains were resistant to most of the other fluoroquinolone antibiotics (Ciprofloxacin, Sparfloxacin, Ofloxacin, Levofloxacin, Norfloxacin) were tested by Rafaque, Ihsan *et al*.,2015. These isolates showed an elevated level of resistance against these five selected drugs. (Ciprofloxacin=61.37%, Sparfloxacin=60.60%, Ofloxacin=71.70%, Levofloxacin=80%, Norfloxacin=57.20%) (Ihsan, Rafaque *et al*.,2015). The selected drug belongs to the same family but is used for livestock (Farms) not directly intake by humans but 95% of human samples show resistance against this drug. The Isolates that were positive for the screened β-lactam gene (*CITM*) showed 100% resistance against this fluoroquinolone drug. The detailed result of the MIC is shown below in tables and figures forms. The ESBL producer among these CITM-positive isolates also showed resistance against the ENR drug. The resistance breakpoint of enrofloxacin for animal pathogens is ≥ 4.0 µg/ml the susceptible breakpoint is ≤ 0.5 µg/ml and the intermediate range is >0.5 µg/ml to <4.0 µg/ml. (Booth *et al*.,2006)

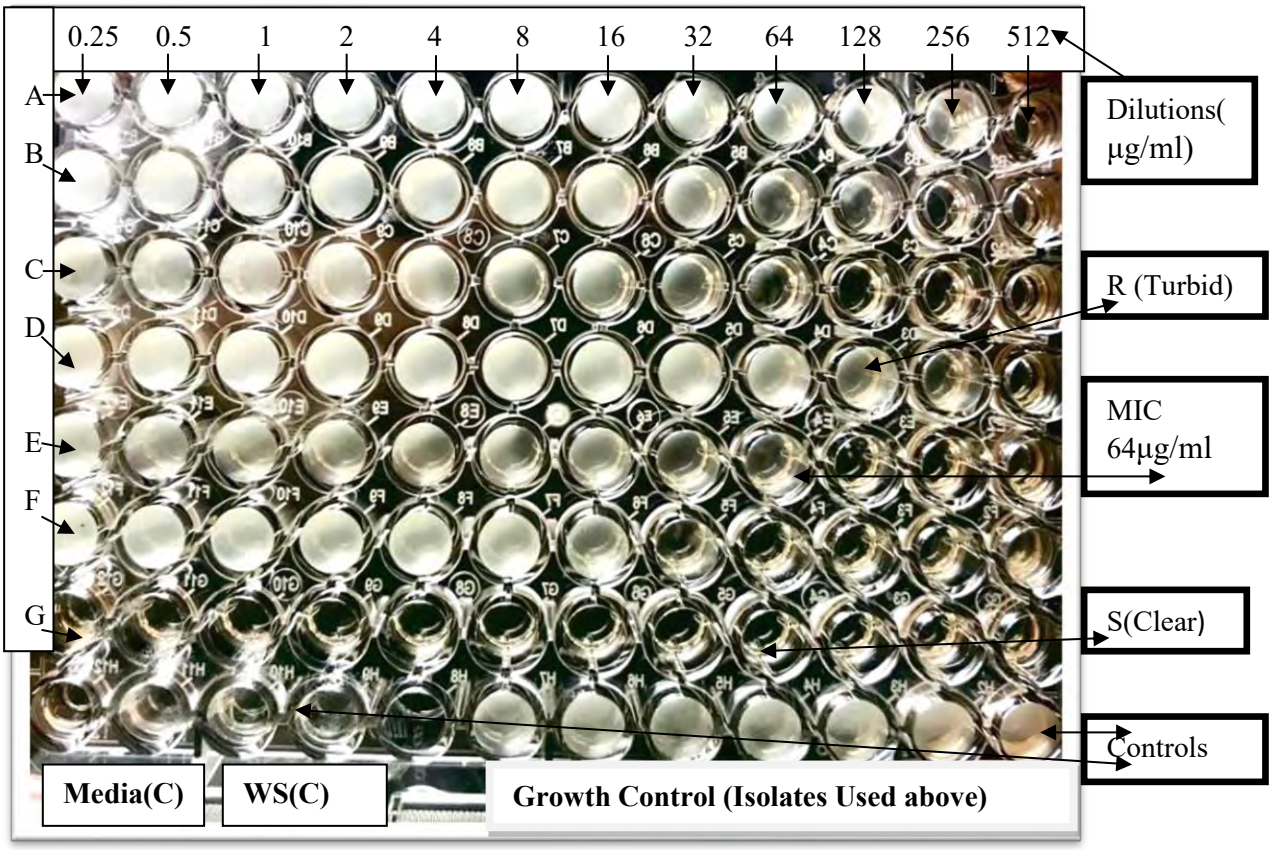


Figure 4.10.1. Minimum inhibitory concentration of Enrofloxacin for 7 isolates (A to G). Rows represent antibiotic dilutions ranging from 512µg/ml in well 12 (Right side) to 0.25µg/ml in well 1 (Left side). The last row contains Controls, 2 wells contain Media Control (MC), 2 wells contain working solution control (WS), and growth control in the last 7 wells of the last row. The A to G isolates showing different MIC values as A=≤256µg/ml, B=≤128µg/ml, C=≤64µg/ml, D=≤256µg/ml, E=≤64µg/ml, F=≤32µg/ml, G=<0.25µg/ml. From A to F all were resistant(R) to antibiotics, and the last G is Susceptible (S) to antibiotics and shows sensitivity.

Table 4.10.2. Antibiogram of Enrofloxacin against Uropathogenic *Escherichia coli* (n=100)

Antibiotic	Enrofloxacin (ENR)												
MIC values in µg/ml (12 Dilutions)	≤512 µg/ml	≤256 µg/ml	≤128 µg/Ml	≤64 µg/ml	≤32 µg/Ml	≤16 µg/Ml	≤8 µg/ml	≤4 µg/ml	≤2 µg/ml	≤1 µg/ml	≤0.5 µg/ml	≤0.25 µg/ml	<0.25 µg/ml
No. of isolates (Results)	2	7	21	27	27	8	1	2	0	1	0	1	3
%age	2%	7%	21%	27%	27%	8%	1%	2%	--	1%	--	1%	3%

