Chapter II

REVIEW OF LITERATURE

Multidrug resistant enteropathogenic *Escherichia coli* associated with urinary tract infections
2.1. *Escherichia coli*

*Escherichia coli* is a common bacterium that normally inhabits the intestinal tracts of humans and animals, but can cause infection in other parts of the body, especially the urinary tract. It is the most common member of the genus *Escherichia*, named for Dr. Theodor Escherich, a German physician (Escherich, 1885; Neill *et al.*, 1994). Subsets of *E. coli* have evolved possessing virulence properties and the association of these organisms with worldwide outbreaks of many enteric/diarrhoeal cases is well established.

*E. coli* is a gram-negative, rod-shaped bacterium propelled by long, rapidly rotating flagella. It is part of the normal flora of the mouth and gut and also helps to protect the intestinal tract from bacterial infection. It also aids in digestion of food. The bacterium, which is also found in soil and water, is widely used in laboratory research and it is said to be the most thoroughly studied life form. In genetic engineering *E. coli* is the microorganism preferred for use as a host for the gene-splicing techniques used to clone genes.

*E. coli* are the predominant facultative anaerobe of the human colonic flora. The organism typically colonizes the infant gastrointestinal tract within hours of life, and, thereafter, *E. coli* and the host derive mutual benefit.
Escherichia coli usually confined to the intestinal lumen and remains harmlessly however, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even normal “non-pathogenic” strains of E. coli can cause infection. Moreover, even the most robust members of species may be susceptible to infection by one of several highly adapted E. coli strains which together have evolved the ability to cause a broad spectrum of human diseases. Infections due to pathogenic E. coli may be limited to the mucosal surfaces or can disseminate throughout the body. Three general clinical syndromes result from infection with inherently pathogenic E. coli strains: (i) They are urinary tract infection, (ii) sepsis/ meningitis and (iii) enteric/ diarrhoeal disease.

Urinary tract infection is one of the most important causes of morbidity and mortality. Forty to 50% of women experience at least one UTI, leading to an estimated 8 million annual physician visits in the United States alone (Schappert, 1999; Zielske et al., 1981). E. coli is by far the most common etiological agent of all UTIs (50-90%) affecting all age groups in India. (Acharya, 1992)

Urinary tract infection is a serious health problem affecting millions of people each year. It is estimated that there are more than 10 million cases in Europe alone per year. The recurrence rate is high, and often the infections tend to become chronic with many episodes. Urinary tract infection
usually starts as a bladder infection but often evolves to encompass the kidneys and ultimately can result in renal failure or dissemination to the blood. UTI is the most common infection in patients with a chronic indwelling bladder catheter, bacteriuria is essentially unavoidable in this patient group (Foxman, 2002).

2.2. SOURCE

_E. coli_ is a common inhabitant of the intestinal tract of humans and animals. The number of bacteria varies in different animals, but an animal will excrete from 130 million to over 18 billion _E. coli_ each day (Salyers et al. 2004). Hence, there is a close association between _E. coli_, faecal material and, possibly, enteric pathogens. The organism can be found on plants, although usually not in great abundance. The inoculation of cells onto dry soil resulted in a 99.00% cent reduction in 24 hr.

2.3. PREVALENCE OF _Escherichia coli_

Member of the Enterobacteriaceae (Edwards and Ewing, 1986) are the cause of the majority of urinary tract infection (UTI). In developing countries, where socioeconomic conditions do not allow many individuals access to a clean water supply or adequate sewage disposal, UTI and gastroenteritis are common infections. These are often caused by multiple drug resistant organisms. (Atif et al. 2010).
Drug resistant *E. coli* from UTI patients may enter environment and may thereby enter water bodies for public utility. These *E. coli* if carry the toxin producing genes like *stx1* and *stx2* or other genes like *hly*, *est* or *elt* may result into enteric diseases in human or animal beings (Gunkel et al., 1985).

Urinary tract infection is among the most common infections in the United States. Each year, at least 4 million women seek treatment for UTIs, and ~$1.6 billion spent in the diagnosis and treatment of UTIs (Foxman and Brown, 2003). UPEC are the cause of 70–90% of all community-acquired UTIs (Foxman and Brown, 2003).

Clinicians are frustrated by the rise in antibiotic resistance among organisms that cause UTIs and by frequent recurrences in healthy adult females after an initial UTI. Many women use antibiotics daily to reduce the risk of recurrence only to suffer recrudescence when this prophylactic regimen is discontinued. The use of *in vitro* and *in vivo* models has allowed the description of events that delineate the acute and chronic stages of UTI. (NKUDIC, 2005).

Rodriguez et al. (2001) while undertaking a comparative study of antimicrobial resistance of *E. coli* strains isolated from UTI patients from Carcas (Venezuela) and Lima (Peru) observed that out of 13 antimicrobials tested against a total of 885 strains of *E. coli*, 525 from Lima and 360 from Carcas; 361 were resistant to ampicillin; 278 (31.4%) to ampicillin/sulbactum;
307 (34.7%) to cephalothin; 136 (15.3%) to gentamicin; 217 (24.5%) to ciprofloxacin and 498 (56.3%) to trimethoprim-sulphamethoxazole.

