Prevalence and molecular characterization of *Escherichia coli* O157:H7 and Shiga-toxin producing *E. coli* (STEC) in cattle related sources

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CERTIFICATE

This is to certify that the research work embodying the results reported here in this thesis entitled **"Prevalence and molecular characterization of *Escherichia coli* O157:H7 and Shiga-toxin producing *E. coli* (STEC) in cattle related sources "** by **Mafruha Nazneen** has been carried out in the Laboratory of Mycology and Plant Pathology, Department of Botany, University of Dhaka under our supervision and guidance. It is further certified that the work presented here is original and suitable for submission in partial fulfillment for the Degree of Doctor of Philosophy in Botany.

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DEDICATION

This piece of work is dedicated to my Family

DECLARATION

I hereby declare that this dissertation is based on entirely my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of another degree or diploma at any other University.

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Abstract

Shiga toxin producing *E. coli* are important food safety issue worldwide. They cause illness ranging from mild diarrhea to severe Hemorrhagic colitis (HC) and hemolytic Uremic Syndrome (HUS) (Brett, *et. al* 2003). Among the STEC, *E. coli* O157:H7 has been reported as the most predominant as it causes many outbreaks and sporadic cases of hemorrhagic Uremic Syndrome in U.S.A, U.K, Japan and Europe (C.D.C. 1982, Coombes *et. al*. 2011). It produces syndrome like bloody diarrhea, hemorrhagic colitis, hemolytic uremic syndrome and even cause death.

Cattle are considered to be the natural reservoir of STEC and *E. coli* O157:H7 and isolated from their feces. People can be infected because of the consumption of contaminated water, undercooked meat, milk, vegetables and other product. Since Bangladesh is an agricultural country, there is a great chance of release of these pathogen and to contaminate water, meat, vegetables and other food staff.

Our study was aimed to detect and isolate the STEC and *E. coli* O157:H7 from cattle related sources such as cow-dung, beef, goat meet, raw milk, cowshed soil and goat dropping and characterization of *E. coli* O157:H7 strains isolated from all these samples. The samples were collected from different meat shop and local market places in Dhaka city. The method followed was both cultural and molecular technic based. After following the procedure of pre-enrichment, the samples were plated on C.T SMAC. Then the presumptive isolates were cultured on EMB and MUG media and subjected to biochemical tests.

Through serological tests and PCR amplification the pathogens were isolated and confirmed as *E. coli* O157:H7. The isolates those containing *eaeA*, *stx-1* or *stx-2* or both, *rfbE* and *fliC* genes were considered as *E. coli* O157:H7. 16S rRNA analysis and sequencing of the isolates were also done. Enterotoxicity, Hemolytic activity, Invasiveness and antibiogram were done to characterize the isolates.

Total 33 samples of cow-dung were tested. Among them 14 samples (42.4%) were found STEC positive and 10 isolates from 6 samples (18% of total sample) were *E. coli* O157:H7. Out of 48 goat meat samples 09 isolates from 05 samples were identified as *E. coli* O157:H7

(10%) and 12 samples (25%) were identified as STEC. Likewise 10% beef was STEC positive of which only one (2%) was *E. coli* O157:H7. From 43 milk samples 04 samples were STEC positive which was 9% of total sample and no *E. coli* O157:H7 was found in milk samples. Out of 23 samples of goat dropping, 26% were STEC and only *one* sample was positive for *E. coli* O157:H7 (4%), from 22 samples of cowshed soil no STEC and *E. coli* O157H7 was found.

All the *E. coli* O157:H7 isolates were found to be non-invasive, and also non-hemolytic. About 86% of the *E. coli* O157:H7 isolates were found as entero-toxin producing in Rabbit-ilial loop test because they had *stx1* or *stx*2 or both genes. In case of antibiotic sensitivity 81% of isolated *E. coli* O157:H7 were sensitive to kanamycin and 68% were sensitive to streptomycin. On the other hand 100% isolates were resistant to novobiocin and 87% were resistant to ampicillin.

From the above discussion it is alarming that there are huge sources of *E. coli* O157:H7 and other STEC which can contaminate inland water, meat, milk and vegetables. So, the findings emphasize the need for proper cattle handling, sanitation and meat processing in the market.

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List of Abbreviations

%	:	Percentage
µl	:	Microliter
bp	:	Base pair
e.g.	:	For example
EHEC	:	Enterohemorrhagic *E. coli*
et al.	:	And others
gm	:	Gram
h	:	Hour
HC	:	Hemorrhagic Colitis
HUS	:	Hemolytic Uremic Syndrome
kDa	:	Kilodalton
kg	:	Kilogram
L	:	Liter
M	:	Molar
mg	:	Milligram
min	:	Minute
ml	:	Milliliter
mM	:	Millimolar
MW	:	Molecular weight
nm	:	Nanometer
^{o}C	:	Degree Celsius
OD	:	Optical density
PBS	:	Posphate buffer solution
pH	:	Negative logarithm of hydrogen ion concentration
pmol	:	Picomole
rpm	:	Rotation per minute
sec	:	Second
spp.	:	Species

stx	:	Shiga toxin
TTP	:	Thrombobiotic thrombocytopenic purpura
UV	:	Ultra violet
V	:	Volts
VTEC	:	Verotoxin Producing *E.coli*

INTRODUCTION

Theodor Escherich, a German bacteriologist discovered *Escherichia coli* in 1885 which was named as the colon bacillus *Bacterium coli commune* (Escherich 1885). He isolated a variety of Gram negative bacteria from infant fecal samples. The name *Escherichia coli* was proposed by Castellani and Chalmers (1919) which was not officially recognized until 1958. It is now classified as part of the gamma-proteobacteria under the family Enterobacteriaceae (Taxonomy Browser).

Escherichia coli is a Gram-negative, short rod, motile, non-spore forming bacterium which is a common inhabitant of the lower gastrointestinal tract of humans and warm-blooded animals. *Escherichia coli* benefits its hosts by producing vitamin k2 and by preventing establishment of pathogenic bacteria within the intestine. In general, *E. coli* exist in a beneficial symbiotic relationship with its host and plays important roles in promoting the stability of the luminal microbial flora and in maintaining normal intestinal homeostasis (Yan and Polk 2004). Normally *E. coli* is harmless and occasionally causes a disease, but in case of debilitated or immune-suppressed host, nonpathogenic commensal strains of *E. coli* can cause infection (Kaper *et al*. 2004).

A few strains *E. coli* are pathogenic and cause serious food poisoning, septic shock, meningitis or urinary tract infections in humans (Vogt 2005). Pathogenic variety of *E. coli* produces toxins and other virulence factors that enable it to reside in parts of the body, and to damage host cells. These pathogenic traits are encoded by virulence genes carried only by the pathogens (Mobley 2004).

Human intestinal strains of *E. coli* are of six categories, such as, Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC) or Shiga toxin-producing *E. coli* (STEC) Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and Diffusely Adherent *E. coli* (DAEC) (Nataro and Kaper 1998). In addition, there are Uropathogenic *E. coli* (UPEC), Meningitis-associated *E. coli* (MNEC),

Extraintestinal pathogenic *E. coli* (ExPEC), and Avian pathogenic *E. coli* (APEC) (Kaper *et al.* 2004).

Enterohaemorrhagic *E. coli* (EHEC) was first reported in 1982 as a causal agent of human disease. EHEC causes bloody diarrhea, non-bloody diarrhoea and haemolytic uremic syndrome (HUS). The bovine intestinal tract was identified as the principal reservoir of EHEC and the first initial outbreak was noticed with consumption of undercooked hamburgers. A wide variety of food items including sausages, unpasteurized milk, lettuce, cantaloupe melon, apple juice and radish sprouts have been linked with disease. The radish sprouts were found to be responsible for an outbreak of 8,000 cases including school children and teachers in Japan (Michino *et al.* 1998). Varma *et al.* (2003) reported that *E coli* O157:H7 is the most important EHEC pathogens in North America, the United Kingdom and Japan, but several other serotypes, particularly O26 and O111 serogroups can cause disease and are more prominent than O157:H7 in many countries (Kaper *et al.* 2004).

Shigatoxin is the key virulence factor for EHEC, which is also known as verocytotoxin (VT). This stx has five identical B subunits. B subunits are responsible for binding the holotoxin to the glycolipid globotriaosylceramide (Gb3) on the target cell surface, and a single A subunit that cleaves ribosomal RNA, causing protein synthesis to cease (Melton-Celsa and O'Brien 1998). The stx family has two subgroups i.e.stx-1 and stx-2. The stx is produced in the colon and travels to the kidney through circulation. In kidney it damages renal endothelial cells and induce local cytokine and chemokine production, resulting in renal inflammation (Andreoli *et al*. 2002). This damage can lead to HUS, which is characterized by haemolytic anaemia, thrombocytopoenia and potentially fatal acute renal failure. This stx also mediates damage of colon, which results in bloody diarrhoea, haemorrhagic colitis, necrosis and intestinal perforation.

Shiga toxin producing *E. coli* (STEC) are the most important cause of food borne diseases (Kaufman *et al.* 2006). They cause illness ranging from mild diarrhea to severe conditions such as Hemorrhegic colitis (HC) and Hemolytic Uremic Syndrome (HUS)(Brett *et al.*2003). This STEC strains have several sero-groups such as O157:H7, and non-O157:H7. *E. coli* O157:H7 has been reported as the most predominant serotype which causes many outbreaks and sporadic cases of HUS in USA, UK, Japan and Europe (CDC 1982; Coombes *et al.* 2011; Pennington 2010). Hemolytic uremic syndrome can lead to acute or chronic renal failure mostly in children (Siegler *et al.* 1993; Hussein *et al* 2005).

Shiga toxin producing *Escherichia coli* (STEC) has been a major health problem for the last few decades. More than 200 STEC serotypes were reported which cause human infection (Eklund *et al.* 2001). Sporadic cases and outbreak have been reported from Latin America, India and some developing countries (Kadu-Mulido *et al*. 2001; Leelaporn 2003). In 1994, Non-O157 STEC strains was first documented in USA, which caused acute diarrhea than the better-known O157 strains and have the potential for large outbreaks. Valilis *et al*. (2018) identified 129 serogroups as well as 262 different O and H antigen combinations of STEC in cases of epidemic and sporadic disease worldwide. They reported frequency of dysenteric illness in patients was 26% for epidemic disease and 25% for sporadic cases. A single large outbreak occurred in Germany and France caused by STEC O104:H4 in 2011(Kalita *et al.* 2014)

There are numerous non-O157 STEC serogroups that often cause illness in people in many countries. The most common serogroups reported to cause foodborne illness are O26, O111, O103, O121, O45 and O145 in the United States, O26, O63, O103, O111, O145 and O146 in Europe, O26, O103, O111, O145, O146 and O174 in South and Central America, O26, O103, O111, O113 and O172 in Australia, and O65, O103, O111, O121, O145, O165 in Japan. Among those 026 serotype was common in all the countries except Japan (Valilis *et al*. 2018).

Escherichia coli O157:H7 is one of the predominant foodborne pathogen worldwide (Tarr *et al.* 2005). It was first recognized as a pathogen in 1982 during an outbreak investigation of hemorrhagic colitis (Riley 1983). About 350 outbreaks were reported in 49 states of USA in 20 years from 1982. In this period 8,598 cases were reported where 1,493 person were hospitalized with 354 cases of Hemolytic Uremic Syndrome (HUS) and 40 deaths (CDC 93, 96, Rodrigue *et al.* 1995). In majority of the cases, food was suspected to be main vehicle. Most reported foodborne pathogen outbreaks of *E. coli* O157:H7 infection have been associated with beef, dry-cured salami (CDC 1995), and milk (Upton and Coia 1994). Waterborne and person-to-person transmission also may occur (Swerdlow *et al.* 1992; Keene *et al.* 1994; Belongia *et al.* 1993). Several outbreaks of *E coli* O157:H7 infections associated with consumption of raw fruit and vegetable produce have been reported worldwide by different groups (Hilborn *et al*. 1999; Davidson *et al*. 1996; Ackers *et al.* 1998,;Mermin *et al*. 1996; Hahn *et al*. 1996; Besser *et al*. 1993; CDC 1993, 1996, 1997). An outbreak started when several people in Germany were infected with enterohemorrhagic *E. coli* (EHEC), leading to hemolytic-uremic syndrome (HUS). Outbreaks also reported from 15 other countries including North America (WHO 2011). Center for Disease control and prevention (CDC) estimated more than 20,000 *E. coli* O157:H7 infection per annum with 250 death in USA (Boyce *et al.*1995)

Cattle feces are reported as natural reservoir of STEC and *E. coli* O157:H7 (Gansheroff and O' Brien 2000; Molina *et al*. 2003; Caprioli *et al*. 2005). Contaminated and inadequately cooked meat and raw milk with *E. coli* O157:H7 may be bio-burden to people (WHO 1997). Contaminated non-pasteurized apple cider, drinking and swimming water, vegetables, mayonnaise, curd, salami, cheese, lettuce and direct contact of animal to person or person to person contact are many other sources of *E. coli* O157:H7 infection (Alam *et al.* 2006).

The pathogenesis of *E. coli* O157:H7 involves many factors and several levels of interactions between the virulence factors of bacterium and the host. These include two different types of Shiga toxins stx-1 and stx-2 and interaction of toxins with host tissues. Another factor is 'intimin' protein encoded by '*eae*' gene. This intimin forms attaching and effacing (A/E) lesion in the intestinal mucosa (Kaper *et al*.1998). These genes are found in the locus of enterocyte effacement (LEE), lamboid phages and a large virulence associated plasmid (Khan *et al.* 2003).

Bangladesh is an agriculture based country. The hygienic condition is compromised with poor sanitation system. Living with domestic or farm animals in close proximity is common scenario in both urban and rural areas. So, there are several possibilities of sources of STEC and *E. coli* O157:H7. In previous studies STEC has been isolated from cattle, calves and children (Nazir *et al.* 2005, 2007; Talukdar *et al.* 2013; Munshi *et al.* 2012), chicken (Mamun *et al.* 2016) and water (Talukdar *et al.* 2013). Islam *et al.* (2010) reported presence of STEC in 34% buffalo meat, 66% beef, 10% raw milk and 8% fresh juice samples. Rectal content of slaughtered animals in Dhaka city were found to be STEC positive in 80% of buffalo, 72% cow and 11.8% goat samples (Islam *et al.* 2016). Islam *et al.* (2007) found shiga toxin producing *E. coli* in stool samples of 2.2% hospitalized diarrheic patients and 6.9% community diarrhea patients using multiplex PCR technique. STEC has also been isolated from stool samples of diarrheic children admitted at Mymenshing Medical college hospital (Islam *et al.* 2016). Study of Fazley *et al.* 2014 on E. coli O157:H7 from cow dung established that these isolates are capable of producing same degree of illness as the clinical strain.

The effect of illness due to STEC and *E. coli* O157:H7 infection can be very serious and may even cause death. Furthermore, there are no vaccines available till to date to prevent the diseases owing to *E. coli* O157:H7. Some experimental approaches, such as stx-based toxoid and toxoid intimin based vaccine are being investigated in animals (Khan *et al.*

2003). So, pathogens associated with this illness are concern of the scientists to get remedy.

Bangladesh is an endemic zone for diarrheal diseases and every year, more than 5% of deaths of children under 5 years of age are attributed to diarrhea. But unfortunately, there is a few official reports on isolation of *E. coil* O157:H7 and on the burden of *E. coli* O157:H7 and other STEC associated diarrhea in Bangladesh. In a developing country like Bangladesh there are several sources of STEC and *E. coli* O157:H7. Many farms in and around Dhaka city might harbor the bacterium probably being contaminated with feces of animals. Meat though sterile itself, can be contaminated from various sources such as feces of animals, water and utensils used in processing, persons handling the meat and some other factors. So, raw consumption of meat, personal hygiene of the meat handling personnel, houseflies (as a vehicle of transmission) can cause a significant morbidity and mortality rate. A low infectious dose of *E. coli* O157:H7 can pose serious threat to public health if proper precautions are not be taken. Raw milk and houseflies can serve as vehicles in transmitting the pathogen to susceptible individuals and can cause a significant morbidity and mortality. In Bangladesh the investigation on the isolation and identification of *E. coli* O157:H7 from cattle related source is insignificant. On the basis of above factors an effort has been taken to isolate *E. coli* O157:H7 and other STEC in cattle related sources using conventional and molecular methods.

The survey of literature indicates that no systematic approach has been made to study various aspects for the detection and isolation of *E. coli* O157:H7 and other STEC from cattle related sources. Therefore, an attempt has been taken to design a complete protocol for the detection and isolation of *E. coli* O157:H7 and other STEC from cattle related sources including cattle dung, raw beef and mutton meats, beef burgers, raw and pasteurized milk etc. In the present investigation the following aspects will be studied in detail:

- Isolation of *E. coli* O157:H7 and STEC from cattle based sources on selective enrichment of the samples followed by plating on selective culture media.
- Cultural and biochemical identifications of the isolates.
- Serological identification of the isolates using specific anti-sera.
- Nucleic acid based identification of the isolates by detection of *eaeA*, *rfbE*, *fliC*, *stx1* and *stx-2* genes by PCR.
- Phenotypic characterization of the isolates by observing the hemolytic activity, enterotoxicity, invasiveness and sensitivity to antibiotics.
- 16s ribosomal RNA genes analysis of selected isolates for authentication of the isolates as *E. coli* O157:H7 and STEC.

LITERATURE REVIEW

2.1 Historical Background

Escherichia coli is a gut bacterium, which cover about 0.1% (Eckburg *et al.* 2005) of human gut flora and benefits humans providing nutrient supplements, enhancement of nutrient acquisition, and preventing the adaptation of bacterial pathogens within the gut (Reid *et al.* 2001). Pathogenic strains of *E. coli* able to cause gastrointestinal disorders, neonatal meningitis and urinary tract infection.

After discovery*, E. coli* (Escherich1885) had drawn attention to Microbiologists and Doctors as a potential indicator and some strains as potential causal agents of human ailments. Shiga toxin producing *E. coli* (STEC) is most important example of pathogenic *E. coli* (Kaper *et al.* 2004).

Shiga toxin producing *E. coli* (STEC) exhibits cytotoxic activity on vero-cells and other cell types in the human body (Karch *et al.* 1999). STEC strains were recognized as human enteric pathogens. This group belongs to a broad range of 'O' serogroups and able to cause diarrhea, hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) (Orden *et al*. 2008). Among 200 reported STEC serotypes, more than 100 serotypes cause human infections (Eklund *et al*. 2001).

The first outbreak of sero-group STEC O157:H7 was documented in the USA in 1982 (Riley 1983) and then in U.K, Japan, New Zealand, Africa, Continental Europe (CDC 1982; Coombes *et al.* 2011; Pennington *et al.* 2010). Non O157:H7 STEC is also recognized as causative agent of hemolytic uremic syndrome and hemorrhagic colitis (Brett *et al.* 2003). Diagnosis and epidemiological studies have given priority to *E. coli* O157:H7 and serotype O111 as these are capable of causing many serious human illness (Patton and Patton 1999; Dos Santos *et al.* 2007). In May 2011, *E. coli* O104:H4 outbreak leading to hemolytic-uremic syndrome ((HUS) was reported in Germany (Kalita et al. 2014) and these outbreaks were found to occur in other countries including some

regions in North America. Riley *et al.* (1983) reported two outbreaks of a particular gastrointestinal illness characterized by
(1) severe abdominal cramp, and watery diarrhea and (2) by bloody diarrhea and little or no fever.

As stated earlier, serotype O157:H7 are able to cause hemorrhagic colitis (HC), and their source was contaminated and undercooked hambergers of a fast-food restaurant. Karmali *et al.* (1983) reported periodic cases of hemolytic uremic syndrome (HUS) linked with cytotoxin producing *E. coli* in stools. The hemolytic uremic syndrome results acute renal failure, microangiopathic hemolytic anemia and thrombocytopenia which was characterized by a bloody diarrheal illness indistinguishable from hemorrhagic colitis.

