ABSTRACT

Extraintestinal infections caused by *Escherichia coli* cause serious illness and death. Every year 130-175 million cases occur globally; >80% are associated with *E. coli*. To investigate the prevalence of *E. coli* causing urinary tract infections in humans, a cross-sectional study was conducted by taking 100 urine samples from 100 suspected patients visited to a local hospital at Chattogram during the period of December 2021 to April 2022. A mid-stream urine sample was collected from each of the suspected patient following standard procedures and tested for the presence of *E. coli* using conventional bacteriological procedures. Culture-positive *E. coli* isolates were confirmed by detecting a housekeeping gene *adk* by polymerase chain reaction (PCR). The *E. coli* isolates obtained were subsequently investigated to determine their susceptibility against a panel of 14 antimicrobials by disc diffusion method and by assessing the minimum inhibitory concentration (MIC) of colistin using broth microdilution method. A subset of strains displaying resistance to colistin, fluoroquinolones and third generation cephalosporins were characterized by PCR to determine the genetic determinants of resistance. The results of the study revealed that the prevalence of *E. coli* in UTI patients was 37% (95% Confidence Interval 28.2% - 46.8%). *In vitro* antimicrobial susceptibility of *E. coli* isolates revealed that 75.7% isolates displayed resistance to Azithromycin and 54.1% to Ampicillin while 37.8% and 29.7% to Tetracycline and Trimethoprim-sulfamethoxazole, respectively. More than 25% isolates displayed resistance to Cefotaxime, Cefoxitin, Ceftriaxone, Ciprofloxacin and Nalidixic Acid. About 52% of the total isolates showed resistance to ≥3 antimicrobials. The minimum inhibitory concentration of colistin in *E. coli* isolates ranged from ≤1 to ≥128 μg/mL, and 1 out of 2 colistin-resistant *E. coli* isolates harbored *mcr-1* gene. All fluoroquinolone-resistant isolates contained *gyrB* gene while 9 isolates harbored *gyrA* gene. None of the isolates examined harbored *parC* gene. Third-generation cephalosirin-resistant isolates harbored extended-spectrum beta-lactamases (ESBLs)-encoding genes, namely bla_{TEM} (37.5%) while 41.7% and 8.3% isolates harbored $bla_{\text{CTX-M}}$, bla_{SHV} genes, respectively. This study highlights the circulation of MDR *E. coli* in UTI patients in Chattogram region.

Keywords: Antimicrobial resistance, Bangladesh, *E. coli*, Multi-drug resistance, Urinary tract infection.

CHAPTER - 1
INTRODUCTION

Urinary tract infection (UTI) refers to the inflammatory disorders of the urinary tract characterized by fever, dysuria, and lower abdominal pain (Hoberman et al., 2003). It is one of the common causes of morbidity, mortality, and health care costs in humans. Overall, 150 million cases of UTIs occur yearly worldwide (Flores-Mireles et al., 2015). Women are more predisposed to UTI than men due to the structural characteristics of female urethra (Foxman, 2000). It has been estimated that 50–60% of adult women experience at least one UTI at some point in their lifespans (Medina and Castillo-Pino, 2019). The major risk factors for UTI include the use of spermicidal agents, frequent sexual intercourse, a prior UTI episode and having a first-degree female family member with a history of UTI (Hooton, 2012). Other factors like age, diabetes, abnormalities in genitourinary tract, immunosuppression and use of urological instruments may contribute to the prevalence of UTIs (Iqbal et al., 2010).

Several bacterial and some fungal pathogens are responsible for causing urinary tract infections. *Escherichia coli* is one of the principal bacterial pathogens frequently implicated in UTI; >80% of UTIs are associated with this bacterium (Russo and Johnson, 2003). *E. coli* is a Gram-negative bacterium belonging to the family *Enterobacteriaceae*, normally found in the gastrointestinal tracts of humans and animals. More than a billion of *E. coli* cells are carried by healthy humans in their intestines. Some strains have the potential to cause a wide spectrum of intestinal and extra-intestinal infections, including UTIs, septicemia, meningitis, and pneumonia in humans and animals (Donnenberg, 2002). The *E. coli* that successfully invade the urinary tract harbor specific factors that enable them to survive. This type of *E. coli* is commonly called uropathogenic *E. coli* (UPEC). The gut of individuals can be a reservoir for *E. coli* and can act a source of UTI (Nielsen et al., 2014). UPEC strains can be able to colonize both the gut and urinary tract (Chen et al., 2013), and thus the dissemination of *E. coli* from the intestine into the bladder via the urethra causes UTI (Hooton, 1990). The prevalence of UTIs due to *E. coli* has been reported from different geographical regions. However, such information in humans in Bangladesh is limited, if not absent.

Antimicrobial treatment is an effective approach to reduce the duration of UTI symptoms. Trimethoprim-sulfamethoxazole, ciprofloxacin, cephalosporins, semi-synthetic penicillins with or without inhibitors, and fosfomycin are the most commonly used antimicrobials for the treatment of UTIs (Hryniewicz et al., 2001). First-line antimicrobials such as trimethoprim and some β-lactams, or second-line quinolones and amoxicillin in combination with clavulanic acid, are considered effective treatments for UTIs. Fluoroquinolones, particularly ciprofloxacin, are used as the drug of choice for UTI in countries where the level of resistance to other antimicrobials such as ampicillin or trimethoprim-sulfamethoxazole is high (Maraki et al., 2013). In addition, carbapenems are considered as the last resort treatment for infections caused by extended-spectrum β-lactamases-producing *Enterobacterales*.

The global rise of resistant *E. coli* to commonly prescribed antimicrobials is of serious concern (de Been et al., 2014). Development of resistance in *E. coli* to commonly prescribed antimicrobials result in decreased effectiveness of some standard regimens (Karlowsky et al., 2001). The emergence of extended-spectrum beta-lactamases (ESBLs) has threatened the empirical use of cephalosporins and ciprofloxacin in UTIs (Pondei et al., 2012). Due to irrational use of antimicrobials, the problem of antimicrobial resistance is compounding in Bangladesh. Detection of UTI caused by *E. coli* and resistance of this pathogen to commonly prescribed antimicrobials in clinical set up is essential and helpful in improving the efficacy of empirical treatment. There is no national surveillance on antimicrobial resistance and insufficient information is available to quantify the problem in Bangladesh.

Aim and objectives of the study

The overall aim of the study was to evaluate the antimicrobial resistance pattern of *E. coli* isolated from humans with urinary tract infections.

The specific objectives included:

1. To estimate the frequency of *E. coli* in urine samples from humans suspected to have urinary tract infections
2. To assess phenotypically the antimicrobial resistance spectrums of *E. coli* isolated from humans with UTIs
3. To determine the genetic basis of multidrug resistant *E. coli* isolated from urine

CHAPTER - 2
REVIEW OF LITERATURE

2.1 Urinary tract infection

Urinary tract infection (UTI) is a common infectious disease with potentially severe complications (Padmavathy et al., 2012). UTIs comprise ranges of disorders manifests either solely in the lower urinary tract (urethritis, cystitis, urethrocystitis) or affects the renal pelvis and kidneys (cystopyelitis, pyelonephritis), which are defined by the presence of microorganisms in urinary tract (Kayser et al., 2005). Asymptomatic bacteriuria could be presented by bacterial attenuation or stable colonization of bacteria (Hooton, 2000). UTI may be asymptomatic, acute, or chronic in both males and females. Acute UTI is more frequently seen in females of all ages; these patients are usually treated on an outpatient basis and are rarely admitted to hospital (Anvarinejad et al., 2012). Chronic UTI in both males and females of all ages is usually associated with an underlying disease (e.g., pyelonephritis, prostatic disease, or congenital abnormally of the genitourinary tract) and these patients are most often hospitalized (Vandepitte et al., 2003). UTI could be further categorized to uncomplicated and complicated infection as part of region of infection depending on several factors which may play a role in distinguishing between these two categories, including the type and the duration of antimicrobial treatment as well as an extension of evaluation of the urinary tracts (Hooton, 2000)

2.2 Etiology of urinary tract infection

The most common cause of UTI is *E. coli*; *Staphylococcus saprophyticus* is the second most common cause of these infections in sexually active females between the ages of 13 and 40 years (Chamberlain, 2009). In complicated cases of UTIs such as those resulting from catheterization or from anatomic obstructions, the most frequent causes are *E. coli, Klebsiella pneumoniae, Proteus mirabilis*, *Enterococcus*, and *Pseudomonas aeruginosa* (Padmavathy et al., 2012). Hematogenous infection of the urinary tract is restricted to a few relatively uncommon microbes, such as *S. aureus, Candida spp., Salmonella spp.* and *Mycobacterium tuberculosis,* which cause primary infections elsewhere in the body (Grabe et al., 2008). Certain bacterial strains within a species are uniquely equipped with specialized virulence determinants provide a selective

advantage to those strain possessing them with regard to colonization and infection (Hooton, 2000).

2.3 Relationship between *E. coli* and UTIs

Escherichia coli is a commensal bacterium causing infection in human and animal and serves as one of most frequents causes of UTI in humans (Paterson and Bonomo, 2005), being responsible for as many as 80% of UTI in otherwise healthy people (Poey et al., 2012). Uropathogenic *E. coli* (UPEC) are most commonly associated with human disease. These bacteria are the primary cause of community acquired UTI (70-95%) and a large portion of nosocomial UTIs (50%), accounting for substantial medical costs and morbidity worldwide (Foxman, 2002). Recurrent or relapsing UTIs are especially problematic in many individuals. The primary reservoir of UPEC believed to be the human intestinal tract and employ a diverse repertoire of virulence factors to colonize and infect the urinary tract in an ascending fashion (Pitout, 2012). Extra-intestinally, particular *E. coli* strains can cause UTIs due to ownership of virulence traits that allow them to induce disease (Koreň et al., 2013). As these *E. coli* strains could adapt to and colonize new environment outside the original niche of the gastrointestinal tract, they defined as ExPEC. UPEC is one example of ExPEC (King-sun, 2007)

2.4 Pathogenesis of urinary tract infection

Microorganisms can reach the urinary tract by hematogenous or lymphatic spread, but there is abundant clinical and experimental evidence to show that the ascent of microorganisms from the urethra is the most common pathway leading to a UTI, especially organisms of enteric origin (*i.e.*, *E. coli* and other *Enterobacteriaceae*) (Grabe et al., 2008). Contamination of the periurethral region with fecal organisms leads to colonization of the distal end of the urethra. Ascending of the organisms up the urethra to the bladder is the most common means of acquiring a UTI (Chamberlain, 2009). Relatively minor trauma or the mechanical effect of sexual intercourse has been shown to allow bacteria access to the bladder. In most instances, these bacteria are purged by the flushing action of voiding. Factors that violate bladder integrity (urinary catheters) or that obstruct urine outflow (enlarged prostate) are also associated with infection (Ryan and Ray, 2004). A single insertion of a catheter into the urinary bladder in ambulatory patients results in urinary infection in 1-2% of cases. In dwelling catheters with open drainage systems result in bacteriuria in almost 100% of cases

within 3-4 days. It is believed that bacteria migrate within the mucopurulent space between the urethra and catheter, and this leads to the development of bacteriuria in almost all patients within about 4 weeks (Grabe et al., 2008).

