Chapter IV.

Isolation and characterization of

*Escherichia coli*

4.1 Introduction
The presence of pathogenic bacteria in milk is of considerable public health concern, particularly for individuals drinking raw milk (Mendelson, 2011). The true prevalence of milkborne disease remains obscure. A number of microorganisms including *Escherichia coli* can contaminate milk and products. *Escherichia coli* is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). *E. coli* inhabits the intestinal tract of animals and humans. Though most *E. coli* are harmless, some are known to be pathogenic, causing both severe intestinal and extraintestinal diseases in man (Kaper *et al.*, 2004; Fairbrother and Nadeau, 2006). Recovery and counting of *E. coli* is used as reliable indicator of fecal contamination and indicates a possible presence of enteropathogenic and/or toxigenic microorganisms. *Escherichia coli* was first described by Theodore Escherich in 1885. It is the type species of genus *Escherichia*, which contains mostly motile Gram negative bacilli within the family *Enterobacteriaceae*. Pathogenic *E. coli* have been classified into different pathotypes that cause a common disease by using common and an assortment of virulence factors (Kaper *et al.*, 2004). One of these pathotypes, called Shiga toxin-producing *E. coli* (STEC), also called verotoxin-producing *E. coli* refers to those strains of *E. coli* that produce at least 1 member of a class of potent cytotoxins called Shiga toxin. The terms Shiga toxin (Stx) and verotoxin (VT) used interchangeably are derived from similarity to a cytotoxin produced by *Shigella dysenteriae* serotype 1 (O’Brien *et al.*, 1982) and cytotoxicity for Vero cells (Konowalchuk *et al.*, 1977).

Dairy cattle are considered as the primary reservoir of *E. coli* particularly Shiga toxin producing *E. coli* (STEC) and the main route of STEC
infections in humans is via consumption of contaminated food (Hussein and Sakuma, 2005; Meng et al., 2007). STEC strains have been isolated from a large variety of different foods including raw-milk cheeses (Caro and Garcia-Armesto, 2007; Stephan et al., 2008). STEC cause severe clinical syndromes in humans such as haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) (Solomakos et al., 2009). The main virulence factors of STEC are the cytotoxins, Shiga toxins 1 and 2 (encoded by the stx1 and stx2 genes, respectively) and the protein intimin (encoded by the chromosomal gene eae) responsible for the intimate attachment of the bacteria to intestinal epithelial cells and the enterohaemolysin, encoded by the ehxA gene (Solomakos et al., 2009). Earlier studies documented the presence of E. coli possessing virulence markers from raw milk and its products (Jayarao and Henning 2001; Holko et al., 2006; Paneto et al., 2007). Recent outbreak of non-O157 E. coli in more than 14 European countries has caused 50 deaths and more than 4000 people ill (WHO 2011). Schaffzin et al. (2011) summarized two outbreaks caused by non-O157 STEC in USA. Characterization of the recent outbreak strain STEC O104:H4 responsible for nation wide outbreak of HUS in Germany revealed an unusual combination of virulence factors of STEC and enteroaggregative E. coli. This very rare combination has been previously described in strains of serotype O111:H2 (Beutin et al., 2004; ECDC, 2011). These reports highlight the ability of non-O157 STEC to cause outbreaks and calls for concerted efforts for their effective control.

Systems of serotyping, subtyping, and virulence typing of STEC are used to aid in epidemiology, diagnosis, and pathogenesis studies (Gyles, 2007).
The STEC have been characterized by serotyping which is used extensively to categorize strains of *E. coli* (Blanco et al., 2004a; Prager et al., 2005).

Analysis of virulence genes has been reported to be a very useful method for subtyping STEC (Beutin et al., 1994). The Shiga toxin genes (*stx*), the *E. coli* attaching and effacing gene (*eae*), and the gene for hemolysin (*ehly* or *ehxA*) are frequently investigated (Roldgaard et al., 2004; Nielsen et al., 2006; Rivas et al., 2006). In a study, *stx* subtyping and phage typing were used to compare the subtypes of O157:H7 STEC from Danish cattle with those recovered from humans with severe disease (Roldgaard et al., 2004).

Guaranteeing a greater food safety level for consumer products warrants an integrated approach to controlling food safety throughout the entire food chain (Stefan, 1997; Valeeva et al., 2005). Raw unpasteurized milk is consumed directly by a large number of people in rural areas and indirectly by a much larger segment of the population via consumption of several types of products. A number of regulations have been developed and introduced to assure food safety at different stages of the food production chain. Given the many potential and emerging hazards along the chain, it is of practical importance to prioritize attributes.

Livestock production including dairy plays a multipurpose role in the agriculture systems of India. Dairy plays a dynamic role in India’s agro-based economy. Today, India ranks the first in the world in terms of milk production. In India, limited information is available regarding the STEC in animals including cattle (Pal et al., 1999), sheep (Bhat et al., 2008), fish (Sanath Kumar et al., 2001), beef (Khan et al., 2002) and human faeces (Khan et al., 2002).
In this study, attempts were made to isolate \emph{E. coli} from milk samples collected at different levels of collection and processing. The \emph{E. coli} isolates were characterized employing standard microbiological methods and further tested for virulence genes and Shiga toxin (\emph{stx}) genes by PCR. The isolates were also characterized for their serotypes and \textit{XbaI} digestion patterns of total DNA separated by PFGE. The \emph{E. coli} isolates were also subjected to antibiotic susceptibility testing.