There is a question about the emergence of *E. coli* O157:H7 whether it is new or old or old but not detected. To answer this question many studies were conducted. The prevalence of *E. coli* O157:H7 was found limited. The Centers for Disease Control and Prevention (CDC) reviewed over 3,000 *E. coil* isolates between 1973 and 1983, from which only one isolate was identified as *E. coli* O157:H7 (Riley *et al*.1983). In other studies carried out between 1978 and 1982 in the Public Health Laboratory, United Kingdom also showed only one isolate as *E. coli* O157:H7 among 15000 *E. coli* isolates. The Laboratory Centre for Disease Control in Canada reported six O157:H7 strains among 2,000 isolates obtained from diarrheal patients between 1978 and 1982 (Nataro *et al*. 1998). Although Shiga toxin producing *Shigella dysenteriae* type-I strains were clearly associated with HUS, stool cultures obtained during many HUS outbreaks yielded only *E. coli*. This event suggesting that in addition to *Shigella dysenteriae* type-I strains, *E. coli* O157:H7 is associated with HUS.

2.2 Importance of Food-borne pathogens

Foodborne illness is a common, costly preventable public health problem, yet sometimes life threatening. Foodborne illness is caused by the ingestion of contaminated foods either

with biological, or chemical or physical hazards, of which biological causal agents is being considered for this study. Biological hazards include bacteria, viruses, and parasites.

Food borne sickness a major concern of the world is now burden of Food Microbiologists. It is estimated that the diarrheal diseases accounts for 4.1% of the total daily global burden of diseases. These diarrheal diseases are cause for the death of 1.8 million people every year and 90% of them are children under the age of 5 years (Islam *et al*. 2006). According to the Centers for Disease Control and Prevention (CDC), approximately 48 million Americans get sick, 128,000 are hospitalized and 3,000 die each year from food poisoning (Barbara, 2019). Sickness associated with the intake of fresh foods, vegetables and meat has increased in the United States during past decades (Sumathi *et al.* 2004). Estimated 12, 600 deaths of children under 5 years old per day for diarrheal diseases had been reported in Latin America, Africa, Asia and especially in developing countries (Alikhani *et al.* 2006). Fast foods prepared with inadequate temperature are very susceptible to bacterial contamination.

Here are some of the important report of shiga toxin producing *E. coli* and *E. coli* O157:H7 as food borne pathogen:

- Serotype O157:H7 has been reported high potent foodborne pathogen worldwide (Tarr *et al.* 2005).
- The first outbreak with 26 cases of infection of *E, coli* O157:H7 with 19 patients hospitalized was reported in USA in 1982 (Riley *et al.* 1983). Then 196 outbreaks or series of *E. coli* O157:H7 infections were documented through 1998 (Griffin and Tauxe 1991).
- Center for Disease Control and Prevention (CDC) estimated 20,000 infection and 250 death for *E. coli* O157:H7 infection in USA (Boyce *et al.* 1995). CDC also reported 73480 cases of infection with 61 deaths and 37,740 illness with 30 deaths annually in USA for *E. coli* O157:H7 and non-O157 STEC, respectively (Mead *et al*. 1999).

- From 1983 to 1996, 55 outbreaks of *E. coli* O157:H7 with no causalities and from 1991 to 1995, 29 outbreaks were reported in England and Wales, and Japan, respectively. In 1998, more than 11,842 cases of *E. coli* O157:H7 infection with 12 death were reported in Japan (Michino *et al*.1998).
- In 1996 a large outbreak of *E. coli* O157:H7 involving 501 cases with 21 elder people's death were reported in Central Scotland (Ahmed 1998). In another epidemic outbreaks of *E. coli* O157:NM occurred from drinking of contaminated surface water reported in Swaziland and south Africa (Issacson *et al.* 1993).

Major outbreaks of *E. coli* O157:H7 infection is shown in Figure 1.

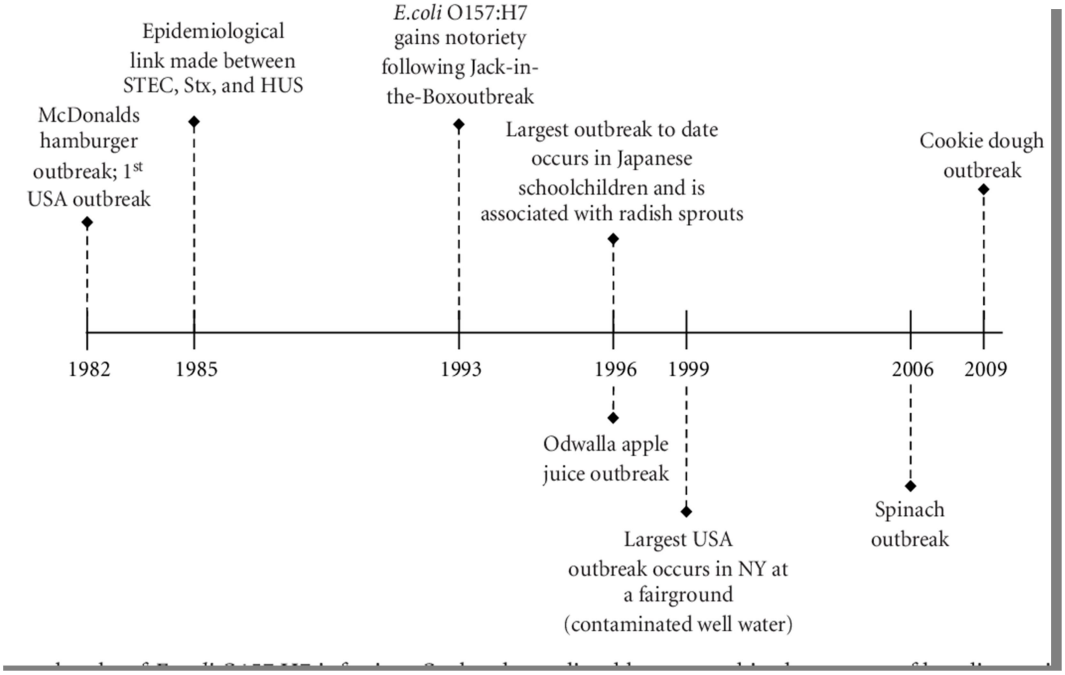

Fig. 1: Major outbreaks of *E. coli* O157:H7 infection. Outbreaks are listed by year and in the context of key discoveries that linked stx with development of HUS. (Source: Mouhak and O' Brien 2011)

- Non-O157 EHEC infections occurred frequently in continental Europe, Australia and Latin America. In 1995 an outbreak of *E. coli* O111:H8 with 23 cases and in1999, another outbreak with 58 cases have been reported in south-Australia and Taxas, respectively (CDC 2000).
- A severe outbreak occurred in Germany in 2011 from May to September which caused 3842 cases of infection with 53 deaths (Wu *et al*. 2011)

2.3 Evolution of *E. coli* O157:H7

E. coli O157:H7 might be derived from the non-toxigenic and less virulent strain *E. coli* O55:H7 (Wick *et al.* 2005). *E. coli* O157:H7 has emerged through four sequential events such as (i) acquisition of an *stx*-2 containing bacteriophage, (ii) acquisition of pO157 and

rfb region, (iii) acquisition of the *stx* containing bacteriophage and loss of ability to ferment D-sorbitol and loss of beta lucourinidase (GUD) activity.

Evolution of *E. coli* O157:H7 is diagrammatically represented in Figure 2.

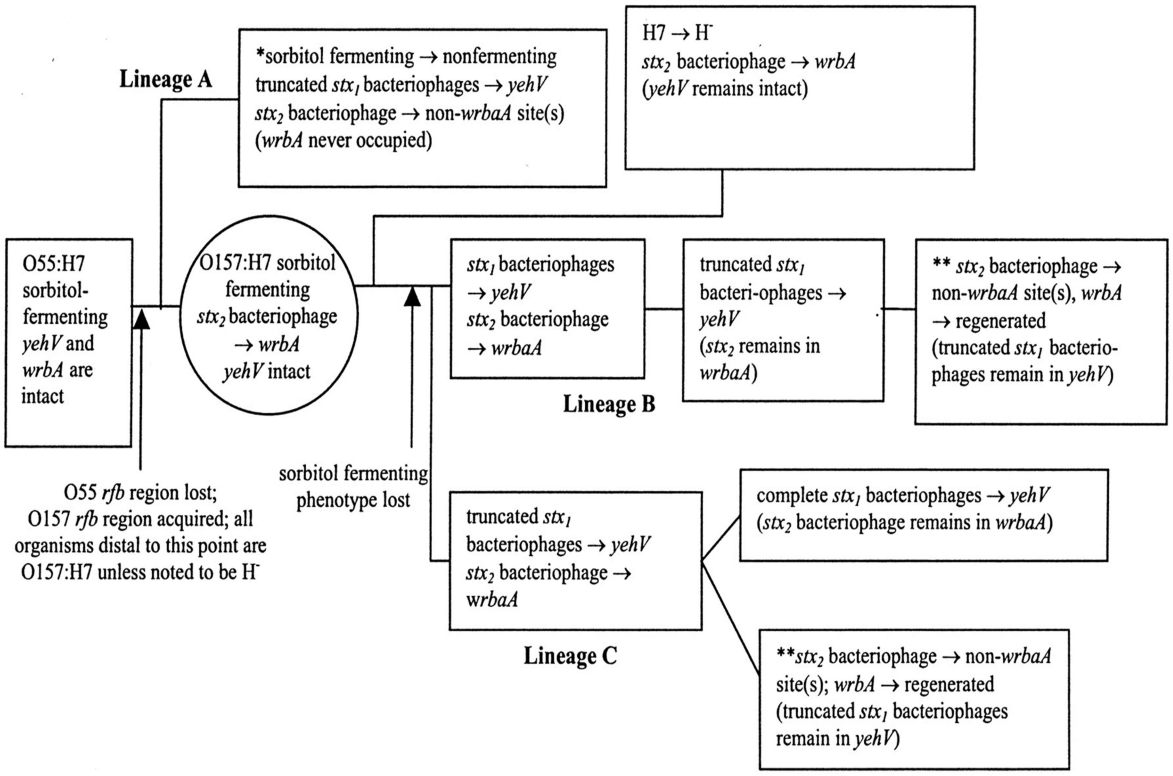

Fig. 2: Diagrammatic presentation of evolution of *E. coli* O157:H7 (source: jb. Asm. Org.)

2.4 Evolution of terms: STEC, EHEC and VTEC

Ten years after the discovery of Shiga toxin (stx), O'Brien *et al.* (1983) discovered certain strains of *E. coli* which are able to yield cytotoxin that can be neutralized by anti-stx antibody. In 1898, Kioshi Shiga described *Shigella dysenteriae* type-1 which was capable of producing shiga toxin. Some *E. coli* strains able to produce the shiga-like toxin which are named as shiga toxin producing *E. coli* (STEC). *E. coli* (STEC) produces cytotoxins

which were same at the genetic and protein levels to the stx produced by *S.dysenteriae* type1.

In 1977, Konowalchuk discovered diarrhoeagenic *E. coli* which produce vero cell killing cytotoxins *in vitro* and named it as Verotoxin producing *E. coli* (VTEC). Later verotoxin was recognised as shiga toxin and *E. coli* O157:H7 can produce shiga toxin (O'Brien *et al.,* 1983).

The enterohemorrhagic *E. coli* (EHEC) was named for its ability to cause HC and HUS; expressing *stx*; *stx* causing attachment; effacing lesions on epithelial cells; and possessing an approximately 60-MDa plasmid. All EHEC strains are human pathogens (Nataro *et al.* 1998).

2.5 Characteristics of *E. coli* O157:H7

Mentioned earlier that *E. coli* O157:H7 is a Gram-negative, rod-shaped bacterium. The "O" refers to the cell wall (somatic) antigen number and the "H" refers to the flagella antigen numbers (the "O" stands for *ohne Hauch* (German word meaning "without huff" or "without film" and "H" for *Hauch*).

E. coli O157:H7 has several characteristics uncommon to most other *E. coli* which are given below:

Acid tolerance: *E. coli* O157:H7 is exceptionally tolerant to acidic environment. *E. coli* cells in stationary phase of growth are significantly more acid tolerant than in the exponential phase. This Acid tolerance is expressed with genes regulated by the RpoS sigma factor operon (Cheville *et a*l. 1996, Rowbury 1998). They examined three mechanism of acid tolerance that is oxidative-arginine dependent and glutamate dependent. All those three mechanism contribute to the microorganisms' overall acid tolerance. Introduction of acid tolerance in *E. coli* can enhance survival of *E. coli* O157:H7 in acidic foods (Cheville *et al* 1996; Layer *et. al.* 1995).

Antibiotic resistance: Initially *E. coli* O157:H7 was susceptible to most antibiotics active against Gram-negative bacteria. But from latest evidences it is apparent that clinical *E. coli* O157:H7 isolates and isolates obtained from foods have developed multidrug resistance of which streptomycene-sufisoxazole-tetracycline being the most common resistance profile.

Capability to produce Shiga toxin: *E. coli* O157:H7 able to produce shiga toxin, which similarates with the shiga toxin produced by *Shigella dysenteriae* type 1. *E. coli* O157:H7 may have stx1or stx2 or both of which stx1 displays 98% sequence homology, while stx2 shares approximately 55% amino acid identity (Obrig 2010).

Possession of *eae* gene: Most of the *E. coli* O157:H7 posses "eae" genes. eae means *E. coli* attaching and effacing. This gene encodes an outer membrane protein 'intimin' that produce AE lesion. Intimin forms intestinal colonization in the animals. The intimin helps *E. coli* O157:H7 cells to attach to intestinal cells, with effacement of the underlying microvilli and accumulates filamentous actin (F-actin) in the subjacent cytoplasm.

Carriage of a 60-MDa plasmid: *E. coli* O157:H7 of human ailment harbors a plasmid (pO157) of approximately 60 MD and it is assumed to play a role in the pathogenicity of disease, but it**s** function is not clear.

Inability to ferment sorbitol within 24 h: *E. coli* O157:H7 cannot ferment the carbohydrate sorbitol with in 24 hour like other *E. coli,* for which it is easy to separate this from other sorbitol fermenting *E. coli* growing in MacConkey agar supplemented with 1% sorbitol. In sorbitol MacConkey agar *E. coli* O157:H7 forms colorless colonies where other *E. coli* form pink colonies.

Growth temperature: VTEC/STEC grow well at above 44^{O}C and its minimum growth temperature is approximately 8 to 10^{o}C.

Thermal inactivation: *E. coli* O157:H7 is a heat labile bacterium that is why this bacterium will be killed at 63^{o}C in food. Pasteurization temperature (72^{o}C for 16.2s exposure) is able to kill more than 10^4 *E. coli* O157:H7 cells per ml (D'Aust *et al.*1988).

Inability to produce B-glucuronidase: *E. coli* O157:H7 strains do not utilize 4-methylumbelliferyl-D-glucuronide, because of inability of producing B-glucuronidase; other *E. coli* strains produce this enzyme.

2.6 Geographical distribution

There are certain geographic distributions of EHEC infection. Sporadic infections of *E. coli* O157:H7 are very common in Canada (Griffin 1995), United states (Josefa *et al.* 2005) and in some countries such as Argentina, Australia, Brazil, Chile and South Africa. *E. coli* O157:H7 is extensively distributed in the United Kingdom, particularly in England, Wales, Scotland and Northern Ireland over the last decade. *E. coli* O157:H7 epidemics are also reported from Turkey (Zeynep *et al.*, 2006). Except Japan, EHEC is not yet a major health problem in most of the Asian countries. A few reports on *E. coli* O157:H7 are available in Hong Kong, Thailand, Malaysia, India, Sri Lanka and Bangladesh (Khan *et al.* 2003).

2.7 Animal reservoir

Prevalence of STEC have been found high (60%) in bovine herds in many countries but in most cases this rate is 10% to 25%. Findings from a study in dairy farms in Canada shown that 36% of cows and 57% of calves were STEC positive. Cattle is the main source of *E. coli* O157:H7 in the food chain. These bacteria existed in other domestic animals mainly ruminants (Kaufman *et al.* 2006) and wildlife, such as sheep, goats, deer, dogs, horses, swine, cats, seagulls and rats (Meng *et al*. 2001). Prevalence of *E. coli* O157:H7 and STEC in cattle rise during warmer period of the year (Elder *et al.* 2000).

2.8 Environmental Survival

E. coli and other STEC can survive in soil, water, food as well as in animal reservoirs. *E. coli* has been found to survive for a couple of years in cow dung mixed soil and for a couple of months in raw manure (Jiang *et al.* 2002). During composting manure it is found effective in destroying *E. coli* O157:H7 if the temperature is maintained above 50^{o}C for 6 days. These organisms can survive for a long time in cold water. Water trough deposits polluted with bovine feces serve as a long term reservoir of *E. coli* O157:H7, which may be a source of infection (Le. June *et al.* 2001).

E. coli O157:H7 may have capability to adapt in extreme changes in temperature, pH and osmotic conditions. The exopolysaccharide (EPS) imparts heat and acid tolerance features of *E. coli* O157:H7 (Yuk and Marshal 2004). These categories of environmental adaptations of *E. coli* O157:H7 help in the endurance and spreading of this organism in farms and the increasing transfer from cattle to cattle. For enduring attitudes of *E. coli* O157:H7 outside the host reservoir increases the risk of pollution of crops and water via-bovine manure contamination, irrigation or direct contact with infected animals.

2.9 Modes of transmission

2.9.1 Food-borne transmission

A variety of foods are identified as vehicles for *E. coli* O157:H7 and STEC transmission including ground beef, roast beef, cooked meats, cake, salami, raw milk, raw apple juice, cheese, cheese curds, yoghurt, pasteurized milk, ice cream, mayonnaise lettuce, potatoes, radish sprouts, alfalfa sprouts and fruits or vegetable salad (Meng *et al.* 2001). Doyle *et al*. (1987) isolated 3.7%, 1.5%, and 2.0% *E. coli* O157:H7, from retail beef, pork and poultry, and lamb samples, respectively. The first recognized outbreak of *E. coli* O157:H7 infection occurred in Oregon in 1982 which was related with eating undercooked hamburgers (Wells *et al*. 1983). Beef donor kebabs sold in cars are also source of *E. coli* O157:H7 in Turkey (Zeynep *et al.* 2006; Ulkanli *et al.* 2006). Many outbreak of *E. coli* O157:H7 were reported for consumption of contaminated apple coder

in Masachusetts, California, Colorado, British Columbia, Canada (CDC 1996). In May 1996 multistate outbreak of U.S.A. was associated with lettuce (Hilborn *et al.* 1999). In Japan largest outbreak in1996 was associated with white radish sprouts (Michino *et al*.1998). The model of transmission of *E. coli* O157:H7 is illustrated in Figure 3.