2.5 Strategies of *E. coli* in UTI

Uropathogenic *E. coli* have developed a number of strategies to escape immune responses, enabling the pathogens to more successfully colonize the urinary tract and persist (Wiles et al., 2008). Siderophores improves iron uptake and thus promote the survival and growth of bacteria within the urinary tract (Abraham, 2011). The ability of UPEC to attach host tissues is one of the paramount factors that facilitate UPEC colonization of the urinary tract, allowing the bacteria to withstand the mass flow of urine and promoting UPEC invasion of urothelial cells (King-sun, 2007). UPEC strains act as opportunistic intracellular pathogens, making them less susceptible to many antibiotics and perhaps less immunogenic. The rearrangement of actin filaments inside epithelial cells during terminal differentiation of the infected immature cells may act as a trigger for increased intracellular multiplication of UPEC and the recrudescence of clinical symptoms (Wiles et al., 2008). UPEC is able to produce a polysaccharide capsule, which considerably increases bacterial survival within the urinary tract and boosts resistance to serum and to phagocytosis (Abraham, 2011). In the existence of indwelling catheters, bacteria form biofilms, multicellular communities, which protect them from attacks of the immune system and exogenous antibiotics (King-sun, 2007).

2.6 Risk factors of UTIs

Urinary tract infection is one of the most common diseases in human, its most frequent community-acquired infection that is associated with significant morbidity and mortality (Poovendran et al., 2013). In the United States, UTI rank the second to respiratory infections in their incidence that about 8 million cases recorded annually in emergency department, clinic, and office practices (Chamberlain, 2009), including more than two million visits for cystitis. Approximately 15% of all community-prescribed antibiotics in the USA are dispensed for UTI. Moreover, the direct and indirect costs associated with community acquired UTIs in the USA alone exceed an estimated US $1.6 billion (Grabe et al., 2008). The majority of UTI cases occur in a woman (female to male ratio 30:1). Age and the sexual activity states are strongly associated with increased the incidence of UTI in female. About 40% - 50% of all

females have as a minimum one UTI at some time in their lives (Chamberlain, 2009). In addition, previous report of World Health Organization (WHO) revealed that the UTIs are common causes of febrile illness in 3-8% of girls and 1% of boys (Momtaz et al., 2013). Furthermore, about 25% to more than 40% of patients with UTIs experience recurrent UTI within weeks or months (Luo et al., 2012). The patient's own intestinal flora act as a reservoir of their infection, which communicate the urethral and perineal area. The urinary tract obstruction or instrumentation, environment sources assume some important (Ryan and Ray, 2004). The male in the fifth decade experiences a rapid increase in the incidence of UTI because of obstruction of urethra following the development of benign prostatic hypertrophy (Chamberlain, 2009).

2.7 Antimicrobial resistance against *E coli*

The managing of infections caused by uro-pathogenic *E. coli* has been complicated by the emergence of antimicrobial resistance, particularly since the late 1990s (Pitout, 2012). In the last 30 years, antibiotic resistance in pathogenic bacterial strains has rapidly increased with the alarming latest appearance of multiple resistant strains, frequently known as superbugs (Venturini, 2011). The occurrence of resistance within *E. coli* strains now is a very current matter due to increasing administration of antibiotics and following selection of mutated resistant strains (Koreň et al., 2013). *E. coli* typically acquires AMR genes through mobile genetic elements (MGE), such as plasmids, insertion sequences, transposons, and gene cassettes/integrons (Calhau, 2015). A large number of resistance-encoding mobile elements, in particular plasmids, are shared between different members of the Enterobacteriaceae and thus further promote the spread of resistance genes (Partridge, 2018) MGE can also encode for virulence factors, and there may be interplay between virulence and antimicrobial resistance (Calhau, 2015). A review study found highest antimicrobial resistance rates against the antibiotic class of tetracyclines in 69.1% of articles followed by sulphonamides in 59.3%, quinolones in 49.4%, beta-lactams in 36.9%, aminoglycosides in 28.7%, nitrofurans in 20.0%, and fosfomycin in 8.4% (Bunduki, 2021). When a bacterial strain resistant to three or more different antimicrobial classes defined as multi-drug resistant (MDR) bacteria (Magiorakos et al., 2012). The increase of MDR bacteria is results of unscrupulous antibiotic use in medicine and agriculture over the last several decades (Partridge et al., 2009). The careless antibiotics usage, without antibiotic sensitivity testing, is the most important factor for promoting the

emergence of MDR, which cause selection and dissemination of antibiotic resistant pathogens in clinical medicine. The strong association between the resistances to different antimicrobial classes has a great impact on the rationale for using antimicrobial policies to reduce MDR in the hospital environment and even in the community (Hall et al., 2003). The continuous used of even a single antibiotic over a period of weeks or months will select bacteria resistant to a different type of antibiotics in addition to the one in use (Livermore, 2007). The risk of MDR bacteria that resistant to only a couple of antibiotics can greatly complicate treatment. Frequently such bacteria are resistant to the primary antibiotic preferred for treatment, requiring the use of secondary and tertiary drugs instead, which may be less effective and more toxic to the patient (Partridge et al., 2009). Recently, MDR bacteria afford numerous challenges and problems for healthcare providers, including increases in hospital-acquired infections, reduced treatment options, higher morbidity, and mortality rates, and healthcare cost increases due to longer hospital stays (Partridge et al., 2009). At a molecular level, MDR may develop through clustering of resistance genes on mobile genetic elements. The physical linkage of plasmid-borne resistance genes in integrons and transposons has been shown to assist the co-transfer of resistance genes (Rijavec et al., 2006). Both antibiotic resistances and virulence factors can be encoded by mobile elements. If cointegration occurs, antibiotic pressure also selects for virulence factors, which in turn could initiate more virulent antibiotic resistant strains (Venturini, 2011).

2.7.1 Resistance to β-Lactams

β-lactam antibiotics, particularly the third generation cephalosporins, are the most important drug class used to treat serious community-onset or hospital-acquired infections caused by *E. coli* (Pitout, 2012). Resistance to β-lactam antimicrobial agents in *E. coli* is principally mediated by β-lactamases, which hydrolyze the β-lactam ring and thus inactivate the antibiotic (Briñas et al., 2005).

2.7.2 Extended Spectrum β-Lactamases (ESBLs) producers

Extended spectrum β-lactamases-producing strains can increase morbidity and mortality rates, in part as a result of linked resistance to other antibiotic families, which restrict therapeutic options and raises healthcare costs (Inwezerua et al., 2014). ESBL, hydrolyse the oxyimino β-lactams like ceftazidime, cefotaxime, ceftriaxone and monobactams, but have no outcome on the cephamycins, carbapenem and related

compounds have emerged as an important mechanism of resistance amongst pathogens (Mukherjee et al., 2013). ESBL are inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Pitout, 2012). ESBL can be divided into four major groups: TEM, SHV, CTX-M, and OXA types. The majority of ESBLs identified in clinical isolates during the 1980s–1990s were the SHV or TEM types, which evolved from parent enzymes such as TEM-1, TEM-2, and SHV-1 (Jacoby and Munoz-Price, 2005). Since the middle of 2000s CTX-M βlactamases had been demonstrated in different members of the *Enterobacteriaceae*, but particularly in *E. coli*, and have become a major type of ESBL in the world of antimicrobial resistance (Pitout, 2012).

2.7.3 Plasmid-Mediated AmpC β-Lactamases

Like ESBL-producing bacteria, bacteria with plasmid-mediated AmpC enzymes have mostly been responsible for nosocomial outbreaks on a worldwide basis during the late 1980s and 1990s (Pitout, 2012). Chromosomal genes in many Gram-negative bacilli encode AmpC βlactamases, frequently inducible by β-lactams. Mutations that increase their expression are in charge for the ready emergence of broad-spectrum cephalosporin resistance in *Enterobactercloacae*. The AmpC enzyme in *E. coli* in general poorly expressed, but plasmid-mediated AmpC enzymes can give these organisms the same resistance profile as a βlactam-resistant *Enterobacter* isolate (Jacoby and Munoz-Price, 2005). Plasmid-mediated AmpC genes are derived from the chromosomal ampC genes of several members of the family *Enterobacteriaceae* (Pitout, 2012). AmpC β-lactamases make available resistance to cephamycins as well as to oxyimino-β-lactams and are resistant to inhibition by clavulanic acid. CMY-2 is the most commonly encountered plasmid mediated AmpC β-lactamase, often found in *E. coli* (Doi et al., 2009).

2.7.4 Carbapenemases

The most recent development in β-lactam resistance is the emergence of carbapenemases, enzymes that hydrolyze carbapenem of β-lactam antibiotics, imipenem and/or meropenem, which are resistant to degradation by other ESBLs (Hilbert, 2011). Plasmid-mediated IMP and VIM-type carbapenemases, identified in clinical isolates during early and late 1990s, respectively which have a wide geographic distribution in Europe, South America, and the Far East and have been found in the

United States (Jacoby and Munoz-Price, 2005). Metallo-β-lactamase classified as class B requiring zinc ion as cofactor for the maximal activity (Hilbert, 2011). A few classes A enzymes (clavulanic acid-inhibited carbapenemases) which is a class of serine β-lactamases, remarkably the plasmid-mediated KPC enzymes, are effective carbapenemases as well. As well as some of class D, OXA-type βlactamases have carbapenemase activity, augmented in clinical isolates (Pitout, 2012).

