CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 Background of the study

Escherichia coli is a gram negative, rod shaped, facultative anaerobic and coliform bacterium belonging to the genus *Escherichia* which are commonly found in lower intestine of warm blooded animals (Tenaillon et al 2012, Singleton 1999). In case of UTI, fecal bacteria colonize urethra and spread up the urinary tract and finally to the bladder while sometimes to the kidneys causing pyelonephritis or the prostrate in males (Nicolle 2008). They are non-sporulating bacteria which are about 2.0 µm long and 0.25-1.0 µm in diameter (YU et al 2014). Motile strains of *E. coli* have flagella which show peritrichous arrangement (Darnton et al 2007). *Eschericia coli* are grouped into enterotoxigenic, enteroinvasive, enteropathogenic and enterohaemorrhagic *E. coli*. At present around 190 strains of *E. coli* have been identified (Stenutz et al 2006). Strains of *E. coli* that cause UTIs are known as uropathogenic *E. coli* (UPEC). Factors that contribute to the pathogenesis of UPEC *E. coli* include hemolysins, secreted proteins, specific lipopolysaccharides and capsular types, iron acquisition systems and fimbrial adhesions (Michael et al 2009). Hemolysin production is considered to be an important virulence factor in urinary tract infection caused by *E. coli* (Hughes et al 1983).

Urinary tract infection is one of the most common bacterial infections. UTI often leads to significant morbidity and mortality (Ramesh et al 2008). During their lifetime approximately 10% of the humans acquire UTI at sometime (Karki et al 2004). UTI is the condition in which urine contains large number of multiplying bacteria. Acute cystitis is the most common type of UTI which is referred as bladder infection. Pyelonephritis, an infection of upper urinary tract or kidney is however more serious type. Similarly, the incidence of UTI is age and sex dependent. Women are more prone to UTI than men as they have shorter urethra and close proximity to perianal region (Forbes et al 2002). Females falling within the age group 21-30 years experience UTI more frequently (Baral et al 2012). Among all the members of Enterobacteriaceae

family, *E. coli* is the most common pathogen (80-85%) involved in UTI (Nicolle 2008, Bhatta et al 2012).

Biofilm formation is a phenomenon which is produced by microorganisms to survive in harsh environment or for establishing bacterial infection in humans (Hung et al 2009, Neupane et al 2016). Biofilm protects bacteria from antibiotics and host defenses which as a result makes the treatment of infection more difficult (Anderson et al 2003). The interaction between the bacterial cells within a biofilm can lead to the exchange of plasmid, drug resistance marker genes and hence enhances antimicrobial resistance (Kostakioti et al 2013, Watnick et al 2000). Thus, biofilm mode of living is advantageous for uropathogens to withstand stress in urinary tract environment (Pramodhini et al 2012). Biofilm formation by *E. coli* is closely associated with the antimicrobial drug resistance and rise in the chronicity of urinary tract infection. UTI caused by biofilm producing *E. coli* may enhance the colonization and incidence rate of UTI as well (Ponnusamy et al 2012).

According to the centers for disease control and prevention, multidrug resistant (MDR) is defined as non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al 2011). The emergence of multidrug resistance *E. coli* in urinary tract infection has become a global concern (Mashwal et al 2017). Several studies have reported *E. coli* being resistant against trimethoprim-sulfamethoxazole, fluoroquinolones and other antibiotics including ciprofloxacin (Karlowsky et al 2006 and Park et al 2006).

E. coli is the major cause of urinary tract infection. The prevalence rate of *E. coli* in urine sample was 30% while the isolation rate was higher in females in comparison with males (Zahera et al 2011). Women belonging to the age group 20-40 are sexually active and they are at the highest risk of UTI. The diagnosis of UTI is usually based on a quantitative urine culture yielding greater than 10^5 colony forming units per ml (Kass et al 1957). However, several studies suggest that more than one third of symptomatic women show CFU counts below this level (low-coliform-count infection) and that a bacterial count of 100 CFU per ml of urine has a high positive predictive value for cystitis in symptomatic women (Kunin et al 1993 and Komaroff et al 1986). Certain types of contraceptives can also invite the risk of UTI (Yadav

et al 2015). *E. coli* showed highest frequency of resistance against cefotaxime, ceftazidime, amoxicillin, ciprofloxacin and norfloxacin while moderate resistance towards gentamicin and low resistance to nitrofurantoin (Bigu et al 2016). Prolonged antibiotic exposure, overstay in hospitals, severe illness, improper use of third generation cephalosporin and increased use of intravenous devices and catheters are the important factors for infection with MDR *E. coli* (Chaudhary et al 2004). Shrestha et al (2015) reported that 50.8% of MDR *E. coli* were isolated from the urine samples from surgical wards. Hospital acquired urinary tract infection has been associated with the indwelling catheters and devices which can provide entry or place for colonization of microorganisms (WHO 2002). Pramodhini et al (2012) demonstrated 63% of *E. coli* isolates to be biofilm producer. A similar rate (67.5%) of biofilm producing isolates of *E. coli* was reported by Sharma et al (2009). Resistance to antibiotics such as ampicillin (83.3% vs 60%), cefotaxime (73.3%vs 35%), norfloxacin (80% vs 60%) and nalidixic acid (93.3% vs 70%) were comparatively higher among biofilm producer than non-biofilm producer (Pramodhini et al 2012). This study showed significant correlation between biofilm production and multidrug resistance, where 80% of strains producing biofilm were MDR phenotypes. According to the research done by Ponnusamy et al 2012, biofilm formation is not only related to the resistance of *E. coli* against antimicrobial drugs but also with the chronicity of UTI disease. However, Khatri et al (2017) and Neupane et al (2016) reported only 15.5% and 14.1% of *E. coli* isolates obtained from total urine samples. Strains of *E. coli* causing urinary tract infection produce hemolysins which may be important in pathogenesis of UTI and such strains show resistance to large number of antibiotic drugs (Onanuga et al 2005).

1.2 Objectives

1.2.1 General objectives:

- To study antibiogram of *E. coli* isolated from urine samples of UTI suspected patients at tertiary hospital, Morang.

1.2.2 Specific objectives:

- To study the prevalence of *E. coli* among UTI suspected patients.
- To perform the antibiotic susceptibility test.
- To determine the ability of biofilm production by *E. coli* isolated from urine sample of patients.

CHAPTER II

LITERATURE REVIEW

2.1 *E. coli*

E. coli are non-sporing, facultative anaerobe, Gram-negative bacteria usually motile peritrichous flagella. They produce gas as they can ferment some carbohydrates. They constitute about 0.1% of the gut microbiota along with other facultative anaerobes (Eckburg et al 2005). They give methyl red reaction positive but Voges-Proskauer reaction negative. Several strains of *E.* can produce polysaccharide capsules. They produce indole, fail to hydrolyse urea and H_2S gas on triple sugar iron agar (TSI) or Kingler's iron agar (KIA).

Other species of *E.* include *E. fergusonii* (Farmer et al 1985), *E. hermannii* (Brenner et al 1982a), *E. vulneris* (Brenner et al 1982b) and *E. blattae* (Burgess, McDermott and Whiting 1973). *E. coli* is the normal flora present in the intestine of mammals and birds. The bacterium is excreted along with the feces but it's survival outside the body seems to be rare. This is the reason that *E. coli* is known as indicator organism of fecal contamination in food and water hygiene.

There are several strains of *E. coli* which are responsible for causing diseases outside the intestinal tract of humans and mammals and they are mostly characterized by the presence of specific virulence factors. Infections caused by such strains are either developed by the endogenous route (urinary tract, gall bladder infections, septicaemia etc.), or in the hospitals and clinics via contaminated equipments and hands of medical staffs (wound infections, meningitis, respiratory tract infections etc.). The intestines of humans are the natural reservoir of enteropathogenic strains (EPEC, ETEC, EIEC, EAgg, EC etc.) of *E. coli*.

E. fergusonii, E. hermannii and *E. vulneris* have been isolated from hospital samples and intestinal tract of warm blooded animals. They are opportunistic pathogens and have been associated with few wound infection cases in

humans. The atypical species of *E.,'E'. blattae* is a part of the intestinal flora of cockroaches.

2.2 Classification

E. is the genus of *E. coli* which belongs to the family Enterobacteriaceae. Kauffman characterized *E. coli* strains on the basis of surface antigens in 1940. This scheme was later modified and was able to recognize over 175 serologically distinct O antigens, 56 H antigens and 80 K antigens (Orskov et al 1992). The serogroup of a particular strain is defined by its O antigen while its serotype is a combination of all three antigens (Mobley et al 2009). Among the 175 known O-antigen serotypes, approximately 70% of isolates causing UTI belong to only eight serogroups (O1, O2, O4, O6, O7, O16, O18 and O75) (Lindberg et al 1975).

2.3 Morphology

E., as member of the family enterobacteriaceae form rod-shaped cells of 2.0-6.0 µm and 1.1-1.5 µm in width with rounded ends (Orskov 1984). Their shape may vary from coccal to long filamentous rods (Gross and Holmes 1990). When usual staining methods are performed, cells appear without spores. *E.* strains except 'inactive' biovar of *E. coli* bear peritrichous flagella which make them motile. These structures are proteinaceous in nature and are long, slender appendages of 19-24 nm diameter which extend about 15-20 µm from the cell surface (Silverman and Simon 1997). According to Orskov and Orskov 1997, all H antigens of *E. coli* have morphologically distinct surface pattern that is identical within each strain and among H antigens associated with different O groups. On the basis of morphology, they can be grouped into six related morphotypes.

E. coli have capsules and microcapsules generally made up of acidic polysaccharides. They have various sizes and can be detected by light microscopy whereas microcapsules only by serological or chemical techniques (Orskov and Orskov 1984). Extracellular slime is produced by mucoid strains of *E. coli* in some cases which is either a polysaccharide of certain K antigen specificities or a common acid polysaccharide formed in many strains of *E.*

coli and other enterobacteriaceae. The latter is explained as M antigen and composed of colonic acid (Orskov and Orskov 1984).

2.4 Metabolism, cultural characteristics and growth requirements

E. coli are facultatively anaerobic, chemo-organotrophic organisms having both a respiratory and a fermentative type of metabolism. Their growth is however, less copious in anaerobic condition. They grow well on ordinary media which contains 1% peptone as carbon and nitrogen source under an optimum temperature of 37°C (Holt et al 1994). Their growth on solid media depends upon the state of lipopolysaccharide of the outer membrane and can be either glistening, smooth (S), or dry, wrinkled, rough (R) colonies, respectively. Homogeneous turbid growth is formed in liquid media within 12-18 h by S whereas agglutination is seen spontaneously which sediments at the bottom of the test tubes in case of R (Orskov 1984). If prolongated incubation (>72 h) is done at 37°C then pellicle can be seen on the surface of liquid media in heavily fimbriated strains or can be induced by serial subcultures under these conditions.

