STUDIES ON ISOLATION AND CHARACTERIZATION OF *Escherichia coli* FROM SHEEP, GOATS AND THEIR HANDLERS

By

Irfan Ashraf

(J-14-MV-378)

Thesis submitted to Faculty of Postgraduate Studies
in partial fulfillment of the requirements
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Division of Veterinary Public Health and Epidemiology

Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu
Main Campus, Chatha, Jammu –180009

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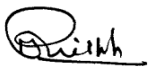
CERTIFICATE–I

This is to certify that the thesis entitled **"Studies on isolation and characterization of *Escherichia coli* from sheep, goats and their handlers''** submitted in partial fulfillment of the requirements for the degree of **Master of Veterinary Science** in **Veterinary Public Health and Epidemiology** to the Faculty of Post-Graduate Studies, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu is a record of bonafide research, carried out by **Mr. Irfan Ashraf**, Registration No**. J-14-MV-378** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that help and assistance received during the course of thesis investigation has been duly acknowledged.



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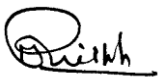

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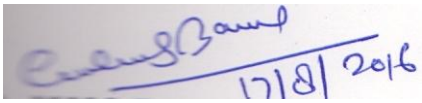

Dr. M.M.S. Zama
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Wisdom is the part and parcel of my religion, Knowledge my weapon, patience my dress, Faith my diet and Sincerity my Companion

Prophet Mohammad (PBUH)

Finally once again and above all I express my gratitude to Almighty Allah for giving me this life for His obedience and to serve mankind. It is His name which will last forever.

Needless to say, all omissions and errors are mine.

Irfan Ashraf

Dated:13-06-2016

Place: R.S. Pura, Jammu

ABSTRACT

Title of thesis	**:**	**Studies on Isolation and Characterization of *Escherichia coli* from sheep, goats and their handlers**
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Major Subject	**:**	Veterinary Public Health and Epidemiology
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A total of 170 samples comprising of 30 faecal samples and 30 rectal swabs of each sheep and goats, 25 stool samples and 25 hand swabs of their handlers were collected from in and around Jammu district and processed for the isolation of *E. coli.* Sixty five biochemically confirmed isolates including 27 of sheep, 26 of goats and 12 of their handlers were obtained. In vitro virulence characterization of isolates exhibited that 55.5% of isolates of sheep and 50% isolates of goats took colour of Congo red dye, similarly 25.9% and 34.6% isolates of sheep and goats respectively were haemolytic on blood agar, but none of human isolate was found pathogenic on both of the in vitro pathogenicity assays. Target specific primers of mPCR for *stx1*, *stx2*, *eaeA* and *hlyA* detected STEC in 28 isolates, with prevalence rate of 55.5%, 50%, and 0% in sheep, goats and their nomadic handlers respectively. All the isolates of handlers were negative for all genes and none of the sheep and goat isolate was found positive for *eaeA* gene. The antibiogram screening showed differential susceptibility pattern among *E. coli* isolates. Norfloxacin was found most potent drug with 84.6% sensitivity followed by Chloramphenicol (80%), Co-trimoxazole (75.3%) and Nalidixic acid (70.7%). However 60% of *E. coli* isolates were resistant to Ampicillin and Amikacin.

Keywords: *Escherichia coli*, STEC, In vitro pathogenicity assays, Handlers, mPCR, Antibiogram.

Signature of Major Advisor **Signature of Student**

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ABBREVIATIONS AND SYMBOLS USED

@	At the rate of
A/E	Attaching and effacing
A.H	Animal Handler
ARG	Antibiotic Resistance Genes
bp	Base pairs
°C	Degree Centigrade
CTSMAC	Cefixime Tellurite Sorbitol MacConkey agar
DAEC	Diffusely adherent *E. coli*
DAE	Diarrheagenic *E. coli*
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
e.g.	*exampli gratia* (for example)
et.al.	*et alii*/ *Alia* (and other people)
etc.	*et cetera* (and other things)
eaeA	*E. coli* attaching and effacing gene
E. coli	*Escherichia coli*
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EMB	Eosin methylene blue
ETEC	Enterotoxigenic *E. coli*
EPEC	Enteropathogenic *E. coli*
F	Faecal
Fig.	Figure
F.V.Sc	Faculty of Veterinary Sciences
g	Gram
H	Flagellar antigen
HC	Haemorrhagic colitis
HCl	Hydrochloric acid
hylA	Enterohaemolysin gene

HUS	Heamolytic uraemic syndrome
i.e.	That is
IMViC	Indole, Methyl Red, Voges Proskauer and Citrate
Ibs	Pounds
µg	Microgram
µl	Microlitre
$MgCl_2$	Magnesium Chloride
MHA	Mueller Hinton agar
min.	Minute
ml	Mililitre
MLA	Mac Conkey Lactose agar
mm	Millimeter
mM	Millimoles
MR	Methyl red
mPCR	Multiplex polymerase chain reaction
MTCC	Microbial Type Culture Collection
n	Number of samples
No.	Number
NSS	Normal saline solution
NTEC	Necrotoxigenic *E. coli*
O	Somatic antigen
%	Percent
PBS	Phosphorous buffer saline
PCR	Polymerase chain reaction
pH	Hydrogen ion concentration
R	Rough
spp.	Species
STEC	Shiga toxin- producing *Escherichia coli*.
Stx	Shiga toxin
stx	Shiga toxin gene
Taq	*Thermus aquaticus*

TBA	Tris borate EDTA
TSI	Triple sugar iron
VP	Voges Proskauer
Vt	Verotoxin
VTEC	Verotoxin- producing *Escherichia coli*
UV	Ultra violet
U	Unit
UT	Untypeable
WHO	World Health Organization

CHAPTER – I

INTRODUCTION

CHAPTER-I

INTRODUCTION

Escherichia coli has been the focus of immense International Research after its recognition as a major cause of large scale epidemics of gastrointestinal illness in animals and man (Deshmukh and Karpe, 2006). Livestock (Ruminants) are known to harbour not only strains pathogenic to animals but also strains which cause asymptomatic infections in animals and which can pass through the food chain to cause clinical disease in man (Arshad *et al*., 2006). Since, the sheep and goats have been the widely accepted source of meat, the association of *E. coli* in sheep and goats has a significance for human infection. Sheep and goats meat can transmit infections and diseases either through handling during preparational procedures or as a result of ingestion by the consumer.

Domestic and wild animals are sources of *E. coli* but ruminants primarily sheep, goats and cattle have been identified as major reservoirs and source for human infection (Kiranmayi *et al*., 2010; Rahimi *et al*., 2012). Many serotypes of EHEC associated with human infection like O91, O157, and O146 have been isolated from sheep (Urdahl *et al.,*2003; Ramachandran *et al.,*2001). O157 has also been isolated from goats (Pritchard *et al.,*2000). *E. coli* is transmitted by ingestion of contaminated food and water, direct contact with animals, faeces and contaminated soil and directly from one person to another. Consumption of raw milk has been found to be of high risk for *E. coli* O157:H7 infections (CDC 2008; Denny *et al.,* 2008). Fermented sausage containing sheep meat was reported as a source of an STEC O103:H25 outbreak in Norway (Sekse *et al*., 2009), while fermented sausage containing beef was the cause of an STEC O26:H11 outbreak in Denmark (Ethelberg *et al.,* 2009). The dispersion of untreated manure in the environment can cause the contamination of different items which can act as secondary source for human infection (Mc Dowell and Sheridan, 2001).

E. coli bacteria was discovered from the human colon in 1885 by German bacteriologist Theodor Escherich (Feng *et.al*.,2002). Dr. Escherich also showed that certain strains of the bacterium are responsible for gastroenteritis and infant diarrhoea, an important public health discovery. Although *E. coli* bacteria was initially called

Bacterium coli, the name was later changed to *Escherichia coli* to honour its discoverer (Feng *et.al*.,2002). *E. coli* is a non-fastidious organism and easy to grow and isolate in laboratory. The species *E. coli* comprises Gram – negative, motile, non-sporulating, rod shaped, facultative anaerobic bacteria which is 0.5µm in diameter and 1.0–3.0µm in length belonging to the family *enterobacteriaceae*, produces rose pink colonies on MacConky agar and metallic sheen on EMB agar.

E. coli is oxidase negative, glucose, lactose, sucrose fermenting with an optimum growth pH of 6.0 -7.0 and temperature of 37^0C. Some diarrheagenic *E. coli* strains have the ability to tolerate exposure to pH 2.0. Eosin Methylene Blue medium is suitable for isolation of *E. coli* from faeces and foods because of the ability to produce distinctive colonies having greenish metallic sheen and not produced by any other member of *Enterobacteriaceae.*

E. coli is a genetically and phenotypically diverse species whose strains are identified on the basis of 'O', 'H' and sometimes 'K' antigens. Over 700 serovars of *E. coli* are recognized on the basis of O, H, and K antigens. The serovars of *E. coli* isolate is based on 'O' antigen determined by the polysaccharide portion of cell wall lipopolysaccharide (LPS) and the 'H' antigen due to flagellar protein.

Escherichia coli is one of the common microbial flora of gastrointestinal tract. The lower intestine conditions are generally more favourable for *E. coli* of animals, human beings and birds but some may become pathogenic to them (Jawetz *et al*., 1984; Levine, 1987). *Escherichia coli* lives a fecal-oral lifestyle and can comprise up to 1% of the gastrointestinal population of mammals. Although most isolates of *E. coli* are nonpathogenic but they are considered as indicator of faecal contamination, particularly in foods of animal origin that may lead to spoilage of food and pathogenic one may cause food borne outbreaks (Donnenberg and Whittam, 2001). One of the most notable features of *E. coli* is broad diversity of disease-causing genotypes. The diseases can encompass different symptoms and gastrointestinal tract pathologies, but there are also diseases at extra intestinal sites. These different genotypes and their disease-causing abilities lead to categories of *E. coli* often referred to as Pathotypes.

The enteric pathogenic *E. coli* are classified into six groups on the basis of their virulence properties such as enterotoxigenic *Escherichia coli* (ETEC) causative agent of diarrhoea in humans, sheep, goats, cattle, dogs, pigs and horses, (Bern *et*

al.,1992). ETEC produce heat-labile (LT) and/or heat-stable (STa and STb) toxins that are frequent cause of diarrhoea in both humans and animals. ETEC cause watery diarrhoea that can be mild in nature or in some instances can be a severe, cholera-like illness where rapid dehydration can be life-threatening. In endemic areas of ETEC-mediated diarrhoea, infants and children under the age of five are the most commonly affected. ETEC exposure in endemic areas is one of the most common causes of traveler's diarrhea. The heat-labile enterotoxin (LT) of ETEC shares structural and functional similarity to the *Vibrio cholera* toxin (Spangler, 1992). Enteropathogenic *Escherichia coli* (EPEC) causative agent of diarrhoea in humans, rabbits, dogs, cats and horses, the pathogenesis of EPEC is in some way unique for enteric bacterial pathogens since it is essentially non invasive and produces no toxins. The attachment of EPEC to the epithelial cell, described as localized adherence, results in attaching and effacing lesion (A/E) (Celli *et al.*, 2000). EPEC deliver its bacterial effector proteins like EspA and EspB into the host cell to alter the cytoskeleton (Knutton *et al*., 1998). However, the most fascinating aspect of EPEC pathogenesis is that it inserts its own receptor into the host cell through the type III secretion system. Rather than searching for a receptor it provides its own receptor and uses it when needed. Thus, EPEC is able to insert the Tir receptor into the host cell membrane where it serves as the receptor for the bacterial protein intimin after it is phosphorylated on tyrosine by the host cell (Deibel *et al*., 1998). There is a specific pathogenicity island, termed the "locus of enterocyte effacement" (LEE), that encodes the genes responsible for the A/E lesion. The LEE encodes a type III secretion system that provides the intimate adhesion (intimin) its receptor (which is injected into and then presented on the surface of the host cell), and the injected proteins responsible for changes in host cell signalling mechanisms, including actin pedestal formation (Jerse, 1990).

Enteroinvasive *Escherichia coli* (EIEC) found only in humans, the pathogenesis of disease caused by EIEC like Shigella involves cellular invasion and spread, and requires specific chromosomal and plasmid borne virulence genes (Nataro & Kaper, 1998). EIEC isolates are nonmotile and 70% are nonlactose fermenters (Silva et al., 1980). Enterohaemorrhagic *Escherichia coli* (EHEC) found in human, goats and cattle. Enteroaggregative *Escherichia coli* (EAEC) found only in human, EAEC express a fimbrial adhesin called "aggregative adherence fimbriae" ("AAF").

EAEC isolates often produce a mucinase called "Pic" whose gene has the ability to express from its nonencoding DNA strand a smaller gene that encodes an enterotoxin, and diffusely adherent *E. coli* (Tamaki *et al*., 2005). The pathogenesis of DAEC diarrhoea is not as yet elucidated, but several virulence-related characteristics have been identified (Nataro & Kaper, 1998; Servin, 2005). Most DAEC strains express a surface fimbria designated F1845 that may be encoded either by the chromosome or a plasmid.

Among them enterohaemorrhagic *E. coli* is much of public health significance which is a subset of serovars of shiga toxin producing *E. coli* (STEC) and is characterised by the production of potent cytotoxins that inhibit protein synthesis within eukaryotic cells. These toxins are either termed as verotoxins (Vt), because of their activity on vero cells, or Shiga toxin (Stx), because of their similarity with the toxin produced by *Shigella dysenteriae*. Therefore, these strains are either termed as Shiga toxin producing *E. coli* (STEC) or Vero toxin producing E. coli (VTEC).

Pathogenicity of *E. coli* strains are due to the presence of one or more virulence factors including invasiveness factors like invasins, heat labile, heat stable enterotoxins, verotoxins and colonization factors or adhesins that are accountable for a variety of intestinal and extraintestinal diseases including diarrhoea, acute inflammation, hemorrhagic colitis, urinary tract infections, septicemia, and neonatal meningitis (Kaper *et al.,* 2004). *E. coli* producing Stx1 and/or Stx2, may cause complications in immunocompromised hosts and children such as haemolytic uraemic syndrome (HUS) an important cause of acute renal failure in children and morbidity and mortality in adults and haemorrhagic colitis (HC) (Isobe *et al.,* 2012; Myrnas and Castegren, 2013; Mijatovic *et al.,* 2014; Peron *et.al.,*2016). Most cases of these diseases are caused by the ingestion of foods and drinks contaminated with faeces, especially ground beef, undercooked hamburgers, salami, raw milk and home made raw milk cheese (Nataro and Kaper, 1998).

Local intestinal effects cause the development of bloody diarrhoea as the toxin internalizes the cells of the gut where it blocks cellular protein synthesis and may lead to apoptosis. HUS results from microvascular disease when the toxins enter the blood stream and bind to receptors on endothelial cells abundant in kidneys and brain (Nataro and Kaper, 1998).The *E. coli* sometimes cause severe lethal infections such as

meningitis, endocarditis, urinary tract infection, septicaemia, epidemic diarrhoea among adults and children (Daini *et al.,* 2005).

With the change in food habits and farming practices, a number of diseases have emerged across the world. During the past two decades, several outbreaks of gastrointestinal illness have occurred by food borne pathogenic *E. coli* especially enterohaemorrhagic *E. coli* serovars because of their low infective dose is reported as low as 50 organisms (Armstrong *et al.,* 1996; Tilden *et al*., 1996; Isobe *et al.,* 2012; Jourdan-da Silva *et al.*, 2012).

The presence of *E. coli* in animal faeces provides the potential for these organisms to enter the food chain via faecal contamination of milk, contamination of meat with intestinal contents during slaughter or contamination of fruit and vegetables by contact with contaminated manure. Contaminated water, used for irrigating or for washing vegetables, can also be source of infection for humans or animals. Infection can also arise from animal to human contact, both direct and indirect, human-to-human contact (Yim *et. al.*, 2010).

Domestic food animals, particularly sheep, goats and cattle have been incriminated to be the major source of STEC (Caprioli *et al*., 2005). Sheep, goat and cattle lack shiga toxin receptors (Globotriaosylceramide) and therefore can be asymptomatic carrier of bacterium (Pruimboom-Brees *et al*., 2000). Shedding of STEC of serovars other than O157:H7 is usually observed in sheep and goats (Beutin *et al*., 1993).

It continues to be the challenge for the people residing in the rural areas where the peoples live in close proximity of sheep, goats & cattle have least knowledge about the pathogenecity of the bacteria. As it continually adopts to different conditions and environments, the organism can remain viable for months together in the soil. It can survive and replicate in both stagnating and free-flowing water. Unlike many other bacteria, pathogenic *E. coli* can survive and replicate in aerobic as well as anaerobic environments. It can respond and adapt to differences in environmental changes such as chemicals, pH, and temperature in remarkable ways. Worldwide, virulent strains of *E. coli* are emerging, as they have the potential to cause food borne illness.

In Jammu & Kashmir Scientists and Researchers have earlier isolated the *Escherichia coli* from different sources like from foods of animal origin, milk and milk products, lambs and calves with the disease causing significance of *Escherichia coli* as identified by Rashid *et.al*.(2013), Sheikh *et.al*.(2013) and Wani *et.al*.(2013) respectively. The present study however was conducted for the isolation of *Escherichia coli* from sheep, goats and their handlers of nomadic community considering the contribution of this community toward meat industry as these people rear the sheep and goats in large numbers and thus these animals built the base for meat industry in Jammu and Kashmir state.

Livestock farmers rearing sheep and goats in general and nomads in particular lack the knowledge of transmission of diseases from animals and are unaware of dynamics of zoonotic diseases and the zoonotic potential of diseases, thus are at the high risk of contracting such diseases.