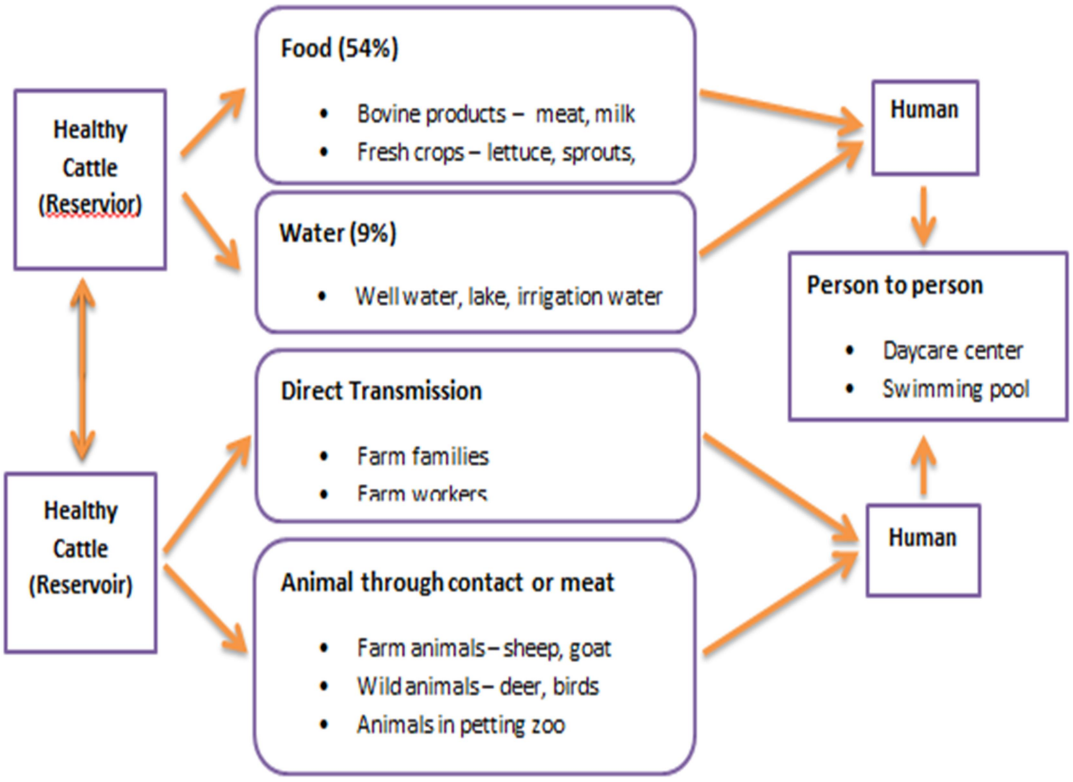

Fig. 3: The model of transmission of *E. coli* O157:H7, which is updated from the diagram by Gansheroff and O` Brien (2000).

Different serogroups of STEC have been found to be associated with food. In India sea food was suspected as vehicle of transmission of STEC. Kumar *et al.* (2001) detected non O157 STEC in fresh fish, shellfish and meat. STEC O157 have been isolated from raw

minced beef 9% (Dutta *et al*. 2000), milk 2.4% (Manna *et al.* 2006) in India. STEC O157 and non-O157 (O111, O113) were isolated from camel milk (Njage *et al*. 2012).

2.9.2 Transmission from bovine to human

Generally direct transmission of *E. coli* O157:H7 from bovines to humans is very rare. In Canada a case of transmission of *E. coli* O157:H7 between calves and a human has been reported (Renwick *et al*. 1993; Beilaszewska *et al.* 2000). Direct transmission from cattle to human also stated by Karch *et al.* (1999) and Renwick (1993). However, such spread appears to be rare.

2.9.3 Transmission through water

Four water-borne outbreaks of EHEC infection were reported in the United States during 1982 to 1994 and sources are mainly contaminated swimming pool water and drinking water (Khan *et al.* 2003). Contaminated drinking water from bovine feces, was associated to EHEC outbreaks in Scotland (Dev *et al.*1991), and Sothern Africa (Isaacson *et al.* 1993) and with well water in Japan (Akashi *et al.* 1994). EHEC was isolated from sea water of Ohio (Francy *et al.* 2003), water of lake Michigan (Haack e*t al.* 2003) and water of Great lake (Byappanahalli *et al.*2006).

2.9.4 Person to person transmission

Person to person transmission of the *E. coli* O157:H7 is very common route of infection. Fecal shedding of *E. coli* O157:H7 by patients with hemorrhagic colitis or HUS is usually the main reason of spreading of the pathogen. However, in some cases, the pathogen can defecate in feces, from which there is a very chance of secondary transmission, which may involve direct hand to hand contact, particularly among children in day care centers (Karch *et al.*1995).

2.10 Characteristics of diseases

As stated earlier, the range of human illness due to *E. coli* O157:H7 infection includes non-bloody diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP); asymptomatic cases are also reported (Besser *et al.* 1999). The incubation period of EHEC diarrhea is generally 3 to 4 days, but exceptionally it may be as long as 5 to 8 days or as short as 1 to 2 days. The initial indications of the disease are usually non-bloody diarrhea accompanied by cramp. Many patients may have abdominal pain and a short-lived fever. Vomiting may occur in about half of the patients during the period of non-bloody diarrhea or at other times of the illness. Within 1 or 2 days bloody stool appeared with the severe abdominal pain which usually lasts for 4 to 10 days; in some severe cases whole stool becomes bloody (Riley *et al.* 1983).

HUS largely affects children and is the leading cause of acute renal failure (Gransden *et al.*1986). The syndrome is characterized by the features of acute renal insufficiency, hemolytic anemia, microangiopathic and thrombocytopenia. Significant pathological changes include swelling of endothelial cells, widened sub-endothelial regions and hypertrophied mesangial cells between glomerular capillaries. These changes combine to narrow the lumina of the glomerular capillaries and afferent arterioles and result in thrombosis of the arteriolar and glomerular microcirculation. Complete blockade of renal microvessels can results glomerular and tubular necrosis with an increased possibility of subsequent hypertension or renal failure (Moake 1994).

TTP mainly affects adults particularly elderly people and resembles HUS histologically. It results definite neurological abnormalities and clots bloods in the brain (Kovacs *et al.*1990). This may also cause fever. A considerable numbers of (50%) cases in elderly people can be fatal (CDC, 2007).

2.11 Infectious dose

The infectious dose is very low. For example, between 0.3 to 15 CFU of *E. coli* O157:H7 per gram was enumerated in lots of patties made from frozen ground beef in a multistate outbreak in the Western United States in 1993. Like-wise 0.3 to 0.4 CFU *E. coli* O157:H7 per gram was detected in salami that were associated with a food borne outbreak (Meng *et al*. 1997).

2.12 Mechanism of disease production

The mechanism of pathogenicity of STEC and *E. coli* O157:H7 had given importance for numerous studies. These studies stated that production of one or more shiga toxins is the essential factors responsible for development of disease. In addition, adherence to host cell membrane, colonization in large intestine and possessing of pO157 correlate with disease production. The role of potential virulence factors are stated below.

2.12.1 Shiga toxin

Production of shiga toxin is the main virulence factor and a distinguishing characteristic of *E. coli* O157:H7. The cytotoxic activity of the toxin was first documented in Vero-cells in 1977 by Konowalchuk *et al*.

The Shiga toxin (stx) consists of stx-l and stx-2 (Obrig *et al*. 2004). A single EHEC strain may express either or both toxins or even multiple forms of stx-2. Shiga toxin of EHEC is identical to the shiga toxin of *S. dysenteriae*1 (Nataro *et al.*1998). Toxin stx-l display 98% and Stx-2 toxin has 55% sequence identity to A subunit and 57% sequence identity to B subunit (Jackson *et al.* 1987). While stx-l is highly preserved, sequence variation exists within stx-2. The different variants of shiga toxin are observed such as stx-2c, stx-2v, stx-2vhb, stx-2e etc. (Calderwood *et al.*1996). The toxin stx-2 is associated with clinical isolates of STEC (Ho *et al*. 2013).

The basic A-B subunit structure is conserved across all members of the stx family. For the prototype toxin of the Shiga toxin family, the single 32-kDa A subunit is proteolytically nicked to yield an approximately 28-kDa peptide (Al) and a 4-kDa peptide (A2); these two peptides are linked by a disulfide bond. The Al peptide performs the enzymatic activity and the A2 peptide serves to bind the A subunit to B subunits (pentamer of five identical 7.2-kDa subunits). The B subunit binds the toxin to a specific glycolipid receptor, globotriaosylceramide or Gb3 on the surface of eukaryotic cells. Gb3 is the main receptor for stx, the stx-2e variant uses Gb4 as its receptor.

After binding, the holotoxin is endocytosed by the coated pits and is transported to the endoplasmic reticulum via the golgi apparatus. The A subunit is translocated to the cytoplasm, where it acts on the 60s ribosomal subunit. The Al peptide is an N-glycosidase which inhibits protein synthesis by removing a single adenine residue from the 28S rRNA of eukaryotic ribosomes, resulting the cause of the death of renal endothelial cells, intestinal epithelial cells, Vero or any other cells those have Gb3 (or Gb4 for stx-2e) receptor.. The *stxAB* genes encoding the A and B subunits are also called *sltAB* and *vtxAB.* Apart from stx-2, production of stx-l from *E. coli* and *S. dysenteriae* is thought to be repressed by iron and low temperature (Nataro *et al.*1998).

The involvement of stx in enterocolitis was confirmed when fluid accumulation and histological destruction occurred after injecting purified toxin into ligated rabbit intestinal loops. The fluid accumulation resulted due to destruction of absorptive villus tip in intestinal epithelial cells by stx (Louise *et al.* 1995).

Direct cytotoxic action of stx on renal endothelial cells is noticed in HUS, but some studies have supported a role for cytokines in this process. Purified stx has been reported to induce the expression of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and IL-6 from murine peritoneal macrophages (Tesh *et al.* 1994) as well as specific synthesis of TNF in the kidney (Harel *et al.*1993). From *in vitro* analysis, it

was found that the TNF-α and IL-1β enhances the cytotoxic effect of stx on human vascular endothelial cells (Melton-Celsa *et al.* 1996) and in addition to these two cytokines, TNF-β and bacterial LPS, have been shown to induce the expression of Gb3 and intensify the binding of stx to human endothelial cells (van de Kar *et al.* 1992). Neurological indications in patients and laboratory animals infected with *E. coli* O157:H7 were found to be caused by secondary neuron disturbances resulting endothelial cell damage by stx (Fujii *et al.* 1994).

2.12.2 Pathogenicity island- locus of enterocyte effacement (LEE)

Genetic studies have shown that the gene responsible for attaching and effacing (A/E) lesions map to 13 region, which is titled as locus of enterocyte effacement (LEE). EHEC may acquire this pathogenicity island by horizontal gene transfer from other species. The LEE region composed of 41 different genes organized into three major segments. These are (i) The middle segment includes the *eae* gene, which encodes intimin and the *tir* gene, and a translocated receptor for intimin. (ii) Downstream of *eae* are the *esp* genes, which encodes secreted proteins responsible for inducing epithelial cell signal transduction events leading to the A/E lesion. (iii) Upstream of *eae* and *tir* are several genes (*esc* and *sep*) those encode a type III secretion system that is involved in extracellular secretion of proteins encoded ese gene.

Intimin is an outer membrane protein (94-kDa to 97-kDa) encoded by *eae* (Donnenberg *et al.* 1993). Intimin is the only potential adherence factor of *E. coli* O157:H7 that plays a role in intestinal colonization *in-vivo* in an animal model. *E. coli* O157:H7 strains produce extensive A/E lesions in the large intestine for close adherence of the bacteria to the epithelial cells. The mutated *eae* gene of *E. coli* O157:H7 strains lacks A/E lesions producing and colonization forming capabilities in any intestinal site (Nataro *et al.*1998). Anti-intimin immune response is found in HUS patients (McKee *et al.*1996).

The Tir protein (78 kDa prot) is encoded in the locus of enterocyte effacement (LEE), upstream from the *eae* gene. It is translocated via type III secretion pathway into the

eukaryotic cell membranes where it serves as intimin receptor (Rosenshine *et al.*1996). Using the type III secretion system, EHEC secrete several Esp proteins such as EspA, EspB, EspC, and EspD, of which EspB and EspB form integral membrane protein (Wolff *et al.* 1998). This integral membrane protein forms a pore structure through which other bacterial effectors gain access to the host cell.

2.12.3 *E. coli* heat-stable enterotoxin 1 (EAST1)

Heat-stable enterotoxin (EAST1) producing *E. coli* was first described in EAggEC (Savarino *et al.*1993). Later it was found in 75 O157:H7 EHEC strains. The toxin is encoded by the *astA* gene (Savarino *et al.* 1996). Disease caused by EHEC is unknown but it could possibly account for some of the non bloody diarrhea commonly seen in persons infected with these strains.

2.12.4 60-1VIDa Plasmid (pO157)

E. coli O157:H7 has a plasmid (pO157) of approximately 60MDa, its size may differs in size from 93.6 to 104kb and contains DNA sequences common to the plasmid present in other serotypes of EHEC obtained from HC patients (Hofinger *et. al.*1998). pO157 contains potential virulence genes, including those encoding a enterohemolysin and catalase-peroxidase (Schmidt *et al.*1996).

Enterohemolysin is found in both O157:H7 strains and in non-O157 Shiga-toxin producing *E. coli* strains (Beutin *et al.* 1994). Hemolysin encoding gene is *ehxA*, which is approximately 60% identical to the *hlyA* gene encoding hemolysin expressed by uropathogenic *E. coli* (Nataro *et al.*, 1998). Breakdown of erythrocytes *in vivo* would release heme (iron source) and hemoglobin, which improve the growth of *E. coli* O157:H7. The hemolysin toxin break-down bovine leukocytes but not human leukocytes (Bauer *et. al.* 1996).

The catalase-peroxidase is a bifunctional periplasmic enzyme of EHEC encoded by *katP*, This enzyme protects the bacterium against oxidative stress, a possible defense mechanism of mammalian cells during bacterial infection (Brunder *et. al.* 1996)

2.13 Detection of *E. coli* O157:H7 and other STEC

Escherichia coli O157:H7 has inability to ferment sorbitol, lack of β–glucuronidase enzyme and weak or no growth at temperatures above 44°C which are unique characters of *E. coli* O157:H7 from other *E coli* (Meng *et al.* 1997).

2.13.1 Culture method:

The most frequently used agar medium for the detection and isolation of *E. coli* O157:H7 and STEC is Sorbitol-MacConkey agar (SMAC). This medium contain 1% sorbitol in place of lactose suger in the standard MacConkey medium. The US Department of Agriculture (USDA) recommended 24 h enrichment on selective medium at 35°C that stimulate growth of *E. coli* but are inhibitory to other species. Sorbitol Mac-Conkey Agar (SMAC) was recommended as the best selective medium for preliminary identification of *E. coli* O157:H7, in which *E. coli* O157:H7 forms colourless colonies (lack of sorbitol fermentation), while other *E. coli* forms red coloured colonies (March and Ratnam 1986). Enrichment and selective procedures are used for isolation of *E. coli* O157:H7 for its occurrence in low number in foods. The selective specific culture media for *E. coli* O157:H7 and selective enrichment medium (Doyle and Schoeni 1987, Padhye and Doyle 1991, Chapman *et al*. 1991) are added with bile salts, novobiocin, cefsulodine and cefixime as selective agents.

SMAC added with 5-bromo-4-chloro-indoxyl-β-D-glucuronide (BCIG) is used to distinguish strains that lack β-glucuronidase (like *E. coli* O157:H7). These organisms develops white colonies, while the colonies which possess β-glucuronidase activity turn green or blue (Okrend *et al*. 1990, Tesh *et al*. 1991). A rapid fluorescent test (Thompson *et al.* 1990) is used recently for detecting *E. coli* O157:H7, by using 4-

methylumbelliferyl-β-glucuronide (MUG). Here β-glucuronidase produces a fluorescent hydrolysis compound (Rippey *et al*. 1987). Positive colonies are fluorescent after ultraviolet light exposure, while the negative *E. coli* O157:H7 give no fluorescence.

Now a days SMAC with added cefixime and tellurite (CT-SMAC) is used, where low concentration of tellurite allows the growth of *E. coli*, and O157 STEC strains, but inhibit the growth of other non-sorbitol fermenters like *Aeromonas* spp., *Morganella*spp., *Providencia* spp., and most other *E. coli* strains (Zadik *et al*. 1993). *E. coli* O157:H7 cannot ferment rhamnose on agar plate. So SMAC is supplemented with rhamnose and tellurite (CR-SMAC) that increase the growth of *E. coli* O157:H7 (Chapman *et al*. 1991).

Recently a new selective media was developed by Biologinc, called "Rainbow agar O157", which is more specific than SMAC for detecting *E. coli* O157:H7. It is more useful for isolating and differentiating other STEC serotypes from non-toxigenic *E. coli*. In this medium most bacteria, other than O157 and non O157 STEC, are repressed, if grow form white or cream color colonies. *E. coli* O157:H7 colonies are unique, with a distinctive blackish color, whereas typical non-O157 STEC colonies are blue or purple. In this media most non-toxigenic *E. coli* colonies are reddish (Meng and Doyle 1998).

2.13.2 Immunological technic:

The enzyme-linked immunosorbent assay (ELISA) is very suitable for rapid screening of *E. coli* O157:H7 and non O157 in food and stool samples. Several immunological methods have been developed to detect O and H antigens to confirm presumptive O157 isolates from culture methods. These methods are fast (from 15 min. to 2 h) and are employed after a pre-enrichment step (24 h). According to Khan *et al.* (2003) cytotoxic activity of shiga toxin existing in stool on vero cell line is also a very sensitive method to detect *E. coli* O157H7 although this method is relatively time intense.

2.13.3 Molecular technic:

Molecular techniques have made it possible to have real-time identification with high sensitivity and specificity. Molecular methods are modern methods that offer extremely sensitive and intensive techniques. These technics are able to detect and to quantify pathogenic bacteria with a sensitivity and specificity not achievable by culture techniques and biochemical or serological tests. Several genes and DNA sequences have been beset to develop molecular methods for detecting STEC, particularly *E. coli* O157:H7, such as: attaching-and-effacing (*eae*) gene (Louie *et al*. 1994, Yu and Kaper 1992), shiga toxin (*stx)* genes (Karch and Meyer1989, Newland and Neill 1988), the β-glucuronidase (*uidA*) gene (Cebula *et al*. 1995, Feng 1993), the DNA sequence upstream of the *eae* gene (Zhao *et al*.1995, Meng *et al*. 1996), the 60-MDa plasmid (Johnson *et al*. 1995), and the haemolysin (*hlyA*) gene (Levine *et al.* 1987 ; Schmidt *et al.* 1995).

The hybridization technique consists in developing specific DNA oligonucleotides (DNA probes) labeled by radioactive isotopes, or enzymatic markers. These probes undergoes hybridization with single stranded DNA from target bacteria, fixed onto nitrocellulose or nylon membranes. The presence of homologous sequences allows the probe to match to the target DNA section, allowing the detection of the gene of interest. DNA probes able to detect *stx1* and *stx2 S*TEC genes were developed (Karch and Meyer 198; Newland and Neill 1988;Willshaw *et al*. 1987).

The suitability, time-saving and relatively low-cost PCR techniques makes potential to develop functional and specific assays to detect *E. coli* O157:H7. Some PCR assays are now available commercially as gene detection diagnostics. The first PCR experiment on *E. coli* O157:H7 was performed by Karch and Meyer (1989) with degenerated primers (a mix of oligonucleotides able to amplify DNA fragment without knowing the exact sequences of the annealing sites) built up to detect *stx1* and *stx2* gene. Read *et al*. (1992) designed a PCR with primers developed on the conserved region of *stx1*, *stx2* and *stxE* genes, in order to detect STEC in food and feces samples.

Meng *et al.* (1997) used primers that amplify a DNA sequence upstream of the *eae* and *stx* genes. This multiplex assay revealed a better specificity than the ones based only on *eae* gene. Fratamico *et al.* (1995) shared Meng *et al.* (1997) principle and developed a multiplex PCR based on three genes (*eae*, *stx* and a portion of a 60 MDa plasmid). This multiplex gives three positive reactions only for *E. coli* O157:H7 and O157:NM. Feng (1993) established a molecular probe on *uidA* gene, specific for *E. coli* O157:H7 (called PF-27).