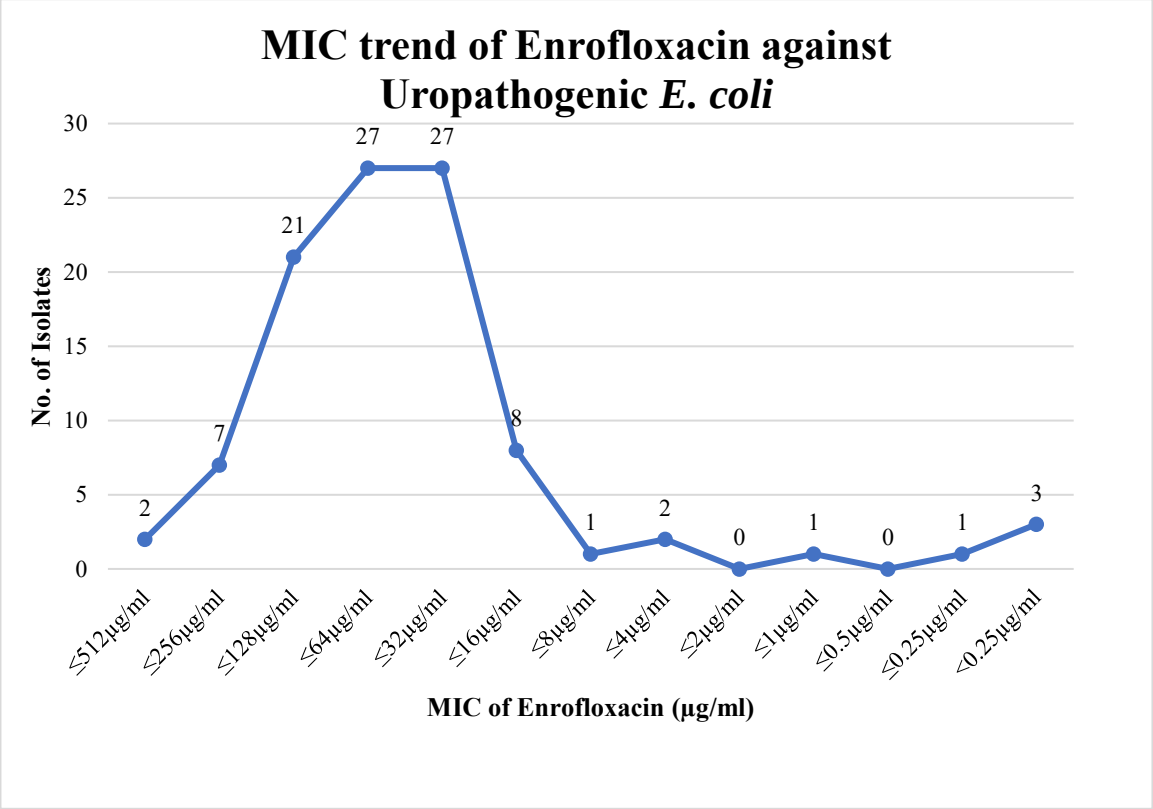


Figure4.10.3. The line graph shows the MIC trend of Enrofloxacin against the UPEC. (n=100)

Table 4.10.4. The table showed the Breakpoint of the enrofloxacin drug and the results (S=susceptible) (IS=Intermediate) (R=resistant). 95% of isolates were resistant to the selected antibiotic.

AB	**Breakpoint (µg/ml)**			**Results**		
---	S	IS	R	S	IS	R
ENR	≤ 0.5 µg/ml	>0.5 to<4.0µg/ml	≥ 4.0 µg/ml	**4** Isolates 4%	**1** Isolate 1%	**95** Isolates 95%

DISCUSSION

This study is about UTI that is caused by Uropathogenic *E. coli* Because many urinary tract infections including cystitis, pyelonephritis and infectious complications causative agent is uropathogenic *E. coli*. UTI is one of the most common infections globally, with significant morbidity, mortality, and high medical costs. UTIs affect both men and women (1:8) but are most common in women. Among these young women, approximately 90% of infections are caused by a gram-negative, rod-shaped, extraintestinal bacterium of normal flora. This high prevalence in females may be due to the difference in anatomical structures and nature of hormonal levels, low socioeconomic status and lack of personal hygiene that keep them more susceptible to UTIs (sotto *et al*., 2001). Severe UTIs involve several virulence genes and antibiotic resistance genes. (Momtaz H, *etal*.,2013; Johnson JR, *etal*.,1991). Many genes have already been discovered with their significant roles in different geographical regions.

