MOLECULAR EPIDEMIOLOGICAL STUDY ON SHEDDING PATTERN OF *E.COLI* O157:H7 IN BOVINES WITH RESPECT TO BREED, AGE AND SEASON

By

PRADEEP KUMAR
M.V.Sc.
RVD/2012-06

THESIS SUBMITTED TO THE
SRI P.V.NARSIMHA RAO TELANGANA STATE UNIVERSITY FOR VETERINARY, ANIMAL AND FISHERY SCIENCES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

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(VETERINARY PUBLIC HEALTH)

DEPARTMENT OF VETERINARY PUBLIC HEALTH & EPIDEMIOLOGY
COLLEGE OF VETERINARY SCIENCE
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SRI P.V.NARSIMHA RAO TELANGANA STATE UNIVERSITY FOR VETERINARY, ANIMAL AND FISHERY SCIENCES, HYDERABAD-500 030
(TELANGANA) INDIA

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2016

DECLARATION

I, **PRADEEP KUMAR, RVD/2012-06** hereby declare that the thesis entitled **"MOLECULAR EPIDEMIOLOGICAL STUDY ON SHEDDING PATTERN OF *E.COLI* O157:H7 IN BOVINES WITH RESPECT TO BREED, AGE AND SEASON"** submitted to **SRI P.V.NARSIMHA RAO TELANGANA STATE UNIVERSITY FOR VETERINARY, ANIMAL AND FISHERY SCIENCES, HYDERABAD** for the degree of **DOCTOR OF PHILOSOPHY** is the result of original research work done by me. It is further declared that the thesis or any part thereof has not been published earlier in any manner.

Date: **(PRADEEP KUMAR)**

CERTIFICATE

Mr. PRADEEP KUMAR has satisfactorily prosecuted the course of research and that the thesis entitled **"MOLECULAR EPIDEMIOLOGICAL STUDY ON SHEDDING PATTERN OF *E.COLI*O157:H7 IN BOVINES WITH RESPECT TO BREED, AGE AND SEASON"** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any University.

Date: **(Dr. N. KRISHNAIAH)**

Place: Hyderabad **Major Advisor**

CERTIFICATE

This is to certify that the thesis entitled **"Molecular epidemiological study on shedding pattern of *E.coli* O157:H7 in bovines with respect to breed, age and season"** submitted in partial fulfilment for the degree of **DOCTOR OF PHILOSOPHY** of the SRI P.V.N.R. TELANGANA STATE UNIVERSITY FOR VETERINARY, ANIMAL AND FISHERY SCIENCES, HYDERABAD, is a record of the bona fide work carried out by D**r. PRADEEP KUMAR, RVD/2012-06** under my guidance and supervision. The subject of the thesis has been approved by student's advisory committee.

No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged. All the assistance and help received during the course of investigation have been duly acknowledged by the author of the thesis.

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(*PRADEEP KUMAR*)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Cont…)

	of bovines

LIST OF FIGURES (Cont…)

LIST OF ABBREVIATIONS

%	: Percent
µL	: Microliter
°C	: Degree Celsius
AFLP	: Amplified fragment-length polymorphisms
bp	: Base pair
CDC	: Centers for disease control and prevention
CFU	: Colony forming unit
CNS	: Central Nervous System
CT	: Cholera toxin
CT- SMAC agar	: Cefixime Tellurite Sorbitol MacConkey agar
DAEC	: Diffusely adherent *E. coli*
DNA	: Deoxyribonucleic acid
dNTPs	: Deoxynucleoside tri phosphate
E. coli	*: Escherichia coli*
E.coli O157:H7	: *Escherichia coli* O157:H7
EAEC or EAggEc	: Enteroaggregative *E. coli*
EAST	: Enteroaggregative heat-stable toxin
EC O157	: *Escherichia coli* O157
EC O157:H7	: *Escherichia coli* O157:H7
EHEC	: Enterohaemmorhagic *E. coli*
EHEC O157	: Enterohaemmorhagic *E. coli* O157
EHEC O157:H7	: Enterohaemmorhagic *E. coli* O157:H7
EIEC	: Enteroinvasive *E. coli*
ELISA	: Enzyme Linked Immuno Sorbent Assay
EMB	: Eosin methylene blue agar
EPEC	: Enteropathogenic *E. coli*
et al	*: et alii*
ETEC	: Enterotoxigenic *E. coli*
FLASH-PCR	: Fluorescent amplification-based specific hybridization PCR flight mass spectrometry
G3b	: Globotriaosylceramide
GIT	: Gastro Intestinal Tract
H antigen	: Flagellar antigen
HC	: Hemorrhagic Colitis

hly	: Haemolysin
HUS	: Hemolytic Uremic Syndrome
IMS	: Immunomagnetic separation
IMVC	: Indole test, Methyl Red test, Voges- Proskauer test, Citrate utilization test
K antigen	: Capsular antigen
kDa	: Kilo Dalton
LPS	: Lipopolysaccharide
LSPA	: Lineage-specific polymorphism assay
LT	: Heat-labile
MALDI-TOF-MS	: Matrix-assisted laser desorption/ionization time-of-flight
$MgCl_2$	: Magnesium chloride
ml	: Milliliter
mPCR/multiplex PCR	: Multiplex polymerase chain reaction
mTSB	: Modified tryptone soy broth
MUG	: 4-methyl umbeliferone glucoronide
O antigen	: Somatic antigen
P value	: Probability value if null hypothesis is true
PCR	: Polymerase chain reaction
PFGE	: Pulse-field gel electrophoresis
pO157	: Plasmid O157
RAJ	: Recto Anal Junction
RFLP	: Restriction fragment length polymorphisms
sec	: Seconds
SMAC agar	: Sorbitol MacConkey agar
SNP	: Single nucleotide polymorphism
ST	: Heat-stable
STEC	: Shiga toxin producing *E. coli*
stx1	*:* Shiga toxin 1
stx2	: Shiga toxin 2
TAE	: Tris acetate EDTA buffer
Taq	*: Thermus aquaticus*
TTP	: Thrombotic Thrombocytopenic Purpura
TTSS	: Type III secretion system
UV	: Ultraviolet
vis-a-vis	*:* compared to

viz	*:*	Videlicet (namely)
VTEC	:	Verocytotoxigenic *E. coli*
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ABSTRACT

Escherichia coli O157:H7 is an emerging zoonotic pathogen. Bovines are thought to be primary reservoir of this pathogen. Animal based foods contaminated with the faeces of bovines would cause foodborne infections in humans with this pathogen emphasising its public health importance. Conventional culture based method for the identification of *E. coli* O157:H7 is time consuming, needs a lot of media and supplement components and there is a possibility to get false results. And research work on epidemiology of shedding pattern of *E. coli* 0157:H7 with respect to age, breed, sex and seasons, genetic and physiological factors of animals and environmental factors is very meagre.

Hence, the present study was undertaken to standardize multiplex PCR technique for the identification of this pathogen by targeting its six virulence genes; *fliCh7, rfbE, eaeA, hlyA, stx1,* and *stx2* genes to study the epidemiology of shedding pattern of *E. coli* 0157:H7 with respect to age, breed, sex and seasons, genetic and physiological factors of animals and environmental factors.

The standardized multiplex PCR produced amplicons of the size, 625 bp, 397bp, 296 bp, 166 bp, 210 bp and 484 bp, respectively for the *fliCh7, eaeA, rfbE, hly, stx1,* and *stx2* virulent genes. The best DNA bands were obtained at MgCl2 concentrations of 3mM, denaturation at 94˚C for 45 sec and annealing temperature of 60°C and agarose concentration of 1.5%. The threshold sensitivity of standardized multiplex PCR was found to be 0.1 CFU/ml for reference culture of *E. coli* O157:H7, 1 CFU/g for spiked faecal samples and 0.1CFU/g for spiked faecal samples with one step enrichment. High

absolute specificity of the standardized multiplex PCR was observed as none of the six DNA bands was found in the PCR product of negative control cultures.

A total of 932 (n) fecal samples were collected from cattle and buffalo farms in different parts of Karnataka from July 2014 to January 2016 and analyzed for the identification and molecular characterization of E. *coli* O157:H7 by using cultural identification, cultural isolation with biochemical characterization and multiplex PCR. When compared to latex agglutination test, the epidemiological sensitivity and specificity of cultural identification were 100% and 83.35%, respectively, of cultural isolation with biochemical characterization were 100% and 92.51%, respectively, of multiplex PCR were 95.60% and100%, respectively when faecal samples were directly analysed and of multiplex PCR were 100% and 98.69%, respectively when faecal samples with one step selective enrichment were analyzed.

The prevalence of shedding of *E. coli* O157:H7 in bovines was found to be10.94%. The percent of cattle and buffaloes shedding *E. coli* O157:H7 was 12.10% and 5.55%, respectively. In cattle, the percent of exotic breeds, crossbreds and indigenous breeds shedding *E. coli* O157:H7 was 18.56%, 13.88% and 4.41%, respectively. Age wise, the percent of shedding of *E. coli* O157:H7 in calves, heifers and adult animals was 14.80%, 12.12% and 8.60%, respectively. Sex wise, the percent of male and female animals that showed shedding of *E. coli* O157:H7 was 15.17% and 9.95%, respectively.

The percent of bovines shedding *E. coli* O157:H7 in winter, monsoon and summer seasons was 14.75%, 10.85% and 7.86%, respectively. Maximum percentage of shedding was observed in the month of January and least in the month of May. Depending on environmental temperature, highest percentage of bovines shedding *E. coli* O157:H7 in faeces was in the temperature range of 20.1-25.0°C and at particular temperature of 22.1-23.0°C indicating that biologically favourable environmental temperature supports the shedding of this pathogen.

The percent of healthy and diarrhoeic animals shedding *E. coli* O157:H7 was 12.06% and 2.68%, respectively, indicating that healthy bovines are more prone for shedding of this pathogen. There was no significant difference in shedding of *E. coli* O157:H7 at *P* value of <0.05 among lactating and pregnant (10.66%), lactating and non pregnant (8.54%), and dry pregnant (9.68%) animals. In contrast to these three groups, the shedding of *E. coli* O157:H7 was significantly very low in non lactating and non pregnant animals (only 1.45%).

Apart from 102 *E. coli* O157:H7 positive samples, the percent of animals that showed shedding of *E. coli* O157 in the faeces was 13.41% and the percent of animals that showed shedding of STEC was 16.52 %.

Highest percent of *E. coli* O157:H7 positive samples were resistant for Nitrofurantoin (100%) followed by Carbenicillin (92.16%), Co-Trimazine (87.25%), Tetracycline (75.49%), Kanamycin (69. 61%), Amikacin (46.08%), Streptomycin (23.53%) and Ciprofloxacin (only 2.94%). The *E. coli* O157 positive samples showed highest resistance against Nitrofurantoin (100%) followed by Carbenicillin (88.80%), Co-Trimazine (83.20%), Tetracycline (65.60%), Kanamycin (60.00%), Amikacin (46.4%), Streptomycin (20.8%) and Ciprofloxacin (only 2.4%) and STEC positive samples showed highest resistance against Nitrofurantoin (99.35%) followed by Carbenicillin (88.31%), Co-Trimazine (73.38%), Tetracycline (69.48%), Kanamycin (52.60%), Amikacin (40.91%), Streptomycin (20.78%) and Ciprofloxacin (only 3.25%). The pattern is similar to that of *E. coli* O157 except the difference in the percentages.

The results found in the present study, give enough indication about epidemiological factors influencing the shedding pattern of *E. coli* O157:H7 in bovines. The association of these factors suggests strategies to reduce its shedding by applying the findings in to the principles of hazard analysis critical control points (HACCP) to strategise pre-harvest preventive and control measures so that the incidence of foodborne outbreaks of this foodborne pathogen in humans could be reduced to great extent.

CHAPTER I

INTRODUCTION

Escherichia coli are Gram negative rods (bacillus), facultative anaerobic bacteria in the family *Enterobacteriaceae* that readily grow on simple bacteriological media. The organism was discovered by Dr. Theodor Escherich in 1885. According to the modified Kauffman scheme, *E. coli* are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profile (Lior, 1996; Nataro and Kaper, 1998). A specific combination of O and H antigens defines the "serotype" of an isolate (Nataro and Kaper 1998).

Based on possession of virulence factors such as exotoxins, pathology they cause and their interactions with eukaryotic cells, six pathotypes of *E. coli* have been distinguished: Enterotoxigenic *E. coli* (ETEC) (Dalton *et. al*., 1999), Enteropathogenic *E. coli* (EPEC) (Ruchaud-Sparagano *et. al.*, 2007), Enteroinvasive *E. coli* (EIEC) (Renata, 2013), Enterohemorrhagic *E. coli* (EHEC)/Shiga toxin-producing *E. coli* (STEC) (Takeda, 2011), Enteroaggregative *E. coli* (EAEC or EAggEc) (Beauchamp and Sofos, 2010), and diffusely adherent *E. coli* (DAEC) (Scaletsky, 2002). The *E. coli* which produce shiga toxins (*Stx)* have been referred as shiga toxin producing *E. coli* (STEC). A subgroup of STEC cause watery diarrhoea in humans which can progress to haemorrhagic colitis and potential systemic complications due to the action of *Stx* (Kaper *et. al.*, 2004). These are referred as Enterohaemmorhagic *E. coli* (EHEC).

EHEC strains are defined based on virulence genes; *Lee* (*locus of enterocyte effacement) Pai* (41 kb) virulence plasmid (*viz;* pO157), type III secretion system (TTSS) and shiga toxin I (*stx1*) and/or shiga taxin II (*stx2*) production (Paton and Paton, 2002 and Wang *et. al.*, 2002). Phenotypically these *E. coli* show "attaching and effacing" phenomenon characterized by the elimination of the microvilli and intimate

enterocyte attachment (Kaper *et. al.,* 2004, Tobey *et. al.,* 2006 and Pennington, 2010). The Shiga toxins identified in EHEC are classified in two distinct subgroups: *Stx*1 and *Stx*2.

About 200 EHEC serotypes have been isolated from animal and food sources (Denis *et. al*., 2012). The most significant EHEC of humans is *E. coli* O157:H7. After the first outbreak of bloody diarrhea in Oregon and Michigan, U.S.A. in 1982, *E.coli* O157:H7 has become the most widely known EHEC strain (Karmali *et. al.,* 1983, Riley *et. al.,* 1983, Wells *et. al.,* 1983). Bovines are thought to be reservoir of this pathogen (Borczyk *et. al.,* 1987; Gannon *et. al.,* 2002; Soderlund *et. al.,* 2012).

Escherichia coli O157:H7 is mainly pathogenic to humans (Capriola *et. al.,* 2005; Pennington, 2010; Soderlund *et al.,* 2012). It will not cause any clinical disease except diarrhoea (rarely) in cattle and other animals (Brown *et. al.,* 1997) due to the difference in distribution of Gb3 receptors between cattle and humans (Smith *et. al.,* 2002). For humans, a minimal cell count of about 10–100 is sufficient to cause serious complications (Keene *et. al.,* 1994). The infection is transmitted to humans by faecal-oral route through contaminated food or water. Contaminated undercooked ground beef and hamburgers have been the most frequently identified vehicle of *E.coli* O157:H7 in human disease outbreaks (Griffin and Tauxe, 1991). The first major outbreak of *E. coli* O157:H7 occured in the year 1982 in US caused by hamburger consumption (Wells *et. al.,* 1983 and Riley *et. al.,* 1983).

Many other foods have acted as vehicles for the transmission of *E. coli* 0157:H7; ready-to-eat cold meats including poultry, pork and beef products, cheese, milk (Bhat *et. al*., 2007 Guh *et. al.,* 2010 and Ebrahim *et. al*., 2012); butter (Rangel *et. al*., 2005); yoghurt (Besser *et. al*., 1993), apple juice, coleslaw; lettuce (Hilborn *et. al*., 1999), spinach, sprouts, melons (Rangel *et. al.,* 2005) and fresh vegetables (Ongeng *et. al*., 2013 and Duncan *et. al*., 2014) and ice cream. Drinking water and recreational waters

have also been the sources of *E. coli* O157:H7 that caused human illness outbreaks (Bruce *et. al.*, 2003; Davis *et. al.*, 2005).

The clinical signs and symptoms of *E.coli* O157:H7 infections in humans include bloody or non-bloody diarrhoea, abdominal cramps, vomiting and little or no fever - Hemorrhagic Colitis (HC), post- diarrheal Hemolytic Uremic Syndrome (HUS) and Thrombotic Thrombocytopenic Purpura (TTP) (Gyles, 2007, Karmali *et al* ., 2010; Pennington H. 2010 and Jeshveen *et al* ., 2012). The illness resolves in 5-10 days (Tarr, 1995). Hemolytic ureamic syndrome (HUS), a life threatening complication, is developed by about 10% of patients, mostly in elderly people and children (Blackall and Marques, 2004 and Reiss *et al.*, 2006; Faten and Afaf, 2013). Thrombotic Thrombocytopenic Purpura (TTP) is considered to be a manifestation of HUS, in elderly, where renal failure is normally mild but neurological involvement is greater with a mortality rate as high as 50% (Griffin, 1995; Pennington, 2010).

The primary reservoir of this serotype is the cattle (Elaine and James, 2010; Eppinger *et al*., 2011; Soderlund *et al.*, 2012). It has been reported that a region of lymphoid follicle-dense epithelium a short distance proximal to the recto-anal junction is the principal site of *E. coli* O157:H7 persistence in adult cattle (Pablo *et al.*, 2008; Michael *et al.*, 2011). Cattle with *E. coli* O157:H7 colonized at this site shed higher numbers of organisms for a longer period than those colonized at other sites (Cobbold and Desmarchelier, 2004, Cobbold *et al.*, 2007). Such cattle are designated as "supershedders". Research studies investigating the frequency of *E. coli* O157:H7 serotype in buffaloes is limited.

Conventional culture method for the identification of *E. coli* O157:H7 is time consuming, needs a lot of media and supplement components (Visetsripong *et al.*, 2007) and there is a possibility to get false results (Orth *et al.*, 2009). Several immunological tests are being used for the detection of *E. coli* O157:H7, i.e. ELISA based

Immunobiosensor Chips (Chuanmin Ruan *et. al.,* 2002**),** described integrating waveguide biosensor based on a fluorescent sandwich immunoassay (Peixuan *et al*., 2005), antibody-based fiber-optic biosensor (Tao Geng *et al.* 2006), latex agglutination test (Divya *et. al.,* 2013; Taye, *et al.*, 2013).

Traditional microbiological culturing techniques are being replaced by polymerase chain reaction (PCR) based techniques, as it is less laborious, time saving, and more sensitive compared to immunology based techniques. Various researchers have developed simple PCR targeting single genes (Abdulmawjood *et al.*, 2003; Bindu and Krishnaiah, 2010). However, multiplex PCR is more specific and eliminates the possibility of false positives. Several multiplex PCR protocols have been developed to detect two genes (Sahilah *et. al.*, 2010), three genes (Osek *et al.* 2002), four genes (Puttalingamma *et al.*, 2012), five genes (Divya *et. al.,* 2013) and six genes (Jeshveen *et. al.*, 2012) of *E. coli* O157:H7.

There are studies, where in *E.coli* 0157:H7 shows varied susceptibility and resistance for different antibiotics routinely used in veterinary and human medicine practice (Sinisa Vidovic and Darren, 2006; Raji *et al.*, 2008).

In India, there is paucity of information on STEC particularly *E. coli* O157:H7. For the first time, the occurrence of *E. coli* O157:H7 was reported in buffalo meat kebabs, sausages, buffalo milk, cow milk and khoa sweet in 1966 by Singh *et. al.* (1996). Few scientists worked on the incidence of this pathogen in various livestock products (Bindu and Krishnaiah 2010), human patients (Khan *et al*., 2002a; Khan *et al*., 2002b) and environmental samples (Hazarika *et. al*., 2007).

There are very few reports and very few researchers have worked on ecology and epidemiology of shedding pattern of *E. coli* 0157:H7 especially with respect to age, breed, sex and seasonal variation. Studies in cattle indicate that fecal shedding of *E. coli*

O157:H7 is typically low in the winter, increases in the spring, peaks during the summer and tapers off in the fall (Rangel *et al.*, 2005; Edrington *et al*, 2006; Milnes *et al.*, 2009; Gautam *et. al.*, 2011; Aseel *et al.*, 2013). There are research findings that indicate that breed may act as predisposing factor for higher shedding of *E. coli* O157:H7 **(**Riley *et al.* 2003; Jeon *et al.* 2013). However, not much work was done in Indian native and cross bred cattle.

There are research findings showing higher prevalence of EHEC O157:H7 in postweaned calves and heifers than in older animals (Hancock *et al*., 1997; Witold and Carolyn 2011). a research work by Jeon *et al.*, (2013) indicated that sex of the animal also contributes towards the ecology of shedding of *E. coli* O157:H7. As per the findings of Heuvelink *et. al., (*1998) *E.coli* O157:H7 were isolated more frequently from diarrheic calves than from normal calves**.**

Because of its public health importance, *E. coli* O157:H7 impacts production security, trade, and consumer confidence for beef and bovine derived foods in a significant way. In the nearly 30 years since *E. coli* O157:H7 became recognized as a foodborne pathogen, research has revealed much about the occurrence of this pathogen in cattle, the production environment, and the factors that affect its prevalence, levels, and persistence in cattle. Despite this, questions remain unanswered and foodborne disease caused by this pathogen continues to occur. Diagnostic techniques should help in faster diagnosis of the causative agent.

Minimizing the levels or concentrations of the pathogen in cattle production will have substantial impact on its prevalence in cattle and on its occurrence in final beef products. The pathogen reduction efforts applied throughout the animal production and processing chain should reduce the risk of *E. coli* O157:H7 occurrence in the final beef products. A better understanding of the ecology, epidemiology and shedding pattern of

E. coli O157:H7 could offer possible strategies to reduce the carriage and shedding of the organism by cattle that in turn could reduce the risk of human infection. Genetic and physiological factors of animals and environmental factors significantly affect the prevalence of *E. coli* O157:H7.

Further, identification of the shedding pattern represents an attractive target for pre-slaughter intervention as a means of reducing risk to humans. Implementation of hazard analysis and critical control point policies in diary animals and both pre-slaughter and post slaughter in meat industry are important to reduce the frequency that the food from these animals is contaminated with this foodborne pathogen (Callaway *et al.*, 2009; Jeong *et al*., 2011).

With the above facts in view, the present research work was undertaken with the following objectives;

1. To isolate *E.coli*O157:H7 in fecal samples collected from cattle and buffaloes of different age group, of different sex and in different seasons and in cattle of different breeds-exotic, crossbreds and indigenous breeds by conventional cultural method.
2. To develop a rapid, sensitive, species-specific and reliable multiplex PCR procedure for the detection of pathogenic *E. coli* 0157:H7 by targeting the six virulence genes; *fliCh7*, *rfbE*, *eaeA*, *hlyA*, *stx1*, and *stx2* genes. And to perform standardized PCR technique for the detection of *E.coli*O157:H7 in faecal samples.
3. To study the comparative efficacy of PCR technique *vis-a-vis* conventional isolation and identification method for the detection of *E.coli*O157:H7 in fecal samples.
4. To do statistical analysis to know the variation in shedding pattern of *E.coli*O157:H7.
5. To do antibiotic sensitivity test for the isolates using selected antibiotics.

CHAPTER II

REVIEW OF LITERATURE

2.1. The *Escherichia coli*

Escherichia coli are Gram negative rods (bacillus), facultative anaerobic bacteria in the family *Enterobacteriaceae* that readily grow on simple bacteriological media. The organism was discovered by Dr. Theodor Escherich in 1885. They ferment lactose and grow best under mesophilic temperatures with an optimum at 37°C. They are generally motile, non acid-fast, do not form spores, are oxidase negative and catalase positive and can reduce nitrate to nitrite. Most *E. coli* have the b-glucoronidase enzyme that breaks down complex carbohydrates (Adamu *et. al.,* 2014).

Most *E. coli* are normal commensals and non pathogenic (Drasar and Hill, 1974; Drasar and Barrow, 1985) found in the intestinal tract whose niche is the mucous layer of the mammalian colon (Kaper *et. al.*, 2004; Songer and Post, 2005). However, there are some strains that have evolved into pathogenic *E. coli* by acquiring virulence factors through plasmids, transposons, bacteriophages, and/or pathogenicity islands and cause a wide spectrum of diseases, ranging from self-limiting to life-threatening intestinal and extra-intestinal illnesses; enteritis, enterotoxaemia, cystitis, pyelonephritis, meningitis, mastitis, arthritis, and septicaemia (Kaper *et. al.*, 2004, Gyles and Fairbrother, 2010; Nataro *et. al.*, 2011). Three general clinical syndromes result from infection with inherently pathogenic *E. coli* strains: (i) urinary tract infection, (ii) sepsis/meningitis and (iii) enteric/diarrheal disease (Nataro and Kaper, 1998).

2.1.1 Serotyping of *Escherichia coli*

According to the modified Kauffman scheme, *E. coli* are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profile (Lior, 1996; Nataro and Kaper, 1998). A total of 173 different O antigens, each defining a serogroup

and 56 H antigens have been recognized currently (Verma *et. al.,* 2013). A specific combination of O and H antigens defines the "serotype" of an isolate (Nataro and Kaper, 1998).

Pathogenic strains of this organism are distinguished from normal flora by their possession of virulence factors such as exotoxins, pathology they cause and their interactions with eukaryotic cells. Six pathotypes of *E. coli* have been distinguished: Enterotoxigenic *E. coli* (ETEC) (Dalton *et. al.*, 1999), Enteropathogenic *E. coli* (EPEC) (Ruchaud-Sparagano *et. al.,* 2007), Enteroinvasive *E. coli* (EIEC) (Renata, 2013), Enterohemorrhagic *E. coli* (EHEC - Shiga toxin-producing *E. coli* or STEC) (Takeda, 2011), Enteroaggregative *E. coli* (EAEC or EAggEc) (Beauchamp and Sofos, 2010), and diffusely adherent *E. coli* (DAEC) (Scaletsky, 2002). An extra-intestinal pathotype (ExPEC) has also been described for strains that cause urinary disease (uropathogenic *E. coli*, UPEC) and meningitis and sepsis (meningitis-associated *E. coli*, MNEC) (Kaper *et. al.,* 2004).

All the intestinal pathotypes cause watery diarrhoea, often in young infants and children and the severity of disease varies with host factors and the combinations of virulence factors of the strain involved. Small intestinal pathotypes include EPEC which adhere tightly to enterocytes via attaching and effacing lesions damaging the microvilli leading to inflammation; ETEC which also adhere to enterocytes and secretes heat-labile (LT) and/or heat-stable (ST) enterotoxins and DAEC which adhere diffusely to enterocytes and cause elongation of the microvilli. Large intestinal pathotypes include EHEC which attach in the colon creating attaching and effacing lesions like EPEC but in addition secrete Shiga toxin (*Stx*); EIEC strains that invade colonic epithelia and can move laterally through the epithelium. EAEC adhere to epithelia in both the small and large intestine aggregating in a thick biofilm and secreting enterotoxins and cytotoxins

(Kaper *et. al.,* 2004). The relationship between different pathotypes is depicted in the ven diagram (Fig 2.1).

EPEC causes a profuse watery diarrheal disease and it is a leading cause of infantile diarrhoea in developing countries (Tobe *et. al.,* 1999). EPEC outbreaks have been linked to the consumption of contaminated drinking water as well as some meat products. Pathogenesis of EPEC involves intimin protein (encoded by *eae* gene) that causes attachment and effacing lesions (Hicks *et. al.,* 1998; Nataro and Kaper, 1998).

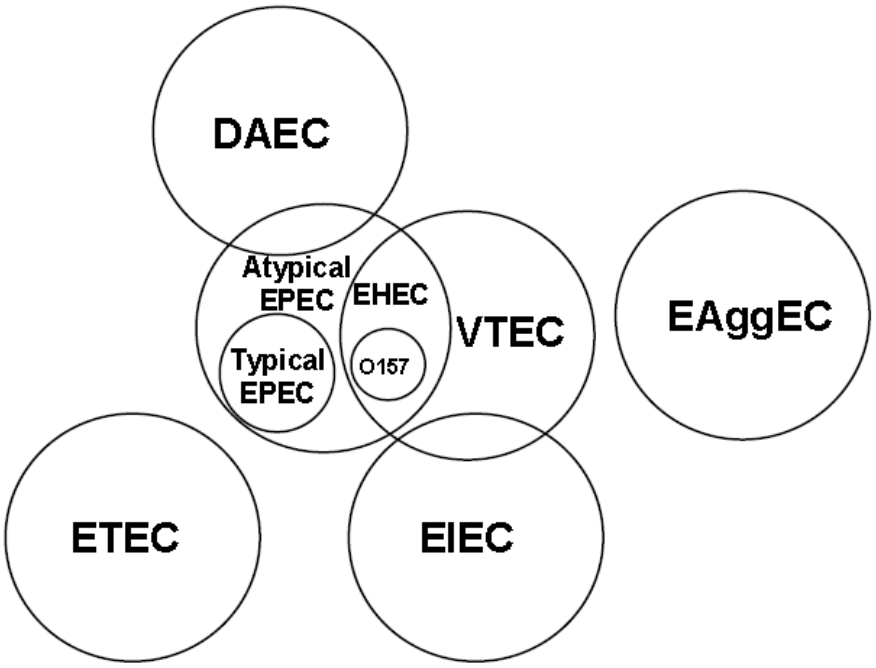
ETEC is recognized as the causative agent of travellers' diarrhoea and illness is characterized by watery diarrhoea with little or no fever. Pathogenesis of ETEC is due to the production of any of several enterotoxins. ETEC may produce a heat-labile enterotoxin (LT) that is very similar in size (86 kDa), sequence, antigenicity and function to the cholera toxin (CT). ETEC may also produce a heat stable toxin (ST) that is of low molecular size (4 kDa) and resistant to boiling for 30 min (Tsen *et. al.,* 1996).

EIEC closely resemble *Shigella* and causes an invasive dysenteric form of diarrhoea in humans. Unlike typical *E. coli*, EIEC are non-motile, do not decarboxylate lysine and do not ferment lactose, so they are anaerogenic. Pathogenicity of EIEC is primarily due its ability to invade and destroy colonic tissue (Mehlman *et. al.,* 1982).

EAEC (also known as EAggEC) are associated with acute or persistent diarrhoea, especially in developing countries. Infection is typically followed by a watery, mucoid, diarrhoeal illness with little to no fever and an absence of vomiting. EAEC strains are characterised by their ability to aggregatively adhere to tissue culture cells in a distinctive "stacked, brick-like" manner. Aggregative adherence in EAEC is mediated by either aggregative adherence fimbrae I (AAF/I) or AAF/II. EAEC also produce an enteroaggregative heat-stable toxin (EAST1) (Sullivan *et. al.,* 2007).

DAEC are a major cause of urinary tract infections worldwide, but its role as

Figure 2.1: Venn diagram illustrating the relationships between *E. coli* pathotypes causing diarrhoeal disease (modified from Donnenberg, 2002)



a causative agent of diarrhoea is controversial. DAEC infection is characterised by the growth of long finger-like cellular projections that wrap around the adherent bacteria. A 75% of DAEC strains produce the F1845 fimbrial adhesin or a related adhesin. Sources implicated in outbreaks of DAEC include contaminated food, especially undercooked ground beef, contaminated water and contact with livestock and other animals (Sullivan *et. al.*, 2007).

2.1.1.1 Enterohaemmorhagic *E. coli* (EHEC/STEC)

Konowalchuk *et. al.* (1977) and O'Brien *et. al.* (1977) were the first to independently report a toxin from several *E. coli* strains active on cultured vero cells (hence the name verocytotoxin) and HeLa cells respectively. The cytopathic effect of the cytotoxin was quite different from the non-cytopathic effect of the classical heat-labile enterotoxin of enterotoxigenic *E. coli* (ETEC). This cytotoxicity was neutralized by an immune serum specific for the Shiga toxin from *Shigella dysenteriae* (*S.*

dysenteriae) type1, hence the name Shiga-like toxin. These *E. coli* have been referred as verocytotoxigenic/shiga toxin producing *E. coli* (VTEC/STEC). A subgroup of STEC cause watery diarrhoea in humans which can progress to haemorrhagic colitis and potential systemic complications due to the action of *Stx* (Kaper *et. al.*, 2004). These are referred as Enterohaemmorhagic *E. coli* (EHEC).

EHEC strains are defined based on virulence genes; *Lee* (*locus of enterocyte effacement) Pai* (41 kb) virulence plasmid (*viz;* pO157), type III secretion system (TTSS) and *stx1* and/or *stx2* production (Paton and Paton, 2002; Wang *et. al.,* 2002). Phenotypically these *E. coli* show "attaching and effacing" phenomenon characterized by the elimination of the microvilli and intimate enterocyte attachment (Dean-Nystrom *et. al.*, 1999; Griffin *et. al.*, 2002; Kaper *et. al.*, 2004; Tobey *et. al.*, 2006; Pennington, 2010). The Shiga toxins identified in EHEC are classified in two distinct subgroups: Stx1 and Stx2. These cytotoxin(s) block protein synthesis and are associated with disease presentation.

About 200 EHEC serotypes have been isolated from animal and food sources, although not all are implicated in illness. The most significant pathogen of humans is *E. coli* O157:H7. After the first outbreak of bloody diarrhea in Oregon and Michigan, U.S.A. in 1982, *E.coli* O157:H7 has become the most widely known EHEC strain (Karmali *et. al.*, 1983; Riley *et. al.*, 1983; Wells *et. al.*, 1983; Tarr *et. al.*, 2005). *E. coli* O157:H7 is capable of producing Shiga toxin 1 (*Stx1*), Shiga toxin 2 (*Stx2*), or both. The other commonly isolated EHEC serotypes include *E. coli* O26:H11, O91:H21, O103:H2, O111:NM, O111:H8, O113:H21, O104:H4, and O157:NM (Ji Youn *et. al* ., 2010; Mellmann *et. al*., 2011; Denis *et. al*., 2012).

EHEC are sub classified into 5 seropathotypes *viz;* A, B, C, D and E based on incidence of the disease and severity of the disease caused. Seropathotype A EHEC

cause a very severe form of the disease and are commonly reported in the disease incidences. *E. coli* O157:H7 belongs to Seropathotype A EHEC.

2.2. The *E. coli* O157:H7

E. coli O157:H7 is the most commonly occurring food borne EHEC. *E. coli* O157:H7 is able to form vero toxins; shiga toxin 1 (*stx1)* and shiga toxin 2 (*stx2). Stx*1 is very similar to the type 1 toxin of *Shigella dysenteriae.* Whereas, *Stx*2 is genetically and immunologically distinct with 55–60% similarity in genetic and amino acid sequences of type 2 toxin of *Shigella dysenteriae*. Virulent isolates of *E. coli* O157:H7 can express *Stx1* only, *Stx2* only, or both toxins.

The vero toxins (*stx1* and *stx2*) produced by *E. coli* O157:H7 are encoded by *stx1* and *stx2* genes respectively. The gene *eaeA* encodes intimin, responsible for adherence of this pathogen to the intestinal lining and causing human illnesses. Hemolysin is encoded by *hlyA* gene, 0157 antigen by *rfbE* gene and flagellar antigen by *fliCh7* gene (Jeshveen *et. al.,* 2012).

Shiga toxins are A-B-type toxins that inhibit protein synthesis. The B5 subunit will bind to the glycolipid G3b (globotriaosylceramide) receptor on the surface of vascular cells in different tissues including the intestine, kidney and nervous system (Engedal *et. al.,* 2011) and the A1 gets translocated into the cell interior and will inhibit protein synthesis by the specific removal of a single adenine residue from the 28S rRNA of the 60S ribosomal subunit leading to death of host cells and consequential haemorrhage. High levels of G3b are found in human kidney which is 1000 times more sensitive to the cytotoxic action of *Stx2* than that of *Stx1* (Mead and Griffin, 1998; Baker *et. al.*, 2007; Ritchie *et. al.*, 2003). *Stx2* is more cytotoxic than *Stx1* in cell culture and animal models (Siegler *et. al.,* 2003).

Several variants of *Stx2* exist (Fuller *et. al.,* 2011; Pennington, 2010); *Stx2, Stx2c* (Lindgren *et. al.,* 1994), *Stx2d* (Pierard *et. al.,* 1998), *Stx2e* (Marques *et. al.,* 1987) and *Stx2f* (Gannon *et. al.,* 1990; Schmidt *et. al.,* 2000). *Stx2c* is the variant most commonly found in O157:H7 strains. *Stx2* and *Stx2c* have the same biological function and possess identical A subunits and B subunits that share at least 97% identity (Sablet *et. al.,* 2008). The possession and expression of the Stx2 gene and the variant Stx2c correlate strongly with the causation of bloody diarrhoea and haemolytic uraemic syndrome (Persson *et. al.*, 2007).

E. coli O157:H7 contains a highly conserved plasmid, named pO157. The pO157 is a nonconjugative F-like plasmid with a range size from 92 to 104 kb. The genes present on this plasmid are hemolysin (ehxA) (Schmidt *et. al.,* 1994), a catalaseperoxidase (katP) (Brunder *et. al.,* 1996), a type II secretion system apparatus (etp) (Schmidt *et. al.,* 1997), a serine protease (espP) (Brunder *et. al.,* 1997), a putative adhesin (toxB) (Tatsuno *et. al.,* 2001), a zinc metalloprotease (stcE) (Lathem *et. al.,* 2002) and an eae conserved fragment (ecf) (Yoon *et. al*., 2005).

Based on sequence length polymorphisms at eleven distinct loci within the O157:H7 genome, a refined classification system, termed the lineage-specific polymorphism assay (LSPA), partitioned *E. coli* O157:H7 strains into three groupings—lineages I, I/II, and II (Eppinger *et. al.,* 2011). These 11 distinct genetic regions are present in 80% of strains of lineage I and absent from 92%of lineage II; strains of lineage I are more often associated with human disease than the strains of lineage II that typically occur in cattle and other ruminants (Zhang *et. al.,* 2007).

The variation in *E. coli* O157:H7 strains is shown in the Table 2.1 (Witold and Carolyn, 2011).

Table 2.1. The serotypic, biochemical and virulence characteristics of the categories of *E. coli* O157:H7

Character	**Category**				
	EC O157	**EC O157:H7**	**EHEC O157**	**EHEC O157:H7**	**STEC *E. coli***
O157 antigen	Yes	Yes	Yes	Yes	?
H7 antigen	?	Yes	?	Yes	?
Growth on selective media[a]	Yes	Yes	?	Yes	?
stx	?	?	Yes	Yes	Yes
eae, tir, hly, ehx, or 92-Kb plasmid	?	?	Yes[b]	Yes[b]	?

[a]Standard selective media used to isolate typical *E. coli* O157:H7.

[b]One or more of the virulence factors detected in an isolate.

?=uncertain/Undetermined or not reported.

2.2.1 Emergence of *E. coli* O157:H7

E. coli O157:H7 strain, designated 2886-75, was first isolated in 1975 from a woman with bloody diarrhoea but was not considered a significant human pathogen. In the year 1978 Konowalchuk *et. al.* (1978) reported for the first time that strains of *E. coli* produced a potent toxin that killed Vero cells. In the year 1982 in US, a hemorrhagic colitis outbreak caused by hamburger consumption resulted in *E. coli* O157:H7 to be first recognized as an important animal borne human pathogen (Wells *et. al.*, 1983 and Riley *et. al.*, 1983). Since then, numerous (more than 200) foodborne cases throughout the world for example in countries like Scotland, Japan, Canada and the UK have been linked with this pathogen (Hodges and Kimball, 2005; Jeshveen *et. al.*, 2012; WHO, 2014). Adamu *et. al.* (2014) has termed the organism as super pathogen.

The chromosomal size of *E. coli* O157:H7 is 5.5 Mb. This genome includes a 4.1 Mb backbone sequence conserved in all *E. coli* strains. The remaining are specific to *E. coli* O157:H7 (1.4Mb). The majority of *E. coli* O157:H7-specific DNA sequences (1.4 Mb) are horizontally transferred foreign DNAs such as prophage and prophage-like elements. *E. coli* O157:H7 contains 463 phage associated genes compared with only 29 in non-pathogenic *E. coli* K-12 (Wick *et. al.,* 2005). Putonti *et. al.* (2006) estimated that at least 53 different species have contributed to these unique sequences in *E. coli* O157:H7.

There are few scientific hypotheses stating how *E. coli* O157:H7 got evolved. The scientific studies have stated that acquisition of VT converting phages from more mildly pathogenic O55:H7 (Wick *et. al.,* 2005; Leopold *et. al.,* 2009; Ogura *et. al.,* 2009), an enteropathogenic strain of *E. coli* (EPEC) and *E. coli* O127:H6 progenitors is believed to be largely responsible for the emergence of *E. coli* O157:H7 as a new pathogenic mutant (Whittam, 1995; Feng *et. al.,* 1998; Beutin, 2006; Feng *et. al.,* 2007; Asadulghani *et. al.,* 2009; Leopold *et. al.,* 2009). *E. coli* O55:H7 acquired the genes for shiga toxins from *Shigella* bacteria through the *bacteriophage λ* (Shaikh and Tarr, 2003; Beutin, 2006; Lim *et. al.,* 2010).

2.3. Public health importance of *E. coli* O157:H7

E. coli O157:H7 is mainly pathogenic to humans though bovines are thought to be reservoirs (Caprioli *et. al.,* 2005; Pennington, 2010; Soderlund *et. al.,* 2012). It will not cause any clinical disease, except diarrhoea (rarely) in cattle and other animals (Brown *et. al.,* 1997) due to the difference in distribution of Gb3 receptors between cattle and humans (Smith *et. al.,* 2002). *E. coli* O157:H7 infection is transmitted by faecal-oral route through contaminated food or water. The incubation period is around 3 to 4 days, but can range from one to ten days (CDC, 2014).