Cebula *et al.* (1995) developed a multiplex PCR for *stx1*, *stx2* and *uidA* genes capable of distinguishing both *E. coli* O157:H7 and *E. coli* O157:NM. In this case it is possible to distinguish these two serotypes from other *E. coli* and to show the existence of one or both shiga toxins. Nagano *et al.* (1998) coupled a PCR reaction with primers for *rfb* gene and for *stx* genes in order to detect and distinguish between O157:H7 able to produce Shiga toxins and non-toxigenic strains. Multiplex PCR technic was also used to detect *E. coli* O157:H7 along with other bacteria by Li. Y *et al*. (2005). A multiplex PCR procedure for six genes fliC, stx-1, stx-2, eaeA, rfbE and hylA was developed by Bai *et al.* (2010).

Real-time PCR technic reduces the time and cost of PCR procedure. As it monitors the amplification of targeted DNA molecule during PCR, one can understand the presence of expected organism during PCR time rather an completion of PCR and gel electrophoresis. Leo *et al*. (2006) used real-time PCR methods for detection of E. coli O157:H7 using shiga toxin genes (stx-1 and stx-2)

2.14 Strain sub typing

As the O157:H7 clone is so highly conserved, a variety of techniques have been used to differentiate strains of this serotype for epidemiological studies.

2.14.1 Plasmid profiling

Plasmid profiles have been used to differentiate strains of O157:H7.. There are three basic plasmid profiles, such as profile-I (68.7 and 4.3 MDa), profile-II (66.2 and 1.8 MDa) and profile-III of 62.5 MDa (Ratnam *et al.* 1988). Plasmid profiles are only useful as epidemiological indicator in fresh bacterial isolates those carry a number of different plasmids.

2.14.2 Phage typing

The phage typing is used for *E. coli* O157:H7 in Canada, the United States, Japan, Australia, England, and some European countries. Around 82 phase types are identified till now. This technique is available only in reference centers that possess the typing phages (Nataro *et al.* 1998).

2.14.3 Biotyping

Biotyping denotes to the pattern of metabolic activities expressed by isolate. Biotyping distinguishes isolates based on their ability to produce different enzymes, colony characteristics, utilization of carbohydrates (sugar fermentation); utilization of amino acids (decarboxylation or deamination); standard enzymetic tests such as IMViC, ureas; tolerance to pH, chemicals and dyes; growth on different media and some others factors. Biotyping may be performed manually or using automated systems (Rao 2006).

2.14.4 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing determines the pattern of resistance to selected individual antibiotics or groups of antibiotics.

2.14.5 Restriction fragment length polymorphism (RFLP)

RFLP analysis is based on the use of a suitable DNA probe in the southern hybridization of digested DNA. The genomic DNA is cut using restriction enzymes such as *PstI*, *Pvull*, *EcoRI*or *HindIII* or others. Probing the restriction fragments with DNA is supposedly

more differentiating than probing with rRNA (ribotyping) or *stx* fragments (Sarnadpour *et. al.*1993). Analysis of STEC strains using RFLP is a sensitive and stable method, easy to perform and can reliably identify outbreak strains from sporadic cases. RFLP has been applied to study the molecular epidemiology of a number of food-borne outbreaks of STEC (Khan *et. al.* 2003).

2.14.6 Ribotyping

Ribotyping encompasses restriction enzyme digestion of the genomic DNA, which is then probed using a plasmid containing the *E. coil* rRNA operon (Grimont *et al.* 1986). This identifies DNA polymorphism (Martin *et al.*1996). However, success of this method depends with the restriction enzyme used. A set of O157:H7 and O157: NM isolates, for example, discriminated by *stx* probe were found to show identical ribotyping results (Martin *et al.*1996). This evidence suggests that the rRNA genes are too conserved to allow discrimination of various O157 strains. The 16S ribosomal RNA (rRNA) gene is highly conserved within a species and among species of same genus. For this reason rybotyping can be used as the new gold standard for the specification of bacterium (Woo 2008). To study bacterial phylogeny and taxonomy, 16S rRNA gene sequence is very useful. Using the 16S rRNA sequences, numerous bacterial genera and species have been re-classified and renamed. Classification of uncultivable bacteria have been determined, and the discovery and classification of novel bacterial species have been facilitated by ribotyping (Woo 2008, Boudewijns 2006)

2.14.7 Pulsed field gel electrophoresis (PFGE)

PFGE has been used to examine the molecular epidemiology of *E. coli* O157:H7 infections by several groups of scientists. In this process macro restriction analysis of the genome is carried out using an infrequent cutting restriction enzyme (e.g. *Xba*I). The enzyme cleaves the genome into 10 to 20 fragments ranging in size from 20 to 700 kb. These larger DNA molecules are separated by agarose DNA electrophoresis. In 1995, the Centres for Disease Control and Prevention (CDC) set up a national electronic database

of PFGE subtypes known as "Pulsenet" to facilitate recognition of outbreaks (Lingwood *et al.* 1996).

2.14.8 Random amplification of polymorphic DNA (RAPD)

RAPD is more efficient and discriminatory than ribotyping and is quicker and less technically demanding than PFGE. In this method, the dendrograms can be made by RAPD-PCR products.

2.14.9 Other subtyping methods

Other PCR based subtyping methods such as repetitive DNA element PCR (rep-PCR),enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) or amplified fragment length polymorphism (AFLP) are available currently to subtype the EHEC strains (Khan *et al.* 2003). Kimura *et al*. (2000) described restriction site-specific PCR (RSS-PCR) which is a technic based on the principle of restriction fragment length polymerization (RFLP) but it does not involves use of endonuclease. This method is based on the use of primers that are 10 to 18 bp long and homologous to specific restriction enzyme recognition sequences. Primers are designed in such a way that they will only amplify genomic DNA fragment that lie between the restriction site sequences on which primers are based on. The basis for this technique is that genetically different bacteria exhibit variations in number and locations of different restriction site sequences throughout the genome. The application of this technique allows amplification of fragments of various lengths, yielding a unique collection of DNA fragments or “finger prints” pattern for each different serotype. Thus the RSS-PCR method can be used as a rapid and specific screening assey for *E. coli* O157:H7 isolated from food and clinical samples.

2.15 Treatment

Treatment of disease due to EHEC infection is limited mainly to supportive care. Although the enterohemorrhagic strains are usually susceptible to a variety of antibiotics,

there is no potential studies showing convincingly that the use of antibiotics has positive effects on disease treatment.

In a study, Proulx et al.(1992) demonstrated a trend toward a lower incidence of HUS in those receiving antibiotics. Follow up investigation performed during the 1996 outbreak in Japan showed that early treatment with fosfomycin, was associated with a reduced risk of HUS (Takeda et al.1998). However, retrospective studies suggest that patients may have great risk to develop HUS if they receive antibiotics (Nataro et al. 1998).

Treatment of renal dysfunctioning due to EHEC is primarily supportive although some experimetal therapies being practiced in clinical trials. Recent treatment may include dialysis, hemofiltration, transfusion of packed erythrocytes, platelet infusions and other interventions as clinically indicated. Severe disease may require renal transplant. In clinical trial synsorb-PK is used in treatment. This synsorb-PK is consist of a chemically synthesized analog of Gb3, the receptor of shigatoxin coupled with diatomaceous earth. This compound is ingested by patients with bloody diarrhoea hopping thatit could absorb toxin from the intestine and prevent the development of HUS. Initial phase I trials have been promising and phase II trials to assess efficacy are in progress (Armstrong *et al.* 1995). There is no vaccines available to prevent disease due to EHEC infection but a number of experimental approaches are being investigated in animals. Lack of an appropriate animal model hampered vaccine development. Parenteral stx toxoid vaccines have shown protective effects in rabbits (Bielaszewsha *et al.*1994) and in pigs (Bosworth *et al.* 1996). Attenuated *Vibrio cholerae* (Nataro *et al.*1998) and *Salmonella typhimurium* (Tzschaschel *et at.*1996) vaccine strains that express stx-B have been developed. The *V. cholerae* constructs have been applied orally to rabbits and have generated neutralizing serum antibodies and partial protection from the enterotoxic effects of shiga toxin. (Acheson *et al.*1996). The intestinal adherence factor intimin has also been expressed in attenuated *V. cholerae* strains (Nataro *et al.* 1998). A parenteral vaccine specific for O157 EHEC has been developed based on O157 polysaccharide conjugated to protein carriers (Konadu *et al.*1994). An ideal broad-spectrum EHEC vaccine should probably develop

both systemic immunity against stx and local intestinal immunity against intimin and other intestinal colonization factors.

2.16 STEC and *E. coli* O157:H7: Bangladesh Perspectives:

Although Bangladesh is a diarrhea prone country there are no statistics about the burden of shiga toxin producing *E. coli* and *E. coli* O157:H7 in total diarrhea cases. Previous reports revealed that several investigations have been performed on isolation, identification and molecular characterization of STEC in Bangladesh. Islam *et al. (*2010) found STEC in 34% of buffalo meat, 66% of beef 10% of raw milk and 8% of fresh fruit juice samples. Talukdar *et al.(*2013) also reported the presence of STEC in Broilar chicken. Rectal content of slaughtered animals in Dhaka city were found to be positive for stx-1 or stx-2 or both in 82% of buffalo, 72.7% of cow and 11.8% of goat. Among these STEC isolates, 14.4% of buffalo, 7.2% of cow and 9.1% of goat samples were STEC O157 and were positive for *stx2, eaeA, katP, etpD and* hly_{EHEC} virulence genes (Islam *et al*. 2008).

According to Quaadri *et al.* (2005) the enterotoxigenic E. coli are the prominent group associated w ith childhood diarrhea in Bangladesh and this accounting for approximately 20% of all diarrhoeal cases. Islam *et al*. (2016) found the presence of STEC in stool sample of 1.2% diarrheic children admitted at Mymensingh Medical college hospital. They reported antibiotic resistance of *E. coli*. Shiga toxin-producing *E. coli* associated diarrhea in Bangladesh has been investigated among hospitalized patients with diarrhea including children and the urban slum community of Dhaka city. Shiga toxin genes were detected by multiplex PCR in 2.2% of hospitalized patients and 6.9% community patients (Islam *et. al.* 2007). In this case they detected the serotype of the isolated STEC strains such as O32:H25, O2:H45, O76:H19, ONT:H19. Jahura *et al*.(2017) detected by using multiplex PCR technique that 66% of STEC isolated from livestock and poultry in Bangladesh were positive for Shiga toxin *(stx1, stx2*), heat stable and heat liable *(sta &*

stb) genes. Their serotype results showed that the STEC strains isolated were serotype O76:H19, O43:H2, O87:H16, O110:H16 and O152:H8.

In Bangladesh, *E. coli* O157:H7 associated infection has not been reported yet. The reasons might be the lack of proper surveillance for *E. coli* O157:H7 or this pathogen may be present but the infections due to this pathogen occur in very few numbers because of the acquired immunity in the population.

MATERIALS AND METHODS

3.1 Samples

Several types of samples were collected for the investigation, such as cow-dung, goat droppings, beef, goat meat, raw milk etc. In each case maximum aseptic measures were followed. Samples were collected from different markets and places of Dhaka city.

3.2. Reference strain

An American Type Culture Collection strain of *Escherichia coli* O157:H7 (ATCC-12079) was used as a reference strain where necessary in the tests for comparison. This reference strain was reconfirmed following the cultural, biochemical tests, fermentation tests, serological and molecular tests. The organism was then preserved in T_1N_1 agar media.

3.3 Collection of samples

About 20–25 gm of each of samples of cow-dung, goat droppings, soils from cowshed and about 250g of each of samples of beef and goat meat were obtained from different areas and markets, respectively, of Dhaka city. The samples were collected using aseptic technic in sterile zip-lock bags and were carried straightway to the laboratory and analyzed on the same day.

20 ml of milk sample from each cow was collected aseptically in a sterile 50 ml Falcon tube from milk-man and brought into the laboratory in thermal box within two hours and analyzed in the same day. The microbiological analyses of all categories of samples were done in the Department of Microbiology, and Department of Botany, University of Dhaka. Part of the study was carried out in the Laboratory of Center for Advanced Research in Sciences (CARS) University of Dhaka and animal house and laboratory of ICDDR, B Dhaka.

The details of the samples collected and their sources are given in the Table 1

Table 1: Area of sampling and number of samples

Area of sampling	Types and Number of sample					
	Cow dung	Beef	Milk	Soils of cowshed	Goat meat	Goat dropping
Malibagh Bazar	05	12	--	05	10	04
Rampura, Banasri	5	10	11	10	--	--
Polashi/ Chankharpul	04	4	10	--	10	--
Thatari bazar	--	--	08	--	--	--
NewMarket kacha bazar	--	5	--	--	8	--
Shantinagar bazar	03	4	--	--	8	--
Goran bazar	04	12	--	5	4	05
Mohammadpur	08	--	9	--	--	04
Jagannath Hall area	04	--	05	02	--	--
Anondo bazar/ Nilkhet	--	03	--	--	8	--
Total	**33**	**50**	**43**	**22**	**48**	**23**

Grand total samples: 229

3.4 Methodology

The methods used for the isolation, identification and molecular characterization of *E. coli* O157:H7 and STEC was designed in the light of FDA approved guidelines where the isolates were screened based on the unique properties and confirmed by detection of

virulence and other marker genes as well as by sero-diagnosis. The overall work-plan is outlined in the following figure.

Overall work plan

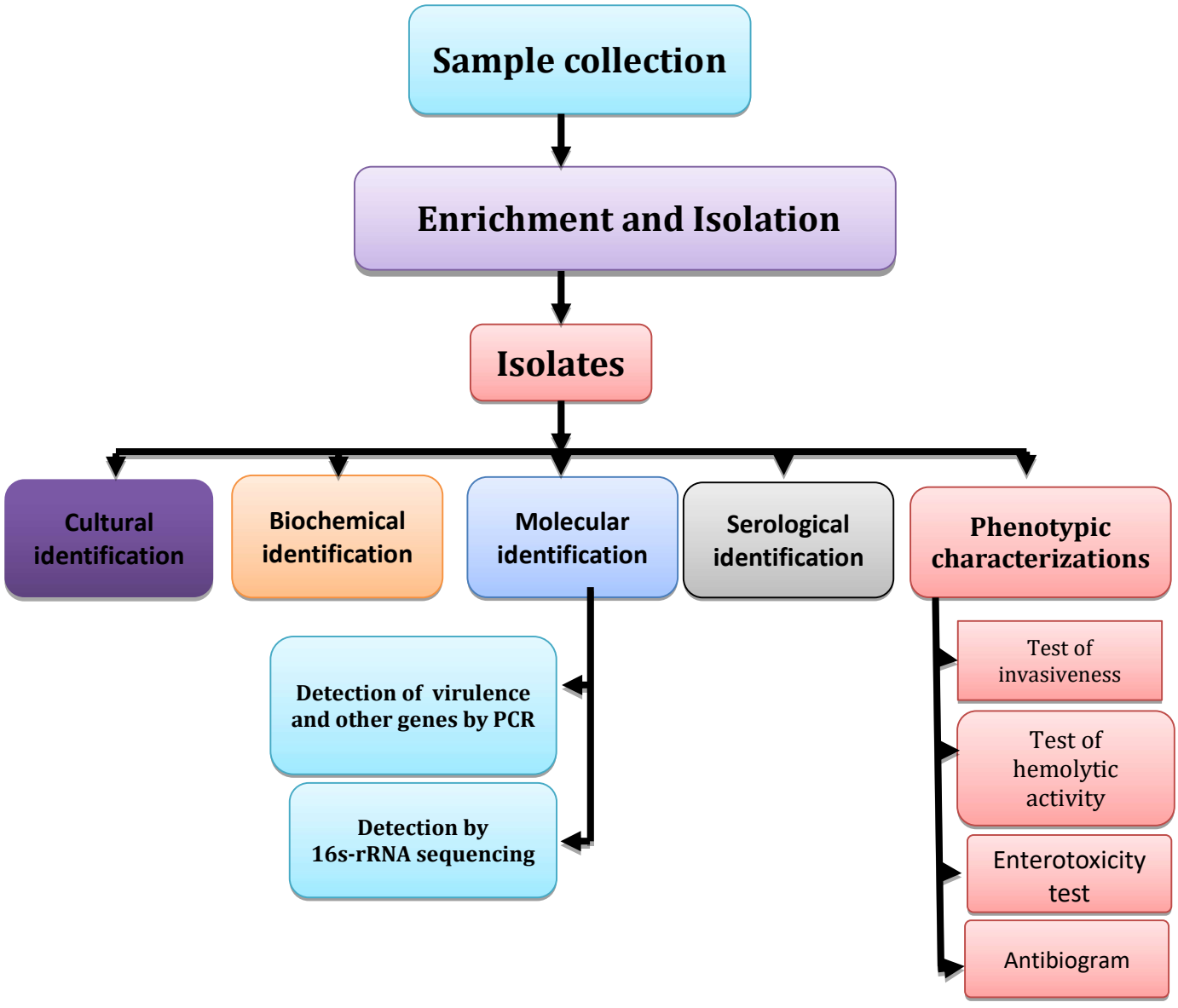

Figure 4 Schematic presentation of over all work-plan

3.5 Media used for the study

3.5.1 Modified Trypticase Soy Broth (mTSB)

Trypticase Soy Broth (TSB) supplemented with 1.5 gm/L of bile salt and 20 mg/L of novobiocin is a Modified Trypticase Soy Broth (mTSB), which is an enrichment broth medium widely used-for STEC and *E. coli* O157:H7. Bile salt inhibits Gram positive and non-enteric bacteria and novobiocin selectively allows the development of *E. coli* O157:H7 colony (Nataro *et al.* 1998).

3.5.2 Media used for primary isolation

CT-SMAC agar medium

CT-SMAC is the worldwide used selective medium for isolation of *E. coli* O157:H7 and other STEC from enriched medium, in which lactose suger has been eplaced by sorbitol. *E. coli* O157:H7 is incapable of fermenting sorbitol and produces colorless colonies on CT-SMAC agar plates; on the other hand, 80% of *E. coli* strains other than *E. coli* O157:H7 ferment sorbitol and thus form pink coloured colonies within 24 hours of incubation. CT-SMAC added with potassium Tellurite (2.5 mg/L) and antimicrobial cefiximie (0.05mg/L) becomes more selective, where cefixime inhibits *Proteus* spp. and tellurite inhibits *Providencia* spp. and *Aeromonas* spp (Nataro *et al.* 1998).

3.6 Preparation of stock solutions

3.6.1 Preparation of stock solution of novobiocin

One hundred milligram of novobiocin sodium salt was dissolved in 5 ml of deionized water. The solution was filter-sterilized and aliquots of 600 µl were made in sterile Eppendorf tubes. The tubes were covered with aluminum foil and stored at -20^{o}C. Five hundred microlitre of this solution was added to 500 ml of TSB to give a final concentration of 20mg/L.

3.6.2 Preparation of CT (Cefixime and potassium tellurite) supplement

The lyophilized CT was melted in the original vial by adding 1 ml of sterile distilled water to prepare CT supplement. The dissolved content of 1 vial, having 2.5 mg/L cefixime and 0.05 mg/L potassium tellurite, was then added to 500 ml of autoclaved, warm liquid SMAC medium, cooled to 45^{o}-50^{o}C for preparation of CT-SMAC agar medium.

3.7 Isolation of *E. coli* O157:H7 and other STEC from samples

Twenty five grams (25 g) of chopped meat was mixed with 225 ml of mTSB in a 400 ml stomacher bag and was stomached in a stomacher machine (400 CIRCULATOR, Seward) at the rate of 250 rpm. After that, the stomached bag was kept in incubator at 37^{o}C for 6-8 h. Then 1.0 ml of enriched broth was taken in 9.0 ml of PBS (10^{-1}) and further diluted to tenfold dilution series upto10^{-4} in PBS. From each dilution 0.1 ml of suspension was spreaded onto duplicate CT-SMAC plates and the plates were incubated at 37^{o}C overnight.