In Pakistan, there is a lack of studies regarding infection data monitoring and prevalence strategy, which results in an inadequacy of the data concerning the prevalence of UTIs. Most physicians treat UTI patients with empirical therapy without any standard laboratory protocol. This blind therapy leads to the emergence of antibiotic-resistant bacteria. That may cause an increase in antibiotic use and prolong the hospital stay of the patient. (Navidinia *et al.*, 2013). Resistance to a particular antibiotic can be a selection and co-selection marker for the same or different antibiotics. This study was conducted to determine the association of phenotypic and genotypic resistance traits in the uropathogenic *Escherichia coli* (UPEC).

The increased antibiotic resistance leads to ineffective antimicrobial therapies, especially β-Lactam and fluoroquinolone antibiotics, which are widely recommended antibiotics as a treatment regime. In recent years, an alarming increase in resistance against these antibiotics has been observed. Antimicrobial resistance is the most urgent public health issue that has been continuously affecting global healthcare, livestock, and agricultural settings, endangering the attainment of imperishable development objectives (WHO, Geneva, *et al*.,2018). Uropathogenic *E. coli* is an emerging MDR pathogen that shows resistance to almost all currently available antibiotics. In Pakistan, high resistance of UPEC to penicillin

groups and modified penicillin has been reported from different areas. Like Islamabad, the resistance of UPEC to Ampicillin and Amoxicillin-Clavulanic acid (Augmentin) were 86% and 76% respectively (Ihsan *et al*., 2014). WHO recommends that trimethoprim-sulfamethoxazole as the first choice of drugs for the treatment of UTIs (Wolff and MacLennan *et al*., 2007). So, increasing resistance to all these antibiotics reduced its use, as reported by Faisalabad 85% of UPEC were resistant to trimethoprim-sulfamethoxazole. (Bashir, S., *et al*., 2011)