### 4.2 Materials and Methods

#### 4.2.1 Bacteria

The standard strain of \emph{Escherichia coli} (MTCC 443) was obtained from microbial type culture collection and gene bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India.

#### 4.2.2 Samples

A total of 767 milk samples from dairy cows were collected at different levels of collection and processing (udder (126), milking utensils (126), milk collection centres (126), receiving dock (269) and market (120)). All the samples were collected aseptically, transported to the laboratory under chilled conditions and processed for microbiological analysis within 24 h of collection.

#### 4.2.3 Isolation of \emph{E. coli}

#### 4.2.3.1 Enrichment
The samples were inoculated into MacConkey’s broth tubes (HiMedia, Mumbai, India) and incubated at 37°C for 18-24 h.

4.2.3.2 Plating on selective media

A loopful inoculum from MacConkey’s broth was streaked onto Eosin Methylene Blue (EMB) agar (HiMedia, Mumbai, India) and plates were incubated at 37°C for 18-24 h and observed for the characteristic metallic sheen of *E. coli*. The well separated pure colonies were picked up on nutrient agar slants as pure culture and subjected for standard morphological and biochemical tests (Cruikshank *et al.* 1975).

4.2.3.3 Confirmation of the isolates

Morphologically typical colonies were verified by Gram staining, IMViC tests, fermentation of sugars like glucose, lactose, xylose, sucrose and maltose, Triple sugar iron agar test, oxidase test and catalase test.

4.2.4 Characterisation of *E. coli*

4.2.4.1 Serotyping

*E. coli* cultures were referred to National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, Himachal Pradesh, India, for serotyping. The isolates were serotyped for ‘O’ antigen.

4.2.4.2 Genotypic characterization

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E. coli isolates were characterized genotypically with reference to virulence marker genes employing polymerase chain reaction.

4.2.4.2.1 Isolation of genomic DNA

The genomic DNA of the isolates was prepared as per Wilson (1987). The isolates were grown in 2 ml of BHI broth overnight at 37°C. Cultures were harvested by centrifugation (8000 g for 10 min) and suspended in 400 µl of TE solution (10 mM Tris HCL; 1 mM EDTA, pH 8.0). Bacteria were lysed by addition of 10 µl (20 mg/ml) proteinase K and 100 µl of 10% SDS followed by incubation at 37°C for 1 h. The cell wall debris, denatured proteins, polysaccharides and polymeric matrix were eliminated by precipitation with addition of 80 µl of 5 M NaCl and 64 µl of CTAB solution (10 % CTAB in 0.7 M NaCl) and incubation at 65°C for 10 min. DNA was purified by two extractions with phenol : chloroform (1 : 1) and chloroform : isoamylalcohol (24 : 1). DNA was precipitated by adding 100 µl of isopropanol and placed at -20°C for 30 min and later centrifuged at 8000 g for 15 min. The pellet was washed in 70% ethanol, air dried and resuspended in 50 µl TE buffer. Suspended pellet (10 ng) was used as the template.

4.2.4.2.2 Detection of the stx1 and stx2 genes

The PCR was carried out using 2 sets of oligonucleotide primers for the stx1 and stx2 genes. The PCR primers for stx1 and stx2 genes were previously described by Cebula et al. (1995). The primers used to amplify the stx1 gene 5’-CAG TTA ATG TGG TGG CGA AGG-3’ and 5’-CAC CAG ACA ATG TAA CCG CTG-3’ and the stx2: 5’-ATC CTA TTC GGA GTT TAC GA-
3’ and 5’-GCG TCA TCG TAT ACA CAG GAG E-3’ were synthesized by Sigma Aldrich, USA. The expected size for PCR-amplified products was 348 bp for the stx1 and 584 bp for the stx2. In brief, the PCR mixture of 25.0 μl contained 1X PCR buffer, 1.5 mM of MgCl₂, each primer within the 2 primer sets at a concentration of 40 nM, 200 μM each of dNTPs, 1.0 U of Taq DNA polymerase and 75ng of template DNA. The PCR reaction was performed in a thermal cycler (Eppendorf, Germany). The samples were initially denatured at 94°C for 3 min and then subjected to 35 cycles, each consisting 1 min at 94°C, 2 min at 65°C and 2 min at 72°C. The synthesis was completed at 72°C for 10 min. After the reaction, PCR products were kept at –20°C until further analysis by agarose gel electrophoresis. The amplicons were separated on 1.2% agarose gels, followed by ethidium bromide staining. Standard molecular size marker (100 bp DNA ladder) was included in each gel. DNA fragments were observed by ultraviolet transilluminator and photographed in a gel documentation system (Alpha Imager, USA).