At present, antimicrobial therapy is one of the primary control measures for reducing morbidity and mortality in animals and humans infected with *E. coli* infection. However prescription of antimicrobials precedes the antimicrobial sensitivity test and the indiscriminate use of these drugs has therefore lead to the development of resistance. It is therefore necessary to monitor the drug resistance pattern of *E. coli* both for effective treatment and also to prevent the emergence of the drug resistance.

Keeping in view the above facts the present study was carried out with the following objectives:

1) Isolation and characterization of *Escherichia coli* from sheep, goats and their nomadic handlers.
2) In vitro pathogenicity of selected *Escherichia coli* isolates.

CHAPTER – II

REVIEW OF LITERATURE

CHAPTER-II

REVIEW OF LITERATURE

2.1 Historical background

Escherichia coli (*E. coli*) originally called *"Bacterium coli"* was first isolated from the stool of a 2-3 days old new-born baby and subsequently from young calves in 1885 by Theodore Escherich (Buxton and Fraser, 1977; Sousa, 2006).The name of bacteria was later changed to honour its discoverer (Feng *et.al*., 2002).

2.2 The organism and its characteristics

2.2.1 Growth and inactivation

Escherichia coli is a facultative anaerobe that can grow from 7ºC to 50ºC with an optimum temperature of 37ºC, although there have been reports of some ETEC strains growing at temperatures as low as 4ºC (Adams and Moss 2008; Xia, 2010). A near neutral pH is optimal for its growth but growth is possible down to pH 4.4 under otherwise optimal conditions. The minimum water activity for growth is 0.95 (Adams and Moss, 2008).

2.2.2 Biochemical properties

E. coli can be differentiated from other members of the *Enterobacteriaceae* on the basis of a number of sugar-fermentation and other biochemical tests. Classically an important group of tests used for this purpose are known by the acronym IMViC. These tested for the ability to produce: indole from tryptophan (I); sufficient acid to reduce the medium pH below 4.4, the break point of the indicator methyl red (M); acetoin (acetylmethyl carbinol) (V); and the ability to utilise citrate (C) (Adams and Moss, 2008). Despite *E. coli* can be identified with a variety of biochemical reactions, the indole test remains the most useful method to differentiate lack of production of β-glucuronidase. Sorbitol non fermenting strains of *E. coli* O157:H7 have been associated with colitis and haemolytic uremic syndrome (HUS) (Besser *et al*., 1999).

2.2.3 Acid and salt tolerance

Escherichia coli is an acid resistant food borne pathogen that survives in the acidic environment of stomach and colonise the gastrointestinal tract (Price *et al*., 2004). Furthermore, it also increases the survival of *E. coli* particularly STEC

O157:H7 in acidic foods, enabling survival for extended periods, especially at refrigeration temperature (Meng *et al*., 2007). Hence, contaminated cultured and fermented foods such as yoghurt and cheese have also been implicated in sporadic cases and the disease outbreaks (Baylis, 2009; Farrokh *et al*., 2012).

3 Reservoir hosts

Domestic and wild animals are sources of *E. coli* but ruminants primarily sheep, goats and cattle have been identified as major reservoirs and source for human infection (Kiranmayi *et al*., 2010; Rahimi *et al*., 2012; Rehman *et.al.,* 2013). Many serovars of EHEC associated with human infection like O91, O157, and O146 have been isolated from sheep (Urdhal *et al.*,2003; Ramachandran *et al.*,2001). O157 has also been isolated from goats (Pritchard *et al.*,2000).

4 Mode of transmission

E. coli is transmitted by ingestion of contaminated food and water, direct contact with animals, faeces, contaminated soil and cross contamination directly from one person to another. Consumption of raw milk has been found to be of high risk for *E. coli* O157:H7 infections (CDC 2008; Denny *et al.,* 2008). Fermented sausage containing sheep meat was reported as a source of an STEC O103:H25 outbreak in Norway, while fermented sausage containing beef was the cause of an STEC O26:H11 outbreak in Denmark (Sekse *et al*., 2009; Ethelberg *et al.,* 2009). VTEC O157 infection was associated with ready-to-eat foods, such as lemon-and-coriander chicken wraps (Whittaker *et al*.,2009). The dispersion of untreated manure in the environment can cause the contamination of different items which could act as secondary source for human infection (Mc Dowell and Sheridan, 2001).

5 Prevalence of *E. coli* in sheep and goats.

5.1. Work done abroad

Beutin *et al.* (1993) screened 720 faecal samples from healthy domestic animals representing seven different species (sheep, goats, cattle, pigs, chickens, dogs, and cats) for verotoxin producing *Escherichia coli* (VTEC). VTEC were isolated from 208 (28.9%) animals, most frequently from sheep (66.6% VTEC carriers), goats (56.1%), and cattle (21.1%). VTEC were isolated less frequently from pigs (7.5%),

cats (13.8%) and dogs (4.8%) but were not found in chickens (<0.7%). Forty-one different O: H serovars and 23 untypeable were isolated.

Heuvelink *et al.* (1998) collected and examined a total of 1038 faecal samples from different animals in Dutch Western Europe, for isolation and characterization of verocytotoxin producing *E. coli. E. coli* O157 strains isolated were 2 (3.8%) of 52 ewes, and 2 (4.1%) of 49 lambs, 57 (10.6%) of 540 adult cattle and 2 (0.5%) of 397 veal calves. Immunomagnetic separation with O157-specific-antibody-coated beads appeared to be significantly more sensitive than conventional plating for detection of the organism in faeces.

Fuente *et al.* (2002) examined 1013 faecal samples from healthy and diarrhoeic sheep and goats for the prevalence of attaching and effacing strains of *Escherichia coli,* samples were plated on MacConkey agar and incubated for 18 hours at 37 C. Four colonies with the typical appearance of *E. coli* from each sample were randomly chosen and tested by biochemical tests to identify *E. coli* isolates. Detection of *eae* and *espB* sequences was performed by use of colony blot hybridization. Results revealed that prevalence of *eae* is higher in healthy sheep and goats than that of diarrhoeic animals.

Blanco *et al.* (2003) examined faecal swabs that were obtained from 1,300 healthy lambs in 93 flocks in Spain for Shiga toxin-producing *Escherichia coli* (STEC). STEC O157:H7 strains were isolated from 5 (0.4%) animals in 4 flocks, and non-O157 STEC strains were isolated from 462 (36%) lambs in 63 flocks. A total of 384 ovine STEC strains were characterized. PCR showed that 213 (55%) strains carried the *stx1* gene, 10 (3%) possessed the *stx2* gene, and 161 (42%) carried both the *stx1* and the *stx2* genes. Enterohemolysin (*ehxA*) and intimin (*eae*) virulence genes were detected in 106 (28%) and 23 (6%) of the STEC strains, respectively. The STEC strains belonged to 35 O serogroups and 64 O:H serotypes.

Cortes *et al*. (2005) screened faecal samples from 222 healthy dairy goats on 12 farms in Spain, as well as bulk tank milk samples of these farms, for the presence of verotoxin-producing *Escherichia coli* (VTEC) and enteropathogenic *E. coli* (EPEC). VTEC and EPEC were isolated in 47.7% and 7.7% of the animals, respectively. VTEC were isolated more frequently from adults and replacement animals than from goat kids. In contrast, EPEC were detected more frequently from

goat kids than from replacement animals and adults. The most frequent serovars among the 106 VTEC strains isolated from goats were O5:H, O76:H19, O126:H8, O146:H21, ONT:H and ONT:H21. None of the VTEC strain was *eae* positive.

Novotna *et al*. (2005) examined 93 rectal swabs of lambs and young goats from extensively and intensively managed herds in Jordan for Shiga toxin-producing *Escherichia coli* (STEC). The STEC O157:H7 strains were demonstrated in 8 of 32 diarrheic lambs in one sheep herd with intensive milk production. In the remaining three herds, serogroups O128, O78, O15 and serovar O128:K85 of STEC strains were the most frequent findings. The presence of *stx2, ehlyA* and *eaeA* genes in all STEC O157:H7 isolates was confirmed by PCR. In two untypable STEC isolates, *stx2* and *ehlyA* genes were detected. In other STEC non-O157 isolates, only *stx1* and *ehlyA* genes were found. All STEC O157:H7 isolates were resistant against sulphonamides and chloramphenicol, five were also resistant against ampicillin and streptomycin, one against co-trimoxazole.

Lenahan *et al*. (2007) examined a total of 1600 faecal, carcass swabs and fleece samples from lambs for the presence of *E. coli* O157:H7 in Ireland. *Escherichia coli* O157:H7 was isolated from 5.75% (23⁄400) of fleece samples, 1.5% (6⁄400) of pre- and 1% (4⁄400) of post chill carcass swabs but was not isolated in faeces (0⁄400). Polymerase chain reaction analysis showed that both the *vt1* and *vt2* genes associated with clinical illness were carried by 05 (five) of the *E. coli* O157:H7 isolates, while 24 of the remaining isolates carried the *vt2* gene only.

Abdullah *et al.* (2010) analysed a total of 150 faecal samples of goats of which 90 from clinical cases from goats with the symptom of diarrhoea and 60 from apparently healthy goats in different areas of Bangladesh. The collected samples were primarily cultured onto Nutrient broth (NB) and then pure culture was performed on Eosin Methylene Blue (EMB) agar, Salmonella-Shigella (SS) agar, MacConkey (MC) agar, Brilliant Green (BGA) agar and Blood agar (BA). Among the samples examined, 65(72.22%) and 25(41.67%) were found to be positive for *E. coli* in clinical and healthy cases respectively.

Ahmed *et al*. (2010) examined 127 faecal samples from diarrhoeic lambs in Nigeria to determine the prevalence of pathogenic bacteria causing diarrhoea and found 36.84 % samples were positive for *E. coli.*

Mersha *et al.* (2010) studied total of 711 samples comprising of faecal samples (172), skin swabs (172), carcass swabs (344) from sheep and goats and 23 water samples in Ethiopia. Samples were enriched in tryptic soya broth and streaked on Sorbitol MacConkey agar colourless colonies of *E. coli* (O157:H7) were further confirmed using biochemical tests. Out of the total of 711 different samples examined, 53 (7.5%) were found positive for *E. coli* O157:H7, of the 53 isolates 16 (30.2%) were from goats, 36 (68.0%) were from sheep and 1 (1.8%) was from a water sample. *Escherichia coli* O157:H7 was isolated in goats from faecal samples (3.3%), skin swabs (10%) and carcass swabs before washing (5%) and after washing (8.3%). In sheep, 5.4% of the faecal samples, 8.0% skin swabs, 9.8% carcass swabs before washing and 8.9% after washing were *E. coli* O157:H7.

Purkayastha *et al.* (2010) examined 90 faecal samples, 36 from diarrhoeic and 54 from apparently healthy sheep collected from different areas in and around Bangladesh Agricultural University (BAU) campus, Bangladesh and 15 (41.67%) and 21 (38.38%) were found to be positive for *E. coli*. The cultural characterization of all positive sheep *E. coli* revealed greenish black colony with metallic sheen in Eosin Methylene Blue agar, bright pink colour smooth transparent colony in MacConkey agar, green colour colony in Brilliant green agar, slight pinkish smooth colony in Salmonella-Shigella agar and colourless colony with hemolysis in blood agar.

Zaki *et al.* (2010) analysed 130 faecal samples 100 from diarrhoeic kids and 30 from healthy kids, bacteriological examination of the faecal samples revealed the presence of *E. coli* (58%), *Salmonella*, (27%), and *Shigella*, as the main causative agents of diarrhoea.

Aklilu *et al*. (2013) assessed the prevalence of *E. coli* in diarrhoeal lambs in Ethiopia they collected and processed samples from diarrhoeal lambs of different age group, result revealed 84 % of cases were positive for *E. coli*.

EL-Alfy *et al.* (2013) screened a total of 384 samples from human, animal and environmental sources in Ismailia, Egypt. *E. coli* isolates (n = 283) were identified by conventional microbiology culture and were phenotypically characterized using biochemical and motility tests. Multiplex PCR (mPCR) was applied for the detection of virulence genes (*stx1, stx2, eaeA* and EHEC *hlyA*). From the overall number of *E. coli* isolates, 31.4% (89/283) were isolated from stools of humans with diarrhoea,

17.3% (49/283) from faecal samples of sheep, cattle and chicken with diarrhoea, 16.6% (47/283) from urine of humans with urinary tract infection.

Osman *et al.* (2013) screened faecal samples of diarrhoeic sheep, goats and calves, enriched samples were plated on sorbitol MacConkey agar and blood agar, and *E. coli* strains were identified by biochemical tests. A total of 132 *E. coli* isolates were recovered from faeces of diarrhoeic animals (goats n = 36 sheep n = 12, and calves n = 84, isolates in a prevalence rate of 27.3% and 9.1%, 63.6%, respectively). Congo red (CR) assay was used as a phenotypic marker for the invasive and non-invasive *E. coli*, all of the 132 tested serovars for the CR binding affinities and for haemolytic activity were 100% positive. The binding activity of the CR dye was found to be variable in their affinity according to their serovars.

Shahzad *et al*. (2013) analysed faecal samples of sheep, goat, cattle, buffalo, chicken, and faeces of monkey as well as aseptically collected samples of milk and beef in Pakistan. Sorbitol-non fermenting (SNF) biotype was detected in faeces of sheep (52 %), goat (56 %), buffalo (92 %) and cattle (84 %). However, *E. coli* (SNF) was not detected in droppings of rural chickens, faeces of monkeys and fresh aseptically collected milk and beef samples.

Suheyla *et al*. (2013) isolated a total of 107 *E. coli* strains from animals of 43 farms. Specific virulence genes were determined by multiplex and uniplex polymerase chain reaction 39 (36.4%) enterovirulent *E. coli* strains were identified and of this 19 (48.7%) were shiga toxigenic, 12 (30.8%) enterotoxigenic and 8 (20.5%) enteropathogenic.

Turkyilmaz *et al.* (2013) investigated 43 farms in Turkey and isolated 107 *E. coli* isolates from 79 lambs and 28 goat kids, faecal samples inoculated onto McConkey agar. Isolates were identified biochemically and confirmed with the Vitek II system. Multiplex PCR was performed for the detection of *stx1*, *stx2* and *eae* genes; uniplex PCR was used for detection of *lt, st, bfp, ial* and *aafII* genes.

Abdul-Ratha and Nadhom (2014) collected and analysed 284 samples (182 samples of mastitic milk and 102 from sheep, goats, cows and calves suffering from diarrhea) from Salahaddin Governorate, College of Veterinary Medicine-University of Baghdad, College of Agriculture- University of Baghdad, Radhwanya zoon, Dora

zoon and Abu-Ghraib zoon. Samples were cultured on MacConkey agar and Eosin Methylene Blue agar, after purification of cultured bacteria, biochemical tests showed that 91 out of 102 faecal samples were positive for *E. coli* and 54 out of 182 milk samples were positive for *E. coli.*

Adefarakan *et al*. (2014) processed 400 rectal swabs from goats and rams at three different locations in Ile-Ife (Nigeria) for isolation and identification of *E. coli*. The amplification of virulence (*eae* and stx_1) and resistance (tetM, tetK and bla (480 CTX bp) genes in isolates was carried out by polymerase chain reaction. 166 *E. coli* isolates from rams (96) and goats (70) were recovered. The susceptibility of the isolates to antibiotics was carried out by disk diffusion technique. Susceptibility of isolates to antibiotics varied greatly.

Ghoneim *et al.* (2014) screened a total of 461 faecal samples collected from sheep (192) goats (76) and human (93) buffalo (40), dairy cattle (86), feedlot (beef) cattle (67), in Egypt using Enzyme-linked Immunosorbent Assay (ELISA) to detect shiga toxins (stx) and then positive samples were cultured onto selective chromogenic media for STEC, when positive isolates were subjected to biochemical tests to be proved as *E. coli* and confirmed molecularly by the polymerase chain reaction (PCR) for the presence of *stx*-encoding genes The occurrence of STEC in both sheep and goat were 2.6% and 5.4% in human samples.

Nasr *et al.* (2014) examined a total of 1200 faecal samples of lambs in Egypt, bacteriological examination of faecal samples revealed that 190 (29 %) samples were positive for pathogenic bacterial culture and 460 (71 %) samples were negative. The isolated bacteria were *E. coli* of pathogenic form from 65 cases (34.20 %) which was the most predominant bacterial isolate. Other bacteria isolated were *salmonella* from 10 cases (5.26 %), *Clostridia* isolated from 15 cases (7.89 %), *Proteus* species isolated from 25 cases (13.10 %), *Shigella* isolated from 20 cases (10.52 %), *Klebsiella* isolated from 15 cases (7.89 %) and mixed infection was reported in 40 cases (21 %). Serotyping of *E coli* revealed that O55/K50 (B5) (2 isolates), while O78/K80 (B-), O125/K70 (B15), O101/K99 and O22/K11 (L) and each of them (1 isolates), while as the untypable isolates were four.

Yousif and Fadhel al-Taii (2014) collected 150 faecal samples (50 stool samples from diarrheal children, 50 from sheep and 50 from cows) for isolation of *E.*

coli (O157: H7). All samples were cultured on MacConkey and Eosin Methylene blue agar. Each *E. coli* growth was confirmed by Gram stain and biochemical tests. All isolated *E. coli* were sub cultured on Sorbitol MacConkey agar plus cifixime potassium tellurite (SMA-CT). Latex agglutination test was used for all isolates of *E. coli* O157: H7 to confirm the serotype. The results showed that *E. coli* were isolated in 40 out of 50 diarrheal children stool samples, where only 2(4%) from these isolates were confirmed as *E. coli* O157:H7. The number of *E. coli* isolates from sheep and cows samples were 45 out of 50 and 48 out of 50 respectively , where only 10 (20%) and 12 (24%) isolates were *E. coli* O157:H7 respectively.