In this study, the collected bacterial samples from non-hospitalized patients were identified using various techniques. Of the isolated samples for the current study 82% from female patients. The study in Bangladesh reported that 80.95% of cases were derived from females and 38.10% of cases were found in the age group of 21-30 years (Rahman, M. *et al*.,2022). All samples were screened to identify uropathogenic *E. coli*. Morphological and biochemical testing of these samples confirmed that 60% of the isolates were uropathogenic *E. coli*. Molecular testing further confirmed that 56% of the isolates were UPEC, with a mean difference of 4%. This reflects the accuracy and sensitivity of routine laboratory detection methods compared to molecular techniques used for identification. Our results for the identification of UPEC show greater accuracy between morphological, biochemical, and molecular tests than in another study conducted in Islamabad. The result is 44% and 32% for biochemical and molecular tests, respectively, with a mean difference of 12%. (Ihsan *et al*., 2016)

The second part of the study involves the screening and optimization of the housekeeping gene (*RecA, Adk*), β- lactam gene (*CITM*) and two novel genes (*A1, B1*). In our study for UPEC phylogenetic analysis, we included the screened and optimized two housekeeping genes (*RecA, Adk*) with previously other five screened and optimized genes (*fumC, icd, gyrB, mdh, purA*) that were processed by our research fellows. Of the UPEC isolated samples out of 152, 30% were *RecA* (46) positive and 43% were *adk* (66) positive. For this, we selected the genotyping method, which is based on DNA sequencing, the multilocus sequence typing method (MLST). Previously UPEC was thought to be a separate clone of *E. coli* which possesses characteristic features such as distinct O antigens, virulence-associated traits,

MDR and genotypes. (Zhang *et al*., 2000. The housekeeping genes, sometimes referred to as reference genes, are very stable, exhibit fewer mutations, and are crucial for fundamental cellular processes. Almost all *E. coli* species include these basic metabolic proteins, which the organism needs for essential cellular functions. (Maidan *et al.,* 2003) MLST database available worldwide enables bacterial population characterization on a large scale. MLST has also been used for the characterization of different *E. coli* pathotypes. (Petersen *et al.,* 2009). The screened and optimized genes in our study were used to perform MLST. Similar genes were used for UPEC molecular phylogenetic and epidemiological analysis in the UK (Toraff *et al*., 2005). There are more MLST protocols available for epidemiological studies. Due to its strong discriminating abilities and the ease with which its data may be electronically altered and sent over the internet. One can match their experimental data to the results provided by the MLST protocol by comparing it to the online data available through the MLST project (Nemoy *et al*., 2005). The versatility of the MLST approach can be utilized for any clinical isolation directly makes it a crucial tool for epidemiological research as well as for standard clinical diagnosis (Diggle, Bell, and Clarke, *et al*., 2003; Spratt *et al*., 1999). The subject of evolution and structural biology of several pathogenic bacteria is also greatly influenced by MLST. It has been used to research a variety of issues, including how drug-resistant clones of dangerous microbes are emerging (Nemoy *et al*., 2005). Other than that, these screened housekeeping genes (*RecA, Adk*) are particularly important for bacterial survival and virulence. *RecA* encodes a universal essential key protein that is involved in SOS (Save-our-soul) response to tolerate DNA damage. *RecA* is a potentially effective therapeutic target for preventing the SOS response in bacteria to improve the effectiveness of traditional antibiotics and continue to successfully treat bacterial infections. *RecA* is one of the most significant and conserved proteins in eubacteria, has 350 amino acids and is involved in DNA damage repair. (Kaushik, V., *et al* 2022; Eisen J.A, *et al*., 1995). *RecA* is a multifunctional protein that is involved in homologous recombination, DNA repair pathway through Nucleotide Excision Repair (NER), SOS regulation and mutagenesis, biofilm formation and DNA synthesis. (McGrew, D. A.*et al*., 2003; Maslowska, K. H., *et al* 2019). In UPEC strains *RecA* gene is crucial for preserving

genomic integrity and facilitating DNA damage repair imposed by various stressors, including antibiotic treatment and host immune responses. Similarly, the *Adk* gene encodes for the adenylate kinase enzyme that is involved in the interconversion of adenine nucleotide (ATP, ADP, AMP) and plays a significant role in cellular homeostasis. ATP synthesis and regulation are important for the survival and cellular process in the urinary tract where they face a lot of metabolic challenges during infection. (Takaine, M., *et al*., 2019). The comparison of housekeeping genes and 16s rRNA shows that housekeeping genes are responsible for basic cellular function and are constitutively expressed in all cells but 16s rRNA is responsible for protein synthesis and used for taxonomic classification and identification.