Reinthaler et al. (2003) conducted a study to evaluate the resistant pattern of *E. coli* in waste water treatment plants without an evaluation of basic antibiotic resistance mechanism. The investigation was done in sewage, sludge and receiving waters from three different treatment plant in southern Austria. A total of 767 *E. coli* isolates were tested regarding their resistance to 24 different antibiotics. The highest resistance rates were found in *E. coli* strains of a sewage treatment plant which treats not only municipal sewage but also sewage from a hospital. Among the antimicrobial agents tested, the highest resistance rates in the penicillin group were found for Ampicillin (upto 18%) and Pipercillin (up to 12%); in the cephalosporin group for Cephalothin (up to 35%) and Cefuroxime-Axetil (up to 11%); in the group of quinolones for Nalidixic acid (up to 15%) and for Trimethoprim/Sulfamethoxazole (up to 13%) and for Tetracycline (57%).

In a study in the University of Maryland (Meng et. al., 1998) involving antibiotic resistance of *E. coli* from animals, food and humans; among the 125 strains isolated, 30 (24%) were resistant to atleast one antibiotic and 24 (19%) were resistant to three or more antibiotics. Cattle isolates had the highest rate (34%) of antibiotic resistance.
In an another study carried out by Sahm et al. in The Surveillance Network Database-USA where 38,835 isolates of *E. coli* from urine were isolated and tested for antibiotic sensitivity that showed the isolates, 7.1% (2,763 of 38,835) were resistant to three or more agents and considered multidrug resistant. Among the multidrug resistant isolates, 97.8% were resistant to ampicillin, 92.8% were resistant to trimethoprim-sulfamethoxazole, 86.6% were resistant to cephalothin, 38.8% were resistant to ciprofloxacin, and 7.7% to nitrofurantoin.

Urinary tract infection (UTI) is the major cause of bacterial infection in infants younger than 90 days. The estimated incidence of UTI in this age group is about 1% (Rushton, H. G., 1997) and UTI is responsible for up to 10% of cases of fever (Crain and Gershel, 1990). UTI shows a marked male predominance during this period of age, and bacteremia occurs in one-quarter of cases, compared to less than 5% in older subjects (Bachur and Caputo, 1995; Ginsburg and McCracken, 1982; Wiswell and Hachey, 1993).

Community-acquired extraintestinal infections with *Escherichia coli* range in frequency from 6 to 8 million cases of uncomplicated cystitis per year to 127,500 cases of sepsis per year in the United States (Russo, 2003). Urinary tract infections (UTIs) caused by *E. coli* are one of the most common extraintestinal infections in women and, because of their high incidence, are the focus of most epidemiologic studies. The source of *E. coli* for these infections
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is a person's intestinal tract; however, how these *E. coli* are acquired by the gut is unclear. Risk factors that lead to intestinal colonization with extraintestinal *E. coli* differ from factors associated with development of infection.

Young, otherwise healthy, sexually active women have the highest risk for community-acquired UTIs. The main risk factors for UTI are recent and frequent sexual intercourse, contraceptive use, and a history of UTIs (Foxman et al., 1995; Remis et al., 1987).

Treatment for UTIs usually involves a short course of an antimicrobial drug, such as trimethoprim-sulfamethoxazole (TMP-SMZ). Over the past decade, the prevalence of drug resistance in *E. coli* has increased dramatically, complicating management of these infections. Across the United States and Canada, urinary tract isolates of *E. coli* from outpatient clinics showed increased resistance to TMP-SMZ and ampicillin (Zhanel et al., 2006).

A more serious concern has been the gradual increase in fluoroquinolone (e.g., ciprofloxacin) resistance among UTI isolates (Karlowsky et al., 2006).

There is increasing evidence that the *E. coli* that causes UTI and other extraintestinal infections may be responsible for community-wide epidemics. In 1986–1987, *E. coli* O15:K52:H1 caused an outbreak of

Other outbreaks of UTI caused by *E. coli* have been described and include a cluster of UTI cases in Copenhagen, Denmark, caused by *E. coli* O78:H10 and a larger outbreak in Calgary, Alberta, Canada, caused by extended-spectrum β-lactamase (ESBL)-producing *E. coli* (Olesen et al., 1994).

Identification of outbreak strains of *E. coli* that cause extra intestinal infections suggests that point sources, possibly contaminated food, may be responsible for local spread of genetically related *E. coli* strains in the community. Recent work in the United Kingdom has focused on a possible link between the increase in ESBL-producing *E. coli* and food animal production. An estimated 30,000 cases of human infection with ESBL-producing *E. coli* occur each year in the United Kingdom, and studies have found epidemic strains of ESBL-producing *E. coli* in the United Kingdom and throughout the world (Coque et al., 2008).

*E. coli* are a multipotent pathogen that has the ability to cause disease in different animal species and man involving several systems and at
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last in the gut. *E. coli* seems to be the most predominant isolate among the pathogenic bacteria recovered from diarrhoeic animal and man. Evidence that animals are a reservoir for *E. coli* found in human was published by Cooke *et al.* (1971) in the early 1970s.

A total of 78 strain of *E. coli* were isolated from 179 samples of faeces and intestinal contents of young animals (newborn calves, lambs and piglets) with diarrhoea resulting in a mortality rate of 50.00% or more. Regardless of the animal species, *E. coli* was found to predominant bacteria associated with diarrhoea in newborn animals (Ramisse *et al.*, 1979).

*E. coli* was isolated from 70% of cases of diarrhoea from the jejunal and ileal contents of 163 piglets of a pig farm, which died of diarrhoea and 45 live piglets with diarrhoea (Sikdar, 1991).