2.7.5 Resistance to Aminoglycosides

Aminoglycosides interfere with bacterial 16S rRNA function by binding at the site where codon-anticodon accurateness is assessed (Davis et al., 2010). In Gram-negative pathogens, mainly enzymes that modify the drug by acetylation, adenylylation, or phosphorylation and less frequently by other manners, including efflux mechanisms, mediate resistance to aminoglycosides (Davis et al., 2010). Aminoglycoside modifying enzymes, including acetylaminotransferases (AACs), nucleotidyltransferase (ANTs), and phosphotransferases (APHs) perform co-factor dependent drug modification in the bacterial cytoplasm. Modified aminoglycosides bind weakly to the ribosome and fail to activate energy-dependent phase II allowing the bacteria to survive in the presence of the drug (Lim et al., 2013). Aminoglycoside-producing bacteria (*Streptomyces* and *Micromonospora* spp.) have fundamental resistance to aminoglycosides through methylation of nucleotides within the A site of 16S rRNA, preventing disruption of translation by the aminoglycoside. The earliest reports of clinical Gram-negative isolates with plasmid-borne rRNA methylase aminoglycoside resistance genes were from Japan (Davis et al., 2010). The acetyltransferases are a most important class of resistance enzymes for the reason that of their ability to inactivate many of the medically useful aminoglycosides, such as gentamicin, tobramycin, amikacin, and netilmicin (Schwocho et al., 1995).

2.7.6 Resistance to Quinolone

First-line antibiotics for UTIs include quinolones and fluoroquinolones. Recent surveys across Europe and the United States showed that the frequency of isolation of UPEC strains resistant to fluoroquinolones is rising year by year (Kawamura-Sato et al., 2010). In Iraq, different studies found high resistance rate to fluoroquinolones among UPEC isolates (Fayroz-Ali, 2012). Resistance to quinolones has been a problem ever since nalidixic acid was introduced into clinical medicine more than 40 years ago. Three

mechanisms of resistance to quinolones are now recognized: mutations that modify the drug targets, mutations that reduce drug accumulation, and plasmids that protect cells from the lethal effects of quinolones. The targets of quinolone action are the vital bacterial enzymes DNA gyrase and DNA topoisomerase (Jacoby, 2005). Quinolone resistance in *Enterobacteriaceae* is frequently the result of chromosomal mutations, leading to alterations in target enzymes or drug accumulation. In recent times, plasmid-mediated quinolone resistance has been reporting by the acquisition of the qnr, qepA, and aac(6´)-Ib-cr genes (Carattoli, 2009). Another plasmid mediated quinolone resistance mechanism was described in 2006, aac(6′)-Ib-cr encodes a variant of aminoglycoside acetyltransferase and is responsible for reduced susceptibility to ciprofloxacin and norfloxacin by N-acetylation of a piperazinyl amine (Ruiz et al., 2012; Paltansing et al., 2013). The aac(6′)-Ib gene, which typically confers resistance to tobramycin and amikacin, but not to gentamicin (Jones et al., 2008). The aac(6′)-Ib gene found on the plasmid in question revealed two codon changes (Ruiz et al., 2012), providing new enzyme with the ability to acetylate fluoroquinolones harboring an unsubstituted piperazinyl group, such as ciprofloxacin and norfloxacin (Jones et al., 2008). The aac(6')-lbcr, which lacks the restriction site present in the aac(6′)-Ib gene (Inwezerua et al., 2014). The cr variant of aac(6´)-Ib encodes an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin by N-acetylation of its piperazinyl amine. Changes in two amino acids has aac(6´)-Ib-cr, Trp102Arg and Asp179Tyr, which together are essential and sufficient for the enzyme's ability to acetylate ciprofloxacin (Park et al., 2006; Fayroz-Ali, 2012). Several studies on clinical *E. coli* have revealed relationship between antibiotic resistance and low prevalence of virulence factors and suggested that quinolone resistance may be directly associated with the loss of virulence (Mokracka et al., 2012).

2.8 Diagnosis of *E. coli*

2.8.1 Specimen collection

The importance of the method of collection of urine specimens, their transport to the laboratory, and the initial efforts by the laboratory to screen and culture the urine cannot be overemphasized (Ryan and Ray, 2004). It is the responsibility of the laboratory to provide the physician with sterile, wide-mouthed, glass or plastic jars, beakers, or other suitable receptacles. They should have tight-fitting lids or be covered with aluminium

foil prior to sterilization by dry heat or autoclaving (Ryan and Ray, 2004). Urine specimens may have to be collected by a surgical procedure, e.g. supra-pubic aspiration, cystoscopy, or catheterization. If not, the laboratory must insist on a clean-catch midstream urine specimen, particularly in females and children. Since urine itself is a good culture medium, all specimens should be processed by the laboratory within 2 hours of collection or be kept refrigerated at 4°C until delivery to the laboratory and processed no longer than 18 hours after collection (Vandepitte et al., 2003; Ryan and Ray, 2004). Whenever possible, urine specimens for culture should be collected in the morning. It is advisable to ask the patient the night before to refrain from urinating until the specimen is to be collected. The examination procedure includes the following steps: first, examination of a Gram-stained smear. Second, a screening test for significant bacteriuria. Third, a definitive culture for urine specimens found to be positive in the screening test, and for all specimens obtained by cystoscopy, suprapubic bladder puncture (SBP), or catheterization. Fourth, Susceptibility tests on clinically significant bacterial isolates (Vandepitte et al., 2003; Ryan and Ray, 2004).

2.8.2 Laboratory examination

Preparation and examination of a Gram-stained smear is a necessary part of the laboratory process. Using a sterile Pasteur pipette (one for each sample), place one drop of well-mixed, uncentrifuged urine on a slide. Allow the drop to dry without spreading, heat-fix and stain. Examine under an oil-immersion lens for the presence or absence of bacteria, polymorphonuclear leukocytes, and squamous epithelial cells (Vandepitte et al., 2003). One or more bacterial cells per oil-immersion field usually implies that there are 10^5 or more bacteria per millilitre in the specimen. The presence of one or more leukocytes per oil-immersion field is a further indication of UTI. Non-infected urine samples will usually show few or no bacteria or leukocytes in the entire preparation (Vandepitte et al., 2003; Ryan and Ray, 2004). In specimens from females, the presence of many squamous epithelial cells, with or without a mixture of bacteria, is strong presumptive evidence that the specimen is contaminated with vaginal flora and a repeat specimen is necessary, regardless of the number of bacteria per oil-immersion field. If results are required urgently, the report of the Gram-stain findings should be sent to the physician with a note that the culture report is to follow (Vandepitte et al., 2003; Ryan and Ray, 2004).

2.8.3 Isolation and identification of *E. coli*

2.8.3.1 Culture

The MacConkey agar or Eosin methylene blue (EMB) agar (or both) are specific media for detection of *E. coli*. On MacConkey agar, deep red colonies are produced, as the organism is lactose-positive, and fermentation of this sugar will cause the medium's pH to drop, leading to darkening of the medium. Growth on EMB agar produces black colonies with a greenish-black metallic sheen (Paton et al., 1998).

2.8.3.2 Biochemical tests for *E. coli*

Escherichia coli ferment glucose and lactose producing acid and carbon dioxide. Acid causes the phenol red indicator in the agar to turn yellow. Carbon dioxide is observed as bubbles or cracks in the agar. There is no hydrogen sulfide production, as indicated by the lack of black precipitate in the agar (Carter, 1986). In IMViC test *E. coli* is indole, methyl red positive and Voges –Prauskeur negative and citrate negative. *E. coli* are lysine positive, and grows on TSI slant (Triple super Iron
test) with yellow color slant and butt and lack of blackening (no hydrogen sulfide gas).

2.8.3.3 Molecular diagnosis and typing

Although serotyping is still widely used for epidemiological investigation of *E. coli* disease, a number of molecular methods for *E. coli* strain characterization are now available and increasingly employed. However, no single method currently available is capable of definitively distinguishing between all pathogenic and non-pathogenic subtypes. Multilocus enzyme electrophoresis and PCR-based methods can be used to assign strains to major phylogroups, A, B1, B2, D and E (Boyd and Hartl, 1998; Clermont et al., 2000). In general, ExPEC strains belong to phylogroups B2 or D whereas intestinal pathogenic strains and commensals belong to groups A and B1. Multilocus VNTR analysis (MLVA) methods are used for epidemiological analysis of intestinal disease caused by *E. coli*, in particular outbreaks of EHEC in humans (Köhler and Dobrindt, 2011). A number of MLST schemes are also available.

CHAPTER - 3
MATERIALS AND METHODS

3.1 Sampling

A cross-sectional survey was conducted to assess the frequency of *Escherichia coli* in urine samples from humans suspected to have urinary tract infections (UTIs) who had been visited at a hospital located at Chattogram during the period between December 2021 and April 2022. Patients with UTIs (i.e., >1 of the following symptoms: frequent urination, painful urination, cloudy urine, or pain in pelvic area, flank, or low back and fever) were enrolled for the investigation. Approximately 10 mL clean-catch midstream urine was collected in a sterile container from each UTI suspected patient and kept refrigerated at 4°C until shipped to the Microbiology Laboratory of Chattogram Veterinary and Animal Sciences University. All procedures were carried out under an approval of the Ethics Committee of CVASU [Approval no. CVASU/Dir (R&E)EC/2019/126(5)]

3.2 Isolation and identification of *Escherichia coli*

Conventional bacteriological procedures were followed for the isolation and identification of *E. coli*. Briefly, the sample was inoculated into a test tube containing buffered peptone water (BPW; Oxoid Ltd., England) and incubated at 37°C for 18 hours for primary enrichment. Inoculum was taken from BPW using sterile inoculating loop and streaked on to MacConkey agar (Oxoid Ltd., England) and incubated at 37°C for 24 hours. Bright pink-colored large colonies yielded on

to MacConkey agar plate were suspected as the growth of *E. coli.* Then suspected colonies were streaked onto Eosin methylene blue (EMB) agar (Merck, India) and incubated at 37°C for 24 hours. Those colonies having characteristic appearance of greenish metallic sheen were considered as *E. coli*. All suspected *E. coli* from EMB agar plates were streaked on to blood agar and incubated overnight at 37°C, and positive cultures were stored in brain heart infusion broth (BHI; Oxoid Ltd., England) at -80°C using 50% glycerol until tested further.