The study showed a high level of resistance against fluoroquinolone antibiotics, about 58.62% of these isolates were resistant to more than one fluoroquinolone, this shows the threatening level of resistance against almost all drugs of fluoroquinolones which are recommended on the usual basis by physicians (Ali, I., Rafaque., *et al* 2015) AmpC β-lactamases play an important role in drug resistance scenario. AmpC may be chromosomally encoded, or plasmid-mediated. In the present study, we are concerned with the plasmid-mediated β-lactam *CITM* gene. Limited data are available regarding the prevalence of this gene, but enough data is available regarding other AmpC β-lactam genes. Recently a study by Zara Rafaque and Zurva on the same isolates, showed the frequency of AmpC-producing UPEC strains is 23% comparable with other studies a study from Egypt shows a 30% AmpC prevalence (El-Hady & Adel *et al*., 2015), another study by Wasef *et al*., 2014 showed 26.9% prevalence of AmpC genes. Prevalence of the FOX gene family was 23.2% (34/152) positive and DHA was 3.2% (5/152) positive confirmed by Zurva and in the current study 2.6% (4/152) were *CITM* positive from 152 confirmed UPEC isolates. Out of these 2.6% (4/152) *CITM-positive* isolates, 25% (1/4) were ESBLs formers. A study from Tehran reported that from 200 human-isolated *E. coli* confirmed samples 1% (2/200) were *CITM* positive. (Farrokhnazar, *et al*., 2016). Among β-lactamases, ESBL is the most prevalent and highly studied. In a study conducted by Ali *et al*., with the same isolates used for the current study, a total of 152 UPEC-confirmed isolates were used. About 42% of ESBL formers (64/152)

were found in these UPEC strains (Ali, Rafaque *et al*., 2015). The prevalence of ESBL-producing strains is higher across the globe as in Western Europe it is reported to be 15% (Balode, Punda-polić&Dowzicky, 2013), China 13%-35% isolates were ESBL producers. (Hawkey, P. M. *Et al*., 2008) and Africa 65% reported by (Ruth, Damian, Romanus & Charles, *et al*., 2011) and alarmingly higher resistance of up to 80% was reported in India (Nasa, Juneja, Singh, Dang, & Singh, *et al*., 2012). We were able to establish a correlation between plasmid-mediated AmpC and ESBL co-occurrence by combining data from earlier investigations with the most recent findings. These ESBL-positive strains for the current study also contain AmpC β-lactam genes, and screened *CITM* genes were present in 1.5% (1/64) of ESBL producers.

About 33% of the AmpC-ESBLs in our isolates are co-produced. Twelve of the total 36 isolates with PABL genes were making ESBLs. In addition, research from India found higher co-production rates of AmpC-ESBL at 42.37% (Ghosh & Mukherjee, 2016). Different research from Japan showed that coproductions accounted for 20% of the total, with 4 of the 19 PABLs also creating ESBLs (Matsumura *et al*., 2012). When two forms of β-lactamases are present in the same isolation, a condition known as co-prevalence, it can be difficult to detect and can provide false positive findings when each enzyme is being tested for diagnostic purposes. Due to the lack of published CLSI recommendations and the possibility of its existence being concealed in microorganisms that are also ESBL-resistant, it is challenging to accurately identify AmpC beta-lactamase synthesis in the laboratory. The worrisome nature of this problem makes therapy extremely difficult. The current study's findings indicated that ESBLs with *CITM* genes are uncommon in our area but a recent study on the same isolates by Zurva *et al*., 2016 shows co-prevalence of other β-lactam genes with PABLs or ESBLs are common in our area.