Seventy *E. coli* strains were isolated from 440 swabs collected from various pathological conditions of poultry by Panneerselvam *et al.* (1988). Gowda *et al.* (1996) could isolate *E. coli* from various pathological conditions of poultry like colisepticemia, ophoritis, peritonitis, omphalitis, entuitis and coligranuloma. Jones *et al.* (2000) examined 250 samples from diseased chickens, which yielded 168 strains of *E. coli*.

Fifty strains of *E. coli* were isolated from 250 clinical specimens of poultry including heart blood, intestinal contents, liver, lungs, ovaries,
peritoneal fluid, spleen and unabsorbed yolk by Mishra et al. (2002). Goswami et al. (2002) isolated 65.00% *E. coli* from 350 samples from dead and sick chickens.

Toxigenic strains like EHEC can potentially enter the human food chain most commonly through contamination with faeces or intestinal contents after slaughter. The organism may also be transmitted from person-to-person through the faecal-oral route (Yamasaki et al., 1999).

### 2.3. PATHOGENESIS OF *E. coli* INFECTION

*Escherichia coli* is the most frequently isolated pathogen from cases of urinary tract infection (UTI) and are rapidly developing multiple drug resistance. Grouping of *E. coli* from UTI cases into a distinct pathotype has been of controversy since different members of pathotypes has been isolated from samples of UTI. The concern is mostly related to the potentially serious clinical outcomes of the infections and possible transmission to human from different sources. Infections due to pathogenic *E. coli* may be limited to the mucosal surfaces or can disseminate throughout the body. Multiple virulence factors like the ability to adhere, proliferate and colonize the small intestine as well as the capacity to produce enterotoxins (Fairbrother, 1992) contribute to the pathogenicity of these organisms. Several classes of enteropathogenic *E. coli* have been recognized on the basis of the types of enterotoxins they
produce. *E. coli* producing heat labile (LT) or heat stable (ST) enterotoxins coded by the *est* and *elt* genes are termed as ETEC (Sears and Kapers, 1996). Both LT and ST occur in two different forms and they disrupt the host cell functions by disturbing the intercellular ion concentrations, leading to intestinal fluid accumulation resulting in osmotic diarrhoea (Sears and Kaper, 1996; Guth, 2000). *E. coli* possessing the *hly* gene termed as EHEC produces haemolysins, which act as spore forming cytolysin on the eukaryotic cells and result in cell lysis (Schmidt et al., 1995). Shiga like toxins (Stx), which exist in two antigenically distinct forms; Stx1 and Stx2, encoded by the *stx1* and *stx2* genes associated with hemorrhagic colitis and hemorrhagic uremic syndrome (O'Brien and Holmes, 1987) in humans and inhibiting protein synthesis are elaborated by *E. coli* strains named as STEC and are recognized as important food borne pathogens of human. Disease outbreaks caused by these enteropathogenic groups of *E. coli* are frequently reported from all over the world causing great concern.

The major problem in identifying and characterizing pathogenic strains of *E. coli* is that they predominantly resemble commensal *E. coli* in many aspects apart from producing toxins (Bettelheim and Beutin, 2003). Therefore molecular methods like polymerase chain reaction (PCR) have been widely preferred for detection of the virulence genes to identify the enteropathogenic *E. coli* strains (Paton and Paton, 1997; Osek et al., 1999;
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Galane and Roux, 2001; Rahman, 2002; Murugkar et al., 2004) due to its sensitivity and specificity. Prevalence studies of these toxigenic genes are of importance in pathogenic characterization of the isolates. A single strain of E. coli may express any of the stx genes or both together, any of the heat stable or labile gene or both together and also may harbor hlyA. Keeping in view the public health importance and the hazards caused by the enteropathogenic strains of E. coli, which can be present even in normal and un-diseased human and animals, the present study was undertaken to isolate and identify these organism from a wide variety of sources and detect the presence of five virulence genes viz, stx1, stx2, estI, eltI and hlyA in them by PCR method.

2.3.1. Classification of pathogenic E. coli

Pathogenic strains of E. coli have been divided into different pathotypes and each pathotype cause diseases using different combinations of the virulence factors, with different molecular pathways.

Depending upon the virulence markers (virotyping), six virotypes of E. coli have been distinguished (Nataro and Kaper, 1998). Their description is given below:
2.3.1.1. Enterotoxigenic *E. coli* (ETEC)

It is the causative agent of traveller's diarrhoea and the illness is characterized by watery diarrhea with little or no fever. These strains produce one or both of the two distinct enterotoxins, which are responsible for diarrhoea. The enterotoxins based on heat stability are distinguished into (i) heat labile enterotoxin (LT) (ii) heat stable enterotoxin (ST). The genes for ST and LT production and colonization factors are located in plasmid. ST binds to a glycoprotein receptor that is coupled to a guanylate cyclase on the surface of intestinal epithelial cells. Activation of guanylate cyclase stimulates the production of cyclic guanosine monophosphate (cGMP), which leads to the secretion of electrolytes and water into the lumen of the small intestine, manifested as the watery diarrhoea characteristic of an ETEC infection. LT binds to specific GM₁ gangliosides on the epithelial cells and activates membrane-bound adenylate cyclase, which leads to increased production of cyclic adenosine monophosphate (cAMP), through the same mechanism employed by cholera toxin, resulting in hypersecretion of electrolytes and water into the intestinal lumen resulting in diarrhoea.