3.3 Identification of *E. coli* by polymerase chain reaction (PCR)

The suspected *E. coli* were confirmed by the detection of a house keeping gene *adk* (adenylate kinase), by PCR. The primers used were as follows: Adk F: 5'-ATTCTGCTTGGCGCTCCGGG-3' and AdkR: 5'-CCGTCAACTTTCGCGTATTT-3' (Wirth et al., 2006).

3.3.1 Extraction of DNA

The isolated *E. coli* stored at -80°C were thawed gently in room temperature. Then the isolates were inoculated separately into blood agar and incubated at 37°C for 24 hours, and 5-6 well isolated colonies from each blood agar plate were used for DNA extraction. Conventional boiling method was used for the extraction of DNA from the obtained *E. coli* isolates. Firstly, 200 µL deionized water was taken into a 2 mL Eppendorf tube and a loopful of fresh colonies (about 5-6) was picked up from the agar plate and transferred to the Eppendorf tube. Then the tubes were vortexed for few seconds to make a homogenous cell suspension and boiled at 99°C for 15 minutes. Immediately after boiling, the suspensions were placed at -20°C for 6-7 minutes for cooling. Finally, the Eppendorf tubes along with the cell suspension were centrifuged at 10,000 rpm for 5 minutes. About 100 µL of supernatant containing bacterial DNA was collected in another sterile Eppendorf tube. The extracted DNA of *E. coli* were preserved separately at -20°C until further testing.

3.3.2 PCR of *E. coli*

PCR reactions were conducted with a 25 µL reaction volume. PCR amplification was performed with the following cycling conditions: 95°C for 2 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 2 min, and a final extension of 72°C for 5 min (Wirth et al., 2006). A previously identified strain of *E. coli* was used as positive control while master mix with nuclease-free water was used as negative control. A product length of 583 bp was considered for the presence of the gene (Wirth et al., 2006).

3.3.3 Visualization of the PCR products by agarose gel electrophoresis

Amplification products were separated by agarose gel electrophoresis (1.5% agarose in 1X TAE). A gel tray was accumulated in a proper set-up by setting comb in the tray. Then, 1.5% agarose solution (Seakem® LE agarose, Lonza) was made and kept in a

water bath at 50°C for cooling, and 5 µL ethidium bromide was added. Finally, the agarose was poured into the gel tray. To solidify the gel, it was kept in room temperature for 20 minutes. Then the gel was transferred into an electrophoresis chamber filled with 1X TAE buffer. An amount of 6 µL of each PCR product for a gene was loaded into a gel-hole and 100 bp DNA marker was used (GeneRular 100 bp DNA Ladder, ThemoFisher Scientific, USA) for comparing the amplicon size of a gene product. Electrophoresis was run at 110 volts, 80 Amp for 25 minutes. Finally, DNA fragments were viewed by UV illumination and photographed (BDA digital, biometra GmbH, Germany).

3.4 Antimicrobial Susceptibility Testing

Susceptibility of *E. coli* isolates against different antimicrobials was performed using Kirby-Bauer disk diffusion method following the guidelines described by the Clinical and Laboratory Standards Institute (CLSI, 2022). Firstly, the preserved *E. coli* was cultured on blood agar and incubated at 37°C for 24 hours. Using a sterile inoculating loop, 2 to 4 individual colonies from the blood agar were transferred into a tube containing 3 mL of sterile normal saline solution. Emulsification of the inoculums was done to avoid clumping of the cells inside test tube by using vortex machine. After that, the bacterial suspension was adjusted to 0.5 McFarland turbidity standards (equivalent to growth of 1.5×10^{8}CFU/mL). Within 15 minutes of preparing the inoculum, a sterile cotton swab was dipped into the inoculum and rotated several times inside of the test tube wall with firm pressure for removing excess fluid from the swab. Then the swab was streaked over the entire dry surface of Mueller Hinton agar (Oxoid Ltd., England) in three directions rotating the plate with an angle of 60°. Finally, the swab was spread around the rim of the agar surface, and after 15 minutes of inoculation; the discs were placed on the agar surface using sterile forceps. Fourteen antibiotic discs were dispensed to the agar surface with optimum distance between two adjacent discs after inoculation of Mueller-Hinton agar plate and incubated at 37°C for 18 hours. The following antimicrobials (with respective disc potencies) were used - Ampicillin (10 µg), Amoxicillin-clavulanic acid (30 µg) Azithromycin (15 µg), Cefotaxime (30 µg), Cefoxitin (30 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Imipenem (10 µg), Levofloxacin (5 µg), Nalidixic acid (30 µg), Sulfamethoxazole-trimethoprim (25 µg) and Tetracycline (30µg). After incubation the

size of zone of inhibition (in mm) around a disc was measured using a ruler and the isolates were defined as sensitive, intermediate or resistant according to CLSI guidelines (CLSI, 2022) (Table 1). Isolates displayed resistance to ≥3 different classes of antimicrobials, was defined as ‘multi-drug resistant’ (Tenover, 2006).

Table 1. Concentrations and diffusion zone breakpoints for resistance for antimicrobial agents tested in this study

Antimicrobial agent	Disc code	Disc concentration	Diffusion zone breakpoint (mm)		
			Sensitive	Intermediate	Resistant
Penicillins					
Ampicillin	AMP	10 µg	≥17	14-16	≤13
Beta lactam combination					
Amoxicillin-clavulanic acid	AMC	30 µg	≥18	14-17	≤13
Cephems					
Cefotaxime	CTX	30 µg	≥26	23-25	≤22
Cefoxitin	FOX	30 µg	≥18	15-17	≤14
Ceftazidime	CAZ	30 µg	≥21	18-20	≤17
Ceftriaxone	CRO	30 µg	≥23	20-22	≤19
Carbapenems					
Imipenem	IPM	10 µg	≥23	20-22	≤19
Aminoglycosides					
Gentamicin	CN	10 µg	≥15	13-14	≤12
Macrolides					
Azithromycin	AZM	15 µg	≥13	-	≤12
Tetracyclines					
Tetracycline	TE	30 µg	≥15	12-14	≤11
Quinolones and Fluoroquinolones					
Ciprofloxacin	CIP	5 µg	≥26	22-25	≤21
Levofloxacin	LEV	5 µg	≥21	17-20	≤16
Nalidixic acid	NA	30 µg	≥19	14-18	≤13
Folate Pathway Antagonists					
Trimethoprim-sulfamethoxazole	SXT	25 µg	≥16	11-15	≤10

3.4.1 Determination of minimum inhibitory concentration (MIC) of colistin

Minimum inhibitory concentration (MIC) values for colistin for all *E. coli* isolates was determined by the broth microdilution method in cation-adjusted Mueller–Hinton broth according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2022). Briefly, cation-adjusted Mueller–Hinton broth with ca. $5x10^5$ CFU/mL inoculum in each well was used. Colistin was tested over a range of dilutions from 0.125 µg/mL to128 µg/mL. *E. coli* ATCC 25922 was used for quality control purpose. Isolates with a colistin MIC ≤4µg/mL were categorized as susceptible and those with a colistin MIC >4 µg/mL were categorized as resistant.

3.5 Detection of the *mcr-1* gene

Screening for the presence of *mcr-1* gene was performed in each phenotypically colistin-resistant isolate by PCR with the primers CLR5-F (5′-CGGTCAGTCCGTTTGTTC-3′) and CLR5-R (5′-CTTGGTCGGTCTGTA GGG-3′) (Liu et al., 2016). PCR amplification was performed with the following cycling conditions: 1 cycle of denaturation at 94°C for 15 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 90 s and elongation at 72°C for 60 s, and a final cycle of elongation at 72°C for 10 min. The amplification was visualized by electrophoresis using 1.5% agarose gel (SeaKem® LE Agarose, Lonza).

3.6 Detection of quinolone resistance genes by PCR

E. coli isolates showing resistance to quinolones were tested for the presence of *gyrA*, *gyrB* and *parC* genes by PCR using the recommended primers, as shown in Table 2. A concentration of 100 pmol/µl was prepared from each primer stock by adding molecular grade deionized water, by following the manufacturer's instructions. To run PCR assays 20 pmol/µl concentration of each primer was used. PCR reactions were carried out in a total volume of 10 µL consisting of 2 µL of nuclease free water, 5 µL dreamtaq master mix (Fermentas, Sweden), 0.5 µL of each primer and 2 µL of DNA template. The PCR conditionsare described in Table 2. Master mix without any DNA template was used for a negative control, and three *E. coli* in-house strains carrying the tested genes were used as the positive controls.