3.8 Identification of *E. coli* O157:H7 and STEC isolates

3.8.1 Cultural properties on CT-SMAC plate

Morphological features of colonies such as size, shape, elevation, color, consistency, and opacity of colonies developed on CT-SMAC plates were carefully observed and recorded. Each suspected *E. coli* O157:H7 colonies was subcultured onto fresh CT-SMAC plate and was incubated overnight at 37^{o}C for development of individual colony (pure culture). An inoculum from the individual colony of suspected *E. coli* O157:H7 and STEC isolates on CT-SMAC plate was transferred onto CT-SMAC slant and kept at 4^{O}C for further study.

3.8.2 Screening of suspected isolates on EMB plate

Assumed *E. coli* O157:H7 and STEC isolates from the CT-SMAC plate were streaked onto Eosin Methylene Blue (EMB) agar plate for primary identification. These isolates

form black colonies with green metallic sheen on EMB medium. EMB agar medium contains lactose sugar and the dyes eosin and methylene blue make difference between enteric lactose fermenter and non-fermenter. The metallic green sheen caused by precipitation of large amount of acid that is produced– onto the growth's surface (Cappuccino *et al.* 1996).

3.8.3 Screening of isolates by 4-methylumbilliferyl-β-D-glcuronide (MUG) test

The isolates which form green metallic sheen on EMB agar plate were selected for further study. Each isolate was streaked on MUG agar the plate surface and incubated overnight at 37^{o}C. The MUG test is based on the enzymatic activity of β-glucuronidase (GUD), which breaks the substrate 4-methylumbelliferyl-D-glucuronide (MUG), to release 4-methylumbelliferone (MU). After incubation, the culture plate was exposed to long-wave (365 nm) UV light, MU displays a bluish fluorescence that is easily envisioned in the medium or around the colonies. GUD non-producer isolates are enterohemorrhagic *E. coli* (EHEC) of serotype O157:H7 and GUD producer isolates are other *E. coli* strains. The lack of GUD phenotype in O157:H7 is often used to dishtinguished this serotype from other, although GUD positive variants of *E. coli* O157:H7 do exist (Feng *et al.* 2002).

3.8.4 Biochemical identification

Biochemical tests were accomplish with EMB positive and MUG negative isolates according to the methods described in Microbiology Laboratory Manual (Cappuccino *et al.* 1996). The biochemical tests include citrate utilization test, indole production test, methyl-red test, Voges-Proskauer test and triple sugar iron agar test.

3.8.4.1 Citrate utilization test

The citrate test defines the ability of microorganisms to use citrate as the sole source of carbon and energy. Simmon's citrate agar was used for citrate utilization test. This medium is a chemically defined medium which comprises of sodium citrate as the carbon

source, NH_4^+ as the nitrogen source and bromophenol blue as the pH marker. Citrate utilizing microorganisms remove the acid from the medium, which raises the pH and turns the pH marker from green to blue. A color change in the medium from green to blue indicates that the test organisms can utilize citrate as its only carbon source.

3.8.4.2 Indole production test

A tryptophan medium is used for indole production test. Tryptophan is a component of most of the proteins and is therefore available to microorganisms as a result of protein breakdown. Some bacteria are able to catalyzes the removal of the indole residue from tryptophan by an enzyme called tryptophanase that is produced within the media. Indole accumulates in the culture media while the rest of the tryptophan molecule (pyruvate and NH_3) is used to satisfy nutritional requirements. The production of indole from tryptophan by *E. coli* O157:H7 and STEC can be detected by growing them on tryptophan rich medium. The accumulation of indole in the medium can be identified by adding Kovac's reagent. This reagent reacts with indole and give a water-insoluble bright red compound on the surface of the medium.

3.8.4.3 Methyl red test

MR-VP medium was used for methyl red test. This is a mixed acid fermentation test. Methyl red is pH marker which is used to detect acidity in media. This reagent is red at pH 4.4 or below and yellow at 6.2 or above. Some bacteria ferment glucose and increase acidity in the medium resulting in the lessening of pH below 5.0. The bacteria are grown in MR-VP broth and after 24 h incubation some methyl-red reagent was added to the culture medium. A red color will develop if the fermentation occurs and considered as MR- positive.

3.8.4.4 Voges-Proskaure test

The Voges-Proskaure test is used to detect a specific organism that carry out 2,3-butanediol fermentation. A portion of bacterial culture grown on MR-VP broth was used

for VP test. VP positive organisms ferment sugars to produce 2,3-butanediol as a major end product in the medium. The addition of 40% KOH and 5% alpha-naphthol in absolute ethanol in the broth culture reveal the presence of acetoin (acetyl methyl carbinol), a precursor in the synthesis of 2,3-butanediol, which in the presence of KOH, develops pink color imparting a rose color to the medium. The reaction occurs in the presence of alpha-naphthol catalyst and a guanidine group of the peptone of the MR-VP medium. Results of Voges-Proskaure test are recorded.

3.8.4.5 Triple Sugar Iron (TSI) agar test

The triple sugar iron test distinguishes different groups or genera of the Enterobacteriaceae. It also differentiate the Enterobacteriaceae from other Gram-negative intestinal-bacilli. This difference is made on the basis of variation in patterns of carbohydrate fermentation and hydrogen sulfide production by the different groups of intestinal organisms. TSI agar medium contains 1% of lactose, 1% of sucrose and 0.1% of glucose. This medium also contains sodium thiosulfate and ferrous sulfate for detection of hydrogen sulfide production which is indicated by blackening of medium. To facilitate observation carbohydrate TSI agar medium is made in slant and butt.

Table 2 Three types of results are observed in TSI test

Reaction	Result
Alkaline slant (red) and acid butt (yellow) with or without gas production (breaks in the agar butt)	Small amount of acid production from glucose fermentation and peptone utilization have caused alkaline reaction on the slant surface. In the butt, acid reaction is maintained due to reduced condition and slow bacterial growth.
Acid slant (yellow) and acid butt (yellow) with or without gas production.	Large amount of acid production from lactose and /or sucrose fermentation has caused acid reaction on the slant surface.
Alkaline slant (red) and alkali butt (red) or no change (orange-red) butt.	No carbohydrate fermentation has occurred. Instead peptone has catabolized, resulting in alkali reaction.

3.9 Polymerase chain reaction (PCR) using *eae*A, *stx*-1, *stx*-2, *rfb*E, *fli*C gene primers

PCR is an enzymatic method of making multiple copies of a pre-selected segment of DNA. The amplification process is accomplished with two synthetic oligonucleotide primers, a thermo-stable enzyme DNA polymerase (*Taq polymerase*). This process involves three major steps: denaturartion, annealing and extension of DNA segment which are repeated for 25-40 cycles. This whole process is performed on an automated cycler, which can heat and cool the tubes with the PCR mixture in a very short time.

- **Denaturation**: This step usually occurs at 94^{o}C. In this step the base pair of DNA segment is broken and release single-stranded DNA to act as templates for the next round of DNA synthesis.
- **Annealing**: At this stage, the primers attach to the templates. The temperature is estimated by determining the melting temperature of primer-template hybrid.

- **Extension:** At this stage, DNA synthesis occurs by the enzymatic action of *Taq polymerase*. The temperature is usually set at 72^{o}C, just below the optimum temperature for *Taq polymerase*.

3.9.1 Preparation of template DNA

Each isolate was incubated into 5 ml of Luria Bertani (LB) broth and incubated overnight at 37^{o}C. The cell pellet was harvested by centrifuging 500 µl of broth culture at 10,000 rpm for 5 min. The supernatant was discarded and the pellet was washed with 500 µl of phosphate buffered saline (PBS) by centrifuging at 10,000 rpm for 5 min. The cell pellet was re-suspended in 200 µl of Tris-EDTA (TE) buffer (pH 8.0) and then kept in boiling water for 10 min. The boiling cell pellet was put on ice immediately. After cooling on ice for 5 min, the suspension was centrifuged at 10,000 rpm for 5 min and 2 µl of the supernatant was used as template DNA

3.9.2 Preparation of reaction mixture

Sterile 1.5 ml micro centrifuge tubes (Eppendorf, Germany) were taken and a mastermix was prepared which include PCR grade water, 10x buffer, 25 mM MgCl2, 2.5mM dNTPs, 200nM forward and reverse primers, and *Taq* polymerase for PCR reaction. The amount of master mix was set according to the number of DNA template. The master mix was aliquoted into PCR tubes before adding extracted DNA from different samples. After adding the template DNA, the PCR tube containing reaction mixture was capped and centrifuged briefly. The PCR tubes were placed in thermal cycler (BioRad, USA).

3.9.3 The sequences of the primers used for PCR

The sequences of the primers used for the detection of *eaeA, rfbE,fliC, stx-1* and *stx-2* are stated below (table 3) with their amplicon size.

Table 3: Primer pair used for PCR of *eaeA, rfbE, fliC, stx*$^{-1}$ and *stx*-^{2} genes

Target gene	Primer name	Primer Sequence (5'→3')	Amplicon size (bp)	Reference
rfbE	O157-F	5'-CGGACATCCATGTGATATAGG-3'	259	Paton and Paton, 1998
	O157-R	5'-TTGCCTSTGTACAGCTAATCC-3'		
fliC	FLICH7-F	5'-GCGCTGTCGAGTTCTATCGAG-3'	625	Gannon *et al.*1997
	FLICH7-R	5'-CAACGGTGACTTTATCGCCATTC-3'		
eaeA	VS8	5'-GGCGGATTAGACTTCGGCTA-3'	150	Kawasaki *et al.* 2005
	VS9	5'-CGTTTTGCCACTATTGCCC-3'		
Stx-1	LP30	5'-CAGTTAATGTGGTGGCGAAGG-3'	348	Vidal *et al.* 2004
	LP31	5'-CACCAGACAAATGTAACCGCTC-3'		
Stx-2	LP41	5'-ATCCTATTCCCGGGAGTTTACG-3'	584	Vidal *et al.* 2004
	LP42	5'-GCGTCATCGTATACACAGGAGC-3'		
16s rRNA	27F	5'- AGAGTTTGATC**M**TGGCTCAG -3'	1460	Lane 1991
	1492R	5'- CGGTTACCTTGTTACGACTT- 3'		

3.9.4 PCR conditions

The PCR reaction condition was specified for each amplification, mentioned in the table 4. A final extension at 72^{o}C for 1:30 minutes and after all the cycle run final extension was kept at 72^{o}C. After this, PCR tubes were stored at -20^{o}C until further analysis. Post – PCR detection of amplified DNA by electrophoretic analysis.

Table 4: PCR conditions for detection of *rfbE, fliC, eaeA, stx-1* and *stx-2* genes

Stage	PCR assays			
	rfbE	*fliC*	*eaeA*	*stx-1* and *stx-2*
Initial denaturing	94°C: 10 min	94°C: 10 min	94°C: 10 min	94°C: 10 min
Denaturing	94°C: 1 min	94°C: 30 sec	94°C: 20 sec	94°C: 1 min
Annealing	56°C: 1 min	65°C: 30 sec	60°C: 30 sec	55°C: 1 min
Extension	72°C: 1 min	72°C: 75 sec	72°C: 30 sec	72°C: 1 min
No. of Cycle	35 cycles	35 cycles	35 cycles	35 cycles
Final extension	72°C: 7 min	72°C: 7 min	72°C: 7 min	72°C: 5 min

3.9.5 Agarose Gel Electrophoresis (AGE) of amplified DNA products

The amplified PCR products were observed by running the PCR products on 1.5% agarose gel in 1X TB buffer (pH 8.2). The agarose gel was prepared by dissolving agarose (Sigma, USA) in 1X Tris-borate EDTA (TBE) buffer (Appendix) to give a final concentration of 1.5% agarose and was heated in a microwave oven for about 2.5 - 3.0 minutes. The melted agarose was allowed to cool to about 50°C. Two microliters of Ethidium Bromide (EtBr)(concentration 0.5 µg/ml) was added in the melted agarose and mixed completely by gentle agitation. The melted agarose with EtBr was poured onto gel electrophoresis unit (Sigma, USA) with spacers and comb. After solidification of the gel, the comb was removed. Then the gel was submerged in 1X TBE buffer in a gel electrophoresis unit. Five microlitter of PCR product was mixed with 1µl loading 6X gel loading dye and loaded into the well and run on 1.5% agarose gel in 1X TBE buffer (pH 8.2). Marker DNA of known size (100bp ladder) (Bioneer) was loaded in one well to determine the size of the PCR products. Electrophoresis was carried out at 95 volts for approximately 45 minutes.

The DNA bands intercalated with Et-Br were observed on a UV trans-illuminator (Vilber Lourmat, France). Photographs were taken using a gel documentation system (Vilber Lourmat, France) and the bands were analyzed. The PCR tubes with amplified DNA were stored at -20^{o}C until further analysis is needed.

3.10 Multiplex PCR

The multiplex PCR was performed with the primers for *eaeA*, *stx*-^{1} and *stx*$^{-2}$ genes in a single tube. For this reason total volume of the PCR mixture was increased up to 50 µl and PCR conditions were set considering the annealing temperature of all three sets of primers (table 5 and 6).

Table 5: Reaction mixture for multiplex PCR of *eaeA*, *stx1* and *stx2* genes

Reagent	**Volume (µl)**
PCR grade water	17.375
10X PCR buffer	3
$MgCl_2$	1.5
dNTPs mixture	3
Primer- VS8	0.15
Primer- VS9	0.15
Primer- LP30	0.25
Primer- LP31	0.25
Primer- LP41	0.25
Primer- LP42	0.25
Taq polymerase	0.125
Template	3.5
Total	30

Table 6: Multiplex PCR conditions for the detection of *eaeA*, *stx1* and *stx2* genes

Stage	Temperature	Time
Initial denaturing	94°C	10 min
Denaturing	94°C	1 min
Annealing	55°C	1min
Extension	72°C	1min
Cycle no. (Denaturing, Annealing, Extension)	35 cycles	
Final extension	72°C	10 min

3.11 Serological detection of *E. coli* O157:H7

Serological detection was performed with isolates presumptively identified as *E. coli* O157:H7 by biochemical tests and was confirmed by PCR of required genes. This test was carried out by Wellcolex^{TM} latex agglutination test kit. (Remel, USA). This latex agglutination test for the detection of serogroup O157 and serotype H7 antigen was done by allowing the reaction between the antigens and their specific antibodies onto the surfaces of polystyrene spheres (latex beads). The reaction can be revealed by observing agglutination of the sensitized latex beads onto the surface of paper slides. The advantage of this method is that the immune-complexes are more discernible than they are by means of precipitation reaction.

For the detection of O157 antigen, a homogeneous suspension was made with one colony *of E. coli* O157:H7 isolate from SMAC plate and one drop of physiological saline on the specified circle of the supplied paper card. One drop of anti-O157 sensitized latex suspension (test latex) was added to the saline mixed antigenic suspension and mixed well by moving the paper card back and forth for 30 sec. Agglutination observed within 30 sec was regarded as positive and delayed or weak agglutination was regarded as

negative. For a control, a similar suspension was made on another circle of the same card and a drop of control latex reagent was added and mixed thoroughly. No agglutination within the same time period indicates negative result.

For the detection of H7 antigen, *E. coli* O1 57:H7 isolate was grown on TSB. One drop of broth culture was taken on the specified circle of the supplied paper card. After that, the anti-H7 latex reagent was mixed by moving the paper card back and forth and agglutination observe within 10-15 sec was regarded as positive. Delayed or weak agglutination was regarded as negative. Same procedure was done in case of negative control where the latex control reagent was added and no agglutination resulted.

3. 12 Phenotypic characterization of the isolates

3.12.1 Enterotoxicity test

This test was performed by rabbit ileal loop assay using the isolates. Live bacteria were used as inocula.

Preparation of live cells: Ten ml of Trypticase soy broth (TSB) was used as culture medium and inoculated with 5-6 colonies from pure culture of the selected isolates. The broth was shaken at 100 rpm and incubated at 37^{o}C for 4 hrs. Then 1.0 ml of broth culture was used as inoculum.

Rabbit Ileal Loop Assay: A pair of adult albino rabbit (New Zealand strain) of 1.5 – 2.0 kg body weight were selected for testing. The rabbits were starved for 24 hrs allowing only water. After proper anesthesia with a lower dose of sodium pentobarbital (0.5 ml/kg body weight, intravenous), the intestine was exposed by cutting abdominal muscle. Intestinal loops of 6–8 cm in length with 2–3 cm intervals between each were made using nylon thread. One ml of sample was inoculated into one loop. The animals were kept in post operative chamber after closing the abdomen. The animals were sacrificed after 18 hrs with excess sodium pentobarbital. The length of each loop and volume of fluid accumulated were measured to determine the amount of fluid accumulation per unit

length of gut. The loop was considered positive if the fluid accumulation is 0.4 ml/cm of loop, otherwise the loop was considered negative.

3.12.2 Test for invasiveness by Congo red binding ability

Congo red binding is linked directly to virulence and pathogenicity, although the biochemical and physical mechanisms involved in determining the virulence remain unclear. Nutrient agar containing 0.01% (w/v) Congo red was used to study the pigment binding ability of the isolates. Plates streaked with the test isolates were incubated at 37^{o}C for 18 hrs. Colonies with dark red center was considers as positive and colonies without dark red center was considered as unable to bind congo red (Tiwari *et. al*. 2002).

3.12.3 Determination of hemolysis activity

Hemolysis means the destruction of erythrocytes by bacterial enzyme, hemolysin. Hemolytic activity is identified by growing the bacteria on a blood agar medium (Cappuccino *et al.*, 1996). There are three types of hemolytic reactions could be observed.

1. α –hemolysis, an incomplete form of hemolysis where a green zone is produced around the colony.
2. β – hemolysis, a complete destruction of red blood cells, exhibits a clear zone around the colony.
3. γ –hemolysis is the indicative of the absence of any hemolysis as no zone is formed in culture plate.

E. coli O157:H7 isolates were screened for hemolysis activity by streaking on sheep blood agar plate followed by incubation at 37˚C for 18 hrs.

3.12.4 Antibiotic Sensitivity Test

Antibiogram of the pathogens were determined using disc diffusion method onto Mueller-Hinton agar. Inoculum was prepared by growing the bacteria for 4-6 hrs in

Mueller Hinton broth so that they are in log phase of growth and then adjusted to the 0.5 McFarland standards. The tests were performed following Clinical and Laboratory Standard Institute (CLSI) guideline. Antibiotics discs (Oxoid Ltd. Basingstock Hamshire, England) used were ampicillin 10µg, streptomycin 0µg, chloramphenicol 30µg, ciprofloxacin 5µg, kanamycin 30µg, nalidixic acid 30µg, novobiocin 30µg were used. The diameter of the zone of inhibition was measured and the isolates were classified as 'resistant', 'intermediate' and 'sensitive' based on CLSI guideline.

3.13 Amplification and sequencing of 16S rRNA gene

Isolates which were positive by biochemical, serological assay and also analyzed for the presence of virulence genes were subjected to 16S rRNA gene sequencing.

DNA extraction from isolates was done by boiling method as mentioned earlier. Then PCR was done with universal primers for 16S rRNA (name and sequence of primers is mentioned in the table 3). The reaction mixture is stated in table 7.

Table 7 Reagent mixture with quantities for amplification of 16S rRNA gene.

Reagents	Quantity (µl)
PCR grade water	26.75
10x Buffer	5.0
$MgCl_2$ (25mM)	2.0
dNTPs	4.0
27F	1.0
1492R	1.0
Taq Polymerase	0.25
Template	10

3.13.1 DNA Sequencing

PCR amplified 16S rRNA gene fragment of the isolates obtained from PCR assay was subjected to sequencing. There are three basic steps in DNA sequencing:

1. Purification of PCR products
2. Cyclic sequencing
3. Purification of cycle sequencing product and detection of nucleotide sequence.