2.3.1.2. Enteroinvasive *E. coli* (EIEC)

These strains cause diarrhoea by penetrating and multiplying within the intestinal cells. The ability to invade the epithelial cells is associated
with the presence of a large plasmid; EIEC may also produce a cytotoxin and an enterotoxin.

2.3.1.3. Enteropathogenic *E. coli* (EPEC)

These strains attach to the brush border of intestinal epithelial cells and cause a specific type of cell damage called effasing lesion. Effasing lesions or attaching-effasing (AE) lesions represent destruction of brush border microvilli adjacent to adhering bacteria. This cell destruction leads to subsequent diarrhoea. As a result of this pathology, the term AE *E. coli* is used to describe true EPEC strains. It is now known that AE *E. coli* is an important cause of diarrhoea in children residing in developing countries.

2.3.1.4. Enterohemorrhagic *E. coli* (EHEC)

These strains carry the genetic determinants for attaching-effasing lesions and Shiga toxin production. The attaching-effasing lesions cause hemorrhagic colitis with severe abdominal pain and cramps followed by bloody diarrhoea. The Shiga toxins 1 and 2 have also been implicated in two extraintestinal diseases; haemolytic uremic syndrome and thrombocytopenic purpura. It is believed that these toxins kill vascular endothelial cells. A major form of EHEC is the *E. coli* O157:H7 that has caused many outbreaks of hemorrhagic colitis in the United States since it was first recognized in 1982.
2.3.1.5. Enteroaggregative *E. coli* (EAggEC)

These strains adhere to epithelial cells in localized regions, forming clumps of bacteria with a ‘stacked brick’ appearance. Conventional extracellular toxins have not been detected in EAggEC, but unique lesions are seen in epithelial cells, suggesting the involvement of toxins.

2.3.1.6. Diffusely adhering *E. coli* (DAEC)

These strains adhere over the entire surface of epithelial cells and usually cause disease in immunologically naive or malnourished children.

2.4. SEROTYPING

A serotype or serovar is a group of microorganisms, viruses, or cells classified together based on their cell surface antigens. The process of determination of serotypes is called serotyping. Serotyping depends on factors, including virulence, lipopolysaccharides (LPS) in Gram-negative bacteria, presence of an exotoxin (such as pertussis toxin in *Bordetella pertussis*), plasmids, phages, genetic profile (such as determined by polymerase chain reaction), or other characteristics which differentiate two members of the same species (Baron1996; Ryan and Ray, 2004) allowing the epidemiologic classification of organisms to the sub-species level (Baron, 1996). A group of serovars with common antigens is called a serogroup. Serotyping plays an
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esential role in determining species and subspecies. The salmonella genus of bacteria, for example, has been determined to have over 4400 serotypes, including Salmonella enterica serovar Typhimurium, S. enterica serovar Typhi, and S. enterica serovar Dublin. Vibrio cholerae, the species of bacteria that causes cholera, has 139 serotypes, based on cell antigens. Only two of them produce an enterotoxin and are pathogens: 0:1 and 0:139. Serotypes were discovered by the American microbiologist Rebecca Lancefield in 1933 (Lancefield RC, 1933). Over 700 antigenic types or serotypes of E. coli have been recognized based on O, H, and K antigens. Serotyping is important in distinguishing the small number of strains that actually cause disease.

Several serotypes of E. coli such as O1, O2, O4, O6, O8, O16, O18, O22, O25 and O75 are preferentially associated with uropathogenic E. coli (UPEC) strains and each of the uropathogenic virulence factors is significantly associated with these serotypes (Hughes et al., 1982; Vaisanen-Rhen et al., 1984; Yamamoto et al., 1995). E. coli strains isolated from dogs with UTI have high prevalence of hly and clustered in 5 serotypes including O2, O4, O6, O7 and O18 (Wilson et al., 1998). These serotypes except for O7 are shared with human UPEC strains. Further, E. coli strains isolated from dogs with UTI were similar to E. coli strains isolated from human UTI (Low et al., 1988; Yuri et al., 1998). Serotypes viz: 01, 02, 03, 06, 07, 09, 015 are the main uropathogens in man seen in our country (Asscher et al., 1969).
2.5. ANTIBIOGRAM

An antibiogram is the result of a laboratory testing for the sensitivity of an isolated bacterial strain to different antimicrobial agents. It is by definition an *in vitro* sensitivity. In clinical practice, antibiotics are most frequently prescribed on the basis of general guidelines and knowledge about sensitivity *e.g.* uncomplicated urinary tract infections can be treated with a first generation antibiotic such as quinolone, etc. This is because *E. coli* is the most likely causative pathogen and it is known to be sensitive to quinolone treatment.

Antimicrobial resistance has increased drastically in recent years in both developed and developing countries and it has rapidly become a leading public health concern (Jordi and Tibor, 2010).

Several factors favour the development of bacterial resistance to antibiotics in developing countries as reported as follows:

1. Less potent activity: Some of the antibiotics provided in developing countries have decreased potency because of degradation or adulteration of the drug, or because of the presence of a lower concentration of active substances (Land, 1992; Pecoul et al. 1999). For instance, substandard
concentrations of ampicillin and tetracycline have been found in Nigeria (Okeke and Lamikanra, 1995; Agom, 1990).