In a study conducted by Islam *et. al.* (2007), a total of 410 diarrhoeal samples were tested for STEC *E. coli* using sensitive culture and PCR methods in an urban slum community of Dhaka city. The prevalence of Shiga toxin-producing and its characteristics were determined in 11 children. In another research work in Tanzania, 275 human patients with diarrhoea were screened for presence of *E. coli* O157:H7 out of which, 10 faecal samples were positive for o *E. coli* O157:H7 (Raji *et. al.,* 2008).

The clinical signs and symptoms of *E.coli* O157:H7 infections in humans include bloody or non-bloody diarrhoea, abdominal cramps, vomiting and little or no fever - Hemorrhagic Colitis (HC), post- diarrheal Hemolytic Uremic Syndrome (HUS) and Thrombotic Thrombocytopenic Purpura (TTP) (Gyles, 2007; Karmali *et. al.,* 2010; Pennington, 2010; Jeshveen *et. al.,* 2012). The illness resolves in 5-10 days (Tarr, 1995). Abdominal tenderness associated with *E.coli* O157:H7 infection may contribute to misdiagnoses, such as appendicitis or intususscception.

Hemolytic ureamic syndrome (HUS), a life threatening complication, is developed by about 10% of patients, mostly in elderly people and children (Blackall and Marques, 2004; Reiss *et. al.,* 2006; Faten and Afaf, 2013). It is characterized by microangiopathic haemolytic anaemia, oliguric renal failure, thrombocytopenia and CNS symptoms followed by death (Tarr *et. al.,* 2005). Thrombotic Thrombocytopenic Purpura (TTP) is considered to be a manifestation of HUS, in elderly, where renal failure is normally mild, but neurological involvement is greater with a mortality rate as high as 50% (Griffin, 1995; Pennington, 2010).

As per Tarr (2009) approximately 8% of those infected with EHEC O157:H7 developed HUS. Children less than 5 years of age have a higher incidence of HUS. They express higher levels of the Gb3 receptor present on the renal endothelial cells. In a research work, largest number of *Escherichia coli* O157:H7 isolates were obtained from children of 1-4 years age, adults of 60-69 years age and others with under

developed immunity (Slutsker *et.al.*, 1997). Mohammad and Maryam (2015) found that children < 2 years of age were at highest risk of infection with *E. coli* O157:H7. In humans, females have been reported to be at a significantly greater risk of developing haemolytic anaemia after infection with *E. coli* O157:H7 than males (Rowe *et. al.*, 1991).

Antibiotic treatment is contraindicated for human *E. coli* O157:H7 infections, because certain antibiotics, such as fluoroquinolones, induce Shiga toxin-encoding bacteriophages in vivo and lead to increased expression of Shiga toxin genes (Zhang *et. al.,* 2000). Antibiotics also may cause bacterial lysis, which could increase free Shiga toxin in the intestinal tract (Wong *et. al.,* 2000).

For humans, a minimal cell count of about 10–100 is sufficient to cause serious complications (Keene *et. al.,* 1994). *E.coli* O157:H7 strains that lack *stx*1, but carry one or two *stx*2 alleles are more likely to cause infections resulting in HUS (Orth *et. al.*, 2007). Though secondary spread is also common, humans represent a dead-end target host of O157:H7. Adults typically shed the bacteria for one week after infection, while young children can excrete the bacteria for more than three weeks (Fitzpatrick, 1999). As per the research of Pennington (2010), the shedding of O157:H7 in children has a median duration of 13 days (range 2 to 62 days) in patients with diarrhoea or HC and 21 days (range from 5 to 124 days) in patients with HUS.

2.3.1 *E. coli* 0157:H7 as a food borne pathogen

The food borne infection by *E. coli* O157:H7 have been reported with increasing frequency from all parts of the world in the form of food poisoning outbreaks. The authority of the Federal Meat Inspection Act, FSIS (Food Safety and Inspection Service) declared *E. coli* O157:H7, an adulterant in raw ground beef and enforced “zero tolerance” (USDA-FSIS, 1998).

E. coli O157:H7 is recognized as one of the most significant food borne pathogen relating to public health especially in South Africa, Europe, Japan and the US (Hodges and Kimball, 2005; Rangel *et. al.,* 2005). The first recognized report of food borne infection with *E. coli* O157:H7 in humans was the hemorrhagic colitis outbreak caused by hamburger consumption. Ground beef is responsible for more human outbreaks of *E. coli* O157:H7 than any other source (Rangel *et. al.,* 2005; Ashgan *et al.,* 2015).

Majority of the times *E. coli* 0157:H7 is transmitted to humans through the food. Infection with *E. coli* O157:H7 can be achieved at least in part through the intake of contaminated foods, in which dairy products and meats contaminated with animal faeces or intestinal contents during/after slaughtering (Hussein and Sakuma, 2005). Contaminated undercooked ground beef and hamburgers have been the most frequently identified vehicle of *E.coli* O157:H7 in human disease outbreaks (Griffin and Tauxe, 1991). The entry of the bacilli into the meat by carcass contamination can be through transfer of pathogen from the intestines during the evisceration procedure, from the hide onto the carcass during skinning, contaminated equipment and tools used during skinning, contaminated operator hands, or contaminated dust particles and water droplets spread by aerosols generated in the production process (Avery *et. al.,* 2004; Narvaez-Bravo *et. al.,* 2013).

Many foods and dairy products have acted as vehicles for the transmission of *E. coli* 0157:H7; ground beef hamburgers, ready-to-eat cold meats including poultry, pork and beef products (Ashgan *et al.,* 2015), cheese, milk (Bhat *et. al.,* 2007 Guh *et. al.,* 2010 and Ebrahim *et. al.,* 2013); butter (Rangel *et. al.,* 2005); yoghurt (Besser *et. al.,* 1993) and ice cream. Results from a microbiological study of 90 outbreaks in the UK, Ireland, Denmark, Norway, Finland, USA, Canada, and Japan, occurring between 1982 and 2006, showed that the source of transmission was food in 42.2% of the outbreaks,

dairy products in 12.2%, animal contact in 7.8%, water in 6.7%, environmental in 2.2%, and unknown in 28.9% (Snedeker *et. al.*, 2009). EHEC O157 was isolated from pork, 2.14% (3/140), milk, 1.67% (1/60); and chicken meat, 1.67% (1/60) in Eastern China (Shaohui *et. al.*, 2014).

Milk borne *E. coli* O157:H7 infection is mainly due to faecal contamination (Armstrong *et. al.*, 1996). Lye *et. al.* (2013) investigated the prevalence of *E. coli* O157:H7 in raw cow, goat and buffalo milk samples in Malaysia. MPN-PCR method targeting the major virulence *rfbE* gene and *fliCH*$_7$ gene of *E. coli* O157:H7 was used. Total of 177 raw milk samples were collected from local dairy farms. The highest prevalence of *E. coli* O157:H7 was found in raw cow milk (18.75%) followed by 7.32% and 3.57% in raw goat and buffalo milk, respectively. In India, Suresh, (1999) isolated this organism from kulfi samples. *E. coli* O157:H7 has been isolated from goat's milk by Bielaszewska *et. al.* (1997).

E. coli O157:H7 has also been isolated from apple juice, coleslaw; lettuce (Hilborn *et. al.*, 1999), spinach, sprouts, melons (Rangel *et. al.*, 2005) and fresh vegetables (Ongeng *et. al.*, 2013 and Duncan *et. al.*, 2014). Sprouted seeds were involved in *E.coli* O157:H7 infections as per the finding of Berger *et. al.* (2010). In their review on fresh fruit and vegetables as vehicle for the transmission of human pathogens, the authors emphasized an increasing risk due to proliferation of *E. coli* O157:H7 during the processing and post-harvest handling procedures of contaminated seeds. The massive outbreak of *E. coli* O157:H7 in schoolchildren in 1996 in Sakai, Japan, was undoubtedly related to contaminated radish sprouts (Michino *et. al.*, 1999). Drinking water and recreational waters have also been the sources of *E. coli* O157:H7 that caused human illness outbreaks (Bruce *et. al.*, 2003; Davis *et. al.*, 2005).

2.4. *E. coli* 0157:H7 outbreaks

The first major outbreak of *E. coli* O157:H7 occured in the year 1982 in US caused by hamburger consumption (Wells *et. al.,* 1983; Riley *et. al.,* 1983). Since then, numerous (more than 200) foodborne cases have been reported throughout the world. More than 30 countries on six continents *viz;* Scotland, Japan, Canada and the UK have been linked with this pathogen (Hodges and Kimball, 2005; Jeshveen *et. al.,* 2012; WHO, 2014).

In 1994, an outbreak of *E. coli* O157:H7 infection linked to commercially distributed dry-cured salami product in Washington and California was reported (MMWR, 1995) which led to voluntary recall of 10,000 pounds of implicated product and suspended the sale of all the products until the source of contamination was determined.

The largest *E. coli* O157:H7 outbreak took place in Sakai City of Japan in 1996 in which the radish sprouts in school lunches were contaminated, causing a total of 9451 cases with 12 deaths (Michino *et. al.,* 1999; Fukushima *et. al.,* 1999). The incidence of all Shiga-toxin-producing organisms (including *E coli* O157) in Japan from April, 1999, to October, 2004 was 2.74 % (Sakuma *et. al.,* 2006). In Europe, 14,000 cases in over 24 countries have occurred from 2000 to 2005, of which 62% belong to the O157 serogroup (Fisher and Meakins, 2006).

In September and October of 2006, *E coli* O157:H7 isolate, TW14359, caused an outbreak associated with contaminated spinach that sickened 205 individuals in the USA. In this outbreak, a total of 15% of the afflicted individuals developed HUS (California Food Emergency Response Team, 2007; Manning *et. al.*, 2008)

In 2006, the incidence of *E. coli* 0157:H7 infection per 100 000 in the European countries was 2.1 in England and Wales, 2.87 in Ireland, 4.7 in Scotland (from 1998 to

2007 the mean yearly rate was 4.28), 0.43 in Germany, and 0.08 in France. In the same year, the incidence was about 1.3 per 100 000 in Canada (CDC, 2014).

In Asia, other than Japan, relatively few outbreaks of *E. coli* O157:H7 have been reported. In Mainland China, *E. coli* O157:H7 was first identified from patients suffering from haemorrhagic colitis in Jiangsu Province in 1986. One major outbreaks of *E. coli* O157:H7 has been reported in Jiangsu Province, China in 1999, which involved 95 *E. coli* O157:H7 infected patients developing acute renal failure with a case fatality rate of 87%. In Taiwan, EHEC is a notifiable disease but as of August 2009, only one confirmed case had been recorded in 2001 (Taiwan CDC, 2014).

2.5. The reservoir of *E. coli* 0157:H7

The primary reservoir of this serotype is the cattle (Borczyk *et. al.,* 1987; Chapman *et. al.,* 1993; Jackson *et. al.,* 1998; Chapman *et. al.,* 2001; Gannon *et. al.,* 2002; Susan Sanchez *et. al.,* 2002; Ogden *et. al.,* 2004; Synge *et. al.,* 2003; Al-Saigh *et. al.,* 2004; Mainil and Daube 2005; Hussein, 2007; Elaine and James, 2010; Eppinger *et. al.,* 2011; Soderlund *et. al.,* 2012).

Studies have shown that up to 30% of all cattle are asymptomatic carriers of *E. coli* O157:H7 (Callaway *et. al.,* 2006; Stanford *et. al.,* 2005). Early outbreaks of *E. coli* O157:H7 were traced to undercooked ground beef and consumption of unpasteurised milk and the majority of *E. coli* O157:H7 outbreaks are traced to cattle products or vegetable products contaminated with cattle waste (Chapman *et. al.,* 1997). Clustering of human cases has been observed in areas with highest cattle density and there was a significant association between the numbers of human cases and the ratio of beef cattle to humans (Chase-Topping *et. al.,* 2008; Valcour *et. al.,* 2002).

As per the findings of Dunn *et. al.* (2004) and Cho *et. al.* (2006), between 1% and 50% of healthy cattle carry and shed *E. coli* O157:H7 in their faeces at any given

time. Cattle faeces is a major source of contamination of beef, other food products and water (Cergole *et. al.,* 2006; Pennington, 2010; Olesen and Jespersen, 2010). This organism can be found in 80% of populations in few herds of cattle and 49% of beef carcasses (Elder *et. al.,* 2000). It does not appear to cause disease in adult cattle, although it can cause diarrhoea in neonates (Blanco *et. al.,* 1996; Hancock *et. al.,* 1997; Besser *et. al.,* 1997). The illness in humans is often related to the consumption of contaminated (Jamshidi *et. al.,* 2008) and undercooked ground beef (Jamshidi *et. al.,* 2012). Numerous *E. coli* O157:H7 outbreaks resulting from contact with cattle or their manure on farms, at fairs, and at petting zoos have also been reported in the USA (CDC, 2005; Durso *et. al.,* 2005; Rangel *et. al.,* 2005).

E. coli O157:H7 was most prevalent in the lower GIT digesta, specifically the cecum, colon and rectum of cattle, as per the experimental inoculation studies done by Luke *et. al.* (2002). It has been reported that a region of lymphoid follicle-dense epithelium a short distance proximal to the recto-anal junction is the principal site of *E. coli* O157:H7 persistence in adult cattle (Naylor *et. al.,* 2003; Francis *et. al.,* 2004; Low *et. al.,* 2005; Naylor *et. al.,* 2005; Pablo *et. al.,* 2008; Michael *et. al.,* 2011). Intimin is an important colonization factor for *E. coli* O157:H7 in intestine (Dean-Nystrom *et. al.,* 1998; Woodward *et. al.,* 2003). This view was supported later by Mauricio *et. al.* (2012).

The data from the study done by Grauke *et. al.* (2002) and Rowland *et. al.* (2007) support an association between levels of fecal excretion of *E. coli* O157:H7 and recto-anal junction colonization in pens of feedlot cattle. Cattle with *E. coli* O157:H7 colonized at this site shed higher numbers of organisms for a longer period than those colonized at other sites (Cobbold and Desmarchelier, 2004; Cobbold *et. al.,* 2007; Moxley, 2004). Such cattle are designated as “supershedders” (Krystyn, 2015). The presence of these animals on a farm is associated with a high prevalence of low-level

shedders and they are likely to infect other animals in the same pen (Chase-Tapping *et. al.,* 2007; Margo *et. al.,* 2007; Margo *et. al.,* 2008).

The "supershedders," have greater *E. coli* O157:H7 transmission potential than other cattle, either through greater incidence or persistence of excretion or excretion of greater number of *E. coli* O157:H7 (Matthews *et. al.,* 2006). The study done by Rowland *et. al.* (2007) supports the hypothesis that the Recto-Anal Junction represents an important colonization site for *E. coli* O157:H7 and suggests that supershedders represent cattle that have persistent colonization of the Recto Anal Junction (RAJ) with high concentrations of *E. coli* O157:H7. The super-shedders are responsible for about 90% of the total number of bacteria in the cattle herd and raise the prevalence of cattle infected with this pathogen on farms, making them a high risk factor at the preharvest level. Utilizing statistical modeling, Matthews *et. al.* (2006a) estimated that, on average, the prevalence of "supershedders" in a population is 4% and that these animals excrete 50 times more *E. coli* O157:H7 than other animals colonized by this organism.

Presently, supershedders are referred to those animals which excrete *E. coli* O157:H7 at levels of $>10^4$ CFU/g of faeces (Davis *et. al.,* 2006; Arthur *et. al.,* 2010, Arthur *et. al.,* 2013; Xu *et. al.,* 2014). This small proportion of super-shedding cattle in a herd is responsible for a large proportion of *E. coli* O157:H7 contamination in a production environment, which may in turn drive the *E. coli* O157:H7 prevalence of cattle in that environment (Chase-Topping *et. al.,* 2007; Chase-Topping *et. al.,* 2008; Cobbold *et. al.,* 2007; Matthews *et. al.,* 2006; Stephens *et. al.,* 2009). The presence of high-level shedders of *E. coli* O157:H7 in feedlot pens was associated with higher prevalence of the pathogen among cattle in the same pen, while cattle that were negative for the pathogen were more likely to have been in a pen that did not have a super-shedding animal (Low *et. al.,* 2005; Cobbold *et. al.,* 2007).

Mathematical modelling of prevalence and transmission dynamics of *E. coli* O157 on cattle farms in Scotland indicates that the distribution of prevalence is best explained when a small proportion of super-shedders are responsible for most of the infections in the rest of the population and that 80% of the *E. coli* O157 infections arise from the 20% of cattle that are shedding high levels of the pathogen (Matthews *et. al.,* 2006b). In a study examining feedlot cattle, Stephens *et. al.* (2009) observed a larger impact of high-level shedders of *E. coli* O157:H7 on hide prevalence than faecal prevalence of penmates. Recently, Fox *et. al.* (2008) reported that the probability of carcass contamination with *E. coli* O157 was significantly associated with the presence of a high-shedding animal within the same truckload of cattle.

In addition, Arthur *et. al.* (2009) have shown that 95% of feedlot pens containing at least one supershedder had *E. coli* O157 prevalence rates on cattle hides exceeding 80%, whereas only 29% of pens without a supershedder exceeded 80% hide prevalence. On contrast, Brandon *et. al*. (2009) hypothesized that those animals which persistently excrete normal levels of *E. coli* O157:H7 over prolonged periods (persistent shedders [PS]) rather than animals that periodically shed abnormally high levels (supershedders) are the most significant source of *E. coli* O157:H7 contamination in the food continuum.

For dairy cattle, the prevalence estimated by testing faeces ranged from 0.2% to 48.8% in the USA (prevalence in calves 0.4–40%) and Canada, Italy, Japan, and the UK (prevalence in calves 1.7–48.8%) (Hussain and Bolinger, 2005). As per the research finding of Sinisa and Darren (2006), of 400 bovine faecal samples, *E. coli* O157 was found in 57 samples (14.2%). Wang *et. al.* (1996) found that the pathogen survived in bovine faeces for up to 56 and 49 days at 22 and 37°C, respectively, despite reductions in moisture content and water activity of the faeces. All these findings illustrate bovines acting as carriers if this public health significant pathogen.

E. coli O157 can also be present in sheep (Indira *et. al.,* 1996; Chapman *et. al.,* 1997; Ogden *et. al.,* 2005; La Ragione *et. al.,* 2009; Soderlund *et. al.,* 2012; Yilmaz, 2014), goat (Fox *et. al.,* 2007; McIntyre *et. al.,* 2002; La Ragione *et. al.,* 2009; El-Jakee *et. al.,* 2012). A study in the UK found *E. coli* O157:H7 contamination to be more common in raw lamb meat products compared to beef (Chapman *et. al.,* 2001). A sum of 8 out of 13 STEC strains isolated from ovine dairy products in Mexico were O157 (Caro *et. al.,* 2007). *E. coli* O157:H7 were isolated from pelts and carcasses in a lamb-processing plant (Kalchayanand *et. al.,* 2007). EHEC O157:H7 were found in 8% of beef and 2% of lamb and goat meat samples in Ethiopia (Hiko *et. al.,* 2008) and were isolated from goats in Spain (Orden *et. al.,* 2008). STEC O157 was more often found in goats (9%) than in cows (7%) in Bangladesh (Islam *et. al.,* 2008). A total of 11 *E. coli* O157:H7 strains were isolated from 300 healthy water buffalos in Turkey (Sekera and Yardimci, 2008). These findings show that healthy domesticated ruminants act as carriers of *E. coli* O157 which was also supported by La Ragione *et. al.* (2009).

EHEC O157:H7 can be highly pathogenic in swine and are rarely found in these animals; a 2-year multifarm survey in the United States did not yield any isolates (Richards *et. al.,* 2006). However, Feder *et. al.* (2003) and Doane *et. al.* (2007) reported isolation of EHEC O157:H7 in pigs. In Great Britain in 2003, intestinal contents of 4.7% of cattle, 0.7% of sheep, and 0.3% of pigs tested positive for *E coli* O157 at slaughter (Milnes *et. al*., 2008).

There are reports of isolation of *E. coli* O157:H7 in chickens (Dipineto *et. al.,* 2006; Doane *et. al.,* 2007). *E. coli* O157:H7 has also been isolated from bear, deer (Fischer *et. al.,* 2001), rabbits (Scaife *et. al.,* 2006), opossums (Renter *et. al.,* 2003) pony, horses, zebra, ostrich, hyena, llama, jaguar (Aseel *et. al.,* 2013) and *Muscidae* and *Calliphoridae* flies (Talley *et. al.,* 2009). In a report by Shaohui *et. al.* (2014) EHEC

O157 was isolated as follows: pig feces, 4% (20/500); cattle feces, 3.3% (2/60); chicken feces, 1.43% (2/140) in eastern china.

2.5.1 *E.coli* 0157:H7 in Buffaloes

Research studies investigating the frequency of *E. coli* O157:H7 serotype in buffaloes is limited. In a study, *Escherichia coli* O157:H7 was isolated from 11 (3.7 %) of 300 faecal samples and 3 (1.4 %) of 213 raw milk samples of Anatolian water buffaloes (*Bubalus bubalus*) in Turkey (Sekera and Yardimci, 2008). It was determined that 8 (73 %) of *E. coli* O157:H7 strains isolated from faecal samples originated from water buffaloes younger than 2 years of age and 3 (27 %) from 2-year-old and older water buffaloes.

2.6 Diagnosis/detection of *E. coli* 0157:H7

2.6.1 Cultural isolation and identification of *E. coli* 0157:H7

Divya *et. al.* (2013) identified *Escherichia coli O157* in human faeces from uremic syndrome patients, small intestine of chicken, ground beef, cattle faeces and raw milk by using routine microbiological cultural tests. Bindu and Krishnaiah (2010) tested 250 samples collected from various sources using cultural methods for the presence of *E. coli* O157:H7 and reported that only 11 samples were positive by cultural methods.

There are three types of enrichment media that are often used when recovering *E. coli* O157:H7: 1) Buffered peptone water supplemented with 8 mg/L vancomycin, 10 mg/L cefsulodin, and 0.05 mg/L cefixime, 2) Modified EC broth (mEC with novobiocin), 3) Soyabeen bile broth with 20 mg/L novobiocin or 10 mg/L acriflavin (OIE, 2006). *E. coli* O157:H7 rapidly ferments lactose and is indistinguishable from most other *E. coli* on traditional lactose containing media. However, *E. coli* O157:H7 cannot ferment sorbitol within 24 h, while 90% of *E.coli* can. This character is used as a criterion for differentiating it from other *E. coli* (March and Ratnam, 1986; Sandra and Samuel,

1986; Adamu *et. al.,* 2014; Lee and Choi, 2006), which led to the development of the Sorbitol MacConkey (SMAC) agar used as selective and differential medium for its isolation. As reviewed by Bettelheim (2007), the inability of most *E. coli* O157:H7 to ferment sorbitol has provided a convenient marker for selecting this pathogen.

Conventional culture methods are very useful for the identification of *E. coli* O157H7. However, it is time consuming and needs a lot of media and supplement components (Visetsripong *et. al.,* 2007). There is a possibility to get false results by the classic microbiological diagnostic procedures based on screening with sorbitol MacConkey agar (Orth *et. al.,* 2009). In a research finding of Manna *et. al.* (2009), among 139 sorbitol-negative isolates of 38 *E. coli* serogroups from food and cattle faeces, only 14.6% were O157. More specific media have also been developed, such as Rainbow Agar, CHROM agar, and O157:H7 ID agar (CDC, 2009).

Most *E. coli* have the b-glucoronidase enzyme that breaks down complex carbohydrates. This enzyme is used in a fluorogenic assay that takes advantage of the breakdown of 4-methyl umbeliferone glucoronide (MUG) yielding a fluorescent compound. However, *E. coli* O157:H7 does not have b-glucoronidase (Thompson *et. al.,* 1990) which is used to differentiate this pathogen from other *E. coli.*

To avoid the problem of false negative results from low abundance specimens, Immunomagnetic separation (IMS) techniques have been developed to assist in the isolation of STEC (principally O157) (Rebecca *et. al.,* 2014, Ayaz *et. al.,* 2014). The procedure involves coating magnetic beads with anti-LPS antibody and mixing them with broth cultures or suspensions of faeces or suspect food homogenates. The beads and bound bacteria are then trapped in a magnetic field, the unbound suspension is decanted, and the beads are washed to retrieve *E. coli* O157:H7. After additional binding and washing cycles, the beads are plated and the resultant colonies are checked for reactivity with the appropriate O157 antiserum and, more importantly, for *Stx*

production. The principal drawback of IMS is, of course, its serogroup specificity. However, it is an extremely valuable enrichment technique, particularly under circumstances where STEC strains of known serogroups are being deliberately targeted, for example, when testing foods/water samples suspected of being the source of an outbreak (Karch *et. al.,* 1996).

Other efficient and less time consuming confirmatory methods and techniques have to be explored and researched to detect *E. coli* O157:H7.

2.6.2 Immunological tests for the confirmation of *E. coli* 0157:H7:

Chuanmin Ruan *et. al.* (2002) developed ELISA based Immunobiosensor Chips for Detection of *E. coli* O157:H7. Peixuan *et. al.* (2005) described integrating waveguide biosensor for the detection of water-borne *E. coli* O157, based on a fluorescent sandwich immunoassay performed inside a glass capillary waveguide. The biosensor allows for quantitative detection of as few as 10 cells per capillary (0.075 ml volume) and can be used in conjunction with cell amplification, PCR and microarray technologies to positively identify this pathogen.

Carvalho *et. al.* (2014) determined the occurrence of *E. coli* O157:H7 by using ELISA (VIDAS ECO O157®, bioMérieux, Lyon, France) test. *E. coli* O157:H7 was detected in 6.67% samples collected in open air markets using ELISA and 23.33% with PCR, which indicates that ELISA is less sensitive compared to PCR.

Tao Geng *et. al.* (2006) developed an antibody-based fiber-optic biosensor to rapidly detect low levels of *Escherichia coli* O157:H7 cells in ground beef. The principle of the sensor is a sandwich immunoassay using an antibody which is specific for *E. coli* O157:H7. The biosensor was able to detect as low as 10^3 CFU/ml pure cultured *E. coli* O157:H7 cells grown in culture broth. Artificially inoculated *E. coli*

O157:H7 at concentration of 1 CFU/ml in ground beef could be detected by this method after only 4 hours of enrichment.

2.6.2.1 Latex agglutination test

Divya *et. al.* (2013) confirmed presence of *E. coli* O157 in human faeces from uremic syndrome patients, small intestine of chicken, ground beef, cattle faeces and raw milk by using latex agglutination test by employing "LK13 HiE.coli^{TM}157 Latex Test Kit" .

A cross sectional study was conducted from October 2010 to March 2011, on apparently healthy slaughtered cattle in Haramaya University Slaughter House to determine prevalence of *E. coli*, with special emphasis on *E. coli* O157:H7. From a total of 113 samples collected, 3 (2.65%) were found to be *E. coli* O157:H7. The confirmation was done by using latex agglutination test (Taye, *et. al.*, 2013). Similarly, Mohamed *et. al.* (2013) confirmed the presence of *E. coli* O157:H7 isolated from 100 raw milk, 70 rectal swabs from apparently healthy and diarrheic calves and 30 stool samples from children using latex agglutination test. Mohmmed *et. al.* (2012) used latex agglutination test to confirm *E. coli* O157:H7 isolates and reported 5.7% isolates from stool of diarrhea patients, 7.3% isolates from sheep feces, 11.1% isolates from milk were positive for *E. coli* O157:H7. None of drinking water samples were positive for *E. coli* O157:H7.

2.6.3 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a robust approach for the rapid identification of microorganisms. The identification mechanism is based on the protein MS pattern obtained by MALDI-TOF MS matching microbial sequence data in available databases.

The use of MALDI-TOF-MS for bacterial identification typically attempt to detect as many bacterial proteins as possible in order to obtain as unique an MS "fingerprint" as possible (Fenselau and Demirev, 2001; Lay, 2001; Demirev and Fenselau, 2008; Fagerquist *et. al.,* 2009; El-Bouri *et. al.,* 2012; Loff *et. al.,* 2014; Ojima-Kato *et. al.,* 2014). Various researchers have used MALDI-TOF-MS for the identification of various organisms. Haag *et. al.* (1998) used MALDI-TOF-MS for rapid identification and speciation of *Haemophilus* bacteria. Mandrell *et. al.* (2005) used MALDI-TOF-MS for the speciation of *Campylobacter coli, C. jejuni, C. helveticus, C. lari, C. sputorum,* and *C. Upsaliensis*.

Barbuddhe *et. al.* (2008) used MALDI-TOFMS for the identification and typing of *Listeria* species. There are very few reports of using MALDI-TOF-MS for the detection of *E. coli* O157:H7. In one of the studies, the expression of a specific bacterial virulence factor most often linked to cases of severe foodborne illness caused by genomically sequenced strain of *E. coli* O157:H7 (Uchida *et. al.,* 1999) was induced and the α-subunit of Shiga toxin 2 (*α*-Stx2) produced was identified by applying top-down MALDI-TOF-TOF-MS/MS technique (Clifton and Omar, 2010). In this study, MALDI-TOF-MS of the furin-digested/disulfide-reduced sample showed a peak at mass-to-charge (*m/z*) 5286 that corresponded to the A2 fragment.

In a research work on discrimination of *E. coli* O157, O26 and O111 from other serovars by MALDI-TOF MS based on the *S10*-GERMS (*S10-spc-alpha* operon gene encoded ribosomal protein mass spectrum) method, three biomarkers, ribosomal proteins S15 and L25, and acid stress chaperone HdeB, with MS *m/z* peaks at 10138.6/10166.6, 10676.4/10694.4 and 9066.2, respectively, were identified as effective biomarkers for O157 discrimination (Ojima-Kato *et. al.,* 2014).

Clifton and Omar (2010) identified the *α*-subunit of Shiga toxin 2 (*α*-Stx2) from *Escherichia coli* O157:H7 using matrix assisted laser desorption/ionization time-of-

flight-time-of-flight tandem mass spectrometry (MALDI-TOF-TOF-MS/MS) which showed a peak at mass-to-charge (*m/z*) 5286 that corresponded to the A2 fragment. In anoher research work, Fagerquist *et. al.* (2010) were able to distinguish *E. coli* O157:H7 from a nonpathogenic *E. coli* by top-down proteomics using MALDI-TOF-TOF-MS/MS. The six proteins targeted were the acid stress chaperone-like proteins, HdeA and HdeB; the cold shock protein, CspC; the YbgS (or homeobox protein); the putative stress-response protein YjbJ (or CsbD family protein); and a protein of unknown function, YahO.

2.6.4 Molecular identification and characterization of *E. coli* O157:H7

Molecular methods remain the most popular and most reliable techniques for detecting different strains of *E. coli* and differentiating the six pathogenic forms of *E. coli*. Substantial progress has been made both in the development of PCR methods as well as nucleic acid-based probe technologies for the detection of different serotypes of *E. coli*. According to Shah *et. al.* (2009), PCR assays are proven specific and sensitive in detecting microbial pathogens such as *E. coli* 0157:H7.

2.6.4.1 Extraction of DNA from *E. coli* O157:H7

Several DNA isolation and purification strategies have previously been investigated with variable rates of success (Lakay *et. al.,* 2007; Menon and Nagendra, 2001; Orsini and Romano-Spica, 2001). In general, isolation of bacterial genomic DNA involves three main steps: cell disruption, DNA extraction, and DNA purification. Many strategies are being used for the extraction of DNA from bacterial cells, such as enzymatic, chemical or thermal lysis, mechanical disruption of the cell wall by beads or sonication, or a combination of the above (Rantakokko and Jalava, 2002; Tongeren *et. al.,* 2011).

Each cell disruption approach has specific advantages and disadvantages. Chemical methods use detergents to solubilize cell membranes. Commonly used detergents are SDS, Triton X-100, and CTAB (Ausbel *et. al.,* 1998). The disadvantage of detergent-based cell lysis is that detergents often contaminate DNA samples and inhibit further manipulations. Enzymes attacking the components of cell surface or cytosol are often added to the detergent-based lysis buffers. However, these enzymes lack efficacy against most bacteria (Bollet *et. al.*, 1991).

Methods like sonication, grinding in liquid nitrogen, shredding with rigid spheres or beads and application of mechanical stress such as filtration have been used for difficult-to-lyse samples prior to or in conjunction with lysis solutions (Shahriar *et. al.*, 2011). These methods still have disadvantages, which include laborious manipulations such as four to six changes of microcentrifuge tubes, multiple stages of incubation, precipitation, elution, washing and drying, or requirement of special equipment. The DNA yield and purity are often poor due to the multi-step manipulations.

Simple boiling method that causes lysis of bacterial cells is the best and less cumbersome procedure for DNA extraction (Tunung *et. al.,* 2007; Chai *et. al.,* 2007; Queipo-Ortuño *et. al.*, 2008). Puttalingamma *et. al.* (2012) extracted DNA by boiling method from the overnight cultures for multiplex PCR for the detection of *E. coli* 0157:H7. Jothikumar and Griffiths (2002) extracted bacterial DNA by boiling at 97°C for 10 minutes followed by freezing at -20°C and centrifugation at 10,000 rpm for 3 minutes.

2.6.4.2 Detection of *E. coli* O157:H7 by PCR

E. coli 0157:H7 outbreaks are on the rise; hence it is important to develop a sensitive, rapid, and species-specific method to identify this pathogen. Traditional

microbiological culturing techniques are being replaced by polymerase chain reaction (PCR) based techniques for the identification and detection of *E. coli* 0157:H7, as it is less laborious and saves significant amount of time.

Abdulmawjood *et. al.* (2003) developed simple Polymerase Chain Reaction (PCR) to detect *rfbE* gene of *E.coli* O157:H7. The *rfbE* gene codes for O157 LPS endotoxin. The oligonucleotide primers Gi-O157-I 5V-CGAGTA CAT TGG CAT CGT G-3V(position no. 4596) and Gi-O157-II 5V-ATT GCG CTG AAG CCT TTG-3V(position no. 5079) were selected from a region of the rfbE gene to detect *E. coli* O157. Bindu and Krishnaiah (2010) tested 250 samples collected from various sources using PCR assay for the presence of *E. coli* O157:H7 and reported that 27 samples were positive.

2.6.4.2.1 Detection of *E. coli* O157:H7 by multiplex PCR

Multiplex PCR is more specific and eliminates the possibility of false positives. Several multiplex PCR protocols have been developed in the past to detect the major virulence genes of *E.coli* O157:H7 (Grant, 2003; Gryko *et. al.,* 2002; Maurer *et. al.,* 1999; Osek, 2002; Osek and Dacko, 2001; Wang *et. al.,* 2002; Dianna *et. al.,* 2003 and Bai *et. al.,* 2010).

Sahilah *et. al.* (2010) detected *stx*1 and *stx*2 Genes in *E. coli* O157:H7 Isolated from retail beef in Malaysia by multiplex PCR. The amplicon sizes produced by the two primers were 180 bp and 255 bp, respectively. Multiplex PCRs was also performed by using primer sequences for the genes *stx1, stx2* and *eae* for detection of *escherichia coli* O157:H7 directly from stools by Holland *et. al.* (2000). Ahmad *et. al.* (2013) confirmed the presence of *E. coli* O157:H7 in 8% (of 200 samples) of ground beef hamburger samples using multiplex PCR that simultaneously detected three genes: *fli*C (encoding flagellar antigen), *rfb*E and *uid*A (encoding beta-glucuronidase). Al-Ajmi *et. al.* (2006)

also used these three primers for detection of *rfbE, uidA* and *fliC* gene by multiplex PCR and reported that multiplex PCR employing these set of primers have high sensitivity and specificity to determine the *E. coli* O157:H7.

A rapid and specific multiplex PCR, which detected *rfbO157* gene and main virulence genes (*stx*1, *stx*2 and *eaeA*) of STEC in the faeces were developed by Osek *et. al.* (2002). Pina *et. al.* (1995) developed a multiplex PCR assay for detection of *E. coli* O157:H7, by employing primers specific for the EHEC *eaeA, Stx1* and *Stx2* genes and the 60-MDa plasmid. PCR products of 1,087 bp (*eaeA*), 227 and/or 224 bp (*Stx1* and/or II (*Stx2*) and 166 bp (plasmid) were successfully amplified simultaneously in a single reaction. Dianna *et. al.* (2003) and Ashgan *et. al.* (2015) used PCR for the diagnosis of *E. coli* O157:H7 in patients and environmental isolates from the outbreak of water borne *E. coli* O157:H7 and *C. jejuni* in the United States. These authors used primers for four genes; *hlyA*, *eae*, *stx1*, and *stx2* and obtained the amplicon products of 166 bp, 1087 bp, 130 bp and 397 bp, respectively.

LeJeune *et. al.* (2004) confirmed the *E. coli* O157:H7 isolates by multiplex PCR using primers for four genes; *fliCh7, eaeA, stx*2 and *stx*1 genes. Puttalingamma *et. al.* (2012) standardizaed multiplex PCR (mPCR) assay for simultaneous detection of four important genes associated with the organism *E. coli* 0157:H7 in food *viz., stx1, stx2, eaeA, hlyA*. The mPCR assay was sensitive enough to detect cells as low as 10^3 CFU ml^{-1} or g^{-1} of the food samples. In Iran, mPCR assay was developed for simultaneous detection of the similar four genes to detect *E. coli* 0157:H7 in juice and vegetables by Reza and Sakineh (2013). Jeon *et. al.* (2013) conducted multiplex PCR to confirm *E. coli* O157. Primers were designed to detect *stx1, stx2, hly,* and *rbfE*. Out of 91 samples, 37 samples (40.66%) were found to be positive for *E. coli* O157 with a detection limit of 10 CFU/swab.

Islam *et. al.* (2007) used primers for the genes *rfbE, eae, stx1, stx2, hlyA* for the confirmation of the shiga toxin-producing *E. coli* (STEC) isolated from patients with diarrhoea in Bangladesh by PCR technique. In this study, the PCR products obtained were 259 bp, 629bp, 606 bp 372 bp and 889 bp, respectively. Divya *et. al.* (2013) confirmed presence of *Escherichia coli O157* in human faeces from uremic syndrome patients, small intestine of chicken, ground beef, cattle faeces and raw milk by using molecular characterization by PCR for the presence of virulence genes, namely*; eae, stx1, stx2, hlyA* and *fliCh7* genes.

Puttalingamma and Harshvardhan (2013) designed mPCR by employing five primers: *chu, ehxA, espP, stx1 and wzy,* which are specific for the EHEC, to detect *E. coli* serotype O157:H7 in food and water samples. The optimized mPCR protocol simultaneously and successfully amplified all genes targeted and is suitable for rapid analysis of the enterohaemmorhagic *E. coli* O157:H7 strains. Using this mPCR, 120 different food samples (animal source) were evaluated for the detection of *E. coli* O157:H7 and four *E. coli* O157:H7 positive strains were detected.

Brandon *et. al.* (2009) also confirmed the identities of presumptive colonies of *E. coli* O157:H7 using a multiplex PCR assay that screened for gene fragments *rfbE, fliC*h7, *eae*, *stx*1, and *stx*2. Dhanashree and Shrikar (2008) used PCR for the detection of shiga-toxigenic *Escherichia coli* (STEC) in diarrhoeagenic stool and meat samples in Mangalore, India. They performed PCR using the primer pair for *stx1,* stx2*, rfb* O157*, hlyA* and *eaeA* genes. Similarly, Ebrahim *et. al.* (2012) detected *E. coli* O157:H7 in raw bovine, camel, water buffalo, caprine and ovine milk in Iran by using PCR. The authors used primres targeting five genes; *fliCh7*, *stx1*, *stx2*, *eaeA* and *ehly* that produced amplicons of size; 629, 210, 484, 166 and 397, respectively.

Jeshveen *et. al.* (2012) optimized a multiplex PCR protocol for the detection of the pathogen *E. coli* O157:H7 targeting its endotoxin (*fliCh7)* and the virulence genes

(*eaeA, rfbE, hly, stx1,* and *stx2*), which produced species-specific amplicons at 625 bp, 397bp, 296 bp, 166 bp, 210 bp and 484 bp, respectively. Mori *et. al.* (2014) did PCR for 7 virulence factor genes: *stx1, stx2, eaeA,* Bundle forming pilus (*bfp), fliC, rfbO157* and *hlyA* to confirm different pathotypes of *E coli* in Alpacas species in Peru.

Edismauro *et. al.* (2014) detected *E. coli* O157:H7 by performing PCR to detect *16SrRNA, stx1, stx2, rfbO157, fliCh7, eae, ehxA, saa (STEC agglutinating adhesion), cnf1,* (cytotoxic necrotizing factor) *chuA, yjaA* and *TSPE4.*C2 genes in rectal swab samples from 52 healthy dairy cattle belonging to 21 farms in Mid-West of Brazil. The amplicons obtained were 401, 338, 255, 248, 119, 1.551, 259, 498, 279, 211, 152 and 625 bp, respectively. Gehua *et. al.* (2002) developed mPCR to detect *E. coli* O157:H7 targeting ten genes, *viz*, *stx*1, *stx*2, *stx*2c, *stx*2d, *stx*2e, *stx*2f, EHEC *hlyA*, *eaeA*, *rfbE*O157 and *fliC*H7. The amplicons obtained were 338, 115, 124, 175, 303, 150, 569, 248, 327 and 247 bp, respectively.