4.2.4.2.3 Multiplex PCR for virulence associated genes

The primers for detection of genes for P-fimbriae (papC), iron-repressible protein (irp2), temperature-sensitive hemagglutinin (tsh), vacuolating autotransporter toxin (vat), enteroaggregative toxin (astA), increased serum survival protein (iss), and colicin V plasmid operon genes (cvii) used in this study were synthesized by Genei, Bangalore, India. The details of the primer sequences are shown in Table 4.1.
The PCR was standardized for the detection of virulence associated genes namely *papC*, *irp2*, *tsh*, *vat*, *ast A*, *iss*, and *cvi* as per the method described by Ewers *et al.* (2003) with suitable modifications.

The PCR was set for 25µl reaction volume. The reaction mixture for multiplex PCR was optimized as follows:- 2.5µl of 10X PCR buffer (consisting of 100mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM MgCl$_2$ and 0.01% gelatin), 0.2mM dNTP mix, 2 mM MgCl$_2$ and 20pmol of forward and reverse primer of each set, 2 unit of Taq DNA Polymerase, 10 ng of DNA template and sterilized water to make up the reaction volume.

**Table 4.1.** Primer sequences of the virulence genes of *E coli* (Ewers *et al.*, 2004)

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer Sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ast A</em></td>
<td>Forward 5'-TGC CAT CAA CAC AGT ATA TCC -3'</td>
<td>116</td>
</tr>
<tr>
<td><em>iss</em></td>
<td>Forward 5'-ATC ACA TAG GAT TCT GCC G -3'</td>
<td>309</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CAG CGG AGT ATA GAT GCC A -3'</td>
<td></td>
</tr>
<tr>
<td><em>irp2</em></td>
<td>Forward 5'-AAG GAT TCG CTG TTA CCG GAC -3'</td>
<td>413</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-AAC TCC TGA TAC AGG TG G C -3'</td>
<td></td>
</tr>
<tr>
<td><em>papC</em></td>
<td>Forward 5'-TGA TAT CAC GCA GTC AGT AGC -3'</td>
<td>501</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CCG GCC ATA TTC ACA TAA -3'</td>
<td></td>
</tr>
<tr>
<td><em>tsh</em></td>
<td>Forward 5'-ACT ATT CTC TGC AGG AAG TC -3'</td>
<td>824</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CTT CCG ATG TTC TGA ACG T - 3'</td>
<td></td>
</tr>
<tr>
<td><em>vat</em></td>
<td>Forward 5'- TCC TGG GAC ATA ATG GTC AG-3'</td>
<td>981</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GTG TCA GAA CGG AAT TGT -3'</td>
<td></td>
</tr>
<tr>
<td><em>cvi</em></td>
<td>Forward 5'-TGG TAG AAT GTG CCA GAG CAA G -3'</td>
<td>1181</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GAG CTG TTT GTA GCG AAG CC - 3'</td>
<td></td>
</tr>
</tbody>
</table>
The reaction was performed in a thermal cycler (Eppendorf, Germany) with a pre-heated lid. The cycling conditions included an initial denaturation at 94\(^0\) C for 3 min. followed by 25 cycles each of 30 seconds denaturation at 94\(^0\) C, 30 seconds annealing at 58\(^0\) C and 3 min extension at 70\(^0\) C. It was followed by final extension of 10 min. at 72\(^0\) C. After the reaction, PCR products were kept at –20\(^0\)C until further analysis by agarose gel electrophoresis. For visualization of PCR products, 0.5 µl of reaction mixtures were further analyzed by agarose gel electrophoresis (1.5%) low melting temperature agarose) stained with ethidium bromide (0.5µg/ml) and visualized by a UV trans-illuminator.

### 4.2.4.3 Pulsed Field Gel Electrophoresis

Genomic DNA fingerprints of the STEC isolates were determined using pulsed-field gel electrophoresis (PFGE) according to a standard protocol developed by PulseNet for *E. coli* O157:H7 (Gautom, 1997).

#### 4.2.4.3.1 Preparation of culture

Overnight grown cultures (2 ml) of *E. coli* at 37\(^0\)C were taken in microcentrifuge tube and centrifuged at 7500 for 5 min to pellet the cells. The pellet was washed by adding 2 ml of Phosphate Buffered saline (PBS) and vortexed and centrifuged again at 7500 rpm for 5 min. The supernatant was discarded. This step was repeated twice and the pellet was resuspended in 2 ml PBS. The optical density of culture was adjusted in the range of 1.3 to 1.4 with the help of PBS.
4.2.4.3.2 Casting of Plugs

Equal volumes (400 μl) of each adjusted bacterial suspension and 1.8% agarose (Biorad, USA) were mixed with 50 mg /ml proteinase K, 10 mg/ml lysozyme and 10% SDS were immediately added into block formers without allowing air bubbles to form. Three plugs per specimen were made. The plugs were allowed to solidify at room temperature for 10-15 min.