Moreover, expired drugs, with altered or removed expiry dates, have also been detected in developing countries (Gustafsson and Wide, 1981; Kigotho, 1997). Some drugs produced in industrialized countries have been found to have expired on distribution in developing countries (Okeke et al. 1999). Finally, the antibiotics provided to these countries may be poorly transported and stored, leading to drug inactivation (Hogerzel et al. 1992; Ballereau, 1997).

2. Lack of diagnostic laboratories: Most of the hospitals in developing countries do not have clinical microbiology laboratories to perform routine analyses for microbiological diagnosis. Even if some services are in place, international guidelines and quality control for susceptibility testing are often not available or require methods not affordable locally. Therefore, they have no information about either the etiology of the infectious diseases or antimicrobial susceptibility, both essential for clinical practice. Bacterial infections are often treated empirically with broad-spectrum antibiotics. For control, an accurate diagnosis including proper susceptibility data is a must, supplemented with capacities to type organism. This needs properly standardized, quality controlled methods
complemented with the collection and compilation of data into appropriate databases on antibiotic resistance. A hierarchical structure of local, national, regional and supranational laboratories with well defined and controlled competences can only meet these tasks.

3. Over-the-counter availability: In most developing countries, antibiotics can be purchased without prescription in pharmacies, general stores, markets, and from street hawkers. Since many drugs are expensive, some patients purchase incomplete regimens whenever possible and discontinue treatment when the symptoms disappear (Lansang, 1990). As in industrialized countries, unnecessary prescriptions of antibiotics, mainly in cases of acute infantile diarrhea and respiratory infections, have been reported in developing countries (Guyon et al., 1994). Although the problem is not exclusive to the developing countries, in many hospitals in these regions antibiotic policies and infection control practices are suboptimal, nonexistent, not appropriately enforced or compromised due to the lack of resources and properly trained personnel (Meers, 1988).

4. Use of antimicrobials in animals. Although it may not seem to be an important problem in developing countries, where the practice is less applied, the excessive use of antimicrobials, especially as growth promoters in animals destined for human consumption, presents a growing
risk to human health due to the emergence of bacteria resistant to the antibiotics fed to animals, which can then be transferred to humans through the food chain (Rasniraul et al. 1988; Barza and Gorbach, 2002). Spread of antibiotics and resistant bacteria into the environment is also a contributor to the problem.

So the indiscriminate use of antimicrobial agents in humans during infections has resulted in the development of resistance amongst bacteria, including *E. coli*. Antibiotic usage selects for resistance not only in pathogenic bacteria but also in the endogenous flora of exposed individuals (animals or humans) or population.

Frequent testing for drug susceptibility of *E. coli* against the commonly used antibiotics and chemotherapeutic agents is necessary to aid in the treatment of disease caused by this organism and also to prevent emergence of drug resistance strains. A number of data are available in literature, where indiscriminate use of antimicrobials and other antimicrobial agents led to the development of single or multiple drug resistance in bacteria. (Dan, 2002; Goossens, 2005; Arnold and Straus, 2005).

Singh *et al.* (1995) isolated a total of 7 *E. coli* strains from the milk samples of 5 cows and 2 buffaloes. All the 7 *E. coli* isolates were resistant
to 3 or more antibiotics and two untyped isolates, resistant to penicillin, tetracycline and doxycycline were capable of producing heat labile enterotoxin.

Singh and Kulshrestha (1994) tested the antibiotic sensitivity of *E. coli* isolated from aquatic foods from different regions of India. A total of 17 *E. coli* strains were isolated and their antibiotic sensitivity was tested by disc method using 10mcg discs of streptomycin, ampicillin, gentamicin, ledermycin, norflox, doxycycline; 30 mcg of nalidixic acid and chloramphenicol; 100 and 50mcg discs of nitrofurantoin and trimethoprim-sulphamethoxazole, respectively. Antibiotic sensitivity pattern indicated that all the 17 isolates were sensitive to norflox, while 16 to chloramphenicol, gentamicin, ampicillin and trimethoprim-sulphamethoxazole. 15 isolates were sensitive to ampicillin and doxycycline, while 13 to nalidixic acid, nitrofurantoin and ledermycin. A total of 7 isolates were sensitive to all 10 antibiotics tested, while 5 isolates were resistant to 2 antibiotics, 3 to 1 antibiotic and 1 each to 3 and 4 antibiotics.

Picozzi *et al.* (2005) screened milk samples originated from goats and cows for the presence of *E. coli* O157 with culture methods. Sorbitol-negative or slow fermenting strains were subjected to phenotypic characterization, antibiotic resistance profiles, PCR reactions for detection of toxins (*stx1* and *stx2*) and intimin (*eae*GEN and *eae*O157) genes and clustering by pulsed field gel electrophoresis (PFGE). Only one strain revealed to be
O157. Susceptibility to 11 antibiotics highlighted the high resistance to tetracycline (50%), sulphonamide and streptomycin (33%) 

Khan *et al.* (2001) isolated 35 strains of *E. coli* from various foodstuffs and identified them on the basis of cultural, morphological and biochemical characteristics. These isolates were further tested for their antibiotic susceptibility with commonly used antibiotics/drugs. The isolated *E. coli* strains exhibited sharp peaks of resistance to antimicrobial agents such as tetracycline (72%), doxycycline (60%) and nalidixic acid (48%). Forty-four per cent of the *E. coli* strains were resistant to nitrofurantoin and penicillin-G respectively. Among the 13 antibiotics/drugs tested for resistance, seven different resistance patterns were observed in the *E. coli* isolates from various foodstuffs.