Ali (2015) screened 50 faecal samples of sheep suffering from diarrhea in Diwanyia province and 32 isolates (64%) of *Escherichia coli* were detected and diagnosed by routine laboratory tests. The results showed only 13 positive isolates out of 32 in PCR technique proved to carry haemolysin toxin gene (40.62%), which were characterized as Enterohemorrhagic *Escherichia coli*.

Ferreira et al. (2015) collected and analysed rectal swabs of healthy sheep for *E. coli*. Of the 115 *E. coli* isolates obtained, 78.3% (90/115) were characterized as STEC, of which 52.2% (47/90) carried stx_1 gene, 33.3% (30/90) stx_2 and 14.5% (13/90) carried both genes. In search of virulence factors, 47.7% and 32.2% of the isolates carried the genes as *saa* and *cnf1*.

Oluyege and Famurewa (2015) investigated total of 722 faecal samples from goats (49), cattle (361) and poultry (19) for the presence of *E. coli.* Samples were streaked on EMB and SMAC agar and characteristic colonies of *E. coli* were confirmed by biochemical tests and overall 316 isolates of *E. coli* were recovered from 10.1% goats, 62.3% cattle, 19.6% local chicken, 4.1% broiler, 2.9% layers, and 0.9% cockerels. Virulence gene stx_1 was detected only in non-O157 strain from goat and local chickens.

5.2. Work done in India

Wani *et al*. (2005) isolated a total of 220 *E. coli* strains belonging to 60 different‘O’ serogroups from 206 local non migratory and 69 migratory goats. Samples were inoculated on MacConkey and EMB agar and colonies showing metallic sheen on EMB were subjected to biochemical tests for identification and

confirmation of *E. coli*. Twenty-eight strains (16.47%) (belonging to 13 different serogroups) from local goats carried *stx*$_1$ gene alone or in combination with *stx*$_2$ gene, while as only one strain (2%) from migratory goats possessed *stx*$_2$ gene alone.

Bhat *et al.* (2007) screened 101 diarrhoeic and 135 healthy lambs and isolated total of 120 and 164 *E. coli* isolates respectively. All the 284 isolates were screened for presence of *stx*$_1$, *stx*$_2$, *eae* and *ehxA* genes using multiplex polymerase chain reaction (m-PCR). Forty four (36.67%) isolates from lambs with diarrhoea carried at least one virulence gene. Twenty one (17.5%) and 15 (12.5%) isolates from diarrhoeic lambs were STEC and EPEC, respectively. Thirty (18.3%) isolates from healthy lambs possessed at least one virulence gene. Fourteen (8.53%) and 16 (9.75%) isolates from healthy lambs were detected as STEC and EPEC, respectively.

Wani *et al.* (2008) screened 338 lambs with (n = 230) and without (n = 108) diarrhoea and recovered 75 (seventy five) *Escherichia coli* isolates with at least one targeted virulence gene. Isolates belonged to 36 different serogroups. Shiga toxin-producing *E. coli* (STEC) was isolated from 9.6% of lambs with and 24.1% of lambs without diarrhoea. Enteropathogenic *E. coli* (EPEC) was isolated from 6.1% of lambs with and 11.1% of lambs without diarrhoea. Of 26 EPEC isolates, seven were typical (positive for *bfpA*), and of 34 *stx*$_1$ positive isolates 25 were subtyped as *stx*$_1$*c*. Five of 29 *stx*$_2$ positive isolates were subtyped as *stx*$_2$*d* and two as *stx*$_2$*c*.

Bandyopadhyay *et al.* (2010) collected a total of 107 faecal samples from diarrhoeic lambs of Arunachal Pradesh, India. 234 *Escherichia coli* were isolated which were further subjected to PCR for the study of virulence characteristics of Shiga toxin-producing *E. coli* (STEC) and enterotoxigenic *E. coli* (ETEC). Out of the 234 isolated *E. coli*, 32% were found positive for STEC and 9% were carrying virulence gene for ETEC.

Sharma *et al.* (2010) studied 40 diarrhoeic and 31 healthy lambs for the prevalence and characterization of STEC on the basis of virulence genes. A total of 50 *E. coli* isolates belonging to 13 different serogroups were isolated. All the isolates were screened for the presence of *stx*$_1$, *stx*$_2$, *eae* and *hlyA* genes using multiplex polymerase chain reaction (PCR) and 15 (30%) (9 diarrhoeic and 6 healthy) *E. coli* strains were designated as Shiga toxigenic *E. coli*. All STEC were further

characterized by the production of turbid zones of hemolysis on Soyabean casein digest agar supplemented with 5% sheep erythrocytes and 10 mM $CaCl_2$.

Kiranmayi *et al.* (2011) collected and analysed a total of 201 samples (104 sheep faecal samples and 97 sheep farm water samples) from different sheep farms and lairages in and around Hyderabad, India. Samples were enriched in 90 ml of modified Escherichia coli (mEC) broth and modified Tryptic Soy broth (mTSB) both supplemented with novobiocin. All the enriched samples were subjected to PCR analysis for the presence of STEC. Out of 201 samples, 93 showed presence of STEC (57 faecal and 36 water samples). Of the 93 STEC positive isolates, 63 (67.74%) showed presence of stx_1, 32 (34.4%) showed stx_2, 47 (50.53%) showed *hlyA* gene and 16 (17.2%) showed both stx_1 and stx_2. Among 58 STEC positive isolates, 7.52% (4 faecal and 3 water samples) isolates possessed all the 3 virulent genes.

Wani *et al*. (2013) examined 728 faecal samples 324 from lambs (230 diarrhoeic, 94 apparently healthy) and 404 from calves (286 diarrhoeic, 118 apparently healthy calves) in Kashmir, India for the presence of enterotoxigenic *Escherichia coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and *Salmonellae*. 23 ETEC isolates were obtained from the diarrhoeic calves and 12 from diarrhoeic lambs.

Mahanti *et al.* (2015) studied 125 faecal samples of goat in west Bengal, India and 245 isolates were identified as *Eschericha coli* positive. 36 (14.7%) of the 245 *E. coli* isolates were positive for any of shiga toxins (stx_1/stx_2). These STEC strains belonged to 22 different serogroups (O2, O5, O20, O21, O22, O25, O41, O44, O45, O60, O71, O76, O84, O85, O87, O91, O103, O112, O113, O120, O156, and O158) and three were untypeable. Resistance was observed most frequently to erythromycin (80.5%), amikacin (52.7%), cephalothin (50%), kanamycin (41.6%), neomycin (36.1%) and gentamycin (36.1%) and less frequently to norfloxacin (2.7%), enrofloxacin (2.7%), and ciprofloxacin (2.7%). Multidrug resistance was observed in eleven STEC isolates.

Manzoor *et al.* (2015) collected and screened a total of 200 faecal samples from lambs and calves in Kashmir J&K, for the enterotoxigenic *E. coli.* Samples were inoculated on MacConkey agar and Eosin Methylene Blue agar and characterstic colonies were subjected for biochemical tests. Out of 200 *E. coli* isolates, 30 were

detected to be ETEC on basis of *est* amplification. The overall prevalence of ETEC was recorded as 15%. Out of 130 *E. coli* isolates from lambs, 24 (18.46%) isolates carried *est* virulence gene and were designated as ETEC. Twenty one of the ETEC isolates belonged to eight different serogroups, 2 isolates were rough and the remaining one isolate was untypeable. Serogroup O8 was the most prevalent among the lamb isolates.

Mishra *et al.* (2016) screened 240 faecal samples comprising 60 each of sheep, cattle, buffalo, and deer from Mathura districts and Kanpur zoo for the presence of *E. coli* and VTEC genes positive by polymerase chain reaction (PCR).Out of 240 faecal samples, 212 *E. coli* strains were obtained. All the *E. coli* isolates were screened by PCR to detect virulence genes stx_1, stx_2, *eaeA* and *hlyA*. Of these, 25 isolates were identified as VTEC. The prevalence of VTEC in sheep was found as 6.67% (4/60).

Neher *et al*. (2016) collected total of 226 different samples viz., faecal, intestinal content, rectal swab and heart blood from different clinically affected/healthy animals and birds which were streaked on McConkeys lactose agar and Eosin Methylene Blue agar for isolation of *E. coli*, confirmed by staining characteristics and biochemical tests. Out of all the samples 138 (61.06%) were found to be positive for *E. coli.* All the isolates were tested for the presence of virulence genes viz. stx_1, stx_2 and *eae* by Multiplex-PCR. Among the 138 isolates a total of 36 (26.08%) isolates were identified as STEC. Among the STEC isolates, 15 (41.67%) exhibited *eae* gene alone, 14 (38.89%) were positive for stx_2 alone, both stx_1 and stx_2 were found in 4 isolates (11.11%), 2 (5.56%) isolates exhibited stx_2 and *eae* together while 1(2.78%) isolate showed stx_1 alone.

6. Prevalence of *E. coli* in humans.

6.1. Work done abroad

Franzolin *et al.* (2005) tested 169 stool samples for *E. coli*, *Salmonella*, *Shigella*, *Yersinia*, *Aeromonas*, *Vibrio*, and 124 for *Campylobacter*, 139 for virus, 158 for *Cryptosporidia*, and 164 for helminthes/other protozoa. *E. coli* was suspected in 138 samples (i.e. 81.7% of all samples examined for bacteria) and of these, 119 were confirmed positive (86.2%) and 30 were found to contain diarrheagenic strains. The most frequently identified DEC were EPEC (10.1%), followed by ETEC (7.5%), and EAEC (4.2%). Nine ETEC strains were identified.

Raji *et al.* (2008) screened 278 samples of diarrhoeal patients in Tanzania for presence of *E. coli* O157:H7. A total of 96 *E. coli* isolate were identified, of these 10 isolates were grouped into sorbitol non-fermenting and glucuronide negative and 49 isolates were sorbitol positive and glucuronide positive. The remaining 37 were sorbitol negative and glucuronide positive. Using the polymerase chain reaction techniques, a total of ten verotocytocin producing *E. coli* were identified. The overall 02 (15%) and 01 (7%) of the isolates of *E. coli* possessed both attaching and effacing (*eae A*) and enterohemolysin (*ehly A*) genes respectively.

Smith *et al.* (2009) analysed 132 stool samples and 90 environmental samples in Zaira Nigeria. 42 (31.8%) out of 132 stool samples were found positive for EHEC 0157:H7, out of 90 environmental samples, 11 (12.2%) were positive for EHEC 0157:H7. Most of the isolates were resistant to Ampicillin, nitrofurantoin and tetracycline, cotrimoxazole, nitrofurantoin and tetracycline.

Bonyadian *et al.* (2010) screened 200 stool samples of diarrhoeal patients in Shahrekord- area Iran by microbiological and biochemical examinations to detect *E. coli.* Serological tests were carried out to identify the O157 or O157:H7 serotypes. Of the 58 *E. coli* isolates, 16 (27.6%) were detected as Stx_1 carrying *E. coli*, 4 (6.9%) carrying Stx_2, eight (13.8%) carrying both Stx_1 and Stx_2, and 12 (20.7%) were *Hly* carrying *E. coli*. None of the isolates were *E. coli* O157 or O157:H7 serovars.

Abd-Elhamed *et al*. (2012) analysed 80 stool samples and 110 urine samples which were collected from diarrhoeal patient in Cairo, Egypt. *E. coli* were isolated in 8 of 80 (10%) collected faecal samples and in 100 of 110 (91%) urine samples. Among the 100 *E. coli* strains analysed, the somatic antigen (serogroup) belonged to 3 O serogroups, O 164 from the stool (8/80, 10%) and O86 (12/110, 11%) and O126 (44/110, 40%) from the urine.

Ademokoya *et al.* (2013) collected 1800 stool samples of diarrhoeic patients in Southwest Nigeria while using standard microbiological techniques for the identification of thee *E. coli* (O157:H7). From this investigation, 78 (4.3%) out of the people sampled were positive for the organism and this organism was found to be more frequent in females (4.5%) than males (4.2%). In respect to age group, the age group 61-70 years showed the highest occurrence rate (8.3%) while the lowest (2.7%) was found within the age group 21-30 years.

Isibor *et al.* (2013) collected 1000 faecal specimens from consenting persons of both sexes and of all age groups from different areas of Nigeria, reporting with cases of diarrhoea and other gastrointestinal complaints as well as apparently healthy individuals. The samples were inoculated on MacConkey, Sorbitol MacConkey, Eosin Methylene Blue and Blood agar and the results revealed that 316 (32.9%) were positive for *E. coli.*

Kalantar *et al.* (2013) examined 466 rectal swabs from children with acute diarrhoea and 125 frozen foods of animal origin to isolate and identify *E. coli* strains based on standard procedures. 87 strains of *E. coli* were detected from 466 rectal swabs of children with acute diarrhoea and 40 strains of *E. coli* strains were detected from the 125 frozen food samples of animal origin. Test results indicated a 5.0% and 2.5% prevalence of Shiga toxin (*stx*) and enterotoxin (*estA*) genes respectively among *E. coli* strains isolated from frozen foods of animal origin. Similarly, 5.7% and 4.5% prevalence of Shiga toxin (*stx*) and enterotoxin (*estA*) genes respectively were identified from *E. coli* strains isolated from children.

Khanjar and Alwan (2014) analysed 198 human stool samples that were collected from patients suffering from bloody and non-bloody diarrhea and urinary tract infections of both genders with variable age. The samples were cultured aerobically on routine media and selective media at 37^0C for 24-48 hrs, then the isolates were identified by biochemical tests and they were confirmed by PCR assay. The results showed that 10 (5%) out of 198 human stools samples were *E. coli* O157:H7 positive.

Achi *et al.* (2015) collected stool specimens from 200 infants and children under two years of age suspected with diarrhoeal disease from five hospitals in Aba Nigeria. Stool samples were also collected from healthy children and all samples were analysed for the EPEC by culturing on sorbitol-MacConkey agar (SMAC), MacConkey agar and Deoxycholate citrate agar. Colonies showing typical *E. coli* growth were tested using classical biochemical methods. Atypical EPEC was diagnosed in 19.5 % of the patients as compared to 6% in the healthy control group.

6.2. Work done in India

Dhanashree and Mallya (2008) analysed 192 diarrhoeic stool samples and 103 meat samples for *E. coli* (STEC) in Mangalore in which 2 out of 192 stool samples

and1 out of 103 meat samples were found positive which showed high isolation rate of *eaeA* positive *E. coli* resembling atypical EPEC.

Gaddad *et al*. (2011) investigated the presence of shiga toxin producing *E. coli* (STEC) in stool samples (n=885) of diarrhoeic patients working in health centres in Gulbarga, India. PCR revealed STEC in 65 samples, stx_1 (86.2%) was found more frequently than stx_2 (12.5%) and both the genes were present in only two human stool samples (3.1%).

Shetty *et al.* (2012) studied 115 stool samples of children and adults having acute diarrhoea in Manglore, India. PCR amplification of *eae*, *bfp*, *stx*, *ehx* genes were used for detection of enteropathogenic (EPEC) and shigatoxigenic *E. coli* (STEC), *lt* and *st* genes were used for enterotoxigenic *E. coli* (ETEC) and *ast*A gene for enteroaggregative *E. coli* (EAEC). The most prevalent DEC was atypical EPEC accounting for 12 (10.4%) cases followed by 4 cases of EAEC (3.4%) and 4 of STEC (3.4%). No ETEC strains were isolated from any of the examined stool samples.

Virpari *et al*. (2013) collected and examined a total of 100 stool samples from diarrhoeal patients, with history of raw milk consumption from primary health centres in and around Anand city, under aseptic conditions. MacConkey broth was used for the enrichment of all the samples and inoculation was done on MacConkey agar and EMB agar. 59 (59%) *E. coli* isolates from 100 stool samples were recovered which were confirmed using biochemical tests. Detection of virulence genes was done using PCR technique.

Kumar *et al.* (2014) collected 120 diarrhoeal samples from the cases admitted in hospitals of Mathura, UP, samples were inoculated on MacConkey agar and EMB agar 39 samples were found positive for *E. coli*. On blood agar 23 isolates revealed hazy wider zone of haemolysis depicting 58.9% incidence of haemolytic *E. coli*. Antibiogram showed that *E. coli* isolates had 100% resistance against most of commonly used antibiotics.

7. Pathogenicity

7.1. Congo red binding assay

7.2. Haemolysis

Berkhoff *et al.* (1986) studied 144 *E. coli* isolates from internal tissue of diseased birds and 170 isolates from poultry house environment to distinguish

between invasive and non invasive *E. coli* using congo red medium as phenotypic marker and found that all of the isolates from diseased birds were CR positive, where as half of isolates from poultry house were CR negative.

Sharma *et al* (2006) examined 97 *E .coli* isolates for the detection of invasive *E .coli* using Congo red dye agar test (CR test) and the results revealed that out of 97 isolates 46 showed CR positive reaction, while 51 were CR negative.

Olowe *et al.* (2008) cultured 82 isolates from, urine, cerebrospinal fluid, peritoneal fluid, blood culture, wound exudates for haemolysis testing using from different patients in Nigeria. Among the 82 isolates tested for haemolysis 22 (26.8%) isolates produced haemolysis while 60 (73.2%) were non-haemolytic isolates. Of the haemolytic isolates 16 (72.1%) were isolated from the urine samples, 4 (18.2%) from stool samples and 2 (9.1%) from blood samples.