4.2.4.3.3 Lysis of Cells in Agarose Plugs

Polypropylene screw-capped tubes (15 ml capacity) were labelled with culture numbers. Proteinase K/Cell Lysis Buffer, 5 ml (50mM Tris:50mM EDTA, pH8.0+1% Sarcosyl) was added to each tube. The tape from reusable mold was removed and with the help of a dispenser, the solidified agarose plug was dropped into the 15 ml tube containing 5 ml of Proteinase K/Cell Lysis Buffer. Care was taken to see that the plug was dipped into the buffer. The tubes were placed in a rack and incubated in a 50-54°C shaker water bath for 15-30 min with constant and vigorous agitation (175-200 rpm). The water level in water bath was kept above the level of lysis buffer in tubes.

The tubes were removed from the water bath, and lysis buffer was carefully poured off. Sterile reagent grade water (10-15 ml) was added to each tube. Then the water was discarded after 15 min. Again 10-15 ml of sterile reagent grade water that had been pre-heated to 50-54°C was added to each tube and the tubes were shaken vigorously in a 50-54°C water bath for 10-15 min. The water was discarded. The plugs were then washed twice with 10-15 ml TE buffer pre-heated at 50-54°C. TE buffer was decanted from the last
wash. The plugs were stored in tubes containing 5 ml sterile TE buffer at 4°C until ready for restriction digestion.

4.2.4.3.4 Restriction digestion of agarose plugs

Microcentrifuge tubes (1.5 ml capacity) were labelled with culture numbers. 200 μl of 1X enzyme buffer was added to the labeled 1.5-ml microcentrifuge tubes. The plug was carefully removed from TE buffer with spatula and placed in a sterile disposable Petri dish. 2.0 mm-wide slice was cut from test and standard samples with a single edge razor blade and transferred to a tube containing diluted 1x buffer. Care was taken to see that the plug slice was dipped in buffer. The remaining part of the plug was replaced in the original tube that contained 5 ml TE buffer and stored at 4°C. The plug slices were incubated in 25°C water bath for 5-10 min or at room temperature for 10-15 min. After incubation, the buffer from the plug slice was aspirated using a pipette with a fine tip taking care not to damage the plug slice. 200 μl restriction enzyme mixture was added to each tube. Agarose-embedded DNA was digested with 50 U of XbaI for 4 h in a water bath at 37°C.

4.2.4.3.5 Casting Agarose Gel

The gel casting tray was assembled and the comb was fitted. 1% agarose in 1X TE buffer was prepared and poured when it was 45°C into the gel casting tray and allowed to solidify. After solidifying, the comb was removed and the plug slices were loaded into the wells of the gel. The plug slices were sealed in the wells by using molten 1% agarose. The gel was then placed in the electrophoresis chamber containing 2.5 L freshly prepared 0.5X
TBE pre-cooled to 14°C. DNA fragments were separated by electrophoresis in 0.5X Tris-borate-EDTA buffer at 14°C for 18 h on a CHEF-II Mapper electrophoresis system (Bio-Rad Laboratories), with a pulse time of 2.2 s to 54.2 s at constant voltage of 6 V/cm. A PFGE Marker I (New England Biolabs) was included in each agarose run.

4.2.4.3.6 Staining and Documentation of PFGE Agarose Gel

When the electrophoresis run was over, the equipment was turned off. The gel was removed and stained with ethidium bromide by diluting 40 μl of ethidium bromide stock solution (10 mg/ml) with 400 ml of reagent grade water by placing on a rotary shaker for 20 min. The gel was destained in approximately 500 ml reagent grade water for 60 - 90 min. The image was photographed in a gel documentation system (AlphaImager, USA).

4.2.5 Antibiotic Sensitivity testing

Antibiotic sensitivity profiles of E. coli isolates were studied against 9 different antibiotics according to methods of Bauer et al. (1966) following National Committee for Clinical Laboratory Standards (NCCLS) guidelines, 1997. Briefly, bacteria were grown in nutrient broth for 18 h. Mueller-Hinton Agar (Hi-media) supplemented with 5% sterile defibrinated sheep blood was used as a medium to study the susceptibility to antibiotics. Different antibiotic discs namely tetracycline (30μg/disc), cephaloridine (30 μg/disc), amikacin (10μg/disc), streptomycin (25μg/disc), carbenicillin (100μg/disc), doxycycline (30 μg/disc), ampicillin (25μg/disc), enrofloxacin (10 μg/disc) and colistin (25μg/disc) were placed on nutrient agar plates and incubated at 37°C for 18-
24 hours. Zones of inhibition were recorded. Results in terms of sensitive or resistant were interpreted as per the manufacturer’s guidelines (HiMedia, 2009). *Escherichia coli* ATCC 25922 was used as control in all assays.

### 4.3 Results and Discussion

The bacteriological examination of milk samples (n=767) revealed characteristic metallic sheen indicating the presence of *E coli* in 77 (10.04%) samples. The isolates were characterized morphologically and biochemically. Maximum isolates (29.87%) were detected from the samples collected at market level followed by at receiving dock (20.78%) (Table 4.2).