Lihan *et al.* (1999) analysed 95 *E. coli* strains isolated from raw milk for plasmid profile and antimicrobial resistance, and typed by RAPD fingerprints. All the *E. coli* strains were resistant to 4 or more of the antibiotics tested. However, none were resistant to ceftazidime, gentamicin and norfloxacin. The multiple antibiotic resistances (MAR) index values of the *E. coli* strains ranged from 0.25 to 0.81, indicating that all were isolated from raw milk samples that were exposed to high risk sources.
Singh et al. (1995) isolated enterotoxigenic *E. coli* (2 isolate each) from orange juice and sugarcane juice from roadside vendors, suspected to be vehicles of a diarrhoeal outbreak at Faridpur in Bareilly District in June 1992. The antibiotic sensitivity of the bacterial isolates was determined by standard disc method using standard 10mcg discs of streptomycin and gentamicin; 30mcg discs of neomycin, carbenicillin, nalidixic acid, tetracycline, doxycycline and kanamycin; 25mcg discs of cotrimoxazole; 300mcg discs of nitrofuradentoin; 50mcg discs of compound sulphonamides and 300 I.U. discs of polymycin B sulphate. The *E. coli* isolates from orange juice were resistant to cotrimoxazole, carbenicillin, doxycycline, streptomycin, and tetracycline, compound sulphonamides while the isolates from sugarcane juice were resistant to streptomycin, tetracycline, carbenicillin and nitrofuradentoin.

Smith and Crabb (1956) showed that frequent use of chemotherapeutic agents including tetracycline in the prevention and treatment of white scour in calves led to the development of drug resistant *E. coli* flora in intestinal tract. In the following years, they observed high proportion of tetracycline resistant *E. coli* in the faeces of pig and chicken, which had been fed diet containing low level of tetracycline than those, which have never been fed tetracycline. A similar observation was made by Loken *et al.* (1971). They found multiple drug resistance among *E. coli* to tetracycline, streptomycin,
neomycin, kanamycin and ampicillin, after calves were given feed with neomycin for one week. A varying degree of resistance to the drugs in current use such as tetracycline, ampicillin, furazolidone etc. particularly in herds where these drugs had been given previously, was reported by various workers (Bartos et al., 1967; Bartos and Lebduska, 1968; Polakova, 1968).

A study carried out by Kapoor and Kulshrestha (1994) on drug sensitivity pattern of \textit{E. coli} recorded from human patients to 7 antimicrobial agents revealed that the isolates showed highest degree of sensitivity to gentamicin (80%), followed by furadentoin (68%) and nalidixic acid (59%). The isolates were resistant to tetracycline (80%), chloramphenicol (94.54%), streptomycin and ampicillin (100%). Goswami \textit{et al.} (2002) isolated 63 \textit{E. coli} from dead and sick chicken and tested the drug sensitivity pattern to 11 antimicrobial agents. The order of resistance recorded as penicillin (100%), tetracycline (98.30%), ampicillin (62.74%), cloxacillin (15.68%), erythromycin (7.84%), kanamycin, gentamicin, chloramphenicol and streptomycin (5.88%) and nalidixic acid (1.96%) was also recorded.

Tabatabaei and Nasirian (2003) isolated 50 \textit{E. coli} strains from chicken in Teheran and tested them against standard antimicrobial agents. They observed 94% resistance against tetracycline, 80% resistance against oxytetracycline and 48% resistance against neomycin.
Chulain et al. (2005) carried out antimicrobial resistance in *E. coli* associated with urinary tract infection in the west of Ireland by disc-diffusion method, where he found, out of 934 isolates of *E. coli*, more than 50% of *E. coli* were resistant to ampicillin, more than 40% were resistant to sulphonamide and more than 30% were resistant to trimethoprim. In general practice most *E. coli* remain susceptible to nitrofurantoin (96.7%), nalidixic acid (93.9%) and ciprofloxacin (94.7%). For all agents rates of resistance were higher in hospital as compared with general practice isolates.

Chitra et al. (2004) carried out another study in the Department of Microbiology, Regional Institute of Medical Sciences (RIMS), Imphal, Manipur, India to determine the antibiotic sensitivity pattern of the pathogens isolated from UTI. A total of 1,109 mid-stream urine samples were collected during the period of January 2001 to April 2004 and organisms were identified by doing standard biochemical tests. Antibiotic susceptibility testing was done according to Kirby-Bauer's disc-diffusion method for all the isolates. Out of 1,109 samples tested, 459 (40.4%) samples showed bacterial pathogens responsible for UTI. *Escherichia coli* was the commonest organism followed by *Klebsiella* and others. Most of the strains of *E. coli*, *Klebsiella* species, coagulase negative *Staphylococci*, *Pseudomonas* species showed resistance to Tetracycline and Norfloxacin. Most of the strains isolated were sensitive to Gentamicin whereas Cotrimoxazole showed resistant to most of the *E. coli* strains. Most of the strains of *E. coli* also showed resistance to Ciprofloxacin.
Multiple drug resistance pattern in Urinary Tract Infection patients in Aligarh was studied by Asad Mohd. in the year 2006 where 168 urine samples were collected from patients with community-acquired UTI and 102 *E. coli* isolates were identified and subjected to antibiotic sensitivity test by Bauer-Kirby disc diffusion method. Data showed that 90% isolates were resistant against ampicilin. Present studies also showed that 60-79% isolates were resistant against chloramphinicol, erythromycin, rifampicin, sulphamathizole, tetracycline. Norfloxacin showed intermediate resistance. The most effective antibiotics in our study against *E. coli* were found to be kanamycin and streptomycin.