Table 2. Primer sequences used in polymerase chain reaction (PCR) to detect *gyrA*, *gyrB*, *parC* and beta lactamase genes

Gene	Primer name	Primer sequence (5´-3´)	Amplicon size (bp)	PCR condition	Reference
gyrA	GyrA-184-F	TACCGTCATAGTTATCCACGA	312	Initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min	Wiuff et al., 2000
	GyrA-185-R	TTCCTGTTTTTGCTCACCCA			
gyrB	GyrB-303-F	GTCCGAACTGTACCTGGTGG	281	Initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min	
	GyrB-304-R	AACAGCAGCGTACGAATGTG			
parC	ParC-305-F	CTATGCGATGTCAGAGCTGG	261	Initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min	
	ParC-306-R	TAACAGCAGCTCGGCGTATT			
bla$_{TEM}$	*bla*$_{TEM}$-L	GCGGAACCCCTATTTG	964	Initial denaturation at 94°C for 3 min, 25 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min	Hasman et al., 2005
	bla$_{TEM}$-R	TCTAAAGTATATATGAGTAA ACTTGGTCTGAC			
bla$_{CTX-M}$	*bla*$_{CTX-M}$-F	ATGTGCAGYACCAGTAARGT KATGGC	593	Initial denaturation at 95°C for 15 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 90 sec, extension at 72°C for 1 min and final extension at 72°C for 10 min	Fang et al., 2008
	bla$_{CTX-M}$-R	TGGGTRAARTARGTSACCAGA AYCAGCGG			
bla$_{SHV}$	*bla*$_{SHV}$-F	CTTTATCGGCCCTCACTCAA	237		
	bla$_{SHV}$-R	AGGTGCTCATCATGGGAAAG			
bla$_{OXA}$	*bla*$_{OXA}$-F	ACACAATACATATCAACTTCG C	813		
	bla$_{OXA}$-R	AGTGTGTTTAGAATGGTGATC			

3.7 PCR amplification for detection of beta lactamase gene

Multiplex PCR was performed to determine the presence of *bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{OXA}$. Primers used for these genes and the PCR conditions are listed in Table 2. The PCR mix of this reaction included 10 µl of master mix, 3 µl of primers mix, 1 µl of DNA and 5 µl of nuclease-free water.

3.8 Statistical analysis

All data were entered into a spreadsheet of Microsoft Excel 2007 and transferred to EpiTools epidemiological calculators (http://epitools.ausvet.com.au.) for data summary and descriptive statistics. The 95% confidence interval of the prevalence values were calculated by the modified Wald method using the GraphPad software QuickCalcs (https://www.graphpad.com/quickcalcs/).

CHAPTER - 4

RESULTS

4.1 Identification of *Escherichia coli*

A total of 100 urine samples from patients suspected with urinary tract infections were investigated during the five-month period (December 2021 to April 2022), 37 (37%, 95% Confidence Interval 28.2% - 46.8%) of these samples were found positive with *E. coli* based on colony appearance on MacConkey and EMB agar plates. The characteristic colonial growth of *E. coli* as observed onto MacConkey and EMB agars are displayed in Figures 1 and 2, respectively. Typical colonies were confirmed by detection of a housekeeping gene *adk* by PCR. All isolates which were phenotypically positive for *E. coli*, were also positive for the presence of the *adk* gene (Figure 3).

4.2 Antimicrobial susceptibility testing of *E. coli* isolates

The overall results of antimicrobial susceptibility testing of *E. coli* isolates obtained from urine samples are shown in Table 3. More than 75% and 50% isolates were resistant to Azithromycin and Ampicillin, respectively, but 37.8% and 29.7% to Tetracycline and Trimethoprim-sulfamethoxazole, respectively. In addition, more than 25% isolates displayed resistance to Cefotaxime, Cefoxitin, Ceftriaxone, Ciprofloxacin and Nalidixic Acid. On the other hand, the isolates exhibited highest sensitivity to Imipenem (94.6%) and Colistin Sulphate (94.6%) followed by Gentamicin (75.7%). Individual antibiogram profiles of all the isolates are illustrated in Figure 4.

4.3 Colistin susceptibility

The broth microdilution test results for *E. coli* isolates are shown in Figure 5. The minimum inhibitory concentration (MIC) of colistin in *E. coli* isolates ranged from ≤1 to ≥128 µg/mL. The MIC values of the two individual resistant isolates were 8 and 128 µg/mL.

Figure 1: Typical colony morphology of *E. coli* on MacConkey agar

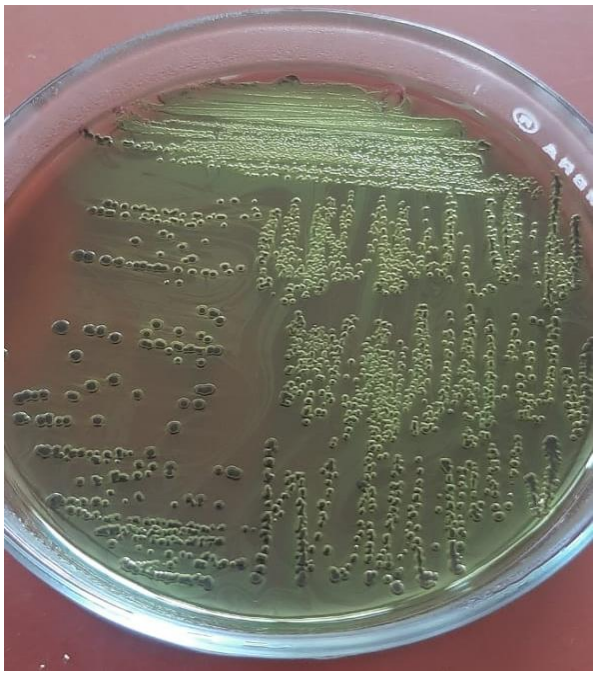

Figure 2: Growth of isolated *E. coli* colony showing greenish metallic sheen on EMB agar

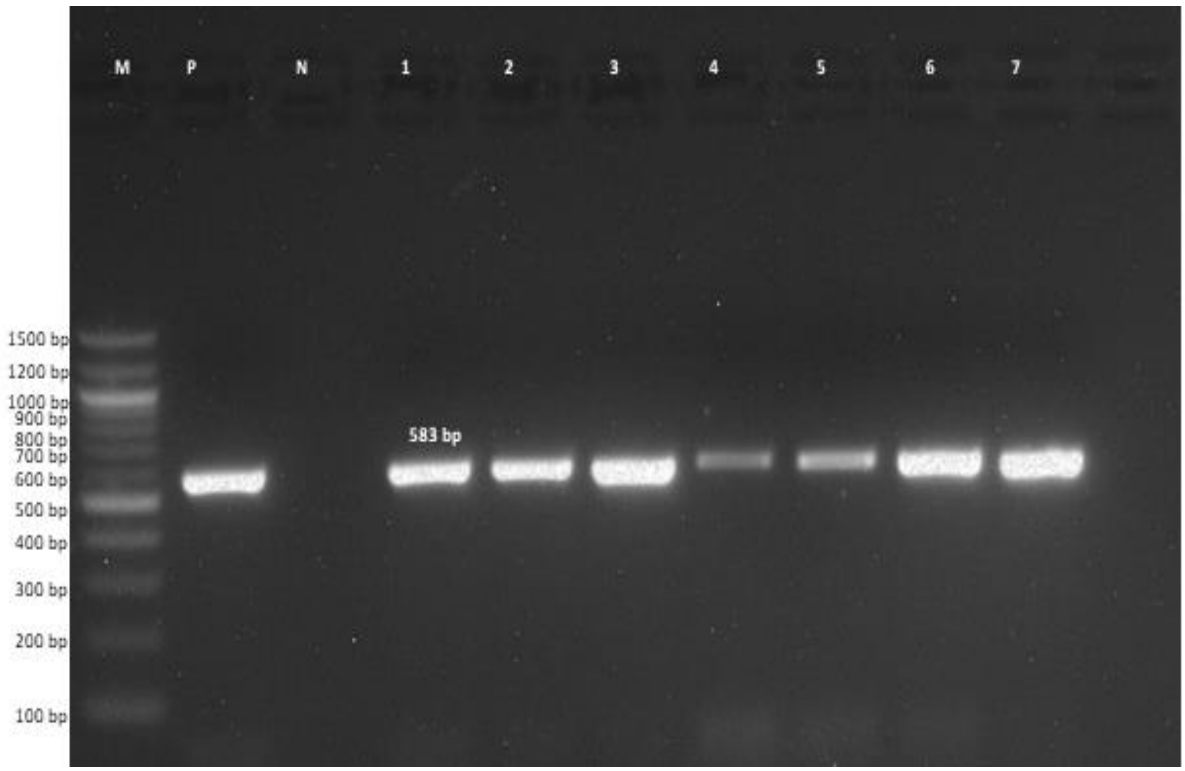

Figure 3: This figure illustrates fragments specifically amplified by PCR by means of the primers adk F and adk R. A product length of 583 bp was considered for the presence of the housekeeping gene *adk* (adenylate kinase). Lane M: 100 bp DNA ladder, Lane P: Positive control, Lane N: Negative control, Lane 1-7: *adk* gene sized (583 bp) amplicon.

Table 3. Antimicrobial susceptibility pattern of 37 *E. coli* isolates obtained from urine samples of humans suspected with urinary tract infections

Antimicrobial agents	**Number of isolates**		
	Sensitive (%)	**Intermediate (%)**	**Resistant (%)**
Penicillins			
Ampicillin (AMP)	12 (32.4)	5 (13.5)	20 (54.1)
Beta lactam combination			
Amoxicillin-clavunate (AMC)	24 (64.9)	6 (16.2)	7 (18.9)
Cephems			
Cefotaxime (CTX)	16 (43.2)	9 (24.3)	12 (32.4)
Cefoxitin (FOX)	24 (64.9)	02 (5.4)	11 (29.7)
Ceftazidime (CAZ)	26 (70.3)	06 (16.2)	05 (13.5)
Ceftriaxone (CRO)	24 (64.9)	03 (8.1)	10 (27)
Carbapenems			
Imipenem (IPM)	35 (94.6)	01 (2.7)	01 (2.7)
Lipopeptides			
Colistin (CT)	35 (94.6)	-	02 (5.4)
Aminoglycosides			
Gentamicin (CN)	28 (75.7)	07 (18.9)	02 (5.4)
Macrolides			
Azithromycin (AZM)	09 (24.3)	-	28 (75.7)
Tetracyclines			
Tetracycline (TE)	23 (62.2)	-	14 (37.8)
Quinolones and Fluoroquinolones			
Ciprofloxacin (CIP)	21 (56.8)	05 (13.5)	11 (29.7)
Levofloxacin (LEV)	25 (67.6)	04 (10.8)	08 (21.6)
Nalidixic acid (NA)	23 (62.2)	03 (8.1)	11 (29.7)
Folate Pathway Antagonists			
Trimethoprim-sulfamethoxazole (SXT)	26 (70.3)	-	11 (29.7)