Metabolic activity of *E. coli* is usually high between 15 to 45°C (Holmes and Gross 1990) and generation time is 20 minutes under optimal conditions. Enterotoxins and hemolysins are exotoxins which are best produced by *E. coli* at 37°C. Since they are translocated into periplasmatic space, they may be liberated in higher levels when exposed to 2000 IU ml^{-1} of polymyxin B for 30 minutes (Bockemuhl 1992). Unlike other coliform bacteria, *E. coli* ferments lactose and produces indole at 44°C which is used to identify the bacterium in food and water bacteriology. *E. coli* forms large (2-3 mm) circular, convex and non-pigmented colonies on nutrient and blood agar after 18-24 hours of incubation while some strains produce hemolysins as well. Large red colonies are grown by *E. coli* on MacConkey agar and they are resistant to low concentration of bile salts (e.g. 0.05% of sodium deoxycholate). As compared to other species of enterobacteriaceae, *E. coli* is more heat resistant and survives at 60°C for 15 minutes or at 55°C for 60 minutes (Holmes and Gross 1990). However, *E. coli* is sensitive to certain dyes like brilliant green and to

higher concentration of deoxycholate (0.25%) which are hence used to make media selective for the isolation of other species of enterobacteriaceae such as *Salmonellae* and *Shigellae.*

E. strains can be preserved for years in tightly closed nutrient agar stabs or on Dorset egg medium kept in the dark at room temperature. But such strains can mutate to R form and lose virulence plasmid even without further subcultures. If the cultures are important then they should be preserved in tryptic soy broth containing ≥10% glycerol at -70°C or in liquid nitrogen (Rowe and Gross 1984).

2.5 Cell wall composition

The cell surface structure of *E. coli* was first studied by Kauffmann in 1940s using serological methods (Kauffmann 1943, 1944). During his investigation cultures did not agglutinate with antisera prepared but became agglutinable only after heating at different temperatures. Hence, Kauffmann categorized several surface antigens with different physical and serological properties and named them as A, L and B antigens (Kauffmann 1965). Further study of chemical composition of gram-negative cell wall and its outer layers (Luderitz, Staub and Westphal 1966, Luderitz et al 1973, Jann and Westphal 1975, Jann and Jann 1987, Rietschel et al 1987) not only described the serological differences and cross-reactions between antigens but also helped to recognize the pathophysiological importance of certain compounds which are now called as endotoxins.

The outer layers of *E. coli* contain the outer membrane with phospholipids, lipid A and proteins from which the polysaccharide (LPS) chains arise, overlayed by capsular polysaccharides (CP) (Jann and Jann 1987). LPS and CP contribute to the pathogenicity of the bacterium and are the chemical basis for O and K antigens respectively. According to the review done by Jann and Jann (1987, 1992) both LPS and CP are produced at cytoplasmic site of cytoplasmic membrane of the bacteria then they are transported to the outer membrane. Here, CP are involved to form capsule of bacteria while LPS are

integrated into cell wall and contains lipid A as hydrophobic moiety linked to the O-specific polysaccharides through a core region.

Lipid A is the only chemical structure in *E. coli* which acts as endotoxin responsible for several in vivo activities such as pyrogenicity, local Shwartzman reactivity, lethal toxicity in mice, adjuvant activity, induction of interferon and tumor necrosis factor. This endotoxin produces various pathophysiological effects in humans including fever, hypotension, disseminated intravascular coagulation and septic shock (Rietschel et al 1987).

166 serological specificities of the polysaccharide chains and five different core structures (Jann and Jann 1987) have been identified. If there is loss of O-specific polysaccharides then it results in mutation from smooth (S) to rough (R) form of the bacteria. Such R mutants consist of only lipid A and the core oligosaccharide in their R-LPS. In *E. coli* the polysaccharide chains have oligosaccharide repeating units that are neutral in general but may have acidic components as well (Jann and Jann 1987).

The capsular (K) antigens of *E. coli* are acidic polysaccharides. On the basis of physical, chemical and microbiological properties, CPs has been divided into two groups. Group I CPs is of high molecular weight (> 100,000) and is co-expressed with O groups 8,9,20 and 101 of *E. coli*. At pH 6, they are stable and correspond to the capsular antigen type A of Kauffmann (1965) when heated to 100°C. As far as group II capsular antigens are concerned, they have lower molecular weight (<50,000), heat-labile (B- and L-type capsular antigens, Kauffmann 1965), do not resist pH 6 and co-expressed with many *E. coli* O groups.

2.6 Antigenic structure of *E. coli*

E. coli has mainly three different types of antigens; O, K and H based on complete serotyping. But, if there is an additional virulence factor expressed by fimbriae then it is O: K: H: F. A number of different K and H antigens can be associated with certain O groups and, moreover, a number of O groups are complex antigenically with a need of further subdivisions (Topley and Wilson 1999).

2.6.1 O Antigens

O antigens of *E. coli* are not species-specific but rather a large number of cross-reactions are identified between individual *E. coli* O antigen and the O antigens of *Salmonella, Citrobacter, Shigella, Providencia* and *Yersinia* (Ewing 1986, Holmes and Gross 1990). Hence, to determine O antigen, a proper biochemical identification should be performed for the isolate. According to Kauffmann, 1965, O antigens are heat stable and not inactivated by heating at 100°C for 2.5 hours. In rabbits, these preparations are used for the production of antiserum but cultures need to be heated for 2 hours at 120°C if it carries A-type capsular antigens. As per the rule, *E. coli* O antigen can be determined when strain suspension is heated at 100°C for 1 hour. Although typing of all defined O antigens is very laborious and restricted to only reference laboratories, it may be useful for screening of certain pathotypes like EPEC, EIEC or EHEC. Virulence factors may be absent in such isolates or may be present in other O groups since they are expressed by plasmids or phages (Topley and Wilson 1999).

2.6.2 K antigens

According to the heat treatment done by Kauffmann (1965), there are 3 serological groups of *E. coli* envelopeantigens. More or less mucoid colonies were grown by strains with A-type capsular antigens which were thermostable at 100°C and after heating at 120°C for 2 hours, it became O-agglutinable. These antigens are now included in class I capsular polysaccharides (Jann and Jann 1987). On the basis of agglutinin binding power and inactivation at 100°C, heat-labile antigens L and B were defined. Results were inconsistent repeatedly for L- and K-type antigens till Orskov found that a variety of surface structures such as fimbriae, flagellae, outer membrane proteins and capsular polysaccharides were included in K antigens and their quantity depended upon the culture conditions. The capsular (K) antigens of *E. coli* are acidic polysaccharides. On the basis of physical, chemical and microbiological properties, CPs has been divided into two groups. Group I CPs is of high molecular weight (> 100,000) and are co-expressed with O groups 8,9,20 and 101 of *E. coli*. At pH 6, they are stable and correspond to the capsular antigen

type A of Kauffmann (1965) when heated to 100°C. As far as group II capsular antigens are concerned, they have lower molecular weight (<50,000), heat-labile (B- and L-type capsular antigens, Kauffmann 1965), do not resist pH 6 and co-expressed with many *E. coli* O groups.

2.6.3 F antigen

F antigens are important virulence factors and some of the fimbrial antigens are involved in the adhesion process. They are heat-labile proteins chemically and develop at 37°C but not at 18°C. A variety of erythrocytes are agglutinated by fimbrial antigens which can be used for their characterization. A typical type of fimbriae is produced by most *E. coli* strains and its haemagglutinating capacity is suspended in the presence of mannose (type I fimbriae). Furthermore, fimbriae may still haemagglutinate in the presence of mannose which are produced by those strains associated with diarrhoeal or extraintestinal disease (Topley and Wilson 1999).

2.6.4 H antigen

Flagellar antigens are heat-labile proteins. Slide or tube agglutinations are performed using broth cultures or growth on semisolid media from actively motile strains for the identification of H antigens. When *E. coli* are first isolated, they are poorly motile. Hence, to obtain well flagellated bacteria, organisms need to be sub cultured for several times in semisolid media (e.g. in U-shaped tubes). Motility is sometimes better at 30°C than at 37°C. H antigens are mostly type-specific and are important for a precise phenotypic characterization of *E. coli* strains. However, their performance is generally limited reference laboratories (Topley and Wilson 1999).

2.7 Pathogenicity

During urinary tract infection, Uropathogenic (UPEC) *E. coli* pathogenesis includes: UPEC colonization in periurethral, urethral and vaginal areas, ascending into the bladder lumen and growth as planktonic cells in urine, adherence to the surface and interaction with the bladder epithelium defense system, biofilm formation, invasion and replication by forming bladder

intracellular bacterial communities (IBCs) and kidney colonization and host tissue damage with increased risk for bacteremia/septicemia (Maria et al 2017). Replication of bacteria in IBC can easily reach as many as 10^5 bacteria per cell; furthermore bacteria in IBC undergo morphological change, flux out of the infected cells and go to infect neighboring cells (Dhakal et al 2008; Spaulding and Hultgren 2016).

2.8 Virulence factors of *E. coli*

Uropathogenic *E. coli* has been identified on the basis of genomic pathogenicity Island (PAI) and the expression of virulence factors such as adhesins, toxins, surface polysaccharides, flagella and iron-acquisition system (Bien et al 2012). All of these components are widely used for the development of drugs and vaccines (Werneburg et al 2015; O'Brien et al 2016).

2.8.1 PAIs

PAIs are the regions within DNA which contain genes that encode potential virulence factors. Hacker and colleagues were first to define PAIs in order to include DNA regions greater than 30 Kb that bear virulence associated genes and they are not usually present in the genome of fecal *E. coli* (Hacker et al 1990). PAIs have mobility genes such as transposons, insertion elements and degraded bacteriophage genome. They also have G+C content different from the genome DNA and are frequently inserted within or adjacent to tRNA genes. As a result of site-specific recombination and under specific environmental conditions, some PAIs can be deleted and have been found to be unstable (Middendorf et al 2004). All of these properties suggest that UPEC have got PAIs inserted into its chromosome by the means of lateral transfer mechanism (Hacket et al 1990, Bidet et al 2005 and Dobrindt et al 2003). The known four models of uropathogenic *E. coli* strains (536, J96, CFT073 and UTI89) have been found to bear multiple PAIs (Tullus et al 1984, Hacker et al 1990, Dobrindt et al 2002, Guyer et al 1998, Kao et al 1997, Knapp et al 1986, Oelschlaeger et al 2002, Schneider et al 2004 and Swenson

et al 1996) and other extraintestinal isolates to carry homologous sequences (Parham et al 2005 and Rasko et al 2001).

When PAIs I and II encoding hemolysin and P fimbriae of *E. coli* strain 536 got deleted spontaneously or intentionally then it not only resulted to the reduction of hemolytic activity and cell binding but also to decreased serum resistance, mannose-resistant hemagglutination (Knapp et al 1986) and virulence in mice (Chen et al 2006 and Knapp et al 1986). In comparison, copies of the *pap* operon-encoding P fimbriae or gene related to hemolytic activity showed no any effect in CFT073 strain (Mobley et al 1990 and Mobley et al 1993). Hence, all these events suggest that other PAIs-encoded genes may play a role in virulence of uropathogenic *E. coli.*

2.8.2 Adhesins

Different surface structures are used by bacteria to bind to the targets on the host cell surface. The word "fimbriae" is derived from the Latin term meaning thread or fringe while pilli, Latin for hair are used to describe these structures. Similarly, some related but thinner wiry structures like fimbrillae and others which don't have recognizable morphology are known as afimbrial adhesions. P, F1C, S, M, Dr and type 1 fimbriae are a number of different pilli and related adhesins expressed by uropathogenic *E. coli* (Topley and Wilson 1999).

2.8.3 Type 1 fimbriae

They are on average 0.5 to 2 μm in length with a diameter of 7 nm and a central axial hole of 0.2-0.25 nm in diameter (Brinton, 1965). A fimbrillar structure containing *Fim*H adhesin responsible for binding and hemagglutination, projects roughly at the distal end of the fimbria which is 16 nm in length. A single bacterium usually expresses 500 fimbriae per cell and it accounts for nearly 8% of the total protein content of the bacterium. An operon consisting of nine separate genes present on the chromosome and arranged in the order: *fimB, fimE, fimA, fimI, fimC, fimD, fimF, fimG* and *fimH* encode type 1 fimbriae (Hull et al 1981, Hultgren et al 1991 and Schilling et al 2001).