2.6. ENDOTOXIN

Endotoxins are part of the outer membrane of the cell wall of gram-negative bacteria. They are invariably associated with gram-negative bacteria whether the organisms are pathogens or not.

Although the term ‘endotoxin’ is occasionally used to refer to any cell associated bacterial toxin, it is properly reserved to refer to the lipopolysaccharide complex associated with the outer membrane of gram-negative bacteria such as *E. coli, Salmonella, Shigella, Pseudomonas, Neisseria, Haemophilus* and other leading pathogens.
2.6.1. Shiga toxins (stx)

The *E. coli* strains showing resistance to antibiotics and carrying enterotoxin synthesizing genes are the cause of concern to health scientists especially because they show variations in their serotypes.

One of the enterotoxins produced by *E. coli* is the Shiga toxin. The natural hosts of Shiga toxin-producing *E. coli* (STEC) are farm and wild life ruminants.

In human, STEC can cause disease, although the clinical picture may vary from uncomplicated diarrhea to hemorrhagic colitis (HC) to hemolytic uremic syndrome (HUS) (Meng et al., 1998). Large outbreaks and cases of HC and HUS in humans were mainly associated with STEC strains belonging to serogroup O157. Human infections with STEC O157 are under nation wide surveillance in a number of countries, but the detection of non-O157 STEC infections is often limited to a small number of specialized laboratories because STEC O157 colonies are more easily detectable on some culture media than non-O157 STEC types which are thus often missed in laboratory diagnosis of stool specimens. However, humans are likely more exposed to non-O157 STEC because these strains are more prevalent in animals and as contaminants in foods than STEC O157. Infections with some non-O157 STEC types, such as O26 and O111, are associated with illness in
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humans, but the role of other non 157 STEC type in human disease needs further examination.

2.6.2. Heat Stable Enterotoxins (ST)

Another type of *E. coli* enterotoxin a heat stable Enterotoxin (ST) also causes diarrhea in human. There are two slightly different toxins designated as STIa and STIb, both having a molecular weight of around 2000 and are resistance to 100 degree Celsius for 15 minutes, water soluble, resistance to proteolytic enzymes and acid but not alkaline resistant. ST binds to a heterogenous group of specific glycoprotein receptors on the brush borders of intestinal epithelial cells and excretion resulting in the inhibition of Na+ coupled Cl− secretion. Thus there is a rapid release of fluids and electrolytes into the intestinal lumen causing diarrhea. A role has also been suggested for arachidonic acid, prostaglandins and leucotrienes in the upsetting of the host fluid balance caused by STI.

The genes for STI generally plasmid mediated, often associated with genes for colonizing fimbriae, drug resistance and colicinogeny.

2.6.3. Heat labile enterotoxins

These are produced by some Enterotoxigenic *E. coli* (ETEC). LT is an oligomeric protein of 88kDa comprising one A submit of 30kDa above a
central aqueous channel formed by B subunits each of 11.5kDa.

Iron starvation and the presence of bile salts and trypsin in the concentrations normally present in the intestine stimulates release of LT from the *E. coli*. While the structural genes of Lt are on plasmids, chromosomal genes may affect levels of expression. These plasmids often also harbour resistance factors to antimicrobial agents as well as genes coding for adherence factors associated with ETEC. There is strong homology between LT causing human illness and LT from porcine and also cholera toxin, suggesting that the LT genes were derived from *Vibrio cholerae* in 1956 by De and colleagues.

2.7. PCR BASED DETECTION OF TOXIN GENES (*stx1, stx2, est, elt* and *hlyA*) OF *E. coli*

2.7.1 Virulence genes of *E. coli*

Like most mucosal pathogens, *E. coli* can be said to follow a requisite strategy of infection:

i. colonization of the mucosal site  
ii. evasion of the host defence  
iii. multiplication  
iv. host damage

The most highly conserved feature of diarrhoegenic *E. coli* strain is their ability to colonize the intestinal mucosal surface despite peristalsis and competition for nutrients by the indigenous flora of the gut.
The virulence of any organism is dependent on an array of virulence factors that play a vital role in the pathogenic process of the organism viz., attachment, invasion, toxicity etc. According to Nataro and Kaper (1998), expression of these factors is controlled by virulence genes present in the *E. coli* genome which is conferred mainly by two pathogenic configurations—virulence related plasmid and chromosomal pathogenicity islands (PI). All six virotypes described above carry at least one virulence related property upon a plasmid.

One such virulent factor, the enterotoxins produced by *E. coli* can be classified into two, heat labile enterotoxins (LT) and heat stable enterotoxins (ST) that causes increased secretions of isotonic fluid by the small intestines (Moon, 1974), which is encoded by *elt* and *est* genes respectively (Sears and Kaper, 1996). The haemolsyn toxin producing *E. coli* penetrate and multiplies within epithelial cells of the colon causing wide spread cell destruction. The major virulent factors of enterohaemorrhagic *E. coli* (EHEC) is the Shiga toxin (Stx) production that is absorbed into the blood and causes systematic vascular damage resulting in edema disease (Moon *et al.*, 2003) and haemorrhagic colitis. This toxin exists in two antigenic forms: Stx1 and Stx2 and are encoded by *stxl* and *stx2* genes respectively (Sears and Kaper, 1996).