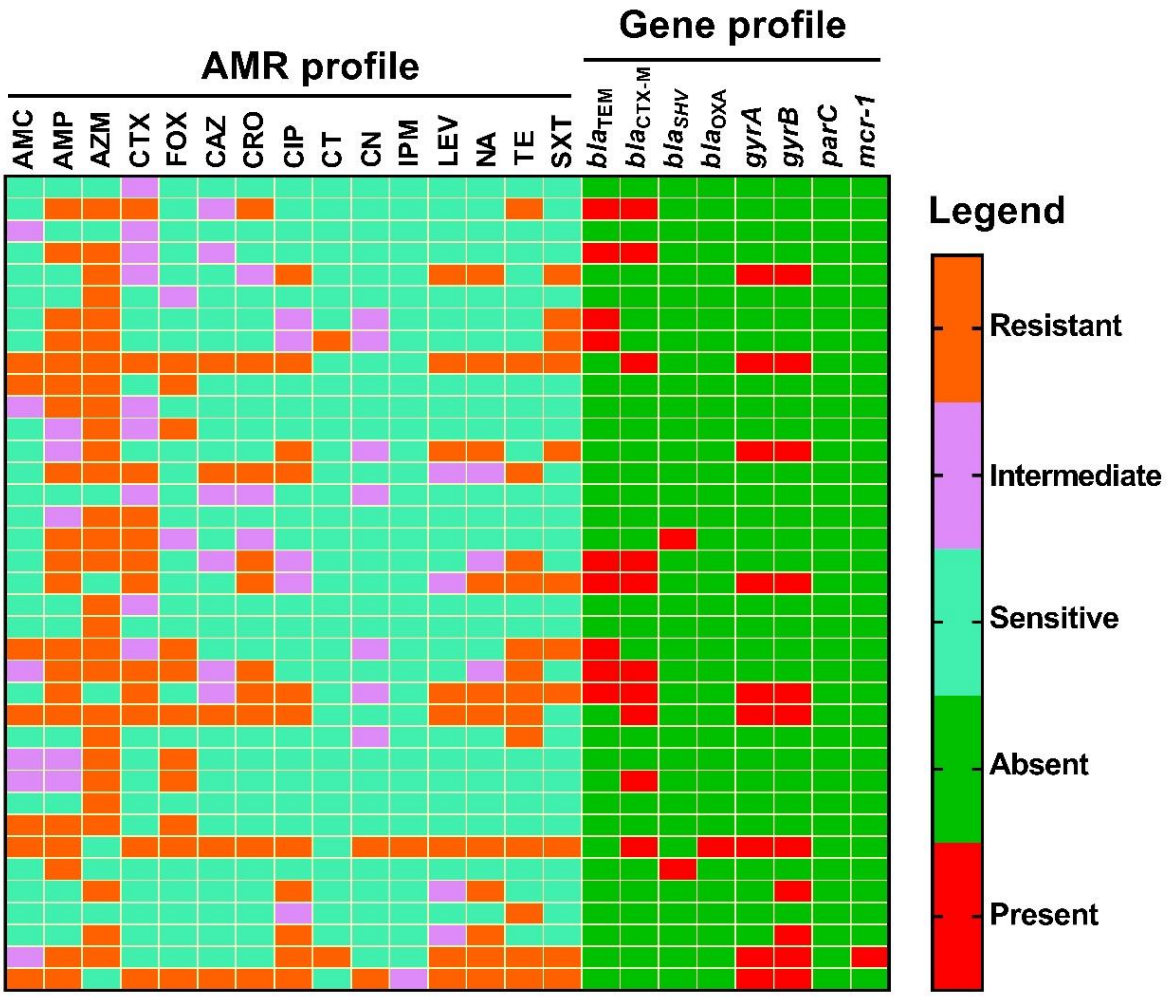

Figure 4: Heat map showing the distribution of antimicrobial resistance phenotype and gene profile of *E. coli* isolates, where AMC = Amoxicillin-clavunate, AMP = Ampicillin, AZM = Azithromycin, CTX = Cefotaxime, FOX = Cefoxitin, CAZ = Ceftazidime, CRO = Ceftriaxone, CIP = Ciprofloxacin, CT = Colistin, CN = Gentamicin, IPM = Imipenem, LEV = Levofloxacin, NA = Nalidixic acid, TE = Tetracycline, SXT = Trimethoprim-sulfamethoxazole

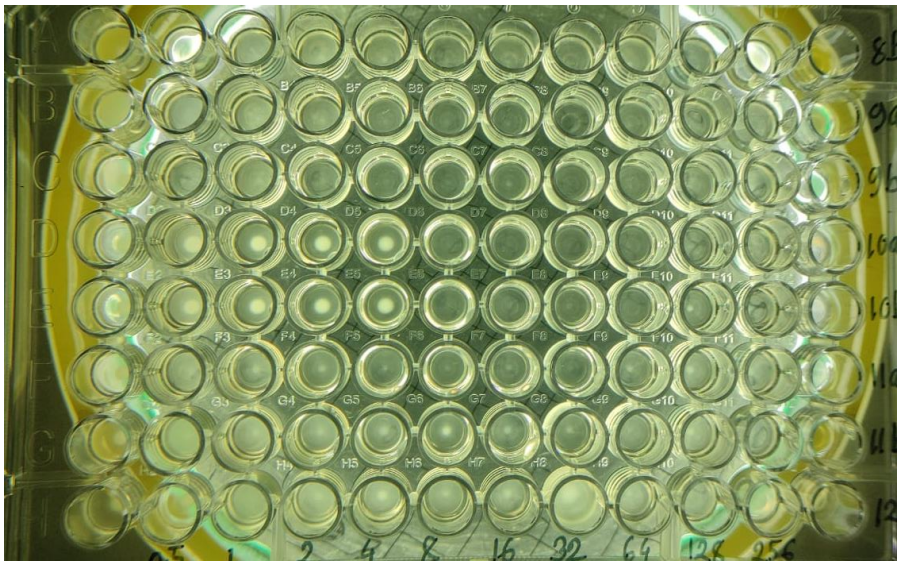

Figure 5: The microtiter plate showing the results of broth microdilution test to determine the minimum inhibitory concentration (MIC) value of colistin for *E. coli* isolates recovered from urine samples of humans.

4.4 Multidrug resistance pattern

Diversity of resistant phenotypes among the 37 *E. coli* isolates is presented in Table 4. A total of 24 resistance patterns with different combination of antimicrobial agents were observed. About 52% of the total isolates showed multi-drug resistance (*i.e.* resistance to ≥3 antimicrobial classes) with a range from 3 to 8 different antimicrobials (Table 5). Approximately 3% of the resistant isolates were resistant to eight antimicrobial classes.

4.5 Detection of *mcr-1* gene

The two phenotypically colistin resistant *E. coli* isolates were tested for the presence of *mcr-1* gene by PCR. Of them, one isolate was found positive for the possession of *mcr-1* gene. The amplicon showing *mcr-1* gene in the isolate tested is illustrated in Figure 6.

Table 4. Antimicrobial resistance profile of *E. coli* isolated from urine samples of humans

Sl. no.	Resistance phenotype	No. of isolates displaying resistance
1.	TE	01
2.	AMP	01
3.	AZM	04
4.	AZM, TE	01
5.	AZM, FOX	03
6.	AZM, CTX	01
7.	AMP, AZM	02
8.	AZM, CIP, NA	02
9.	AMP, AZM, SXT	01
10.	AMP, AZM, CTX	01
11.	AMP, AZM, CT, SXT	01
12.	AMC, AMP, AZM, FOX	02
13.	AZM, CIP, LEV, NA, SXT	02
14.	AMP, AZM, CTX, CRO, TE	02
15.	AMP, CTX, CRO, NA, TE, SXT	01
16.	AMP, AZM, CTX, FOX, CRO, TE	01
17.	AMC, AMP, AZM, FOX, TE, SXT	01
18.	AMP, AZM, CTX, CAZ, CRO, CIP, TE	01
19.	AMP, AZM, CIP, CT, LEV, NA, TE, SXT	01
20.	AMP, CTX, CRO, CIP, LEV, NA, TE, SXT	01
21.	AMC, AMP, AZM, CTX, FOX, CAZ, CRO, CIP, LEV, NA, TE	01
22.	AMC, AMP, CTX, FOX, CAZ, CRO, CIP, CN, LEV, NA, TE, SXT	01
23.	AMC, AMP, AZM, CTX, FOX, CAZ, CRO, CIP, LEV, NA, TE, SXT	01
24.	AMC, AMP, CTX, FOX, CAZ, CRO, CIP, CN, IPM, LEV, NA, TE, SXT	01

Table 5. Number and percentages of *E. coli* isolates exhibiting resistance to various number of antimicrobial classes

Number of antimicrobial classes to which isolates were resistant	Number (%) of resistant isolates
3	5 (13.5)
4	5 (13.5)
5	4 (10.8)
6	2 (5.4)
7	2 (5.4)
8	1 (2.7)

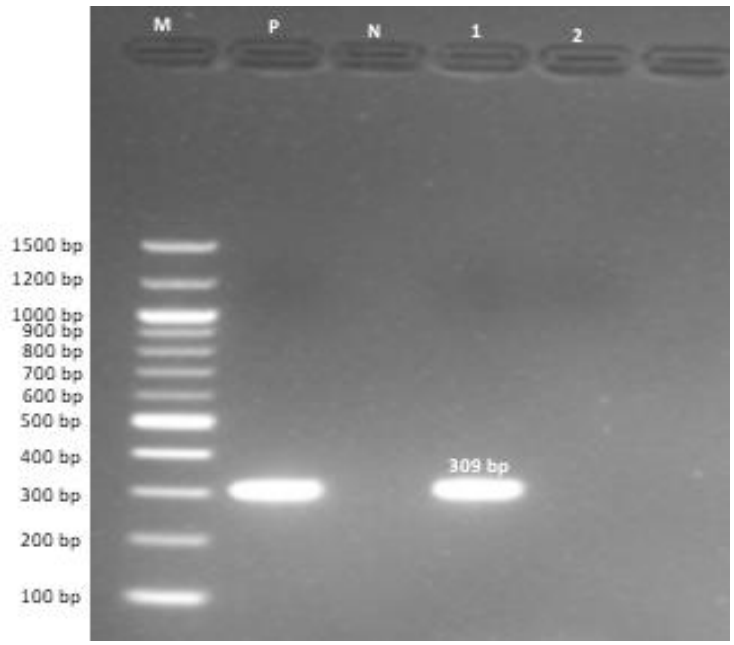

Figure 6. Result of PCR assay for *mcr-1* gene of *Escherichia coli* isolates obtained from urine samples from humans. Lane M: 100 bp DNA ladder; Lane P: Positive control; Lane N: Negative control; Lane 1 showing the amplicon (309 bp) of *mcr-1* gene of the one *mcr-1* gene positive isolates of *E. coli* and Lane 2 showing the colistin-resistant *E. coli* isolate without harboring *mcr-1* gene.