2.6.4.2.2 Detection and characterization of *E. coli* O157:H7 by other types of PCR

A variety of molecular subtyping methods have been developed to improve the understanding of the epidemiology of *E. coli* O157:H7 outbreaks apart from simple PCR and multiplex PCR. These methods include random amplified polymorphic DNA (RAPD) (Ji-Yeon *et. al.,* 2006), pulse-field gel electrophoresis (PFGE), restriction fragment length polymorphisms (RFLP), amplified fragment-length polymorphisms (AFLP), Fluorescent amplification-based specific hybridization PCR (FLASH-PCR) and phage typing (Shima *et. al.,* 2006).

Pulsed field gel electrophoresis (PFGE) is a highly discriminatory technique for the molecular typing of bacteria (Thomson-Carter *et. al.,* 1993) which has been widely used for examination of strains of *E. coli* O157 isolated from a wide variety of sources (Böhm and Karch, 1992; Barret *et. al.,* 1994; Johnson *et. al.,* 1995; Krause *et. al.,* 1996;

Conedera *et. al.,* 2001). Hiroshi *et. al.* (2014) used PCR targeting *eaeA* and *hlyA* (*ehxA*) genes and PFGE with *Xba*I endonuclease to trace the dissemination routes of shiga toxin-producing *Escherichia coli* o157 in bovine offal at slaughter in Japan.

Fluorescent amplification-based specific hybridization PCR (FLASH-PCR) is one of selective methods for rapid detection and confirmation of *E. coli* O157:H7. Khatami *et. al.* (2012) developed FLASH–PCR assay to detect the *Stx*-encoding gene *Stx1* of *E. coli* O157:H7 where in PCR product of 336 bp was successfully amplified.

Khaldoon *et. al.* (2008) did molecular characterization of *E. coli* O157:H7 strains using Random Amplified Polymorphic DNA (RAPD) employing 53 decamer primers. 56 different RAPD patterns were observed consequently. *E. coli* O157:H7 appeared to be genetically diverse based on the RAPD patterns. In another research study, *E.coli* O157:H7 were subtyped using RAPD-PCR employing OPAR8 and OPAR20 primers. Primer OPAR8 produced 8 RAPD-PCR fingerprintings namely P1 to P11, whereas, OPAR20 produced 16 RAPD-PCR fingerprintings; Q1-Q18. Combination of two primers showed that all the 20 isolates of *E.coli* O157:H7 differentiated into 20 individual isolates which may suggest the high level of local geographical genetic variation (Sahilah *et. al.,* 2010).

Shaohui *et. al.* (2014) did molecular characterization of enterohemorrhagic *E. coli* O157 isolated from animal faecal and food samples in eastern China. The primer, RAPD-2 (5'-TGCCCAGCCT-3'), was used for RAPD-PCR. RAPD analysis showed that the isolates formed 10 RAPD types (R1–R10) with bands ranging from approximately 200 to 2100 bp. A total of 9 (30%) EHEC O157 isolates were grouped into RAPD type R1. RAPD types R2, R3, and R4 were composed of 7 (23.3%), 4 (13.3%), and 4 (13.3%) isolates, respectively. The other RAPD types, R5–10, were composed of a single isolate. Patricia *et. al.* (1997) developed a PCR-restriction fragment length polymorphism (PCR-RFLP) to identify and characterize the gene *fliC*

in *E. coli* O157:H7. The restriction pattern was different for O157:H7 isolates and O157: NM isolates.

Yang *et. al.* (2013) developed a quantitative PCR assay using SYBR Green I, based on the published sequences of the *rfbE* and *fliC* genes from *E. coli* O157:H7. This method detected the *E. coli* O157:H7 O somatic antigen gene and the flagellar antigen gene simultaneously, with good specificity, sensitivity, and repeatability. The sensitivity of the assay was 2.95x10 copies/μL, which is 10^3 times more sensitive than obtained with a conventional PCR.

George *et. al.* (2013) employed Single Nucleotide Polymorphism (SNP) analysis for precise dissection of an *E. coli* O157:H7 outbreak in the USA. They suggested that backbone ORF (open reading frame) SNP set sequencing offers pathogen differentiation capabilities that exceed those of PFGE (Pulsed field gel electrophoresis) and MLVA (multiple-locus variable-number tandem-repeat analysis).

2.6.4.2.3 Detection and characterization of *E. coli* O157:H7 by gene and genome sequencing

Apart from PCR based techniques, whole genome sequencing could be used to identify and characterize the pathogen *E. coli* O157:H7. Perna *et. al.* (2001) sequenced the genome of *E. coli* O157:H7 to identify candidate genes responsible for pathogenesis, to develop better methods of strain detection and to advance our understanding of the evolution of *E. coli*, through comparison with the genome of the non-pathogenic laboratory strain *E. coli* K-12. They found that lateral gene transfer is far more extensive than previously anticipated. In fact, 1,387 new genes encoded in strain-specific clusters of diverse sizes were found in O157:H7.

Hayashi *et. al.* (2001) did complete chromosome sequencing of an *E. coli* O157:H7 strain isolated from the Sakai outbreak. The chromosome is found to be 5.5

Mb in size, 859 Kb larger than that of K-12 (non pathogenic form). They identified a 4.1-Mb sequence highly conserved between the two strains, which may represent the fundamental backbone of the *E. coli* chromosome. The remaining 1.4-Mb sequence comprises of O157:H7-specific sequences, most of which are horizontally transferred foreign DNAs.

Kulasekara *et. al.* (2009) sequenced the genome of the strain responsible (TW14359) for 2006 outbreak of *E. coli* O157:H7 caused by consumption of contaminated spinach. The TW14359 genome contains 70 kb of DNA segments not present in either of the two reference O157:H7 genomes. They identified seven putative virulence determinants, including two putative type III secretion system effector proteins, candidate genes that could result in increased pathogenicity or, alternatively, adaptation to plants, and an intact anaerobic nitric oxide reductase gene, norV.

As per the review of Eppinger *et. al.* (2013), the genome of 26 enterohemorrhagic *E. coli* O157:H7 strains has been sequenced. These bioinformatic resources will enable detailed whole-genome comparisons and permit investigations of genotypic and phenotypic plasticity in future studies.

In 2006, in an outbreak of *E. coli* O157:H7 caused by consumption of contaminated spinach, there was a notably high frequency of HUS. Bridget *et. al.* (2009) sequenced the genome of the strain responsible (TW14359) for this outbreak with the goal of identifying candidate genetic factors that contribute to an enhanced ability to cause HUS. The TW14359 genome contained 70 kb of DNA segments not present in the two reference O157:H7 genomes. In addition they found that presence of *norV* gene and absence of *stx1* gene may serve as a marker of a greater propensity for HUS. Sanjar *et. al.* (2014) sequenced *Escherichia coli* O157:H7 Strain 2886-75, associated with the first reported case of human infection in the United States and deposited genome sequence in GenBank under the accession number AVRR00000000.

2.7Antimicrobial Resistance

The most frequently reported resistance phenotype of *E.coli* 0157:H7 and non-0157 isolates are found to resist streptomycin, sulfixoxazole and tetracycline. Increasing resistance for fosfomycin, the drug of choice for paediatric gastrointestinal infections due to shiga toxin *E. coli* infection, has been documented in Japan (White *et. al.,* 2002). Antimicrobial susceptibility testing of the *E. coli* O157 isolates revealed that 10 were multidrug resistant and 73 and 5 were resistant to sulfisoxazole and tetracycline, respectively (Sinisa and Darren, 2006).

In a study by John *et. al.* (2001), *E. coli* O157:H7 isolates were found resistant for 86% antibiotics that could be used to treat *E. coli* infections in animals. Most isolates were susceptible to other antibiotics, such as trimethoprim-sulfamethoxazole and ceftiofur. All isolates were susceptible to ciprofloxacin, an antibiotic used widely in human medicine. The occurrence of *E. coli* O157:H7 multiple antibiotic resistant profiles may show a risk for public health and food safety as well as animal health and production (Von Muffling *et. al.*, 2007). In a research work, 10/14 *E. coli* O157:H7 isolates were resistant to tetracycline (Kim *et. al.,* 2012).

In a study on prevalence of antimicrobial resistance among *E. coli* O157 isolates by Manna *et. al.* (2006), among three isolates from the slaughtered cattle, two were resistant to nitrofurantoin, tetracycline and co-trimoxazole. Resistance to gentamicin and norfloxacin was also recorded. Prevalence of antimicrobial resistance was also common in the calf diarrhoea isolates. Excepting amikacin, chloramphenicol and nalidixic acid resistance was recorded against all other agents. Overall, occurrence of resistance was most frequent against nitrofurantoin (8 strains) followed by co-trimoxazole (4 strains), tetracycline (3 strains), ampicillin (3 strains), gentamicin (2 strains), norfloxacin (2 strains), cephalothin and ciprofloxacin (1 strain each).

In a study done by Taye *et. al.* (2013) majority of *E. coli* O157:H7 were found to be susceptible to chloramphenicol (30 μg), kanamycin (30 μg), spectinomycin (100 μg). In another study, the VTEC O157 isolates were 100% resistant to oxytetracycline, chloramphenicol, streptomycin and amoxycillin (Raji *et. al.*, 2008). Among the isolates of *E. coli* O157:H7, very high resistance was detected against ampicillin(100%), tetracycline(93.75%), cefuroxime and co-trimoxazole (75%), ceftriaxone, chloramphenicol and ciprofloxacin (68.75%). Highest susceptibility was detected against gentamicin (25%) (Naik and Desai, 2012).

2.8 Ecology/shedding pattern of *E. coli* 0157:H7

There are very few reports and very few researchers have worked on ecology and shedding pattern of *E. coli* 0157:H7 especially with respect to age, breed, sex and seasonal variation. Genetic and physiological factors of animals and environmental factors might affect the prevalence of *E. coli* O157:H7, suggesting potential intervention practices to reduce this pathogen entering to the food production chain.

2.8.1 Season

The season has been demonstrated to influence the shedding of this pathogen by cattle by many research workers (Chapman *et. al.*, 1997; Shere *et. al.*, 1998; Barkocy-Gallagher *et. al.*, 2003; Conedera *et. al.*, 2001; Hussain and Bolinger 2005; Edrington *et. al.*, 2006; Milnes *et. al.*, 2009).

Numerous studies in cattle indicate that faecal shedding of *E. coli* O157:H7 is typically low in the winter, increases in the spring, peaks during the summer and tapers off in the fall (Edrington *et. al.*, 2006; Hancock *et. al.*, 2001; Hussein *et. al.*, 2005; Gautam *et. al.*, 2011), although year-round shedding has also been identified (Lahti *et. al.*, 2003; Smith *et. al.*, 2010). Chapman *et. al.* (1997) observed highest prevelance of *E. coli* O157:H7 in summer months in his one year study of *Escherichia coli* O157 in

cattle, sheep, pigs and poultry. Similarly, Elder *et. al.* (2000) isolated *E. coli* O157 from 28% of faeces samples collected from fed beef cattle at slaughter during July and August. In a survey of ground beef samples in the US showed that they were 3 times more likely to be contaminated with *E. coli* O157:H7 from June – September (Chapman *et. al.*, 2001).

A year-long survey of 93 abattoirs in the United Kingdom showed peak carriage of *E. coli* O157:H7 from June to August (Milnes *et. al.,* 2009). Barkocy-Gallagher *et. al.* (2003) observed a peak *E. coli* O157:H7 fecal prevalence rate of 12.9% in the summer months, in comparison with 6.8%, 0.3%, and 3.9% in the fall, winter, and spring months, respectively. The same authors found that the prevalence of *E. coli* O157:H7 on cattle hides (a known risk factor for beef contamination) was highest from spring through fall.

Edrington *et. al.* (2004) studied variation in the faecal shedding of *Salmonella* and *E. coli* O157:H7 in lactating dairy cattle and found that *E. coli* O157:H7 was isolated only in the summer sampling times with no positive samples found on any farm in January. In a field survey of *E. coli* O157 ecology on a cattle farm in Italy carried by Conedera *et. al.* (2001), overall, *E. coli* O157 was isolated from 138 (10.7%) of 1293 bovine rectal swabs and monthly excretion rates ranged from 2.7 to 23.7%. A seasonal pattern of excretion was detected with peaks in July to August 1996 and in July 1997. In another study by Hancock *et. al.* (1997), the rates of excretion showed seasonal peaks in the summer months.

One hundred forty-four steers were assigned randomly to 24 pens on arrival at the feedlot. Samples of rectal faeces were obtained from each steer four times (October and November 2003, and March and April 2004) during finishing. On arrival (October 2003), 2 (1.4%) of 144 cattle were shedding *E. coli* O157:H7. The shedding increased significantly to 10 (6.9%) of 144 after 28 days (November 2003), to 76 (53%) of 143 at

the third sampling (March 2004) (Khaitsa *et. al.*, 2006). Fernandez *et. al.* (2009) studied the seasonal variation of Shiga toxin-producing *E. coli* (STEC) O157 in cattle in Argentina using multiplex PCR and reported that stx1 + stx2 positive *E. coli* O157 shedding occurs more in warm season.

As per Van Donkersgoed *et. al.* (1999) the number of animals shedding the organism in their faeces is usually higher in the summer months, which correlates with an increased incidence of human disease. Similarly, Elder *et. al.* (2000) isolated *E. coli* O157 from 28% of faecal samples collected from fed beef cattle at slaughter during July and August. Variation in the occurrence of *E. coli* O157:H7 was observed even in zoo animals, with animals being culture positive only in the summer months, but not in the spring, autumn, or winter (Aseel *et. al.*, 2013).

In a study on microbial flora of sheep, Indira *et. al.* (1996) found that the incidence of *E. coli* O157:H7 was transient and ranged from 31% of sheep in June to none in November. In another study, collection of positive isolates of *E. coli* O157:H7 was 15 times more likely from June through September than in other months (Stanford *et. al.*, 2005). These findings are similar to the bulk of studies conducted in the northern hemisphere, in which peak shedding for the organism occurs in summer or fall (Hancock *et. al.*, 1997; Mechie *et. al.*, 1997; LeJeune *et. al.*, 2004).

Human outbreaks of *E. coli* O157:H7 mirror the seasonal shedding patterns in cattle, occurring predominantly in the summer months (Besser *et. al.*, 1999; Rangel *et. al.*, 2005). A review of *E. coli* O157:H7 diarrhea in the US by Slutsker *et. al.* (1997) found that *E. coli* O157:H7 was isolated most frequently from patients during the summer months. Results from an epidemiological review of *E. coli* O157:H7 outbreaks in the US (1982-2002) showed that outbreaks involving ground beef peaked in summer months (Rangel *et. al.*, 2005). In Scotland, HUS and *E. coli* O157:H7 infections peaked

in patients less than 15 years of age in July/August, followed by a plateau from June to September (Douglas *et. al.,* 1997).

Edrington *et. al.* (2006) did correlation and regression analysis and revealed that there was strong positive correlation existed between day length (r =0.67; R^2 = 0.45; *P* = 0.0009) and, to a lesser extent, ambient temperature (r = 0.43; R^2 = 0.19; *P* = 0.05) and *E. coli* O157:H7 prevalence. The authors have concluded that increase in the ambient temperature has been considered as a potential cause of the increased prevalence of the shedding of *E. coli* O157:H7 during the warmer seasons. However, clear effects of temperature on the shedding of the pathogen by cattle have not been demonstrated (Brown-Brandl *et. al.,* 2009; Edrington *et. al.*, 2004; Fitzgerald *et. al.*, 2003).

However, there are studies that have not observed similar seasonal pattern of *E. coli* O157:H7 shedding (Alam and Zurek, 2006; Ogden *et. al.,* 2004; Sargeant *et. al.*, 2004; Synge *et. al.,* 2003). These studies have shown that cooler temperatures can enhance the persistence of *E. coli*, including *E. coli* O157:H7, in manures and soils (Ishii *et. al.,* 2006; Topp *et. al.,* 2003). In determining the effect of seasonal factors on the shedding patterns in calves of food borne pathogenic *E.coli* O157:H7, Bayko (2011) found that the number of CFU/g being higher in the cold rooms when compared to the warm rooms. The prevalence in Scottish beef cattle at slaughter was found to be highest during the winter as per the research findings of Ogden *et al,* (2004).

2.8.2 Breed

There are research findings that indicate that breed may act as predisposing factor for higher shedding of *E. coli* O157:H7. Jeon *et. al.* (2013) evaluated animal genetic/breed and physiological factors that affect the prevalence of *E. coli* O157 in cattle. Cattle were assigned to six breed groups according to the following breed

composition ranges: calf breed group 1 =100% to 80% of Angus and 0% to 20% of Brahman; calf breed group 2= 79% to 60% of Angus and 21% to 40% of Brahman; calf breed group 3= 62.5% of Angus and 37.5% of Brahman; calf breed group 4 = 59% to 40% of Angus and 41% to 60% of Brahman; calf breed group 5 = 39% to 20% of Angus and 61% to 80% of Brahman; and calf breed group 6 = 19% to 0% of Angus and 81% to 100% of Brahman. The breed group 6 excreted the lowest number of *E. coli* O157 among the groups indicating that Brahman breed cattle showed lowest prevalence of *E. coli* O157, when compared with other crosses in an Angus-Brahman multibreed herd. The researchers concluded that breed variation in the prevalence of *Escherichia coli* O157 in cattle as critical to reduce outbreaks of this pathogen in humans. Similarly, in a study by Riley *et. al.* (2003), lesser prevalence of *E.coli* O157:H7was observed in Brahman (0.034 proportion), Romosinuano (0.023 proportion) than in Angus breed (0.058) representing the evidence of breed-to-breed genetic variation in *E. coli* O157 shedding.

2.8.3 Age

Generally, calves have a higher prevalence of VTEC O157:H7 colonisation and excrete the agent at greater concentrations than adult cattle (Hancock *et. al.,* 1997) and the shedding declines with increasing age (Shaw *et. al.*, 2004). In a study, the higher proportion of positive samples among cows in their prime production years than in older cows appears to support the generalisation of presence of an age-dependent variation in prevalence (Riley *et. al.,* 2003). In one of the research works, the likelihood of positive isolates was 2.6 times higher in calves and heifers compared with mature cows (Stanford *et. al.,* 2005). In another research finding, prevalence of EHEC O157:H7 in cattle was higher in postweaned calves and heifers than in younger and older animals (Witold and Carolyn, 2011).

Studies conducted by Garber *et. al.* (1995) reported higher *E. coli* O157 excretion rates in weaned than unweaned calves. In one of the research works, highest prevalence of *E.coli* O157:H7 was found in calves (older) (22.9%) followed by calves (milk-fed) (18.1%), heifers (13.9%) and adult cows (9%) (Stanford *et. al.,* 2005). In Japan, overall prevalence of *E.coli* O157:H7 was 5%–10%, but 32%–46% among heifers (n>400) (Ezawa *et. al.,* 2004). EHEC shedding on eight cattle farms in Denmark was higher among postweaned than nonweaned calves, and it was reduced among calves 1–4 months old, if they suckled colostrums or stayed >2 days with their dams after birth (Rugbjerg *et. al.,* 2003).

In England and Wales within-herd prevalence of STEC O157 in cattle farms was 1%–51% and the shedding was the lowest among calves <2 months old and highest in calves 2–6 months (Paiba *et. al.*, 2003). Young weaned calves were found to shed *E.coli* O157:H7 at higher levels and longer period than adult cattle in experimental studies, which could be due to dietary stress during weaning and because they do not yet have a fully formed rumen to help suppress *E. coli* O157:H7 growth (Cray and Moon, 1995; Hussain and Bolinger, 2005).

Serologic evidence suggests that most calves are exposed to *E. coli* O157:H7 (Laegreid *et. al.,* 1999). Heuvelink *et. al.* (1998) found that *E.coli* O157:H7 were isolated more frequently from calves less than 3 months old than from adult cows/ buffaloes. Cray and Moon (1995) reported that calves shed *E. coli* O157:H7 longer period than adult cattle given the same level of *E. coli* O157:H7 inoculums. In a research work on *E. coli* O157:H7 isolated from sheep, cattle and human patients by Soderlund *et. al.* (2012), verotoxin-producing O157:H7 was found in 11/597 (1.8%) of samples from sheep in Swedish slaughterhouses comprising of 9/492 faecal (1.8%) and 2/105 ear samples (1.9%). All positive sheep were < 6 months old.

However, the rate of excretion was not affected by the age of the animals at sampling as per the findings of Conedera *et. al.,* (2001).

2.8.4 Sex/gender and other factors

The number of super-shedders was significantly different (P = 0.022) between bulls (27.27%, n= 11) and steers (3.75%, n= 80) in a research work by Jeon *et. al.* (2013), indicating bulls are more susceptible to become super-shedders. Finishing cattle and female breeding cattle are also often under dietary stress and finishing cattle are normally housed in mixed groups from multiple farms, which could lead to increased transmission (Gunn *et. al.,* 2007).

In a study by Stanford *et. al.* (2005) on ecology of *E. coli* O157:H7 in commercial dairies in Southern Alberta, dry cows (11.1%) showed higher shedding than lactating cows (10.7%). Similarly, in a research work by Riley *et. al.* (2003), non lactating dairy cows (0.033 proportion) were shown to have a slightly higher prevalence of *Escherichia coli* O157:H7, but not significant, than lactating cows (0.024 proportion). Similarly, Wilson *et. al.* (1993) found increased shedding of *E. coli* O157:H7 in dry cows compared to lactating cows. In contrast, Fitzgerald *et. al.* (2003) reported a higher incidence of shedding in lactating compared with nonlactating animals.

As per the findings of Heuvelink *et. al. (*1998) *E.coli* O157:H7 were isolated more frequently from diarrheic calves than from normal calves.

2.9 Research on *E. coli* O157:H7 in India

In India, there is paucity of information on STEC particularly *E. coli* O157:H7. It has not been identified as a significant etiologic agent of diarrhoea for humans in India. However, isolation of O157 and non-O157 serogroups of *E. coli* that exhibiting the cytotoxic activity in vero cells has been reported from human patients with

diarrhoea in India, but these strains have not been well characterized, and their origin is uncertain. Similarly, there are not many reports of isolation of STEC from various animal species in India (Wani *et. al.*, 2004).

In India, for the first time the occurrence of *E. coli* O157:H7 was reported in buffalo meat kebabs, sausages, buffalo milk, cow milk and khoa sweet in 1966 (Singh *et. al.*, 1996). In 2002, researchers in Calcutta (present Kolkata), India, reported finding of non-O157 STEC isolates in 1.4% of stool samples from humans suffering from bloody diarrhea (Khan *et. al.*, 2002a; Khan *et. al.*, 2002b). They concluded that STEC was not an important cause of diarrhea in India. However, Steve *et. al.* (2007) isolated *Escherichia coli* serotype O157:H7 in water samples collected from the Ganges River.

Manna *et. al.* (2006) studied the occurrence of *E. coli* O157 in cattle stool in West Bengal, India, and the virulence properties and antimicrobial resistance of the *E. coli* isolates. *E. coli* serotype O157 was isolated from faecal samples from two (2.04%) slaughtered cattle and six (7.59%) diarrhoeic calves. In another research work, duplex assay using two specific molecular beacons by real-time PCR was used to target *rfb* gene of *E. coli* O157:H7. In 60 market samples, 3 samples (raw milk, kulfi, and paneer) were positive for *E. coli* O157:H7 (Singh *et. al.*, 2009).

Hazarika *et. al.* (2007) isolated *E. coli* from 112 beef samples out of which 7 were STEC and 1 *E.coli* O157:H7. The authors employed vero cell cytotoxicity, PCR targeting three genes; *stx1, stx2* and *eae* and enterohemolysin production to detect *E.coli* O157:H7. In a report by Dhanashree and Shrikar (2008) 192 diarrhoeagenic stool samples and 103 meat samples were screened for STEC, using conventional culture methods and PCR from December 2003 to 2006 in the department of Microbiology, Kasturba Medical College, Mangalore. Of the 40 *eae* positive *E. coli* isolates from meat sample, one was positive for all the STEC genes, namely *stx1, stx2, rfb* O157 and EHEC *hlyA*. This isolate belonged to O157 serogroup.

E.coli O157 was isolated from both bovine and ovine faecal samples as per the report of Wani *et. al*. (2003). A 10 year epidemiological survey of *E. coli* O157 across India was conducted in 5678 human samples and 11,415 non human samples. There were only 30 (0.5%) human samples which were positive for *E. coli* O157. The *E. coli* O157 was seen in meat 0.9%, (13/1376), milk and its products 1.8%, (10/553), seafood 8.4%, (16/190) and water 1.6%, (8/486). The isolates obtained were widely distributed among both domestic and wild animals (Rakesh *et. al.,* 2008; Verma *et. al.,* 2013).

Bindu and Krishnaiah (2010) tested 250 samples collected from various sources using PCR assay for the presence of *E. coli* O157:H7 and reported that 27 samples were positive. In an analysis, 500 samples were collected in a 2 years' time from five different sources such as human faeces from uremic syndrome patients, small intestine of chicken, ground beef, cattle faeces and raw milk for the prevalence and characterization of *E. coli* O157:H7 in Chennai, India by Divya *et. al.* (2013). The authors found only 5 samples were positive for *E. coli* O157:H7. The molecular characterization was done by PCR for the presence of 5 virulence genes, namely *eae, stx1, stx2, hlyA* and *fliC* genes.

Naik and Desai (2012) worked on isolation of *E. coli* O157:H7 from diarrheagenic stool sample of patients in Surat (India) and determination of their antimicrobial resistance pattern. *E. coli* O157:H7 was isolated from 13.91 % samples emphasising the presence of the pathogen in humans in India and its public health significance. Thenmozhi (2010) conducted a study to know the frequency of *E. coli* O157:H7 and studied characteristics in drinking water sources, collected from different areas in Namakkal district, Tamil Nadu. *E. coli* O157:H7 was found to be higher in stagnant water on the rocks than the flowing water. Shanta *et. al.* (2000) identified beef marketed in Calcutta, as a source of *E. coli* O157:H7 indicating prevailing unhygienic

practices in slaughter houses in Calcutta. Seven of the ten STEC were identified as *E. coli* O157:H7 by using PCR (primers for *stx1* and *stx2*) and bead ELISA.

2.10 Strategies for the prevention of *E. coli* O157:H7 shedding and infection

The development of control strategies to reduce *E. coli* O157:H7 will require the identification of biological, environmental, and/or management factors that affect its incidence in cattle and their production environments. Research investigations and epidemiological studies have identified a number of risk factors or management practices that can or may contribute to the occurrence of this pathogen, and that may be exploitable to reduce its numbers, persistence and transmission in cattle (Sargeant *et. al.,* 2007).

The best way to prevent *E. coli* O157:H7 infection is by adequate cooking of food especially beef. All meat, in particular hamburgers, should be thoroughly cooked to reach a centre temperature of at least 70°C for two minutes in order to inactivate the bacteria (The Food Standards Agency, UK, 2014).

The testing and segregation of *E. coli* O157:H7-positive cattle near or immediately before slaughter until the animals test negative has been considered as a method to limit the number of cattle contaminated with *E. coli* O157:H7 entering the food chain.

2.10.1 *Vaccination*

Vaccination is used to prevent *E. coli* O157:H7 colonization and faecal excretion in ruminants and it is based on inducing the animal's immune system to protect itself from antigens expressed by *E. coli* O157:H7 (Van Donkersgoed *et. al.,* 2005). Cattle receiving the vaccine were less likely to be colonized at the terminal rectum (Peterson *et. al.,* 2007b; Smith *et. al.,* 2009b) and less likely to shed *E. coli* O157:H7 in faeces (Moxley *et. al.,* 2009; Peterson *et. al.,* 2007a; Smith *et. al.,* 2009a). A commercial

vaccine is fully licensed for use in Canada and Bioniche Life Sciences, Inc. is working to meet the requirement for the U.S. conditional license (Bioniche Life Sciences, Inc., 2008).

In a recent clinical vaccine trial, commercially fed cattle were used to test the effect of a two-dose regimen of a vaccine against type III secreted proteins of *E. coli* O157:H7 (Naylor *et. al.,* 2005; Smith *et. al.,* 2008). The study found that pens of vaccinated cattle were less likely to test positive for *E.coli* O157:H7. In a study by Potter *et. al.* (2004) vaccination of cattle with proteins secreted by *E. coli* O157:H7 significantly reduced the numbers of bacteria shed in faeces, the numbers of animals that shed, and the duration of shedding in an experimental challenge model.

A vaccine targeting siderophore receptor and porin proteins (Epitopix, LLC, Wilmar, MN) recently received a conditional license for use in cattle in the United States (Epitopix LLC, 2009). The vaccine tended to reduce *E. coli* O157:H7 faecal prevalence and faecal concentrations of the pathogen in *E. coli* O157:H7-inoculated calves.

Immunization of the sows using a vaccine containing *E. coli* O157:H7 intimin protected the suckling piglets from *E. coli* O157:H7 infection and intestinal damage (Dean-Nystrom *et. al.,* 2002). Vaccination of experimentally infected calves with a combination of type III secreted proteins (EspA, intimin, and Tir) reduced *E. coli* O157:H7 concentrations in feces and the total load of the pathogen that was shed (McNeilly *et. al.,* 2010). In contrast, vaccination of calves with EspA alone induced an immune response, but did not protect against *E. coli* O157:H7 intestinal colonization (Dziva *et. al.,* 2007). Intramuscular immunization with H7 flagellin reduced colonization rates and delayed peak shedding of *E. coli* O157:H7 in orally inoculated calves, but did not reduce total pathogen shedding (McNeilly *et. al.,* 2008).

2.10.2 Other measures

Experiments with individual Holstein calves found that the addition of chitosan microparticles (CM) to feed decreased *E. coli* O157:H7 shedding. Feeding of chitosan microparticles resulted in statistically significant reductions in the numbers recovered from rectal swab samples ($P < 0.05$) and the duration of shedding ($P < 0.05$) (Kwang *et. al.*, 2011).

As a direct-fed microbial, selected *L. acidophilus* strains, alone or in combination with *P. freundenreichii*, (probiotics) have been the most thoroughly studied and often are very effective at reducing the prevalence of fecal shedding of *E. coli* O157:H7 (Peterson *et. al.*, 2007c; Tabe *et. al.*, 2008; Younts-Dahl *et. al.*, 2005). *Lactobacillus acidophilus* culture, has repeatedly demonstrated effectiveness at reducing *E. coli* O157:H7 in feedlot cattle by up to 50% (Brashears *et. al.*, 2003; Elam *et. al.*, 2003; Younts-Dahl *et. al.*, 2005). This product is currently available commercially in the United States and is being used in many, if not most, large US feedlots (Callaway *et. al.*, 2004).

The prevalence of *E. coli* O157:H7 got reduced when animals were fed with *E. coli* O157:H7-infecting bacteriophages (Sheng *et. al.*, 2006; Niu *et. al.*, 2009; Oot *et. al.*, 2007). Research indicates that neomycin sulfate could be an effective intervention. Administration at therapeutic doses reduced faecal shedding of *E. coli* O157:H7 compared to controls (Elder *et. al.*, 2002; Keen *et. al.*, 2006). However, concerns about the development of antibiotic resistance may hinder the approval of neomycin sulfate for this use (Callaway *et. al.*, 2009). When sodium chlorate was administered in feed and drinking water, it reduces *E. coli* O157:H7 populations in the faeces and in the intestinal content of cattle (Callaway *et. al.*, 2002)

Numerous studies have demonstrated that processing practices and antimicrobial intervention procedures applied at slaughter; including hide washes, steam pasteurization, organic acid washes, hot water washes, or combinations of these treatments, substantially reduce *E. coli* O157:H7 from cattle carcasses (Barkocy-Gallagher *et. al.,* 2003; Elder *et. al.,* 2000). High-level fecal shedding of *E. coli* O157:H7 is associated with increased hide contamination, and hides are an important source of beef carcass contamination at harvest (Arthur *et. al.,* 2009).

It would be accepted that reducing the carriage or prevalence of *E. coli* O157:H7 in cattle would reduce the risk of human exposure to this pathogen. Implementation of hazard analysis and critical control point policies in diary animals and both pre-slaughter and post slaughter in meat industry are important to reduce the frequency that ground beef is contaminated with this foodborne pathogen (Callaway *et. al.,* 2009; Jeong *et. al.,* 2011). Analytical and theoretical models of preharvest control of *E. coli* O157:H7 have likewise predicted significant impacts of on-farm intervention strategies on human cases of this foodborne bacterium (Jordan *et. al.,* 1999a; Jordan *et. al.,* 1999b; LeJeune and Wetzel, 2007). Although risk factors on farms have been extensively studied from the bacterial perspectives, information regarding animal factors and environmental factors that may contribute to the prevalence of this pathogen is lacking.

CHAPTER III
MATERIAL AND METHODS

The present study was undertaken to standardize multiplex PCR for the detection of *E.coli* O157:H7 in faecal samples and to isolate and detect *E.coli* O157:H7 from faecal samples of cattle and buffaloes by cultural identification, biochemical characterization, latex agglutination and the standardized multiplex PCR and to find the variation in epidemiology of shedding pattern of *E.coli* O157:H7 in bovines between buffaloes and cattle, different age groups, sex, healthy and diarrhoeic animals and among seasons and in cattle among breeds.

The following chemicals, media, detection kits and equipment were used at different stages of the present study.

3.1 Equipment

The various equipment used in this study are as shown in the Table 3.1 and Fig 3.1a and Fig 3.1b.

3.2 Glassware and plastic ware

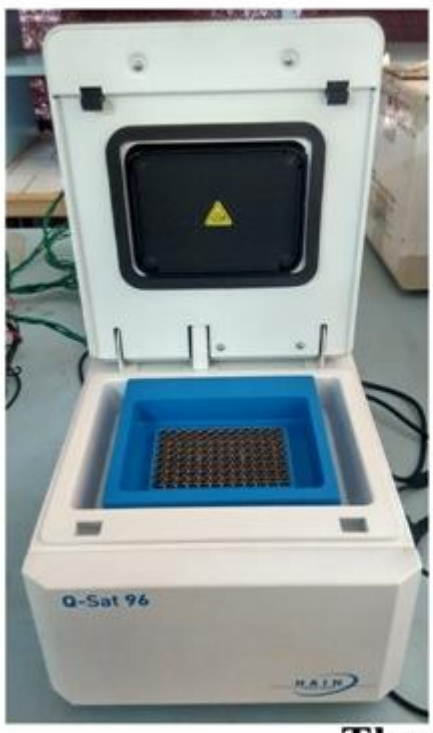
Glassware like petri dish plates, beakers, conical flasks, pipettes (1 to 10 mL volume), measuring cylinders, centrifuge tubes, glass slides and plastic ware like eppindorf tubes, micropipette tips, micro centrifuge tubes (0.5 to 2.0 mL), PCR reaction tubes (minimum reaction volume of 25 µL), spatula, test tube racks, sterile tongs, bunsen burner, nitrile gloves, face mask, re-sealable plastic sachets and other usual laboratory glass ware were used in this study.

3.2 Chemicals and media

The various media utilized in this study, viz; Modified Trypticase soya broth supplemented with novobiocin, Sorbitol MacConkey agar (SMAC), Tellurite-cefixime supplement [potassium tellurite (1.25 mg) and cefixime (0.025 mg)], Eosin methylene blue agar (EMB agar).

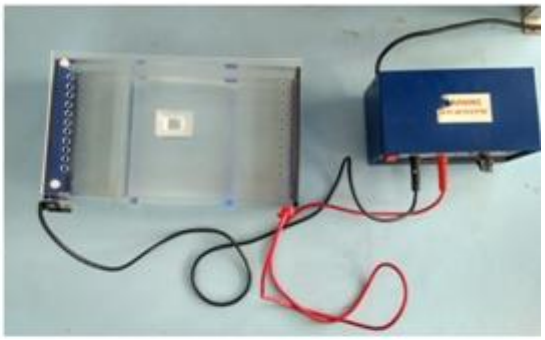
Table 3.1: List of equipment used in the present study

Name of the equipment	Trade name	Manufacturing firm
Refrigerated Centrifuge	Eppindorf Centrifuge® Model 5430 R	Eppindorf, Germany
Water bath	Model no. BEA-13	Biocraft Scientific Systems Pvt. Ltd, India
Micro centrifuge	Laboratory Table Top Centrifuge (micro)	Micro Teknik Pvt. Ltd., India
Deep Freezer	Blue Star Chest Freezer® Model CHF300B	Blue Star Ltd., India
Gel documentation system	Molecular Imager® Gel Doc™ XR+	BioRad, USA
Gel electrophoresis	Biotech Submarine Gel System	Biotech, Salem, India
Biosafety cabinet	Biological Safety Cabinet **(**BSL II) – 1300 series A_2	Thermo Fisher Scientific, USA
Laminar air flow	Laminar air flow	Deccan Techno, India.
Weighing balance	Weighing Balance Scale Tec® Model SAB 303	HiMEDIA, India
Hot air oven	Yorco Hot Air Sterilizer (Oven)	York Scientific Industries Pvt.Ltd., India
Bacteriological incubator	Bacteriological Incubator	Micro Teknik, India
Autoclave	Yorco Vertical Autoclave	York Scientific Industries Pvt. Ltd., India
Vortex Mixer	Cyclomixer® Model CM 101	Rajendra Electrical Ltd., India
Microscope	Binocular Microscope, Model CH20BIMF	Olympus India Pvt. Ltd.
Micropipettes	Micropipettes	Thermo fisher Scientific, USA
Thermocycler	Q-Sat 96 Thermal Cycler®	Quanta Biotech Ltd. UK
Spectrophotometer	SP-UV 2005 Spectrophotometer	Spectrum Instruments, Aust, Pty, Ltd, Australia
Thermometer	Laboratory Thermometer	Texla Scientific Instruments, Hyderabad, India
Colony counter	Digital Colony Counter	Biocraft Scientific Systems Pvt. Ltd, India
Inoculation loop	Inoculation loop	HiMEDIA, India.



Thermocycler

Gel documentation system



Submarine gel electrophoresis system

Refrigerated centrifuge

Fig 3.1a: Various equipment used for multiplex PCR

Fig 3.1b Biosafety cabinets used in the present study

Fig 3.2: Wellcolex® *E.coli* O157:H7) Latex Agglutination test kit

MacConkey Agar (CT-SMAC), the contents of 1 vial of Tellurite-Cefixime supplement were rehydrated with 5 ml of sterile distilled water, mixed gently to dissolve the contents completely. The contents were added to 495 ml of sterile, molten, cooled Sorbitol MacConkey agar base, mixed well and poured into sterile petri plates. Sterility of the media was checked by incubating plates at 37°C for 24 hours. The composition of media and chemical reagents used for cultural and biochemical identification of *E.coli* O157:H7 is appended in Annexure 1.

Anti-O157 and anti-H7 latex reagent (Wellcolex® *E.coli* O157:H7) was procured from Thermo Fisher Scientific India, Pvt. Ltd., (REF No. R30959601), manufactured by Remel Europe Ltd, UK (Fig 3.2) for the purpose of Latex Agglutination test. All the chemicals and molecular reagents for PCR were procured from Merk GeNei^{TM}, Bengaluru *viz;* dNTPs, 100 bp DNA ladder, *taq* polymerase, magnesium chloride and buffer. The PCR primers for the six virulent genes namely; *fliCh7, rfbE, stx1, stx2, eaeA* and *hly* genes were also synthesized by Merck GeNei^{TM}, Bengaluru.

3.3 Standardization of multiplex PCR

The standard culture of *E.coli* O157:H7 – US FDA strain used for the standardization in this study was obtained from Department of Microbiology, College of Fishery Sciences, KVAFSU, Mangalore. The strain was stored at -20˚C in Soyabeen bile broth with novobiocin added with 25% glycerol. For experiment purposes, an inoculum of the standard culture was inoculated in to Soyabeen bile broth with novobiocin then incubated at 37˚C for 24 hours for enrichment. An inoculum of the culture from this broth is streaked on to MacConkey Sorbitol agar with potassium tellurite and cefixime supplement and incubated at 37˚C for 24 hours. This culture was used for the standardization of multiplex PCR.

Table 3.2: Sequence of the primers used in the present study

Target gene	Primer	Sequence	Amplicon size (bp)	Reference
fliCh7	F	GCGCTGTCGAGTTCTATCGAGC	625	Sarimehmetoglu *et. al.* (2009)
	R	CAACGGTGACTTTATCGCCATTCC		
rfbE	F	CAGGTGAAGGTGGAATGGTTGTC	296	Sarimehmetoglu *et. al.* (2009)
	R	TTAGAATTGAGACCATCCAATAAG		
Stx1	F	TGTAACTGGAAAGGTGGAGTATACA	210	Sarimehmetoglu *et. al.* (2009)
	R	GCTATTCTGAGTCAACGAAAAATAAC		
Stx2	F	GTTTTTCTTCGGTATCCTATTCC	484	Sarimehmetoglu *et. al.* (2009)
	R	GATGCATCTCTGGTCATTGTATTAC		
eaeA	F	ATTACCATCCACACAGACGGT	397	Sarimehmetoglu *et. al.* (2009)
	R	ACAGCGTGGTTGGATCAACCT		
hly	F	ACGATGTGGTTTATTCTGGA	166	Sarimehmetoglu *et. al.* (2009)
	R	CTTCACGTCACCATACATAT		

3.3.1 DNA template preparation

The extraction of the genomic DNA of *E. coli* 0157:H7 was done by using the modified boiled cell method (Queipo-Ortuño *et. al.*, 2008). For the extraction of the genomic DNA from the standard culture, overnight culture of *E. coli* O157:H7 in trypticase soya broth was centrifuged for 2 min at 15,000 rpm and the supernatant was discarded. The cell pellet was resuspended in 500 µl of nuclease free water followed by

vigorous vortexing. The homogenized cell suspension was boiled for 10 min at 100°C. The boiled cell lysate was immediately cooled at -20˚C for 10 min and centrifuged again at 15000 rpm for 2 min. The supernatant was used as template DNA for multiplex PCR.