Yousseff *et al*. (2008) examined 85 *E. coli* isolates which were isolated from diarrhoeic hens for their pathogenicity in Egypt. Virulence tests were done on isolates and it was found that all *E. coli* isolates bind Congo red (100%), β-hemolysin was observed in 48 isolates (56.47%) and enterotoxins assays revealed that 02 *E. coli* strains recovered from birds were O26 (22.86%) and O157 (5.71%), while invasiveness assay revealed negative results.

Ahmad *et al*. (2009) analysed 86 water samples from 55 poultry farms in Pakistan and 73 produced growth on MacConkey's agar. Biochemical tests identified 26 of these as *E. coli* organism. Pathogenicity test for *E. coli* was conducted by using congo red binding activity. Out of 26 isolates of *E. coli*, 20 resulted in the growth of brick red coloured colonies indicative of pathogenic nature where as the remaining 6 produced greyish white colonies after 96 hours of growth and were considered as non pathogenic.

Ranjan *et al*. (2010) studied a total of 220 symptomatic cases from urinary tract infections and 50 stool samples from apparently healthy individuals. The colonies identified as *Escherichia coli* were screened for virulence factors, i.e haemolysin, Mannose Resistant and Mannose Sensitive Hemagglutination (MRHA, MSHA), Cell surface hydrophobicity, and Serum resistance. Among the 220 cases, 91 (41.36%) were haemolytic, 68 (30.90%) showed MRHA, 58 (26.36%) were cell surface hydrophobicity positive, and 72 (32.72%) were serum-resistant. In 50

controls, 03 (6%) were haemolytic, 06 (12%) showed MRHA, 09 (18%) showed cell surface hydrophobicity, and 12 (24%) were serum-resistant.

Bashar *et al*. (2011) examined a total of 60 faecal samples and *E. coli* were isolated and identified by conventional cultural, biochemical and motility test. The heat stable toxins were determined by Suckling Mice Assay (SMA). Among the isolates of *E. coli*, only 45% and 14% isolates showed β hemolysis and α hemolysis, respectively. Congo red binding has been used as a potential virulence marker and 69 % *E. coli* isolates showed congo red binder, among which, 59% isolates were haemolytic.

Gupta *et al*. (2013) investigated 54 isolates of *E. coli* which were isolated from raw fish and ready to eat fish products in Ludhiana, Punjab for the in vitro pathogenicity using congo red assay, haemolysis on blood agar, and gelatinase activity on gelatine agar. In vitro virulence characterization of isolates exhibited that all *E. coli* isolates were haemolytic while indicators of plausible cytotoxicity (lecithinase, protease and gelatinase production) were in the range of 16.67% to 35.19% indicated that though the isolates were haemolytic they were perhaps less likely to be cytotoxic. Congo red binding assay for *E. coli* isolates revealed that majority (88.89%) of the isolates failed to uptake the dye and only few (11.11%) could bind the dye.

Lamey *et al.* (2013) screened 63 isolates of *E. coli* isolated from mastitis milk samples of buffaloes in Egypt to determine the pathogenicity of *E. coli* isolates. Virulence tests were performed on *E. coli* serogroups isolated from mastitis cases. It was found that 24 (38.1%) had congo red binding activity, 20 (31.75%) were invasive and 8 (12.7%) had haemolytic activity and 50 strains (79.37%) were serum resistant.

AL-Saiedi *et al.* (2014) investigated the in vitro and in vivo virulence of 47 *E. coli* isolates which were obtained from naturally infected broiler birds. Congo red binding activity showed that 60% were positive, whereas motility test displayed that 51.85% were motile. The ability of isolated *E. coli* to produce haemolysin was found that 44.6% of these isolates were haemolytic. Hemagglutination test indicated that 3.70% of the present isolates were positive. The results of in vitro testing indicated that 03 isolates were classified as highly, moderately and slightly virulent according to their characteristics of pathogenicity.

Hassan and Bakeet (2014) examined 19 isolates of *E. coli* isolated from tissues of infected pigeons to detect the virulence factors associated with pathogenic *E coli* in various parts of Assiut province using blood agar for haemolysis and congo red medium. The results revealed that all *E. coli* isolates (100%) were found positive for the congo red binding activity and (84.2%) of the isolates were positive for haemolytic activity.

Parul *et al.* (2014) collected and studied a total of 90 samples (70 faecal samples of cattle and 20 soil samples) for the isolation of *E. coli* and the isolates were confirmed using various biochemical tests like IMViC. Confirmed isolates were subjected to congo red dye assay and hemolysis assay to assess their virulence properties. Antibiotic sensitivity pattern of pathogenic isolates was studied by Disc diffusion method. The percent positivity on Congo red dye assay was 44.28% for faeces and 5% for soil while only faecal *E. coli* (4.28%) were found to be positive for haemolysis assay.

Sayed (2014) studied the pathogenicity of 18 strains of *E. coli* obtained from mastitis milk samples of cows and it was found that 13 isolates (72.2%) showed congo red binding activity, 6 isolates (33.3%) were invasive, one isolate (5.6%) had haemolytic activity while as 15 isolates (83.3%) were serum resistant.

Yadav *et al.* (2014) examined 70 isolates of *E. coli* for their pathogenicity using congo red medium, and results revealed that 65 (92.86 %) isolates showed a congo red binding ability while 5 (7.14 %) isolates did not bind congo red dye up to 72 hours post inoculation (PI).

Ali *et al*. (2015) studied pathogenicity of 23 *E. coli* isolates using congo red binding activity which were isolated from broilers birds and the results revealed that 6 of isolates were positive on congo red medium with incidence of 26.08%. Isolates that produced red colour colonies on congo red medium were considered as CR positive.

Atere *et al*. (2015) analyzed 97 (ninety seven) recently dead chicken from 23 different farms for the presence of pathogenic *E. coli*. A total of forty eight (48) pathogenic *E. coli* were isolated. 43 out of the 48 isolated *E. coli* showed haemolysis on sheep blood agar, which constituted 89.6% of the total *E. coli* isolated. In vitro

susceptibility of the isolates against antimicrobial agents was determined using disk diffusion method.

8. Antimicrobial sensitivity pattern of *Escherichia coli* isolates.

Chattopadhya *et al.* (2001) observed that all the *E. coli* (STEC) isolates (12 of animals, 1 of human and 4 of food samples) from a total of 876 samples (330 of animals,184 of humans and 362 food samples) were uniformly sensitive to common antibiotics, except tetracycline, dicloxicillin, erythromycin, cephalaxin and linomycin.

Leelaporn *et al.* (2003) performed antimicrobial susceptibility tests of *E. coli* isolates in Bangkok, by disc diffusion method. All the isolates were found susceptible to cefaclor, ceftriaxone, imipenem, netilmicin, norfloxacin, ciptofloxacin, nalidixic acid, and forfomycin. More than 90% of the isolates were susceptible to cefdinir, gentamycin, neomycin and chlorophenicol. Resistance rates to ampicillin, co-trimoxazole and tetracycline were 17, 39, and 65 percent respectively.

Cergole-Novella *et al*. (2006) isolated a total of 107 STEC from human infections, cattle and foods in Brazil. They observed that the highest frequencies of susceptibility to antimicrobial agents were among food (100) and bovines (87%) strains while 47.6% of the human isolates were resistant to at least one drug. The antimicrobials to which resistance most frequently observed were tetracycline (90%) and streptomycin (75%) among human strains and sulphazotrin (88%) in animal strains.

Rashid *et al.* (2006) isolated 21 strains of *E. coli* from 120 samples of bovine mastitic milk over a period of about two years of which 20 isolates were typed and one isolate was untypeable. Multiple drug resistance (MDR) was recorded being the highest against piperacillin (100%), Ampicillin (71%) Cephoxitin (71%), Enerofloxacin (33.33%) and Nalidixic acid (61.90%), the most commonly used antibiotics in the field. The sensitivity was highest against Chloramphenicol (80.95%), Ciprofloxacin (76.20%), Norfloxacin (76.20%) and gentamycin (71.42%).

Mora (2007) examined 722 *E. coli* (STEC) isolates recovered from humans, sheep, and food in Spain to determine antimicrobial resistance profiles. Fifty eight (41%) out of 141 STEC O157:H7 isolates and 240 (40%) out of 581 non-O157 STEC isolates showed resistance to atleast one of the 26 antimicrobial agents tested. Shiga

toxin- producing *E. coli* O157:H7 showed a higher percentage of resistant strains recovered from bovine (53%) and beef meat (57%), human (23%) and ovine (23%) sources. Sulfisoxazole (36%) had the most common antimicrobial resistance followed by tetracycline (32%), streptomycin (29%), amplicillin (10%), trimethoprim (8%), cotrimazole (8%), chloramphenicol (7%), kanamycin (7%), piperacillin (6%), and neomycin (5%). Ten (7%) STEC O157:H7 and 17 (21%) non- O157 isolates were resistant to five or more antimicrobial agents. The multiple resistance pattern most often observed was that of streptomycin, sulfisoxazole and tetracycline.

Naidu *et al*. (2007) compared the antibiotic resistance pattern of STEC isolates from different sources. Highest resistance was recorded with ampicillin (73%) and cephalexin (69.23%). Chloramphenicol and gentamycin showed a comparatively low incidence of resistance i.e., 21.54 and 12.3% respectively. Half of the strains isolated from human diarrhoeic samples were resistant to nine or more antibiotics.

Yadav *et al.* (2007) assessed 49 isolates of *Escherichia coli* isolated from mutton in Gujarat state of India for the drug susceptibility and the percentage of isolates resistant to antibacterial agents was recorded as sulphadiazine (93.33%), cephaloridine (80.00%), cephalexin (33.33%), penicillin G, ceftiofur and norfloxacin, carbenicillin and enrofloxacin (26.67% each), and oxytetracycline and amoxycillin (20.00% each).

Olatoye and Olufemi (2010) tested 71 isolates of *E. coli* O157:H7 which were isolated from beef in Nigeria for antibiotic sensitivity. Antibiotic susceptibility profile showed that all the isolates were resistant to one or multiple antibiotics. Tetracycline resistance was the highest in 91.4% of the isolates, while 72.9% resistance to nitrofurantoin and Chloramphenicol, 65.7% to cefuroxime, 44.3% resistance to cotrimozole, 35.7% resistance to nalidixic acid, 11.4% resistance to gentamycin.

Rigobelo *et al*. (2010) determined the sensitivity pattern of 120 *E. coli* (STEC) isolated from an abattoir in Brazil to ten antimicrobial drugs. Resistance was seen against cephalothin (84.0%), streptomycin (45%) Nalidixic acid (42%), Tetracyclin (20%), and less frequently to Trimethoprim (9%), Cephlothin (8%), and Amikacin (6%). Multidrug resistance (MDR) was seen in 38.4% of the isolates and resistance to 2 or 3 antibiotics was common.

Rashid (2011) studied antibiotic sensitivity of *E. coli* from clinical cases and foods of animal origin in Jammu. 47 of 120 isolates revealed multidrug resistance to four or more antibiotics. Fifty percent of the isolates were resistant to Amoxycillin, 44.16 % to ampicillin, 40% to cefotexime, 19.16% to amikacin, 6.66% to chloramphenicol and 13.33% to gentamycin.

Aly *et al.* (2012) isolated a total of 147 *E. coli* strains from clinical specimens and food samples. The antibiotic resistance profile of these strains was determined against 7 classes of antimicrobial agents (26 different members). Almost 90% of *E. coli* strains were resistant to at least one of the tested antibiotics. The highest antibiotic resistance was recorded against conventional Beta-lactams. The highest sensitivity of the isolates was to imipenem and polymyxin-B where all isolated *E. coli* strains were sensitive to imipenem. The resistance to tetracyclines, macrolides and sulfonamides/trimethoprim was almost in the same order of magnitude of 30-37%. The resistance to quinolones and aminoglycosides was 19 and 10 % respectively.

Hiroi *et al*. (2012) determined the antimicrobial resistance patterns of 138 *E. coli* isolated from humans in Japan. 31 isolates showed the resistance to one or more antimicrobial agents. 24 of the isolates were resistant to tetracycline, 23 to streptomycin, 12 to ampicillin, 7 to chloramphenicol and kanamycin, 3 to nalidixic acid, 1 to gentamycin and 1 to cefuroxime.

Moses *et al.* (2012) studied 18 *E. coli* O157 isolates from human stool (12) from cattle faeces, unpasteurized milk (5) and water (1) using agar disk diffusion method to determine the drug resistance in Nigeria. Resistant rate among human strains was low against gentamycin (8.3%), streptomycin (8.3%), chloramphenicol (25.0%) and sulphamethoxazole-trimethoprim (25.0%). Increasing resistant pattern against tetracycline, ampicillin, cephalexin and clavulanic acid-potentiated amoxicillin was observed in about 50% to 80% of human and cattle isolates.

Sasaki *et al*. (2012) studied the antimicrobial resistance in O157 and O26 strains of STEC. Resistance to dihydrostreptomycin was detected most frequently followed by Oxytetracycline and ampicillin. Resistance to one or more antimicrobial agents was detected in 13.3% of the O157 isolates and 54.5% (6/11) of the O26 isolates. The antimicrobial resistance rate in the O26 STEC isolates was significantly higher that in the O157 isolates.

Dey *et al.* (2013) tested 112 samples from poultry to determine the prevalence of antimicrobial resistance. The antibiotic sensitivity pattern showed that *E. coli* isolates were sensitive to erythromycin, ciprofloxacin, kanamycin, nalidixic acid and resistant to amoxicillin, tetracycline and sulphamethaxazole.

Mahmoud *et al.* (2013) tested 12 isolates of EHEC which were isolated from lambs (4), calves (4) and fish (4) in Egypt to determine the frequency of resistance to commonly used antimicrobial agents in Veterinary field. Results showed that among the antimicrobial discs tested, ampicillin was the most common antibiotic that the isolates were resistant to (91.6%), followed by tetracycline (83.3%).

Rajput *et al.* (2013) tested *E. coli* isolates which were isolated from clinical cases of diarrhoea in kids of Mathura area of U.P for their sensitivity to the commonly used antibiotics. About 67% of the isolates were resistant to Oxytetracycline, Gentamicin, and Ceftriaxone. Only antibiotic groups like fluoroquinolones and the Chloramphenicol have fared well in effectively inhibiting the in vitro growth of *E. coli*. Chloramphenicol has given the lowest percentage of resistance at zero and the next best was Enrofloxacin at 34%.

Mahanti *et al.* (2014) studied the antibiogram of 363 isolates of *E. coli* which were isolated from faecal samples of buffloes in West Bengal, India. The antibiotics used were amikacin (30 µg), gentamicin (30 µg), kanamycin (30 µg), neomycin (30 µg), oxytetracycline (30 µg), co-trimoxazole (25 µg), ceftazidime (30 µg), levofl oxacin (5 µg), cefepime (30 µg), ciprofl oxacin (5 µg), ceftriaxone (30 µg), enrofloxacin (5 µg), pefloxacin (5 µg), amoxycillin (25 µg), chloramphenicol (30 µg), cefuroxime (30 µg) and norfloxacin (10 µg) (Hi Media, India). The antibiotic resistance of ETEC isolates was observed most frequently towards amikacin (56%), kanamycin (44%), gentamicin (40%) and neomycin (36%).

Mahanti *et al.* (2015) screened 36 *E. coli* (STEC) isolates isolated from faecal samples of healthy goats for antibiotic sensitivity and found that resistance was observed most frequently to erythromycin (80.5%), amikacin (52.7%), cephalothin (50%), kanamycin (41.6%), neomycin (36.1%) and gentamycin (36.1%) and less frequently to norfloxacin (2.7%), enrofloxacin (2.7%), and ciprofloxacin (2.7%). Multidrug resistance was observed in 11 STEC isolates.

Mohammed *et al.* (2014) cultured a total of 384 meat samples for detection of *E. coli* and which were also tested for antimicrobial susceptibility. Investigation revealed a 15.89% overall prevalence of *E. coli* in the meat samples. All the *E. coli* isolates were found insensitive to penicillin, ampicillin, doxicycline and erythromycin but sensitive for tetracycline.

Radwan *et al.* (2014) recovered 83 *E. coli* isolates from 200 broiler chicken suffering from colibacillosis. The disc diffusion method was used to determine antibiotic susceptibility of the isolates for 10 antibiotics comprising 6 different antimicrobial classes. Antibiogram profiles indicated maximum resistance to ampicillin (100%), high frequency of resistance to amoxicillin (97.6%), sulfamethoxazole/trimthoprim (94%), streptomycin (92.8%) and ciprofloxacin (89.2%). Conversely, the aminoglycoside amikacin was shown effective against 97.6% of the isolates.

Begum *et al.* (2015) tested 12 and 6 isolates of STEC from cattle and pig faecal samples respectively in Guwahati, India for in vitro susceptibility against 15 different antimicrobial agents and showed highest sensitivity towards ciprofloxacin (100%) followed by norfloxacin (91.67%), chloramphenicol (83.33%), nalidixic acid (83.33%), co- trimoxazol (75%) and cephotaxim (66.67%), gentamicin (58.33%), streptomycin (58.33%), enrofloxacin (41.67%), tetracycline (33.33%) and amoxicillin (16.67%) in case of isolates from cattle. While STEC isolates from pigs showed highest sensitivity to ciprofloxacin (100%) and norfloxacin (100%).