<table>
<thead>
<tr>
<th>Levels &amp; Samples</th>
<th>No of strains</th>
<th>Serotypes</th>
<th>Genes detected</th>
<th>Stx 1</th>
<th>Stx 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Udder (n=126)</td>
<td>13</td>
<td>O4, O88, O169, O97, O103, O2, O56, O110, O80, O84, O140, UT</td>
<td>iss, tsh, irp2, astA, papC, vat</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Milking utensils (n=126)</td>
<td>14</td>
<td>O4, O24, O83, O147, O88, O95, O2, O56, O60, O124, O120, UT</td>
<td>iss, tsh, irp2, astA, papC, vat, cvi</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>DCS (n=126)</td>
<td>11</td>
<td>O4, O141, O2, O112, O60, O21, O120, UT, Rough</td>
<td>iss, tsh, irp2, astA, papC, vat</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Dock (n=267)</td>
<td>16</td>
<td>O166, O159, O4, O141, O95, O69, O43, O21, O80, UT, Rough</td>
<td>iss, tsh, irp2, astA, papC, vat</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Market (n=120)</td>
<td>23</td>
<td>O60, O112, O159, O51, O83, O18, O100, O15, O7, O3, O25, O120, O4, O45, O114, O56, UT, Rough</td>
<td>iss, tsh, irp2, astA, papC, vat, cvi</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>
The isolates were sent for serotyping at National Salmonella and Escherichia Centre, Kasauli. Out of 77 E. coli isolates, 64 were typeable and belonged to 35 different O serogroups (O2, O3, O4, O7, O15, O18, O21, O24, O25, O43, O45, O51, O56, O60, O69, O80, O83, O84, O88, O95, O97, O100, O103, O110, O112, O114, O120, O124, O140, O141, O146, O147, O159, O166, O169) (Table 4.2). Eight isolates were considered as O untypeable (UT) and five as rough strains. Milk may get contaminated with E. coli during different stages of production and processing. Therefore, the present work was intended to study the occurrence of E. coli in the milk collected at different levels. In earlier studies (Altalhi and Hassan 2009), E. coli was isolated from 33 (66%) of the 50 raw milk and product samples tested. A study in Pakistan also reported heavy contamination of raw milk and its products by E. coli (Soomro et al. 2002). In this study, eight isolates were untypeable. Many E. coli isolates, including some STEC, have O or H antigens, or both, that are not in the international scheme and, therefore, untypeable (Gyles, 2007). Serotyping is effective in revealing some of the diversity among E. coli and is often the starting point in characterization of E. coli. However, there is limited access to a small number of laboratories certified for E. coli serotyping. Because of the importance of serotype O157:H7 in human disease, it is common to consider STEC serotypes in 2 major categories, O157 and non-O157 (Gyles, 2007). The concept of seropathotypes is useful for investigation of bacterial factors that contribute to disease and transmissibility and may be refined to be of value in a diagnostic setting.
In the present study, a total of 35 different serogroups of *E. coli* were isolated, of which O4 was the predominant. Earlier workers in India (Wani *et al.* 2004) reported the predominance of O9, O8, O60 and O25 in animal cases. Sanath Kumar *et al.* (2001) reported the presence of STEC in seafoods of serotypes other than that of O157. These findings indicate the variable distribution of different serogroups of *E. coli* in different geographical regions in India. Studies in USA reported that *E. coli* O26, O45, O103, O111, O113, O121, O145, and O157 have been the most commonly identified O-serogroups associated with Shiga toxin–producing *E. coli* (STEC) implicated in outbreaks of human illness all over the world (DebRoy *et al.* 2011). Zweifel *et al.* (2010) detected a notable proportion of non-O157 STEC serotypes associated with human infections in semi-hard and hard raw milk cheese. Taking into account these facts and the lack of data on the occurrence of STEC in milk from India, the study assumes importance. Studies in India have confirmed that cattle are the principal reservoir of non-O157 serotypes (Khan *et al.* 2002).