For the extraction of the genomic DNA of *E. coli* 0157:H7 directly from the faecal samples, 1 g of sample was added with 9 ml distilled water, vortexed and centrifuged for 2 min at 15,000 rpm and the supernatant was discarded. The pellet was resuspended in 1 ml of nuclease free water followed by vigorous vortexing. The homogenized suspension was boiled for 10 min at 100°C. The boiled cell lysate was immediately cooled at -20˚C for 10 min and centrifuged again at 15000 rpm for 2 min. The supernatant was used as template DNA for multiplex PCR.

For the extraction of the genomic DNA of *E. coli* 0157:H7 from the faecal samples with one step selective enrichment in mTSB, overnight culture in trypticase soya broth was centrifuged for 2 min at 15,000 rpm and the supernatant was discarded. The cell pellet was resuspended in 500 µl of nuclease free water followed by vigorous vortexing. The homogenized cell suspension was boiled for 10 min at 100°C. The boiled cell lysate was immediately cooled at -20˚C for 10 min and centrifuged again at 15000 rpm for 2 min. The supernatant was used as template DNA for multiplex PCR.

For the extraction of the genomic DNA of *E. coli* 0157:H7 from the culture on the agar (isolate), 2-3 colonies on CT-SMAC agar were suspended in 500 µl of nuclease free water followed by vigorous vortexing. The homogenized cell suspension was boiled for 10 min at 100°C. The boiled cell lysate was immediately cooled at -20˚C for 10 min and centrifuged at 15000 rpm for 2 min. The supernatant was used as template DNA for multiplex PCR.

3.3.2 Multiplex PCR protocol

The multiplex PCR protocol was standardized in a volume of 25 µl of reaction mixture containing 2 µl of *Taq* DNA Polymerase (1 unit/ µl), 2.0 µl of DNA template solution, 2.5 µl of 10 x reaction buffer, 0.5 µM of deoxynucleoside triphosphates (dNTPs), 0.2 µM each of the 12 primers (6 primer pairs) and magnesium chloride (MgCl2) (concentrations were standardized). Sterile nuclease free water was added accordingly to make up the 25 µl reaction mixture. The magnesium chloride (MgCl2) concentration was evaluated for 1.5 mM, 2mM, 2.0mM, 3.0 mM and 3.5mM.

Thermal cycling was performed using the 96-Well Q-Sat 96 Thermal Cycler® and the PCQB software. It consisted of a 5 minute initial denaturation at 94˚C followed by 35 cycles of denaturation at 94˚C (time period standardized), 1 min of annealing (temperature standardized) and extension at 72˚C for 1 min, with a 10 min final extension at 72˚C followed by maintenance at 4˚C. The time period of denaturation temperature evaluated were 20 sec, 45 sec and 1 minute. The annealing temperature was evaluated between 58°C to 63°C by performing gradient multiplex PCR. The mPCR protocol has been developed using primer pairs targeting six specific virulent genes of *E. coli* O157:H7; *fliCh7*, *rfbE*, *stx1*, *stx2*, *eaeA* and *hly*. The primers were synthesised by Merck GeNei™. The sequence of the primers used inthis study is shown in the Table 3.2.

3.3.3 Agarose gel electrophoresis

From each PCR product an aliquot of 5 µl was subjected to agarose (concentration standardized) gel electrophoresis containing 0.5 x TAE buffer (pH 8.0) to visualize the amplicons. The agarose was prepared in 1x TAE buffer and boiled. After cooling for about 2 minutes ethidium bromide was added to the agarose solution at the rate of 1% of the agarose solution to stain the DNA bands. The molten agarose was cast into the tray fitted with the comb. After solidification, the comb was taken out

and the gel is placed into the submarine horizontal electrophoresis chamber filled with 1x TAE buffer sufficient to immerse the agarose gel.

Five µL of PCR product and 5 µL of 100 bp DNA ladder as molecular marker were loaded in to the wells and the electrophoresis was carried out at 80 Volt, 400 mA for 40 min. The DNA bands were visualized under ultraviolet (UV) light using gel documentation system (BioRad) and analyzed by using the Image Lab™ software (BioRad). The agarose concentration evaluated was for 1% and 1.5%.

3.3.4 Threshold sensitivity of multiplex PCR

To estimate the threshold sensitivity of standardized multiplex PCR, 1 ml of ten fold serial dilutions of *E. coli* O157:H7 reference culture viz*;* 10000, 1000, 100, 10, 1, 0.1 and 0.01 CFU were prepared in modified Tryptone Soy Broth (mTSB) supplemented with novobiocin and incubated at 37°C for 1 hour. The dilutions in mTSB broth were subjected for standardized multiplex PCR.

The threshold sensitivity of standardized multiplex PCR was also checked by inoculating (spiking) the reference culture of *E. coli* O157:H7 into the faecal sample which was found to be negative for *E. coli* O157:H7 by cultural characters. 1 g of such faecal sample was spiked with 1 ml of ten fold serial dilutions of *E. coli* O157:H7 reference culture viz*;* 10000, 1000, 100, 10, 1, 0.1 and 0.01 CFU, incubated at 37°C for 1 hour and directly (without selective enrichment) subjected for multiplex PCR.

Also, faecal samples spiked with the above stated dilutions of the reference culture were inoculated into 9 ml of mTSB broth (one step selective enrichment) and incubated at 37°C for 24 hours. These spiked faecal samples with one step selective enrichment were then subjected for multiplex PCR.

3.3.5 Specificity of multiplex PCR

To evaluate the specificity of standardized multiplex PCR, *E. coli* other than *E. coli* O157:H7 (sorbitol positive isolate on CT-SMAC agar) *Staphylococcus aureus, Streptococci, Vibrio parahaemolyticus, Brucella abortus* (strain 19) and *Salmonella* cultures maintained in the Department of Veterinary Public Health and Epidemiology, Veterinary College Bidar, Karnataka and Department of Veterinary Public Health and Epidemiology, College of Veterinary Sciences, Rajendranagar, Hyderabad, Telangana, India, were used as negative controls and subjected for standardized multiplex PCR along with reference culture of *E. coli* O157:H7 to rule out false negative identification of six virulent genes of *E. coli* O157:H7.

3.4 Collection of samples

A total of 932 fecal samples were collected from fresh faecal pats of buffaloes and cattle of different ages (calf, heifer and adult), sex, in different seasons and in cattle of different breeds (indigenous, crossbred and exotic), from different cattle and buffalo farms in Bidar, Gulbarga, Bengaluru and other parts of Karnataka and fresh fecal pats of the diarrheic cattle and buffaloes admitted to the clinics, Department of Teaching Veterinary Clinical Complex, Veterinary College Bidar, Karnataka during the period from July 2014 to January 2016. The ambient temperature was recorded while collecting the faecal samples. The samples were placed in clean and sterile re-sealable plastic sachets, labeled properly and transported to laboratory in thermo-cool container jacketed with ice packs. The samples were processed and analyzed for the isolation of *E.coli* O157:H7 within 24 hours. The samples which could not be analyzed within 24 hours were preserved in the deep freezer at -20°C and analyzed on the next days.

3.5 Processing of samples

3.5.1 Cultural isolation of *E.coli* O157:H7 and biochemical characterization of isolates.

Three grams of each faecal sample was inoculated into 27 ml (1:9 ratio) (modified tryptone soy broth (mTSB) supplemented with novobiocin and incubated overnight at 37°C for 24 hours. A loopful of inoculum from the enrichment medium was inoculated onto selective isolation medium: Sorbitol-MacConkey agar supplemented with cefixime (0.025 mg) and potassium tellurite (1.25 mg) (CT-SMAC). The agar plate was incubated for 24 h at 37°C. Growth of typical colourless colonies (sorbitol negative) indicates positive for *E. coli* O157:H7.

These isolates were further subjected for inoculation on eosin methylene blue (EMB) agar, to confirm the isolate as *E.coli* by observing for the colonies producing metallic sheen, Gram's staining and biochemical tests. The biochemical tests performed were Indole test, Methyl Red test, Voges- Proskauer test, Citrate utilization test (IMVC), Nitrate test and Sugar fermentation test for Lactose, Sucrose and Dextrose. The typical colourless isolates on CT-SMAC agar showing metallic sheen on EMB agar, Gram negative staining character, positive for indole (pink red top line), methyl red (red colour), sugar fermentation (yellow slant and butt with gas production) and nitrate tests (red colour) and negative for Voges- Proskauer test (no pink top line), Citrate utilization test (no change in colour of the medium from green to blue colour) were presumed as positive for *E. coli* O157:H7 and inoculated into modified tryptone soy broth for preservation and confirmative identification and molecular characterization by using multiplex PCR and latex agglutination.

3.5.2 Identification and molecular characterization of *E. coli* O157:H7 in faecal samples by multiplex PCR

The faecal samples directly and the samples with one step selective enrichment in mTSB broth were subjected for standardized multiplex PCR for the detection and molecular characterization of *E. coli* O157:H7. The multiplex PCR products were visualized by agarose gel electrophoresis. The faecal samples that showed presence of

*fliCh*7 and *rfbE* genes were considered as positive for *E. coli* O157:H7 and observed for the four virulent genes, *viz; stx1, stx2, eaeA* and *hly* for further characterization.

Further, the samples showing only presence of *rfbE* gene and/or other virulent genes and no *fliCh*7 gene were considered positive for *E. coli* O157:H$^-$. These samples combined together were classified as *E. coli* O157. The samples showing absence of both of the *rfbE* and *fliCh*7 genes and presence of only one of the or both of the *stx1 and stx2* genes were considered positive for Shiga Toxin producing *E. coli* (STEC) other than *E. coli* O157:H7. The samples positive for *E. coli* O157:H7, *E. coli* O157:H$^-$ and *E. coli* other than *E. coli* O157:H7 positive for anyone or both of the shiga toxin genes together were classified as STEC.

The samples found positive for *E. coli* O157:H7, *E. coli* O157:H$^-$ and *E. coli* other than *E. coli* O157:H7 showing presence of anyone or both of the shiga toxin genes (STEC) were counterchecked and confirmed by cultural inoculation, Gram's staining, biochemical characterization and latex agglutination.

3.5.3 Confirmative identification of *E. coli* O157:H7 by Latex Agglutination Test

The samples found positive for *E. coli* O157:H7 by cultural isolation, cultural isolation with biochemical characterization and multiplex PCR were confirmed by using latex agglutination test (Fig 3.2).

For the confirmation of the samples found positive for *E. coli* O157:H7 by cultural isolation, the isolated colonies on CT-SMAC agar were tested for the presence of O157 and the H7 antigens by latex agglutination. For the confirmation of the samples found positive for *E. coli* O157:H7 by multiplex PCR, 1 g of each faecal sample was mixed thoroughly with 1 ml of mTSB broth by vortexing to prepare suspension and allowed to settle. The supernatant was used to test for the presence of O157 and the H7

antigens by latex agglutination. The procedure followed for latex agglutination test (as per the instruction of the manufacturing company) was as follows;

3.5.3.1 O157 procedure

40µL of saline was placed in first two circles on a reaction card supplied with the product and emulsified with each test sample (sufficient growth on CT-SMAC agar and 40 µL of faecal sample suspension in mTSB broth) using separate mixing sticks provided with the product. One drop of O157 latex test was mixed with the emulsified culture in first circle and one drop of O157 control latex was mixed with the emulsified culture in the second circle so that latex spreads the entire area of the circle. The emulsion was observed for agglutination within 30 seconds of thorough mixing.

3.5.3.2 H7 procedure

40µL of saline was placed in first two circles on a reaction card supplied with the product and emulsified with each test sample (sufficient growth on CT-SMAC agar and 40 µL of faecal sample suspension in mTSB broth) using separate mixing sticks provided with the product. One drop of H7 latex test was mixed with the emulsified culture in the third circle and one drop of H7 control latex was mixed with the emulsified culture in the fourth circle so that latex spreads the entire area of the circle. The emulsion was observed for agglutination within 30 seconds of thorough mixing.

3.5.3.3 Positive control procedure

One drop of each test latex (O157 and H7) was placed in separate circles (fifth and seventh) on the reaction card. One drop of positive control for each antigen (O157 and H7), provided with the product, was placed next to respective test latex and mixed. In these two circles, agglutination was observed definitely and hence positive control.

3.5.3.4 Negative control procedure

One drop of each test latex (O157 and H7) was placed in separate circles (sixth and eighth) on the reaction card. One drop of negative control for each antigen (O157 and H7), provided with the product, was placed next to respective test latex and mixed. In these two circles agglutination was not observed definitely and hence negative control.

3.5.3.5 Interpretation

Agglutination of the O157 test latex accompanied by a lack of agglutination of the control latex indicates positive for *E. coli* O157 antigen in the culture under test. Lack of agglutination in both O157 reagents indicates that the culture under test is negative for *E. coli* O157. Similarly agglutination of the H7 test latex accompanied by a lack of agglutination of the control latex indicates positive for *E. coli* H7 antigen in the culture under test. Lack of agglutination in both H7 reagents indicates that the culture under test is negative for *E. coli* H7.

3.6 Epidemiological Sensitivity and specificity of the cultural isolation, cultural isolation with biochemical characterization and multiplex PCR (Thrusfield, 2005)

The number of positive samples for *E. coli* O157:H7 by cultural isolation, cultural isolation with biochemical characterization and multiplex PCR was compared with the gold standard confirmative diagnostic test – latex agglutination test (CDC, 2015) to estimate the epidemiological sensitivity and the specificity (Thrusfield, 2005). The formulae used for the estimation are described below;

Sensitivity = a/a+c

Specificity =d/b+d

Where a = true positive

b = false positive

c = false negative

d = true negative

3.7 Analysis and interpretation on epidemiology of shedding pattern of *E. coli* O157:H7

In the present study, since the standardized multiplex PCR identification *E. coli* O157:H7 in faecal samples with one step selective enrichment appears more sensitive and specific, it was employed as a technique for the final consideration of the sample as positive for *E. coli* O157:H7 and hence its results was used for the analysis and interpretation on epidemiology of shedding pattern of *E. coli* O157:H7 in buffaloes and cattle.

Analysis was done to know the prevalence of shedding of *E. coli* O157:H7 in bovines and variation in its shedding pattern in buffaloes and cattle. Sex wise variation in shedding pattern of *E. coli* O157:H7 was analysed between male and female bovines. Age wise variation in shedding pattern of *E. coli* O157:H7 was analysed between calves, heifers and adult bovines. The bovines after first pregnancy and above were grouped as adults, between weaning and first pregnancy were grouped as heifers and the young ones before weaning were grouped as calves. To analyse the effect of diarrhoea on shedding of *E. coli* O157:H7, the animals were grouped as diarrhoeic and healthy.

Seasonal variation in shedding pattern of *E. coli* O157:H7 in bovines was analysed among three seasons, *viz;* summer, monsoon and winter. In India, the months March, April, May and June are taken as summer, the months July, August, September and October are taken as monsoon and the months November, December, January and February are taken as winter season (Surrender Singh, 2014). The ambient temperature of the farm was recorded while collecting faecal samples to study its effect on shedding of *E. coli* O157:H7. For the analysis of breed wise variation in shedding pattern of *E.*

coli O157:H7 in cattle, the animals were grouped as exotic, crossbred and indigenous breeds of cattle.

3.9 Antibiotic Sensitivity/Resistance test

In this study, the samples found positive for *E. coli* O157:H7 by mPCR were subjected for antibiotic sensitivity test using Muller Hinton agar. The commercially available antibiotic discs (octadisc) and their concentrations (g) used in this study were Amikacin (Ak) 10µg/ disc, Carbenicillin (Cb) 100 µg/disc, Ciprofloxacin (Cf) 10µg/disc, Co-Trimazine (Cm) 25µg/disc, Kanamycin (K) 30µg/disc, Nitrofurantoin (Nf) 300µg/disc, Streptomycin (S) 10µg/disc and Tetracycline (T) 30µg/disc. The octadiscs were placed on the surface of the inoculated agar plates and the plates were incubated at 37 °C for 18 to 24 h. Zones of inhibition were measured manually and interpreted using standard interpretation chart provided along with the octadiscs by the manufacturer as shown in the Table 3.3.

Table 3.3: Zone of inhibition of antibiotic discs

S. No.	Antibiotic	Concentration per disc	Zone of Inhibition (mm) for *Enterobactericeae*		
			Susceptible		Resistant (≤)
			Sensitive (≥)	Intermediate	
1	Amikacin (Ak)	10µg	06	04-05	03
2	Carbenicillin (Cb)	100 µg	23	20-22	19
3	Ciprofloxacin (Cf)	10µg	22	20-21	19
4	Co-Trimazine (Cm)	25µg	16	14-15	13
5	Kanamycin (K)	30µg	18	14-17	13
6	Nitrofurantoin (Nf)	300µg	17	15-16	14

7	Streptomycin (S)	10µg	08	05-07	04
8	Tetracycline (T)	30µg	15	12-14	11

4.9 Prevalence of *E. coli* O157:H7/H$^-$ (*E. coli* O157) and STEC in faecal samples of bovines

Molecular characterization by using multiplex PCR was used to classify the isolates as *E. coli* O157:H7 or *E. coli* O157:H$^-$ or STEC based on detection of number of genes identified out of the 6 genes targeted. The samples which showed cultural and biochemical characters and presence of only *rfbE* and *fliCh7* genes by multiplex PCR were identified as *E. coli* O157:H7. The samples which showed cultural and biochemical characters and presence of only *rfbE* but not *fliCh7* genes by multiplex PCR were classified as *E. coli* O157:H$^-$. The samples *E. coli* O157:H7/ and *E. coli* O157:H$^-$ together were considered as *E. coli* O157.

Further, if the samples showed cultural and biochemical characters and did not show presence of both *rfbE* and *fliCh7* genes but any one of or both of the *stx1 and stx2* genes by multiplex PCR were considered as Shiga Toxin producing *E. coli* (STEC) other than *E. coli* O157:H7. The isolates *E. coli* O157:H7, *E. coli* O157:H$^-$ and *E. coli* showing presence of any one or both of the *stx1 and stx2* genes together were classified as shiga toxin producing *E. coli* (STEC).

The samples found positive for E. *coli* O157 and STEC were also subjected for antibiotic sensitivity test against Amikacin (Ak) 10µg/ disc, Carbenicillin (Cb) 100 µg/disc, Ciprofloxacin (Cf) 10µg/disc, Co-Trimazine (Cm) 25µg/disc, Kanamycin (K) 30µg/disc, Nitrofurantoin (Nf) 300µg/disc, Streptomycin (S) 10µg/disc and Tetracycline (T) 30µg/disc to find out the antibiotic susceptibility pattern.

3.10 Statistical analysis

In the present study proportion and percentage was used to compare and analyze the shedding pattern of *E. coli* O157:H7. Pearson chi-square goodness-of-fit test was employed to find significance of variation in shedding pattern of *E. coli* O157:H7 (Petrie and Watson, 2013) and were scored as significant if $P<0.05$. For calculating chi-square goodness-of-fit test statistic, Yetes' correction was used to exempt error factor.

CHAPTER IV

RESULTS

The present study was undertaken to standardize multiplex PCR for the identification of *E.coli* O157:H7 from faecal samples of cattle and buffaloes by cultural identification, cultural isolation with biochemical characterization, latex agglutination and the standardized multiplex PCR and to find the variation in epidemiology of shedding pattern of *E.coli* O157:H7 in bovines between buffaloes and cattle, between different age groups, sex, lactating and non lactating, pregnant and non pregnant, healthy and diarrhoeic animals and among seasons in bovines and among breeds in cattle.

A total of 932 fecal samples were collected from fresh faecal pats of buffaloes and cattle from different cattle and buffalo farms in Bidar, Gulbarga, Bangalore and other parts of Karnataka and fresh faecal pats of the diarrheic cattle and buffaloes admitted to the clinics, Department of Teaching Veterinary Clinical Complex (TVCC), Veterinary College Bidar, Karnataka during the period from July 2014 to January 2016. The samples were processed and analyzed for the identification and confirmation of *E.coli* O157:H7 by cultural characters, biochemical characterization, latex agglutination and the standardized multiplex PCR.

4.1 Standardization of multiplex PCR

4.1.1 DNA template preparation

The extraction of the genomic DNA from the grown standard *E. coli* 0157:H7 culture and isolates from the faecal samples was done by using the modified boiled cell method (Queipo-Ortuño *et. al.,* 2008). The results obtained in this standardized multiplex PCR technique proves that boiling cell method for the extraction of genomic

DNA has produced optimum results of multiplex PCR for the identification of *E. coli* 0157:H7.

4.1.2 Multiplex PCR protocol

In the present study, to standardize multiplex PCR for the identification of *E. coli* 0157:H7, 6 pairs of primers targeting *fliCh7*, *eaeA*, *rfbE*, *hly*, *stx1*, and *stx2*, six virulent genes were used. The parameters that were standardized were magnesium chloride ($MgCl_2$) concentration, time period for denaturation, annealing temperature and the concentration of agarose used for agarose gel electrophoresis.

The Fig 4.1, 4.2 and 4.3 show the multiplex PCR amplicons in gel electrophoresis where in MgCl2 concentration was standardized. It was observed that, the bands that were produced by $MgCl_2$ concentration of 1.5 mM and 2 mM were very much faint and indiscernible (lane 5, 6 and lane 3, 4 in Fig 4.1). The concentration of $MgCl_2$ at 2.5 mM produced discernible 6 DNA bands for the 6 genes (lane 1 and 2 in Fig 4.1). However, the band of *fliCh7* gene was very faint. The concentration of $MgCl_2$ at 3 mM (lane 1 and 2 in Fig 4.2) produced discernible bands for 6 genes better than at 2.5 mM and also bright band of *fliCh7* gene. The concentration of $MgCl_2$ at 3.5 mM produced inconsistent and extra bands as shown in the Fig 4.3 (lane 2, 3 and 4). It could be concluded that the optimum concentration of $MgCl_2$ for the multiplex PCR detection of *E. coli* 0157:H7 by targeting 6 genes was 3 mM.

The time period for the denaturation was standardized for 20 sc and 45 sec. The Fig 4.4 shows the agarose gel electrophoresis where in the time period of denaturation was 20 sec and the Fig 4.5 shows the agarose gel electrophoresis where in the time period of denaturation was 45 sec. 20 sec denaturation time period produced inconsistent bands and also the band for the larger gene *fliCh7* of size 625 bp was not found in the gel electrophoresis. Whereas 45 sec denaturation time period produced

consistent and bright bands and also the band for the larger gene *fliCh7* of size 625 bp was clearly visible in the gel electrophoresis.

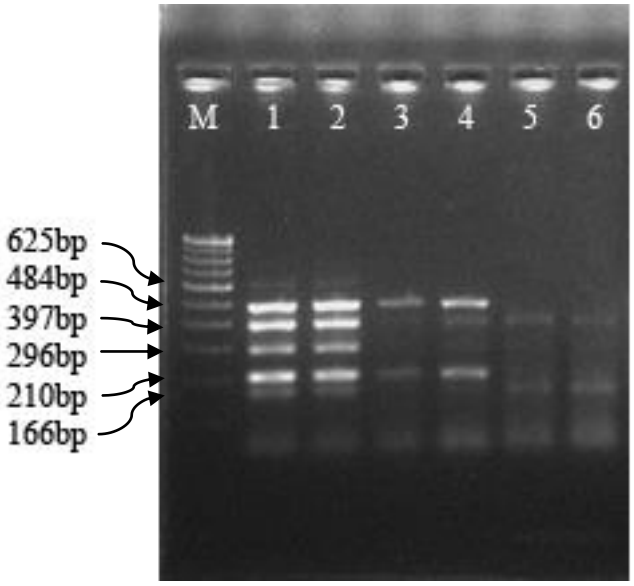


Fig 4.1: Standardization of multiplex PCR for concentration of $MgCl_2$ (1.5 mM, 2mM and 2.5 mM)

Lane L: DNA ladder
Lane 1 and 2: Amplicons of six genes obtained using 2.5 mM $MgCl_2$ concentration.
Lane 3 and 4: Amplicons of six genes obtained using 2.0 mM $MgCl_2$ concentration.
Lane 5 and 6: Amplicons of six genes obtained using 1.5 mM $MgCl_2$ concentration.

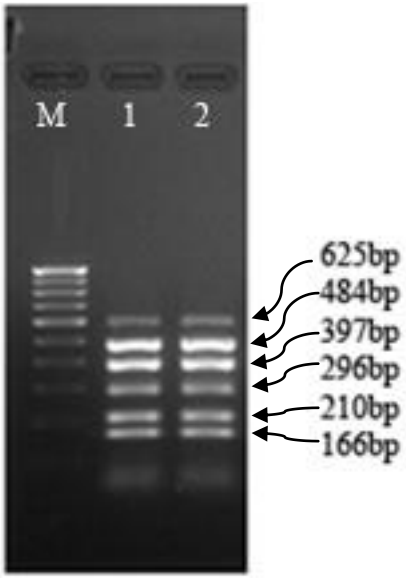


Fig 4.2: Standardization of multiplex PCR for concentration of $MgCl_2$ (3 mM)

Lane L: DNA ladder

Lane 1 and 2: Amplicons of six genes obtained using 3 mM $MgCl_2$ concentration.

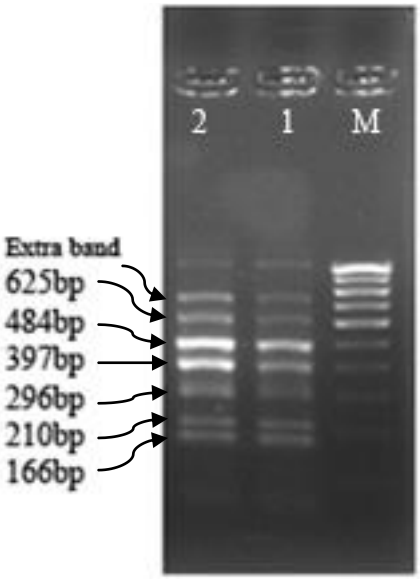


Fig 4.3: Standardization of multiplex PCR for concentration of $MgCl_2$ (3.5 mM)

Lane L: DNA ladder

Lane 1 and 2: Amplicons of six genes obtained using 3.5 mM $MgCl_2$ concentration.

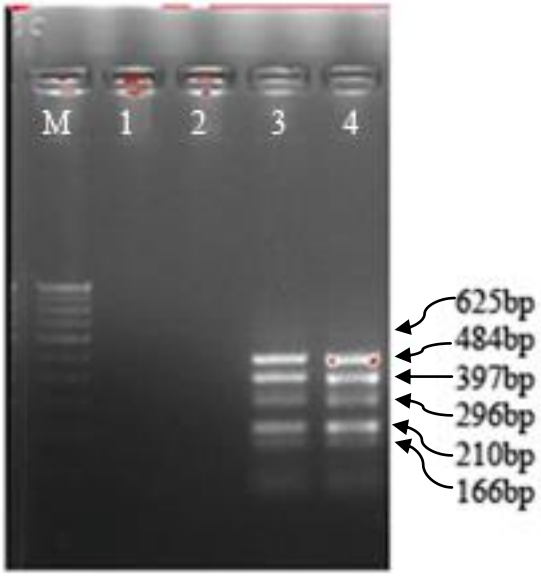


Fig 4.4: Standardization of multiplex PCR for time period of denaturation temperature (94°C for 20 sec)

Lane L: DNA ladder

Lane 1, 2, 3 and 4: Amplicons obtained at denaturation temperature of 94°C for 20 sec. No amplicons in lane 1 and 2 (inconsistency) and band for the larger gene *fliCh7* (625 bp) was not found in lane 3 and 4.

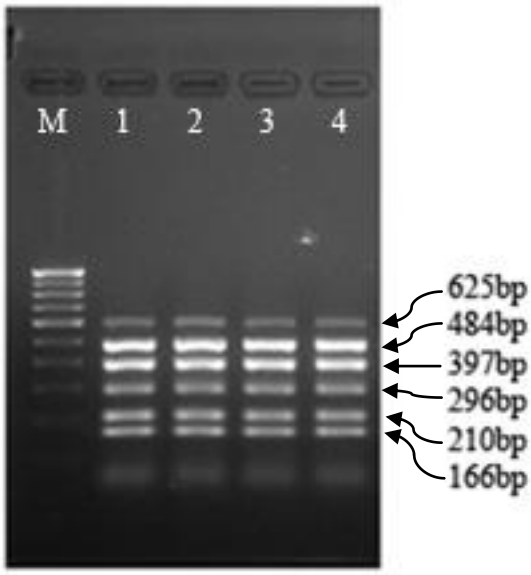


Fig 4.5: Standardization of multiplex PCR for time period of denaturation temperature (94°C for 45 sec)

Lane L: DNA ladder

Lane 1, 2, 3 and 4: Amplicons obtained at denaturation temperature of 94°C for 45 sec. Amplicons for 6 genes were obtained consistently including the larger gene *fliCh7* (625 bp)

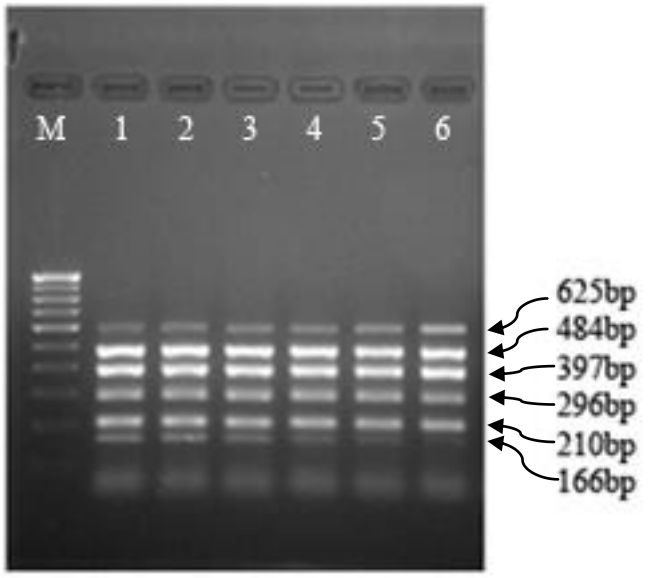


Fig 4.6: Standardization of multiplex PCR for annealing temperature

Lane L: DNA ladder
Lane 1: Annealing at 58°C
Lane 2: Annealing at 59°C
Lane 3: Annealing at 60°C
Lane 4: Annealing at 61°C
Lane 5: Annealing at 62°C
Lane 6: Annealing at 63°C

The annealing temperature was evaluated for six temperatures between 58°C to 63°C by doing gradient mPCR. It was observed that lower annealing temperature gradient multiplex PCR produced bright band of *hly* gene but fainter band of *fliCh7* gene and as the annealing temperature increased from 58°C to 63°C the brightness of the band of the gene *fliCh7* (625 bp) also increased, however, at the cost of decrease in the brightness of the band of the gene *hly* (166 bp) as appreciated in the Fig 4.6. The band of *hly* gene was almost invisible at the annealing temperature of 63°C. Therefore, it could be interpreted that the annealing temperature 60°C amplifies all the 6 genes in optimum way and seems most suitable for multiplex PCR.

The agarose concentration for the agarose gel electrophoresis was evaluated for 1% and 1.5%. The bands visualized at 1 % agarose concentration were very faint and the band of the gene *hly* (166 bp) was not visible because of its low size as depicted in the Fig 4.7. Whereas the bands visualized at 1.5 % agarose concentration were bright and distinct and the band of the gene *hly* (166 bp) was also visible as depicted in the Fig 4.8.

The standardized multiplex PCR for the detection of *E. coli* 0157:H7 in the prsesnt study could be represented as follows.

It consisted of volume of 25 µl of reaction mixture containing 2 µl of *Taq* DNA Polymerase (1 unit/ µl), 2.0 µl of DNA template solution, 2.5 µl of 10 x reaction buffer, 0.5 µM of deoxynucleoside triphosphates (dNTPs), 0.2 µM each of the 12 primers (6 primer pairs) and 3 mM magnesium chloride ($MgCl_2$) and the rest of the volume, sterile nuclease free water to make up the 25 µl reaction mixture volume. The thermal cycling parameters were; 5 minute initial denaturation at 94˚C followed by 35 cycles of denaturation at 94˚C for 45 sec, 1 min of annealing at 60˚C and extension for 1 min at 72˚C, with a 10 min final extension at 72˚C followed by maintenance at 4˚C. The agarose gel electrophoresis was optimized at 1.5 % concentration of agarose. The DNA

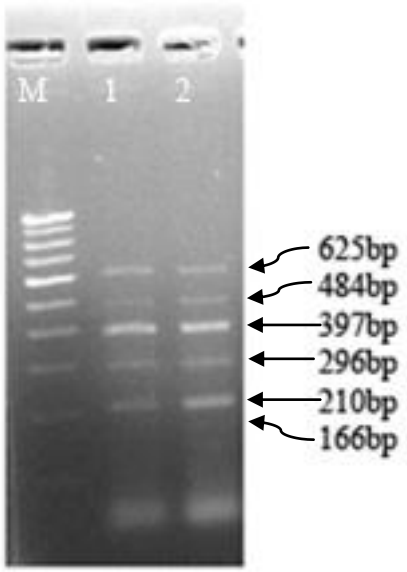


Fig 4.7: Standardization of multiplex PCR for concentration of agarose in agarose gel electrophoresis (1%)

Lane L: DNA ladder

Lane 1 and 2: DNA bands of the amplicons on agarose gel electrophoresis at agarose concentration of 1%. The DNA band of *hly* gene (166 bp) not observed

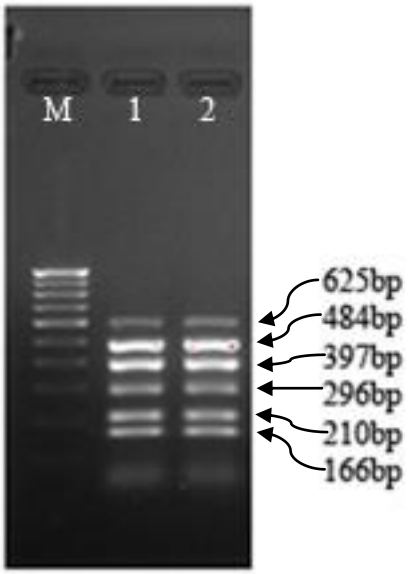


Fig 4.8: Standardization of multiplex PCR for concentration of agarose in agarose gel electrophoresis (1.5%)

Lane L: DNA ladder

Lane 1 and 2: DNA bands of the amplicons on agarose gel electrophoresis at agarose concentration of 1.5 %

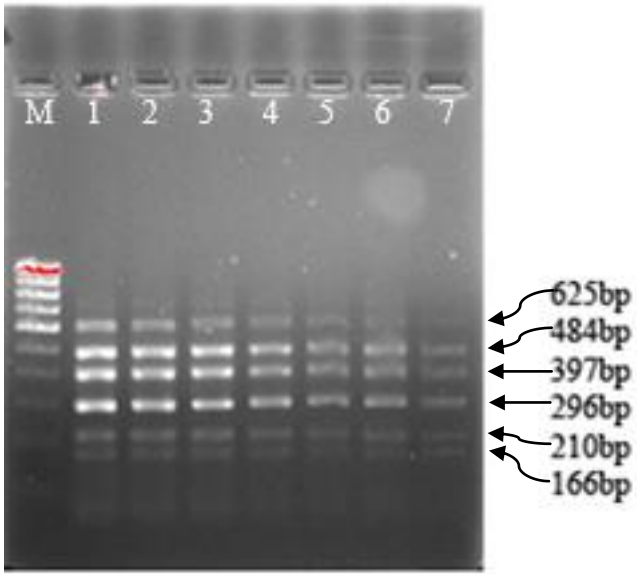


Fig 4.9: Threshold sensitivity of standardized multiplex PCR, when tenfold serial dilutions of *E. coli* O157:H7 reference culture in mTSB were subjected for standardized multiplex PCR.

Lane L: DNA ladder
Lane 1: 10000 CFU/ml
Lane 2: 1000 CFU/ml
Lane 3: 100 CFU/ml
Lane 4: 10 CFU/ml
Lane 5: 1 CFU/ml
Lane 6: 0.1 CFU/ml
Lane 7: 0.01 CFU/ml

bands were visualized under ultraviolet (UV) light using gel documentation system (BioRad) and analyzed by using the Image Lab™ software (BioRad).

With this standardized multiplex PCR protocol, the amplicons produced were of the size, 625 bp, 397bp, 296 bp, 166 bp, 210 bp and 484 bp, respectively for the *fliCh7*, *eaeA, rfbE, hly*, *stx1*, and *stx2* genes. The size of the amplicons is depicted in the Fig 4.8.

4.1.3 Threshold sensitivity of multiplex PCR

The threshold sensitivity of standardized multiplex PCR, when tenfold serial dilutions of *E. coli* O157:H7 reference culture viz*;* 10000, 1000, 100, 10, 1, 0.1 and 0.01 CFU in modified Tryptone Soy Broth (mTSB) were subjected for standardized multiplex PCR, was found to be 0.1 CFU/ml. The DNA bands are less discernible at the dilution of 0.01 CFU/ml. The gel electrophoresis of these tenfold serial dilutions has been depicted in the Fig 4.9.

The threshold sensitivity of standardized multiplex PCR was also evaluated by spiking with tenfold serial dilutions of *E. coli* O157:H7 reference culture viz*;* 10000, 1000, 100, 10, 1, 0.1 and 0.01 CFU into 1 g of faecal samples found to be negative for *E. coli* O157:H7 by cultural characters. When these spiked faecal samples were directly subjected for standardized multiplex PCR, the sensitivity was found to be 1 CFU/g as shown in the Fig 4.10. When spiked faecal samples with one step selective enrichment were subjected for multiplex PCR the threshold sensitivity was found to be 0.1CFU/g in as shown in the Fig 4.11.

4.1.4 Specificity of multiplex PCR

Specificity of standardized multiplex PCR was evaluated by subjecting *E. coli* other than *E. coli* O157:H7 (sorbitol positive isolate on CT-SMAC agar), *Staphylococcus aureus, Streptococci, Vibrio parahaemolyticus, Brucella abortus* (strain

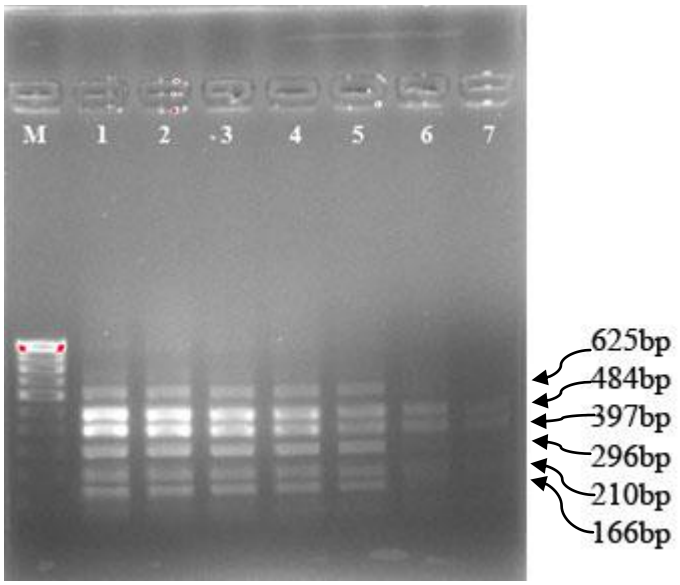


Fig 4.10: Threshold sensitivity of standardized multiplex PCR, when faecal samples spiked with tenfold serial dilutions of *E. coli* O157:H7 reference culture were directly subjected for standardized multiplex PCR.

Lane L: DNA ladder
Lane 1: 10000 CFU/ml
Lane 2: 1000 CFU/ml
Lane 3: 100 CFU/ml
Lane 4: 10 CFU/ml
Lane 5: 1 CFU/ml
Lane 6: 0.1 CFU/ml
Lane 7: 0.01 CFU/ml

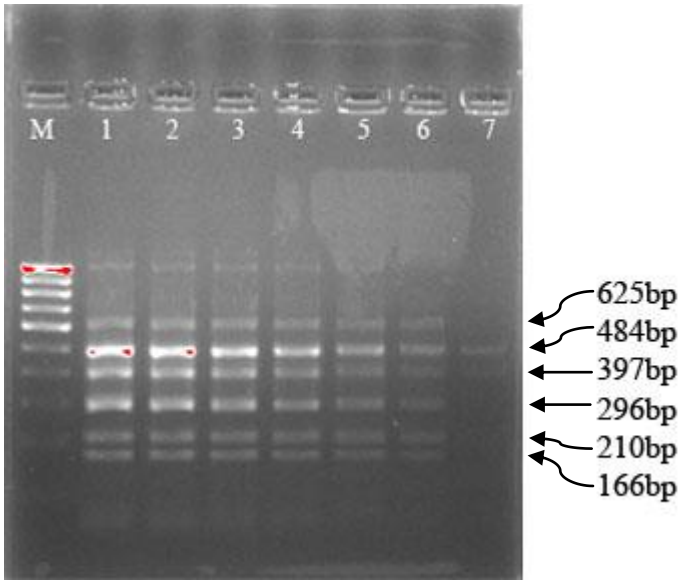


Fig 4.11: Threshold sensitivity of standardized multiplex PCR, when faecal samples spiked with tenfold serial dilutions of *E. coli* O157:H7 reference culture with one step selective enrichment in mTSB were subjected for standardized multiplex PCR.