2.8.4 Dr adhesins

The Dr adhesin consists of both fimbrial and afimbrial structures on the surface of *E. coli.* It binds to the Dr blood group antigen which is a part of the decay-acceleration factor and is also a membrane protein that prevents cell lysis by compliment (Nowicki et al 1990, Nowicki et al 1993 and Nowicki et al 1988). Dr adhesins bind to the bladder epithelium and type IV collagen basement membrane of urinary tract (Westerlund et al 1989). A minority of UTI strain contains these adhesins. However, some studies have shown that the genes for the members of Dr family are more prevalent among cystitis and pyelonephritis strains than among fecal control strains of *E. coli* (Donnenberg et al 1996). Dr adhesins are involved with epithelium invasion. Specifically, domains of DraD and DraE have been described to be important for invasion and other chaperone-subunit interactions have recently been illustrated (Das et al 2005, Zalewska et al 2005, Zalewska et al 2001 and Pietak et al 2005).

2.8.5 F1C and S fimbriae

E. coli strains that express F1C and S fimbriae have highly related biogenesis genes but different adhesin alleles. These fimbriae have been associated with UTI and other extraintestinal infections, in specific, neonatal meningitis. O-antigen serotypes that are overrepresented among pyelonephritis isolates and in those strains that contain P fimbriae and hemolysin commonly bear F1C fimbriae (Pere et al 1985, Zingler et al 1993 and Zingler et al 1992). F1C fimbriae attaches to the vascular endothelium on kidney cross sections, human distal tubular and collecting tubular epithelium (Korhonen et al 1990). They are recognized by immunoflourescence of organisms in the urine of UTI patients so they are expressed in vivo (Pere et al 1987).

2.9 Toxins

Hemolysin, cytotoxic necrotizing factor 1 (CNF-1) and the secreted autotransporter toxins are the three classes of proteins produced by UPEC and may be considered as toxins (Topley and Wilson 1999).

2.9.1 Hemolysin

Uropathogenic *E. coli* consists of *hly* operon (*hly* CABD) usually situated near to the P-fimbrial genes on the same pathogenecity island of the chromosome (Kao et al 1997, Swenson et al 1996, Blum et al 1995 and High et al 1988). The synthesis and secretion of hemolysin requires proteins which are encoded by *hly* genes (Felmlee et al 1985). The prohemolysin protein which is encoded by *hly*A gene lacks biological activity. In the presence of two palmitoyl groups, the HlyC protein changes prohemolysin to produce mature and active hemolysin protein (Issartel et al 1991 and Lim et al 2000). A type 1 secretion system produces hemolysin to the extracellular milieu. This system is composed of HlyB, an ATP-binding cytoplasmic membrane protein; HlyD, a cytoplasmic membrane protein with a large periplasmic domain; and TolC, an outer membrane protein not encoded within the hemolysin operon (Koronakis et al 1996). RfaH positively regulates the expression of hemolysin operon which is also a positive regulator of LPS synthesis (Bailey et al 1992). To bind to the host cell membrane and to assume its functional tertiary structure, hemolysin requires calcium (Boehm et al 1990 and Ludwig et al 1988). Instead of forming a pore through the membrane, hemolysin appears to insert into the outer leaflet of the host cell membrane (Soloaga et al 1999). To a wide number of erythrocytes, nucleated cell types such as leukocytes, fibroblasts and uroepithelial cells, hemolysin is cytotoxic at higher doses (Mobley et al 1990, Soloaga et al 1999 and Cavalieri et al 1982).

2.9.2 CNF-1

It is a 110- KDa protein which is encoded by a single gene (Falbo et al 1993). The secretion of actin stress fibres and membrane ruffling in HEp-2 cells are induced by CNF-1 toxin (Fiorentini et al 1988). CFN-1 is responsible for the deamidation of glutamine 63 of Rho protein into glutamic acid which leads to the constitutive activation of members of the Rho family of small GTP-binding proteins and finally results to the rearrangement of cytoskeleton (Flatau et al 1997 and Schmidt et al 1997). Apoptosis is caused by CFN-1 in the 5637 bladder cell line and this process might explain the exfoliation of the bladder epithelial cells after after infection with UPEC (Mills et al 2000).

2.9.3 Autotransporters

It is a 107-KDa protein which is represented by “Sat” (secreted autotransporter toxin). *E. coli* strains associated with the clinical manifestation of acute pyelonephritis (55%) express more “Sat” than fecal strains (22%) (Guyer et al 2000). An unusually long signal sequence, a secreted passenger domain (the mature protein) and an autotranporter C-terminal domain are the three characteristic domains of Sat protein which are similar to those of SPATE (serine protease autotransporters of *Enterobacteriaceae*) protein. According to several studies on autotransporters, it has been found that the translocation across the inner membrane takes place through *sec*-dependent pathway and across the outer membrane through a β-barrel porin structure formed by the carboxy terminus autotransporter domain. This phenomenon of export is also called type V secretion.

2.9.4 Other toxins

Recently, several other proteins have been detected in uropathogenic *E. coli* which have cytotoxic activity. Nonribosomal peptide synthases (NRPS) and polypeptide synthases (PKS) are group of enzymes secreted by B2 *E. coli* strains that were found to initiate DNA double-stranded breaks in cultured epithelial cells (Nougayrede et al 2006). O6:k13:H1 uropathogenic strain produces soluble unidentified toxins which were found to induce apoptosis in a renal tubule cell line (Chen et al 2003). Furthermore, studies on the complete genomic sequences of CFT073 and other UPEC strains have shown new putative toxins whose functions are yet to be known.

2.10 Urinary tract infection

Urinary tract infection is the infection involving any part of the urinary tract such as the kidneys, ureters, bladder and urethra. Clinically, UTIs are categorized into two groups and they are uncomplicated and complicated. UTIs are caused by a wide range of Gram negative and Gram positive bacteria and some fungi as well. However, uropathogenic *E. coli* are mainly responsible for uncomplicated and complicated UTIs (Topley and Wilson 1999).

2.10.1 Uncomplicated urinary tract infection

Uncomplicated UTIs are associated with healthy individuals or those with no any urinary tract abnormalities (Hooton et al 2012). These infections are further differentiated into lower urinary tract infection or cystitis and upper UTI also known as pyelonephritis (Hannan et al 2012). Female gender, sexual activity, vaginal infection, diabetes, obesity and genetic susceptibility are the factors that affect cystitis (Foxman et al 2014).

2.10.2 Cystitis

It is the inflammation and irritation of urethra and bladder when they become infected with bacteria. It affects people of all ages and both sexes. It is however, more common among women since they have shorter urethras. Nearly 80% of urinary tract infections are caused by normal flora when they enter into the sterile urinary tract. Frequent urination, burning sensation without vaginal discharge, pain above the pubic bone or in the lower back are major symptoms of cystitis (Nicolle et al 2008).

2.10.3 Pyelonephritis

It is the inflammation of kidneys, usually caused by bacteria (Lippincott Williams and Wilkins 2011). Prior urinary tract infection, diabetes, structural problems in the urinary tract, spermicide use and sexual intercourse are the common risk factors associated with pyelonephritis (Lippincott Williams and Wilkins 2011 and Colgan et al 2011). Flank pain, fever, nausea and vomiting in addition to the lower UTI symptoms are the clinical signs of pyelonephritis (Lane et al 2011).

2.10.4 Complicated urinary tract infection

The infection which occurs in those patients who have either metabolically, anatomically and functionally abnormal urinary tract or caused by pathogens that are resistant to antibiotics is known as complicated UTI (Hooton et al 1991 and Ronald et al 1991). The clinical symptoms range from a mild cystitis to life-threatening urosepsis and even a long period of asymptomatic bacteriuria. Complicated UTIs are associated with the factors that compromise

the urinary tract or host defense including, urinary retension caused by neurological disease, urinary obstruction, renal failure, immunosuppression, renal transplantation, pregnancy, presence of calculi and indwelling catheters (Lichtenberger et al 2008; Levison et al 2013).

2.10.5 Recurrent UTI

Approximately 25% of women will have a risk of second symptomatic episode of UTI within 6 months (Foxman, 1990)**.** Vaginal colonization, intestinal colonization and intracellular bacteria residing within transitional epithelial cells are considered to be the reservoir for reinfection (Johnson et al 2005 and Mysorekar et al 2006). Periurethral bacterial colonization and the presence of certain *E. coli* virulence factors are pathogen-related factors while voiding dysfunction, high intercourse frequency, oral contraceptive and spermicide use are the host behavioral risk factors that predispose women to recurrent UTI (Finer et al 2004).

2.10.6 Renal scarring

Some *E. coli* strains may reach to the upper urinary tract when host response fails and cause pyelonephritis. In children, the inflammation due to the infection leads to renal scarring. Renal scarring includes several events among which the influx of neutrophils is the crucial one. As a result, oxidative burst occurs due to the production of granulocytic cytotoxic products such as lysozyme, elastase and myeloperoxidase (Heinzelmann, Mercer-Jonas et al 1999). These mechanisms targeted to destroy the pathogens may be harmful to the host with tissue destruction along with fibrosis (Chen et al 2005).

2.10.7 Asymptomatic bacteriuria

It is defined as the condition where a specified number of bacteria are isolated from an appropriately collected urine sample without any clinical symptoms of UTI. Nearly 40 % of elderly men and women are likely to develop asymptomatic bacteriuria usually among staying in nursing homes. Asymptomatic bacteriuria has been linked with low birth weight and 4 to 60% of immunosuppressed renal graft recipients. Diabetes patients also have high

risk of developing asymptomatic bacteriuria where the incidence is approximately 3 folds more in adult women. Hence, asymptomatic bacteriuria should be treated after renal transplantation, before urological surgery and during pregnancy (Stein and Funfstuck 2000).

2.10.8 Catheter associated UTI

According to the recent studies, bacteria initiate the formation of biofilms as they adhere to the surface of catheters. These biofilms are mainly composed of bacteria, bacterial glycocalices, Tamm-Horsfall protein and urinary salts such as apatite and struvite (Nickel et al 1985). With the help of biofilms, bacteria protect themselves from the action of antibiotics leading to the treatment failure. If catheterization is done for more than 30 days then almost all patients are likely to have bacteriuria (Warren et al 1982). A level of 100 or more colony-forming unit permillilitre isolated from culture specimen of catheters is the proof of infection as this count persists or rises within 48 hours (Stark et al 1984).

2.11 Prevention of UTI

There are different methods for promoting overall urinary health and some of them are given below:

1. Proper hydration and nutrition: dehydration is the condition where body lacks proper amount of water. It leads to concentrated urine and less frequent voiding that eventually support bacterial growth in bladder. Pale-colored urine, moist mucous membrane and normal specific gravity are the indications of adequate hydration. Following strategies can be followed to stay hydrated:
    - Variety of fluids should be taken throughout the day.
    - Fluid intake should be encouraged routinely during social activities.
    - Foods that have high water content should be taken.
    - Awareness program should be conducted on the importance of hydration and urinary health.
    - Diabetic patients should maintain their blood glucose level.
2. Good personal hygiene:

- Personal hygiene should be performed correctly to prevent contact with urine and feces.
- Genital and anal areas should be washed with mild soap and water daily.

3. Healthy voiding habits:
   - Any issues with constipation and fecal impaction should be addressed.
   - Complete emptying of the bladder is possible only in relaxed voiding environment with comfortable toilet seat at appropriate height.
4. Avoid unnecessary urinary catheters:
   - External condom catheters can be used for male patients without urinary retention or bladder outlet obstruction.
   - Intermittent catheterization can be done for those with spinal cord injury or bladder emptying dysfunction.
5. Insertion of urinary catheters using aseptic techniques:
   - Urinary catheters should be inserted using sterile equipments such as gloves, drapes, sponges and sterile solution for cleaning urethral meatus.
   - Catheter should be as small as possible in size in order to prevent leakage.