In this study, PCR assay yielded amplified products of ~348 bp and ~584 bp specific for *stx1* and *stx2* genes, respectively (Fig 4.1; 4.2). Of 77 *E. coli* strains recovered, 25(32.47%) had one or more of the genes responsible for virulence of *E. coli* (Table 4.2). PCR assay revealed that 11 of 77 (14.29%) of the isolates carried the *stx1* gene alone, 3 of 77 (3.9%) possessed the *stx2* alone and 11 of 77 (14.29%) carried both the *stx1* and *stx2* genes.
In this study, the \textit{stx1} gene was the predominant \textit{stx} type (28.57%), in contrast to previous studies in cattle and humans (Padola \textit{et al.} 2004; Rivas \textit{et al.} 2006) and dairy cattle (Irino \textit{et al.} 2005; Fremaux \textit{et al.} 2006). It has been demonstrated that the \textit{stx2} gene is more cytotoxic than the \textit{stx1} gene, and is associated with high virulence in humans (Fremaux \textit{et al.} 2006; Rasooly and Do 2010). Characterization of STEC isolated from Swiss raw milk cheese revealed thirteen of the 24 strains typeable with O antisera belonging to the serogroups O2, O22, and O91 and the \textit{stx2} (86%) was more prevalent than the \textit{stx1} gene (46%) (Zweifel \textit{et al.} 2010). On the contrary, Carneiro \textit{et al.} (2006) could not detect the \textit{stx1} and \textit{stx2} genes from \textit{E. coli} strains isolated from pasteurized milk. The \textit{stx1} and \textit{stx2} genes were detected in 3% and 6.1%, respectively of \textit{E. coli} strains isolated from raw milk samples in Saudi Arabia (Altalhi and Hassan 2009). Montenegro \textit{et al.} (1990) reported that most of the STEC isolates of bovine origin encoded for shiga-toxin 1 gene. In another study, of the 16 strains detected from raw milk cheese samples, 11 were typed
into 7 *E. coli* O groups (O2, O15, O22, O91, O109, O113, O174), whereas 5 strains were nontypeable and the *stx*1 and *stx*2 variants were detected in 1 and 15 strains, respectively (Stephan *et al.* 2008). STEC O83 has been reported to be associated with human illness (Bettelheim 2007) raising the possibility that O83 might be transmitted to humans via milk products. Since not all STEC strains are equally pathogenic to humans, evaluation of virulence-associated factors is necessary to assess the potential of individual isolates to cause human illness.

In the present study, 77 *E. coli* strains isolated from milk samples were screened for the presence of seven virulence marker genes (Fig 4.3). A multiplex polymerase chain reaction (PCR) assay was used to detect presence of the genes for P-fimbriae (*papC*), iron-repressible protein (*irp2*), temperature-sensitive hemagglutinin (*tsh*), vacuolating auto transporter toxin (*vat*), entero-aggregative toxin (*astA*), increased serum survival protein (*iss*), and colicin V plasmid operon genes (*cvi*). The multiplex PCR profile revealed amplification of virulence associated genes with different patterns. Presence of all the seven genes was detected in none of the isolate. The *iss* gene was detected in the highest number of isolates (25) followed by the *tsh* and the *irp2*. Amplification of six genes was observed in one isolate which harboured the *tsh, vat, irp2, papC, iss* and *ast A* genes. Different combinations of five genes were observed in ten isolates, whereas, amplification of different combinations of four and three genes were detected in fifteen and twenty isolates each. Amplification of any one gene was observed in ten isolates. Three isolates did not exhibited presence of any of the seven genes. Number of
*E. coli* isolates showing frequency of virulence marker genes are shown in Table 4.3.

Fig 4.3. Amplification of virulence associated genes in *Escherichia coli*

**Table 4.3.** No of isolates showing frequency of virulence marker genes in *E. coli*

<table>
<thead>
<tr>
<th>Level</th>
<th>iss</th>
<th>tsh</th>
<th>rip2</th>
<th>astA</th>
<th>papC</th>
<th>vat</th>
<th>cvi</th>
<th>stx1</th>
<th>stx2</th>
<th>stx 1 &amp; stx 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Udder</td>
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<td>5</td>
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<td>3</td>
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<tr>
<td>Market</td>
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<td>12</td>
<td>15</td>
<td>14</td>
<td>11</td>
<td>1</td>
<td>2</td>
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</table>
Several virulence factors have been associated with the virulence of *E. coli* like adhesins (F1- and P-fimbriae), iron acquisition systems (aerobactin and yersiniabactin), hemolysins (hemolysin E and temperature sensitive hemagglutinin), resistance to the bactericidal effects of serum and phagocytosis (outer membrane protein, iss protein, lipopolysaccharide, K/1)-capsule and colicin production) as well as toxins and cytotoxins (Ewers et al., 2003). Gram negative bacteria have several methods for transporting their proteins to the external environment (Henderson *et al.*, 2004; Kostakioti and Stathopoulos, 2004). *Tsh* and *vat* function as autotransporters, and these systems are capable of directing their own secretion across the outer membrane. *Tsh* coding for temperature sensitive hemmagglutinin has adhesive and proteolytic properties independently (Dozois *et al.*, 2000).

Large foodborne outbreaks have been linked to STEC, some of which are attributed to dairy products (Fremaux *et al.* 2006). Many factors, such as geographical location and sampling, isolation, and testing methods, make comparisons of different studies difficult (Xia *et al.* 2010). There is a paucity of data on contamination by STEC in raw milk and there is growing concern over the emergence of highly virulent non-O157 STEC serotypes that are globally distributed, several of which are associated with outbreaks and/or severe human illness, such as HUS and HC (Bettelheim 2007; Brooks *et al.* 2005). The non-O157 STEC serotypes recorded in this study are reported to be associated worldwide with disease in humans and represent a risk for the public health (DebRoy *et al.* 2011). For this, any microbiological control in dairy farms should not only be targeted to the search of O157:H7 serotype. Consumption of contaminated food has been considered as the main route of
STEC infections in humans (Meng et al. 2007). STEC strains have been isolated from a large variety of different foods including raw-milk cheeses (Caro and Garcia-Armesto 2007; Stephan et al. 2008; Solomakos et al. 2009).