3.13.1.1 Purification of PCR product

The PCR product was purified by using commercially available centrifugal filter device according to the following way:

PCR products (1450bp) from were purified as a prerequisite for DNA sequencing. Purification was performed using Wizard® SV Gel and PCR Clean-Up System (Wisconsin, USA) according to procedure stated in the manual. Equal volume of membrane binding solution was added to the PCR product. The mixture was transferred to a SV minicolumn which was inserted into a collection tube and incubated at room temperature for 1 minute. It was centrifuged at 16000x g for 1 minute. The flow through was discarded and the minicolumn was reinserted into the collection tube. Then, 700 µl of membrane wash solution was added and centrifuged at 16000xg for 1minute. Again, the flow through was discarded and the minicolumn was reinserted into the collection tube. This step was repeated with 500 µl membrane wash solution and centrifugation at 16000x g for 5 minutes. After that the collection tube was emptied and the column assembly was centrifuged for 1 minute and allow evaporation of any residual ethanol.

Finally, the minicolumn was transferred to a clean 1.5 ml microcentrifuge tube. Fifty five microliters of nuclease free water was added to the minicolumn. After incubating at room temperature for 1 minute it was centrifuged at 16000xg for 1 minute. Then the minicolumn was discarded and purified DNA was stored at -20ºC.

3.13.1.2 Measurement of DNA concentration

Concentration of purified PCR products was measured as ng/µl using Nanodrops (Thermo Scientific, USA). The ratio between the readings at 260 nm and 280 nm (OD 260 /OD 280) provides an estimate of the purity of the DNA. Pure DNA preparations have OD 260/OD 280 values of 1.8.

3.13.2 Cyclic Sequencing reaction

After purification of the PCR products, cycle sequencing was performed using BigDye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystem, USA) according to manufactures instruction. The reaction mixture composition for cycle sequencing was as follows (table 8).

Table 8 Name and quantity of reagents used for cyclic sequencing

Reagents	Quantity (µl)
Termination BigDye	4.0
Template	1.0
Primers(for ward or reverse, 5pmol)	1.0
Deionized water	4.0
Total volume	10

Reaction mixture of cycle sequencing was prepared for either 96-well reaction plates or microcentrifuge tubes to perform cycle sequencing of purified PCR products. The reaction mixture was added to each tube and mixed well followed by brief spinning. The Big Dye® Terminator 3.1 Sequencing Buffer (5X) is supplied at a 5Xconcentration. For a half of the reaction in the final volume of 20 µl the sequencing buffer was used. Tubes were placed in a thermal cycler and the volume was set in the cycler. The cycle

sequencing started with initial denaturation at 96^{o} C for 1 minutes followed by 25 steps of 96^{o}C for 10 seconds, 50^{o}C for 10 seconds and 60º C for 4 minutes. The reactions were held at 4^{o}C until ready to purify the extension products. The product was then spun down in microcentrifuge. The extension products were purified by Ethanol/EDTA precipitation method. Before starting the precipitation method, reaction plate was removed from thermal cycler and spun briefly. Then 5 µl EDTA followed by 60 µl of 100% ethanol was added to each well. The reaction plate was sealed with aluminum tape and mixed by inverting 4 times. After that, reaction plate was incubated at room temperature for 15 minutes. After spinning the plate up to 185×g, 60 µl of ethanol was added to each well. The centrifuge machine was set at 4^{o}Cand spun at 1600 ×g for 15 minutes. In last step the plate was inverted again and spun at 185 ×g for exactly 1 minute. The plate removed from the centrifuge and sealed with aluminum tape at 4°C. The sample was then analyzed by ABI Genetic Analyzer (Applied Biosystems®, USA).

3.13.3 Detection of the nucleotide sequence

The purified cycled sequenced product was analyzed by electrophoresis in the ABI prism 3130 genetic analyzer (ABI prism USA). DNA was separated through the POP7 contained in a capillary and detected by laser beam. When the nucleotides reached a detector window in capillary electrophoresis, the laser beam excited the fluorescence labeled fragments. The emitted fluorescence was detected by CCD camera and the fluorescence intensified data was measured by specific software.

3.13.4 Sequence analysis

Raw sequences available in ABI chromatogram file format from automated DNA sequencer was edited using Chromas 2.31 software. BLAST search was performed with the FASTA format of edited sequence data to find out the sequence homology available in the GenBank.

3.14 Storage of *E. coli* O157:H7 isolates

For short term preservation, 2 ml of T_1N_1 soft agar medium was taken in a vial and was inoculated by stabbing with bacteria isolates grown on SMAC agar plate. Then the vial was incubated at 37^{o}C overnight. After incubation, the surface of the medium was covered with sterile paraffin oil and the vial was stored at room temperature.

For long term preservation, 700 µl of bacterial culture grown in TSB at 37^{o}C for 6 hrs was taken in a sterile eppendorf tube and 300 µl of sterile glycerol was added to the broth culture and stored at -20^{o}C.

RESULTS

This study is intended to isolate and characterize *E. coli* O157:H7 and shiga toxin producing *E. coli* (STEC) from cattle related samples. Samples were collected from different markets and places of animal harboring with in Dhaka city. Enrichment, selective plating, biochemical tests, serological tests and nucleic acid based methods have been applied for isolation and identification of *E. coli* O157:H7 and other STEC. The experimental findings are illustrated below.

4.1 Isolation of *E. coli* and STEC from collected samples

Thirty three (33) cow-dung, fifty (50) beef, forty three (43) milk, forty eight (48) goat meat, twenty three (23) goat dropping and twenty two (22) ground soil samples of cowshed collected from different markets and places of animal harboring around Dhaka city. The samples were enriched in trypticase soy-broth supplemented with antibiotic Novobiocin and bile salt at 37°C for 6 hours. After enrichment the culture broth were subjected to tenfold series dilution. From appropriate dilutions, the broth cultures were spread onto CT-SMAC plates and were incubated at 37°C overnight.

Table 9. Colony characteristics of *E. coli* O157:H7 and STEC on CT-SMAC plate.

Colony characteristics on CT- SMAC plate	Results
Size	Moderate
Shape	Round
Elevation	Raised
Margin	Entire
Color	Colorless with smoky center
Opacity	Opaque
Consistency	Gummy

After incubation, different types of colonies were observed on CT-SMAC plate (**Fig. 5)** Colony characteristics of *E. coli* O157:H7 and STEC on CT-SMAC plate are shown in Table 9. Colonies showing typical characteristics of *E. coli* O157:H7 and STEC were sub-cultured on CT - SMAC plates.

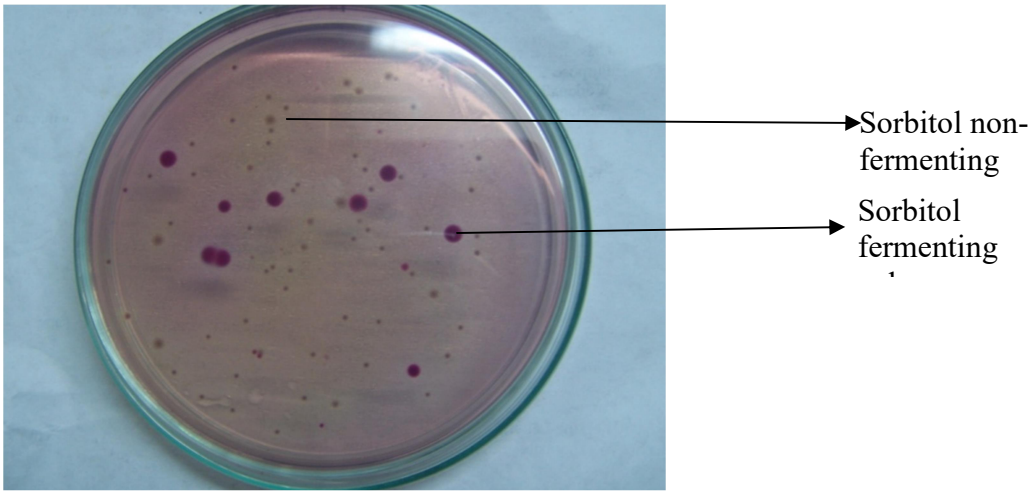

Fig. 5. Different types of colonies on CT-SMAC agar plate

A total of 21 of 33 cow dung samples produced sorbitol non fermenting colonies on CT-SMAC agar plate. Out of these 21 samples, a total of 1210 isolates were selected as suspected *E. coli* O157:H7 and STEC. Likewise, 50 beef samples were included in this study of which 42 produced sorbitol non-fermenting colonies on CT-SMAC agar plate. Of these 42 samples, 252 isolates were found to produce typical *E. coli* O157:H7 like colonies. Similarly, 43 milk samples from different brand and 22 soil samples from various cowsheds were also analyzed in this study. Of them, a total of 503 isolates were found to produce sorbitol non-fermenting colonies from 26 out of 43 milk samples. On the other hand, 55 isolates from 8 of 22 cowshed soil samples were selected as suspected *E. coli* O157:H7 and STEC as they produced sorbitol non-fermenting colonies on CT-SMAC plates. All the isolates were proceeded for further investigation.

In case of goat, a total of 48 meat samples were analyzed, of which 39 samples generated 1523 sorbitol non-fermenting isolates on CT-SMAC plate. Likewise, 56 isolates from 19 of 23 goat- dropping samples were selected as suspected *E. coli* O157:H7 and STEC.

4.2 Identification of suspected *E. coli* and STEC isolates

4.2.1 Growth on EMB plates

Considering colony characteristics of CT-SMAC plates suspected *E. coli* and STEC isolates were streaked onto EMB agar plates and after overnight incubation, isolates showing growth with green metallic sheen were selected for further identification and others were discarded (Fig. 6).

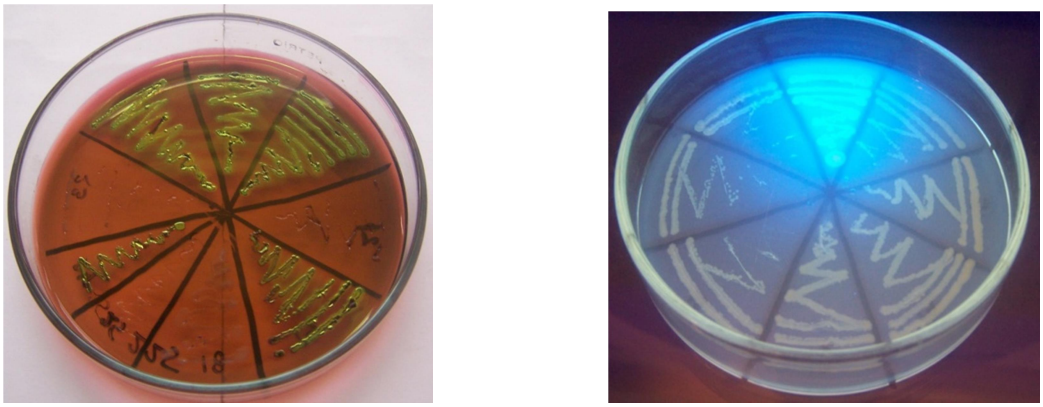

Fig. 6. Growth on EMB plate showing green metallic sheen (Left) and MUG plate with blue fluorescent and non-fluorescent growth (Right).

Of 1210 isolates from cow-dung samples producing sorbitol non-fermenting colonies on CT-SMAC plate, 175 were found to be EMB positive. Among 252 sorbitol non-fermenting isolates from beef samples, 184 showed green metallic sheen on EMB plates, and 187 isolates from milk and 5 isolates from soil of cowsheds showed typical green metallic sheen on EMB plates (table 10).

Similarly, 107 isolates from meat and13 isolates from goat dropping samples were found to be EMB positive showing green metallic sheen in case of goat.

4.2.2 Growth on MUG medium

Suspected isolates from EMB plate were streaked onto MUG plates. After incubation and exposure to long wave UV light (365 nm), 53 isolates from cow-dung, 86 isolates

from beef, 104 isolates from milk and 2 isolates from soil of cowshed showed growth without blue florescence. Therefore they were considered as MUG negative which is a characteristic of *E. coli* O157:H7. The rest showed growth with blue florescence, thereby, MUG positive and was not considered for further investigation (fig. 6).

In goat sample, 46 isolates from meat and 7 isolates from goat dropping were also found as MUG negative. EMB positive and MUG negative isolates are presumed to be *E. coli* O157:H7 and STEC, which were subjected to further investigation (table 10).

Table 10. Number of selected isolates of *E. coli* O157:H7 and STEC based on characteristics on selective media

Types and No. of samples	No. of samples produced SNF colonies	No. of isolates selected on		
		CT-SMAC	EMB	MUG
Cow-dung (33)	21	1210	175	53
Beef (50)	42	252	18	86
Milk (43)	26	503	187	104
Soil of cow-shed (22)	08	55	03	02
Goat meat(48)	39	1523	107	46
Dropping (23)	19	131	15	07

4.3 Biochemical Test

EMB positive and MUG negative isolates were subjected to different biochemical tests to differentiate *E. coli* O157:H7 and STEC from other *E. coli*. Isolates which showed biochemical reactions typical for *E. coli* O157:H7 and STEC were selected for further confirmation (fig. 7). Among 53 EMB positive and MUG negative isolates from cow-dung, all were found to ferment glucose and lactose with the production of gas. All of them were methyl red and indole positive and Voges-Proskaure negative. None of them

produced H_2S. Among the 53, only 45 were motile and 29 were found Simone`s citrate negative. In case of beef, 86 EMB positive and MUG negative isolates were found to ferment glucose and lactose with the production of gas, but were H_2S negative.

Table 11. Biochemical tests results of the isolates of suspected *E. coli* O157:H7 and STEC obtained from bovine samples

Types of samples	Number of isolates showed typical results								
	TSI				Motility	Indole	MR	VP	Simones citrate
	Yellow butt	Yellow slant	Gas	H_2S	(+)ve isolates	(+) ve isolates	(+) ve isolates	(-) ve isolates	(-) ve isolates
Cow-dung	53	53	53	0	45	53	53	53	29
Beef	86	86	86	0	81	86	86	86	20
Milk	104	104	104	0	94	104	104	104	50
Cow shed soil	03	03	03	0	03	03	03	03	02

They showed positive reaction for methyl red and indole tests but 56 isolates out of the 86 were found Vogues-Proskaure negative. Of 86 isolates, 81 were motile and 20 were Simon`s citrate negative. Likewise, 104 MUG negative but EMB positive isolates from milk samples showed typical reaction on KIA and were methyl red and indole positive and Voges-Proskaure negative but only 50 isolates were Simon`s citrate negative. From cow-shed soil, 3 isolates showed methyl red and indole positive and Voges-Proskaure negative and 2 of them were Simon`s citrate negative. Findings of biochemical tests of the isolates derived from bovine samples are presented in Table 11.

In case of goat meat samples, 46 MUG negative isolates were found to ferment glucose and lactose with the production of gas, H_2S negative isolates showed methyl red and indole positive reactions, but 37 of them were motile, while 40 were Voges-Proskaure

negative and only 19 of them were Simone`s citrate negative. Among the 15 isolates from goat droppings all were found to ferment glucose and lactose with gas production, $H_2S$ negative isolates, showed methyl red and indole positive and Voges Proskaure negative reactions. Here, all the 15 isolates were also showed Simone`s citrate negative. Biochemical tests of the non-sorbitol fermenting *E. coli* isolated from goat meat and goat droppings samples are presented in Table 12.

Table 12. Biochemical test results of suspected *E. coli* O157:H7 and STEC isolates obtained from goat samples

Type of samples	**Number of isolates showing typical results**								
	KIA				**Motility**	**Indole**	**MR**	**VP**	**Simone's citrate**
	Yellow slant	Yellow butt	Gas	H_2S	(+)ve isolates	(+)ve isolates	(+)ve isolates	(-)ve isolates	(+)ve isolates
Goat meat	46	46	46	0	37	46	40	46	19
Droppings	17	17	17	0	15	15	15	15	15

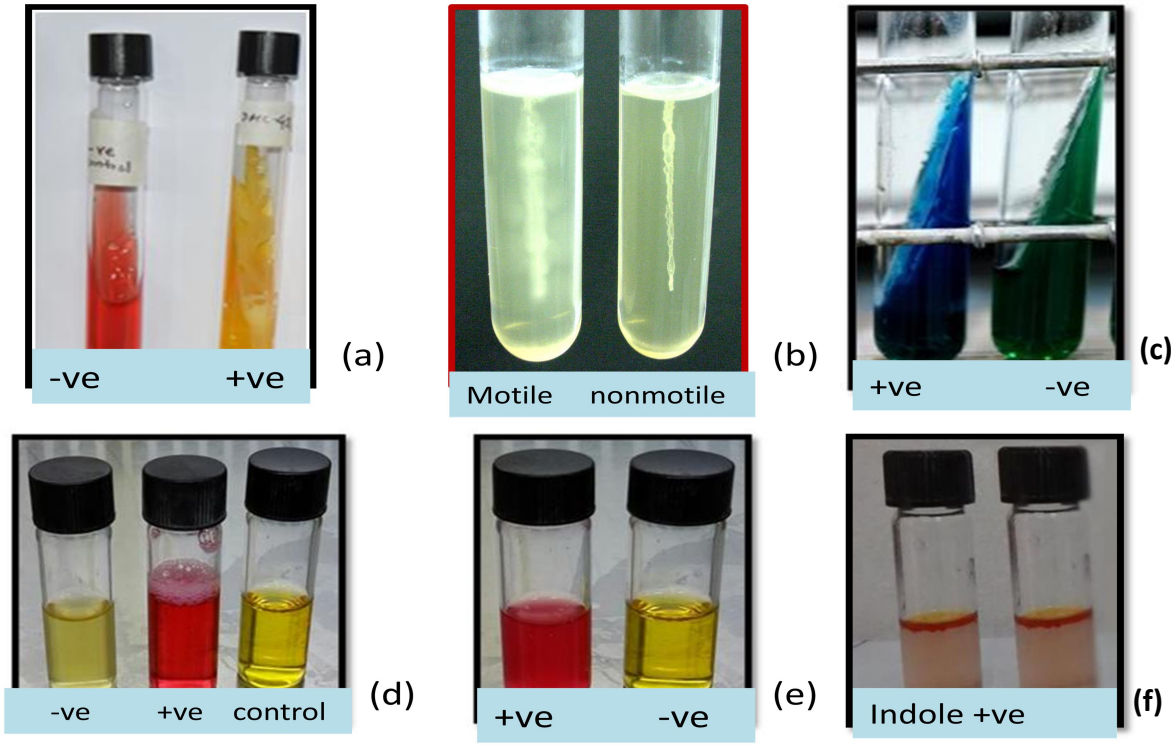

Fig. 7. Results of biochemical tests of the isolates. (a) TSI/KIA test, (b) Motility test, (c)Simone's citrate test, (d) Voges Proskaure (VP) test, (e) Methyl red (MR) test and (f) Indole test.

The *E. coli* O157:H7 and STEC are lactose fermenter, motile, citrate negative, MR negative and indole positive.

The lowest number of isolates that gave expected results in the above mentioned biochemical tests were considered as presumptively identified *E. coli* O157:H7 and or STEC. These presumptively identified as the isolates of *E. coli* O157:H7 and STEC. All these isolates were selected for further molecular and serological analysis.

4.4 Molecular characterization of *E. coli* O157:H7 and STEC by Polymerase Chain Reaction (PCR)

An attempt was taken to amplify genes, which are typical of *E. coli* O157:H7 either singly or in combination. For this purpose, the genes responsible for virulence of O157:H7 and STEC, *e.g.*, *eae*A, *stx*-1, *stx*-2, and also *rfb*E and *fli*C responsible for O157 and H7 sero-groups were selected for PCR amplification. Template DNA was prepared from the isolates, which were presumptively identified as *E. coli* O157:H7 by their cultural and biochemical tests. Two (2) microlitre of extracted template DNA was amplified to detect *eae*A, *stx*1, *stx*2, *rfb*E and *fli*C genes by using specific primer pairs. Isolates those gave bands of expected size were considered to carry the genes tested for. For *eaeA, stx1, stx2, rfb*E and *fli*C genes, 150bp, 384bp, 584 bp, 259bp, 625bp bands, respectively, were expected on agarose gel. The isolates showed specific bands, were considered positive for those genes (fig. 8).