2.12 Treatment of UTI

- Asymptomatic bacteriuria should not be treated since overuse or misuse of antibiotics can result to the emergence of resistant organisms.
- On the basis of organism's sensitivity pattern, antibiotic therapy should be selected.
- Antibiotics should be started only after the arrival of urine culture reports but if antibiotic therapy has already begun then it should be checked whether the antibiotic is appropriate as per the report or should be stopped.
- Foul, smelling or cloudy urine is not an indicative to start antibiotic therapy.
- First-line and narrow spectrum antibiotics are usually preferred for treatment of UTI.
- Some antibiotics used for the treatment of UTI are as below:

a) Nitrofurantoin: it is best recommended for the treatment of cystitis. Female outpatients showed only 0.9% resistance against nitrofurantoin which added to the fact that it is highly active against *E. coli* (Sanchez 2016). However, nitrofurantoin is not preferred for the treatment of pyelonephritis since it does not penetrate well into the renal parenchyma. The use of nitrofurantoin for prolonged suppression of UTI is not considered appropriate in elderly patients because of its adverse effects.

b) Trimethoprim/Sulfamethoxazole: uncomplicated cystitis is treated with trimethoprim and sulfamethoxazole with cure rates of 90-100%. It has also found to be effective in the treatment of UTI in men. Even when the resistance rate is 30%, trimethoprim and sulfamethoxazole may remain effective at a clinical cure rate of 85% (Gupta 2001).

c) Fluoroquinolones: fluoroquinolones such as levofloxacin and ciprofloxacin are generally used for treating uncomplicated pyelonephritis and complicated UTI including urosepsis when the local resistance is less than 10% (Gupta 2011). Nitrofurantoin or amoxicillin/clavulanate are recommended alternative to fluoroquinolones for uncomplicated UTI (Alternatives to fluoroquinolones 2016).

d) Oral β-lactam agents: they are used for the treatment of uncomplicated UTI as alternative agents. Amoxicillin and ampicillin are not preferred currently for empiric therapy due to the increased prevalence of resistance but they can be used for those which show susceptibility, especially to *E. fecalis.*

2.13 Biofilm

Extracellular DNA, exopolysaccharides called extracellular polymeric substances, pilli, flagella and other adhesive fibres create a scaffold to form a multicellular bacterial community that is protected from immune responses, antimicrobial agents and other stresses (Kostakioti et al 2013). The antimicrobial resistance increases on biofilm maturation as the biofilm provides physical barrier to antibiotic entry (Ana et al 2015). Biofilm-like

intracellular bacterial communities (IBCs) formed by UPEC protect their members from neutrophils, antibiotics and harsh environment (Anderson et al 2003).

Type 1 pili, antigen 43 and adhesive surface fibres called curli induce biofilm formation by mediating interbacterial interactions and attachment to surfaces. Transcription of antigen 43 is regulated by oxidative stress regulator (OxyR; also known as hydrogen peroxide-inducible genes activator) (Danese et al 2000), whereas type 1 pilus and curli fibre genes are regulated by polymyxin-resistant protein B (PrmB; also known as BasS) on iron sensing (Foxman et al 2014), leading to phosphorylation of polymyxin-resistant protein A (PmrA; also known as BasR) and quorum sensing regulator B (QseB) (Danese et al 2000). UPEC biofilm formation on catheters is dependent on type 1 pili (Guiton et al 2012).

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

A complete list of materials, equipments, chemicals, reagent, antibiotics and media used for the study are listed in Appendix 1.

3.2 Methods

3.2.1 Place of study

The study was carried out in Biratnagar Metropolitan city, Eastern Nepal from August to December 2018.

3.2.2 Sample size and types

During the study 400 urine samples were analyzed. All the works related to research were performed in microbiology laboratory of tertiary hospital and in central campus of technology. The urine samples were taken from urinary tract infection suspected patients visiting a tertiary hospital, Biratnagar.

3.3 Sample collection

The urine (midstream) samples for the study were collected from UTI suspected patients in sterilized screw-cap propylene bottles following standard guidelines. The samples were then processed in microbiology laboratory as soon as after the collection. The containers were labeled with patient's name, ID number, specimen type and date collected. In case of any delay in processing for more than 2 hours, samples were refrigerated at 4°c or preservatives such as boric acid were added and stored at room temperature until the processing time.

3.3.1 Inclusion criteria

In the inclusion criteria, Patients having symptoms of urinary tract infection were included in the study.

3.3.2 Exclusion criteria

Mixed culture and patients with radiological evidence of other infection as the cause of symptoms, patients with urinary catheterization and asymptomatic ones were excluded from our study.

3.4 Culture of *E. coli*

Urine specimens were cultured by using semi-quantitative culture technique. A loopful of well-mixed sample was inoculated using standard calibrated loop onto CLED agar and incubated aerobically at 37°c for 24 hours. After overnight incubation, colony count yielding bacterial growth of $\geq 10^{5}$ were taken as being significant (Tamalli et al 2013). For identification of isolates, at first colony characteristics of isolated bacteria were observed on agar plates and Gram staining was done. Gram negative isolates were then further identified by performing different biochemical tests including catalase, oxidase, indole utilization test, citrate agar test, methyl red, VP test, carbohydrate fermentation test and triple sugar iron utilization test.

3.5 Quantitative Enumeration of *E. coli*.

Number of colonies formed after incubation will be counted by Colony counter and multiplied with a factor of 100 to get the colonies in 1 ml of a subject's sample.

Number of colonies contained in 4 µl of sample = n

Therefore the number of colonies in 1000 µl

(1 ml)

= n x 1000/4

3.6 Identification of isolates

3.6.1 Identification with staining method

Gram staining was performed as per the standard technique using alcohol as decolorizer. Staining procedures and reagents are given in appendices.

3.6.2 Identification with biochemical tests

Required biochemical tests were performed for the identification of *E. coli* isolates.

i. Catalase test
ii. Indole test
iii. Methyl red test
iv. Voges-Proskauer test
v. Citrate utilization test
vi. Triple sugar iron agar test
vii. Carbohydrate fermentation test
viii. Starch hydrolysis test

The procedures for all the above mentioned biochemical tests are mentioned in the appendix.

3.7 Characterization

The biofilm production and antibiotic susceptibility test were studied.

3.8 Biofilm Assays

3.8.1 Microtitre plate method

Each culture was individually grown overnight in 10ml of Trypticase Soya Broth (TSB) at 37°C for 24 hours and diluted to 1:40 in TSB containing 0.25% glucose. Then 200 microliter of diluted culture was inoculated in a microtitre well. The plates were incubated at 37°C for 24 hrs for biofilm production. After incubation, content of each well was removed by gentle tapping. The wells were washed with 0.2ml of phosphate buffer saline (PBS with pH 7.4) for four times and finally stained with 0.1% crystal violet solution for 30 minutes. After rinsing thrice with the sterile distilled water and subsequent drying, the stain taken up by the adherent biofilm was extracted by using 95% ethanol at 4°C. The content of each well was transferred to another microtitre well and the absorbance was measured at 595nm .The experiment will be performed in triplicate and repeated three times (Borucki et al 2003).

Interpretation was made on OD by subtracting OD of control wells from OD of test wells. The optical density (OD_s) of each strain was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the mean absorbance of negative controls (OD_{nc}). The following classification was used for the determination of biofilm formation: no biofilm production ($OD_s \leq OD_{nc}$), weak biofilm production ($OD_{nc} < OD_s \leq 2.OD_{nc}$), moderate biofilm production ($2.OD_{nc} < OD_s \leq 4.OD_{nc}$) and strong biofilm production ($4.OD_{nc} < OD_s$) (Stepanovic et al 2007).

3.8.2 Tube method

According to Borucki et al 2003, each culture was individually grown overnight in 10ml of Trypticase Soya Broth (TSB) at 37°C for 24 hours and diluted to 1:40 in TSB containing 0.25% glucose. Then the tubes were decanted and washed with PBS (7.2) and dried. Then the tubes were stained by 0.1% crystal violet. Stain was removed by deionized water. Tubes were then dried in inverted position for biofilm formation. When a visible film lined the wall and bottom of the tube, biofilm formation was taken as positive. Ring formation at the liquid interface was not an indicative of biofilm formation. Experiments were performed in triplicate and repeated for three times.

3.8.3 Congo red agar method

Congo red agar medium was prepared with brain heart infusion broth 37g/L, sucrose 50g/L, agar 10g/L and congo red indicator 8g/L. First congo red was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 minutes separately from other constituents. Then it was added to the autoclaved brain heart infusion agar. CRA plates were inoculated with test organism and incubated at 37°C aerobically. Black colonies with a dry crystalline consistency indicated biofilm production. The experiment was performed in triplicate and repeated three times (Freeman et al 1989).

3.9 Antibiotic susceptibility testing

The antibiotic Susceptibility test will be performed by:

Disk Diffusion method: AST of identified *E. coli* was evaluated against different available antibiotics such as ampicillin, chloramphenicol, sulfonamides, tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin, cefotaxime and nalidixic acid by disc diffusion method following CLSI guidelines. Colonies were taken from CLED agar plates and turbid suspension was made as per 0.5 MacFarland standards by emulsifying colonial growth in LB broth. A sterile cotton swab was dipped into lysogeny broth (HiMedia lab, Mumbai, India) and the swab was streaked on the entire surface of Muller Hinton agar three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculums. Finally, swab was done all around the edge of the agar surface. Using sterile tweezers, antibiotic discs were placed aseptically on the surface of MHA plates. The plates were then incubated at 37°c for 24 hours.