Lane L: DNA ladder
Lane 1: 10000 CFU/ml
Lane 2: 1000 CFU/ml
Lane 3: 100 CFU/ml
Lane 4: 10 CFU/ml
Lane 5: 1 CFU/ml
Lane 6: 0.1 CFU/ml
Lane 7: 0.01 CFU/ml

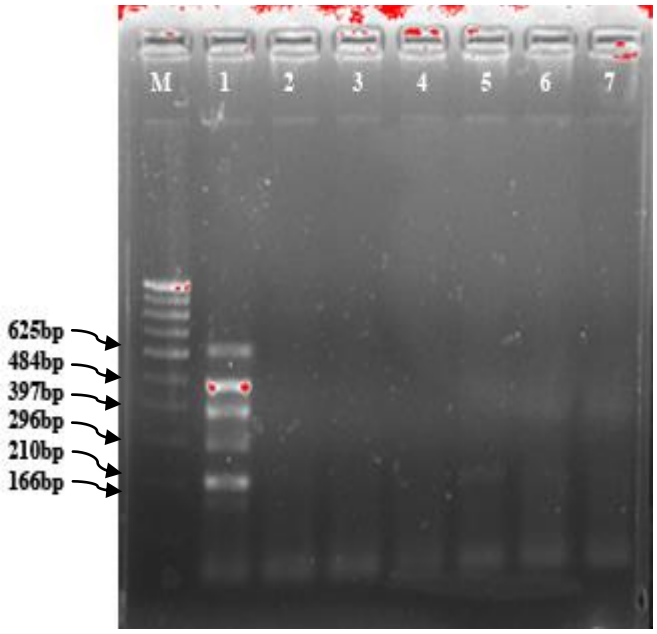


Fig 4.12: Specificity of standardized multiplex PCR, when *E. coli* other than *E. coli* O157:H7, *Staphylococcus aureus, Streptococci, Vibrio parahaemolyticus* and *Salmonella* cultures as negative control along with *E. coli* O157:H7 culture were subjected for standardized multiplex PCR.
Lane L: DNA ladder
Lane 1: *E. coli* O157:H7
Lane 2: *E. coli* other than *E. coli* O157:H7
Lane 3: *Staphylococcus aureus*
Lane 4: *Streptococci*
Lane 5: *Vibrio parahaemolyticus*
Lane 6: *Brucella abortus*
Lane 7: *Salmonella*

19) and *Salmonella* cultures as negative control along with reference culture of *E. coli* O157:H7 for standardized multiplex PCR to analyse for the detection of six virulent genes. None of the six bands was found in the PCR product of negative control cultures as shown in the Fig 4.12.

4.2 Cultural isolation and biochemical characterization of *E. coli* O157:H7 from faecal samples.

E. coli O157:H7 from faecal samples was isolated by enriching in modified tryptone soy broth (TSB) and incubating overnight at 37°C for 24 hours. Selective plating was done on sorbitol-MacConkey agar supplemented with cefixime (0.025 mg) and tellurite (1.25 mg) (CT-SMAC) and incubated for 24 h at 37°C. The sorbitol positive (sorbitol fermenting pink coloured) colonies indicate negative for *E. coli* O157:H7 as shown in the Fig 4.13 and the typical sorbitol negative (sorbitol non fermenting colourless) colonies indicate positive for *E. coli* O157:H7 as shown in the Fig 4.14. The sorbitol negative colonies of *E. coli* O157:H7 appeared small, circular and colourless with smoky centre (1-2) mm in diameter.

The isolated colonies were subjected for gram's staining, inoculation on Eosin methylene blue agar and biochemical tests. The suspected *E. coli* O157:H7 isolates showed gram negative character indicated by pink coloured coccobacilli (take counter stain safranin) under the oil immersion microscope as shown in the Fig 4.15 and produced colonies with metallic sheen on EMB agar as shown in the Fig 4.16. The biochemical tests performed were indole test, methyl red test, vogues-proskauer test, citrate utilization test (IMVC), nitrate test and sugar fermentation test for lactose, sucrose and dextrose. The isolates which showed positive reaction for indole, methyl red, fermentation of sucrose, dextrose and lactose (triple sugar iron agar) with gas production and nitrate tests and negative reaction for vogues proskauer and citrate utilization tests were taken as positive for *E. coli* O157:H7 otherwise negative for *E.*

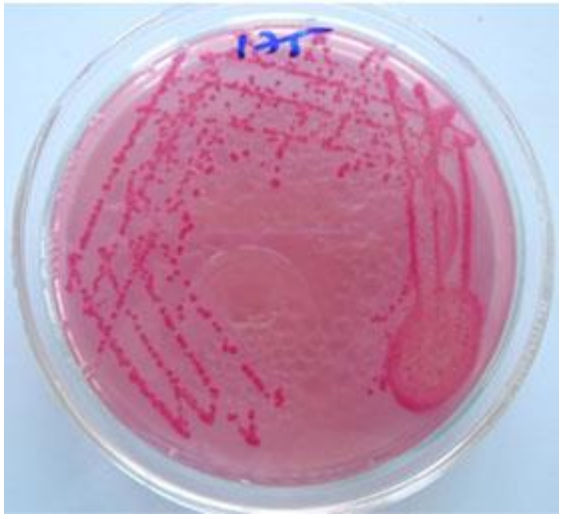


Fig 4.13: CT-SMAC agar plate showing sorbitol positive (pink coloured) colonies indicating negative for *E. coli* O157:H7

Fig 4.14: CT-SMAC agar plate showing sorbitol negative (colourless) colonies indicating positive for *E. coli* O157:H7

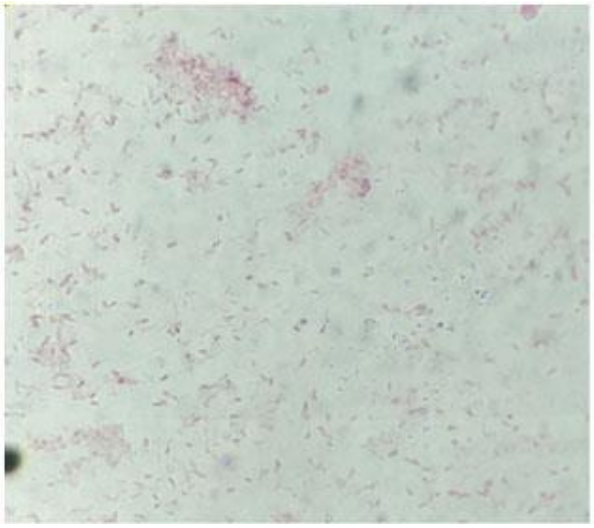


Fig 4.15: Gram's staining of sorbitol negative colonies on SMAC agar plate suspected for *E. coli* O157:H7

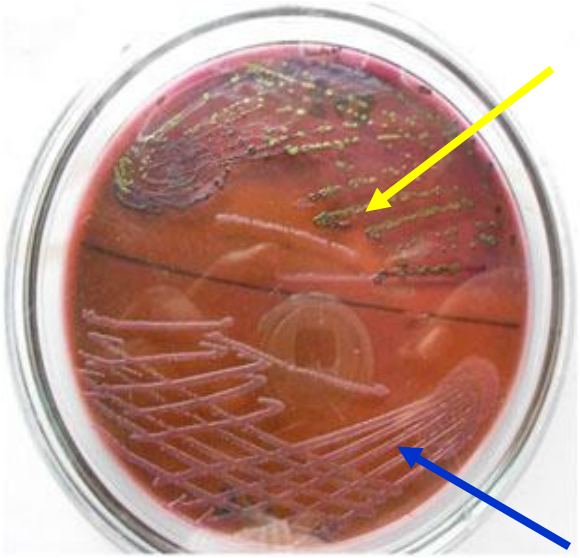


Fig 4.16: Sorbitol negative colonies on SMAC agar plate inoculated on EMB agar. A sorbitol negative culture from one sample showing metallic sheen (above) presumed to be positive for *E. coli* O157:H7. Sorbitol negative culture from another sample (below) not showing metallic sheen on EMB agar presumed to be negative for *E. coli* O157:H7.

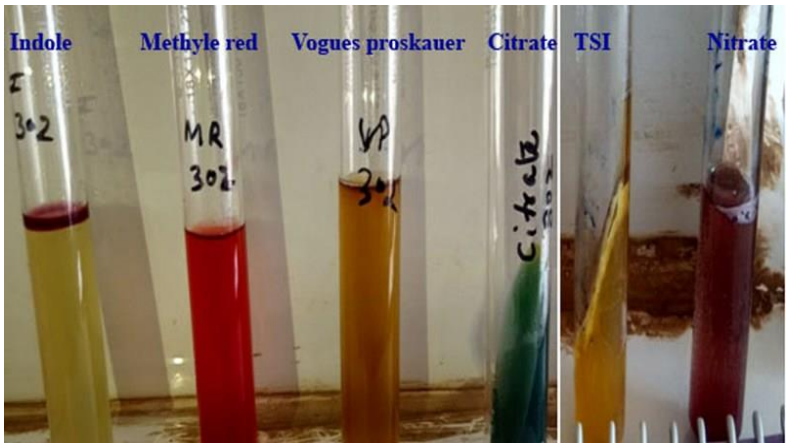


Fig. 4.17 Sample No. 302 showing positive reaction for indole, methyl red, fermentation of sucrose, dextrose and lactose (triple sugar iron agar) with gas production and nitrate tests and negative reaction for vogues proskauer and citrate utilization tests indicating positive for *E. coli* O157:H7.

Fig 4.18: Sample No. 301 showing negative reaction for indole, methyl red and vogues proskauer and positive reaction for fermentation of sucrose, dextrose and lactose (triple sugar iron agar) with gas production, nitrate tests and citrate utilization tests indicating negative for *E. coli* O157:H7.

coli O157:H7. Fig 4.17 shows biochemical characters of a sample positive for *E. coli* O157:H7 and Fig 4.18 shows biochemical characters of a sample negative for *E. coli* O157:H7.

The results of cultural isolation and biochemical characterization of *E. coli* O157:H7 from faecal samples is presented in the Table 4.1. Based on cultural identification (sorbitol negative colonies on CT-SMAC), out of 932 samples analyzed, 231 samples were found to be positive for *E. coli* O157:H7 which accounts for 24.79%. Based on cultural isolation with biochemical characterization, out of 932 samples analyzed, 154 samples were found to be positive for *E. coli* O157:H7 which accounts for 16.52%.

The samples found (presumed) positive for *E. coli* O157:H7 by cultural identification and cultural isolation with biochemical characterization were subjected for multiplex PCR and latex agglutination test to countercheck and confirm the isolates as *E. coli* O157:H7.

4.3 Identification and molecular characterization of *E. coli* O157:H7 in faecal samples by multiplex PCR

The faecal samples were directly as well as the faecal samples with one step selective enrichment were subjected for standardized multiplex PCR for the identification of *E. coli* O157:H7 and molecular characterization based on detection of six virulent genes. The samples that showed presence of *fliCh7* and *rfbE* genes were considered positive for *E. coli* O157:H7. The PCR products which showed presence of *fliCh7* and *rfbE* genes were observed for the presence of the other four genes, viz; *eaeA, hly, stx1,* and *stx2* for the molecular characterization.

The results of identification and molecular characterization of *E. coli* O157:H7 in faecal samples by multiplex PCR is presented in the Table 4.1. When faecal samples

Table 4.1: Identification of *E. coli* O157:H7 in the faecal samples of bovines by using different methods

Diagnostic test / No. Of samples	**Positive**	**Negative**	**Total**	**Percentage (%)**
Cultural identification	231	701	932	**24.79**
Cultural Isolation with biochemical characterization	154	778	932	**16.52**
Multiplex PCR directly on faecal samples	87	845	932	**9.33**
Multiplex PCR on faecal samples with one step selective enrichment	102	830	932	**10.94**
Latex agglutination test	91	841	932	**9.76**

Table 4.2: Confirmative identification of *E. coli* O157:H7 by multiplex PCR

Diagnostic test / No. Of samples	Multiplex PCR on faecal samples with one step selective enrichment		**Total**	**Percentage (%)**
	Positive	**Negative**		
No. of samples positive by Cultural identification	102	129	231	**44.16**
No. of samples positive by Cultural Isolation with biochemical characterization	102	52	154	**66.23**
No. of samples positive by Multiplex PCR directly on faecal samples	87	0	87	**100.00**
Latex agglutination test	91	0	87	**100.00**

Table 4.3: Confirmative identification of *E. coli* O157:H7 by latex agglutination test

Diagnostic test / No. Of samples	Latex agglutination test		**Total**	**Percentage (%)**
	Positive	**Negative**		
No. of samples positive by Cultural identification	91	140	231	**39.39**
No. of samples positive by Cultural Isolation with biochemical characterization	91	63	154	**59.09**
No. of samples positive by Multiplex PCR directly on faecal samples	87	0	87	**100**
Multiplex PCR on faecal samples with one step selective enrichment	91	11	102	**89.22**

were directly subjected for multiplex PCR, out of 932 samples analyzed, 87 samples were found to be positive for *E. coli* O157:H7 which accounts for 9.33%. Among the 87 samples positive for *E. coli* O157:H7 isolates, 74 samples showed presence of all the 6 virulent genes and 13 samples showed presence of only 5 virulent genes, *viz; fliCh7, rfbE, eaeA, hly* and *stx1* but lacking *stx2* gene.

When faecal samples with one step selective enrichment were subjected for multiplex PCR, out of 932 samples analyzed, 102 samples were found to be positive for *E. coli* O157:H7 which accounts for 10.94%. Among the 102 samples positive for *E. coli* O157:H7, 89 samples showed presence of all the 6 virulent genes as shown in the

Fig 4.19 (4.19a, 4.19b and 4.19c) and 13 samples showed presence of only 5 virulent genes, *viz; fliCh7, rfbE, eaeA, hly* and *stx1* but absence of *stx2* gene as shown in the Fig 4.20. Out of these 89 faecal samples, 73 samples were the same samples that showed presence of all the 6 virulent genes of *E. coli* O157:H7 when faecal samples were directly subjected for multiplex PCR. That means, in addition to 73 samples 16 more samples were found to be positive for *E. coli* O157:H7 when faecal samples with one step selective enrichment were subjected for multiplex PCR. Further, the 13 samples that showed presence of 5 virulent genes, *viz; fliCh7, rfbE, eaeA, hly* and *stx1* but absence of *stx2* gene were the same 13 samples that showed presence of 5 virulent genes, *viz; fliCh7, rfbE, eaeA, hly* and *stx1* but absence of *stx2* gene when faecal samples were directly subjected for multiplex PCR.

To countercheck and confirm the identification of *E. coli* O157:H7, the isolates that were found to be positive for *E. coli* O157:H7 by cultural identification and cultural isolation with biochemical characterization were subjected for multiplex PCR. The results are shown in the Table 4.2. Out of 231 samples identified as positive for *E. coli* O157:H7 by cultural identification, 102 samples were confirmed by multiplex PCR.

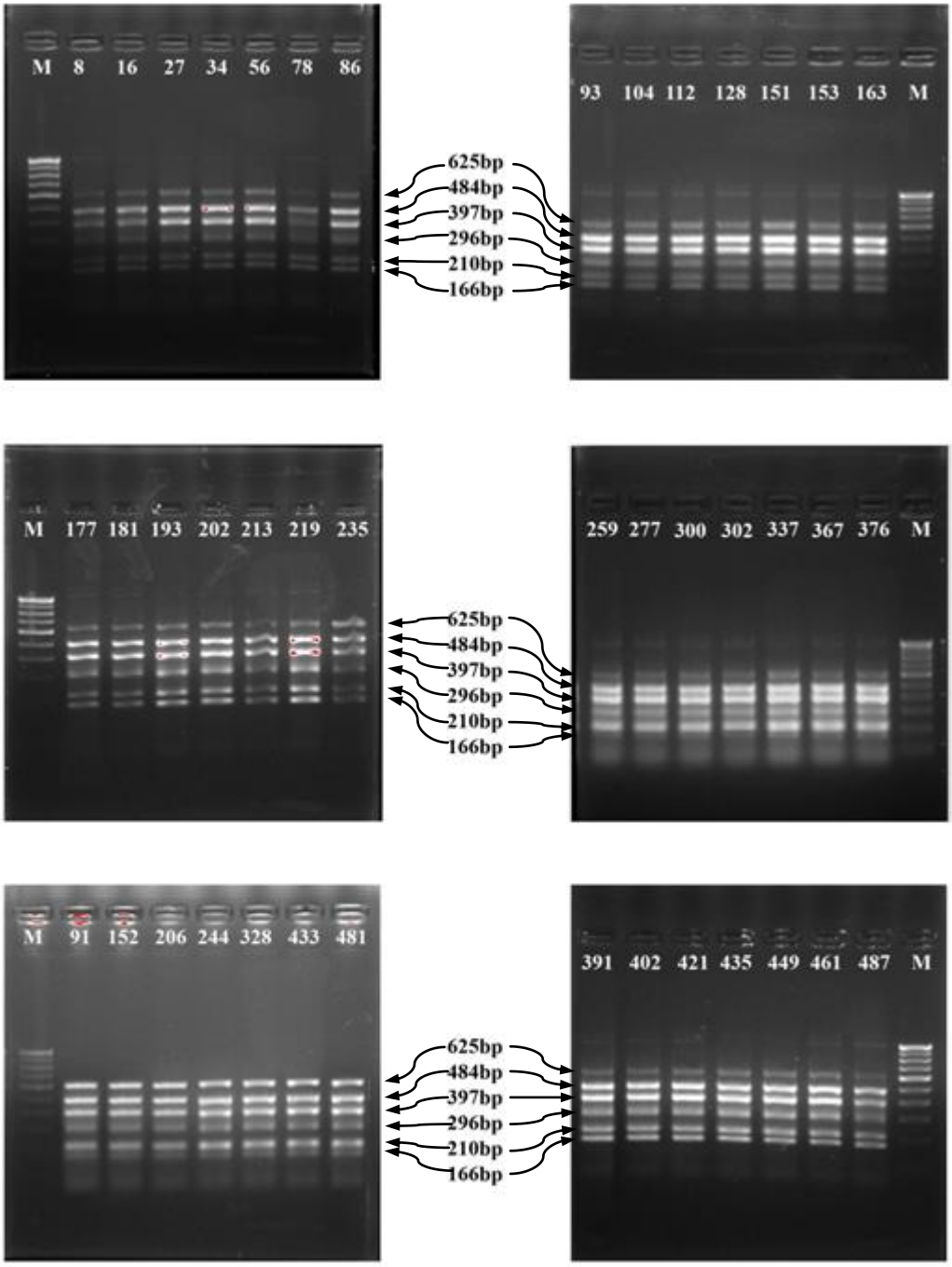


Fig 4.19a: Forty two samples out of the 89 samples found positive for *E. coli* O157:H7 by multiplex PCR when faecal samples were analyzed after one step selective enrichment.

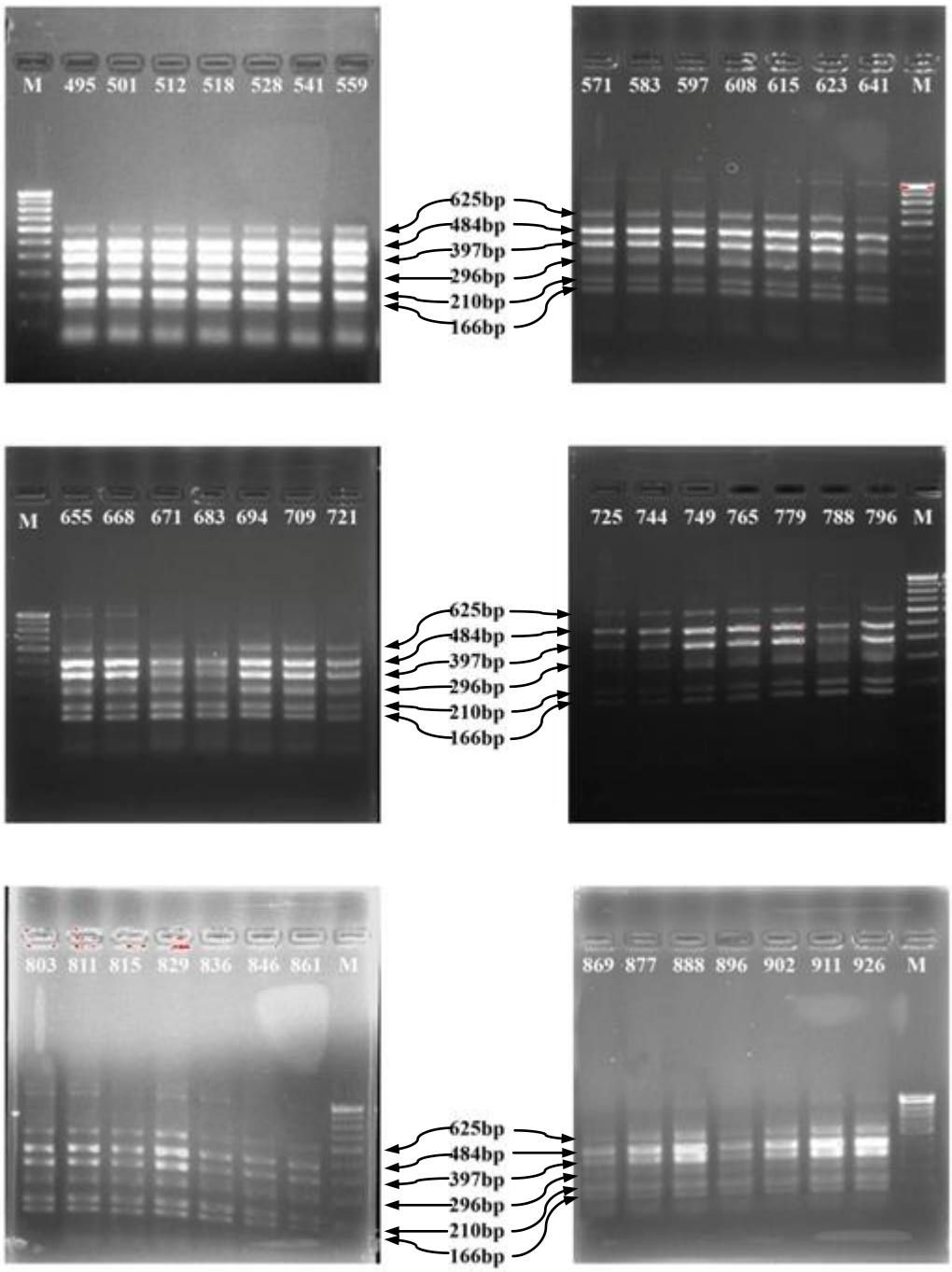


Fig 4.19b: Forty two samples out of the 89 samples found positive for *E. coli* O157:H7 by multiplex PCR when faecal samples were analyzed after one step selective enrichment.

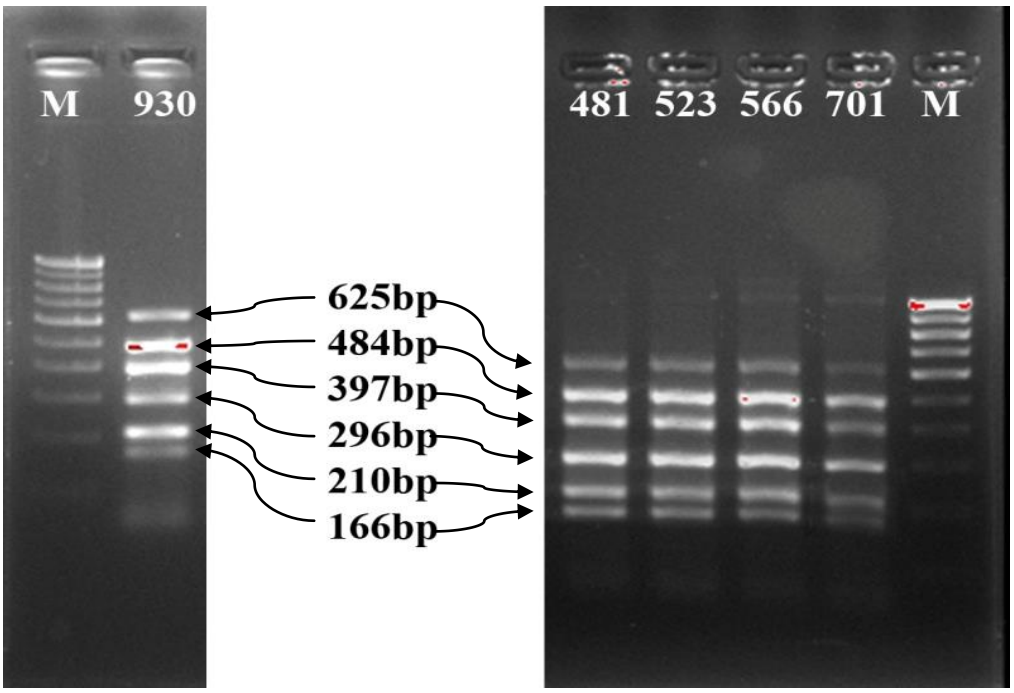


Fig 4.19c: Five samples out of 89 samples found positive for *E. coli* O157:H7 by multiplex PCR when faecal samples were analyzed after one step selective enrichment

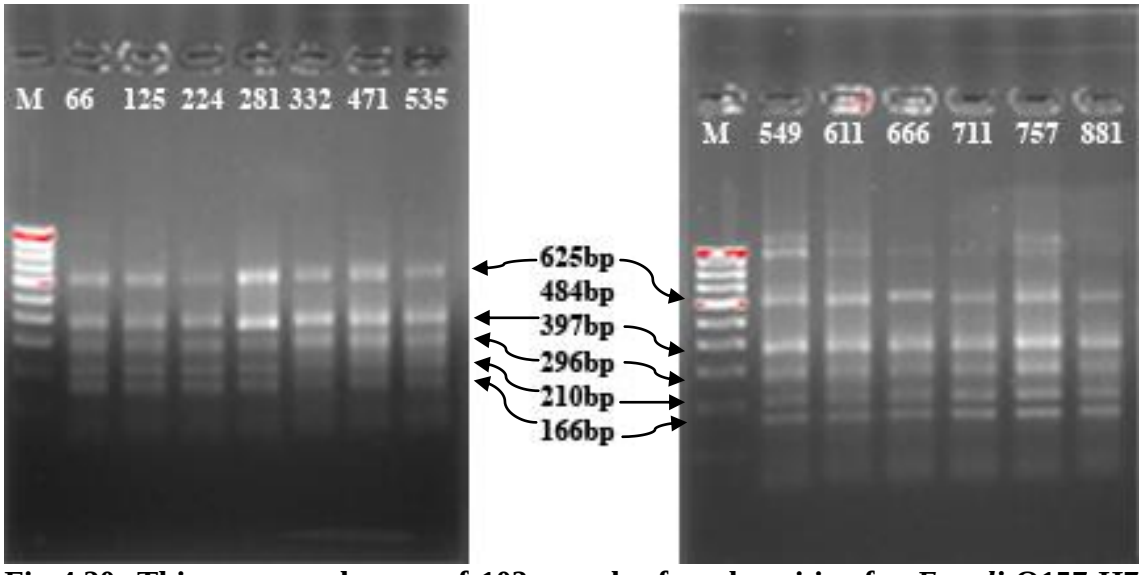


Fig 4.20: Thirteen samples out of 102 samples found positive for *E. coli* O157:H7 by multiplex PCR showing presence of only 5 genes viz; *fliCh7, rfbE, stx1, eaeA* and *hly* and absence of *stx2.*

Out of 154 samples identified as positive for *E. coli* O157:H7 by cultural identification with biochemical characterization, 102 samples were confirmed by multiplex PCR. Again among these, 89 showed presence of all the 6 virulent genes and 13 showed presence of only 5 virulent genes *viz; fliCh7, rfbE, eaeA, hly* and *stx1* but absence of *stx2* gene. All these confirmed samples were the same samples which were identified as positive for *E. coli* O157:H7 when the faecal samples with one step selective enrichment were subjected for multiplex PCR.

4.4 Confirmative identification of *E. coli* O157:H7 by Latex Agglutination Test

The samples identified as positive for *E.coli* O157:H7 by cultural isolation, cultural isolation with biochemical characterization and multiplex PCR were subjected for confirmative identification by observing for the presence of the O157 and the H7 antigens using latex agglutination test (Wellcolex® *E.coli* O157:H7). The samples/isolates positive for O157 and the H7 antigens showed agglutination reaction, respectively, as shown in the Fig 4.21 and the isolates negative for O157 and the H7 antigens, respectively, showed no agglutination as shown in the Fig 4.22**.** The results are shown in the Table 4.3. Out of the 231 samples that were positive for *E.coli* O157:H7 by cultural identification and 154 samples that were positive for *E.coli* O157:H7 by cultural isolation with biochemical characterization, 91 samples were confirmatively identified as positive for *E.coli* O157:H7 by latex agglutination test.

Out of the 102 samples that were identified as positive for *E.coli* O157:H7 when faecal samples with one step selective enrichment were analyzed by multiplex PCR, 91 samples were confirmatively identified as positive for *E.coli* O157:H7 by latex agglutination test and out of the 87 samples that were identified as positive for *E.coli* O157:H7 when faecal samples were directly analyzed by multiplex PCR, all the 87 samples were confirmatively identified as positive for *E.coli* O157:H7 by latex agglutination test

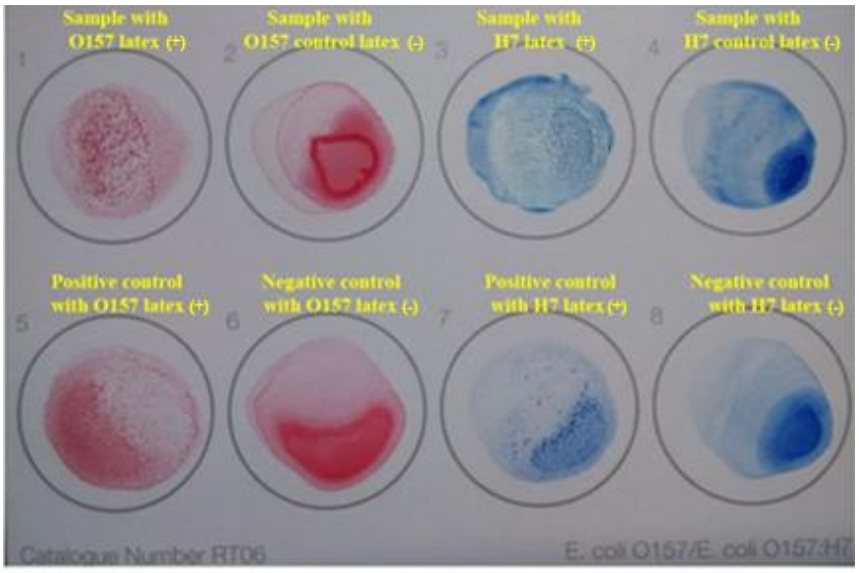


Fig 4.21: Sample No. 302 positive for O157 and the H7 antigens showing agglutination reaction in 1st and 3rd circle similar to positive controls, indicating positive for *E. coli* O157:H7

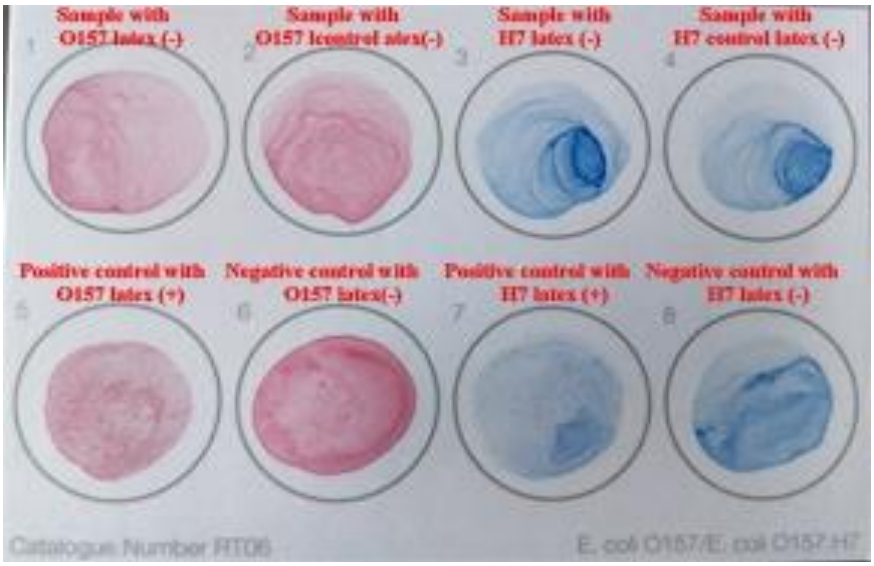


Fig 4.22: Sample No. 19 negative for O157 and the H7 antigens showing no agglutination reaction in 1st and 3rd circles similar to negative controls, indicating negative for *E. coli* O157:H7

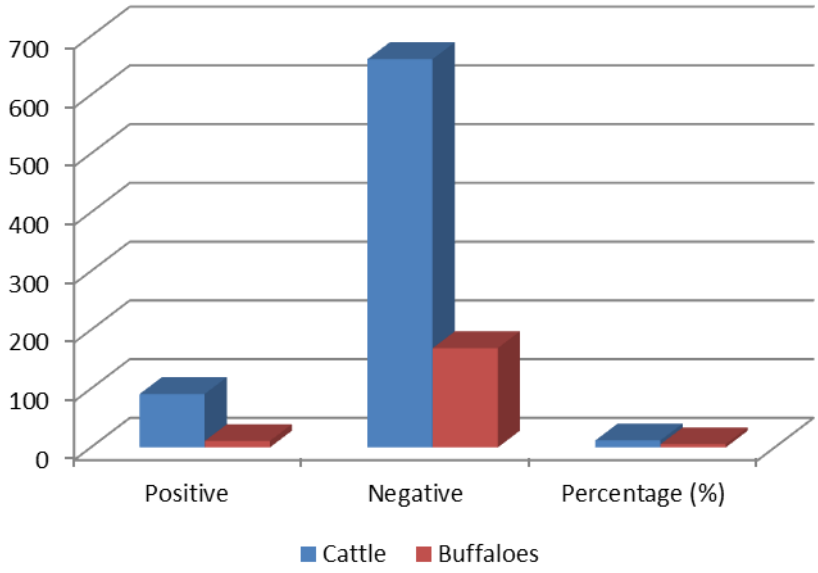


Fig 4.23: Variation in the shedding pattern of E. *coli* O157:H7 in cattle and buffaloes

4.5 Epidemiological sensitivity and specificity of cultural identification, cultural and biochemical identification and multiplex PCR identification of *E. coli* O157:H7

In the present study, the results of identification of *E. coli* O157:H7 by cultural isolation and identification method with that of latex agglutination test as gold standard test was compared. Based on cultural identification, out of 932 faecal samples, 231 samples were positive and 701 samples were negative for *E. coli* O157:H7. And based on latex agglutination test, only 91 samples were positive and 841 samples were negative for *E. coli* O157:H7. The estimated sensitivity of cultural identification *vis-a-vis* latex identification test was 100%. However, the estimated specificity was very less i.e. 83.35%. The results are presented in the Table 4.4.

When we compared the results of identification of *E. coli* O157:H7 by cultural isolation with biochemical characterization method with that of latex agglutination test, out of 932 faecal samples, 154 samples were positive and 778 samples were negative for *E. coli* O157:H7 based on cultural isolation with biochemical characterization. And based on latex agglutination test, only 91 samples were positive and 841 samples were negative for *E. coli* O157:H7. The estimated sensitivity of cultural isolation and biochemical characterization *vis-a-vis* latex identification test was 100%, and the estimated specificity was 92.51%. The results are presented in the Table 4.5.

The results of identification of *E. coli* O157:H7 when faecal samples were directly analyzed by multiplex PCR was compared with that of latex agglutination test. Out of 932 faecal samples, 87 samples were positive and 845 samples were negative for *E. coli* O157:H7 based on multiplex PCR. And based on latex agglutination test, 91 samples were positive and 841samples were negative for *E. coli* O157:H7. The estimated sensitivity of multiplex PCR *vis-a-vis* latex identification test was 95.60%, and the specificity was 100%. The results are presented in the Table 4.6.

Table 4.4. Sensitivity and specificity of cultural identification of *E. coli* O157:H7

Diagnostic test		Identification by latex agglutination test (true status)		Total
		Positive	Negative	
Identification by cultural identification	Positive	91	140	**231**
	Negative	0	701	**701**
Total		**91**	**841**	**932**

Table 4.5. Sensitivity and specificity of cultural and biochemical identification of *E. coli* O157:H7

Diagnostic test		Identification by latex agglutination test (true status)		Total
		Positive	Negative	
Identification by cultural isolation with biochemical characters	**Positive**	91	63	**154**
	Negative	0	778	**778**
Total		**91**	**841**	**932**

Table 4.6. Sensitivity and specificity of identification of *E. coli* O157:H7 when faecal samples were directly analyzed by multiplex PCR

Diagnostic test		Identification by latex agglutination test (true status)		Total
		Positive	Negative	
Identification directly in faecal samples by multiplex PCR	**Positive**	87	0	**87**
	Negative	4	841	**845**
Total		**91**	**841**	**932**

Table 4.7. Sensitivity and specificity of identification of *E. coli* O157:H7 when faecal samples with one step selective enrichment were analyzed by multiplex PCR

Diagnostic test		Identification by latex agglutination test		Total
		Positive	Negative	
Identification in faecal samples with one step enrichment by multiplex PCR	**Positive**	91	11	**102**
	Negative	0	830	**830**
Total		**91**	**841**	**932**

Similarly when we compared the results of identification of *E. coli* O157:H7 when faecal samples with one step selective enrichment were analyzed by multiplex PCR with that of latex agglutination test, out of 932 faecal samples, 102 samples were

positive and 830 samples were negative for *E. coli* O157:H7 based on multiplex PCR. And based on latex agglutination test, 91 samples were positive and 841samples were negative for *E. coli* O157:H7. The estimated sensitivity of multiplex PCR *vis-a-vis* latex identification test was 100%, and the specificity was 98.69%. The results are presented in the Table 4.7.

4.6 Epidemiology of shedding pattern of *E. coli* O157:H7 in bovines

In the present study, since the standardized multiplex PCR identification *E. coli* O157:H7 in faecal samples with one step selective enrichment appears more sensitive and specific, it was employed as a technique for the final consideration of the sample as positive for *E. coli* O157:H7 and hence this results was used for the analysis and interpretation on epidemiology of shedding pattern of *E. coli* O157:H7 in buffaloes and cattle.

4.6.1 Prevalence of shedding of *E. coli* O157:H7 in bovines

The Table 4.1 shows the prevalence of shedding of *E. coli* O157:H7 in bovines estimated by using various identification methods.

The prevalence of shedding of *E. coli* O157:H7 in bovines was found to be 24.79% (231 out of the total of 932 fecal samples) based on the results of cultural identification, 16.52% (154 out of the total of 932 fecal samples) based on the results of cultural identification with biochemical characterization, 9.33% (87 out of the total of 932 fecal samples) when faecal samples were directly subjected for multiplex PCR analysis and 10.94% (102 out of the total of 932 fecal samples) when faecal samples with one step selective enrichment were subjected for multiplex PCR analysis. The prevalence of *E. coli* O157:H7 in bovines was found to be 9.76% (91 out of the total of 932 fecal samples) when the samples were subjected for confirmative identification by latex agglutination test.

In the present study, since the standardized multiplex PCR identification *E. coli* O157:H7 in faecal samples with one step selective enrichment was employed for the final consideration of the sample as positive for *E. coli* O157:H7, it could be concluded that the prevalence of shedding of *E. coli* O157:H7 in bovines was 10.94%.

4.6.2 Shedding pattern of *E. coli* O157:H7 in cattle and buffaloes

In the present study, out of 932 faecal samples analyzed, 752 samples were from cattle and 180 samples were from buffaloes. Based on the multiplex PCR identification of *E. coli* O157:H7, the percent of cattle that showed shedding *E. coli* O157:H7 was 12.10% (91 out of 752 samples). Whereas, the percent of buffaloes that showed shedding of *E. coli* O157:H7 was only 5.55% (11 out of 180 samples). The results are presented in the Table 4.8 and Fig 4.23. The Fig 4.24 shows the agarose gel electrophoresis of multiplex PCR of the 11 faecal samples of buffaloes positive for six genes of *E. coli* O157:H7.

When the data was subjected for chi-square goodness-of-fit test with a *P* value of <0.05, there was high significant difference in shedding of *E. coli* O157:H7 in the faeces by indigenous breeds, crossbreds and exotic breeds. Significant difference was observed in shedding of *E. coli* O157:H7 in the faeces by indigenous breeds, crossbreds and exotic breeds even at *P* value of <0.05.