CHAPTER – III

MATERIALS AND METHODS

CHAPTER-III

MATERIALS AND METHODS

3.1 PLACE OF WORK

The study was conducted at the Division of Veterinary Public Health and Epidemiology, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-J, R.S. Pura, Jammu and Kashmir, India.

3.2 MEDIA, CHEMICALS AND REAGENTS

Media and antibiotics used in the present study were procured from Hi-Media. Chemicals and reagents used were from reputed National and International firms. Glassware and other materials were cleaned and sterilized following the standard procedures. Details of media /chemicals used in the present study are listed below:

NSS (Normal Saline Solution)

Ingredients	**Amount (gm/ litre)**
Sodium Chloride	800 mg
Distilled Water	100 ml

Dissolved 800 mg of Sodium Chloride solution in 100 ml of distilled water and sterilized by autoclaving at 15lbs (121^0C) for 15 minutes.

MacConkey Lactose agar (Hi Media, Mumbai)

Ingredients	**Amount (gm/litre)**
Peptic digest of animal tissue	20.0
Lactose	10.0
Sodium taurocholate	5.0
Neutral red	0.07
Agar	15.13

Final pH (at 25^0c) =7.4±0.2

55.37 grams of the dehydrated medium was added in 1000 ml of distilled water. Heated to boiling to completely dissolve the medium. Sterilized by autoclaving at 15 lbs (121°C) for 15 minutes.

EMB Agar (Hi Media, Mumbai)

Ingredients	**Amount (gm/litre)**
Peptic digest of animal tissue	10.0
Dipotassium phosphate	2.0
Lactose	5.0
Sucrose	5.0
Eosin-Y	0.4
Methylene blue	0.065
Agar	13.50

Final pH at 25^0C =7.2± 0.2

36 grams of dehydrated medium was suspended in 1000 ml distilled water and heated to dissolve the medium completely, overheating was avoided, cooled to 50^0C and the medium was shaken in order to oxidize the methylene blue and suspend the flocculent precipitate. The medium was sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes.

Nutrient Agar (Hi Media, Mumbai)

Ingredients	**Amount (gms/litre)**
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15
Distilled water	1000 ml

Final pH (at 25^oc) = 7.3±1

Dissolved 37.0 grams in 1000 ml in distilled water and sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes.

Simmon's Citrate Agar (Hi-Media,Mumbai)

Composition

Ingredients	**Amount (gm/litre)**
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0

Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0

Final pH (at 25°C) = 6.8± 0.2

24.28 grams of dehydrated media was suspended in 1000ml of distilled water and then heated to dissolve the medium completely. Mixed properly and distributed in test tubes and sterilized by autoclaving at 121°C at 15lbs pressure for 15 minutes.

Triple Sugar Iron (TSI) Agar (Hi Media, Mumbai)

Ingredients	**Amount (gm/litre)**
Peptic digest of animal tissue	10.0
Casein enzyme hydrolysate	10.0
Yeast extract	3.0
Beef extract	3.0
Dextrose	1.0
Lactose	10.0
Sucrose	10.0
Ferrous sulphate	0.2
Sodium chloride	5.0
Sodium thiosulphate	0.3
Agar	12.0
Phenol red	0.024

Final pH = 7.4 ± 0.2 at 25°C

65gm of TSI medium were suspended in 1000 ml of distilled water and boiled to dissolve. Then distributed in test tubes and sterilized at 121°C at 15 lbs pressure for 15 minutes and allowed to set in slopped form with a butt about 1 inch long.

Sheep Blood Agar Base (Hi Media,Mumbai)

Ingredients	**Amount (gm/litre)**
Casein enzymic hydrolysate	14.00
Peptic digest of animal tissue	4.50
Yeast extract	4.50
Sodium chloride	5.00

Agar	12.50

Final pH (At 25°C) = 7.3 ± 0.2

40.5 grams were added in 1000ml of distilled water and Heated to dissolve the medium completely. The medium was sterilized by autoclaving at 15 lbs pressure and 115°C for 15 minutes, cooled to 50°C then 7% sterile sheep blood was poured aseptically.

Muller Hinton Agar (M 173 Hi Media, Mumbai)

Ingredients	**Amount (gm/litre)**
Beef infusion	300
Casein acid hydrolysate	17.50
Starch	1.5
Agar	17.0

38.00 grams were added in 1000 ml of distilled water. Heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Bile Salt Agar

Ingredients	**Amount (gm/litre)**
Peptone	10.00
Meat extract	5.00
Sodium chloride	5.00
Sodium taurocholate	5.00
Agar	15.00

Final pH (at 25^0C) = 8.5±0.2

Suspended 40 grams in 1000 ml of distilled water, heated to dissolve, and sterilized by autoclaving at 15 lbs pressure, at 121°C temperature for 15 minutes.

MacConkey Broth (Hi Media, Mumbai)

Composition

Ingredients	**Amount (gm/litre)**
Peptic digest of animal tissues	20.0
Lactose	10.0
Bile salts	5.0

Sodium chloride	5.0
Neutral red	0.075

Final pH (At 25°C) = 7.4 ± 0.2

Suspended 40 grams in 1000 ml distilled water, heated to dissolve, distributed in test tubes and sterilized by autoclaving at 15 lbs pressure, at 121°C temperature for 15 minutes.

Nutrient Broth (Hi Media, Mumbai)

Ingredients	**Amount (gm/litre)**
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5

Final pH (at 25^0C) = 7.4± 0.2

Suspended 13.0 grams in 1000 ml distilled water and sterilized by autoclaving at 15 lbs pressure (121^oC) for 15 minutes.

Peptone Water (Hi Media, Mumbai)

Ingredients	**Amount (gm/litre)**
Peptone	10 g
Sodium chloride	5 g
Distilled water	1000 ml

Final pH = 7.2± 0.2

Suspended 15 grams in 1000 ml distilled water and heated to dissolve the medium completely. Dispensed in tubes and sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes.

Ethidium bromide (10mg/ml)

Ingredients	**Amount**
Ehidium bromide	50 mg
Double distilled water	5 ml

Store the solution in amber coloured vial at 4^0C.

EDTA (0.5M)

Ingredients	**Amount**
EDTA.2H2O	18.61 g

Double distilled water	100 ml

Adjust the PH to 8.0 with solid sodium hydroxide pellets.

Tris Borate – EDTA (TBS) stock solution (5X)

Ingredients	**Amount**
Tris	54.0 g
Boric acid	27.5 ml
EDTA (0.5)	20 ml
Double distilled water to make	1000 ml

The solution was autoclaved and stored at room temperature. For working solution (1X), dilute the stock TBE in double distilled water.

Tryptone Water/ Broth (Hi Media, Mumbai)

Ingredients	**Amount (gm/litre)**
Casein enzymic hydrolysate	10.00
Sodium chloride	5.00

Final pH (at 25^0C) = 7.5± 0.2

The broth was boiled to dissolve and dispensed in tubes. Autoclaved at 121^0C at 15 lbs pressure for 15 minutes.

MR-VP Medium (Glucose phosphate broth) (Hi Media, Mumbai)

Ingredients	**Amount (gm/litre)**
Peptone	7.0
Glucose	5.0
Dipotassium phosphate	5.0
Distilled water	1000 ml

Final pH = 6.9 ± 0.2

The broth was boiled to dissolve and dispensed in tubes. Autoclaved at 121^0C at 15 lbs pressure for 15 minutes.

Reagents used in the study

Kovac's reagent

Amyl or isoamyl alchohol	150 ml
Concentrated HCl	50 ml
Para-dimethyl amino benzaldehyde	10 g

Dissolved the aldehyde in alchohal and slowly add acid. Store the reagent in refrigerator.

3.3 COLLECTION OF SAMPLES

A total of 170 samples, 30 faecal samples and 30 rectal swabs each of sheep and goats, 25 hand swabs and 25 stool samples of handlers of sheep and goats belonging to nomadic community were collected from in and around of Jammu district.

Table: 3.1: Detail of samples collected

Animal	Faecal Samples	Swabs
Sheep	30	30 Rectal Swabs
Goat	30	30 Rectal Swabs
Handlers	25	25 Hand Swabs

Sampling from rectal swab: Sterile swab stick moistened with sterile normal saline water was inserted in the rectum of the sheep and goats and placed in sterile containers.

Collection of sample from faecal material: About 100 gm of fresh faecal sample was collected aseptically from sheep, goats, and their handlers into sterile container.

Sample from sheep and goats handlers: The samples from the hands of the sheep and goats handlers were collected with the help of sterile swab stick moistened with sterile normal saline. Then transferred immediately to screw caped test tubes containing 10 ml of sterile *E. coli* broth (HiMedia, Mumbai, India) for collection of stool samples sterile containers were provided to the handlers, and were transported to laboratory for further processing.

Transportation of sample: After collection, all the samples were transported to the laboratory immediately in an insulating foam box with ice or ice packs.

3.4 ISOLATION

Enrichment: Samples were placed in enrichment by inoculated into MacConkey's broth tubes (HiMedia, Mumbai, India) at 37ºC for 18-24 hours followed by selective plating.

Plating on selective media: A loopful inoculum from MacConkey's broth was streaked onto MacConkey's agar and incubated at 37ºC for 24 hours for the characteristic pink colonies. Pink colonies from MacConkey's agar were inoculated onto Eosin Methylene Blue (EMB) agar (HiMedia, Mumbai, India) and plates were incubated at 37ºC for 18-24 hours for the characteristic metallic sheen specific of *E. coli*. The well separated pure colonies were picked up on nutrient agar slants as pure culture and used for standard morphological and biochemical tests (Cruikshank *et al.,* 1975).

3.5 IDENTIFICATION

Presumptive *E. coli* isolates were subjected to identification as per procedure described by Hitchins *et al.,*1992) and Cruikshank *et al.,*1975).

3.5.1. Gram's staining

The isolated colonies with metallic sheen on EMB agar plate presumed as *Escherichia coli* were subjected to Gram's staining as per standard procedure. The Gram negative rods after Gram's staining were subjected to further identification by biochemical test.

3.5.2. Biochemical tests

A series of biochemical tests were performed which included Catalase test, Oxidase test, Triple sugar iron agar test, Indole test, Methyl red test, Voges-Proskauer test, Citrate test and sugar fermentation test as stated by Quinn, *et al.,*1994.

3.5.2.1. Catalase test

Catalase test was performed to check the presence of enzyme catalase and hence the ability of the bacteria to oxidize hydrogen peroxide to oxygen and water. The test was performed by thoroughly mixing a loopful of the presumptive *E. coli* with a drop of 3 percent H_2O_2 placed on a clean glass slide. The production of gas bubbles due to liberation of oxygen was taken as a positive test.

3.5.2.2. Oxidase test

The test depends on the presence of certain oxidases (cytochrome oxidase) in bacteria that will catalyse the transport of electrons between electron donors in

bacteria and redox dye tetramethyl-p- pnenylenediamine. The dye is reduced to deep purple colour. The test was performed by soaking filter paper strip with a little freshly made 1 percent solution of tetramethyl-p-phenylene-diamine dihydrochloride dye. A small amount of culture was immediately rubbed on the paper with a platinum loop. Absence of deep purple hue appearing within 5-10 seconds indicated an oxidase negative reaction for *E. coli*.

3.5.2.3. Triple sugar iron test (TSI)

TSI test was performed by inoculating the TSI agar in a test tube with the test organism upto the bottom of the butt. Streaking was also done on the slant surface and the test tube was incubated at 37ºC for 24 hours. The tubes showing acid butt (yellow), acid slant (yellow), with gas production and no H_2S production were interpreted to be positive for *E. coli* (Quinn *et al.*,1994).

3.5.2.4. Indole test

The test based on the ability of bacteria to decompose amino acid tryptophan to pyruvic acid, ammonia and indole. The presence of indole in the medium was detected by inoculating the test organism tryptone water containing tryptophan (pH 7.2) and incubating at 37ºC for 48 hours. Then 0.5 ml of Kovac's reagent was added slowly and the tube shaken gently. Appearance of red ring indicated a positive reaction for *E. coli* (Hitchins *et al.*,1992).

3.5.2.5. Methyl Red (MR) test

The test is employed to detect the production of sufficient acid during the fermentation of glucose which lowers the pH below a value of about 4.5 as shown by change in colour of MR indicator added at the end of incubation period. The test was carried out by inoculating MR-VP medium (Hi Media, Mumbai) with the test organism and incubating at 37ºC for 24 to 48 hours. Appearance of red colour on addition of methyl red indicator, indicated positive reaction (Hitchins *et al.*,1992).

3.5.2.6. Voges – Proskauer test

Certain bacteria produce non acidic or neutral end product such as acytylmethylcarbinol or its reduction product butylenes glycol from organic acid intermediates of carbohydrate fermentation. These substances can be tested by

calorimetric reaction with Barrit's reagent (Alcoholic alpha- nephthol and 40% KOH). Acetymethylcarbinol is oxidized to diacetyl in the presence of alpha- nephthol in an alkaline environment which in turn form pink colour complex in presence of guanidine group present in the peptone of MR-VP medium.

The organism was inoculated in 5ml of MR-VP medium and incubated at 37ºC for 48 hours. Then 1ml of 40% potassium hydroxide and 3ml of 5% alpha-nephthol in absolute ethyl alcohol was added. No change in colour indicated negative reaction for *E. coli* (Hitchins *at el.,* 1992).

3.5.2.6. Citrate utilization test

This test is used to determine the ability of an organism to utilize citrate as sole of carbon and energy for growth and ammonium salts the sole source of nitrogen. The test was carried out by inoculating Simmon's citrate slant with a test organism and incubating for 24-48 hours. No change in green solid slant indicated negative reaction for *E. coli* (Quinn *et. al.,* 1994).

3.6. Serogrouping

Serogrouping of the *E. coli* isolates was done from National Salmonella and Escherichia Centre, Central Research Institute , Kasauli- 173204 (H.P) on the basis of their 'O' antigen.

3.7 Virulence assay

3.7.1. Congo red dye agar test (CR Test).

The test was carried out as per the technique of Berkhoff and Vinal (1986). The colonies of *E. coli* were streaked on Congo red agar and incubated for 24 hours at 37^{o}C and then left at room temperature for next two days (48 hrs). Reaction was recorded at 18, 24, 48 and 72 hours. Appearance of red colonies within 72 hours was recorded as a positive reaction. Negative colonies did not bind the dye and remained white or grey even after 72 hours and were declared as negative.

3.7.2. Haemolysis

The test was performed as per the protocol of Beutin *et al.,* 1989. *E. coli* isolates were propagated on blood agar base supplemented with 5% washed sheep

erythrocytes and incubated at 37°C for 24 hours. Pathogenic strains of *E. coli* produced clear zones of haemolysis and was recorded as haemolysin positive.

3.8 Molecular characterization of *E. coli* isolates using PCR

The isolates were characterized by multiplex polymerase chain reaction targeting four genes (*stx1, stx2, eaeA,* and *hlyA*) as per the method described by Paton and Paton 1998 with slight alteration. The multiplex PCR is standardized using reference strain of *E. coli* (MTCC no. 9537).

Table 3.2: List of primers (5´-3´) used in mPCR

Target gene	Primer	Sequence (5´-3´)	Amplicon size (bp)	References
stx$_1$	*stx*$_1$*F*	ATAAATCGCCATTCGTTGACTAC	180	Paton and Paton, 1998
	stx$_1$*R*	AGAACGCCCACTGAGATCATC		
stx$_2$	*stx*$_2$*F*	GGCACTGTCTGAAACTGCTCC	255	
	stx$_2$*R*	TCGCCAGTTATCTGACATTCTG		
eaeA	*eaeA*F	GACCCGGCACAAGCATAAGC	384	
	*eaeA*R	CCACCTGCAGCAACAAGAGG		
hlyA	*hlyA*F	GCATCATCAAGCGTACGTTCC	534	
	*hlyA*R	AATGAGCCAAGCTGGTTAAGCT		

3.8.1. Preparation of DNA template by boiling snap chilling method

The DNA template was prepared as per the method of Blanco *et al*. (1996) with slight alteration.100µl of phosphorus buffer saline (PBS) was taken in micro centrifuge tubes and a loopful of each isolate was mixed with the nuclease free water thoroughly in each micro centrifuge tube. The suspended isolates in micro centrifuge tubes were then treated with boiling water for 10 minutes. After heat treatment centrifuge tubes were placed immediately in ice for 10 minutes and centrifuged at 1000 rpm for 10 minutes. 2.0µl of the supernatant was taken as DNA template.

3.8.2. Multiplex PCR protocol for the detection of *stx1, stx2, eaeA, and hlyA* genes

Multiplex polymerase chain reaction (mPCR) was carried out as per protocol of Paton and Paton (1998) with slight alteration. The mPCR was carried out in a final reaction volume of 25µl using 0.2ml thin wall sterile and nuclease free PCR tubes (Eppendrof, Germany).The PCR mixture contained a final concentration of 2mM

MgCl2, 0.6mM concentration of each 2□-deoxynucleoside 5□-triphosphate (dNTPs), 5µl of 5X assay buffer,0.5µl of forward and reverse primers, 2.0µl template DNA and 1.0U of GoTaq DNA Polymerase (Promega Corporation, Madison, U.S.A). The optimized components of the reaction mixture are listed in table 3.3.