PFGE of XbaI-digested DNA fragments isolated from the 74 E. coli isolates generated 54 unrelated XbaI-PFGE subtypes at 60% similarity (Figs. 4.4 to 4.10). The isolates were resolved in 14-22 bands ranging from 48.5 to 630kDa. Diverse E. coli strains were isolated from samples collected at different levels. The strains were very diverse. Strains isolated from samples collected at same level clustered together.

Fig 4.4. Dendrogram showing diversity of Escherichia coli strains isolated at udder level.
Fig 4.5. Dendrogram showing diversity of *Escherichia coli* strains isolated from milking utensils.

Fig 4.6. Dendrogram showing diversity of *Escherichia coli* strains isolated at dairy cooperative level.
Fig 4.7. Dendrogram showing diversity of *Escherichia coli* strains isolated from samples collected at receiving dock.

Fig 4.8. Dendrogram showing diversity of *Escherichia coli* strains isolated from market milk.
Fig 4.9. Dendrogram of the 74 *E. coli* strains based on PFGE patterns after digestion with enzyme *Xba*I.
Fig. 4.10. PFGE comparing restriction profiles for *Escherichia coli* isolated from milk samples. Lanes 1 and 12: isolates recovered from bovine milk samples; Lane 12: \(\lambda\) molecular weight markers (numbers on the left are kb weights).

Since the advent of molecular finger printing, there has been a great deal of effort directed towards developing molecular methods suitable for use in clinical and public health laboratories. Subtyping methods that discriminate beyond the level of serotype have found enormous application in epidemiological studies, outbreak investigations, and early detection of geographically dispersed foodborne disease outbreaks. One of the most widely applied methods of subtyping is pulsed-field gel electrophoresis (PFGE). PFGE with *XbaI* endonuclease is currently considered as being a highly discriminatory method for molecular typing of STEC. There is enormous genetic diversity within STEC (Nielsen *et al.*, 2006). For example, in
O157:H7, the diversity is largely due to insertions and deletions in O islands (Kudva et al., 2002; Shaikh and Tarr, 2003).

Comparison of the patterns that are obtained allows investigators to determine the relationships of isolates to each other. In a comparison of PFGE, MLST and repetitive-element PCR, PFGE was the most discriminatory, identifying 72 distinct profiles among 92 O157:H7 isolates, whereas repetitive-element PCR identified 14 groups and MLST distinguished 5 groups (Foley et al., 2004).

In this study, although the isolates belonged to the same serotype, they did not have identical PFGE profiles and the stx genotypes. Isolates of the same serotypes tended to cluster together, however, polymorphism among the isolates of the same serotype was also observed, according to different PFGE patterns. All other isolates had their own specific PFGE profiles, with similarity indexes ranging from 40% to 90%. In a study (Fremaux et al. 2006), PFGE of XbaI-digested DNA fragments isolated from the 118 STEC isolates generated 74 unrelated XbaI-PFGE subtypes. PFGE demonstrated a wide diversity of STEC strains isolated at different levels of collection. This diversity may be linked to the different factors such as location, handling, utensils used, and environmental contamination.

In food animals, antimicrobials are used for the control and treatment of bacterial associated infectious diseases and for growth promotion purposes as well. As a consequence of antimicrobial use in animals, there is potential of development of antimicrobial-resistant zoonotic foodborne bacterial pathogens and subsequent transmission to humans as food contaminants. Non-O157 strains isolated from humans and animals have developed antibiotic resistance
and many are resistant to multiple antimicrobials commonly used in human and veterinary medicine (Gonzalez and Blanco, 1989; Farina et al., 1996). In this study, the antibiotic susceptibility of the *E. coli* isolates was tested against nine commonly used antibiotics. The maximum antibiotic resistance was observed against amikacin and streptomycin (89.6%) followed by tetracyclines (88.1%), ampicillin and doxycycline (86.4%), carbenicillin (70.1%) (Table 4.4; Fig 4.11). All the isolates were sensitive to colistin. Enrofloxacin and cephaloridine showed moderate percent resistance.

Among the Shiga toxin producing strains, resistance against antibiotics was observed most commonly to cephaloridine (72%), tetracycline (36%), ampicillin and carbenicillin (32%), and less frequently to streptomycin and doxycycline (16%) and amikacin (12%) (Fig 4.12). The isolates were sensitive to enrofloxacin, colistin and ciprofloxacin. More than one-third of the strains showed reduced susceptibilities to different antimicrobial agents but was not completely resistant to any of the antibiotics.

Various studies have reported resistance of *E. coli* strains to antibiotics. In a study carried out in Estonia to estimate the distribution of udder pathogens and their antibiotic resistance, ampicillin, streptomycin and tetracycline resistance were observed in 24.3%, 15.6% and 13.5%, respectively among the *E. coli* isolates (Kalmus et al., 2011). While examining the hygienic and sanitary quality of pasteurized cow's milk, *Escherichia coli* was identified in 77.05% of the samples and the highest rates of resistance to antimicrobial agents were observed for ampicillin (19.2%), cephalothin (18.9%), and tetracycline (17.1%) (Zanella et al., 2010)
### Table 4.4. Per cent sensitivity/resistance of *E. coli* isolates.