4.6 Detection of *gyrA*, *gyrB* and *parC* genes

All the quinolone- and fluoroquinolones-resistant *E. coli* isolates were tested for the presence of the *gyrA*, *gyrB* and *parC* genes whose amplicon sizes were 312bp, 281bp and 261bp, respectively. According to the results obtained, 9 isolates harbored the *gyrA* gene, while 11 had the *gyrB* gene. None of the isolates harbored *parC* gene (Figure 4). Amplicons showing the presence of *gyrA* and *gyrB* of the *E. coli* isolates tested are depicted in Figures 7 and 8, respectively.

4.7 Identification of *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{OXA}$ genes

Nine out of 24 isolates (37.5%) gave positive amplicon for the *bla*$_{TEM}$ gene. The *bla*$_{CTX-M}$ gene was detected in 41.7% isolates, whereas *bla*$_{SHV}$ gene in 8.3% isolates. Only one isolate was found positive for *bla*$_{OXA}$ gene. Isolates having the *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{OXA}$ genes as measured by the typical amplicon sizes of the gene products, are portrayed in Figures 9 and 10, respectively.

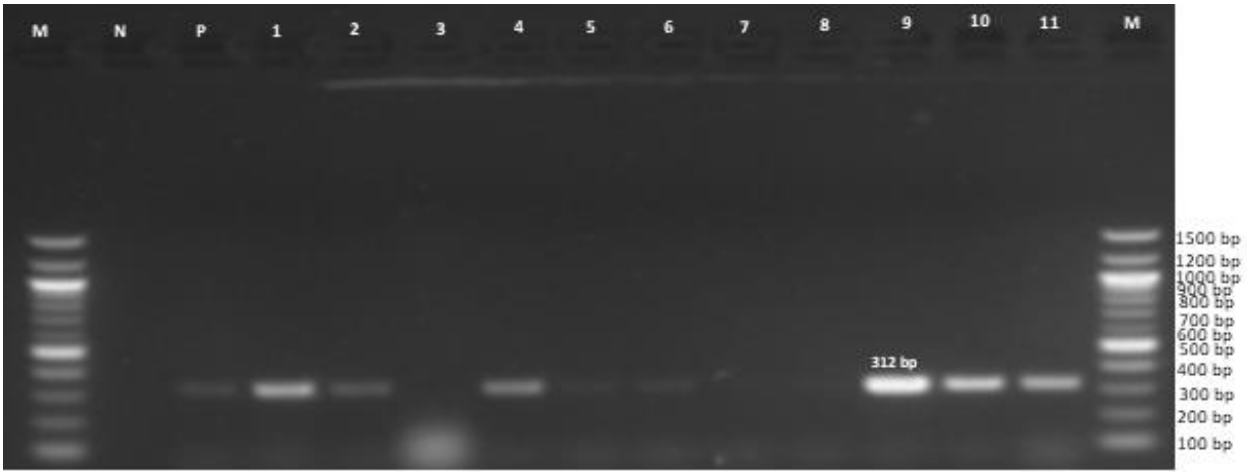

Figure 7: Result of PCR assay for the *gyrA* gene of some of the isolates tested; Lane M: 100 bp DNA ladder; Lane N: Negative Control; Lane P: Positive Control; lane 1, 2, 4, 8, 9, 10,11: PCR products showing the *gyrA* gene sized (312 bp) amplicon; Lane 3, 5, 6, 7: Samples giving negative reactions in PCR.

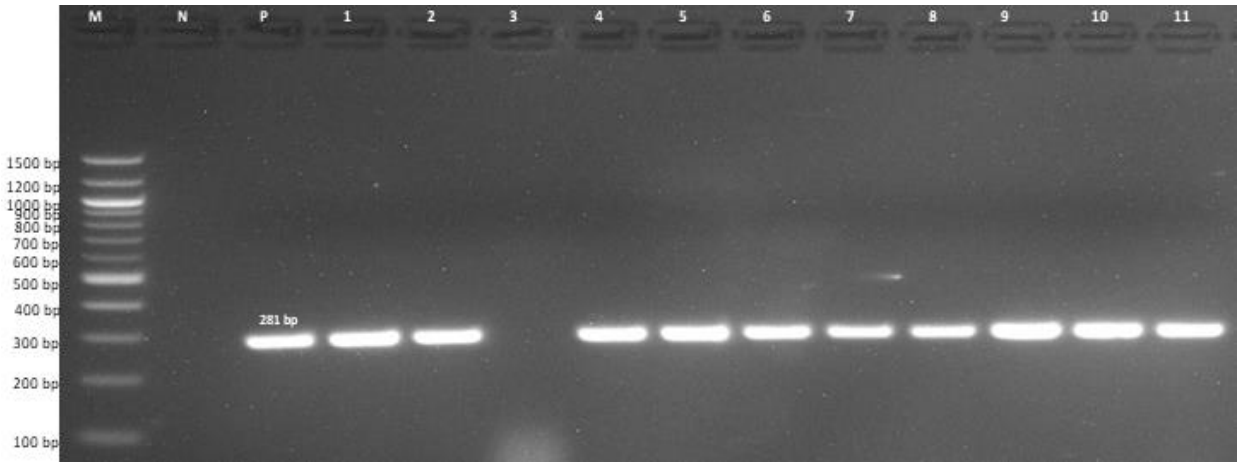

Figure 8. Result of PCR assay for the *gyrB* gene of some of the isolates tested; Lane M: 100 bp DNA ladder; Lane N: Negative Control; Lane P: Positive Control; lane 1, 2, 4, 5, 6, 8, 9, 10: PCR products showing the *gyrB* gene sized (281 bp) amplicon; Lane 3: Sample giving negative reactions in PCR.

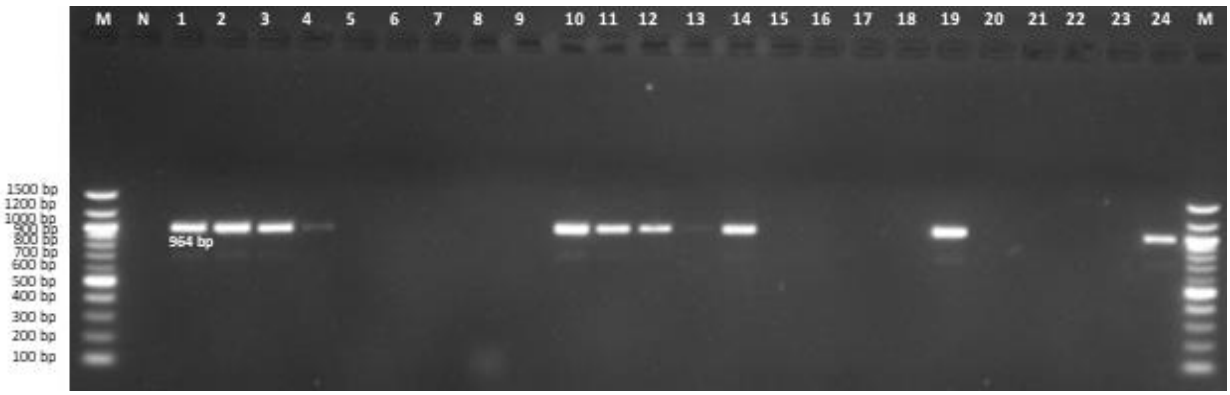

Figure 9: Result of PCR assay for *bla*$_{TEM}$ gene of *Escherichia coli* isolates obtained from urine samples from humans. Lane M: 100 bp DNA ladder; Lane N: Negative control; Lane 1, 2, 3, 4, 10, 11, 12, 14, 19, 24 showing the amplicon (964 bp) of *bla*$_{TEM}$ gene of the positive isolates of *E. coli* and Lane 5, 6, 7, 8, 9, 13, 15,16, 17, 18, 20, 21, 22, 23 showing the samples giving negative reactions in PCR.

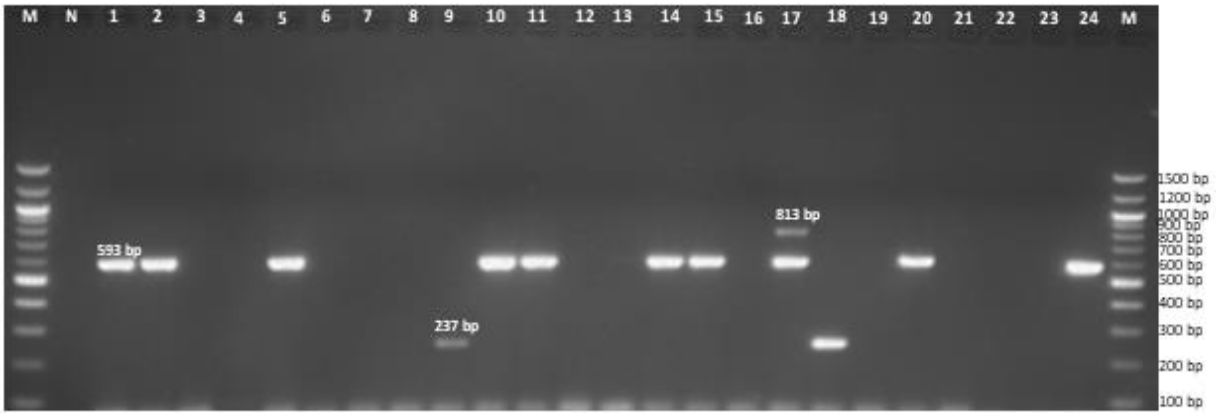

Figure 10. Result of PCR assay for *bla*$_{CTX-M,}$ *bla*$_{SHV}$, *bla*$_{OXA}$ genes of *Escherichia coli* isolates obtained from urine samples from humans. Lane M: 100 bp DNA ladder; Lane N: Negative control; Lane 1, 2, 5, 10, 11, 14, 15, 17, 20, 24 showing the amplicon (593 bp) of *bla*$_{CTX-M}$ gene of the positive isolates of *E. coli*; Lane 9, 18 showing the amplicon (237 bp) of *bla*$_{SHV}$ gene of the positive isolates of *E. coli* isolates; Lane 17 showing the amplicon (813 bp) of *bla*$_{OXA}$ gene of the positive isolate of *E. coli* isolates: Line 3, 4, 6, 7, 8, 9, 12, 13, 16, 18, 19, 21, 22, 23 showing the samples giving negative reactions in PCR.