The enterohaemorrhagic *E. coli* strains harbouring a plasmid encoded enterohaemolysin gene (*hly A*), act as a spore forming cytolysin on
eukaryotic cells and resulting in cell lysis (Schimdt et al., 1995). Tarr (1995) showed an additional virulence factors, lipopolysaccharide (LPS) expressed in some *E. coli* strains (rfb O157 strains) which is commonly associated with severe bloody diarrhoea, haemorrhagic colitis and haemolytic uremic syndrome.

2.7.2. Detection of virulence genes by polymerase chain reaction

Diarrhoeagenic *E. coli* strains were among the first pathogens for which molecular diagnostic methods were developed. Indeed, molecular methods remain the most reliable techniques for differentiating diarrheagenic strains from non-pathogenic members due to their high sensitivity, rapidity and specificity. Apart from colony hybridization assay with oligonucleotide or polynucleotide probes, the polymerase chain reaction (PCR), first conceptualized by Kary Mulliis of Cetus Corporation, USA in 1985 has revolutionized the diagnostic technique especially in the field of molecular microbiology. It is highly sensitive and is a specific molecular method for detection of genes encoding virulence factors of the pathogenic organisms (Rahman, 2002; Moon et al., 2003). This technique is so sensitive, specific and rapid and has greatly improved the ability to differentiate virulent strains from non-virulent *E. coli* (Osek, 2003). This technique is so sensitive that the target gene can be amplified without purifying the organisms or isolating separate
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colonies. Crude lysates or DNA extracts from single colonies, mixed broth cultures, colony sweeps, or even direct extracts of faeces or foods can be used as templates for PCR, though sometime false positive results may obtained due to presence of cryptic target sequences (Wani et al., 2004).

A number of reports are available regarding the detection of *E. coli* virulence gene by PCR. In a study carried out to detect heat stable enterotoxin II by application of PCR, it was found that seven out of thirty eight strains of *E. coli* from diarrhoeic chickens were positive for the gene of heat stable enterotoxin II. Fagen *et al.* (1999) reported that in *E. coli* isolated from healthy and diseased cattle, sheep, pigs and goats, the most commonly encountered virulence genes were *stx1* and *hlyA* as detected by PCR using their specific primers.

Osek *et al.* (1999) observed that labile-enterotoxin gene (*elt1*) only in 9 *E. coli* strains (22.5%), heat stable, enterotoxin gene (*est 1*) in 29 *E. coli* strain (72.5%) and est II was detected in 31 *E. coli* strains (77.5%) out of 40 *E. coli* strains isolated from diarrhoeic piglets by using PCR. However no *stx1* and *stx2* gene was detected.

Paton and Paton (1998) developed two multiplex PCR assays for simultaneous detection and genetic characterization of a total of 52 STEC strains, out of which 28 strains were isolated from human faeces (patients with
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diarrhoea or HUS), 7 were from domestic animals and 17 were from foods. Assay 1 utilizes 4 PCR primer pairs and detects the presence of \textit{stx1}, \textit{stx2} (including variants of \textit{stx2}), \textit{eae A} and enterohaemorrhagic \textit{E. coli hly A}. Assay 2 uses two primer pairs specific for portions of the \textit{rfb} (0-antigen encoding) regions of \textit{E. coli} serotypes O157 and 0111. The two assays were validated by testing 52 previously characterized STEC strains and observing 100% agreements with previous result.

Rahman (2002) reported that all of the ETEC strains harboured one or both \textit{stx} genes (\textit{stx1} and \textit{stx2}) as detected by single and multiplex PCR. In cases of non-EHEC strains tested from patients with diarrhoea and enteritis, 14.28\% were recorded positive for \textit{stx} gene. Out of 37 \textit{E. coli} strains isolated from cases of diarrhoea and gastrointestinal diarrhoea in human patients, 5 (13.5\%) harboured verocytotoxin (VT) gene, two harboured both VT1 and VT2 genes, while two strains harboured only VT1 gene and one strains harboured only VT2 gene.

Pal \textit{et al.} (1999) reported the isolation of STEC from non-diarrhoeic animal sources in India. They collected faecal samples from 67 healthy cattle in a semi-urban community near Calcutta (now Kolkata) and examined STEC by multiplex PCR and culturing on Sorbitol MacConkey Agar (SMA). ETEC was isolated from faeces of seven (10.5\%) animals. A total of 876 samples (330 animals, 184 human and 362 food samples) were screened...
for the presence of STEC by PCR. Seventeen STEC strains were isolated. The isolation rate was higher in diarrhoeic animals (6.02%) followed by diarrhoeic handler (3.12%) and raw beef (1.78%).

A systematic study on strains of STEC in India was carried out by Khan et al. (2002). The study includes antibiotic resistance, virulence gene and 25 free flying pigeons for the presence of toxin genes by using PCR. None of the strains were found to possess Shiga toxin genes. However, presence of eaeA gene was recorded in six isolates and hly A gene in seven isolates.