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Antibiotic with concentration</th>
<th>(n=77) S No. (%)</th>
<th>R No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ampicillin (A) (10µg/disc)</td>
<td>12 15.6</td>
<td>65 86.4</td>
</tr>
<tr>
<td>2</td>
<td>Amikacin (AK) (10µg/disc)</td>
<td>8 10.4</td>
<td>69 89.6</td>
</tr>
<tr>
<td>3</td>
<td>Cephaloridine (30µg/disc)</td>
<td>52 67.5</td>
<td>25 32.5</td>
</tr>
<tr>
<td>4</td>
<td>Doxycycline (30µg/disc)</td>
<td>9 11.7</td>
<td>68 86.4</td>
</tr>
<tr>
<td>5</td>
<td>Colistin (10µg/disc)</td>
<td>77 100</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Carbenicillin (10µg/disc)</td>
<td>23 29.9</td>
<td>54 70.1</td>
</tr>
<tr>
<td>7</td>
<td>Enrofloxacin (EX) (10µg/disc)</td>
<td>62 80.5</td>
<td>15 19.5</td>
</tr>
<tr>
<td>8</td>
<td>Streptomycin (S) (10µg/disc)</td>
<td>8 10.4</td>
<td>69 89.6</td>
</tr>
<tr>
<td>9</td>
<td>Tetracycline (T) (10µg/disc)</td>
<td>9 11.7</td>
<td>68 88.3</td>
</tr>
</tbody>
</table>

**S**- Sensitive, **R**- Resistant
Fig 4.11. Antibiotic susceptibility testing of *Escherichia coli* isolates from milk samples.

Fig 4.12. Antibiotic susceptibility testing of *Escherichia coli* isolates from milk samples.

The antimicrobial susceptibility of Shiga toxin-producing *E. coli* isolates from various farms was compared and resistance to tetracycline was identified in 23% isolates and to sulphadimethoxine in 48% isolates (Cho *et al.*, 2007). Half of the STEC isolates were resistant to at least one antimicrobial agent. Multidrug resistant patterns were more common in non-O157 STEC than O157 STEC (Cho *et al.*, 2007). Resistance patterns in isolates from cattle sources may be affected by the use of antimicrobial agents on farms and by other environmental influences. Shiga toxin-producing *E. coli* isolated from samples of raw minced beef, mutton, pork, and chicken from the Jilin region of China were multi-resistant, exhibiting resistance to ampicillin, ciprofloxacin, tetracycline, sulfamethoxazole-trimethoprim, gentamycin, and streptomycin (Li *et al.*, 2011). An overall high percentage (>65%) of *E. coli* strains resistant to tetracycline, streptomycin, erythromycin, and sulfamethoxazole were isolated from diarrheal lambs, calves and goats (Medina *et al.*, 2011).
Moreover, a high level of resistance (approximately 30%) to ampicillin, chloramphenicol, trimethoprim, and trimethoprim-sulfamethoxazole was also detected. Continued surveillance of emerging antimicrobial resistance among E. coli strains isolated from foods of animal origin is needed to ensure public health.

Conclusions

The morbidity and mortality associated with several large outbreaks of gastrointestinal diseases caused by Shiga toxin-producing E. coli indicates the threat of these organisms to public health (Paton and Paton, 1998). These are commonly recovered from food animals and were found responsible for severe gastrointestinal and systemic diseases such as haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) leading to diarrhoea, especially among the infants in the developing countries (Paton and Paton 1998). Although, in India, reports are available on isolation, identification and characterization of STEC in human and animals (Wani et al. 2004; Chattopadhayay et al. 2001; Sanath Kumar et al. 2001; Khan et al. 2002; Bhat et al. 2008), there appears to be no information on association of STEC in raw milk.

In conclusion, our findings provide the information about the involvement of STEC in raw milk in India. In India, a large number of people in rural areas and a much larger segment of the population consume raw unpasteurized milk directly and indirectly. The fact that the serotypes reported from raw milk samples were common to that reported from animal populations suggests that the source of contamination of milk might be from animals. Once entered into the dairy food processing plants, the foodborne pathogens can lead
to persistence through formation of biofilms, subsequently contaminating the processed milk products and exposure of consumers to pathogenic bacteria (Oliver et al. 2005). Furthermore, recontamination of dairy products may occur with pathogens such as *E. coli* which survive and thrive in post-pasteurization processing. Under these circumstances, unpasteurized dairy products as well as dairy products that become re-contaminated after pasteurization with foodborne pathogens pose a risk to the consumer. The isolation and diversity of STEC serotypes found in this study have confirmed that raw milk can be an important reservoir of STEC. The serotypes carrying genes related to human diseases suggest a risk to the population. This should be taken into account in the control and prevention measures to minimize the risk of STEC foodborne infection in humans.