A recent rise in UPEC strains with MDR and XDR characteristics made treating UTIs more challenging. Ali *et al*. (2014) (Ali, Kumar, Ahmed, & Dasti, 2014) previously showed the multidrug resistance (MDR) phenotypic resistance profile of these isolates. Another research from Canada claims that 33.3% of the AmpC-producing *E. coli* were discovered to be MDR (9/27) (Baudry, Mataseje, Zhanel, Hoban, & Mulvey, 2009).

The *A1* and *B1* genes are two screened novel genes with unknown functions with known DNA sequences and have limited data that are just related to their sequences. The significant frequency found in the current study in 152 UPEC strains that showed the prevalence of *A1* genes in the isolated samples was 24% (37/152) and the occurrence of the *B1* gene was 34% (51/152). The genome sequence of the *A1* gene starts from 72559 and ends at 72781 and contains 223bp length, similarly, it *contains* 397bp length that starts from 23259 and ends at 23655. This sequence of genes (*A1, B1*) is 99.9% like the plasmid sequence of O25b: H4-ST131 shown by DNA blast on NCBI. (Totsika *et al*., 2011) In future, by knocking out these genes we can further determine the function and importance of these genes. In the current study out of 41 ST131 strains of UPEC, 26.8% (11/41) were *A1* positive and 41.4% (17/41) were *B1* positive strains. The co-occurrence of these genes (*A1*, *B1*) was 26.8% (11/41) means all *A1* positive genes also have *B1* genes in ST131 strains. Out of 37 total *A1* positive strains, 11 (30%) were ST131 and 51 *B1* positive strains 17 (33%) were ST131.

In a present research study, we observed that β-lactamase producing isolates that contain the *CITM* gene show 100% resistance to fluoroquinolone-class antibiotics. These were human-isolated UPEC, and the drug showed resistance used for livestock. It shows that through indirect pathways humans are also exposed to livestock antibiotics. A new study published in the California journal *Environmental Health Perspectives* in February 2023 shows the significant impact of livestock antibiotics on human health and is linked with antibiotic resistance to animals as well as humans.

Previous research has shown that there exists a relationship between excessive antibiotic use in livestock and the development of antimicrobial-resistant diseases in humans. According to the annual data such diseases account for 3 million yearly infections and 35,000 deaths. The different routes of transfer of resistant bacteria from animals to humans include contaminated meat, water, air, and direct exposure due to professional responsibilities (Joan *et al*., 2023). A similar study conducted in Turkey revealed that antimicrobial resistance in animals affects their health and is linked with human diseases. In 1951, streptomycin was administered to turkeys which was the first documented and recorded case of antibiotic resistance in food animals (Starr& Reynolds, 1951). Another research on *E. coli* isolated

from 198 cattle in eastern Algeria, suggested that these strains are responsible for the production of extended-spectrum β-lactamases (ESBLs) which cause resistance against tetracycline and ampicillin (Liu *et al*., 2016). Moreover, some studies highlighted that the use of antibiotics in food animals could catalyze the growth and spread of resistant bacterial strains leaving a negative impact on human health. Animals transfer these resistant bacteria to humans through food, water, manure, fertilizers, and mud. There is unquestionable proof that meals made from numerous animal sources and through all phases of food processing include a sizable number of resistant bacteria (Witte*et al*.,2000; Chang *et al*.,2015). In food-borne pathogens such as *E. coli, Salmonella,* and diverse types of enterococci a homologous relationship is found between drug-resistant bacteria in humans and animals.

CONCLUSION

This research study on uropathogenic *Escherichia coli* and its association with UTIs highlights its significance to the global health threat and concludes that the PCR detection of clinical isolates is more decisive than biochemical and other routine-based identification methods. The present study proved that the option of the MLST technique based on the screened housekeeping genes proved efficacious for the phylogenetic analysis of UPEC. The results indicated a high prevalence and susceptibility of UTI in females. Antibiotic resistance is a worldwide health challenge and increasing day by day leads to limited options for treatments. Misuse and overuse of antibiotics lead to those challenges. The screened antibiotic-resistance genes can be helpful for the effective treatment of UTIs. The study also concluded that the AmpC β-lactamases containing isolates showed resistance against fluoroquinolone drugs exhibiting a new level of danger to confer resistance against β-lactam and fluoroquinolone drugs. The evidence of the study suggests that human UPEC isolates showed a prominent level of resistance against livestock drugs (Enrofloxacin). Determined Minimum inhibitory concentration of Enrofloxacin that showed an overall higher level of enfloxacin resistance in clinical isolates. They showed a higher level of 84% of Enrofloxacin resistance in clinical isolates. To reduce the impact, reduce antibiotic use, use herbal medicines, need to develop guidelines for antibiotic use and find other useable ways to treat animals' health problems. Other advanced diagnostic options are required to address this global health threat. Further studies are required to investigate alternative innovative therapeutics and pinpoint potential genetic causes. More surveillance and research are required on a large scale to get a broader and more vivid view of the resistance scenario across Pakistan.