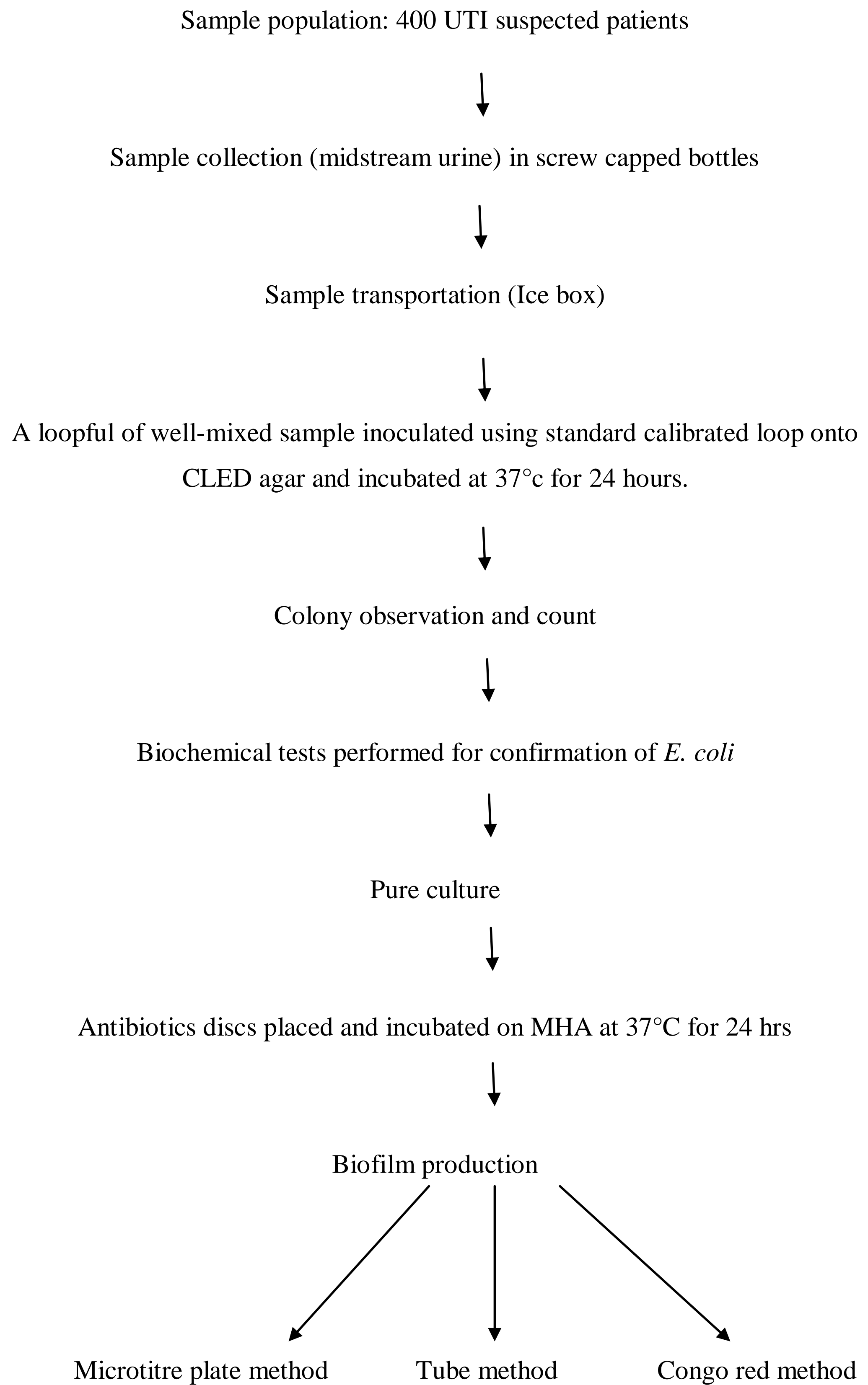
3.10 Quality Control for Tests

During the study, standard procedures were followed for the collection, isolation and identification in order to maintain quality and accuracy of all tests. All the media, antibiotics and reagents were prepared, stored and utilized as per the recommendation by manufacturing company. Antibiotic discs were stored at refrigerator temperature.

3.11 Data Analysis

The information was collected from questionnaire and finally tabulated. The data were analyzed statistically at 5% level of significance by SPSS.

Flow chart for sample processing



CHAPTER IV

RESULT

4.1 Study Population

The study was done in Microbiology Lab of Central Campus of Technology; study period was from August to December 2018. The total 400 study populations included Urinary Tract Infection suspected patients. The study population was composed of 9 (2.25%) male population and 391 (97.75%) female population.

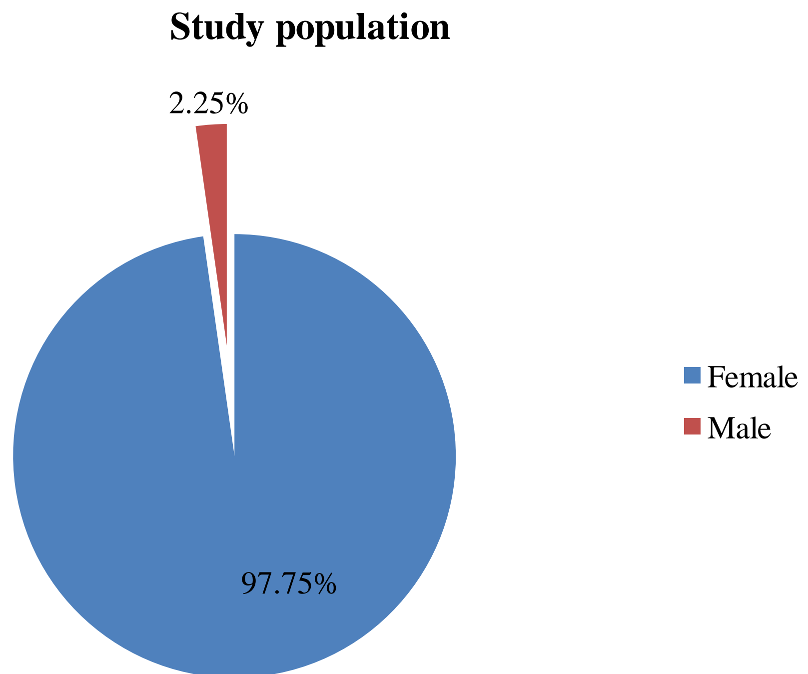


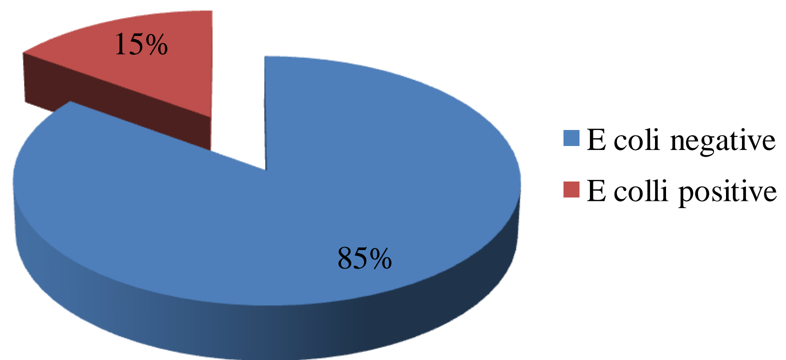
Figure 4.1: Study Population

4.2 Prevalence of *E. coli*

Out of 400 population samples, there were 60 (15%) positive *E. coli* in sample populations as shown in **Figure 4.2.**

Figure 4.2: Prevalence of *E. coli*

Prevalence of *E coli*



4.3 Antibiotic susceptibility pattern of *E. coli* isolates

The table below shows the sensitivity and resistance pattern of isolated *E. coli* strains against different antibiotics. The most sensitive drug for *E. coli* was found to be Chloramphenicol (100%), Cephoxitin (78.33%) and Ofloxacin (78.33%). On the other hand, the isolated strains of *E. coli* were resistant to Ampicillin (100%), Amoxicillin (100%) and Nalidixic acid (65%). The antibiotic susceptibility pattern of *E. coli* isolates is shown in **Table 4.3**.

Table 4.3: Antibiotic susceptibility test of *E. coli*

S.N	Antibiotics	Resistant	Sensitive	P value
1.	Amoxicillin	60(100)	-	-
2.	Ampicillin	60(100)	-	-
3.	Cefotaxime	16(26.66)	44(73.33)	0.00
4.	Ceftriaxone	15(25)	45(75)	0.00
5.	Cephoxitin	13(21.66)	47(78.33)	0.00
6.	Chloramphenicol	-	60(100)	-
7.	Ciprofloxacin	12(20)	48(60)	0.00
8.	Co-Trimoxazole	23(38.33)	37(61.66)	0.018
9	Gentamycin	14(23.33)	46(76.66)	0.00
10.	Nalidixic acid	39(65)	21(35)	0.01
11.	Norfloxacin	31(51.66)	29(48.33)	0.584
12.	Ofloxacin	13(21.66)	47(78.33)	0.00
13.	Tetracycline	26(43.33)	34(56.66)	0.201
14.	Trimethoprim	22(36.66)	38(63.33)	0.06

4.4 Multidrug resistant (MDR) *E. coli*

Uropathgenic *E. coli* which showed resistance to three or more than three drugs were considered as multidrug resistant. Hence, 42 isolates of *E. coli* were MDR out of 60. The prevalence of MDR Uropathgenic *E. coli* was 70% as shown in **Table 4.4**.

Table 4.4: Multidrug resistant (MDR) Uropathgenic *E. coli*

Samples	Uropathgenic *E. coli*
Total samples	60 (100%)
Multidrug resistant	42 (70%)

4.5 Biofilm formation assaying

The biofilm forming ability of *E. coli* was performed by three different methods: Microtitre plate method, Tube method and Congo red agar method which is given in **Table 4.5.**

Table 4.5: Biofilm formation by *E. coli*

SN	Biofilm formation	Microtitre plate method	Tube method	Congo red agar method	P value
1.	High	19 (31.66%)	13 (21.66%)	10 (16.66%)	
2.	Moderate	26 (43.33%)	19 (31.66%)	16 (26.66%)	0.00
3.	Weak/None	15 (25%)	28 (46.66%)	34 (56.66%)	

4.6 Antibiotic resistance of biofilm producer and non-producer *E. coli*

Ampicillin and Amoxicillin were resisted by all types of *E. coli*. The biofilm producing *E. coli* showed high resistance to all antibiotics as compared to biofilm non-producer *E. coli* **Table 4.6.**

Table 4.6: Antibiotic Resistance of biofilm producer and non-producer *E. coli*

SN	Antibiotics	% of Biofilm Producer Resistant	% of non-biofilm Producer Resistant	P-value
1.	Amoxicillin	100	100	
2.	Ampicillin	100	100	
3.	Cefotaxime	31.11	13.33	
4.	Ceftriaxone	31.11	6.66	
5.	Cephoxitin	24.44	13.33	
6.	Chloramphenicol	-	-	
7.	Ciprofloxacin	24.44	6.66	0.00
8.	Cotrimoxazole	42.22	26.66	
9.	Gentamycin	28.88	6.66	
10.	Nalidixic acid	71.11	46.66	
11.	Norfloxacin	53.33	46.66	
12.	Ofloxacin	24.44	13.33	
13.	Tetracycline	44.44	40	
14.	Trimethoprim	42.22	20	

4.7 Sensitivity and specificity of Biofilm assay by Tube method over Microtitre plate method

Among 60 positive samples, 45 were biofilm forming which account for 75%. The sensitivity and specificity of tube method was 68.88% and 93.33% respectively. The parameters like sensitivity, specificity, positive predictive value and negative predictive value are shown in **Table 5.**

Table 4.7: Sensitivity and specificity of Biofilm assay by Tube method over Microtitre plate method

TM Biofilm	MP method Biofilm		Total
	MP Positive	MP Negative	
TM Positive	31	1	32
TM Negative	14	14	28
Total	45	15	60

Sensitivity= 68.88%, Specificity= 93.33%, PPV= 96.87 %, NPV= 50%

4.8 Sensitivity and specificity of biofilm assay by congo red agar (CRA) method over microtitre plate (MP) method

The sensitivity and specificity of Tube method was 48.88% and 73.33% respectively. The parameters like sensitivity, specificity, positive predictive value and negative predictive value are shown in **Table 4.8.**

Table 6: Sensitivity and specificity of biofilm assay by congo red agar (CRA) method over microtitre plate (MP) method

CRA Biofilm	MP Method Biofilm		Total
	MP Positive	MP Negative	
CRA Positive	22	4	26
CRA Negative	23	11	34
Total	45	15	60
Sensitivity= 48.88%, Specificity= 73.33%, PPV= 84.61%, NPV= 32.35%			

4.9 Sensitivity and specificity of biofilm screening methods

The microtitre plate was found to be most efficient standard method for studying biofilm formation as compared to the tube method and congo red agar method. The parameters like sensitivity, specificity, positive predictive value and negative predictive value are shown in **Table 4.9.**

Table 7: Sensitivity and specificity of biofilm screening methods

Biofilm Screening method	Sensitivity (%)	Specificity (%)	Positive Predictive value	Negative predictive value	Accuracy (%)
Tube method	68.88	93.33	96.87	50	68.88
Congo red agar method	48.88	73.33	84.61	32.35	66.66

CHAPTER V
DISCUSSIONS

E. coli are a bacterium which belongs to the normal flora of intestine in human beings and other warm blooded animals. Out of 150 million UTI cases diagnosed each year all over the world, *E. coli* being the most common account for 80-85% of the cases (Nicolle et al 2008, Bhatta et al 2012 and Sahm et al 2001). UTI can lead to other serious clinical conditions if not treated on time. Complicated urinary tract infection, recurrent UTI, cystitis and pyelonephritis are the consequences of UTI. A reliable diagnosis is required for reducing the duration of disease course and for preventing the entry of bacterium into the upper urinary tract (CLSI 2005). However, there are several other strains of *E. coli* that can cause infection outside the intestinal tract. Special virulence factors are present in such strains of *E. coli.*

In order to improve the guidelines for empirical antibiotic therapy, regular monitoring of resistance pattern is necessary due to the increasing antibiotic resistance (CLSI 2005, Grude et al 2001 and Kripke et al 2006). Several microbes including *E. coli* produce biofilms to survive in unfavorable conditions. Biofilm is formed by enclosing bacterial cells in polymeric matrix and adhering to a surface (Soto et al 2014). Uropathogenic *E. coli* which produce biofilms may be responsible for many recurrent UTIs (Rijavec et al 2008). These biofilms make bacteria highly resistant to antibiotics (Soto 2014). Haphazard use of antibiotics has lead to the emergence of multidrug resistant *E. coli* resulting to the death of thousands (Goldstein 2000 and Schwaber et al 2007).