Table 3.3: Multiplex PCR protocol

Component	**Quantity (µl)**
5X assay buffer	5.0
MgCl2	2.0
dNTPs	1.5
Primers Forward Primers reverse	0.5 each
Taq DNA polymerase (1U)	0.2
Template DNA	2.0
Nuclease free water	10.3
Total	**25µl**

PCR was performed in a thermocycler (Eppendrof, Microcycler) with heated lid using the steps and cycle conditions as given in table 3.3; initial denaturation at 95ºC for 2 minutes followed by 15 cycles; each cycle consisting of denaturation at 95ºC for 1 minute, annealing at 65ºC for 2 minutes and extension for 1.5 minutes at 72ºC. A second phase of 20 cycles was followed with each cycle consisting of denaturation for 1 minute at 95ºC, annealing at 60ºC for 2 minutes and extension for 2 minutes at 72ºC. A final extension was done at 72ºC for 5 minutes. The PCR product was analysed by agarose gel electrophoresis for the amplicon sizes of 180bp, 255bp, 384bp and 534bp.

3.8.3 Cyclic conditions

Table 3.4: **Steps and cyclic conditions of mPCR.**

Step	**Action**	**Temperature (ºC)**	**Time (minutes)**	**No. of cycles**
1	Initial denaturation	95	2	1
2	Denaturation	95	1	15
	Annealing	65	2	
	Extension	72	1.5	
3	Denaturation	95	1	25
	Annealing	60	2	
	Extension	72	2	
4	Final Extension	72	5	1

3.8.4. Agarose Gel Electrophorosis

Agarose gel was made by boiling 1.5 gram of agarose in 100 ml of 1X TBE buffer. Agarose solution was cooled to 50ºC then ethidium bromide solution was added to it at the rate 4µl per 100 ml of the agarose solution. The molten agarose solution was then poured into gel casting tray fitted with two combs. After the solidification the tray containing the gel was placed in a submarine horizontal electrophoresis unit filled with 1X TBE buffer upto the level of 1mm above the gel surface. The combs were carefully removed to form the wells in the gel. On a clean paraffin film 10µl of PCR product was mixed with 2µl of DNA loading dye and then loaded into the well. A 100bp DNA ladder was also loaded in the first well of the gel to serve as molecular size marker. Electrophoresis was performed by applying an electric potential of 140 volts across the two ends of the electrophoresis unit and the moment was observed by monitoring the migration of dye. After a sufficient migration the electric potential was stopped and the gel removed from the TBE buffer, drained and observed under UV transilluminator. Molecular sizes of PCR products were estimated by comparison of their mobility with respect to that of standard molecular size marker (100bp DNA ladder). The PCR product was photographed by the gel documentation system (BioDocAnalyze, Biometra, Germany).

3.9. Antibiogram of the *E. coli* isolates

E. coli isolates were studied for their antibiogram pattern by disc diffusion technique as described by Bauer *et al.* (1966) against a panel of 10 antibiotics. The antibiotic discs used were obtained from Hi Media Laboratories Pvt. Ltd. Mumbai. Isolates were tested for against 10 commonly used antibiotics viz. Amikacin (AK) 30µg, Ampicillin (AMP) 10µg, Amoxicillin (AMX) 10µg, Chloramphenicol (C) 30µg, Co-trimoxazole(COT) 25µg, Gentamicin (GEN) 10µg, Nalidixic acid (NA) 30µg, Norfloxacin (NX) 10µg, Tetracyclin (TE) 30µg and Streptomycin (S) 10µg.

Isolates were inoculated in nutrient broth and incubated at 37^{o}C for 16 hrs. Each broth culture was smeared on Muller-Hinton agar (Hi-Media,Mumbai) plates using a sterile cotton swab. Plates were allowed to dry for few minutes and antibiotic discs were placed on the agar surface and plates were incubated for 12-24 hrs at 37^{o}C. The sensitivity or resistance of isolates for a particular antibiotic was determined by measuring the diameter of the zone of inhibition of growth with Antibiotic zone scale (Hi Media, Mumbai). The results were interpreted as sensitive or resistant based on CLSI interpretive standards (CLSI- 2007).

CHAPTER – IV

RESULTS

CHAPTER-IV

RESULTS

In the present study a total of 170 samples comprising of 30 faecal samples and 30 rectal swabs of each migratory sheep and goats, 25 stool samples and 25 hand swabs of their handlers were collected from in and around Jammu District and processed for the isolation of *E. coli*. Isolates were further subjected to characterization, pathogenicity tests and serotyping. The results of study are presented below.

4.1. Isolation of *Escherichia coli* from sheep, goats and their nomadic handlers.

Characterstic pink coloured and lactose fermenting colonies on MacConkey agar with metallic sheen on EMB agar were contingently considered as *E .coli* (Plates 1 and 2). All the isolates were Gram negative small rods (Plate 3), oxidase negative, catalase positive and produced gas but not H_2S in TSI agar (Plate 4). Then all isolates were subjected to biochemical tests and presented the IMViC pattern as + + - - (Plate 5).Out of the total samples processed, 65 samples revealed the presence of *E. coli*, including 53 samples from sheep and goats and 12 samples from their nomadic handlers.

Table 4.1: *E. coli* isolates obtained from sheep goats and their nomadic handlers.

Species	Type of samples	Samples processed	*E. coli* isolates obtained	*E. coli* percentage
Sheep	Faecal samples	30	15	50
	Rectal swabs	30	12	40
Goat	Faecal sample	30	14	46.6
	Rectal swabs	30	12	40
Handlers	Stool samples	25	12	48
	Hand swabs	25	0	0
Total		**170**	**65**	**38.23**

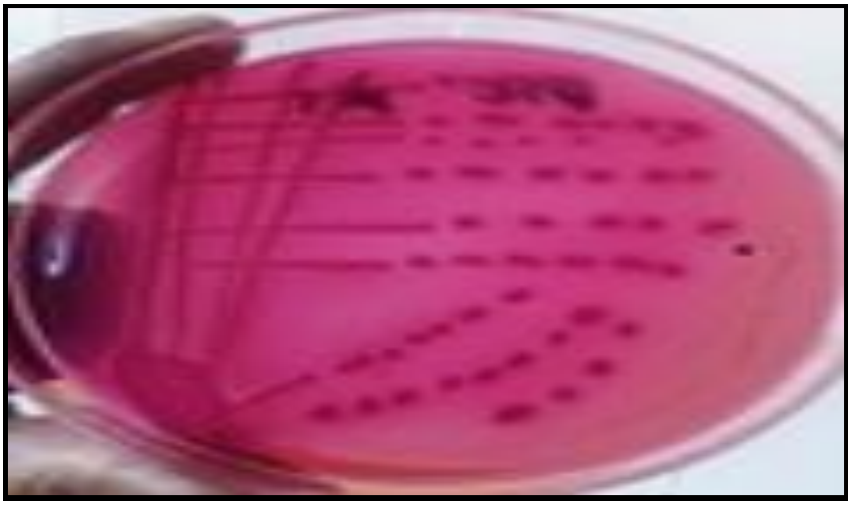

Plate 1: Lactose fermenting *E. coli* colonies on Mac Conkey agar.

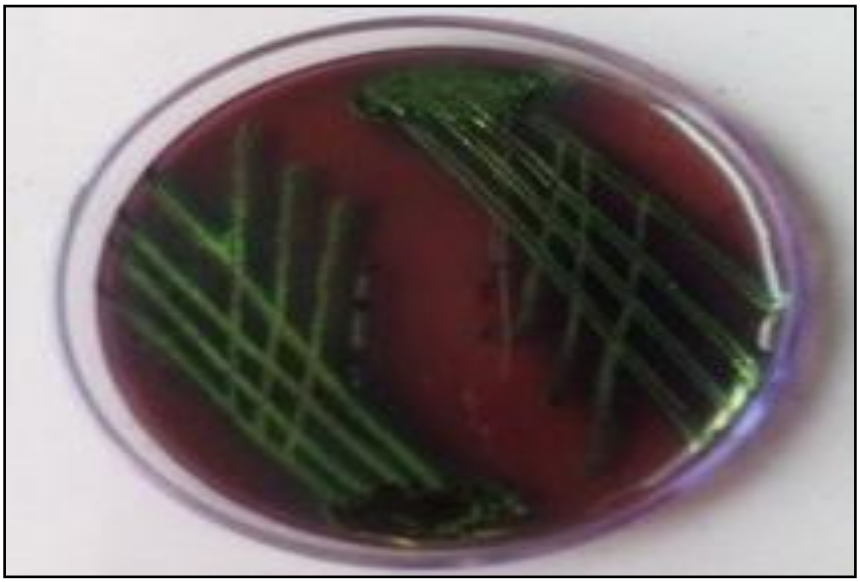

Plate 2: *E. coli* showing metallic sheen on EMB agar.

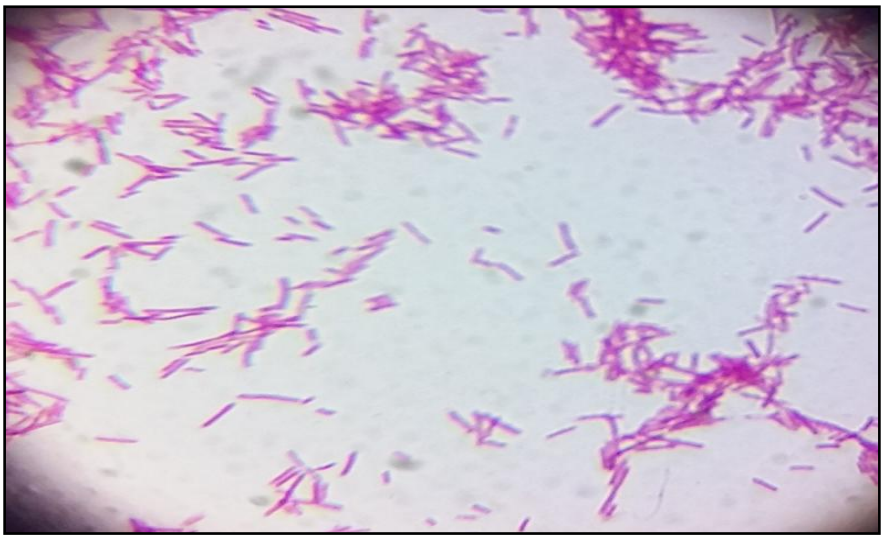

Plate 3: Gram negative rods of *E. coli.*

Plate 4: TSI test of *E. coli* isolates with negative control (-) on left side.

Plate 5: *E. coli* showing (++- -) IMViC test.

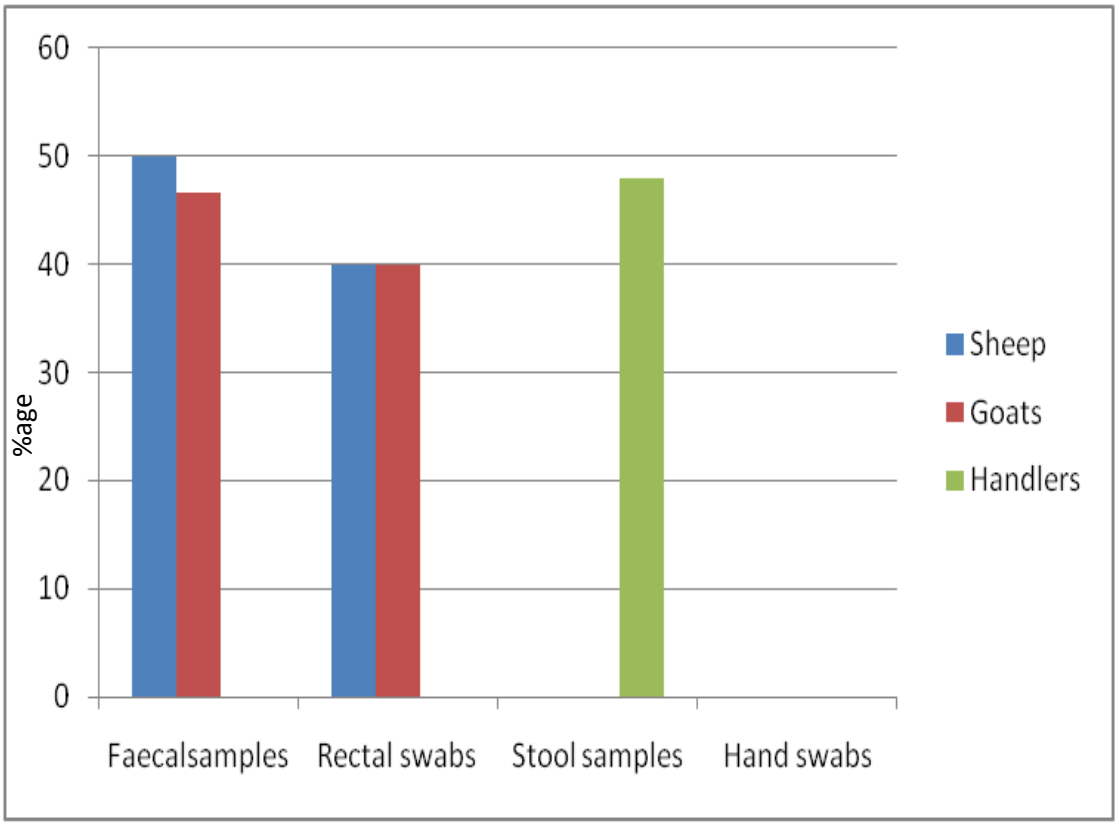

Fig.1: Bar diagram showing prevalence of *E. coli* from sheep, goats and their nomadic handlers

4.2. In vitro pathogenicity of isolated *Escherichia coli*

In vitro pathogenicity of *E. coli* isolates were assessed by using Congo red (CR) dye binding assay and Haemolysis assay. Congo red (CR) dye binding assay is used as a phenotypic marker to distinguish between virulent and avirulent strains of *E. coli*. Virulent strains produced reddish colonies on Congo red medium.

The ability of certain *E. coli* strains to lyse erythrocytes of mammalian species termed as haemolysis. Four different types of haemolysins viz alpha, beta, gamma and enterohemolysin (E-hly) are produced by different pathogroups of *E. coli.*

All 65 isolates (27 of sheep, 26 of goats and 12 of human) were subjected to Congo red dye binding (CR) assay. A total of 28 isolates 15 of sheep and 13 of goats were positive for CR assay (Plate 6), where as none of human isolate was found positive for CR assay. Further, all 65 Isolates (27 of sheep, 26 of goats and 12 of humans) were streaked on sheep blood agar and results revealed that 7 isolates of sheep and 9 isolates of goats produced the desired clear zone of haemolysis (Plate 7) but all of the human isolates were negative for haemolysis. The percent positivity of CR assay was 55.5% for isolates of sheep, 50% for goats and 0% for human isolates while 26.9% isolates from sheep, 36% from goats were found positive for haemolysis assay.

Table 4.2: *E. coli* isolates screened for in vitro pathogenicity.

	Species	**No. of isolates screened**	**Positive isolates**	**Positive %**
Congo red dye Assay	Sheep	27	15	55.55
	Goat	26	13	50
	Handlers	12	0	0
Total		**65**	**28**	**43.15**
Haemolysis Assay	Sheep	27	7	25.9
	Goat	26	9	34.6
	Handlers	12	-	-
Total		**65**	**16**	**24.6**

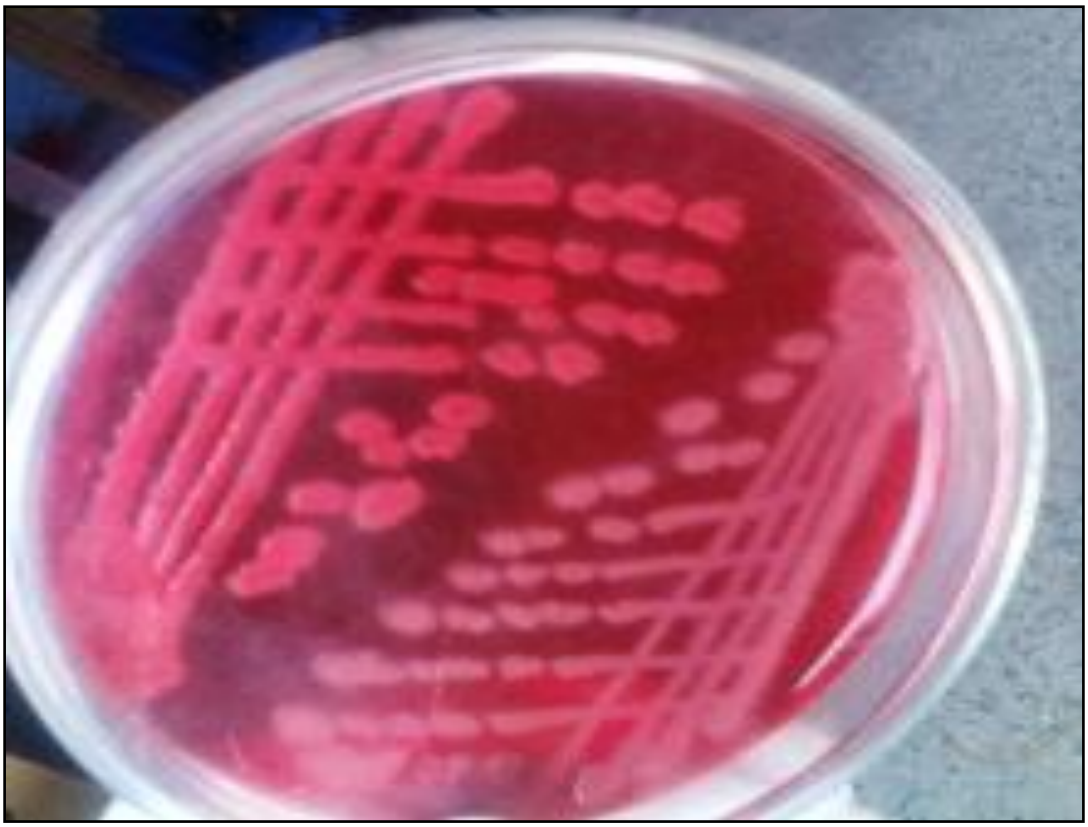

Plate 6: Reddish colonies of *E.* coli on Congo red medium with negative control on right side