4.6.3 Breed wise variation in the shedding of *E. coli* O157:H7 in cattle

Out of the 752 faecal samples collected from cattle and analyzed for the shedding pattern of *E. coli* O157:H7, 264 samples were from exotic breeds, 216

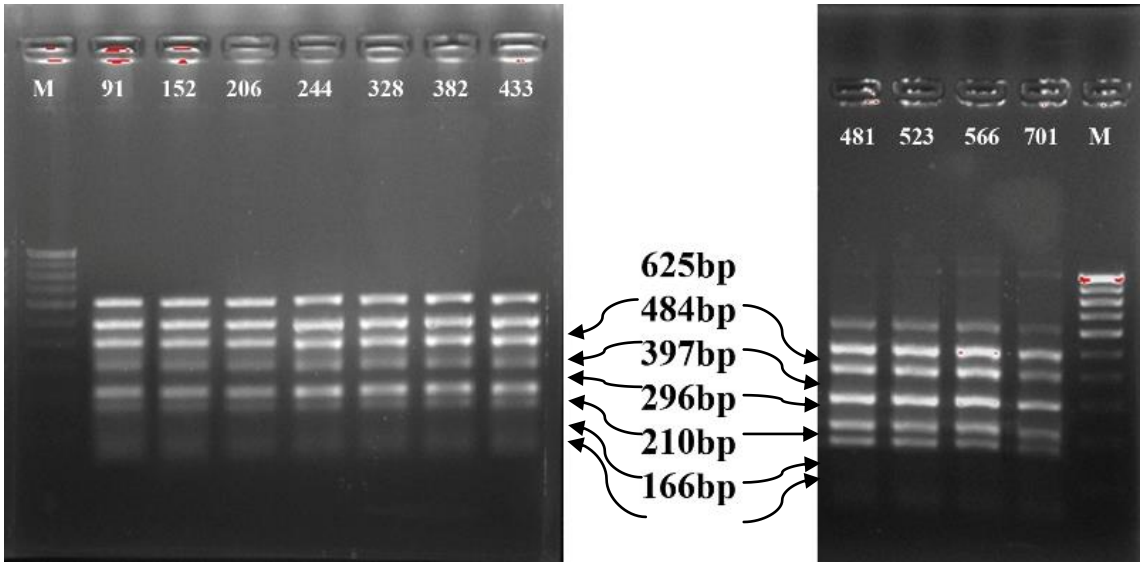


Fig 4.24: Eleven samples from buffaloes positive for *E. coli* O157:H7

Fig 4.25: Breed wise variation in the shedding of E. *coli* O157:H7 in cattle

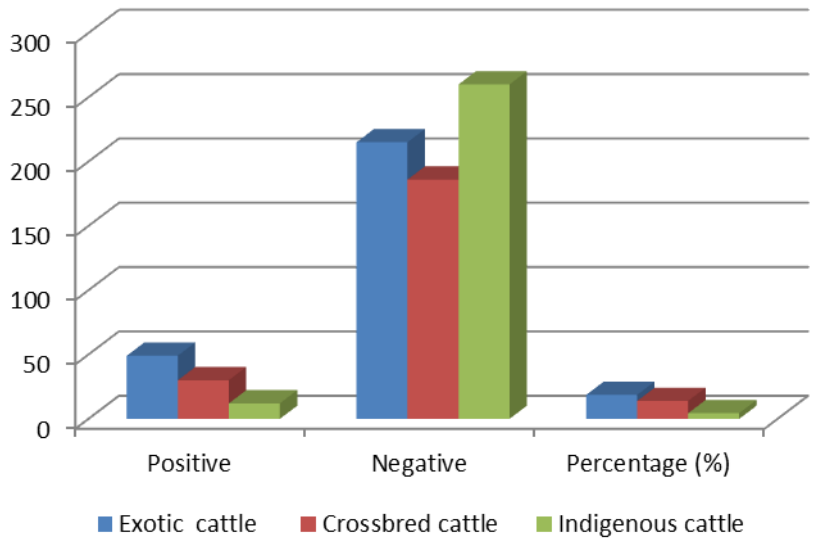


Table 4.8: Variation in the shedding pattern of E. *coli* O157:H7 in cattle and buffaloes

No. of faecal samples	Cattle	Buffaloes	**Total**
Positive	91	11	**102**
Negative	661	169	**830**
Total	**752**	**180**	**932**
Percentage (%)	**12.10**	**5.55**	**10.94**

Table 4.9: Breed wise variation in the shedding of E. *coli* O157:H7 in cattle

No. of faecal samples	Exotic cattle	Crossbred cattle	Indigenous cattle	**Total**
Positive	49	30	12	**91**
Negative	215	186	260	**661**
Total	**264**	**216**	**272**	**752**
Percentage (%)	**18.56**	**13.88**	**4.41**	**10.94**

Table 4.10: Age wise variation in the shedding of E. *coli* O157:H7 in the faecal samples of bovines

Category of animal/ No. of faecal samples	Calf	Heifer	Adult	**Total**
Positive	41	16	45	**102**
Negative	236	116	478	**830**
Total	**277**	**132**	**523**	**932**
Percentage (%)	**14.80**	**12.12**	**8.60**	**10.94**

Table 4.11. Sex wise variation in the shedding of *E. coli* O157:H7 in the faecal samples of bovines

No. of faecal samples	Male	Female	**Total**
Positive	27	75	**102**
Negative	151	679	**830**
Total	**178**	**754**	**932**
Percentage (%)	**15.17**	**9.95**	**10.94**

Table 4.12: Seasonal variation in the shedding of *E. coli* O157:H7 in the faecal samples of bovines

Season/ No. of faecal samples	**Summer Avg temp =36.24°C**	**Monsoon Avg temp =32.48°C**	**Winter Avg temp =23.03°C**	**Total**
Positive	29	28	45	**102**
Negative	340	230	260	**830**
Total	**369**	**258**	**305**	**932**
Percentage (%)	**7.86**	**10.85**	**14.75**	**10.94**

samples were from crossbreds and 272 samples were from indigenous breeds. Based on the multiplex PCR identification of *E. coli* O157:H7, the percent of exotic cattle that showed shedding *E. coli* O157:H7 was high, i.e. 18.56% (49 out of 264 samples). The

percent of crossbred cattle that showed shedding *E. coli* O157:H7 was equally high, 13.88% (30 out of 216 samples). The percent of cattle of indigenous breeds that showed shedding *E. coli* O157:H7 was only 4.41% (12 out of 272 samples). The results are shown in the Table 4.9 and Fig 4.25.

The results indicate that the native breeds of India are less prone for shedding of the pathogen *E. coli* O157:H7 in faeces when compared to cross bred cattle and the exotic breeds of cattle. When the data was subjected for chi-square goodness-of-fit test with a *P* value of <0.05, there was high significant difference in shedding of *E. coli* O157:H7 in the faeces by indigenous breeds, crossbreds and exotic breeds. Significant difference was observed in shedding of *E. coli* O157:H7 in the faeces by indigenous breeds, crossbreds and exotic breeds even at *P* value of <0.001.

4.6.4 Age wise variation in the shedding pattern of *E. coli* O157:H7 in bovines

Age wise variation in shedding pattern of *E. coli* O157:H7 was analysed between calves, heifers and adult bovines. In this study, out of 932 faecal samples analyzed, 277 samples were from calves, 132 samples were from heifers and 523 samples were from adult bovines. Based on the multiplex PCR identification of *E. coli* O157:H7, the percent of calves that showed shedding *E. coli* O157:H7 in faeces was 14.80% (41 out of 277 samples). The percent of heifers that showed shedding *E. coli* O157:H7 was 12.12% (16 out of 132 samples). The percent of adult bovines that showed shedding *E. coli* O157:H7 in faeces was only 8.60% (45 out of 523 samples). The results are presented in the Table 4.10 and Fig 4.26.

The results indicate that age influences the shedding of *E. coli* O157:H7 in faeces and the shedding was more in calves and heifers than in adult bovines. When the data was subjected for chi-square goodness-of-fit test with a *P* value of <0.001, the proportion of calves shedding *E. coli* O157:H7 in the faeces was significantly very high compared to heifers and adult cattle. When the test of significance was applied between

adult animals and heifers, proportion of heifers shedding *E. coli* O157:H7 in faeces was significantly high compared to adult cattle at *P* value of <0.05.

4.6.5 Sex wise variation in the shedding pattern of *E. coli* O157:H7 in bovines

In this study, out of 932 faecal samples analyzed, 178 samples were from male animals and 754 samples were from female animals. Based on the multiplex PCR identification of *E. coli* O157:H7, the percent of female animals that showed shedding of *E. coli* O157:H7 was only 9.95% (only 75 out of 754 samples), whereas, the percent of male bovines that showed shedding of *E. coli* O157:H7 was 15.17% (27 out of 178 samples). The results are presented in the Table 4.11 and Fig 4.27.

This indicates that male bovine animals are more prone for shedding of *E. coli* O157:H7. When the data was subjected for chi-square goodness-of-fit test with a *P* value of <0.05, there was significant difference between male and female animals in shedding of *E. coli* O157:H7 in the faeces.

4.6.6 Seasonal variation in the shedding pattern of *E. coli* O157:H7 in bovines

Seasonal variation in shedding pattern of E. *coli* O157:H7 was analysed between three seasons, *viz;* summer, monsoon and winter. In this study, out of 932 faecal samples collected and analysed from July 2014 to January 2016, 369 faecal samples were collected in summer season, 258 faecal samples were collected in monsoon season and 305 faecal samples were collected in winter season. Based on the multiplex PCR identification of *E. coli* O157:H7, the percent of bovines that showed shedding of*E. coli*

Fig 4.26: Age wise variation in the shedding of E. *coli* O157:H7 in the faecal samples of bovines

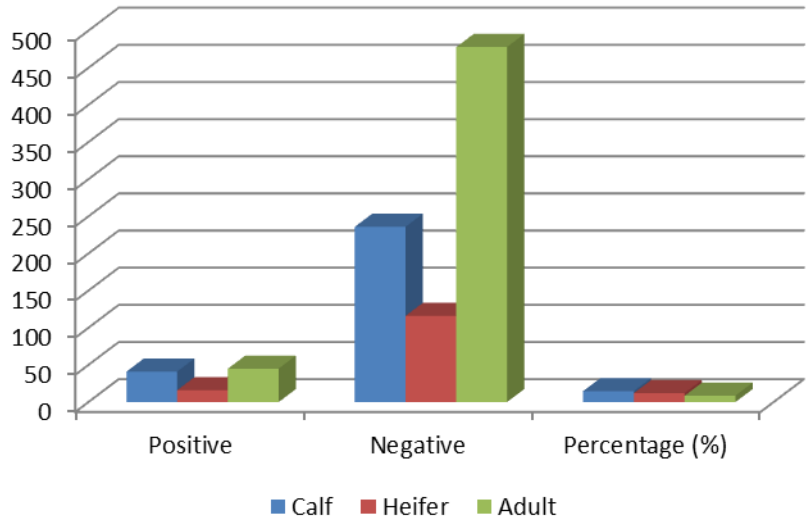


Fig 4.27: Sex wise variation in the shedding of *E. coli* O157:H7 in the faecal samples of bovines

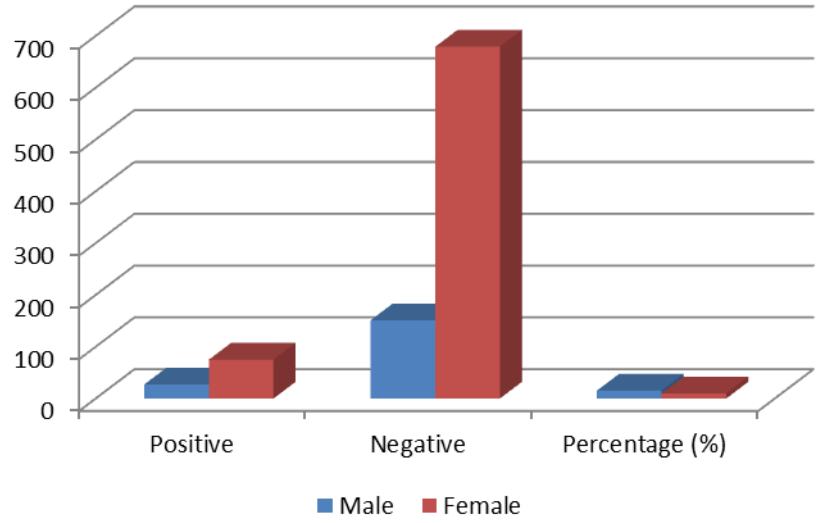
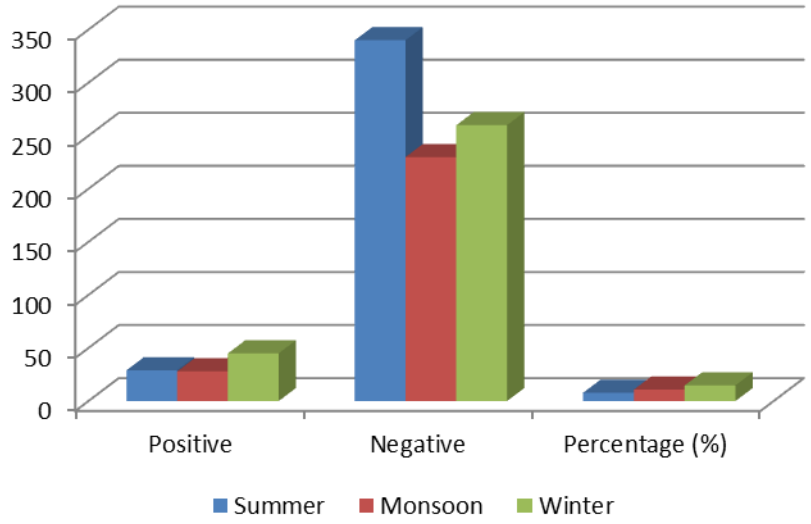


Fig 4.28: Seasonal variation in the shedding of *E. coli* O157:H7 in the faecal samples of bovines



O157:H7 in faeces in winter season was 14.75% (45 out of 305 samples). The percent of bovines that showed shedding *E. coli* O157:H7 in monsoon season was 10.85% (28

out of 258 samples). The percent of bovines that showed shedding *E. coli* O157:H7 in faeces in summer season was only 7.86% (29 out of 369 samples).

The results are presented in the Table 4.12 and Fig 4.28, which indicated that season influences the shedding of *E. coli* O157:H7 in faeces. When the data was subjected for chi-square goodness-of-fit test with a *P* value of <0.05, there was significant difference in the shedding pattern of *E. coli* O157:H7 in the faeces among summer, monsoon and winter seasons. The proportion of animals shedding *E. coli* O157:H7 in the faeces was significantly very high in winter compared to the other two seasons.

When the test statistic was compared between summer and monsoon seasons, there was no significant difference in shedding of *E. coli* O157:H7 in the faeces at *P* value of <0.05, however there was significant difference at *P* value of <0.1 with higher shedding pattern in monsoon than in summer season. When the test statistic was compared between monsoon seasons and winter season, there was significant difference in shedding pattern of *E. coli* O157:H7 in the faeces at *P* value of <0.05. And when test statistic was applied to compare between summer season and winter season, there was highly significant difference in shedding of *E. coli* O157:H7 in the faeces at *P* value of <0.05 and even at *P* value of <0.001.

To further elaborate on the effect of season on shedding of *E. coli* O157:H7 in bovines, monthly trend of shedding of this pathogen was analysed. The results are shown in the Table 4.13 and Fig 4.29. It could be appreciated that maximum percentage of faecal samples positive for *E. coli* O157:H7 were found in the month of January. From January month the trend of percent of animals showing shedding of this pathogen showed decreasing trend up to May and then increasing trend up to the month of December and least percentage of samples positive for *E. coli* O157:H7 were found in the month of May.

4.6.7 Effect of environmental temperature on shedding of *E. coli* O157:H7 in bovines

For the analysis of effect of environmental temperature on shedding of *E. coli* O157:H7 in bovines, the environmental temperature of the farm was recorded at the time of collection of the faecal sample. The samples were grouped into six ranges of temperature for the statistical analysis, *viz;* 10.1-15.0°C (Group I), 15.1-20.0°C (Group II), 20.1 to 25.0°C (Group III), 25.1 to 30.0°C (Group IV), 30.1 to 35.0°C (Group V) and 35.1 to 40.0°C (Group VI).

Based on the multiplex PCR identification of *E. coli* O157:H7, the percent of bovines shedding *E. coli* O157:H7 in faeces in Group I range of environmental temperature was only 8.57 % (9 out of105 samples). The percent of bovines shedding *E. coli* O157:H7 in faeces in Group II range of environmental temperature was, 13.87 % (19 out of 137 samples). The percent of bovines shedding *E. coli* O157:H7 in faeces in group III range of environmental temperature was 17.14 % (24 out of 140 samples).

The percent of bovines shedding *E. coli* O157:H7 in faeces in group IV range of environmental temperature was equally high 17.04 % (23 out of 135 samples). The percent of bovines shedding *E. coli* O157:H7 in faeces in group V range of environmental temperature was low, 7.98 % (17 out of 213 samples). The percent of bovines shedding *E. coli* O157:H7 in faeces in group VI range of environmental temperature was very low, 4.95 % (10 out of 202 samples). The results are shown in the Table 4.14 and Fig 4.30.

This indicates that the environmental temperature has effect on the shedding of *E. coli* O157:H7 in faeces in bovines. In our study, the range of environmental temperature in which animals showed highest shedding of *E. coli* O157:H7 in faeces was 20.1 to 25.0°C. When the data was subjected for chi-square goodness-of-fit test

Table 4.13: Monthly variation in the shedding of *E. coli* O157:H7 in the faecal samples of bovines

No. of faecal samples/month	Positive	Negative	**Total**	**Percentage**
January	13	58	**71**	**18.31**
February	12	64	**76**	**15.79**
March	9	87	**96**	**9.36**
April	8	83	**91**	**8.79**
May	5	87	**92**	**5.43**
June	7	85	**92**	**7.61**
July	6	52	**58**	**10.34**
August	7	60	**67**	**10.45**
September	7	58	**65**	**10.77**
October	8	58	**66**	**12.12**
November	8	70	**78**	**10.26**
December	12	68	**80**	**15.00**
Total	102	830	**932**	**10.94**

Table 4.14: Effect of environmental temperature on shedding of *E. coli* O157:H7 in bovines

Group	No. of faecal samples/ Temperature range	Positive	Negative	**Total**	**Percentage (%)**
Group I	10.1-15.0°C	9	96	**105**	**8.57**
Group II	15.1-20.0°C	19	118	**137**	**13.87**
Group III	20.1-25.0°C	24	116	**140**	**17.14**
Group IV	25.1-30.0°C	23	112	**135**	**17.04**
Group V	30.1-35.0°C	17	196	**213**	**7.98**
Group VI	35.1-40.0°C	10	192	**202**	**4.95**
	Total	102	830	**932**	**10.94**

Table 4.15: Effect of diarrhoea on shedding pattern of *E. coli* O157:H7 in bovines

No. of faecal samples	Diarrhoeic	Healthy	**Total**
Positive	3	99	**102**
Negative	109	722	**830**
Total	**112**	**821**	**932**
Percentage (%)	**2.68**	**12.06**	**10.94**

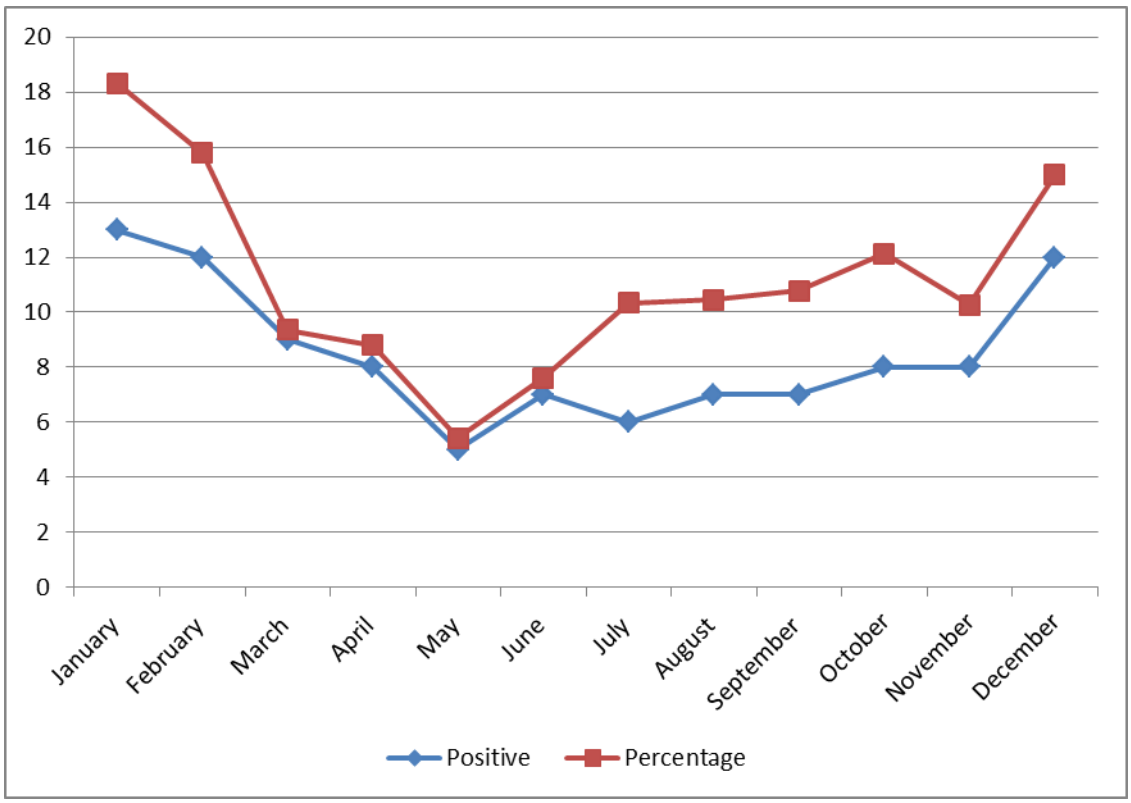


Fig 4.29: Monthly variation in the shedding of *E. coli* O157:H7 in the faecal samples of bovines

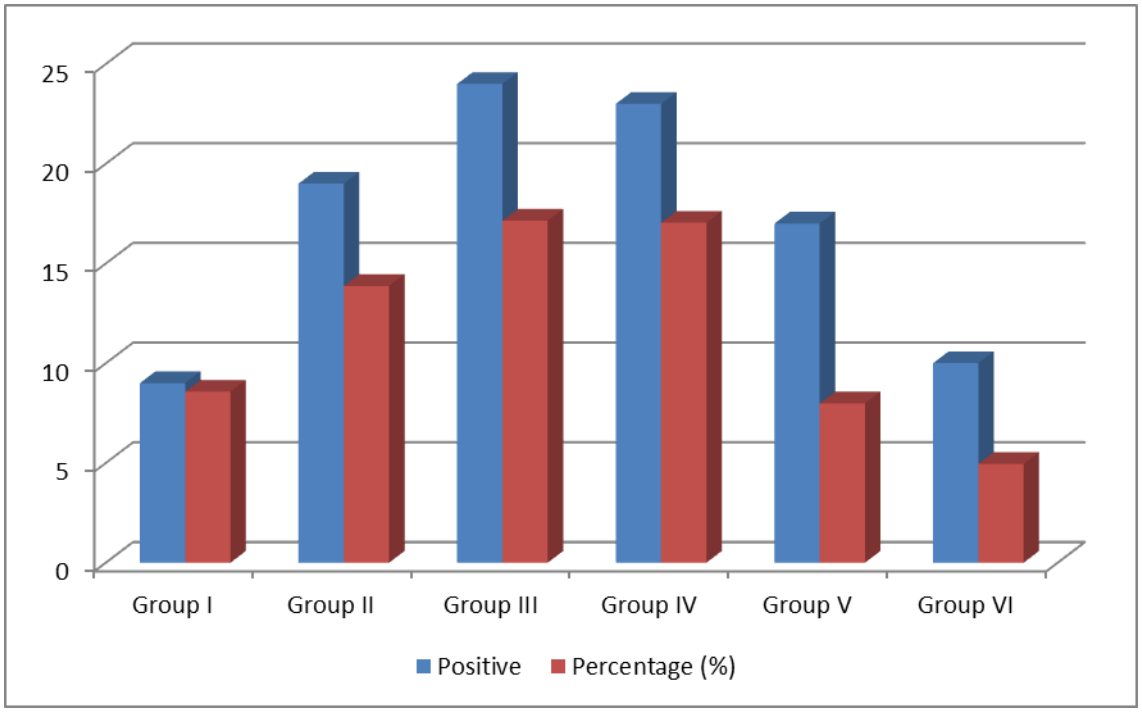


Fig 4.30: Variation in the shedding of *E. coli* O157:H7 in the faecal samples of bovines among six groups of ambient temperature range

with a *P* value of <0.05, there was significant difference in the shedding pattern of *E. coli* O157:H7 in the faeces among the six groups of range of environmental temperature.

When the class interval of the temperature was decreased and analysed, the temperature trend analysis of shedding pattern of *E. coli* O157:H7 has been depicted with line diagram in the Fig 4.31. The highest number of faecal samples positive for *E. coli* O157:H7 were found in the samples that were collected at the environmental temperature of 22.1 to 23.0°C.

4.6.8 Effect of diarrhoea on shedding of E. *coli* O157:H7 in bovines

In this study, out of 932 faecal samples analyzed, 112 samples were from diarrhoeic animals and 821 samples were from healthy animals. Based on the multiplex PCR identification of *E. coli* O157:H7, the percent of diarrhoeic animals that showed shedding of *E. coli* O157:H7 was mere 2.68% (only 3 out of 112 samples), whereas, the percent of healthy bovines that showed shedding of *E. coli* O157:H7 was very high *i.e.*, 12.06% (99 out of 821 samples). The results are shown in the Table 4.15 and Fig 4.32. This indicates that diarrhoea has no influencing effect on the shedding of *E. coli* O157:H7 and healthy animals are more prone for shedding of this pathogen than diarrhoeic animals.

4.6.9 Effect of lactation and pregnancy on shedding of E. *coli* O157:H7 in adult bovines

In this study a total of 523 faecal samples were collected from adult bovines. These samples were grouped into four groups based on physiological status, *viz;* Lactating and pregnant (group I), Lactating and non pregnant (group II), Dry pregnant (group III), and Non lactating and non pregnant (group IV) based on the records available in the farms and as communicated by the farmers, to find out the effect of

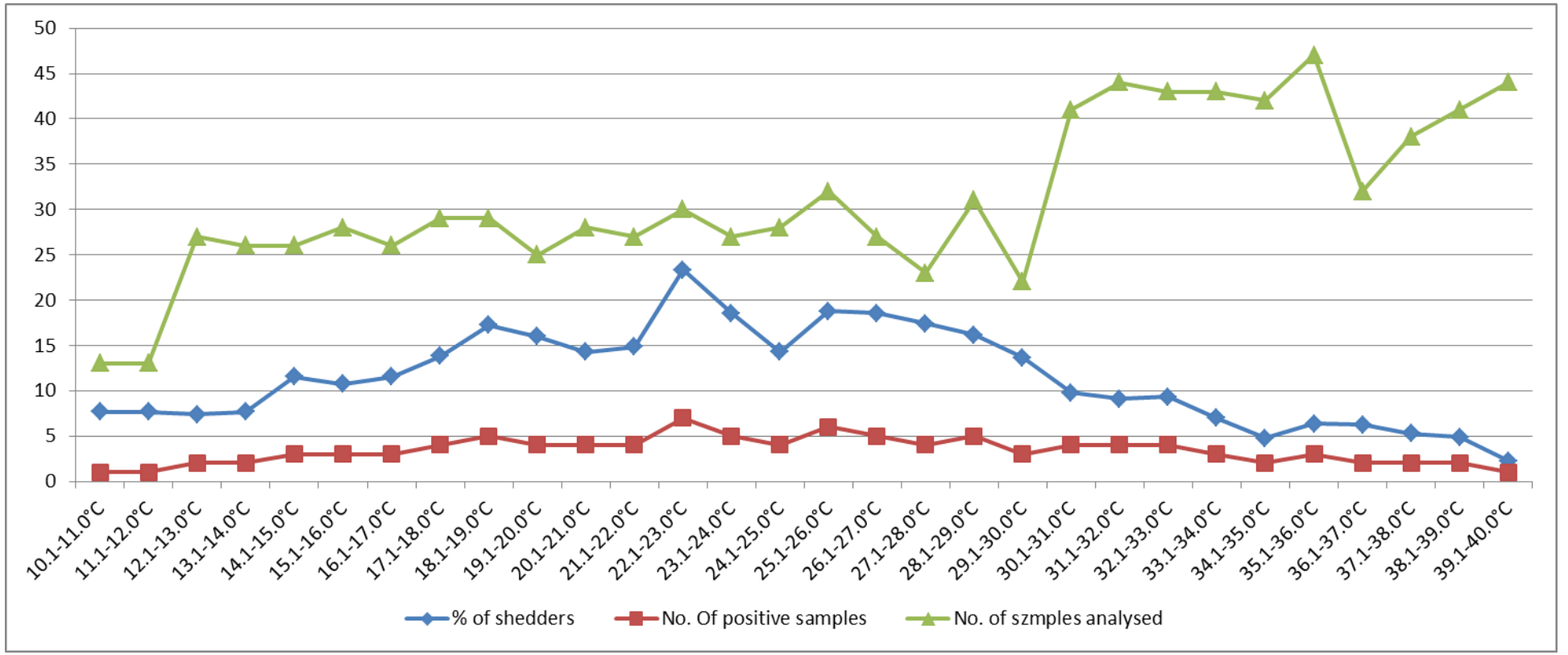


Fig 4.31: Temperature trend of variation in shedding pattern of *E. coli* O157:H7 in the faecal samples of bovines

lactation and pregnancy on shedding of E. *coli* O157:H7 in adult bovines. The results are shown in the Table 4.16 and Fig 4.33.

Based on the multiplex PCR identification of *E. coli* O157:H7, the percent of group I animals that showed shedding of *E. coli* O157:H7 was 10.66% (21 out of 197 samples). The percent of group II animals that showed shedding of *E. coli* O157:H7 was also 8.54% (14 out of 164 samples). The percent of group III animals that showed shedding of *E. coli* O157:H7 was 9.68% (9 out of 93 samples). The percent of group IV animals that showed shedding of *E. coli* O157:H7 was 1.45% (1 out of 69 samples). When the data was subjected for chi-square goodness-of-fit test with a *P* value of <0.05, there was significant difference in the shedding pattern of *E. coli* O157:H7 in the faeces among these four groups. However, when chi-square goodness-of-fit test was applied among the first three groups (Group I, group II and group III), there was no significant difference in shedding pattern of *E. coli* O157:H7 even at *P* value of <0.25. And when chi-square goodness-of-fit test was applied to test the null hypothesis between any of these three groups and the group IV then there was a significant difference in the shedding pattern of *E. coli* O157:H7.

4.7 Antibiotic resistance/sensitivity pattern of *E. coli* O157:H7 positive samples

In this study, the samples confirmed as positive for *E. coli* O157:H7 by mPCR were subjected for antibiotic sensitivity test using Muller Hinton agar for the routinely used antibiotics *viz;* Amikacin (Ak), Carbenicillin (Cb), Ciprofloxacin (Cf), Co-Trimazine (Cm), Kanamycin (K), Nitrofurantoin (Nf), Streptomycin (S) and Tetracycline (T). Zones of inhibition were determined and interpreted using Standard interpretative chart provided with the octadiscs. The results are presented in the Table 4.17. The Fig 4.34 shows the antibiotic sensitivity pattern of one of the samples found positive for *E. coli* O157:H7.

Table 4.16: Effect of lactation and pregnancy on shedding pattern of E. *coli* O157:H7 in the faecal samples in adult bovines

Category of animal/ No. of samples	Lactating and pregnant	Lactating and non	Dry pregnant	Non lactating non pregnant	**Total**

		pregnant			
Positive	16	13	6	1	36
Negative	171	141	77	59	448
Total	**187**	**154**	**83**	**60**	**484**
Percentage (%)	**8.56**	**8.44**	**7.23**	**1.66**	**7.44**

Table 4.17: Antibiotic sensitivity/resistance of samples positive for *E. coli* O157:H7

S. No.	Antibiotic	Sensitive (Percentage - %)	Intermediate (Percentage - %)	Resistant (Percentage - %)
1	Amikacin (Ak)	36 (35.29)	19 (18.63)	47 (46.08)
2	Carbenicillin (Cb)	3 (2.94)	5 (4.90)	94 (92.16)
3	Ciprofloxacin (Cf)	87 (85.29)	12 (11.76)	3 (2.94)
4	Co-Trimazine (Cm)	4 (3.92)	9 (8.82)	89 (87.25)
5	Kanamycin (K)	15 (14.71)	16 (15.69)	71 (69.61)
6	Nitrofurantoin (Nf)	00 (00)	00 (00)	102 (100)
7	Streptomycin (S)	51 (50.00)	27 (26.47)	24 (23.53)
8	Tetracycline (T)	7 (6.86)	18 (17.65)	77 (75.49)

Table 4.18: Prevalence of *E. coli* O157:H7/H$^-$ (*E. coli* O157) in faecal samples of bovines

		No. of samples
Positive	*E. coli* O157:H7	102
	E. coli O157:H$^-$	23
	Total (*E. coli* O157)	**125**
Negative		807
Total		**932**
Percentage (%)		**13.41**

Table 4.19: Prevalence of STEC in faecal samples of bovines

		No. of samples
Positive	*E. coli* O157:H7	102
	E. coli O157:H$^-$	23
	E. coli only with *stx1* and/or *stx2* genes	29
	Total (*STEC*)	**154**
Negative		778
Total		**932**
Percentage (%)		**16.52**

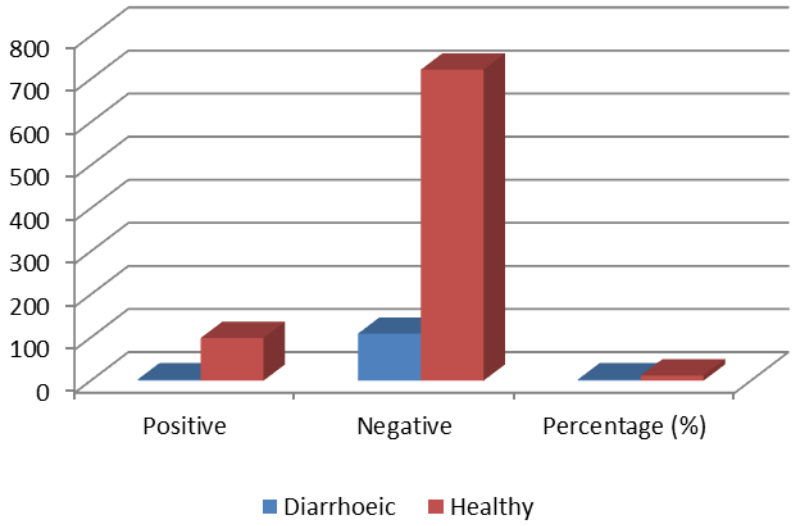


Fig 4.32: Effect of diarrhoea on shedding pattern of E. *coli* O157:H7 in bovines

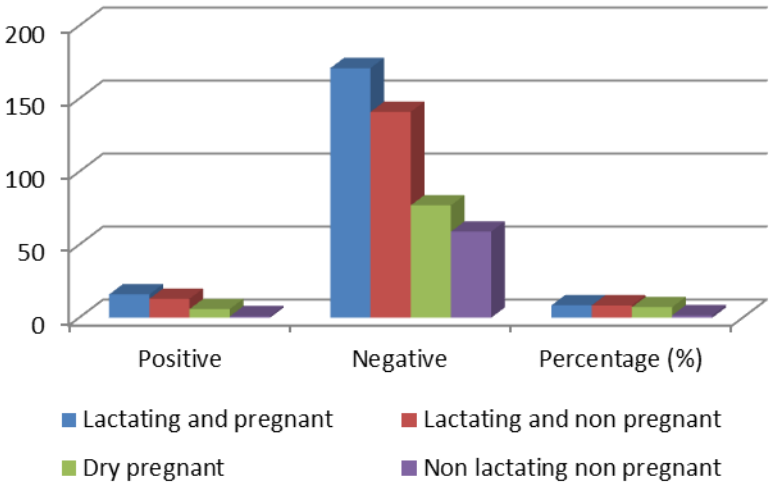


Fig 4.33: Effect of lactation and pregnancy on shedding pattern of E. *coli* O157:H7 in the faecal samples in adult bovines

Fig 4.34: Antibiotic resistance/ sensitivity pattern of *E. coli* O157:H7 positive samples

The results found that, the percent of *E. coli* O157:H7 positive samples showing resistance for the antibiotics Amikacin, Carbenicillin, Ciprofloxacin, Co-Trimazine,

Kanamycin, Nitrofurantoin, Streptomycin and Tetracycline was 46.08%, 92.16%, 2.94%, 87.25%, 69.61%, 100.00%, 23.53% and 75.49% respectively. 18.63%, 4.90%, 11.76%, 8.82%, 15.69%, 0%, 26.47% and 17.65% of the samples showed intermediate susceptibility of the respective antibiotics. And 35.29%, 2.94%, 85.29%, 3.92%, 14.71%, 00%, 50.00% and 6.86% of the samples were sensitive to the respective antibiotics.

4.8 Prevalence of *E. coli* O157:H7/H$^-$ (*E. coli* O157) and STEC in faecal samples of bovines

Few faecal samples which exhibited cultural and biochemical characters showed presence of only *rfbE* but not *fliCh7* genes by multiplex PCR. Such isolates were classified as *E. coli* O157:H$^-$. 23 isolates were characterized as *E. coli* O157:H$^-$ by multiplex PCR. The isolates *E. coli* O157:H7 and *E. coli* O157:H$^-$ together were characterized as *E. coli* O157. The percent of animals that showed shedding *E. coli* O157 in the faeces was 13.41% (125 out of 932). The results are presented in Table 4.18. The Fig 4.35 shows two samples (lane 1 and 2) positive for *E. coli* O157:H$^-$ (presence of only *stx1, stx2, eaeA* and *rfbE* gene and absence of *fliCh7* and *hly* genes).

Further, few samples which showed cultural and biochemical characters did not show presence of both *rfbE* and *fliCh7* genes by multiplex PCR. However, few of these samples showed presence of any one of or both of the *stx1 and stx2* genes. These were considered positive for Shiga Toxin producing *E. coli* (STEC) other than *E. coli* O157:H7. In this research, the isolates *E. coli* O157:H7, *E. coli* O157:H$^-$ and *E. coli* showing presence of any one of or both of the *stx1 and stx2* genes by multiplex PCR were together characterized as shiga toxin producing *E. coli* (STEC). The percent of animals that showed shedding of STEC was 16.52 % (154 out of 932 samples). The

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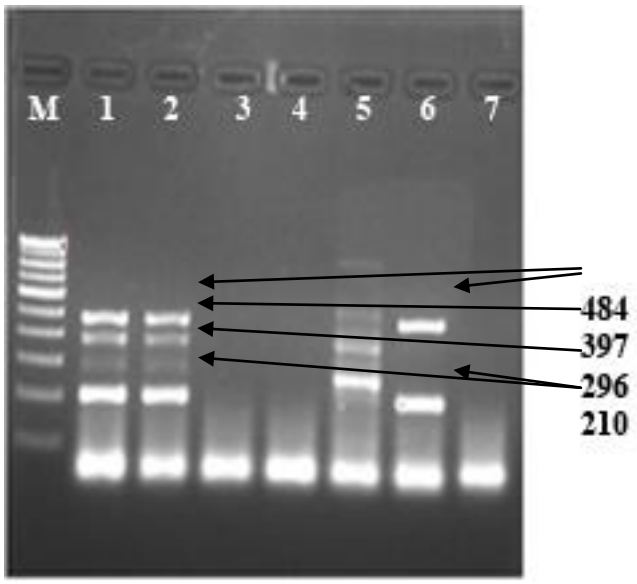

Figures 4.35: Samples positive for *E. coli* O157:H$^-$ (*E. coli* O157) and STEC.
Lane M: DNA ladder
Lane 1 and 2: two samples (sample No. 601 and 612) positive for only four genes; *rfbE, eaeA*, *stx1* and *stx2* and absence of *fliCh7* (625 bp) and *hly* (166bp) genes
Lane 3, 4 and 7: samples negative for all the six genes of *E. coli* O157:H7
Lane 6: sample (sample No. 338) positive for only two genes; *stx1* and *stx2* (STEC)
Lane 5: sample showing non specific bands

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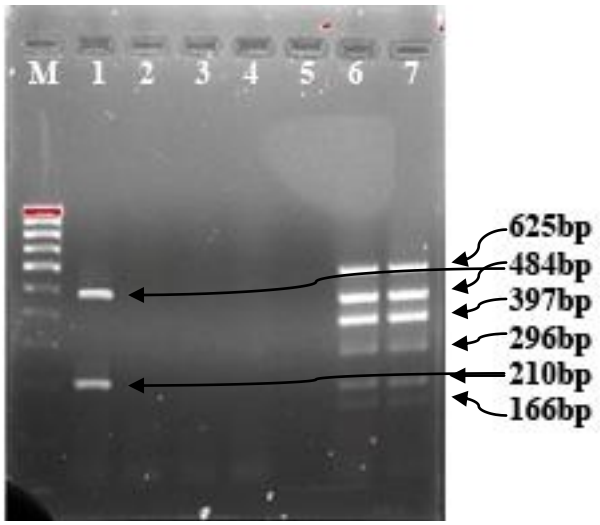
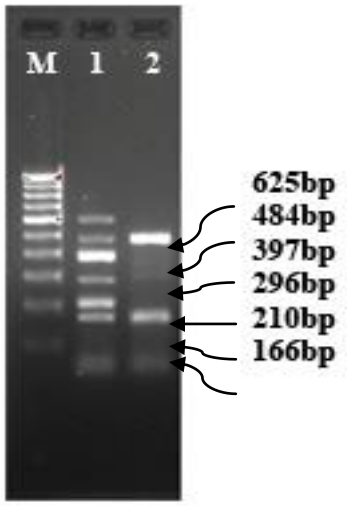


Figures 4.36: Samples positive for *E. coli* O157:H7 and STEC

Lane M: DNA ladder
Lane 1: sample (sample No. 376) positive for only two genes; *stx1* and *stx2* (STEC)
Lane 2, 3, and 4: samples negative for all the six genes of *E. coli* O157:H7
Lane 5 and 6: samples (sample No.721 and725) positive for all the six genes of *E. coli* O157:H7



Figures 4.37: Samples positive for *E. coli* O157:H7 and STEC

Lane L: DNA ladder

Lane 1: sample (sample No. 128) showing presence of Amplicons of six genes (*E. coli* O157:H7)

Lane 2: sample (sample No. 269) showing presence of amplicons of only *stx2* and *hly* genes (STEC).

results are presented in Table 4.19. The Fig 4.35 (lane 6) and Fig 4.36 (lane 2) show samples positive for STEC other than *E. coli* O157:H7 showing presence of only *stx1* and *stx2* genes.