The present study was carried out microbiology laboratory of Central Campus of Technology, Dharan. During the study period a total of 400 urine samples were collected from UTI suspected patients. In the present study the overall prevalence of *E. coli* in sample population was 15% (60/400). This value was probably affected by the month of collection. However, Khatri et al 2017 and Neupane et al 2016 showed very similar report of 15.5% and 14.1% respectively.

Urinary tract infection is more common in women as compared to men owing to the fact that anatomically females have shorter urethra. At some point in their life, 40% of women develop UTI (Tan et al 2016). During their lifetime, women are 30 times more likely than men to develop a UTI with almost half of them experiencing at least one episode of UTI (Foxman et al 2002). As per the reports, one in three women have their first episode of UTI by the age of 24 years (Foxman et al 2002). Even in our research work, only 9 (2.25%) were male while 391 (97.75%) were female patients suspected with UTI symptoms.

In our study out of total 400 samples, 71 (17.77%) showed significant growth ($\geq 10^5$ cfu/ml). Sherchan et al 2016 and Ponnusamy et al 2012 reported comparatively higher percentage (23.49%) and 87.9% of UTI cases. This variation in the data may be due to the difference in socioeconomic aspects of the places. But according a research done by Neupane et al 2016, 18.8% of the sample population showed significant growth of bacteria which is very similar to our result.

For the antibiotic susceptibility test of *E. coli* isolates, 14 antibiotics were used in our analysis. The overall antibiotic resistance pattern is shown in Table 4.3. According to the table resistance percentage was as follows: Amoxicillin and Ampicillin 100% followed by Nalidixic acid 65%, Norfloxacin 51.66%, Tetracycline 43.33%, Cotrimoxazole 38.33%, Trimethoprim 36.66%, Cefotaxime (26.66%), Ceftriaxone (25%), Gentamycin (23.33%), Ofloxacin and Cephoxitin 21.66%, Ciprofloxacin 20% and Chloramphenicol 0. A very close data was revealed by Sharma et al 2013 which included 81.3% resistance towards Ampicillin, Nalidixic acid (78.9%), Norfloxacin (53.2%) and Cotrimoxazole (54.1%). 100% sensitivity to chloramphenicol was also reported by Ouno et al 2013. From the table it is also clear that *E. coli* isolates were significantly sensitive to Cephoxitin, Ofloxacin, Gentamycin, Ceftraixone, followed by Cefotaxime and Co-Trimoxazole while Nalidixic acid was only significantly resistant. Highest resistance to Amoxicillin and Ampicillin may be due to the random use of such drugs without any prescription of a physician.

In our study, out of 60 positive isolates of *E. coli,* 42 (70%) were found to be multidrug resistance (MDR). Baral et al 2012 recorded 41.1% of MDR *E. coli*

isolates in his investigation which was very less in comparison to our work. As per the experimentation done by Poursina et al 2018 and Dehbanipour et al 2016 73% and 68% of *E. coli* were multidrug resistant and it was very close to our analysis. Multidrug resistance has become a major problem in the treatment of diseases. The resistance of UTI causing bacteria towards commonly used antibiotics is escalating both in developing and developed countries (Elsayed et al 2017).

Among 60 *E. coli* isolates, 19 (31.66%) were strong biofilm producers, 26 (43.33%) moderately positive and 15 (25%) were weak ones by using microtitre plate method which were in accordance with the findings of Neupane et al 2016 and Khatri et al 2017. In tube method, 13 (21.66 %) were strongly positive, 19 (31.66%) moderate and 28 (48.66%) were weak or non biofilm producers. A research done by Murugan et al 2011 also showed the similar results for tube method. Similarly, in congo red agar method, 10 (16.66%) were biofilm producers while 34 (56.66%) were non-biofilm producers. These values were related with the study conducted by Ponnusamy et al 2011. Table 4.5 shows the $P<0.05$ which proved the fact that there was significant correlation between the type of method we perform and biofilm production. Biofilm producing microorganisms show resistance to large number of antibiotics increasing antibiotic resistance up to 1000 folds and hence, higher concentration of antimicrobial is required to treat such microorganisms (Stewart et al 2001). Inadequate amount of antibiotics reaching some areas of biofilm and inactiveness of bacteria located at the base of biofilm may be the reason for such resistance (Soto et al 2014).

Number of false positive, false negative, true positive and true negative were recorded in all three methods of biofilm production. Sensitivity and specificity of TM was found to be 68.88% and 93.33% respectively with accuracy of 68.88%. For congo red agar method the sensitivity and specificity was 48.88% and 73.33% with accuracy of 66.66%. The screening of biofilm in our analysis was similar to other findings. Devaraj et al 2015 concluded microtitre method as gold standard technique for screening biofilm as compared to TM and CRA. Even in our study microtitre plate method was considered to be the

efficient method for quantitative screening of biofilm as compared to TM and CRA.

The highest rate of antibiotic resistance among biofilm producing bacteria was towards Amoxicillin (100%), Ampicillin (100%) followed by Nalidixic acid (71.11%), Norfloxacin (53.33%) and Tetracycline (44.44%) whereas the highest rate of susceptibility was seen towards Chloramphenicol (100%), Cephoxitin(75.56%), Ciprofloxacin and Ofloxacin. In our investigation, both the biofilm producing and non-producing *E. coli* were resistant to Amoxicillin and Ampicillin (100%) while both were 100% sensitive to Chloramphenicol. However, resistance to other antibiotics such as Nalidixic acid (71.11% vs 46.66%), Norfloxacin (53.33% vs 46.66%), Cotrimoxazole (42.22% vs 26.66%) was comparatively higher among biofilm producers than non-biofilm producers. It is also shown in the Table 4.6 that the resistant pattern of biofilm producers is significantly more than non-biofilm producers.

The antibiotic resistance among biofilm producing *E. coli* was higher than that of biofilm non-producing *E. coli* which was in accordance to the findings of Neupane et al 2016 and Khatri et al 2017. Furthermore, there was a significant correlation ($p<0.05$) between biofilm formation and multidrug resistance (MDR) which was also reported by Kulkarni et al 2018 and Murugan et al 2011.

High prevalence of Multidrug resistant *E. coli* in UTI suspected patients alarms the need of prescribing antibiotics based only on culture and sensitivity reports. The absence of MDR *E. coli* in some few cases may be due maintenance of personal hygiene, age, gender etc. Transmission of MDR bacteria through sexual contact, irrational use of antibiotics and sales of substandard or falsified antibiotics may contribute to the increasing antimicrobial resistance (Gautam et al 2013).

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The prevalence of *E. coli* in urine samples was found to be only 15% among the UTI suspected patients. Most of the *E. coli* isolates were biofilm producers which comparatively showed resistance to more number of antibiotics than non-biofilm producers. Microtitre plate method was considered to be the best for screening biofilms. High prevalence of Multidrug resistant *E. coli* in UTI suspected patients alarms the need of prescribing antibiotics based only on culture and sensitivity reports. The absence of MDR *E. coli* in some few cases may be due maintenance of personal hygiene, age, gender etc. Thus, the presence of MDR *E. coli* in urine may result to different health issues including urinary tract infection, complicated UTI, cystitis, pyelonephritis etc. The maximum number of *E. coli* were biofilm producers and and maximum of them were resistant to antibiotics used. This correlation may illustrate the ability of biofilm formation with drug resistance in the bacterium and resulting to the failure of antibacterial drugs. In our investigation, Ampicillin, Amoxicllin were resisted by all the positive isolates while Chloramphenciol showed 100% sensitivity against *E. coli* biofilms.

6.2 Recommendations

- Unauthorized distribution of antibiotics without prescription of doctor has increased the prevalence of MDR isolates. Hence, such irrational use of antibiotics should be banned.
- Research in establishing relationship between ESBL and biofilm producers should be done in order to make treatment more efficient.
- The RFLP technique should be used for confirming biofilm formation
- Antibiotics such as Nitrofurantoin and Amikacin should be included in the study which has been found to be effective against urpathogenic *E. coli* in several studies.
- To make clinical therapy effective, drugs should be given only on the basis of culture and sensitivity reports.

REFERENCES

Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ. (2003). Intracellular bacterial biofilm-like pods in urinary tract infections. US National Library of Medicine National Institutes of Health, 301(5629): 105-7.

Ansari S, Nepal HP, Gautam R, Shrestha S, Neopane P, Gurung G, Chapagain ML. (2015). Community acquired multidrug resistant clinical isolates of *E. coli* in a tertiary care center of Nepal. Antimicrobial resistance and infection control, 4:15.

Awasthi TR, Pant ND, Dahal PR. (2015). Prevalence of multidrug resistant bacteria in causing community acquired urinary tract infection among the patients attending outpatient department of seti zonal hospital, Dhangadi, Nepal. Nepal journal of biotechnology, 3(1):55-59.

Baral P, Neupane S, Marasini BP, Ghimire KR, Lekhak B, Shrestha B. (2012). High prevalence of multidrug resistance in bacterial uropathogens from Kathmandu, Nepal. BMC Research Notes, 5:38.

Bhandari P, Joshi DR, Shrama KR, Khanal S, Acharya G, Adhikari N. (2017). High frequency of multidrug resistant urinary isolates in pregnant women in a tertiary care hospital of Nepal. SOJ Microbiology and infectious diseases, 4(4):1-5.

Borucki MK, Peppin JD, White D, Loge F and Call DR. (2003). Variation in biofilm formation among strains of Listeria monocytogenes. APP. Environ Microbial, 69:7336-7342.

Brenner, D. J., A. C. McWhorter, J. K. L. Knutson and A. G. Steigerwalt (1982). "*E. vulneris*: a new species of Enterobacteriaceae associated with human wounds." Journal of Clinical Microbiology 15(6): 1133-1140.

Brenner, D., B. Davis, A. Steigerwalt, C. Riddle, A. McWhorter, S. Allen, J. Farmer, Y. Saitoh and G. Fanning (1982). "Atypical biogroups of *E. coli* found in clinical specimens and description of *E. hermannii* sp. nov." Journal of clinical microbiology 15(4): 703-713.

Burgess, N., S. McDermott and J. Whiting (1973). "Aerobic bacteria occurring in the hind-gut of the cockroach, *Blatta orientalis*." Epidemiology & Infection 71(1): 1-8.