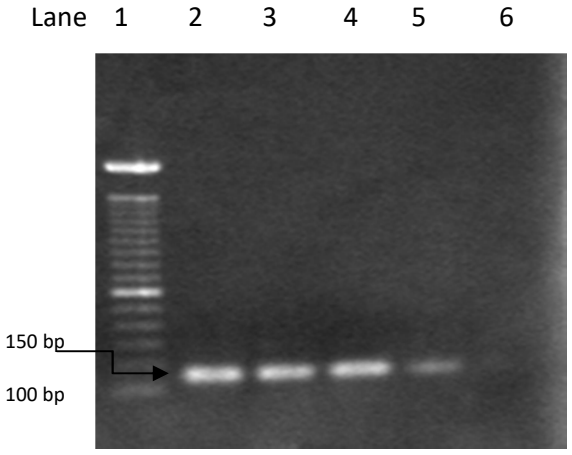

Fig. 8. Agarose gel electrophoresis showing 150 bp amplification products of ***eaeA*** gene specific primers. Lane 1- 100 bp marker, lane 2- positive control, lanes 3 to 5- Isolates, lane 6-no template (negative) control.

From cow-dung, 29 isolates which were presumptively identified as *E. coli* O157:H7 or STEC by biochemical test were subjected to molecular analyses. Of them, 25 isolates were found to be positive for *eae*A gene, rest were negative.

Moreover, 13 isolates were found to be positive for *stx*-1 and 07 isolates for *stx*-2 genes, which were derived from 14 samples. Hence, 42% of the cow dung samples were

characterized as STEC as they contain either or both of *stx* genes. Furthermore, 10 of those isolates were both *rfb*E and *fli*C genes positive. So, from 33 cow dung samples, 10 (ten) *E. coli* O157:H7 isolates belonging to 06 samples (i.e. 18% samples) were identified as *E. coli* O157:H7 positive (table 13).

Table 13. Occurrence of virulence and other marker genes among the isolates of cow-dung samples

Sample type	Isolates	Virulence genes				
		***eae*A**	***stx*-1**	***stx*-2**	***rfb*E**	***fli*C**
Cow-dung	CD-09	+	-	+	-	-
	CD-11	+	-	+	+	+
	CD-16	+	+	-	-	-
	CD-17	+	-	+	+	+
	CD-21	+	+	-	-	-
	CD-22	+	-	-	n.d	n.d
	CD-33	+	-	-	-	-
	CD-37	+	+	-	-	-
	CD-41	+	-	-	n.d	n.d
	S-$8_{(93)}$	+	+	-	-	-
	S-$8_{(116)}$	+	-	-	n.d	n.d
	S-$9_{(43)}$	+	+	-	-	-
	S-$9_{(53)}$	+	+	-	-	-
	S-$10_{(98)}$	+	-	+	-	-
	S-$11_{(05)}$	+	-	+	+	+
	S-$11_{(08)}$	+	-	+	+	+
	S-$12_{(32)}$)	+	-	+	+	+
	S-$12_{(62)}$	+	-	-	n.d	n.d
	S-$12_{(53)}$	+	+	-	-	-
	S-$13_{(99)}$	+	+	-	-	-
	S-$13_{(105)}$	+	+	-	+	+
	S-$13_{(110)}$	+	+	-	+	+
	S-$13_{(143)}$)	+	+	-	+	+
	S-$13_{(149)}$	+	+	-	+	+
	S-$15_{(20)}$	+	+		+	+
Total	**25**	**25 (16/33)**	**13 (8/33)**	**07(6/33)**	**10 (6/33)**	**10 (6/33)**

(+) = isolates positive and (-) = isolates negative for a particular gene. nd = not done

Table 14: Occurrence of virulence and other marker genes among the isolates of Beef samples

Types of sample	Isolates	Virulence genes				
		***eae*A**	***stx*-1**	***stx*-2**	***rfb*E**	***fli*C**
	BF-01	+	-	-	n.d	n.d
	BF-02a	+	-	-	n.d	n.d
	BF-02b	+	-	-	n.d	n.d
	BF-02c	+	-	-	n.d	n.d
	BF-12a	+	+	-	-	-
	BF-12b	+	+	-	-	-
	BF-12c	+	+	-	-	-
	BF-13	+	-	-	n.d.	n.d.
	BF-15	+	-	-	n.d	n.d
Beef	BF-17	+	-	-	n.d.	n.d.
	BF-20a	+	-	-	n.d.	n.d.
	BF-20b	+	+	-	-	-
	BF-20c	+	-	-	n.d.	n.d.
	BF-20d	+	-	-	n.d.	n.d.
	BF-20e	+	-	+	-	-
	BF-28	+	-	-	n.d	n.d
	BF-37	+	-	-	n.d	n.d
	BF-40	+	-		n.d	n.d
	BF-44	+	+	+	+	+
Total		**19 (11/50)**	**5 (3/50)**	**2 (2/50)**	**1 (1/50)**	**1 (1/50)**

(+)= isolates positive, and (-)= isolates negative for a particular gene.

nd= not done.

From 11 of 50 beef samples, 20 isolates presumptively identified as *E. coli* O157:H7 and or STEC previously by biochemical tests. Of them, 19 isolates were found to contain

*eae*A gene. Likewise, 05 isolates were *stx*1 and 02 were *stx*2 gene positive. Altogether, at least one *stx* gene was found to be positive in 06 isolates which belong to 05 samples. Therefore, nearly 10% of the beef samples can be characterized as STEC. None but only one isolate showed positive results in *rfb*E or *fli*C genes, therefore only one isolate is identified as *E.coli*O157:H7 (table 15).

In case of cows' milk, 34 isolates obtained from 11 of 43 samples were found to contain *eae*A gene; among them, 03 isolates had *stx*-1gene and only one (01) isolate had *stx*-2 gene; obtained from four different samples. So, 9% (4/43) of the milk samples were characterized as STEC (table 16). Here none of the isolates was positive for *rfb*E and *fli*C genes; therefore, none can be identified as *E. coli* O157:H7. In case of soil samples from cowshed, only one isolate was positive for *eae*A gene and no isolate was found to be positive for *stx*-1*, stx*-2*, rfb*-E*,* or*fli*-C genes, suggesting that there was no *E. coli* O157 in soil.

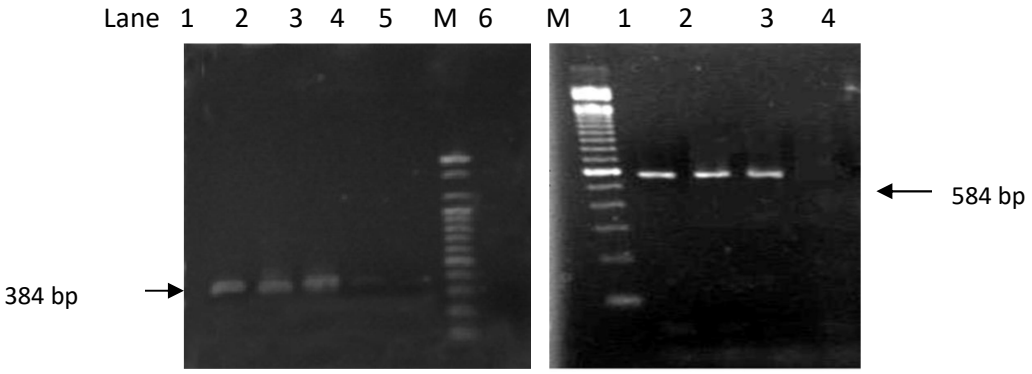

Fig. 9. Agarose gel electrophoresis showing 348 bp and 584 bp amplification products of ***stx*-1** (a) and ***stx*-2** (b) genes specific primers in PCR. (a) Lanes- 1 to 5 - isolates, lane- 6 negative control (b). Lane 1 positive control, 2 to 3 isolates, lane 4- negative control. In both figure M, 100 bp marker.

The genetic markers were characterized in 19 presumptively identified isolates from 14 goat meat samples. Of those 14 samples, *eae*A gene was found to be positive among 18

isolates, but *stx*-1 *and stx*-2 genes were found to be positive in 12 isolates from 08 samples and 08 isolates from equal number of samples respectively. Again, 12 isolates from 8 samples were positive for *rfbE* and 11 isolates from 7 samples were positive for *fliC* genes. Here, 9 isolates can be identified as *E. coli* O157:H7 by molecular analysis among which 5 isolates belonged to a single sample (fig. 8) (table17).

From biochemically identified 15 isolates of 23 goat dropping samples, *eaeA* gene was detected in 10 isolates. Whereas, 04 isolates were *stx-1* positive, 02 isolates were *stx-2* positive, and 04 isolates were *rfbE* positive. Therefore, from goat dropping 06 samples were characterized as STEC (26%) and only one isolate (GDS-1a) was confirmed as *E. coli* O157:H7 as it was positive for *eaeA, stx-1, rfbE* and *fliC* genes (fig. 9) (table 18).

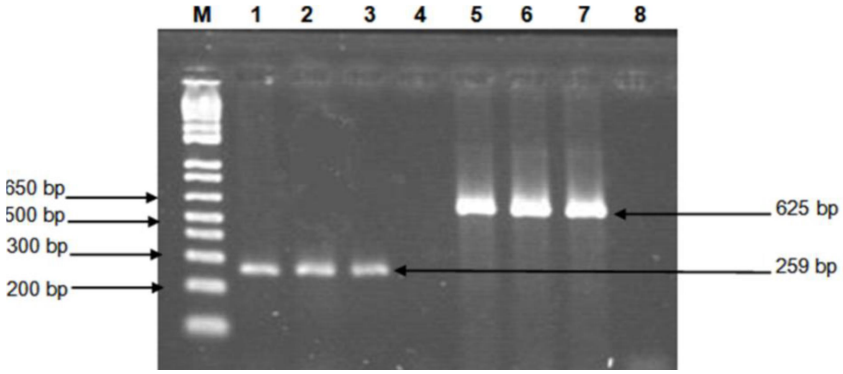

Fig. 10. Agarose gel electrophoresis showing 259 and 625 bp PCR amplification products of *rfb*E and *fli*C genes, respectively. M = 1 kb plus DNA marker; lane 1, 5 = S11; lane 2, 6 = S12; lane 3, 7= *E. coli* O157:H7 NCTC 12079; lane 4, 8 = negative control.

Table 15: Occurrence of virulence and other marker genes among the isolates of milk samples

Sample	Isolates	Virulence genes				
		***eae*A**	***Stx*-1**	***Stx*-2**	***rfb*E**	***fli*C**
Milk	ML-20 (5)	+	-	-	n.d	n.d
	ML-21 (14)	+	+	-	-	-
	ML- 35(1,2,3,8)	+	-	-	n.d	n.d
	ML-36(1,3,5,9)	+	-	-	n.d	n.d
	ML-37(2,3)	+	-	-	n.d	n.d
	ML- 8(1,3,4,5,6)	+	-	-	n.d	n.d
	ML-40(1,5,7,8,10)	+	-	-	n.d	n.d
	ML-41(1,3,4,5)	+	-	-	n.d	n.d
	ML-41(2)	+	+	-	-	-
	ML-42 (1)	+	-	+	-	-
	ML-43 (2)	+	+	-	-	-
	ML-08(4,5)	+	-	-	n.d	n.d
Total		**31 12/43)**	**03 03/43)**	**01(01/43)**	**None**	**None**

(+)= isolates positive, and (-)= isolates negative and nd = not done for a particular gene. The numbers in the parenthesis alongside the sample numbers denote the isolate identification.

Table 16: Occurrence of virulence and other marker genes among the isolates of Goat meat samples

Sample	Isolates	Virulence genes				
		***eae*A**	***Stx*-1**	***Stx*-2**	***rfb*E**	***fli*C**
Meat	GM-07i	+	-	-	n.d	n.d
	GM-01i	+	-	+	-	+
	GM-02g	+	+	+	+	+
	GM-03c	+	+	+	+	+
	GM-04b	-	n.d	n.d	n.d.	n.d
	GM-05c	+	+	-	-	-
	GM-06d	+	-	+	+	-
	GM-08b	+	+	+	+	+
	GM-15k	+	+	-	+	-
	GM-19a	+	-	-	n.d	n.d
	GM-19b	+	+	-	-	-
	GM-24a	+	+	+	+	+
	GM-31k	+	-	+	-	+
	GM-33j	+	-	+	+	-
	GM-35 GM-41a	+	+	-	+	+
	GM-41c	+	+	-	+	+
	GM-41d	+	+	-	+	+
	GM-41e	+	+	-	+	+
	GM-41f	+	+	-	+	+
Total		**18(14/48)**	**12 (8/48)**	**8 (8/48)**	**12(8/48)**	**11(7/48)**

(+) = isolates positive, and (-) = isolates negative for a particular gene.

nd = not done

Table 17: Occurrence of virulence and other marker genes among the isolates of Goat dropping samples

Sample	Isolates	Virulence genes				
		*eae*A	*stx*-1	*stx*-2	*rfbE*	*fliC*
Goat Dropping	GDA-3a	+	-	-	n.d	n.d
	GDA-4a	+	+	-	-	-
	GDA-4b	-	-	-	n.d	n.d
	GDA-6b	+	-	-	n.d	n.d
	GDA-6d	+	-	+	-	-
	GDA-6e	+	-	-	n.d	n.d
	GDA-7a	+	+	-	+	-
	GDA-7b	+	-	-	+	-
	GDA-7d	-	-	-	+	-
	GDD-3d	-	-	-	n.d	n.d
	GDD-4a	+	+	-	-	-
	GDD-4b	+	-	+	-	-
	GDD-4c	-	-	-	n.d	n.d
	GDS-1a	+	+	-	+	+
	GDS-1b	-	-	-	n.d	n.d
Total		**10(10/23)**	**4(4/23)**	**2(2/23)**	**4(4/23)**	**1(1/23)**

(+)= isolates positive, and (-)= isolates negative for a particular gene

nd = not done

4.5 Identification of *E. coli* O157:H7 by serological test

Culturally and biochemically identified isolates which were found to contain the above mentioned genes were subjected to serological identification using commercial rapid latex agglutination test kit *E. coli* O157:H7 (Wellcolex Ramal, USA) (Fig 10). This test

used antibodies against O157 and H7 antigens bound on latex particles which would bind to the respective antigens. A positive result is noticed by the development of an agglutinated pattern showing clearly visible clumping of the latex particles. In a negative result, the latex does not agglutinate and the appearance of the suspension remains markedly unchanged.

Among the 29 biochemically positive isolates from cow-dung, 9 showed agglutination reaction against O157-specificanti-sera and 8 isolates showed agglutination reaction against H7- specific anti-sera. Hence, 8 isolates from cow dung samples confirmed as O157:H7 sero-group. Altogether, from beef, only one isolate showed agglutination reaction (table 19). From milk and soil of cowshed, no isolates showed positive agglutination reaction. Therefore, none of the isolates of these two categories were *E. coli* O157:H7.

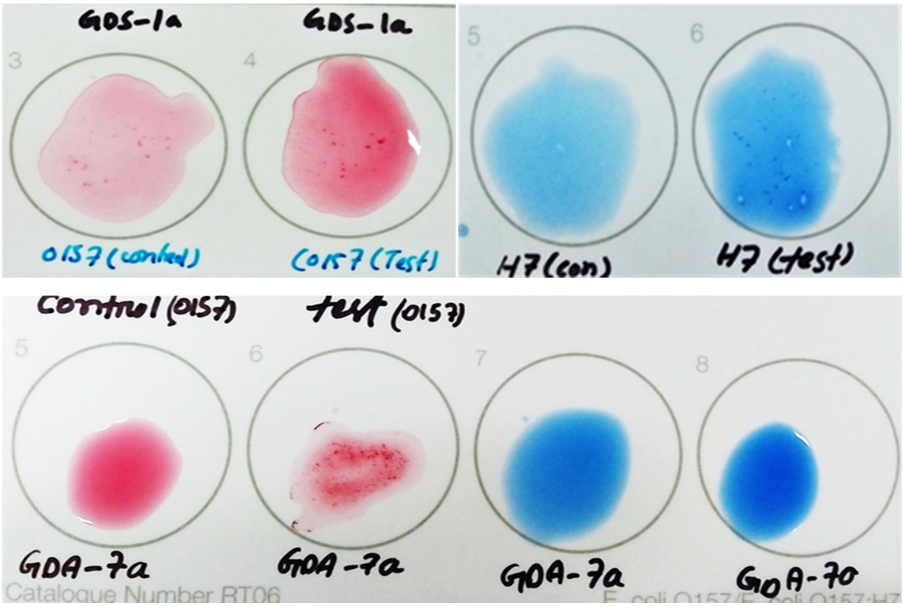

Fig. 11. Results of latex agglutination tests of *E. coli* O157:H:7 isolates. Red and blue agglutination indicate test for O157 and H:7, respectively. The upper panel in this figure shows the reaction with the isolate GDS-1a with latex control and latex test reagents, respectively for O157 (red) and H:7 antigens. In the lower panel, the same reaction with isolate GDA-7a. Note that, for the isolate GDS-1a, both the latex test reagents gave positive results. On the other hand, the isolate GDA-7a gave positive reaction only with test latex -O157 reagent.

Table 18. Latex agglutination test results of the isolates obtained from bovine source

Type of samples	Isolates	O157 antigen	H7 antigen	Both O157 and H7 antigens
Cow-dung	CD-11	+	+	+
	CD-17	+	+	+
	S11- 05	+	--	-
	S11-08	+	+	+
	S12-32	+	+	+
	S13-99	-	-	-
	S13-105	+	+	+
	S13–110	+	+	+
	S13-143	+	+	+
	S13-149	+	+	+
	S15-20	+	+	+
Total	11 (06/33)	09(5/33=15%)	08(5/33=15	08(5/33=15%)
Beef	BF-17a	-	--	-
	BF-17b	-	--	-
	BF-12a	-	--	-
	BF-12b	-	--	-
	BF-12c	-	--	-
	BF-44	+	+	+
	BF-15	--	--	-
	BF-20b	--	--	-
	BF-40	--	--	-
Total	**0 9 (06/50)**	**01(1/50=2%)**	**01(1/50=2%**	**01(1/50=2%)**
Milk	$ML\text{-}21_{(14)}$	--	--	-
	$ML\text{-}41_{(02)}$	--	--	-
	$ML\text{-}42_{(01)}$	--	--	-
	$ML\text{-}43_{(04)}$	--	--	-
Total	04 (04/43)	None	None	None
Soil of cow-shed	Csso-8b	--	--	-
	Csso-9c	--	--	-

(+)= isolates with positive response and (-)= isolates with negative response,
nd = not done

In case of goat samples**,** among the 19 Simon`s citrate negative isolates from meat samples identified through biochemical tests, 12 showed agglutination reaction against O157 anti-sera and 11 isolates showed agglutination reaction against 'H7' anti-sera. In total, 9 isolates were positive for both anti-O157 and anti-H7 anti-sera. On the other hand, 5 isolates showed agglutination against-O157 anti-sera and 1 sample showed positive agglutination against H7 anti-sera from goat droppings.