CHAPTER - 5
DISCUSSION

Urinary tract infections are among the most common infections worldwide and caused predominantly by *E. coli*, which could lead to sepsis, renal damage, or even death (Klein and Hultgren, 2020). *E. coli* is the most common etiological agent of UTIs. In this study, urine samples from suspected patients were tested to determine the frequency and antimicrobial resistance pattern of *E. coli* isolated from urine samples. Presence of possible corresponding genes associated with quinolones and beta-lactam resistance were also investigated. The results of the study revealed that 37% samples, i.e., more than one-third samples were found positive for *E. coli* as an etiological agent of urinary tract infection. *E. coli* is reported as one of the nine bacterial pathogens of global concern that are responsible for some of the common infections that occur in community and hospital settings (WHO, 2014) and most often implicated in UTI.

Majority of the positive isolates were found resistant to Azithromycin and Ampicillin. The high level of resistance to Ampicillin confirms previous studies, indicated that this antimicrobial is less effective against *E. coli* (Almohana, 2004; Hadi, 2008; Al-Hilali, 2010). Several reports were in matching line of the present study. Momtaz et al. (2013) showed, 99.3% of UPEC were resistant to Penicillin group in Iran, and 92.5% were to Amoxicillin (Mukherjee et al., 2013) in India. The high level of resistance developed in *E. coli* against Azithromycin indicates a consequence of overuse of this antimicrobial agent.

The isolated *E. coli* was found resistant to Ciprofloxacin (29.7%), Levofloxacin (21.6%) and Nalidixic acid (29.7%). Mukherjee et al. (2013), reported 80% of UPEC isolates resistance to Ciprofloxacin. In Chinese Hospitals by Cao et al. (2011) who revealed, 75% and 71% of UPEC isolates resistance to Ciprofloxacin and Levofloxacin, respectively. Fluoroquinolones are a critically important antimicrobial listed by World Health Organization (WHO, 2012) which are the most commonly prescribed antimicrobials for UTI and therefore, resistance to these antimicrobials is a great threat to public health. Indiscriminate and over-use of Ciprofloxacin could have roles in developing a high level of resistance in *E. coli* to this critically important antimicrobial.

Quinolone resistance normally arises by mutations in the quinolone resistant determining region (QRDR) of the *gyrA* and *gyrB* genes and less frequently with mutations in the topoisomerase IV encoded by *parC* and *parE* genes, plus to decreased membrane penetrability in conjunction with the over-expression of efflux pumps, with additional low-level resistance mediated by plasmid-mediated quinolone resistance genes (Cao et al., 2011). The presence of *gyrA* and *gyrB* in Ciprofloxacin-resistant *E. coli* isolates indicates that mutations have occurred in these genes to acquire the resistance to Ciprofloxacin and the contribution of other gene *parC* might be little. To find out the possible mutation sites, these genes should have been sequenced, which was not possible in this study due to limitation of resources.

A significant number of isolates was found resistant to third generation cephalosporins. The level of resistance to third-generation cephalosporin most likely due to the acquirement of β-lactamases, which encodes by *bla* genes. In recent decades, the extended-spectrum β-lactamases (ESBLs) of the TEM, SHV, CTX-M, and OXA type have emerged as significant mechanisms of resistance in Gram-negative bacilli (Apisarnthanarak et al., 2008). ESBLs are enzymes able to efficiently hydrolyze extended-spectrum cephalosporins and monobactams and have been associated with therapeutic failures. These enzymes have widely spread to geographic regions, and this is due, in part, to the fact that many resistance genes are often carried on self-transmissible or mobile plasmids that are capable of spreading horizontally between and within species (Bonnet, 2004).

The ESBL-positive *E. coli* isolates investigated here encoded mainly CTX-M (41.7%), followed by TEM-type (37.5%), and SHV-type (8.3%) -lactamases. This occurrence is lower than the level reported in Switzerland (91%) (Lartigue et al., 2007), Norway (90%) (Tofteland et al., 2007), and Austria (85%) (Eisner et al., 2006). The ability to produce ESBL confers resistance against broad-spectrum cephalosporins, which are widely used for the treatment of serious bacterial infections.

Two *E. coli* isolates exhibited resistance to Colistin sulphate. In an Indian study colistin resistant isolates were detected among human patients (Ghafur et al., 2014). This antibiotic is considered a last-resort antibiotic treatment option for infections with

multi-drug resistant pathogens belonging to *Eneterobacteriaceae*. The finding of colistin-resistant *E. coli* in the present study is worrisome.

The common mechanism of colistin resistance is generally thought to be associated with the chromosomally-encoded (Poirel et al., 2017). A plasmid-mediated colistin resistance gene (*mcr-1*) conferring resistance to colistin was identified from *Enterobacteriaceae* in China in 2015 (Liu et al., 2016). Later colistin resistant *E. coli* and *Klebsiella pneumoniae*, carrying the *mcr-1* gene were reported worldwide from different sources (Poirel et al., 2017). In the present study *mcr-1* gene was detected in one *E. coli* isolate. Although high MIC value of colistin was determined in other *E. coli* isolate, the absence of *mcr-1* gene suggested the presence of any of the other *mcr* genes.

On the other hand, the isolates displayed highest sensitivity to Imipenem followed by Gentamicin. Giray et al. (2012), in agreement with Hadi (2008) and Al-Hilli (2010), demonstrated that all (100%) *E. coli* isolates were susceptible to imipenem and meropenem. Still, the carbapenems offer an alternative for treatment, but resistance to cabapenems has been reported, and such multi-resistance poses even more serious problems for treatment (Woodford et al., 2007). Previous literature by Al-Hilali (2010) stated that 100% and 45.5% isolates were sensitive to Amikacin and Gentamicin, respectively. The percentages of aminoglycosides susceptibility described in this study harmonize with those reported by Almohana (2004) in Najaf and Cao et al (2011) in China who reported, 93% of isolates were sensitive to amikacin and 63% to gentamicin.

Lower occurrence of resistance was recorded in the present study to trimethoprim-sulfamethoxazole in opposite to Essen-Zandbergen et al. (2007) in Netherlands, who demonstrated, most of the *E. coli* selected were resistant to sulfamethoxazole (94%) and trimethoprim (81%). In Poland, Mokracka et al. (2012) revealed that 86.6% of *E. coli* isolates were resistant to sulfamethoxazole. The emergence of drug resistance to trimethoprim and sulfamethoxazole by UPEC have limited the choices for selecting the appropriate antibiotic for the treatment of UTIs.

Appropriate antibiotic use is an essential component of any program to slow the emergence and spread of drug-resistant microorganisms in the heath-care setting. A strain of UPEC considered as an MDR if it was resistant to at least three classes of

antimicrobials. The present study demonstrated half of *E. coli* isolates were MDR showing resistance to a minimum of three classes of the antimicrobial agents tested. The result of the present study was supported by a study conducted in India where prevalence of MDR *E. coli* was about 52.9 percent (Hasan et al., 2007). Hadi (2008) also reported that 82.6% of *E. coli* isolates were MDR. Similarly, Al-Hilli (2010) revealed that all *E. coli* isolates obtained from Merjan Teaching Hospital in Hilla, Iraq, were considered as MDR. The level of MDR amongst the UTI isolates was found to vary according to geographical location. In Tabriz, northern west of Iran, 84.2% of tested strains were observed as MDR (Rezaee et al., 2011) and Mukherjee et al. (2013) revealed, MDR strains was detected in 92.5% of isolates obtained from hospitalized patients in Kolkata, India. While, in Dublin, Ireland, 36% isolates were considered as MDR (Cooke et al., 2010). The increase of MDR strains in the present study may be the results of uncontrolled antibiotic use in medicine over the last several years. The careless antimicrobial usage without antimicrobial sensitivity testing is one of the most important factors for promoting the emergence of MDR, which cause selection and dissemination of antimicrobial-resistant pathogens in clinical medicine.

One of the major limitations of the study was small sample size. Moreover, the study had no individual demographic data to compare the laboratory results with the participants. Future investigation is required in large scale covering the other hospital settings.

The findings of this study indicate that there has been a significant increase in antimicrobial resistance in the *E. coli* causing UTI in humans. The general people use antibiotics largely without consulting with the physicians. Due to indiscriminate exploitation of antimicrobial agents, such high incidence of multi drug resistance may apparently be occurred which may ultimately replace the drug sensitive microorganisms from antibiotic saturated environment (Van de Boogard and Stobberingh, 2000).

CHAPTER - 6
CONCLUSIONS

The present study shows an emergence of MDR *E. coli* among the patients with urinary tract infection. Relative high level of antimicrobial resistance was observed among Azithromycin and Ampicillin. However, Cefotaxime, Cefoxitin, Ceftriaxone, Ciprofloxacin, and Nalidixic acid have shown comparatively less resistant. However, ESBL-producing *E. coli* is a growing problem. The resistance to ciprofloxacin in the circulating strains has evolved because of the acquisition of the *gyrA* and *gyrB* genes. Due to higher rates of sensitivity to imipenem, colistin sulphate, and gentamicin may be the effective choice of drugs in case of resistance to β-lactams and fluoroquinolones class of antibiotics to treat UTI. This suggests a reassessment of empirical therapies in urinary tract infections treatment caused by *E. coli*.

CHAPTER - 7
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