Fig 4.30 (lane 2) shows sample positive for STEC other than *E. coli* O157:H7 showing presence of only *stx2* gene and *hly* gene.

4.9 Antibiotic sensitivity/resistance of *E. coli* O157 and STEC positive samples

In this study, the *E. coli* O157 and STEC samples confirmed by mPCR and/or latex agglutination test were subjected for antibiotic sensitivity test using Muller Hinton agar for the routinely used antibiotics. Zones of inhibition were determined interpreted using Standard interpretative chart provided with the octadiscs.

The results found that, the percent of *E. coli* O157 positive samples showing resistance for the antibiotics Amikacin, Carbenicillin, Ciprofloxacin, Co-Trimazine, Kanamycin, Nitrofurantoin, Streptomycin and Tetracycline was 46.40%, 88.80%, 2.40%, 83.20%, 60.00%, 100.00%, 20.80% and 65.60% respectively. 19.2%, 8.8%, 16.0%, 11.2%, 20.00%, 0.00%, 23.20% and 20.80% of the samples showed intermediate susceptibility of the respective antibiotics. And 34.40%, 2.40%, 82.40%, 5.60%, 20.00%, 00.00%, 56.00% and 13.60% of the samples were sensitive to the respective antibiotics. The results are presented in the Table 4.20.

The percent of STEC positive samples showing resistance for the antibiotics Amikacin, Carbenicillin, Ciprofloxacin, Co-Trimazine, Kanamycin, Nitrofurantoin, Streptomycin and Tetracycline was 40.91%, 88.31%, 3.25%, 73.38%, 52.60%, 99.35%, 20.78% and 69.48% respectively. 18.83%, 9.09%, 14.29%, 20.78%, 18.83%, 0.00%, 22.08% and 18.18% of the samples showed intermediate susceptibility of the respective antibiotics. And 40.26%, 2.60%, 82.47%, 5.84%, 28.57%, 0.65%, 57.14% and 12.34% of the samples were sensitive to the respective antibiotics. The results are presented in the Table 4.21.

Table 4.20: Antibiotic sensitivity/resistance of samples positive for *E. coli* O157

S. No.	Antibiotic	Sensitive (Percentage - %)	Intermediate (Percentage - %)	Resistant (Percentage - %)
1	Amikacin (Ak)	43 (34.4)	24 (19.2)	58 (46.4)
2	Carbenicillin (Cb)	3 (2.4)	11 (8.8)	111 (88.8)
3	Ciprofloxacin (Cf)	103 (82.4)	20 (16.00)	3 (2.4)
4	Co-Trimazine (Cm)	7 (5.6)	14 (11.2)	104 (83.2)
5	Kanamycin (K)	25 (20.00)	25 (20.00)	75 (60.00)
6	Nitrofurantoin (Nf)	00 (00)	00 (00)	125 (100)
7	Streptomycin (S)	70 (56.00)	29 (23.2)	26 (20.8)
8	Tetracycline (T)	17 (13.6)	26 (20.8)	82 (65.6)

Table 4.21: Antibiotic sensitivity/resistance of samples positive for of STEC

S. No.	Antibiotic	Sensitive (Percentage - %)	Intermediate (Percentage - %)	Resistant (Percentage - %)
1	Amikacin (Ak)	62 (40.26)	29 (18.83)	63 (40.91)
2	Carbenicillin (Cb)	4 (2.60)	14 (9.09)	136 (88.31)
3	Ciprofloxacin (Cf)	127 (82.47)	22 (14.29)	5 (3.25)
4	Co-Trimazine (Cm)	9 (5.84)	32 (20.78)	113 (73.38)
5	Kanamycin (K)	44 (28.57)	29 (18.83)	81 (52.60)
6	Nitrofurantoin (Nf)	1 (0.65)	00 (00)	153 (99.35)
7	Streptomycin (S)	88 (57.14)	34 (22.08)	32 (20.78)
8	Tetracycline (T)	19 (12.34)	28 (18.18)	107 (69.48)

CHAPTER V
DISCUSSION

The most significant Enterohaemorrhagic *Escherichia coli* (EHEC) of humans is *E. coli* O157:H7. The infection is transmitted by faeco-oral route through contaminated food or water. The primary reservoir of this serotype is the cattle (Elaine and James, 2010; Eppinger *et. al*., 2011; Soderlund *et. al.*, 2012). After the first outbreak of bloody diarrhea in Oregon and Michigan, U.S.A. in 1982, *E.coli* O157:H7 has become the most widely known EHEC strain ((Karmali *et. al.*, 1983, Riley *et. al.*, 1983, Wells *et. al.*, 1983).

In India too there could be foodborne outbreaks because of this pathogen as the people consume milk, milk products and beef. However, systematic diagnosis of the foodborne outbreaks in India is not carried out. Few scientists have worked on the incidence of this pathogen in various livestock products (Bindu and Krishnaiah 2010), human patients (Khan *et. al*., 2002a; Khan *et. al*., 2002b) and environmental samples (Hazarika *et. al*., 2007). Naik and Desai (2012) worked on isolation of *E. coli* O157:H7 from diarrheagenic stool sample of patients in Surat (India) and determination of their antimicrobial resistance pattern. They isolated *E. coli* O157:H7 from 13.91 % samples emphasising the presence of the pathogen in humans in India and its public health significance.

Sensitive, specific and less time consuming diagnostic techniques for the identification of *E. coli* O157:H7 are lacking. Conventional microbiological culture based method, though very useful for the identification of *E. coli* O157:H7, is time consuming (Visetsripong *et. al.*, 2007). In addition, there is a possibility to get false results by these classic microbiological diagnostic procedures (Orth *et. al.*, 2009). Several immunological tests are being used for the detection of *E. coli* O157:H7 (Tao Geng *et. al.* 2006; Divya *et. al.*, 2013; Taye, *et. al.*, 2013).

The present study was undertaken to standardize sensitive and specific multiplex PCR technique for the detection of *E.coli* O157:H7, to isolate and confirm *E.coli* O157:H7 from faecal samples of cattle and buffaloes by cultural identification, biochemical characterization, latex agglutination and the standardized multiplex PCR and to find the variation in epidemiology of shedding pattern of *E.coli* O157:H7 in bovines between buffaloes and cattle, between different age groups, sex, lactating and non lactating, pregnant and non pregnant, healthy and diarrhoeic animals and between seasons and in cattle between breeds.

5.1 Standardization of multiplex Polymerase Chain Reaction (multiplex PCR) for the identification of *E. coli* O157:H7

5.1.1 DNA template preparation

In this standardization technique, we have used modified boiled cell method (Tunung *et. al.,* 2007; Chai *et. al.*, 2007; Queipo-Ortuño *et. al.,* 2008) for the extraction of genomic DNA of *E. coli* 0157:H7. Many other researchers have used boiling cell method to extract genomic DNA for the detection of various bacteria by PCR. The researchers, Abdulmawjood *et. al.* (2003), Bai *et. al.* (2010), Jeshveen *et. al.* (2012), Puttalingamma *et. al.* (2012), Lye *et. al.* (2013), Ahmad *et. al.* (2013) and Einas *et. al.* (2015) have used boiling cell method to extract genomic DNA for the detection of *E. coli* 0157:H7 by PCR. The boiling cell method was used for the preparation of template DNA from other microbes, *viz; Salmonella* (Park *et. al.,* 2009), *Vibrio parahaemolyticus* (Lesley *et. al.,* 2005) and *Campylobacter* spp. (Chai *et. al.,* 2007). The results obtained in this standardized multiplex PCR technique proves that boiling cell method for the extraction of genomic DNA is simple, less harmful, quicker, has few steps and would produce optimum results of multiplex PCR for the identification of *E. coli* 0157:H7.

5.1.2 The primers

In this standardized multiplex PCR, the primers used targeted the 6 specific virulent genes of *E. coli* 0157:H7; *fliCh7, eaeA, rfbE, hly, stx1,* and *stx2.* The genes *stx1* and *stx2* encode vero toxins/shiga toxins (*stx1* and *stx2*, respectively). The gene *eaeA* encodes intimin, responsible for adherence of this pathogen to the intestinal lining and causing attacing and effacing lesions in human illnesses. *hlyA* gene encodes Hemolysin, *rfbE* gene encodes 0157 endotoxin antigen and *fliCh7* gene encodes flagellar antigen (Pennington, 2010; Jeshveen *et. al.,* 2012).

5.1.3 Multiplex PCR protocol

The concentration of $MgCl_2$ is very important to get reliable PCR products (Roux, 1995). The magnesium ions react with free dNTPS to form soluble complexes which will help in synthesizing PCR amplicons. If the concentration is less, the yield of the PCR product will be very meagre or no product and if the concentration is more, then chances of getting unwanted bands and primer dimers will be more which causes confusion in the identification of the organisms and interpretation.

The above scientific principle could be well related to the research findings in the present study. The concentration of $MgCl_2$ routinely used for PCR is 1.5 mM. However, in the present research findings, the multiplex PCR products were very meagre, when the concentration of $MgCl_2$ was less than 2.5 mM. $MgCl_2$ concentration of 3mM produced optimum amount of the amplicons indicated by the clear and discernible DNA bands on agarose gel electrophoresis and the concentration of 3.5 mM produced unwanted DNA bands. Therefore the optimum $MgCl_2$ concentration for the multiplex PCR detection of *E. coli* 0157:H7 was 3 mM. Jeshveen *et. al.* (2012) also concluded that, 3 mM concentration of $MgCl_2$ was the most suitable concentration for the detection of *E. coli* 0157:H7 by multiplex PCR.

The time period of denaturation is one of the parameters for PCR. Generally, the duration would increase as the size of the gene that is to be amplified increases (Green and Sambrook, 2012). In this study the denaturation temperature of 94°C for 20 seconds of time period amplified only five genes; *eaeA, rfbE, hly, stx1* and *stx2*. The gene *fliCh7* could not be amplified. The same gene along with the other five genes got amplified at the denaturation temperature of 94°C for 45 seconds of time period. The larger size could be explained as the reason for the variation in the amplification of the *fliCh7* gene and the other five genes (Roux, 1995). Similar temperature –time combination was used by Ahmad *et. al.* (2013) to confirm *E. coli* O157:H7 using three gene (*rfbE, fliCh7* and *uidA*) multiplex PCR. However, Jeshveen *et. al.* (2012) could achieve amplification of all the 6 genes at the denaturation temperature of 94°C for 20 seconds of time period which is a deviation from the present findings.

Another crucial criterion in the standardization of multiplex PCR is annealing temperature. It depends upon the melting temperature of the primers used (Green and Sambrook, 2012). The melting temperature of the primers used in our study ranged between 53.2°C (*hly* gene) to 64.0°C (*fliCh7* gene). Hence, we standardized multiplex PCR by running gradient PCR at the annealing temperatures between 58°C to 63°C with an increment of 1°C so that all the six genes would be amplified optimally.

The optimum annealing temperature was found to be 60°C where we got optimum amount of amplicons of all the 6 genes was obtained and produced well discernible DNA bands in gel electrophoresis. The four genes *viz; eaeA, rfbE, stx1,* and *stx2* genes produced bright DNA bands at all the 6 gradient annealing temperatures. However, at higher annealing temperature, bright band of *fliCh7* gene but very faint band of *hly* gene was visualized in gel electrophoresis. On contrary, at lower annealing temperature, bright band of *hly* gene but very faint band of *fliCh7* gene was visualized in gel electrophoresis. At 60°C the DNA bands of both *fliCh7*and *hly* genes were bright and discernible though fainter than

the bands of the other four genes. Therefore, 60°C was taken as the annealing temperature in this standardized multiplex PCR for the identification of *E. coli* 0157:H7. Jeshveen *et. al.* (2012) also achieved the best amplicons at the annealing temperature of 60°C.

5.1.4 Agarose gel electrophoresis

After the amplification, visualization of the multiplex PCR amplicons was done by agarose gel electrophoresis. The concentration of agarose used is very important for the optimum visualization of the bands. Generally smaller the molecular size of the amplicons, higher would the concentration of agarose to be used for gel electrophoresis (Green and Sambrook, 2012). When the amplicons of multiplex PCR were subjected for agarose gel electrophoresis at 1% agarose concentration, very faint DNA bands of the amplicons were visualized and the band of the gene *hly* was not visible because of its low size. Whereas the DNA bands visualized at 1.5 % agarose concentration were bright and the band of the gene *hly* was also visible. Hence, 1.5% of agarose concentration was used in this study.

Finally, the multiplex PCR protocol standardized in this study consisted of volume of 25 µl of reaction mixture containing 2 µl of *Taq* DNA Polymerase (1 unit/ µl), 2.0 µl of DNA template solution, 2.5 µl of 10 x reaction buffer, 0.5 µM of deoxynucleoside triphosphates (dNTPs), 0.2 µM each of the 12 primers (6 primer pairs) and 3 mM magnesium chloride ($MgCl_2$) and the rest of the volume, sterile nuclease free water to make up the 25 µl reaction mixture volume. The thermal cycling parameters were; 5 minute of initial denaturation at 94˚C followed by 35 cycles of denaturation at 94˚C for 45 sec, 1 min of annealing at 60˚C and extension for 1 min at 72˚C, with a 10 min final extension at 72˚C followed by maintenance at 4˚C. The agarose gel electrophoresis was optimized at 1.5 % concentration of agarose.

With this standardized technique, the size of the amplicons produced was 625 bp, 397 bp, 296 bp, 166 bp, 210 bp and 484 bp, respectively for the six targeted virulent genes,

viz; *fliCh7*, *eaeA*, *rfbE*, *hly*, *stx1*, and *stx2*. These six virulent genes of *E. coli* 0157:H7 targeted in this study would be a comprehensive set of genes and hence high sensitivity and specificity of the standardized multiplex PCR as discussed in the further paragraphs.

The identification of the *E. coli* 0157:H7 by multiplex PCR saves cost of chemicals and reagents which would be used for simple PCR targeting individual genes in separate PCR reaction mixture. And also the standardized multiplex PCR procedure produces distinct bands for each primer on agarose gel electrophoresis for the quicker detection of *E. coli* O157:H7.

There are previous studies to develop multiplex PCR for the identification of *E. coli* 0157:H7. Sahilah *et. al.* (2010) detected *stx*1 and *stx*2 Genes in *E. coli* O157:H7 isolated from retail beef in Malaysia by multiplex PCR (2 gene multiplex PCR), wherein the multiplex PCR protocol consisted of 35 cycles of denaturation at 94°C for 2 minutes, annealing at 35°C for 1 minute and polymerization at 72°C for 2 minutes which differ from the multiplex PCR protocol standardized in the present study. However, the sequence of primers used by these authors to detect *stx*1 and *stx*2 genes was different from the sequence of the primers used in the present study. It could be noted that identification of only these two genes would characterize the isolate as STEC rather than EHEC O157:H7.

Ahmad *et. al.* (2013) confirmed the presence of *E. coli* O157:H7 in 8% of ground beef hamburger samples using multiplex PCR that simultaneously detected three genes: *fli*C, *rfb*E and *uid*A (encoding). The three gene multiplex PCR used by these authors specifically identifies genes of O157 endotoxin, H7 flagellin and beta-glucuronidase and would characterize the isolate as *E. coli* O157:H7. However, the isolate cannot be characterized as STEC/EHEC. LeJeune *et. al.* (2004) and Puttalingamma *et. al.* (2012) confirmed the *E. coli* O157:H7 isolates by multiplex PCR using primers for four genes; *fliCh7*, *eaeA*, *stx*2 and *stx*1 genes.

Brandon *et. al.* (2009) and Divya *et. al.* (2013) characterized *Escherichia coli O157* isolates by multiplex PCR targeting 5 virulence genes, namely*; eae, stx1, stx2, hlyA* and *fliCh7* genes. Bai *et. al.* (2010) and Jeshveen *et. al.* (2012) also used these same pairs of primers targeting 6 genes to detect *E. coli* 0157:H7. The amplicons after multiplex PCR observed on agarose gel electrophoresis were of the size, 625 bp, 397bp, 296 bp, 166 bp, 210 bp and 484 bp, respectively for *fliCh7, eaeA, rfbE, hly, stx1,* and *stx2* genes.

5.1.5 Threshold sensitivity of multiplex PCR

The threshold sensitivity of standardized multiplex PCR, when ten fold serial dilutions of *E. coli* O157:H7 reference culture in modified Tryptone Soy Broth (mTSB) were subjected for standardized multiplex PCR was found to be very high (0.1 CFU/ml). However, the effect of components of faecal matrix on detection of *E. coli* O157:H7 in faecal samples would have to be considered while affirming the sensitivity of a diagnostic technique (Wang *et. al.*, 1997).

Therefore, the threshold sensitivity of standardized multiplex PCR was also checked by inoculating (spiking) the reference culture of *E. coli* O157:H7 into the faecal sample which was found to be negative for *E. coli* O157:H7 by cultural characters both directly as well as with one step selective enrichment (Arthur *et. al.*, 2005). The threshold sensitivity of standardized multiplex PCR was found to be low (1 CFU/g) in spiked faecal samples without one step selective enrichment compared to spiked faecal samples with one step selective enrichment in mTSB broth (0.1 CFU/g). The rationale here could be the inhibitory effect of components of faecal matrix as supported by the findings of Wang *et. al.* (1997) in spiked food samples. It could be inferred that single step selective enrichment of the faecal samples before multiplex PCR increases the sensitivity by tenfold (Loge *et. al.,* 2002). Nevertheless threshold sensitivity of 1 CFU/g when faecal samples directly analyzed is a good sensitivity.

According to the USDA's Food Safety and Inspection Service (USDA-FSIS, 2002), ground beef is considered adulterated if as little as 1 CFU of EHEC O157:H7 is detected in 25 g of ground beef. The multiplex PCR standardized in the present study was very sensitive enough to detect 1 CFU in suspected sample and 0.1 CFU in the suspected sample with one step selective enrichment.

The threshold sensitivity (detection limit) of the standardized multiplex PCR in this study was better than that of other researchers. Bindu and Krishnaiah (2008) standardized three gene multiplex PCR for the identification of *E. coli* O157:H7 in beef samples where in the threshold sensitivity was 1.7 CFU/ml. Similarly, Arthur *et. al.* (2005) reported the minimum detection limit of 1.7 CFU/ml by PCR after 18hrs of enrichment. Fode-Vaughan *et. al.* (2003) developed 2 gene multiplex PCR where in the threshold sensitivity was 20 cells/ml. Likewise, Ibekwe *et. al.* (2002) and Ibekwe and Grieve (2003) reported a minimum detection level of 20 cells of *E. coli* O157:H7 /ml with real time PCR.

5.1.6 Specificity of multiplex PCR

Specificity of standardized multiplex PCR when evaluated by subjecting *E. coli* other than *E. coli* O157:H7 (sorbitol positive isolate on CT-SMAC agar) *Staphylococcus aureus, Streptococci, Vibrio parahaemolyticus, Brucella abortus* (strain 19) and *Salmonella* cultures as negative control along with reference culture of *E. coli* O157:H7. None of the six DNA bands was found in the PCR product of negative control cultures which indicates that the standardized multiplex PCR is very specific for *E. coli* O157:H7 only**.** This high specificity of the standardized multiplex PCR avoids identification of false positive samples and increases its reliability.

From the above discussion it could be inferred that the multiplex PCR standardized in this study is a rapid, sensitive, highly specific and reliable method for the detection of pathogenic *E. coli* O157:H7 as it targets 6 genes; *eae*, *stx1*, *stx2*, *hlyA*, *rfb*E and *fliCh7*

genes, specific for the pathogen and as it has shown high threshold sensitivity and specificity. Further, the technique would be helpful in characterizing the isolates as STEC and EHEC. The standardized multiplex PCR is open for further use for the diagnosis of *E. coli* O157:H7 during emergencies of biological war and investigation of suspected food borne and water borne outbreaks.

5.2 Cultural Isolation and biochemical characterization of *E. coli* O157:H7 from faecal samples.

In this study, *E. coli* O157:H7 from faecal samples was isolated by enriching in Modified Tryptone Soy Broth (mTSB). Tryptone soya broth as enrichment medium specifically for *E. coli* O157:H7 has been recommended by ISO committee under specification, ISO/DIS16654:1999 (ISO, 2015). There are many references that advocate use of Tryptone Soy Broth supplemented with novobiocin as enrichment medium for *E. coli* O157:H7 (Bindu and Krishnaiah 2010; Ahmad *et. al.,* 2013; Puttalingamma and Harshvardhan, 2013).

E. coli O157:H7 rapidly ferments lactose and is indistinguishable from most other *E. coli* on traditional lactose containing media. However, *E. coli* O157:H7 cannot ferment sorbitol within 24 h, while 90% of *E.coli* can. This character was used as a criterion for differentiating it from other *E. coli* (March and Ratnam, 1986; Sandra and Samuel, 1986; Faten and Afaf, 2013**;** Adamu *et. al.,* 2014; Ahmad *et. al.,* 2013; Sinisa and Darren, 2006**;** Lee and Choi, 2006). Centers for Disease Control and Prevention also recommends Sorbitol-MacConkey as selective indicator media for *E. coli* O157:H7 (CDC, 2009). In addition, Orth *et. al.* (2007) showed the resistance of *E.coli* O157:H7 for tellurite and therefore grows in concentration often that inhibit most other *E. coli.* Therefore, Cefixime Tellurite-Sorbitol MacConkey (CT-SMAC) was used as selective and differential medium in this research work. Only the typical sorbitol negative colonies were considered as positive for *E. coli* O157:H7.

Many other researchers both from India and abroad have used Cefixime Tellurite-Sorbitol MacConkey (CT-SMAC) agar as a selective medium for the isolation of *E. coli* O157:H7. Divya *et. al.* (2013) identified *Escherichia coli O157* in human faeces, small intestine of chicken, ground beef, cattle faeces and raw milk by using routine microbiological cultural tests, wherein they employed CT-SMAC agar. Similarly, Bindu and Krishnaiah (2010) tested 250 samples collected from various sources for the presence of *E. coli* O157:H7 by employing cultural isolation on CT-SMAC agar and reported that only 11 samples were positive by cultural methods. Aseel *et. al.* (2013) used CT-SMAC agar for isolation of *E. coli* O157:H7 strain from faecal samples of zoo animal with satisfactory results. There are researchers who have used CHROMagar O157 with 5 mg/liter of novobiocin and 2.5 mg/liter of potassium tellurite as a selective and differential medium to detect *E. coli* O157:H7 (Arthur *et. al.*, 2013).

The isolated sorbitol negative colonies were further subjected for Gram's staining, inoculation on eosin methylene blue (EMB) agar and biochemical tests. The isolates were presumed to be positive for *E. coli* O157:H7 showed Gram negative character, metallic sheen character on EMB agar and positive reaction for indole, methyl red, fermentation of sucrose, dextrose and lactose (triple sugar iron agar) with gas production and nitrate tests and negative reaction for vogues proskauer and citrate utilization tests (Quinn *et. al.,* 2004).

As such there is no difference in the biochemical characters between *E. coli* O157:H7 and other serotypes of *E. coli* except sorbitol fermentation, i.e., *E. coli* O157:H7 being sorbitol negative and other serotypes being sorbitol positive (Lee and Choi, 2006; Bettelheim, 2007; Adamu *et. al.,* 2014). Nevertheless, the biochemical tests would help in eliminating sorbitol negative isolates on Sorbitol-MacConkey agar other than *E. coli* (Faten and Afaf, 2013; Naik and Desai, 2012). Ahmad *et. al.,* (2013) used biochemical tests for further confirmation of sorbitol negative isolates on Sorbitol-MacConkey agar as *E. coli* O157:H7. Many other researchers have used biochemical tests as additional criteria for

confirmation of suspected colonies on Sorbitol-MacConkey agar as *E. coli* O157:H7 (John *et. al.*, 2001; Osek, 2002; Bindu and Krishnaiah, 2010).

Based on the cultural isolation method, the percent of animals that showed shedding of *E. coli* O157:H7 was 24.79%. Conventional culture methods are very useful for the identification of *E. coli* O157H7. However, these methods are time consuming (Arthur *et. al.*, 2005) and there is a possibility to get false positive results (Orth *et. al.*, 2009) as supported by our findings of identification of *E. coli* O157H7 by using latex agglutination test and the standardized multiplex PCR. Based on the identification by cultural isolation with biochemical characters, 16.52% of the bovines were shedders of the organism *E. coli* O157:H7. However, biochemical reactions cannot differentiate between *E. coli* O157:H7 and other serotypes of *E. coli* (Bettelheim, 2007). Hence, here also there are chances of getting false positive samples as indicated by estimate of its epidemiological specificity as discussed further.

5.3 Identification and molecular characterization of *E. coli* O157:H7 in faecal samples by multiplex PCR

The faecal samples were directly as well as the faecal samples with one step selective enrichment were subjected for standardized multiplex PCR for the identification of *E. coli* O157:H7 and molecular characterization based on detection of six virulent genes.

When faecal samples were directly subjected for multiplex PCR, the percent of animals that showed shedding of *E. coli* O157:H7 was 9.33% (87 out of 932 samples). And when faecal samples with one step selective enrichment were subjected for multiplex PCR, the percent of animals that showed shedding of *E. coli* O157:H7 was 10.94% (102 out of 932 samples).

To countercheck and confirm the identification of *E. coli* O157:H7, the isolates that were found to be positive for *E. coli* O157:H7 by cultural identification and cultural

isolation with biochemical characterization were subjected for multiplex PCR. Out of 231 samples identified as positive for *E. coli* O157:H7 by cultural identification, 102 samples were confirmed by multiplex PCR. And out of 154 samples identified as positive for *E. coli* O157:H7 by cultural identification with biochemical characterization, 102 samples were confirmed by multiplex PCR.

Based on the results of multiplex PCR, it could be noted that only 44.16% and 66.33% of the samples identified as positive for *E. coli* O157:H7 by cultural characters and by cultural isolation with biochemical identification, respectively were true positive samples and the remaining 55.84% and 37.23%, respectively, were false positive samples.

The findings of this research work indicate that not all sorbitol negative isolates from faecal sample on CT-SMAC agar are *E. coli* O157:H7. This is supported by the report of Manna *et. al.* (2009) where in among 139 sorbitol-negative isolates of 38 *E. coli* serogroups from food and cattle faeces, only 14.6% were O157**.** Strockbine *et. al.* (1998) mentioned that *Escherichia hermanii* is biochemically similar to *E. coli* O157:H7 and can produce sorbitol negative colonies on CT-SMAC.

Simultaneously, a review by Treor (2008) states that most, but not all, 0157 VTEC strains do not ferment sorbitol. However, in this research work, no sample that produced sorbitol positive colonies on CT-SMAC showed agglutination against O157 and H7 antibodies by latex agglutination test and yielded the amplicons of the targeted 6 virulent genes by multiplex PCR.

Among the 87 samples positive for *E. coli* O157:H7, when faecal samples were directly subjected for multiplex PCR, 74 samples showed presence of all the 6 virulent genes and 13 samples showed presence of only 5 virulent genes, *viz; fliCh7, rfbE, eaeA, hly* and *stx1* but lacking *stx2* gene. Among the 102 samples positive for *E. coli* O157:H7, when faecal samples with one step selective enrichment were subjected for multiplex PCR, 89

samples showed presence of all the 6 virulent genes and 13 samples showed presence of only 5 virulent genes, *viz; fliCh7, rfbE, eaeA, hly* and *stx1* but absence of *stx2* gene. Out of these 89 faecal samples, 73 samples were the same samples that showed presence of all the 6 virulent genes of *E. coli* O157:H7 when faecal samples were directly subjected for multiplex PCR (Witold and Carolyn, 2011)..

That means, in addition to 73 samples 16 more samples were found to be positive for *E. coli* O157:H7 when faecal samples with one step selective enrichment were subjected for multiplex PCR. Further, the 13 samples that showed presence of 5 virulent genes, *viz; fliCh7, rfbE, eaeA, hly* and *stx1* but absence of *stx2* gene were the same 13 samples that showed presence of 5 virulent genes, *viz; fliCh7, rfbE, eaeA, hly* and *stx1* but absence of *stx2* gene when faecal samples were directly subjected for multiplex PCR. These results indicate that the sensitivity of the multiplex PCR is more when the samples are analyzed after one step selective enrichment when compared to when the samples are directly analyzed.

Of the 102 positive samples all were characterized as EHEC O157:H7 as they showed presence of eaeA, *hly* and anyone of the genes of shiga toxins in addition to *rfbE and* fliCh7 genes (Paton and Paton, 2002; Wang *et. al.*, 2002; Pennington, 2010; Witold and Carolyn, 2011). These samples were further characterized by observing for the presence of other four virulent gens (*eaeA, hly, stx1* and *stx2*). 89 samples showed presence of all the 6 virulent genes and 13 samples showed presence of only 5 virulent genes, *viz; fliCh7, rfbE, eaeA, hly* and *stx1* but absence of *stx2* gene. That means, 89 samples positive for *E. coli* O157:H7 would be characterized as EHEC O157:H7 producing both shiga toxins; *stx1*, and *stx2* and the 13 samples would be characterized as EHEC O157:H7 producing only *stx1* and not producing *stx2.*

E. coli O157:H7 that has genes to produce *Stx2* are more virulent and cytotoxic (Mead and Griffin, 1998; Baker *et. al.*, 2007; Ritchie *et. al.*, 2003; Siegler *et. al.*, 2003). The

possession and expression of the *Stx2* gene correlate strongly with the causation of bloody diarrhoea and haemolytic uraemic syndrome (Persson *et. al.*, 2007). It could be stated that, 87.25% (89 out of 102 samples) of the samples positive for *E. coli* O157:H7 in this study were highly virulent.

5.4 Confirmative identification of *E. coli* O157:H7 by Latex Agglutination Test

In this study, latex agglutination test (Wellcolex® *E.coli* O157:H7) was used for the confirmative identification of *E. coli* O157:H7 by detecting presence of the O157 and the H7 antigens. Based on the results of latex agglutination test, only 9.76% of the bovines were showing shedding of *E. coli* O157:H7. It could be noted that, when compared to the results of latex agglutination test, 60.61% and 40.91% of the samples identified as positive for *E. coli* O157:H7 by cultural characters and by cultural isolation with biochemical identification, respectively, were false positive samples.

On the contrary, only 10.88% of the samples were identified as positive for *E. coli* O157:H7 when faecal samples with one step selective enrichment were analyzed by multiplex PCR in excess to that of latex agglutination. And when the faecal samples were analyzed directly by multiplex PCR, no sample was identified as positive for *E. coli* O157:H7 in excess to that of latex agglutination test. These findings highlight the higher sensitivity and specificity of multiplex PCR when compared to cultural identification and cultural isolation with biochemical characterization.

The samples/isolates positive for O157 and the H7 antigens showed agglutination reaction with latex coated with respective monoclonal antibodies in latex agglutination test. Since monoclonal antibodies react very specifically with their respective antigens latex agglutination test was used for the confirmation of the isolates as *E. coli* O157:H7. USDA FSIS recommends use of latex agglutination test for the confirmation of *E. coli* O157:H7 from meat products and environmental samples (USDA FSIS, 2015). Many authors have

used latex agglutination test for the final confirmation of *E. coli* O157:H7 isolates (Aseel *et. al.*, 2013; Divya *et. al.*, 2013; Taye, *et. al.*, 2013; Mohmmed *et. al.*, 2012; Mohamed *et. al.*, 2013). Faten and Afaf (2013) confirmed prevalence of *E.coli* O157:H7 in intestinal and urinary tract infection in children by latex agglutination test using Wellcolex *E.coli* O157:H7 (Remel) latex agglutination kit which was also used in the present study.

Other diagnostic tests based on immunological principles have been used for the detection of *E. coli* O157:H7 by various research scholars. Chuanmin Ruan *et. al.* (2002) developed ELISA based Immunobiosensor chips for detection of *E. coli* O157:H7 and Carvalho *et. al.* (2014) determined the occurrence of *E. coli* O157:H7 by using ELISA. Peixuan *et. al.* (2005) developed waveguide biosensor for the detection of water-borne *E. coli* O157, based on a fluorescent sandwich immunoassay and Tao Geng *et. al.* (2006) developed an antibody-based fiber-optic biosensor to rapidly detect low levels of *E. coli* O157:H7 cells in ground beef. However, the use of latex agglutination test by numerous authors as cited above and the results obtained in the present study suggests the reliability of this test.

5.5 Epidemiological sensitivity and specificity of cultural identification, cultural and biochemical identification and multiplex PCR identification of *E. coli* O157:H7

In this study, the latex agglutination test, as it is very sensitive and specific for the two specific antigens of *E. coli* O157:H7 was taken as gold standard test to compare and estimate epidemiological sensitivity and specificity of cultural isolation method, cultural isolation with biochemical identification and multiplex PCR. Centres for Disease Control and Prevention, USA recommends latex agglutination test as the confirmative test for *E. coli* O157:H7 (CDC, 2015). Also many research scholars have reported use of latex agglutination test as a confirmative test for *E. coli* O157:H7 (Divya *et. al.*, 2013; Taye, *et.*

al., 2013; Mohmmed *et. al.*, 2012; Mohamed *et. al.,* 2013) in various samples with optimum results.

In the present study, when the results of cultural isolation was compared with latex identification test, though the sensitivity was 100% the specificity was very low (83.35%) indicating that the probability of identifying false positive samples was very much high. When the results of cultural isolation with biochemical characterization was compared with latex identification test, again sensitivity was 100% and specificity was higher than cultural identification (92.51%) and hence avoids identification of many of false positive samples.

When the results of multiplex PCR (directly on faecal samples) was compared with latex identification test, the sensitivity was found to be low (95.60%) but the specificity was highest (100%). Simultaneously, when the results of multiplex PCR (on faecal samples with one step selective enrichment) was compared with latex identification test, the sensitivity was found to be 100% and specificity was also equally high (98.69%). These findings show that, one step selective enrichment before multiplex PCR enhanced its sensitivity though there was slight reduction in its specificity compared to when the faecal samples were directly analyzed. Nevertheless, the specificity was almost closer to 100%. It could be stated that the standardized multiplex PCR was sensitive and specific and was far better than cultural identification method and cultural isolation with biochemical characterization method especially with one step selective enrichment.

In this epidemiological study, in nutshell, out of 932 bovine faecal samples, the number of samples identified *E. coli* O157:H7 by cultural isolation, cultural isolation with biochemical identification, latex agglutination test and by multiplex PCR were 231, 154, 91 and 102 (faecal samples with one step selective enrichment), respectively. It is noteworthy that all the 91 samples identified as positive for *E. coli* O157:H7 by latex agglutination test were identified by multiplex PCR. But 13 more samples which were found to be positive for *E. coli* O157:H7 by multiplex PCR were identified as negative by latex agglutination test.

These seven samples were further confirmed as *E. coli* O157:H7 by re-inoculating the isolate on CT-SMAC agar, eosin methylene blue (EMB) agar, by biochemical characterization and re-subjecting the isolates for multiplex PCR.

The samples produced sorbitol negative colonies on CT-SMAC agar, colonies showed metallic sheen on EMB agar, positive reaction for indole and methyle red tests, negative reaction for vogues proskauer and citrate utilization tests and showed presence of 6 virulent genes in the amplicons by multiplex PCR. These results indicate that the sensitivity of the standardized multiplex PCR (with one step selective enrichment) was higher than latex agglutination test and it could be stated that latex agglutination identified 13 samples false negative samples for *E. coli* O157:H7.

The rationale could be that, latex agglutination test identifies the *E. coli* O157:H7 based on reaction between the latex coated monoclonal antibodies (antibodies against O157 and H7 antigens) with the respective antigens expressed on the surface of the bacteria at the time of testing. These seven isolates might have the genes of these two antigens but have not been expressed. At a given point of time, not all the genes would be expressed by any living organism. This rationale gives the explanation for the high sensitivity of PCR in general and multiplex PCR in particular compared to other diagnostic techniques.

It could be concluded that the multiplex PCR used in this study is highly sensitive and specific for the identification of *E. coli* O157:H7 and hence reliable. Therefore, in the present research work, the results of multiplex PCR on faecal samples with one step selective enrichment was used for the final consideration of sample as positive or negative for *E. coli* O157:H7 and further epidemiological analysis.

5.6 Epidemiology of shedding pattern of *E. coli* O157:H7 in bovines

5.6.1 Prevalence of shedding of *E. coli* O157:H7 in bovines

In this study, out of the total faecal samples tested the prevalence of shedding of *E. coli* O157:H7 in bovines was found to be 10.94% (102 out of 932 samples). As the literature

is perused, it could be said that there is no uniformity in the findings on prevalence of shedding of this pathogen. The prevalence varies both spatially and temporally.

For dairy cattle, the prevalence of *E. coli* O157:H7 estimated by testing faeces ranged from 0.2% to 48.8% in the USA, Canada, Italy, Japan, and the UK (Hussain and Bolinger, 2005). Sinisa and Darren (2006) reported that, based on longitudinal and point prevalence studies, combined prevalence of shedding of *E. coli* O157 in bovines was 15.6% and it ranged from 2.5 to 57.5% in different farms. As per meta-analysis and meta-regression analysis done by Islam *et. al.* (2014) the prevalence estimate of *E. coli* O157 in cattle at the global level was 5.68%. The pooled prevalence estimates in Africa, Northern America, Oceania, Europe, Asia and Latin America-Caribbean were 31.20%, 7.35%, 6.85%, 5.15%, 4.69% and 1.65%, respectively.

Not much work on prevalence of *E. coli* O157:H7 in bovines has been done in India. In a review by Wani *et. al*. (2003), it was stated that STEC O157 was isolated from both bovine and ovine faecal samples. In an investigation, out of 27 *E. coli* isolates from 112 beef samples, only one STEC O157 was detected (Hazarika *et. al.,* 2007). Steve *et. al.* (2007) reported isolation of *Escherichia coli* serotype O157:H7 in water samples collected from the Ganges river. In a research study on the occurrence of *E. coli* O157 in cattle stool in West Bengal, India, *E. coli* serotype O157 was isolated from faecal samples from two (2.04%) slaughtered cattle and six (7.59%) diarrhoeic calves (Manna *et. al.*, 2006).

5.6.2 Shedding pattern of *E. coli* O157:H7 in cattle and buffaloes

In this study, based on the multiplex PCR identification of *E. coli* O157:H7, the percentage of cattle showing shedding *E. coli* O157:H7 in faeces was higher (12.10%) than buffaloes (only 5.55%). These findings indicated that, though bovines are considered as reservoir for *E. coli* O157:H7 (Kaper *et. al.,* 2004; Mainil and Daube, 2005; Capriola *et. al.,* 2005), cattle are more prone for shedding of this zoonotic pathogen than buffaloes. When the data was subjected for chi-square goodness-of-fit test of significance, with a *P* value of

<0.05, the proportion of cattle shedding *E. coli* O157:H7 in the faeces was significantly more compared to buffaloes.

There are lot many reports on incidence of *E. coli* O157:H7 across the world (Borczyk *et. al.,* 1987; Chapman *et. al.,* 1993; Jackson *et. al.,* 1998; Chapman *et. al.,* 2001; Gannon *et. al.,* 2002; Susan Sanchez *et. al.,* 2002; Synge *et. al.,* 2003; Ogden *et. al.,* 2004; Al-Saigh *et. al.,* 2004; Mainil and Daube 2005; Hussein, 2007; Elaine and James, 2010; Eppinger *et. al.,* 2011; Soderlund *et. al.,* 2012). Studies have shown that up to 30% of all cattle are asymptomatic carriers of *E. coli* O157:H7 (Stanford *et. al.,* 2005; Callaway *et. al.,* 2006). As per the findings of Dunn *et. al.* (2004) and Cho *et. al.* (2006), at any given time, 1-50% of healthy cattle carry and shed *E. coli* O157:H7 in their faeces. Elder *et. al.* (2000) reported that this organism can be found in 80% of populations in few herds of cattle and 49% of beef carcasses.

However, research studies investigating the frequency of *E. coli* O157:H7 serotype in buffaloes is limited. In a study, *E. coli* O157:H7 was isolated from only 3.7 % faecal samples and 1.4 % raw milk samples of Anatolian water buffaloes (*Bubalus bubalus*) in Turkey (Sekera and Yardimci, 2008).

The differences in host response and excretion dynamics could be the reason for such a vast gap in shedding of this pathogen between cattle and buffaloes. There could be differences in the innate immune response among the two species that negates bacterial replication on the epithelium and either reduces attachment to or increases detachment from the epithelium of the terminal rectum (Michael *et. al.,* 2011).

5.6.3 Breed wise variation in the shedding of *E. coli* O157:H7 in cattle

In the results obtained in the present study indicate that the native breeds (only 4.41%) of India are less prone for shedding of the pathogen *E. coli* O157:H7 in faeces when compared to cross bred cattle (13.885) and the exotic breeds (18.56%) of cattle. Chi-square

goodness-of-fit test of significance shows that, with a *P* value of <0.05, there was high significant difference in shedding of *E. coli* O157:H7 in the faeces by indigenous breeds, crossbreds and exotic breeds. Significant difference was observed in shedding of *E. coli* O157:H7 in the faeces by indigenous breeds, crossbreds and exotic breeds even at *P* value of <0.001.