Plate 7: Haemolysis on blood agar

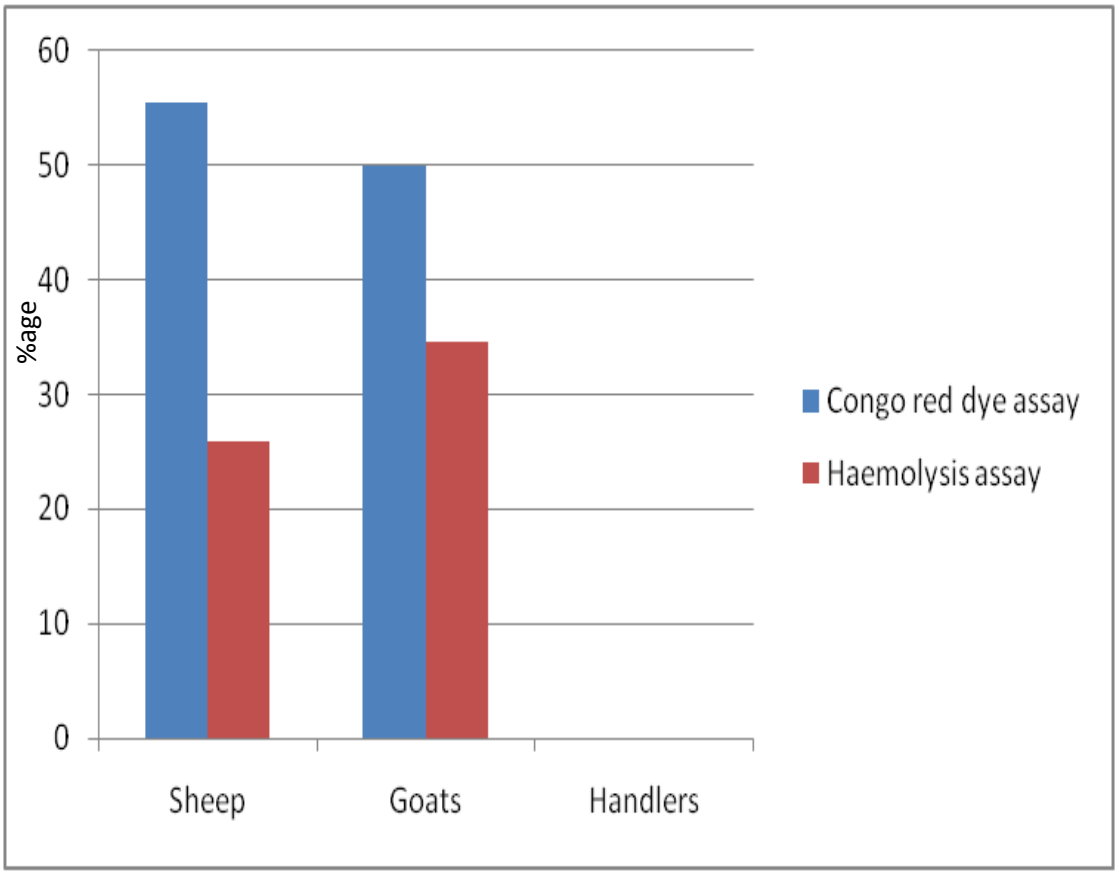

Fig. 2: Bar diagram showing in vitro pathogenicity of *E. coli* isolates, from Sheep goats and their nomadic handlers.

4.3: Serogroups of *E. coli* isolates

Total of 24 *E. coli* isolates (09 of sheep, 13 of goats and 02 of humans) were sent to National Salmonella and Escherichia centre, Central Research Institute Kasauli (HP) for serogrouping. 24 *E. coli* isolates belonged to five different serogroups in addition to untypeable strains were obtained. The most common serogroups were O35 and O126 (each with four isolates) followed by O9, O84, O91 (each with one isolate). Of the total *E. coli* isolates 13 were untypeable.

Table 4.3: Serogroups of *E. coli* obtained from sheep, goats and their nomadic handlers.

Species	No. of isolates serogrouped	Serogroups
Sheep	09	O35 (1), O126 (3), O91 (1), UT (4)
Goats	13	O9 (1), O35 (3), O84 (1), O126 (1), UT (7)
Handlers	02	UT (2)
Total	**24**	**O9 (1), O35 (4), O84 (1), O91 (1), O126 (4), UT (13)**

UT= Untypeable, figures in parenthesis indicate no. of isolates

Table 4.4: Detail of virulence genes in serogrouped *E. coli* isolates from sheep, goats and their handlers.

S.No.	Sample no.	Serogroup	Species	Virulence gene
1	17	O35	Sheep	*hly*A
2	19	UT	Sheep	stx_1
3	23	O91	Sheep	stx_1
4	28	UT	Sheep	*hly*A
5	29	UT	Sheep	stx_2
6	31	UT	Sheep	*hly*A
7	21	O126	Goat	Stx_1, *hly*A
8	24	UT	Goat	stx_1, *hly*A
9	26	O35	Goat	stx_1
10	30	UT	Goat	stx_1,*hly*A
11	36	UT	Goat	stx_2
12	37	UT	Goat	stx_1, *hly*A
13	38	UT	Goat	stx_1
14	39	UT	Goat	Stx_1, *hly*A
15	20	UT	Handler	No gene
16	27	UT	Handler	No gene

4.4. Prevalence of different virulence genes (*stx$_1$, stx$_2$, eaeA, and hlyA*) in *E. coli* isolates from sheep, goats and their nomadic handlers.

A total of 65 *E. coli* isolates, 27 of sheep, 26 of goats and 12 of humans were screened by multiplex polymerase chain reaction (mPCR) (Plate 8) for the presence of *stx$_1$, stx$_2$, eaeA* and *hlyA* genes. Out of 27 *E .coli* isolates of sheep 18 isolates (66.6%) revealed the presence of either of virulence gene which were under study. Genes were detected individually in all the isolates, none of the gene found in combination with other. *stx$_1$* gene was detected in 12 isolates (44.4%), *stx$_2$* and *hlyA* genes were detected in 03 isolates (11.1%). However none of the isolate found positive for *eaeA* gene.

Out of 26 *E. coli* isolates of goats, 13 isolates (50.0%) showed the presence of one of gene which were studied. 12 *E. coli* isolates (46.1%) revealed the presence of *stx$_1$* alone or in combination with *hlyA* gene, where as 01 isolate (3.8%) carried *stx$_2$* alone. Further, *hlyA* gene was detected in eight isolates (30.7%) which was in combination with *stx$_1$* gene while none of the isolates carried *hlyA* gene alone. All the isolates from goats were found negative for *eaeA* gene.

Out of 12 isolates of humans none of them was found positive for any gene.

Table 4.5: Detail of virulence genes (*stx$_1$, stx$_2$, eaeA* and *hlyA*) in *E. coli* isolates from sheep, goats and their nomadic handlers.

Species	No. of isolates screened	Prevalence (%)						
		stx$_1$	***stx$_2$***	***hlyA***	***eaeA***	***stx$_1$* and *stx$_2$***	***stx$_1$*and *hlyA***	**STEC**
Sheep	27	12 (44.4)	3 (11.1)	3 (11.1)	0 (0)	0 (0)	0 (0)	15 (55.5)
Goats	26	4 (15.3)	1 (3.8)	0 (0)	0 (0)	0 (0)	8 (30.7)	13 (50.0)
Handlers	12	0 (0)	0 (0)	0(0)	0(0)	0(0)	0(0)	(0)
Total	**65**	**16 (24.6)**	**4 (6.1)**	**3 (4.6)**	**0 (0)**	**0 (0)**	**8 (12.3)**	**28 (43.0)**

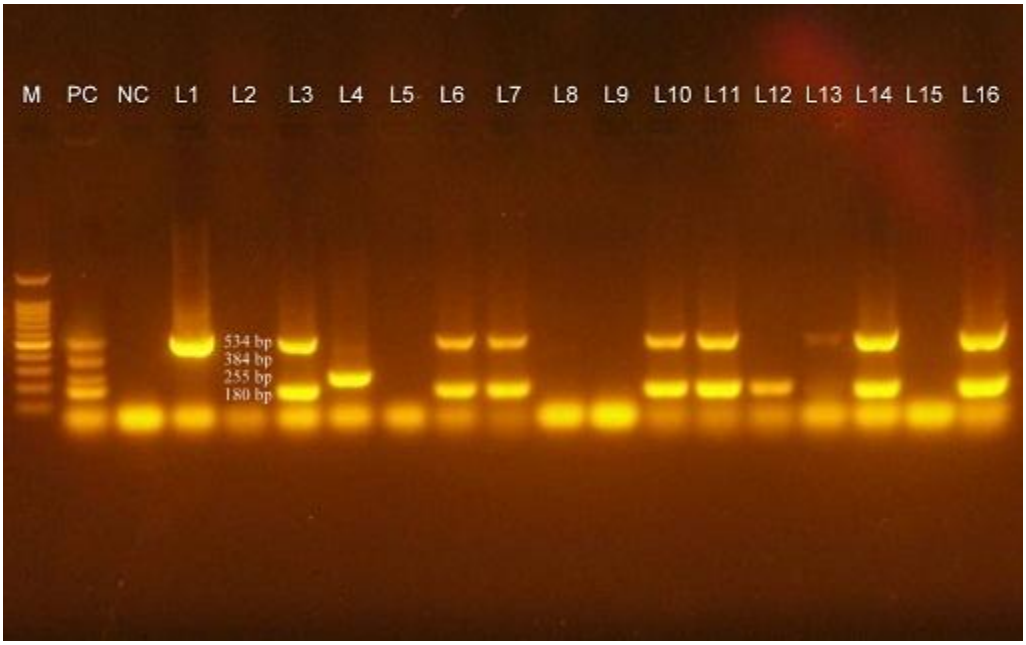

Plate 8: Agarose gel showing mPCR amplification products of *stx*$_1$, *stx*$_2$, and *hly*A genes in *E. coli* isolates

Lane M : 100bp Molecular weight marker

Lane PC : Positive control

Lane NC : Negative control

Lane 1 & 13 : Amplified product of *hly*A gene

Lane 2, 5, 8, 9 & 15 : Negative samples

Lane 3, 6, 7, 10, 11, 14 & 16 : Amplified products of *stx*$_1$ *and hlyA* genes

Lane 4 : Amplified product of *stx*$_2$ gene

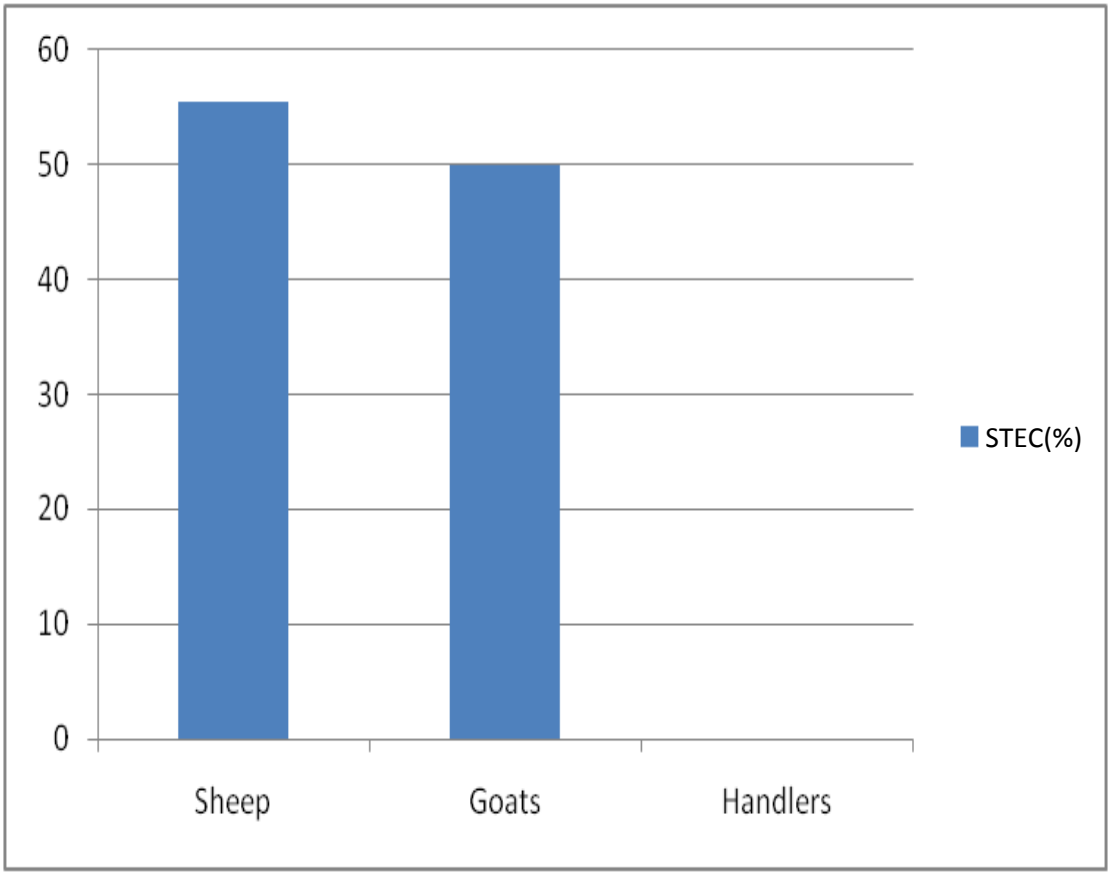

Fig. 3: Bar diagram showing prevalence of STEC in sheep, goats and their nomadic handlers

4.5. Antibiogram pattern of *Escherichia coli* isolates from sheep, goats and their nomadic handlers.

Total of 65 isolates of sheep, goats and their nomadic handlers were analysed for antibiotic sensitivity by disc diffusion method (Plate 9). Results revealed that Norfloxacin was found most potent drug with 84.6% sensitivity followed by Chloramphenicol, Co-trimoxazole, Nalidixic acid and Gentamicin with 80%, 75.3%, 70.7% and 60% sensitivity respectively. Further it was found that 60% of *E. coli* isolates were resistant to Ampicillin and Amikacin, 44.6% to tetracycline, 24.6% to Streptomycin and 13.8% to Nalidixic acid. Also, 50.7% of isolates shown intermediate sensitivity to Amoxicillin, 55.3% to streptomycin, 35.3% to both Gentamicin and Tetracycline, 29.2% to Ampicillin and 24.6% to Amikacin (Table 4.6)

Table 4.6: Antibiotic Sensitivity pattern of *E. coli* isolates sheep, goats and their handlers. (n=20)

S.No.	Antimicrobial Agent	No. of Isolates		
		Sensitive	Intermidiate	Resistant
1	Amikacin (Ak), 30µg	10 (15.3)	16 (24.6)	39 (60)
2	Ampicillin (AMP), 10µg	7 (10.7)	19 (29.2)	39 (60)
3	Amoxicillin (AMX),10µg	26 (40)	33 (50.7)	6 (9.2)
4	Chloramphenicol (C), 30µg	52 (80)	7 (10.7)	6 (9.2)
5	Co-trimoxazole (COT), 25µg	49 (75.3)	6 (9.2)	10 (15.3)
6	Gentamicin (GEN),10µg	39 (60)	23 (35.3)	3 (4.6)
7	Nalidixic acid (NA), 30µg	46 (70.7)	10 (15.3)	9 (13.8)
8	Norfloxacin (NX), 10µg	55 (84.6)	7 (10.7)	3 (4.6)
9	Streptomycin (S),10µg	13 (20)	36 (55.3)	16 (24.6)
10	Tetracycline (TE), 30µg	13 (20)	23 (35.3)	29 (44.6)

Figures in parenthesis indicate percentage out of total number of *E. coli* isolates.