Chaudhari BK, Singh GK, Parajuli KP, Shrestha K. (2016). Incidence and susceptibility of uropathogens isolated among the patients at tertiary care hospital in eastern Nepal. Journal of Nobel medical college, 5(9):51-55.

Clinical and Laboratory Standards Institute, Performance Standards for Antimicrobial Susceptibility Testing, M100 – S25, Vol. 37 No. 1 Jan. 2017, CLSI, Wayne, PA 19087 USA.

Daoud Z, Afif C. (2011). *E. coli* Isolated from Urinary Tract Infections of Lebanese Patients between 2000 and 2009: Epidemiology and Profiles of Resistance. Hindawi Publishing Corporation Chemotherapy Research and Practice, (6).

Darnton NC, Turner L, Rojevsky S, Berg HC. (2007). On Torque and Tumbling in Swimming *E. coli.* . US National Library of Medicine National Institutes of Health, 189(5): 1756-64).

Dehbanipour R, Rastaghi S, Sedighi M, Maleki N and Faghri J. (2016). High Prevalance of multidrug-resistance Uropathogenic *E. coli* strains, Isfahan, Iran. Journal of Natural Science, 7(1):22-26.

Dellavalle, P. D., A. Cabrera, D. Alem, P. Larrañaga, F. Ferreira and M. Dalla Rizza (2011). "Antifungal activity of medicinal plant extracts against phytopathogenic fungus *Alternaria* spp." Chilean journal of agricultural research 71(2): 231.

Devaraj, C. and A. G. Sajjan (2015). "Comparision of Three Different Methods for the Detection of Biofilm in Gram Positive Cocci and

Gram Negative Bacilli Isolated from Clinical Specimens." Journal of Pharmaceutical Sciences and Research 7(11): 952.

Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson and D. A. Relman (2005). "Diversity of the human intestinal microbial flora." science 308(5728): 1635-1638.

Elsayed TI, Ismail HAF, Elgamal SA, Gad AHA. (2017). The Occurrence of Multidrug Resistant *E. coli* which Produce ESBL and Cause Urinary Tract Infections. Journal of Applied Microbiology and Biochemistry, 1(6:8).

Erb, A., T. Stürmer, R. Marre and H. Brenner (2007). "Prevalence of antibiotic resistance in *E. coli*: overview of geographical, temporal, and methodological variations." European Journal of Clinical Microbiology & Infectious Diseases 26(2): 83-90.

Farmer, J., G. Fanning, B. Davis, C. O'hara, C. Riddle, F. Hickman-Brenner, M. Asbury, V. Lowery and D. Brenner (1985). "*E. fergusonii* and *Enterobacter taylorae,* two new species of *Enterobacteriaceae* isolated from clinical specimens." Journal of clinical microbiology 21(1): 77-81.

Flores-Mireles AL, Walker JN, Caparon N, Hultgren SJ. (2016). Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat Rev Microbial. Auther Manuscript ;available in PMC, 13(5):269-284.

Freeman DJ, Falkiner FR, Keane CT. (1989). New method for detecting slime production by coagulase negative Staphylococci. J Clin Pathol, 42:872-874.

Gautam R, Chapagain ML, Acharya A, Rayamajhi N, Shrestha S, Ansari S, Upadhaya G, Nepal HP. (2013). Antimicrobial susceptibility patterns of *E.* coli from various clinical sources. Journal of Chitwan medical college. 3(3):14-17.

Ghoniem, E. M., G. R. El Hendawy, T. M. A. Moteleb, H. A. Hassan and H. A. E. R. Khalil (2014). "Characterization of vancomycin-resistant *Staphylococcus aureus* in the National Liver Institute." Menoufia Medical Journal 27(4): 825.

Goldstein, F. and M. S. Group (2000). "Antibiotic susceptibility of bacterial strains isolated from patients with community-acquired urinary tract infections in France." European journal of clinical microbiology and infectious diseases 19(2): 112-117.

Graber, C. J., M. K. Wong, H. A. Carleton, F. Perdreau-Remington, B. L. Haller and H. F. Chambers (2007). "Intermediate vancomycin susceptibility in a community-associated MRSA clone." Emerging infectious diseases 13(3): 491.

Grude, N., Y. Tveten, A. Jenkins and B.-E. Kristiansen (2005). "Uncomplicated urinary tract infections Bacterial findings and efficacy of empirical antibacterial treatment." Scandinavian journal of primary health care 23(2): 115-119.

Hughes C, Hacker J, Roberts A, Goebel W.(1983). Hemolysin Production as a Virulence Marker in Symptomatic and Asymptomatic Urinary Tract Infections Caused by *E. coli*. Infection and Immunity, 39(2): 546-551.

Karlowsky JA, Kelly LJ, Thornsberry C, Jones ME, Sahm DF (2002). Trends in antimicrobial resistance among urinary tract infection isolates of *E. coli* from female outpatients in the United States. Antimicrob Agents Chemother 46:2540–2545.

Kass, E. H. (1957). "Bacteriuria and the diagnosis of infections of the urinary tract: with observations on the use of methionine as a urinary antiseptic." AMA archives of internal medicine 100(5): 709-714.

Khatri S, Pant ND, Neupane S, Bhandari S, Banjara MR. (2017). Biofilm production in relation to extended spectrum beta-lactamase production and antibiotic resistance among uropathogenic *E. coli*. Janaki Medical College Journal of Medical Sciences, 5(1):61-63.

Komaroff, A. L. (1986). "Diagnostic decision: Urinalysis and urine culture in women with dysuria." Annals of internal medicine 104(2): 212-218.

Kostakioti M, Handjifrangiskou M, Hultgren SJ. (2013). Bacterial Biofilms: Development, Dispersal and Therapeutic Strategies in the Dawn of the Postantibiotic Era. US National Library of Medicine National Institutes of Health, 3(4).

Kripke, C. (2005). "Duration of therapy for women with uncomplicated UTI." American family physician 72(11): 2219.

Kulkarni SR, Peerapur BV. (2018). Analysis of Antibiotic Sensitivity Profile of Biofilm-forming Uropathogenic *E. coli*. Journal of Natural Science, Biology and Medicine, 9(2): 175-179.

Kunin, C. M., T. H. Hua, C. Krishnan, L. Van Arsdale White and J. Hacker (1993). "Isolation of a nicotinamide-requiring clone of *E. coli* 018: K1: H7 from women with acute cystitis: resemblance to strains found in neonatal meningitis." Clinical infectious diseases 16(3): 412-416.

Lindberg, U., L. Hanson, U. Jodal, G. Lidin-Janson, K. Lincoln and S. Olling (1975). "Asymptomatic bacteriuria in schoolgirls: II. Differences in *E. coli* causing asymptomatic and symptomatic bacteriuria." Acta Pædiatrica 64(3): 432-436.

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falages ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Liljequist BO, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definations for acquired resisitance. Clinical Microbiology and Infection, 18(3): 268-281.

Mashwal FA, Safi SHEI, George SJ, Adam AA, Jebakumar AZ. (2017). Incidence and molecular characterization of the extended spectrum betalactamase-producing *E. coli* isolated from urinary tract

infections in Eastern Saudi Arabia. Saudi Medical Journal, 38(8): 811-815.

Mittal S, Shrama S, Chuadhary U. (2015). Biofilm and Multidrug Resistance in Uropathogenic *E. coli.* Pathogens and Global Health, 109(1): 26-29.

Mobley MS, Donnenberg HLT, Hagan EC.(2009). Uropathogenic *E. coli.* EcoSalplus , Page:1-39.

Murugan S, Devi PU, John PN.(2011). Antimicrobial Susceptibilty Pattern of Biofilm Producing *E. coli* Urinary Tract Infections. Current Research in Bacteriology, 4(2): 73-80.

Neupane S, Pant ND, Khatiwada S, Chaudhary R, Banjara MR. (2016). Correlation between biofilm formation and resistance towards different commonly used antibiotics along with extended spectrum beta lactamase production in uropathogenic *E. coli* isolated from the patients suspected of urinary tract infection visiting Shree Birendra Hospital, Chhauni, Kathmandu, Nepal. Antimicrobial resistance and infection control, 5:5.

Nicolle LE. (2007). Short-term Therapy for Urinary Tract Infection: Success and Failure. US National Library of Medicine National Institutes of Health, 315(2008): 540-545.

Ørskov, F. and I. Ørskov (1992). "*E. coli* serotyping and disease in man and animals." Canadian journal of microbiology 38(7): 699-704.

Ouno GA, Korir SC, Cheruiyota JC, Ratemo OD, Mabeya BM, Mauti GO, Mauti EM, Kiprono SJ. (2013). Isolation, Identification and Characterization of Urinary Tract Infectious Bacteria and the Effect of Different Antibiotics. Journal of Natural Sciences Research, 3(6):2224-3186.

Parajuli NP, Maharjan P, Parajuli H, Joshi G, Paudel D, Sayami S, Khanal RJ. (2017). High rates of multidrug resistnace among uropthogenic *E. coli* in children and analyses of ESBL producers in Nepal. Antimicrobial resistance and infection control, 6:9.

Park, C. H., A. Robicsek, G. A. Jacoby, D. Sahm and D. C. Hooper (2006). "Prevalence in the United States of aac (6′)-Ib-cr encoding a ciprofloxacin-modifying enzyme." Antimicrobial agents and chemotherapy 50(11): 3953-3955.

Ponnusamy P, Natarajan V, Sevanan M. (2012). Invitro biofilm formation by uropathogenic *E. coli* and their antimicrobial susceptibility pattern. Asian Pacific Journal Of Tropical Medicine, 210-213.

Poursina F, Sepehrpour S and Mobasherizadeh S. (2018). Biofilm formation in Nonmultidrug-resistant *E. coli* isolated from patients with Urinary tract infection in Isfahan, Iran. Advanced Biomedical Research, 7:40.

Pramodhini S, Niveditha S, Umadevi S, Kumar S, Stephen S. (2012). Antibiotic resistance pattern of biofilm-forming uropathogens isolated from catheterized patients in Pondicherry, India. Australasian Medical Journal, 5(7):344-348.

Rijavec, M., M. Müller-Premru, B. Zakotnik and D. Žgur-Bertok (2008). "Virulence factors and biofilm production among *E.* coli strains causing bacteraemia of urinary tract origin." Journal of medical microbiology 57(11): 1329-1334.

Sharma AR, Bhatta DR, Shrestha J, Banjara MR. (2013). Antimicrobial susceptibility pattern of *E. coli* isolated from urinary tract infected patients attending Bir Hospital. Nepal journal of science and technology, 14(1):177-184.

Sherchan JB, Gurung P, Karkee P, Shrestha N, Ohara H. (2016). Microbiological and clinical profile of uropathogenic *E. coli* isolates in Kathmandu University Hospital. J Nepal Health Res Counc, 14(32):33-38.

Soto, S. M. (2014). "Importance of biofilms in urinary tract infections: new therapeutic approaches." Advances in biology 2014.

Stamm WE, Hooton TM. (1993). Management of Urinary Tract Infections in Adults. The New England Journal of Medicine, Page: 1328-1334.

Stenutz R, Weintraub A, Widmalm G. (2006). The Structures of *E. coli* O-Polysaccharides Antigens. US National Library of Medicine National Institutes of Health, 30(3): 382-403.

Stepanovic S, Vukovic D, Hola V, Bonqventura GD, Djukic S, Clrkovic I and Ruzicka F. (2007). Quantification of biofilm in microtitre plates: Overview of testing conditions and practical recommendation for assessment of biofilm production by *Staphylococci*. Journal compilation 2007 APMIS, 115:891-9.

Stewart, P. S. and J. W. Costerton (2001). "Antibiotic resistance of bacteria in biofilms." The lancet 358(9276): 135-138.