FUTURE PROSPECTS

By the time this research was through, we realized there was still a lot of work to be done in this field of study. The following work needs to be done in this regard.

- The functions of two amplified genes *A1* and *B1* were unknown and can be used for further research and by knocking out these genes their function and importance can be identified.
- Screen other plasmid-mediated AmpC β-lactamase genes (*ACC*, *DHA, CTX-M, MOX, FOX).*
- Establishing a correlation and a comparative study between β-lactamases and fluoroquinolones could be done.
- Use housekeeping genes (*RecA, Adk, fumC, Icd, purA, gyrB, mdh*) to establish MLST.
- Investigate the functions of amplified genes *A1* and *B1* via knockout experiments.
- Antibiotic resistance surveillance and monitoring should be intensified against UPEC to get a clear picture of the resistance scenario in Pakistan. This data can be used to develop even more effective UTI prevention measures.
- Targeting screening genes in the production of vaccines might result in the development of potent vaccinations against UPEC infections, lowering the prevalence and severity of UTIs.
- Similarly, the targeting genes can be used to produce sensitive, efficient, and specific diagnostic tools.
- With the help of one health approach, the genes could be screened from animals and used for the identification of potential reservoir and transmission pathways.
- Researchers can use the targeted genes from different hosts and environments for microbiome studies.
- Phylogenetic studies can be conducted to figure out the most prevalent phylogroups in Pakistan.
- A comparative study between β-lactamases and fluoroquinolones could be done.

- Collaboration between researchers and health workers around the globe can lead to the early detection of emerging resistance patterns.

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Appendix

Appendix-A

List A A-Prepared media Used for a research study.

Sr#	Name of media	Produced by
1	MacConkey agar (MA)	Oxoid England
2	Eosin methylene blue agar (EMB)	Oxoid England
3	Cysteine-, lactose- and electrolyte-deficient agar (CLED)	Oxoid England
4	Nutrient agar (NA)	Oxoid England
5	Luria-Bertani broth & agar (LB)	Oxoid England
6	Tryptone soya agar (TSA)	Oxoid England
7	Muller Hinton broth (MHB)	Oxoid England

Appendix-B

List B- The equipment used during the research study.

Sr#	Equipment	Manufacturer
1	Autoclave	TOMY SX 300E
2	Biosafety cabinet	Dwyer Mark II USA
4	Electronic balance	VWR 203B
5	Hot plate stirrer M-100	Cleaver Scientific
6	Incubator	INNOVA-40
7	Microscope	Micros Austria
8	Micropipettes	Thermospecific
9	Microtiter plates	BrandTech scientific
9	PCR machine	SimpliAmp thermal cycler
10	Refrigerator	Dawlance
12	Vortex	VWR digital vortex mixture

Appendix-C

List C-1. Chemical composition of used chemicals in gram staining.

Sr#	Reagents	Composition	Quantity
1.	Crystal violet gram strain	Deionized water	80ml
		Crystal violet	2.0g
		Ammonium oxalate	0.8g
		95% Ethanol	20ml
2.	Gram iodine solution (Mordant)	Distilled water	300ml
		Potassium	2.0g
		Iodine	1.0g
3.	Acetone alcohol decolorizer	Acetone	50ml
		95% Ethanol	50ml
4.	Safranin (Counter strain)	Stock solution	10ml
		Distilled water	90ml
	Stock solution for safranin	Safranin O	2.5g
		95% Ethanol	100ml

List C-2. Composition of 0.5% McFarland standard.

1.175% barium chloride dihydrate ($BaCl.2H_2O$)	0.05/10ml
1% Sulfuric acid (H_2SO_4)	9.95/10ml