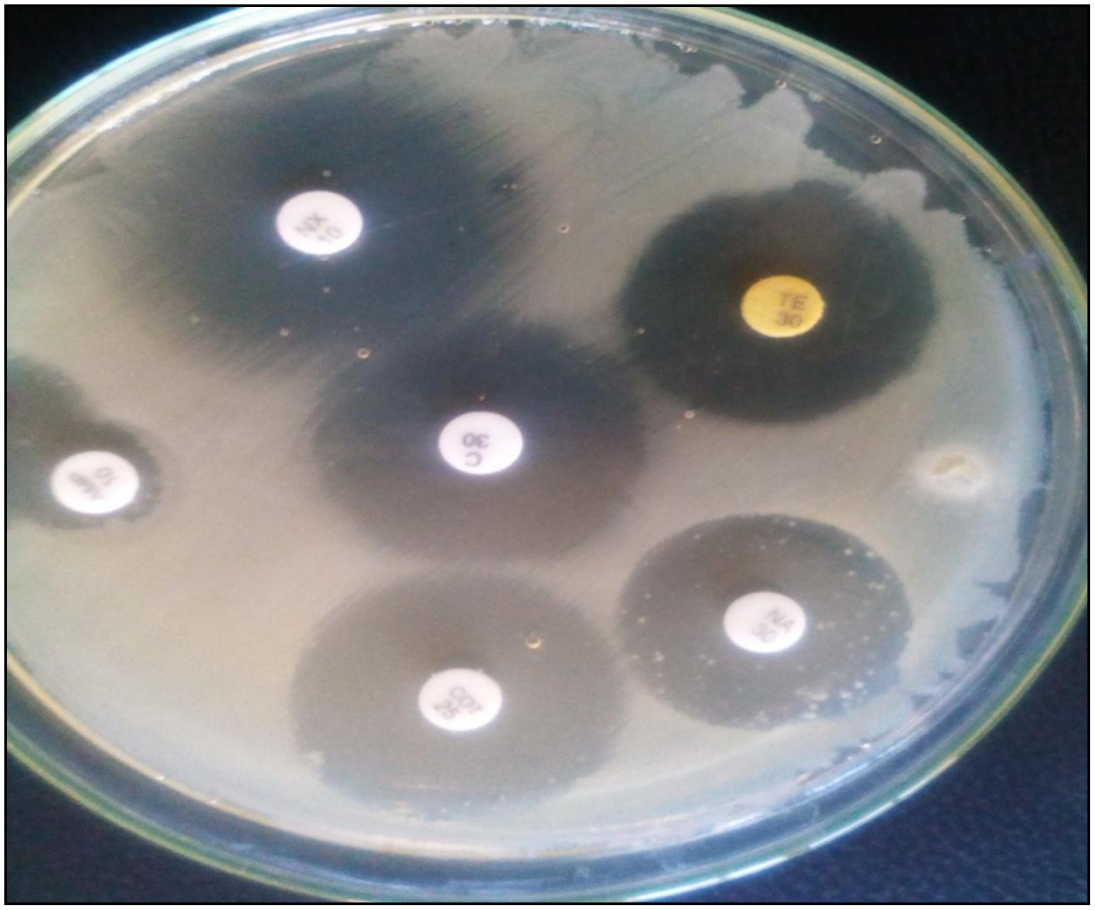

Plate 9: Antibiotic sensitivity test of *E. coli* isolates on Mueller Hinton agar

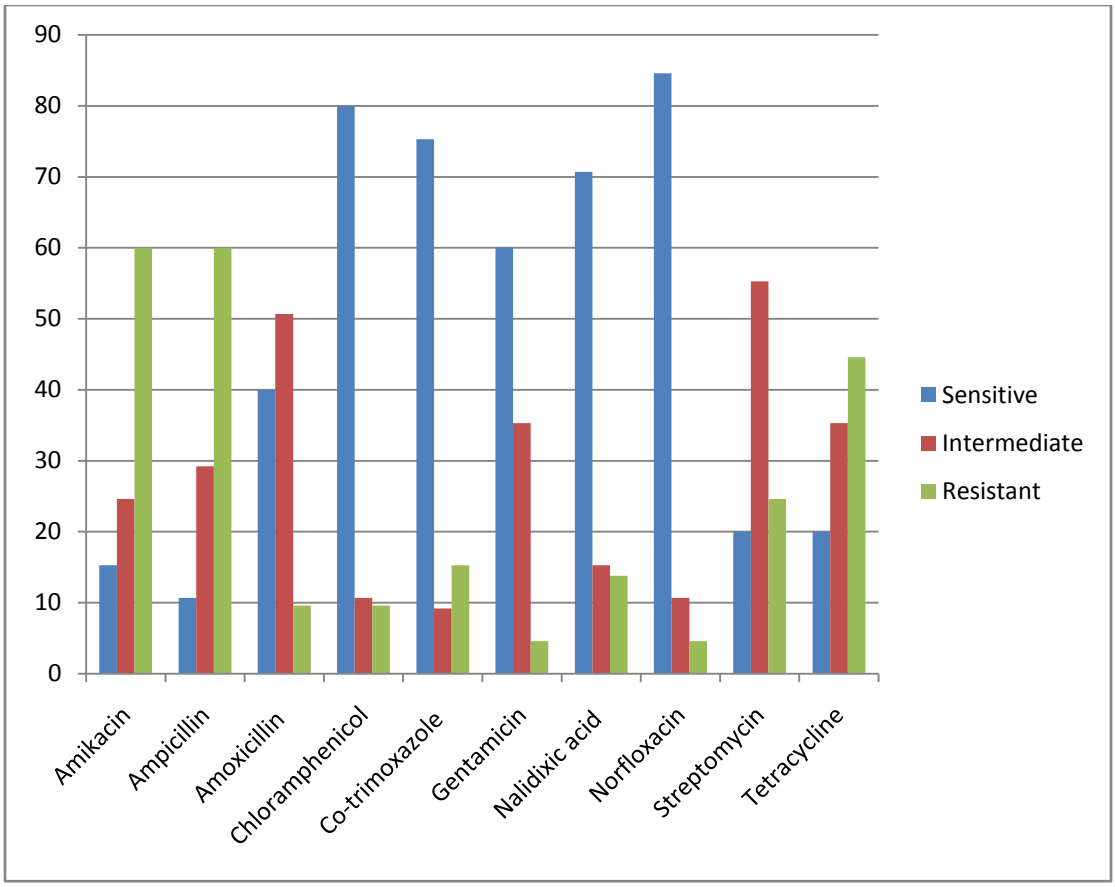

Fig. 4: Bar diagram showing antibiotic sensitivity pattern of *E. coli* isolates

CHAPTER – V

DISCUSSION

CHAPTER-V

DISCUSSION

E. coli has been recognized as a major cause of large scale epidemics of gastrointestinal illness in animals and man (Deshmukh and Karpe, 2006). *E. coli* being an important cause of infant diarrhea and gastrointestinal illness in human, and Shiga-toxin producing *E. coli* infections may result in life threatening sequel such as heamolytic-uremic syndrome an important cause of acute renal failure in children and morbidity and mortality in adults, haemorrhagic colitis and thrombotic thrombocytopenic purpura. Sheep and goats are known to harbour not only strains pathogenic to animals but also strains which cause asymptomatic infections in animals and which can pass through the food chain to cause clinical disease in man (Arshad *et al*., 2006). Sheep and goats have been identified as major reservoirs and food contaminated with faecal material of sheep and goats is a frequent source for human infection (Kiranmayi *et al*., 2010). *E. coli* infection is particularly a challenge for the nomadic peoples as they live in close proximity of sheep and goats, and have no or least knowledge about pathogenicity of bacteria and the transmission of disease.

With this concern the present study was conducted to isolate and characterize the *E. coli* from sheep, goats and their nomadic handlers. To achieve the objective, a total of 170 samples comprising of 30 faecal samples and 30 rectal swabs of each migratory sheep and goats, 25 stool samples and 25 hand swabs of their handlers were collected from in and around Jammu District and processed for the isolation of *E. coli.* Isolates were further subjected to characterization and pathogenicity tests. Of the all 170 samples from sheep, goats and their handlers *E. coli* were isolated from 65 samples, including 27 isolates from sheep, 26 from goats and 12 from their handlers.

In the present study the prevalence of *E. coli* isolates from 60 samples (30 faecal and 30 rectal swabs) of sheep was 45%. These findings are almost similar to that of Purkayastha *et al.* (2010) who reported prevalence of 41.67% of *E. coli* from faecal samples of sheep. Similarly Ahmed *et al*. (2010) observed 36.84% of faecal samples from lambs, positive for *E. coli*. However, Ali (2015) reported 63% of diarrhoeal samples positive for *E. coli*, the probable reason behind this variation may be more shedding of *E. coli* in diarrhoeal cases.

In present study 26 (43.3%) *E. coli* isolates were obtained from 60 samples (30 faecal samples and 30 rectal swabs) of goats. These findings corroborate with Abdullah *et al.* (2010) who reported (41.67%) faecal samples positive for *E. coli* in healthy cases of goats. Similar results were obtained by Adefarakan *et al*. (2014) who also found 42.2% faecal samples from goats, positive for *E. coli.*

In our study, out of 50 samples obtained from sheep and goats handlers, 12 (24%) samples were positive for *E. coli*. These findings are comparable with results of Isibor *et al.* (2013) who reported 32.9% of stool samples of human positive for *E. coli.* Similar results obtained by Kumar *et al.* (2014) who reported 32.5% of stool samples found positive for *E. coli.* However, Virpari *et al*. (2013) found 59% of stool samples positive for *E. coli.* This variation in results may be because in present study hand swabs of handlers were also taken which may have decreased the percentage of *E. coli* isolates because of frequent washing of hands.

All of 65 *E. coli* isolates obtained from sheep, goats and their handlers were tested for in vitro pathogenicity using Congo red (CR) dye binding assay and results revealed that 43.5% isolates were found virulent. These results are comparable with Parul *et al.* (2014) who reported 44.28% of *E. coli* isolates as virulent. Similar results were obtained by Sharma *et al* (2006) who found 47.42% of *E. coli* isolates as virulent on using Congo red dye. However, Sayed (2014) reported 33.3% of *E. coli* isolates were invasive, possible reason could be different source of samples and also may be because of difference within serovars of *E. coli* in our study and that of Sayed (2014).

In present study a total of 65 *E. coli* isolates obtained from sheep, goats and their handlers were tested for haemolysis assay using 5% sheep blood agar and results revealed that 24.6% of isolates produced clear zone of haemolysis on blood agar. These results are in agreement with those of Olowe *et.al.* (2008) who reported 26.8% *E. coli* isolates shown haemolytic activity on blood agar, where as results are in contrast with those of Atere *et al*. (2015) who reported 89.58% *E. coli* isolates positive for haemolytic activity. In a similar study Sayed (2014) reported 5.6% of *E. coli* isolates shown haemolytic activity. Parul *et. al.* (2014) also found 4.28% of *E. coli* isolates had shown haemolytic activity on blood agar. Ranjan *et al*. (2010) reported 41.36% of *E. coli* isolates shown haemolytic activity on blood agar. Osman *et al.* (2012) reported 100% haemolytic activity of *E. coli* isolates on blood agar, these

variations could be because of different strains of *E. coli*, as virulence factors vary among strains of *E. coli*.

Out of total isolates obtained in the present study, 24 *E. coli* isolates were serogrouped and they belonged to five different serogroups in addition to untypeable with the common serogroups being O35 and O126 (each with four isolates) while one isolate each belonged to serogroups O9, O84, O91 and thirteen isolates were untypeable. All of the serogroups obtained in present study have earlier been reported. O9, O35 have been reported by wani *et.al.* (2005) and Fuente *et al.* (2002) from sheep and goats. Serogroup O84 was earlier reported by Mahanti *et al.* (2015) from faecal samples of goats. Serogroup O91 was earlier reported by Manzoor *et al.* (2015) from lambs and calves and by Wani *et.al.* (2005) from goats and serogroup O126 was earlier reported by Lamey *et al.* (2013) and Sayed (2014) from mastitis milk.

In the present study 27 *E. coli* isolates obtained from sheep were subjected to multiplex PCR (mPCR) for detection of shiga toxin producing *E. coli* (STEC) and results revealed that 55.5% of isolates found positive for STEC. These findings are in accordance with that of Kiranmayi *et al.* (2011) who reported 54.80% prevalence of STEC from faecal samples of sheep. However, results are in contrast to that of Ferreira *et al*. (2015) who reported 78.3% of faecal samples from sheep found positive for STEC, it could be because of different geographical conditions. In present study prevalence of stx_1 is 44.4% and that of stx_2 is 11.1%, and these findings are almost similar that of Bhat *et al.* (2007) who reported 42.8% isolates carried stx_1 and 11.4% isolates carried stx_2. Where as prevalence of *hly*A in our study was 11.1% and it is in contrast to that of Bhat *et al.* (2007) who obtained 80% prevalence of *hly*A. It may be because of the fact that the strains of *E. coli* isolated in our study may be different from those obtained by Bhat *et al.* (2007).

In present study 26 isolates of *E. coli* obtained from goats were subjected to multiplex PCR (mPCR) for detection of shiga toxin producing *E. coli* (STEC) and results revealed that 50% of isolates were found positive for STEC. These results are supported by Cortes *et al*. (2005) who reported 47.7% of faecal samples of healthy goats were positive for STEC. Similar results were obtained by Beutin *et al.* (1993) who observed 56.1% prevalence of STEC in faecal samples of goats. In present study mPCR showed that out of total 13 STEC isolates obtained from goats, 04 isolates (15.3%) possessed stx_1 gene alone and eight isolates (30.7%) possessed *stx*1 in

combination with *hly*A gene, one isolate (3.8%) possessed stx_2 gene alone and none of STEC isolates found positive for *eae* genes alone or in combination with others . These findings are almost comparable with that of Cortes *et al*. (2005) and Wani *et al*. (2005) who reported higher prevalence of stx_1 gene than that of stx_2 gene and none of isolates carried *eae* gene. The prevalence of *hly*A in present study which is 30.7% is in contrast to previous study by Mahanti *et al.* (2015) who reported 77.7% of *hly*A gene in faecal samples of goats and probable reason may be difference in the type of the bacterial strains obtained in both studies. However, the prevalence of STEC obtained in our study isn't in line to that obtained by Wani *et al*. (2005) who reported 16.47% of faecal samples from goats found positive for STEC. Similar study was done by Mahanti *et al.* (2015) in west Bengal who reported 14.7% prevalence of STEC. These differences could be because of seasonal variation, diet and different environmental conditions. Moreover, the sampling in our study was carried out in the migratory flocks of sheep and goats reared by nomadic people of J&K, who don't follow the hygienic practices. In addition to this isolation of the sick animals from the flock is very rare in this ethenic group (nomads). These factors may be responsible for the higher degree of prevalence of STEC in our study as compared to some of the other studies conducted.

In present study, 12 human isolates were subjected to mPCR, none of humans isolate was found positive for STEC and it may be because that samples were collected from apparently healthier persons.

In the present study 65 isolates were tested for antibiotic sensitivity pattern. Norfloxacin was found most potent drug with 84.6% sensitivity followed by Chloramphenicol, Co-trimoxazole, Nalidixic acid and Gentamicin with 80%, 75.3%, 70.7% and 60% sensitivity respectively. High sensitivity of *E. coli* isolates to Norfloxacin, chloramphenicol, co-trimoxazole and gentamicin was also reported by Begum *et al.* (2015) and Rashid *et al.* (2006). Results also revealed high resistance of *E. coli* isolates towards Ampicillin and Amikacin which also stands reported by Rashid (2011) and Radwan *et al.* (2014).

CHAPTER – VI

SUMMARY AND CONCLUSIONS

CHAPTER-VI

SUMMARY AND CONCLUSION

The present study was designed to isolate and characterize *E. coli* from sheep, goats and their nomadic handlers from in and around Jammu District. A total of 170 samples comprising of 120 samples of sheep and goats and 50 samples of their handlers were collected and processed for the isolation of *E. coli.* The *E. coli* isolates were isolated and identified on the basis of cultural, morphological and biochemical characteristics. The *E. coli* isolates were subjected for in vitro pathogenicity using Congo red (CR) dye binding assay and haemolysis assay. Multiplex polymerase chain reaction (mPCR) of *E. coli* was done as per the Paton and Paton (1998) to identify the STEC. Antibiotic sensitivity pattern of isolated *E. coli* to various antibiotics was also studied.

In present study a total of 170 samples were processed, *E. coli* was isolated from 65 samples; 27 from sheep, 26 from goats and 12 from their handlers. 24 *E. coli* isolates were serogrouped, the most common serogroups obtained were O35 and O126 (each with four isolates) followed by O9, O84, O91 (each with one isolate). Of the total *E. coli* isolates 13 were untypeable.

All the 65 isolates from sheep, goats and their handlers were subjected to Congo red (CR) dye binding assay and haemolysis assay for in vitro pathogenicity, and results revealed that 43.15% and 24.6% of isolates from all sources were found positive for Congo red medium assay and haemolysis assay respectively.

All the 65 *E. coli* isolates, from sheep, goats and their handlers were screened by multiplex PCR for presence of stx_1, stx_2, *eae* and *hly*A genes. Results revealed that 31 isolates found positive for one or more virulence genes from sheep and goats but none of the isolate from handlers found positive for any pathogenic gene. The stx_1 gene was found more frequent (45.2%) from sheep and goats alone or in combination with *hly*A, followed by *hly*A (20.7%) and stx_2 (7.5%) where as none of the isolate found positive for *eae* gene. Overall prevalence of STEC in sheep and goats was found as 52.8%.

The results of antibiotic sensitivity and resistance pattern of *E. coli* isolates revealed that Norfloxacin was most potent drug with 84.6% sensitivity followed by

Chloramphenicol, Co-trimoxazole, Nalidixic acid and Gentamicin with 80%, 75.3%, 70.7% and 60% sensitivity respectively. 60% of *E. coli* isolates showed resistant to both Ampicillin and Amikacin.

The current study led to the following conclusions and recommendations:

1. High Prevalence of *E. coli* in general and STEC in particular in sheep and goats indicates that sheep and goats are important reservoir for *E. coli.*

2. Prevalence of *E. coli* in both sheep and goats observed in present study were almost similar thus both animals may be equally important source for human infection particularly those who live in close proximity of these animals.

3. Results of in vitro pathogenicity indicate that the apparently healthy animals can carry pathogenic strains of *E. coli.*

4. Resistance to the frequently used antibiotics indicates a need of judicious use of antibiotics in the field.

5. Public Health awareness including safe and hygienic practices, are of prime importance in decreasing the occurrence of *E. coli* infection and its spread to humans especially to the individuals closely associated with rearing and management of sheep and goats.

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CERTIFICATE-IV

Certified that all the necessary corrections as suggested by the external examiner/evaluator and the advisory committee have been duly incorporated in the thesis entitled **"Studies on isolation and characterization of *Escherichia coli* from sheep, goats and their handlers"** submitted by **Mr.Irfan Ashraf**, Registration No. **J-14-MV-378.**

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