Tan CW, Chlebicki MP. (2016). Urinary Tract Infections in Adults. US National Library of Medicine National Institutes of Health, 57(9):485-490.

Tenaillon O, Rodriquez VA, Gaut RL, McDonald P, Bennett AF, Long AD, Gaut BS. (2012). The Molecular Diversity of Adaptive Convergence. US National Library of Medicine National Institutes of Health, 335(6067): 457-61.

Toval F, Kohler CD, Vogel U, Wagenlehner F, Mellmann A, Fruth A, Schmidt MA, Karch H, Bielaszewska M, Dobrindt U. (2014). Characterization of *E. coli* isolates from hospital inpatients or outpatients with urinary tract infection. Journal of clinical microbiology, 52(2):407-418.

Watnick P, Kolter R. (2000). Biofilm, City of Microbes. Journal of Bacteriology, 182(10): 2675-2679.

Yadav, K. K., N. Adhikari, R. Khadka, A. D. Pant and B. Shah (2015). "Multidrug resistant *Enterobacteriaceae* and extended spectrum β-lactamase producing *E. coli*: a cross-sectional study in National

Kidney Center, Nepal." Antimicrobial resistance and infection control 4(1): 42.

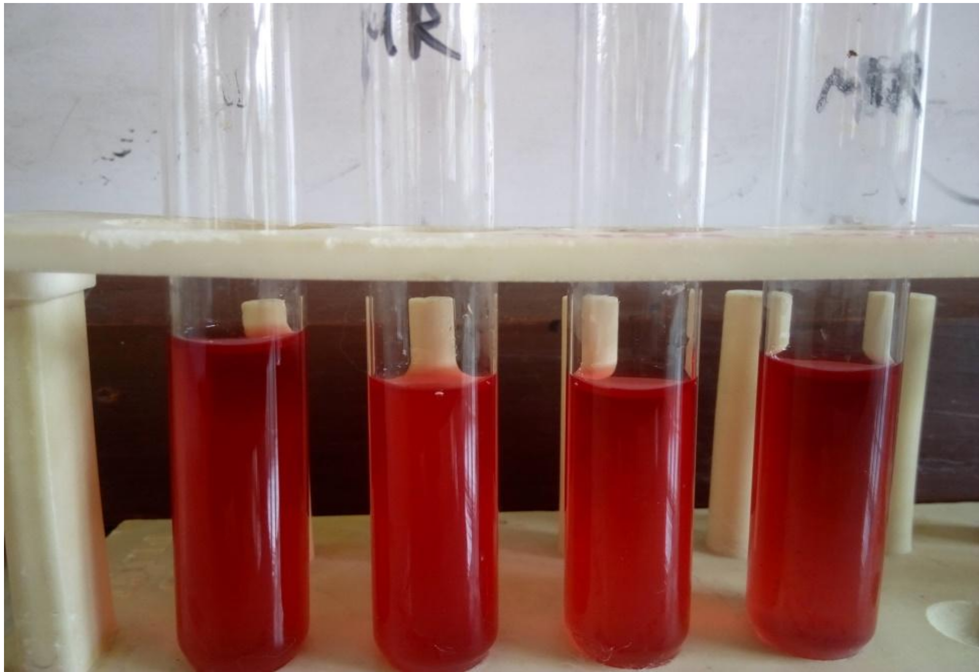
YU S, Fu AZ, Qui Y, Engel SS, Shankar R, Brodovicz KG, Rajpathak S, Radican L. (2014). Disease Burden of Urinary Tract Infections among Type 2 Diabetes mellitus patients in the U.S. US National Library of Medicine National Institutes of Health, 28(5): 621-6.

Zahera M,Rastogi C, Singh P, Iram S, Khalid S, Kushwaha A. (2011). Isolation, identification and characterization of *E. coli* from urine samples and their antibiotic sensitivity pattern. European journal of experimental biology, 1(2):118-124.

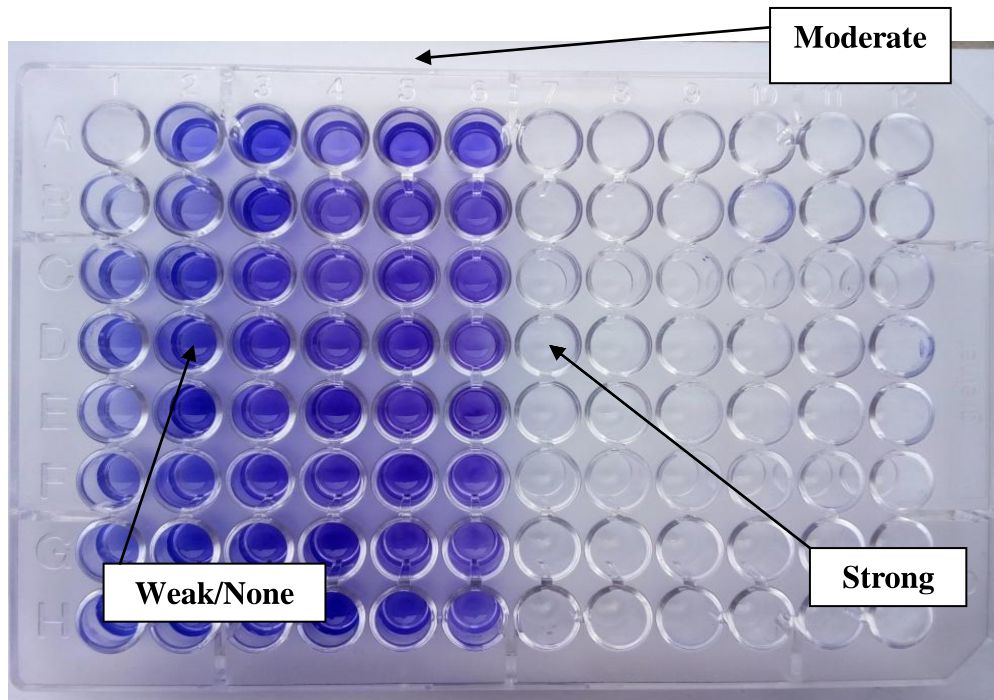
Photographs

Photograph 1: Sample collection

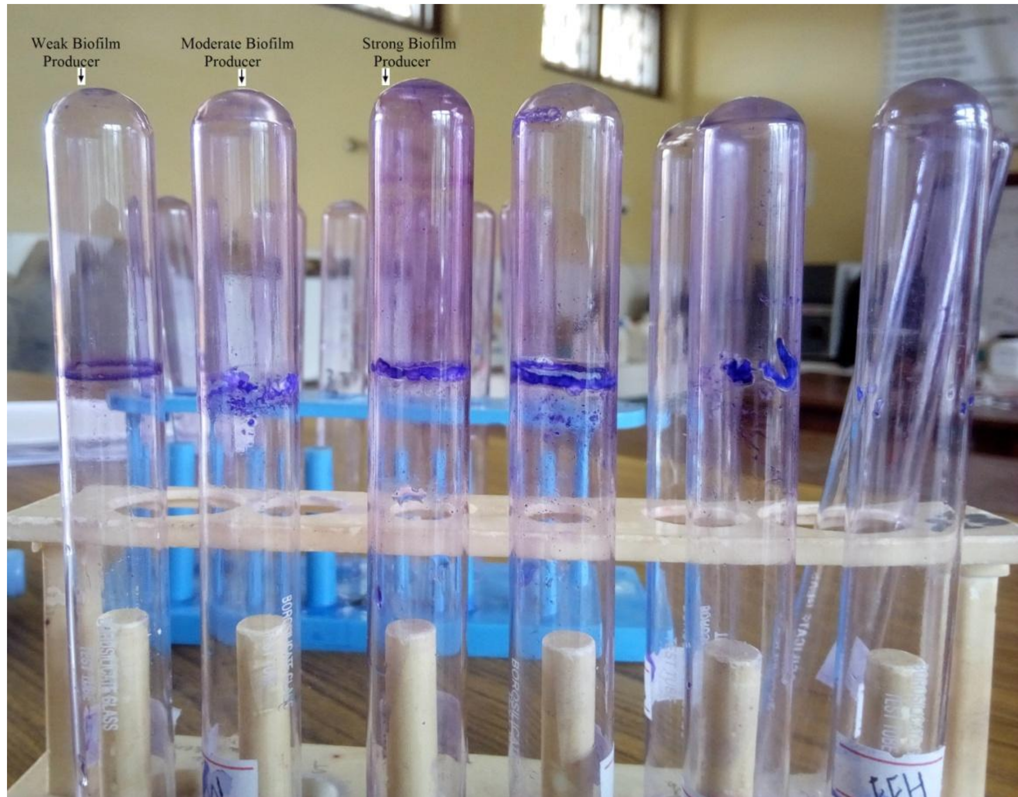
Photogarph 2: Sample processing in the microbiology lab



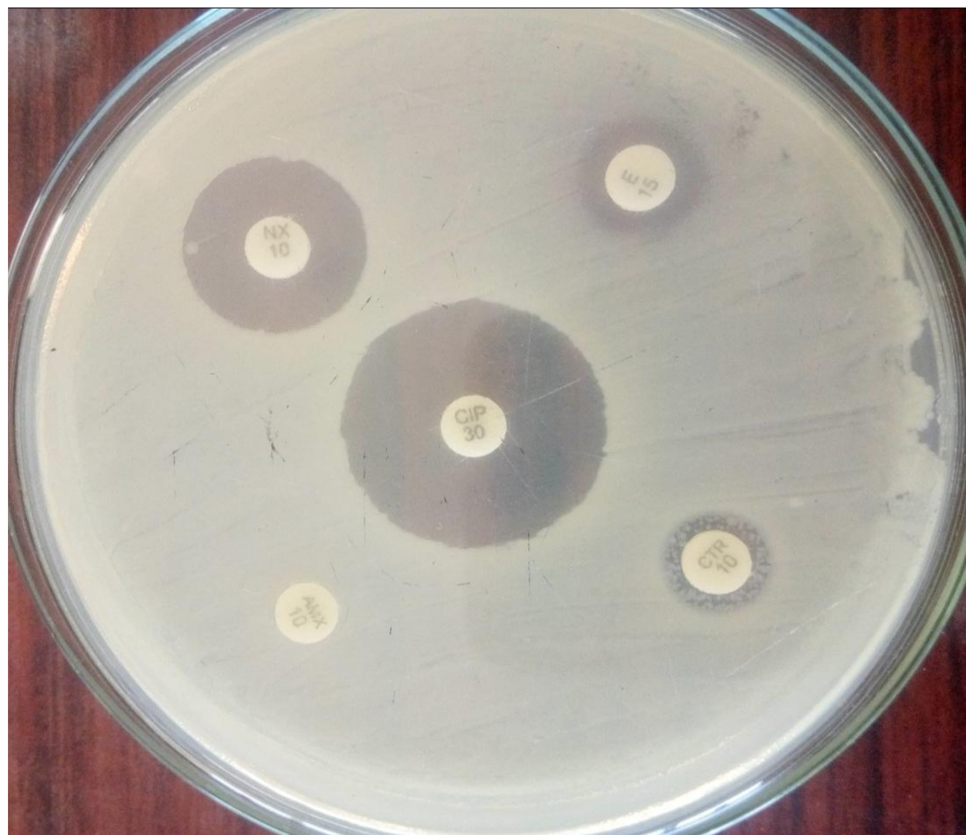
Photograph 3: Biochemical tests of *E. coli*



Photograph 4: Biofilm of *E. coli* in microtitre wells



Photograph No.5: Boiofilm Production Testing by Tube Method



Photograph No.6 Antibiotic susceptibility test