There are research findings that support the findings in this research work that breed may act as predisposing factor for higher shedding of *E. coli* O157:H7. In a research, Jeon *et. al.* (2013) found that the cattle with 19-0% of Angus and 81-100% of Brahman breed genetic makeup excreted the lowest number of *E. coli* O157 when compared with other crosses in an Angus-Brahman multibreed herd, indicating that Brahman breed cattle showed lowest prevalence of shedding of *E. coli* O157. The researchers concluded that breed variation in the prevalence of *E. coli* O157 in cattle as critical to reduce outbreaks of this pathogen in humans.

Similarly, in a study by Riley *et. al.* (2003), lesser prevalence of *E.coli* O157:H7 was observed in Brahman (0.034 proportion), Romosinuano (0.023 proportion) breeds than in Angus breed (0.058) representing the evidence of breed-to-breed genetic variation in *E. coli* O157 shedding. In a meta-analysis and meta-regression analysis on regional variation in the prevalence of *E. coli* O157 in cattle, Islam *et. al.* (2014) concluded that the important factor that might have influenced higher prevalence of this pathogen in the African and Northern American regions and lower in Asia and Latin America-Caribbean was type of cattle in these regions of the world. This indicates that the difference in the genetic makeup of the animals in different regions have an effect on the variation in shedding of this pathogen.

Genetically, indigenous breeds are known for their adaptability and disease resistance pattern. The same genetic factors could be the reason for less percent of indigenous breeds showing shedding of *E. coli* O157:H7 in faeces when compared to

crossbreds and exotic breeds. The results of this study provide a proof of the superiority of genetic makeup of indigenous breeds.

5.6.4 Age wise variation in the shedding pattern of *E. coli* O157:H7 in bovines

In the present study, age wise variation in shedding pattern of *E. coli* O157:H7 was analysed between calves, heifers and adult bovines. The percent of calves showing shedding of *E. coli* O157:H7 in faeces was higher (14.80%) than heifers (12.12%) and adult bovines (8.60%). The results indicate that age influences the shedding of *E. coli* O157:H7 in faeces and the shedding was more in calves and heifers than in adult bovines. Chi-square goodness-of-fit test of significance indicates that the proportion of calves shedding *E. coli* O157:H7 in the faeces was significantly very high (*P* value of <0.001) compared to heifers and adult cattle. When the test of significance was applied between adult animals and heifers, proportion of heifers shedding *E. coli* O157:H7 in faeces was significantly high compared to adult cattle at *P* value of <0.05.

Generally, calves have a higher prevalence of VTEC O157:H7 colonisation and excrete the agent at greater concentrations than adult cattle (Hancock *et. al.,* 1997) and the shedding declines with increasing age (Shaw *et. al.,* 2004). The results in the present study were similar to other reports (Zhao *et. al.,* 1995; Hancock *et. al.,* 1997; Shere *et. al.,* 1998) in which immature animals were more at risk for shedding *E. coli* O157:H7 than mature animals. In one of the research works, the likelihood of positive isolates was 2.6 times higher in calves and heifers compared with mature cows (Stanford *et. al.,* 2005). The prevalence of EHEC O157:H7 in cattle was higher in postweaned calves and heifers than in younger and older animals (Witold and Carolyn, 2011). Manna *et. al.* (2006) also reported higher prevalence of *E. coli* O157 in calves (7.59%) than in adult cattle (2.04%).

Young weaned calves were found to shed *E. coli* O157:H7 at higher levels and longer period than adult cattle in experimental studies, which could be due to dietary stress during weaning and because they do not yet have a fully formed rumen to help suppress *E.*

coli O157:H7 growth **(**Cray and Moon, 1995; Hussain and Bolinger, 2005)**.** The findings in the present study and the above references support the generalisation of presence of an age-dependent variation in prevalence of *E. coli* O157:H7 (Riley *et. al.,* 2003).

5.6.5 Sex wise variation in the shedding pattern of *E. coli* O157:H7 in bovines

In the present study, the percent of male bovines that showed shedding of *E. coli* O157:H7 was comparatively higher (15.17%) than that of female animals (9.95%). This indicates that male bovines are more prone for shedding of *E. coli* O157:H7 than female bovines. The chi-square goodness-of-fit test of significance indicates statistical significant difference between male and female animals in shedding of *E. coli* O157:H7 in the faeces, with a *P* value of <0.05. The results of the present research work is well supported by one of the studies, where in the number of super-shedders was significantly higher (P = 0.022) in bulls (27.27%) (Jeon *et. al.*, 2013). Hormonal differences in male and female animals could be the reason for higher shedding of this pathogen in male bovines compared to females (Schultz *et. al.*, 2005; Schroeder and Sasha, 2005).

5.6.6 Seasonal variation in the shedding pattern of *E. coli* O157:H7 in bovines

Among those factors thought to affect the prevalence of *E.* coli O157:H7, only season has been repeatedly and most consistently demonstrated to influence the shedding of this pathogen by cattle (Chapman *et. al.*, 1997; Shere *et. al.*, 1998; Conedera *et. al.,* 2001; Barkocy-Gallagher *et. al.*, 2003; Hussain and Bolinger 2005; Edrington *et. al.*, 2006; Milnes *et. al.*, 2009).

In the present study, seasonal variation in shedding pattern of *E. coli* O157:H7 was analysed among three seasons, *viz;* summer, monsoon and winter (Surrender Singh, 2014). The percent of bovines shedding *E. coli* O157:H7 in faeces in winter season was higher (14.75%) when compared to monsoon season (10.85%) and summer season (7.86%). The chi-square goodness-of-fit test of significance, with a *P* value of <0.05, indicates that there

was significant difference in the shedding pattern of *E. coli* O157:H7 in the faeces among summer, monsoon and winter seasons and hence influencing effect of season on the shedding of *E. coli* O157:H7 in faeces.

When the test statistic was applied to compare between summer and monsoon seasons, there was no significant difference in shedding of *E. coli* O157:H7 in the faeces at *P* value of <0.05, however there was significant difference at *P* value of <0.1 with higher shedding pattern in monsoon than in summer season. When the test statistic was applied to compare between monsoon and winter seasons, there was significant difference in shedding of *E. coli* O157:H7 in the faeces at *P* value of <0.05 with higher shedding pattern in winter than in monsoon season. And when test statistic was applied to compare between summer and winter seasons, there was highly significant difference in shedding of *E. coli* O157:H7 in the faeces at *P* value of <0.05 and even at *P* value of <0.001.

In the present study, to further elaborate seasonality of shedding of *E. coli* O157:H7 in bovines, monthly trend of shedding of this pathogen was analysed. It was observed that the percentage of animals shedding this pathogen showed decreasing trend from January to May and then increasing trend from June to December with maximum percentage of faecal samples positive for *E. coli* O157:H7 found in the month of January and least in the month of May.

The results obtained in this study are in contrast to the findings of many other researchers (Hancock *et. al.,* 2001; Edrington *et. al.,* 2004b; Hussein *et. al.,* 2005; Edrington *et. al.,* 2006; Gautam *et. al.,* 2011). All these authors interpreted that the prevalence of shedding of this pathogen typically increases during the summer months and is lowest in the winter. Chapman *et. al.* (1997) observed highest prevalence of *E. coli* O157:H7 in summer months. Similarly, Elder *et. al.* (2000) isolated *E. coli* O157 from 28% of faeces samples collected from fed beef cattle at slaughter during summer months.

Barkocy-Gallagher *et. al.* (2003) observed a peak *E. coli* O157:H7 faecal prevalence rate of 12.9% in the summer months, in comparison with 6.8%, 0.3%, and 3.9% in the fall, winter, and spring months, respectively. Edrington *et. al.* (2004b) studied variation in the faecal shedding of *Salmonella* and *E. coli* O157:H7 in lactating dairy cattle and found that *E. coli* O157:H7 was isolated only in the summer sampling times with no positive samples found on any farm in January. Fernandez *et. al.* (2009) studied the seasonal variation of Shiga toxin-producing *E. coli* (STEC) O157 in cattle in Argentina using multiplex PCR and reported that stx1 + stx2 positive *E. coli* O157 shedding occurs more in warm season. Variation in the occurrence of *E. coli* O157:H7 was observed even in zoo animals, with animals being culture positive only in the summer months, but not in the spring, autumn, or winter (Aseel *et. al.*, 2013).

However, there are certain studies that have not observed the similar seasonal pattern of shedding of this pathogen (Sargeant *et. al.*, 2000; Alam and Zurek, 2006). These studies have shown that cooler temperatures can enhance the persistence of *E. coli*, including *E. coli* O157:H7, in manures and soils (Topp *et. al.*, 2003; Ishii *et. al.*, 2006). Synge *et. al.* (2003) found that cattle accounted for the higher *E. coli* O157 prevalence during the winter months compared to summer months. The prevalence in Scottish beef cattle at slaughter was found to be highest during the winter as per the research findings of Ogden *et.al.* (2004). In determining the effect of seasonal factors on the shedding patterns in calves of food borne pathogenic *E. coli* O157:H7, Bayko (2011) found that the number of CFU/g being higher in the cold rooms when compared to the warm rooms.

The probable reasons for these contrasting findings could be the climatic and weather parameters which vary as per the geographical location. Factors suggested as potentially effecting seasonality of prevalence include ambient temperature, rainfall, and other seasonally driven factors (Rasmussen and Casey, 2001); however, temperature is generally implied as the most plausible explanation. As a result of more favourable growth

temperatures, the higher prevalence of *E. coli* O157:H7 in cattle during warmer seasons could be observed (Hancock *et. al.,* 2001; Gunn *et. al.,* 2007; Vidovic *et. al.,* 2007).

Edrington *et. al.* (2006) did correlation and regression analysis and revealed that there was strong positive correlation existed between day length (r =0.67; R^2 = 0.45; *P* = 0.0009) and ambient temperature (r = 0.43; R^2 = 0.19; *P* = 0.05) and *E. coli* O157:H7 prevalence. The authors have concluded that increase in the ambient temperature has been considered as a potential cause of the increased prevalence of the shedding of *E. coli* O157:H7 during the warmer seasons.

Most of the studies that interpreted that summer season favoured higher prevalence of shedding of *E. coli* O157:H7 would have been undertaken in temperate regions. And those studies that interpreted that winter season favours higher prevalence of shedding of *E. coli* O157:H7 would have been undertaken in tropical and subtropical regions. Therefore, in addition to stating that season as an influencing factor on the shedding pattern of *E. coli* O157:H7, it would be apt to state that weather parameters as influencing factors on the shedding pattern of *E. coli* O157:H7, especially environmental temperature.

Therefore, in this study the environmental temperature of the animal farms at the time of collection of the faecal samples was recorded to find out the effect of temperature on shedding pattern of *E. coli* O157:H7. The influence of environmental temperature is discussed in the next paragraphs.

5.6.7 Effect of environmental temperature on shedding of *E. coli* O157:H7 in bovines

In the present study, among the 6 groups of range of environmental temperature, highest percentage of bovines shedding *E. coli* O157:H7 in faeces was found in the temperature range of 20.1-25.0°C, followed by the temperature range of 25.1-30.0°C, 15.1-20.0°C, 10.1-15.0°C, 30.1-35.0°C and 35.1-40.0°C in the sequence of decreasing prevalence. This indicates that the environmental temperature has effect on the shedding of

E. coli O157:H7 in faeces in bovines. The chi-square goodness-of-fit test of significance, indicates that there was significant difference in the shedding pattern between these temperature ranges at *P* value of <0.05 and even *P* value of <0.001.

Further, the temperature trend analysis of shedding pattern of *E. coli* O157:H7 was done by decreasing the width of the class interval. The trend has been depicted with line diagram. It was found that the highest percentage of faecal samples positive for *E. coli* O157:H7 were in the samples that were collected at the environmental temperature of 22.1-23.0°C and least at 39.1-40.0°C.

The findings of shedding of *E. coli* O157:H7 depending on the environmental temperature that was observed in the present study rejects the hypothesis of Edrington *et. al.* (2006), in which states that temperature does not account and rather only day length accounts for the seasonality of *E. coli* O157:H7 shedding and makes to accept the interpretation that the environmental temperature accounts significantly for the seasonality of shedding of *E. coli* O157:H7. In fact the regression analysis on *E. coli* O157:H7 prevalence as a function of temperature done by the above cited authors predicts that maximum prevalence would be observed at the environmental temperature range of 25-30°C.

The temperature dependent variation of shedding of *E. coli* O157:H7 found in the present study is well supported by the findings in a study on microbial flora of sheep by Indira *et. al.* (1996) wherein the incidence of *E. coli* O157:H7 was transient and ranged from 31% of sheep in June to none in November. The maximum temperature recorded were 26°C and 2.2°C in June and November months respectively during the study period indicating that the biologically favourable temperature of 26°C supports shedding of *E. coli* O157:H7 in faeces. Similarly, in a study done by Ferna´ndez *et. al.* (2009) the percentages of *stx* producing *E. coli* in cows were 28% in winter 56% in summer. The range of environmental temperature was 0–12°C in winter, 20–38°C in summer again reflecting the favourable

range of environmental temperature. The findings of Eun *et. al.* (2015) also supports the findings of the present study, where in it was showed that 20% of the frozen meat products contained virulence genes, including verotoxin (VT) 1 and 2 of EHEC O157:H7 over 180 days of frozen storage and after 3 freeze thaw cycles, the population of EHEC O157:H7 did not change regardless of the type of products or initial inoculated concentration, indicating the strong survival ability of EHEC O157:H7.

It could be concluded that biologically favourable environmental temperature supports the shedding of this pathogen rather than extremes of the environmental temperature (summer and winter) (Hughes, 2013). The variation in the environmental temperature in different seasons differs between latitudes and longitudes of geographical areas (Surrender Singh, 2014). Hence contrasting results in the seasonality of shedding of *E. coli* O157:H7 obtained in the present research work and the research findings of different scientists. Further work is needed to confirm the temperature dependent shedding pattern of *E. coli* O157:H7 in cattle.

5.6.8 Effect of diarrhoea on shedding of E. *coli* O157:H7 in bovines

In the present study, the health status of the animals while collecting the faecal samples was also evaluated for its effect on the shedding of *E. coli* O157:H7. The percent of healthy bovines that showed shedding of *E. coli* O157:H7 was significantly very high (12.06%) (P value <0.001) when compared to diarrhoeic animals (2.68%). This indicates that healthy animals are more prone for shedding of this pathogen than diarrhoeic animals. Our findings are supported by the report of Mead and Griffin (1998) who stated that healthy cattle are the major reservoir for *E. coli* O157:H7. In contrast, Heuvelink *et. al. (*1998) concluded that *E.coli* O157:H7 were isolated more frequently from diarrheic calves than from normal calves**.**

The rationale behind low shedding in diarrhoeic animals could be competitive exclusion (La Ragione *et. al.*, 2009). Colonization of the intestines with diarrhoea causing

organisms would competitively exclude *E. coli* O157:H7 and hence no shedding over a period of time. Field studies have shown *E. coli* group O26 to be prevalent in ruminant animals when the prevalence of *E. coli O157:H7* in the same study animals was very low (Aktan *et. al.*, 2004; Fukushima & Seki, 2004). This hypothesis is further supported by Xu *et. al.* (2014) who concluded that non super shedding cattle exhibited less diverse dominant bacterial flora than the super-shedders.

5.6.9 Effect of lactation and pregnancy on shedding of *E. coli* O157:H7 in adult bovines

Based on the results obtained in the present study, the shedding pattern of *E. coli* O157:H7 was almost similar in lactating and pregnant (10.66%), lactating and non pregnant (8.54%), and dry pregnant (9.68%) animals. The chi-square goodness-of-fit test of significance among these three groups showed no significant difference in shedding of *E. coli* O157:H7 at *P* value of <0.05 and even at *P* value of <0.5. Similarly, chi-square goodness-of-fit test of significance between any of these three groups showed no significant difference in shedding of *E. coli* O157:H7 at *P* value of <0.05 and even at *P* value of <0.5. In contrast to these three groups, the shedding of *E. coli* O157:H7 was very meagre in non lactating and non pregnant animals (only 1.45%). The chi-square goodness-of-fit test of significance among the four groups showed significant difference in shedding of *E. coli* O157:H7 at *P* value of <0.05. The chi-square goodness-of-fit test of significance between any one of the first three groups and the group IV animals also showed significant difference in shedding of *E. coli* O157:H7 at *P* value of <0.05.

The results indicate that the physiological state of the adult animal definitely has effect on the shedding of *E. coli* O157:H7 in faeces. In this study, non lactating and non pregnant animals were very less prone for shedding of *E. coli* O157:H7 than pregnant and lactating animals. The rationale here could be the hormonal differences between these animals and the animals of other three groups (Schultz *et. al.,* 2005; Schroeder and Sasha, 2005). The findings of the present study also concludes that the meat of spent animals and

non productive animals is safer than productive animals with respect to this food borne pathogen.

Very few research studies have been done to know the effect of lactation and pregnancy on the shedding of *E. coli* O157:H7. In a study by Stanford *et. al.* (2005) on ecology of *E. coli* O157:H7 in commercial dairies in Southern Alberta, dry cows (11.1%) showed higher shedding than lactating cows (10.7%) but not a significant differnece. Similarly, in a research work by Riley *et. al.* (2003), non lactating dairy cows (0.033 proportion) were shown to have a slightly higher prevalence of *E. coli* O157:H7, but not significant, than lactating cows (0.024 proportion). Similarly, Wilson *et. al.* (1993) found little higher shedding of *E. coli* O157:H7 in dry cows compared to lactating cows. No reference was available on shedding of this pathogen in non productive animals.

5.6 Antibiotic resistance of *E. coli* O157:H7 isolates

One of the objectives of this study was to determine to what extent *E. coli* O157:H7 isolates are resistant to antibiotics. A motivation to this objective was the fact that there has been increasing reports on the isolation and identification of antibiotic resistant *E. coli* O157 strains (Schroeder *et. al.,* 2002; Vali *et. al.,* 2004; Ahmed *et. al.,* 2005; Vidovic and Korber, 2006).

In the present study, when the *E. coli* O157:H7 isolates from the positive samples were subjected for antibiotic sensitivity for the routinely used antibiotics it was found that the isolates showed resistance for almost all the antibiotics except, Ciprofloxacin (2.94%) and Streptomycin (23.53%). Highest percent of isolates were resistant for Nitrofurantoin (100%) followed by Carbenicillin (92.16%), Co-Trimazine (87.25%), Tetracycline (75.49%), Kanamycin (69. 61%), Amikacin (46.08%), Streptomycin (23.53%) and Ciprofloxacin (only 2.94%). All most all (85.29% - sensitive and 11.76% - intermediate) the isolates of *E. coli* O157:H7 were susceptible for ciprofloxacin and about 76% (50% - sensitive and 26.47% - intermediate) of the isolates were susceptible for streptomycin.

A similar type of results was obtained by John *et. al.* (2001), where in *E. coli* O157:H7 isolates were found resistant for 86% antibiotics that could be used to treat *E. coli* infections in animals. Most isolates were susceptible to other antibiotics, such as trimethoprim-sulfamethoxazole and ceftiofur. All isolates were susceptible to ciprofloxacin, an antibiotic used widely in human medicine. As per the findings of Manna *et. al.* (2006), occurrence of resistance of *E. coli* O157:H7 was most frequent against nitrofurantoin (8 strains) followed by co-trimoxazole (4 strains), tetracycline (3 strains), ampicillin (3 strains), gentamicin (2 strains), norfloxacin (2 strains), cephalothin and ciprofloxacin (1 strain each) which was almost similar to the findings of the present study.

In a study in Surat (India) an attempt was made to isolate EHEC from diarrheagenic stool sample of patients and determination of their antimicrobial resistance pattern and found that 13.91 % isolates from 115 *E.coli* isolates were identified as EHEC O157:H7 and the highest resistance was detected against Ampicillin(100%) followed by Tetracycline(93.75%), Cefuroxime and Co-Trimoxazole (75%), Ceftriaxone, Chloramphenicol (68.75%), Ciprofloxacin (68.75%) and Gentamicin (25%) which again correlates with the findings of the present study (Naik and Desai, 2012).

In Japan, the most frequently reported resistance phenotype of *E.coli* 0157:H7 were found to resist streptomycin, sulfixoxazole and tetracycline (White *et. al.,* 2002) which is contrary to the results of the present study. The resistance pattern of this pathogen against tetracycline was similar to our findings in studies carried by various researchers (Kim *et. al.,* 2012; Manna *et. al.,* 2006; Raji *et. al.,* 2008; Naik and Desai, 2012). In antimicrobial susceptibility tests in South Korea, isolates of *E. coli* O157:H7 were mainly resistant to erythromycin (100%), ampicillin (27.2%) and tetracycline (18.2%) and not resistant to Ciprofloxacin (0.00%) and streptomycin (0.00%) (Ji-Yeon *et. al.,* 2006). In the present study majority of the isolates were resistant against Kanamycin. Contrastingly, in a study done by

Taye *et. al.* (2013) majority of *E. coli* O157:H7 were found to be susceptible to Chloramphenicol (30 µg), Kanamycin (30 µg), Spectinomycin (100 µg).

In the present research study, multiple antibiotic resistance (MAR), defined as resistance to three or more different classes of antibiotics, was observed in 69.61% of the *E. coli* O157:H7 isolates.

The susceptibility pattern of microbes, perhaps, varies from one region to another region of the world depending upon the usage of antibiotics in human and animal medicine. The occurrence of *E. coli* O157:H7 multiple antibiotic resistant profiles may show a risk for public health and food safety as well as animal health and production (Von Muffling *et. al.*, 2007). Resistant genetic elements from these isolates could also be transferred to other enteric bacteria of clinical importance and other zoonotic bacteria which may pose threat to public health.

5.7 Prevalence of *E. coli* O157:H7/H$^-$ (*E. coli* O157) and STEC in faecal samples of bovines

In the present study, in addition to the detection of *E. coli* O157:H7, the samples were also analysed for the detection of *E. coli* O157:H7/H$^-$ (*E. coli* O157) and STEC (Witold and Carolyn, 2011) based on the molecular characterization by multiplex PCR. Apart from 102 *E. coli* O157:H7 isolates, 23 isolates were characterized as *E. coli* O157:H$^-$ by multiplex PCR. It could be concluded that not all isolates which exhibited typical cultural and biochemical characters were found positive for *E. coli* O157:H7 by multiplex PCR. The isolates *E. coli* O157:H7 and *E. coli* O157:H$^-$ together were characterized as (Nataro and Kaper, 1998) *E. coli* O157. The percent of animals shedding *E. coli* O157 in the faeces was 13.41%.

In a study, *E. coli* O157 was detected in 52 of the 200 beef and rectal samples (26%) tested. Of the positive samples, 49 (24 in beef and 25 in rectal samples) were *E. coli* O157

and three (2 from beef and 1 from rectal samples) were *E. coli* O157:H7 (Gokhan and Belgin, 2010).

Further, few isolates which showed cultural and biochemical characters did not show presence of both *rfbE* and *fliCh7* genes by multiplex PCR. However, few of these isolates showed presence of any one of or both of the *stx1 and stx2* genes. These were considered positive for Shiga Toxin producing *E. coli* (STEC) other than *E. coli* O157:H7. The isolates *E. coli* O157:H7, *E. coli* O157:H^{-} and *E. coli* showing presence of any one of or both of the *stx1 and stx2* genes together were characterized as n considered as shiga toxin producing *E. coli* (STEC) (Nataro and Kaper, 1998; Wani *et. al.,* 2004). The percent of animals showing shedding of STEC was 16.52 %.

In a study in Riyadh, Saudi Arabia, out of 540 milk samples analyzed, STEC were recovered from 86 samples (15.93%). Serotyping of *E. coli* isolates revealed, 26 (4.81%) strains O157:H7 and the remaining 60 (11.12%) STEC other than O157:H7 [23 (4.26%) strains O111, 20 (3.70%) strains O113: H21, 10 (1.85%) strains O22: H8 and 7 (1.3%) strains O172: H21]. And out of 150 meat samples, STEC were recovered from 17 (11.33%) samples. Serotyping revealed, 6 (4%) strains O157: H7 and the remaining 11 (7.33%) STEC other than O157:H7 [5 (3.33%) strains O111 and 4 (2.67%) strains O174: H2 and only two (1.33%) strains were identified as O22: H8] (Onizan *et. al.,* 2015).

In India, there is paucity of information on STEC. It has not been identified as a significant etiologic agent of diarrhoea for humans in India. However, isolation of O157 and non- O157 serogroups of *E. coli* that exhibited the cytotoxic activity in vero cells has been reported from human patients with diarrhoea in India, but these strains have not been well characterized, and their origin is uncertain (Gupta *et. al.,* 1992). In astudy by Chattopadhyay *et. al.* (2001) a total of 876 samples (330 animal, 184 human, 362 food samples) were screened for the presence of STEC by conventional as well as PCR techniques. Seventeen STEC strains (12, 1 and 4 from animal, human and food samples respectively) were isolated.

Kumar *et. al.* (2001) investigated the occurrence of STEC in fresh fish, shellfish and meat sold in open markets in Mangalore, India. Two out of 60 fish samples and two out of the 48 clam samples were positive for *stx* and *hlyA* genes by PCR. STEC strains belonged to non- O157 serogroups. Wani *et. al.* (2003) reported isolation and characterization of STEC serogroups associated with diarrhoea in calves and lambs in India. STEC strains belonging to different serogroups were detected in 9.73% of calves and 6% of lambs studied. One of the most important serogroups, O157 known to cause certain life-threatening infections in humans, was isolated from both bovine and ovine faecal samples. Similarly, Wani *et. al.* (2004) reported O116 (STEC) isolation from five calves with diarrhoea. The virulence gene profile revealed presence of *stx1*, *eaeA* and *hlyA* genes. Similarly, Kumar *et. al.*(2004) characterized STEC strains isolated from seafood and beef in Mangalore by bead-ELISA, vero cell cytotoxicity assay, PCR and colony hybridization for the detection of *stx1* and *stx2* genes and found 4 strains from seafood, 6 from beef and 1 from a clinical case of bloody diarrhoea were found to carry Shiga toxins.

5.8 Antibiotic resistance of *E. coli* O157:H7/H$^-$ (*E. coli* O157) and STEC isolates

In the present study, the *E. coli* O157 and STEC isolates confirmed by mPCR were isolated and subjected for antibiotic sensitivity test using Muller Hinton agar for the routinely used antibiotics. The results found that, highest percent of *E. coli* O157 isolates that showed resistance was against Nitrofurantoin (100%) followed Carbenicillin (88.80%), Co-Trimazine (83.20%), Tetracycline (65.60%), Kanamycin (60.00%), Amikacin (46.4%), Streptomycin (20.8%) and Ciprofloxacin (only 2.4%).

In a report, all the *E. coli* O157 isolated from cattle faeces from the commercial and communal farms were resistant to erythromycin, tetracycline and sulphamethoxazole. A low percentage (25%) were resistant to chloramphenicol, kanamycin, ampicillin and streptomycin and none were resistant to either neomycin or norfloxacin which is a type of flouroquinolone similar to ciprofloxacin (Collins and Cornelius, 2008). Whereas the

prevalence rate of resistance to streptomycin among the *E. coli* O157 isolates was 2.3% in a study carried by Sinisa and Darren (2006) supporting to the results of the present study.

With respect to antibiotic sensitivity pattern of STEC, highest percent of isolates that showed resistance was against Nitrofurantoin (99.35%) followed Carbenicillin (88.31%), Co-Trimazine (73.38%), Tetracycline (69.48%), Kanamycin (52.60%), Amikacin (40.91%), Streptomycin (20.78%) and Ciprofloxacin (only 3.25%). The pattern is similar to that of *E. coli* O157 except the difference in the percentages.

Chattopadhyay *et. al.* (2001) studied the antibiotic sensitivity pattern of STEC strains from animal, human and food products and reported that STEC strains were uniformly sensitive to common antibiotics except tetracycline, cephalexin, dicloxacillin, erythromycin and lincomycin. The STEC isolates obtained in the present study were also resistant to tetracycline.

5.8 Conclusion

E. coli O157:H7 which is an emerging zoonotic pathogen where in bovines are considered as the primary reservoir. Though it will rarely cause mild form of the disease in bovines, it causes very sever disease in humans. It's mainly a food borne pathogen. Quicker identification of this pathogen is of import to identify the this etiological agent responsible for food borne outbreaks. The multiplex PCR technique standardized in this research work was very sensitive and specific for the identification of this pathogen.

The development of control strategies to reduce *E. coli* O157:H7 will require the identification of biological, environmental, and/or management factors that affect its incidence in cattle and their production environments. Research investigations and epidemiological studies have identified a number of risk factors or management practices that can or may contribute to the occurrence of this pathogen, and that may be exploitable to reduce its numbers, persistence, and transmission in cattle.

The results found in this research, give enough indication about these factors influencing the shedding of *E. coli* O157:H7 in the faeces of bovines. While all or none of these above factors may contribute to shedding of this pathogen, the association of these factors suggests strategies to reduce its shedding by applying the findings in to the principles of hazard analysis critical control points (HACCP) to strategise pre-harvest preventive and control measures so that the incidence of foodborne outbreaks of this foodborne pathogen in humans could be reduced to great extent.

CHAPTER 6
SUMMARY

The most significant Enterohaemmorhagic *E. coli* of humans is *Escherichia coli* O157:H7. Bovines are thought to be primary reservoir of this pathogen. Animal based foods contaminated with the faeces of bovines would cause foodborne infections with this pathogen. It causes Hemorrhagic Colitis (HC), post- diarrheal Hemolytic Uremic Syndrome (HUS) and Thrombotic Thrombocytopenic Purpura (TTP) in humans. A multiplex PCR technique was standardized for the identification of where in *E. coli* O157:H7 by targeting six virulence genes; *fliCh7, rfbE, eaeA, hlyA, stx1,* and *stx2 E. coli* O157:H7–US FDA strain was used as a reference cultur and to study the epidemiology of shedding pattern of *E. coli* 0157:H7 with respect to age, breed, sex and seasons, genetic and physiological factors of animals and environmental factors.

The extraction of the genomic DNA of *E. coli* 0157:H7 was done by modified boiled cell method. The parameters of multiplex PCR protocol standardized were magnesium chloride (MgCl2) concentrations (1.5mM, 2 mM, 2.5mM, 3mM and 3.5mM), time period of denaturation at 94˚C (20 sec and 45 sec), annealing temperature (58°C - 63°C) and agarose concentration (1% and 1.5%). The standardized multiplex PCR consisted of volume of 25 µl of reaction mixture containing 2 µl of *Taq* DNA polymerase (1 unit/ µl), 2.0 µl of DNA template solution, 2.5 µl of 10 x reaction buffer, 0.5 µM of dNTPs, 0.2 µM each of the 12 primers and 3 mM magnesium chloride ($MgCl_2$) and sterile nuclease free water to make the volume to 25 µl. The thermal cycling consisted of 5 minute initial denaturation at 94˚C followed by 35 cycles of denaturation at 94˚C for 45 sec, 1 min of annealing at 60˚C and extension for 1 min at 72˚C, with a 10 min final extension at 72˚C. The agarose gel electrophoresis was optimized at 1.5 % concentration of agarose. The multiplex PCR produced amplicons of 625 bp, 397bp, 296 bp, 166 bp, 210 bp and 484 bp sizes, respectively for the *fliCh7*, *eaeA, rfbE, hly, stx1,* and *stx2* virulent genes.

The threshold sensitivity of standardized multiplex PCR was found to be 0.1 CFU/ml for reference culture of *E. coli* O157:H7, 1 CFU/g for spiked faecal samples and 0.1CFU/g for spiked faecal samples with one step enrichment. None of the six DNA bands was found in the PCR product of negative control cultures; *E. coli* other than *E. coli* O157:H7, *Staphylococcus aureus, Streptococci, Vibrio parahaemolyticus, Brucella abortus* and *Salmonella* indicating high specificity of the multiplex PCR.

In the present study, a total of 932 fecal samples were collected from fresh faecal pats of buffaloes and cattle of different ages (calf, heifer and adult), sex, in different seasons and in cattle of different breeds (indigenous, crossbred and exotic), from different cattle and buffalo farms in different parts of Karnataka from July 2014 to January 2016. The faecal samples were subjected for cultural identification of *E. coli* O157:H7 by enrichment in modified tryptone soy broth (mTSB) followed by selective isolation in Sorbitol-MacConkey agar supplemented with cefixime (0.025 mg) and potassium tellurite (1.25 mg) (CT-SMAC). These isolates were further subjected for inoculation on Eosin methylene blue (EMB) agar, Gram's staining and biochemical tests, viz; Indole test, Methyl Red test, Voges- Proskauer test, Citrate utilization test (IMVC), Nitrate test and Sugar fermentation test for Lactose, Sucrose and Dextrose.

The faecal samples directly and with one step selective enrichment in mTSB broth were subjected for standardized multiplex PCR for the identification and molecular characterization of *E. coli* O157:H7. The samples found positive for *E. coli* O157:H7 by cultural isolation, cultural isolation with biochemical characterization and multiplex PCR were confirmed by using latex agglutination test by observing for the presence of O157 and the H7 antigens.

In the present study, latex agglutination test was taken as confirmative diagnostic test and the results obtained by cultural isolation, cultural isolation with biochemical characterization and multiplex PCR was compared with that of latex agglutination test to estimate the epidemiological sensitivity and the specificity. The sensitivity of cultural identification *vis-a-vis* latex agglutination test was very high i.e., 100%. However, the specificity was low i.e. 83.35%. The sensitivity of cultural isolation with biochemical characterization *vis-a-vis* latex identification test was again very high i.e., 100% and the specificity was also better i.e., 92.51%. The sensitivity of multiplex PCR *vis-a-vis* latex identification test was low, i.e., 95.60% but the specificity was very high, i.e., 100% when faecal samples were directly analysed by multiplex PCR. Similarly, the sensitivity of multiplex PCR *vis-a-vis* latex identification test was very high, i.e., 100% and the specificity was also high, i.e., 98.69% when faecal samples with one step selective enrichment were analyzed by multiplex PCR.

The prevalence of shedding of *E. coli* O157:H7 in bovines was found to be10.94% (102 out of 932 samples). Of these, the percent of cattle [12.10% (91 out of 752 samples)] shedding *E. coli* O157:H7 was significantly higher than buffaloes [5.55% (11 out of 180 samples)]. In cattle, the percent of exotic breeds [18.56% (49 out of 264 samples)] shedding

E. coli O157:H7 was significantly high, in crossbred cattle it was equally high [13.88% (30 out of 216 samples)] and in indigenous breeds it was significantly very low [4.41% (12 out of 272 samples)]. Age wise, the shedding *E. coli* O157:H7 in calves [14.80% (41 out of 277 samples)] and heifers [12.12% (16 out of 132 samples)] was significantly higher than in adults [8.60% (45 out of 523 samples)]. Sex wise, the percent of male bovines [15.17% (27 out of 178 samples)] that showed shedding of *E. coli* O157:H7 was significantly higher than female animals [9.95% (only 75 out of 754 samples)].

Seasonally, the percent of bovines shedding *E. coli* O157:H7 in faeces in winter season was 14.75% (45 out of 305 samples), in monsoon season was 10.85% (28 out of 258 samples) and in summer season it was only 7.86% (29 out of 369 samples). There was significant difference in the shedding of this pathogen among these three seasons. Between summer and monsoon seasons, there was no significant difference in shedding of *E. coli* O157:H7 in the faeces at *P* value of <0.05, however there was significant difference at *P* value of <0.1. Between monsoon and winter seasons, there was significant difference in shedding pattern of *E. coli* O157:H7 in the faeces at *P* value of <0.05. And between summer and winter seasons, there was highly significant difference in shedding of *E. coli* O157:H7 in the faeces at *P* value of <0.05. The monthly trend of shedding of this pathogen showed decreasing trend from January to May and then increasing trend from June to December with maximum percentage of shedding in the month of January and least in the month of May.

The variation in shedding pattern of *E. coli* O157:H7 depending on environmental temperature showed highest percentage of bovines shedding *E. coli* O157:H7 in faeces in the temperature range of 20.1-25.0°C, followed by the temperature range of 25.1-30.0°C, 15.1-20.0°C, 10.1-15.0°C, 30.1-35.0°C and 35.1-40.0°C. The temperature trend analysis of shedding pattern showed that highest percentage of shedding of *E. coli* O157:H7 was at the environmental temperature of 22.1-23.0°C and least at 39.1-40.0°C. The percent of healthy bovines that showed shedding of *E. coli* O157:H7 was significantly higher (12.06%) than diarrhoeic animals (2.68%).

When the effect of lactation and pregnancy on shedding of *E. coli* O157:H7 was analyzed, there was no significant difference in shedding of *E. coli* O157:H7 at *P* value of <0.05 and even at *P* value of <0.5 among lactating and pregnant (10.66%), lactating and non pregnant (8.54%), and dry pregnant (9.68%) animals. In contrast to these three groups, the shedding of *E. coli* O157:H7 was significantly very meagre in non lactating and non

pregnant animals (only 1.45%). The results indicate that the physiological state of the adult animal definitely has effect on the shedding of *E. coli* O157:H7 in faeces.

The samples found positive for *E. coli* O157:H7 by mPCR were subjected for antibiotic sensitivity test. Highest percent of *E. coli* O157:H7 positive samples were resistant for Nitrofurantoin (100%) followed by Carbenicillin (92.16%), Co-Trimazine (87.25%), Tetracycline (75.49%), Kanamycin (69. 61%), Amikacin (46.08%), Streptomycin (23.53%) and Ciprofloxacin (only 2.94%). The occurrence of *E. coli* O157:H7 multiple antibiotic resistant profiles may show a risk for public health and food safety as well as animal health and production.

In the present study, apart from 102 *E. coli* O157:H7 isolates, 23 isolates were characterized as *E. coli* O157:H^{-} by multiplex PCR. The *E. coli* O157:H7 and *E. coli* O157:H^{-} together were characterized as *E. coli* O157. The percent of animals that showed shedding of *E. coli* O157 in the faeces was 13.41% (125 out of 932). Further few of the samples showed presence of any one of or both of the *stx1 and stx2* genes and absence of *rfbE* and *fliC7* genes. These were considered positive for Shiga Toxin producing *E. coli* (STEC) other than *E. coli* O157:H7. These samples together with *E. coli* O157:H^{-} and *E. coli* O157:H7 were characterized as shiga toxin producing *E. coli* (STEC). The percent of animals that showed shedding of STEC was 16.52 % (154 out of 932 samples).

Highest percent of *E. coli* O157 positive samples that showed resistance was against Nitrofurantoin (100%) followed by Carbenicillin (88.80%), Co-Trimazine (83.20%), Tetracycline (65.60%), Kanamycin (60.00%), Amikacin (46.4%), Streptomycin (20.8%) and Ciprofloxacin (only 2.4%). Highest percent of STEC positive samples that showed resistance was against Nitrofurantoin (99.35%) followed by Carbenicillin (88.31%), Co-Trimazine (73.38%), Tetracycline (69.48%), Kanamycin (52.60%), Amikacin (40.91%), Streptomycin (20.78%) and Ciprofloxacin (only 3.25%).

The results found in the present study, give enough indication about epidemiological factors influencing the shedding pattern of *E. coli* O157:H7 in bovines which helps to strategise pre-harvest preventive and control measures so that the incidence of foodborne outbreaks of this foodborne pathogen in humans could be reduced to great extent.

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ANNEXURE 1

Modified trptic soy broth

Casein enzymic hydrolysate	17 g
Papaic digest of soyabean meal	3 g
Bile salt mixture Sorbitol	1.5 g
D-Glucose	2.5 g
Sodium chloride	5 g
K_2HPO_4	4 g
Novobiocin	0.02
pH	7.1±0.2

Sorbitol MacConkey agar

Peptic digest of animal tissue	17 g
Proteose peptone	3 g
Sorbitol	10 g
Bile salts	1.5 g
Sodium chloride	5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	13.5 g
pH	7.1±0.2

Cefixime-tellurite supplement (vial)

Potassium tellurite	1.25 mg
Cefixime	0.025 mg

Rehydrate one vial with 5 ml sterile distilled water and add to 495 ml of sterile molten SMAC

Tryptone water

Tryptone	20 g
Sodium chloride	5 g
DW	1000 ml
pH	7.4

Kovac's reagent

Isoamyl alcohol	150 ml
P-Dimethyl aminobenzaldehyde	10 g
Concentrated hydrochloric acid	50 ml

Triple sugar iron agar

Peptic digest of animal tissue	20 g
Yeast extract	3 g
Beef extract	3 g
Lactose	10 g
Sucrose	10 g
Dextrose	1 g

Ferrous sulphate	0.2 g
Sodium chloride	5 g
Sodium thiosulphate pentahydrate	0.3 g
Phenol red	0.024 g
Agar	12 g
DW	1000 ml
pH	7.4±0.2

Citrate utilization test medium

Sodium citrate	2 g
Dipotassium phosphate	1g
$(NH_4)H_2PO_4$	1g
$MgSO_4.7H_2O$	0.2 g
Bromothymol blue	0.08 g
Sodium chloride	5 g
Agar	13.5 g
pH	6.9±0.2

MRVP medium

Glucose	5 g
K_2HPO_4	5 g
Peptic digest of animal tissue	5 g

Nitrate broth

Peptic digest of animal tissue	5 g
Meat extract	3 g
Potassium nitrate	1 g
Sodium chloride	30 g

Muller Hinton agar

Beef infusion	300 g
Casein acid hydrosylate	17.5 g
Starch	1.5 g
Agar	17 g

Reagents for agarose gel electrophoresis

Tris-acetate EDTA (TAE) buffer (50x)

Tris-base	242 g
Acetate (100% acetic acid)	57.1 ml
EDTA (0.5M)	100 ml

Add distilled water up to one litre. To make 1x TAE from 50X TAE stock, dilute 20ml of stock into 980 ml of DI water

1.5% agarose

Agarose	6 g
TAE buffer (1X)	40 ml