Table 19. Serological test results of the isolates obtained from Goat

Sample	Isolates	O157 antigen	H7 antigen	Both O157 and H7 antigens
	GM-01i	-	+	-
	GM-03c	+	+	+
	GM-08b	+	+	+
	GM-15k	+	-	-
	GM-19a	-	-	-
	GM-19b	-	-	-
	GM-24a	+	+	+
	GM-2g	+	+	+
Goat meat	GM-31k	-	+	-
	GM-33j	+	-	-
	GM-35	-	-	-
	GM-41a	+	+	+
	GM-41c	+	+	+
	GM-41d	+	+	+
	GM-41e	+	+	+
	GM-41f	+	+	+
	GM-6n	+	-	-
Total	**17**	**12 (8/48=16.6%)**	**11 (7/48=14.5%)**	**11 (7/48=14.5%)**
Goat	GDS-1a	+	+	+
dropping	GDA-4a	-	-	
	GDA-6d	+	-	
	GDA-7a	+	-	
	GDA-7b	+	-	
	GDA-7b	+	-	
Total	06	5(5/22=22.7%)	1(1/22=4.5%)	1(1/22=4.5%)

(+)= isolates with positive, and (-) = isolates with negative response, nd = not done

4.6 Molecular identification of the isolates by 16S rRNA gene sequencing

The isolates confirmed as *E. coli* O157:H7 through serological test and molecular detection were subjected to 16s rRNA gene sequencing method to determine the specificity of the strain. Using universal primers an amplicon of more than >1450 bp was obtained which later subjected to sequencing (fig. 12). The raw sequence data was edited using chromas 2.31 software (available at http://www.techneltsium.com.au/chromas.html). After BLAST searching (available at www.ncbi.nlm.nih.gob/blast/), the edited sequencing data of the isolates showed maximum (> 98%) genetic similarity with available *E. coli* O157:H7 data in GenBank. In this study, 11 of 12 isolates (from 8 samples) showed more than 98% similarity with 16S rRNA gene sequence of *E. coli* O157:H7, hence genetically confirmed as O157:H7 strains of *E. coli* (table 20).

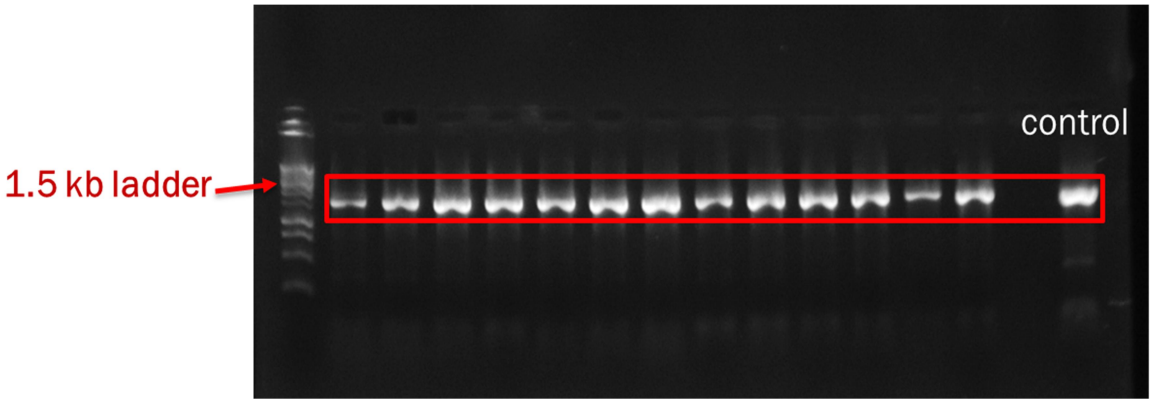

Fig. 12. Agarose gel electrophoresis showing product of PCR amplification of 16s rRNA gene.

Table 20. Per cent similarity of isolates with 16S rRNA gene sequence of reference strain of *E. coli* O157:H7

Types of sample	Isolates	% of similarity with reference strain
(+) control	*E. coli* NCTC12079	99.1
Beef	BF-44	98.6
Goat meat	GM-8b	98.3
	GM-24a	98.4
	GM-2g	98.2
	GM-41a	99.0
	GM-41e	98.8
Goat dropping	GDS-1a	98.5
Cow dung	S-11$_{(5)}$	99.2
	S-12$_{(32)}$	98.6
	S-13$_{(105)}$	99
	S-13$_{(143)}$	Poor result
	S-15$_{(20)}$	98.1

4. 7 Detection of virulence genes by multiplex PCR

In order to lessen the time requirements of PCR amplification of each gene individually, an attempt was taken to set a multiplex PCR for simoultaneous amplification of three genes typical for *E. coli* O157:H7. For this genes for attaching effacing (*eaeA*) and shiga toxins (*stx-1*) and (*stx-2)* were chosen. In the present study multiplex PCR was set in different combination of primer pairs to standardize the amount of required primers as well as the PCR conditions. Bands of expected size and brightness was revealed in multiplex amplification of *eae*A and *stx*-1, *stx*-1 and *stx*-2, and also *eae*A and *stx*-2 genes

(figure not shown). Again, multiplex PCR for *eae*A, *stx*-1,and *stx*-2 genes also showed similar brightness as shown in the PCRs for each gene individually (**Figure 12).** Four isolates (GM-8b, GM-3c, SN-2g,GM-24) were positive for *eae*A, *stx*-1 and *stx*-2 gene. Two isolates GM-31, GM-33) were positive for *eae*A and *stx*-2 gene. One isolate (GM-15k) was positive for *eae*A and *stx*-1gene. Two isolates (GM-7I, GM-6n) were only for *eae*A gene. For *eae*A, *stx*-1, *stx*-2 gene specific primers 150bp, 348bp and 584bp bands were found during gel electrophoresis.

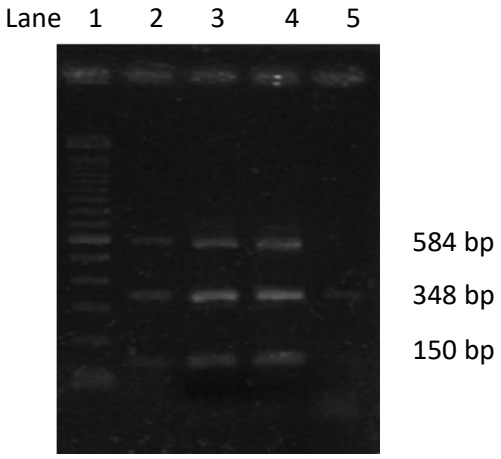

Fig. 13. Agarose gel electrophoresis showing 150 bp, 348 bp and 584 bp amplification products of ***eaeA*, *stx1*** and ***stx2***genes specific primers in multiplex PCR. Lane 1: 1000 bp marker, Lane 2: Isolate, Lane 3: Isolate, Lane 4: Positive control, Lane 5: No template control.

4.8 Phenotypic characterization

After isolation and identification, the isolates were subjected to phenotypic characterization by enterotoxicity test, test of invasiveness, hemolytic activity and response to several antibiotics.

4.8.1 Entero-toxicity test

Detection of enterotoxic ability exerted by production shiga-toxin (*stx*), the isolates identified as *E. coli* O157:H7 and STEC were tested in the rabbit ileal-loop following the

procedure of Sanyl *et al.* (1975). Most of the isolates were found to produce fluids which indicates that the isolates were entero-toxic. A total of 21 isolates having genes for either *stx*-1 or *stx*-2 or both, were subjected to enterotoxicity test. Of them,18 showed accumulation of fluid in amount greater then negative value as referred by Sanyl *et al,* (1975) (table 22). Thereby, 86% of the isolates confirmed by biochemical, genetic marker, and serological test as *E. coli* O157:H7 and STEC revealed to have enterotoxic activities (fig. 13)

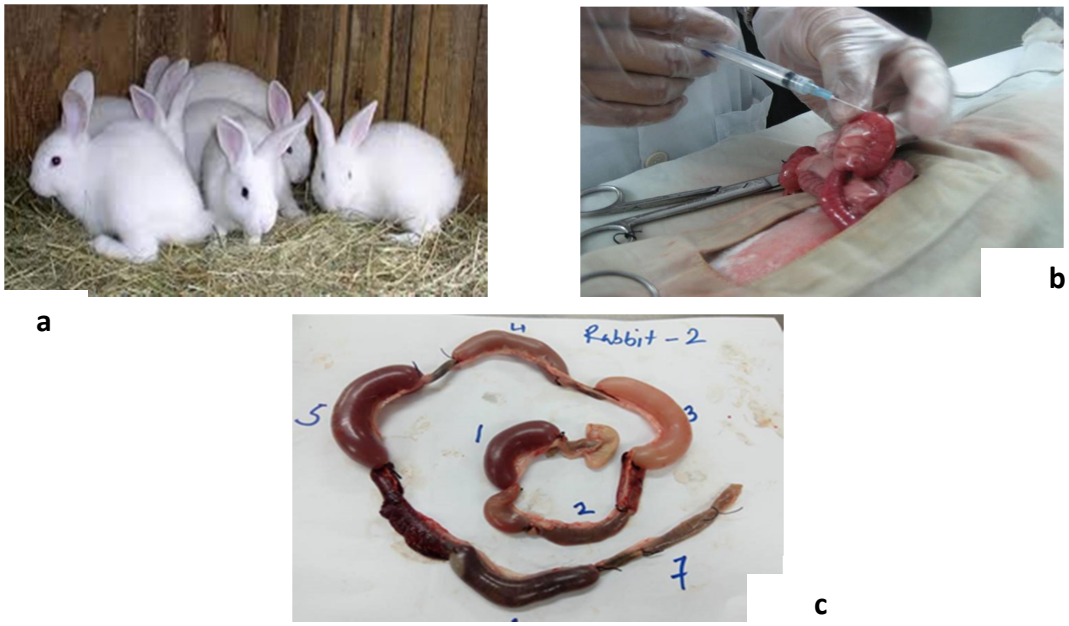

Fig. 14. Enterotoxicity of *E.coli* O157:H7 strains on the rabbit ileal-loop. a: selected rabbits before inoculation. b: injection of inoculum. c: rabbit ileum after 18 hrs of inoculation.

Bacterial whole cell culture grown on TSB were used as inoculum which might contained approximately 10^5 to 10^6 cells per ml of broth. Loop-1 (positive control) contained one ml of broth culture obtained from the *E. coli* NCTC12079; loops 2-6 inoculated with 1.0 ml of samples; loop 7 with PBS(negative control).

Table 21. Enterotoxicity assay of the isolates of *E. coli* of different samples by Rabbit Ileal loop test.

Types of sample	Isolates	Loop length (cm)	Volume of fluid (ml)	Fluid (ml)/ cm
(+) control	*E. coli* NCTC12079	6	9.5	1.58
Beef	BF-44	7.5	9	**1.2**
Goat meat	GM-24	6	11	**1.8**
	GM-2g	6	9	**1.5**
	GM-3c	7	10	**1.4**
	GM-41a	6	10	**1.66**
	GM-41c	8	12	**1.5**
	GM-41d	7	7	**1**
	GM-41e	7	19	**2.7**
	GM-41f	7.5	12	**1.75**
	GM-8b	8	13	**1.6**
Goat dropping	GDS-1a	5.5	9.5	**1.7**
Cow dung	S-11$_{(5)}$	6	0	0
	S-11$_{(8)}$	7	3.5	**0.5**
	S-12$_{(32)}$	6.5	1.2	0.18
	S-13$_{(105)}$	7	9	**1.29**
	S-13$_{(110)}$	7	0	0
	S-13$_{(143)}$	6.5	7.6	**1.17**
	S-13$_{(149)}$	6.5	6.5	**1**
	S-15$_{(20)}$	5.5	3.2	**1.7**
	CD-11	6	13.2	**2.2**
	CD-17	5.5	11	**2**
	(-) control	6	0	0

+ = positive , - = negative control/ or negative results.
Numbers in parenthesis denotes the isolate identification.
Bold numbers are positive values in test samples.

4.8.2 Test for Invasiveness

Invasiveness is considered as an important virulent factor. The isolates were analyzed for invasiveness activity by growing them on Congo red containing media. All the isolates including (+)ve control were colorless after 24 hrs of inoculation, *i.e,* none were invasive in nature (Fig. 15.).

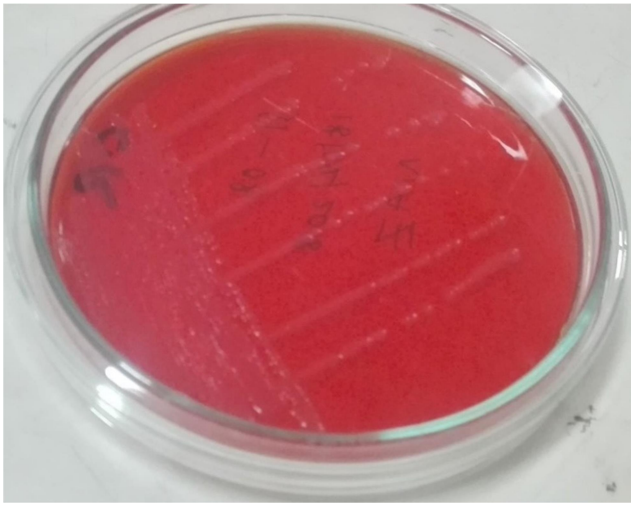

Fig. 15. Congo-Red agar plate showing non-invasive colony of *E. coli* O157:H7

4.7.3 Test for hemolytic activity

Test for hemolytic activity was done by growing all the isolated *E. coli* O157:H7 on sheep blood agar media. None of the isolates were found to be hemolytic as they did not show any zone of lysis of RBC (Fig. 15).

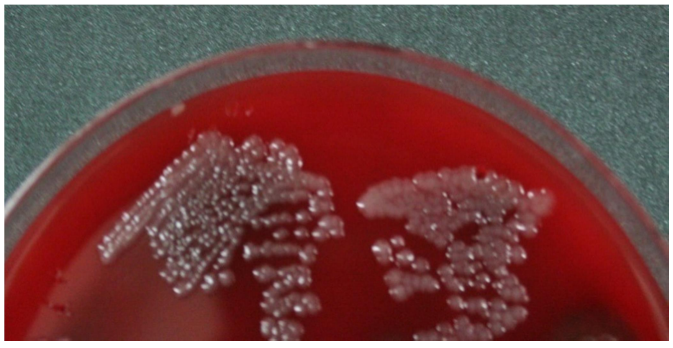

Fig. 16. Growth of isolates on blood agar plate showing non-hemolytic nature of *E. coli* O157:H7

4.8.4 Test for antibiotic sensitivity

To detect antibiotic sensitivity pattern, 16 *E. coli* O157:H7 isolates were subjected to antibiotic sensitivity test. A total of seven commonly used antibiotics which are being used against *E. coli* namely Ampicillin, Streptomycin, Ciprofloxacin, Nalid, Chloramphenicol, and Novobiocin were chosen for sensitivity test. The test was done following Kirby-Bauer method (Disc diffusion). The diameter of inhibition zone was measured with scale and interpreted following guideline chart (Libre Texts) (fig. 16).

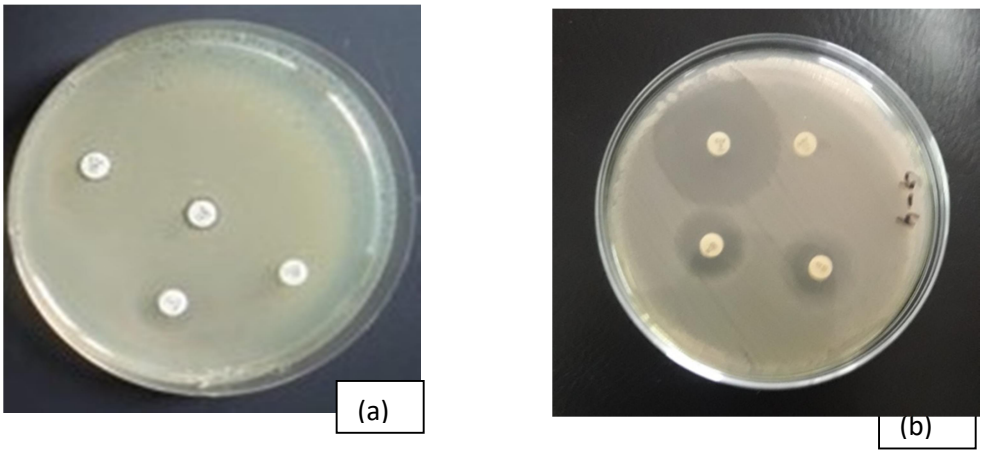

Fig. 17. Antibiotic sensitivity test of isolated *E. coli* O157:H7. a: Muller-Hilton plate with antibiotic disk; b: Muller-Hilton plate showing inhibition zone.

Among the 16 isolates, 13(81.25%) were sensitive to Kanamycin and 11(68.75%) were sensitive to Streptomycin. From 16 isolates, all (100%) were resistant to Novobiocin and 14(87.5%) of them were found to be resistant to Ampicillin. On the other hand, 32.25% of the isolates were resistant to Chloramphenicol and 37.5% isolates were resistant to ciprofloxacin. In case of Nalidixic acid, 43.8% isolates were sensitive and 56.2% isolates showed intermediate response.Among the 16 isolates, 13(81.25%) were sensitive to kanamycin and 11 (68.75%) were sensitive to Streptomycin. From 16 isolates, all (100%) were resistant to Novobiocin and 14(87.5%) of them were found to be resistant to Ampicillin. On the other hand, 32.25% of the isolates were resistant to Chloramphenicol

and 37.5% isolates were resistant to ciprofloxacin. In case of Nalidixic acid, 43.8% isolates were sensitive and 56.2% isolates showed intermediate response (fig. 18).

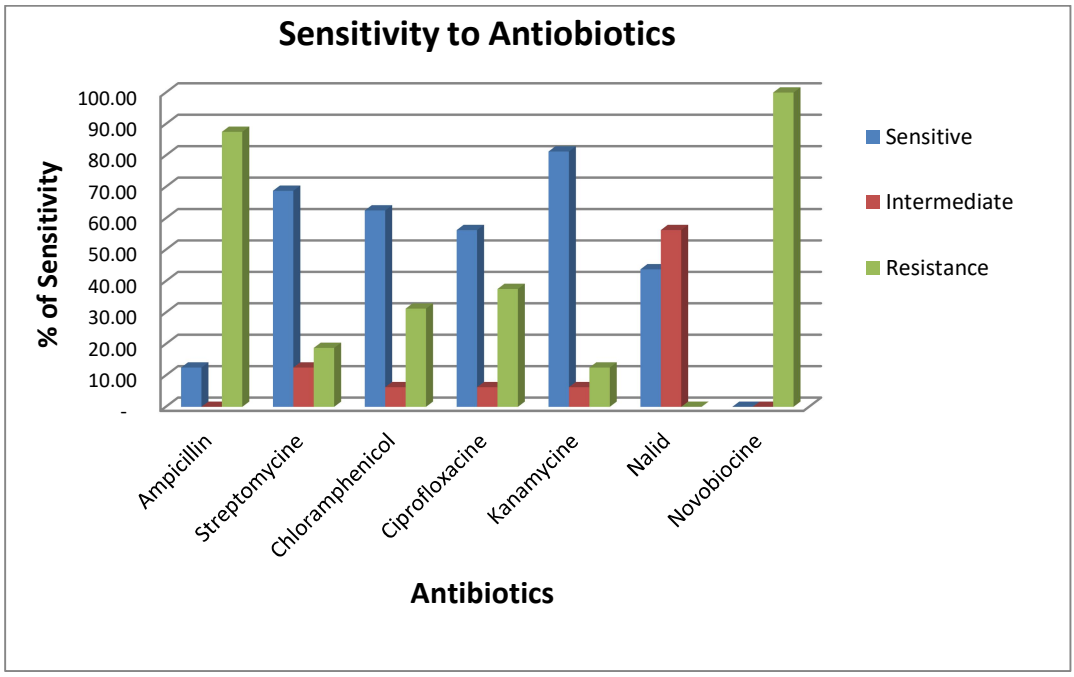

Fig. 18. Response of isolates against different antibiotics

From the whole study it is revealed that both cow and goats are the reservoir of STEC and *E. coli* O157: H7. In the present study, it is found that 42.42% cow-dung samples carried STEC which had at least one *stx* genes in combination with another virulence gene eaeA. At least 18% of the samples were found to carry *E. coli* O157: H7 which were positive for *eaeA, stx, rfbE* and *fliC* genes and also positive for serological reactions.

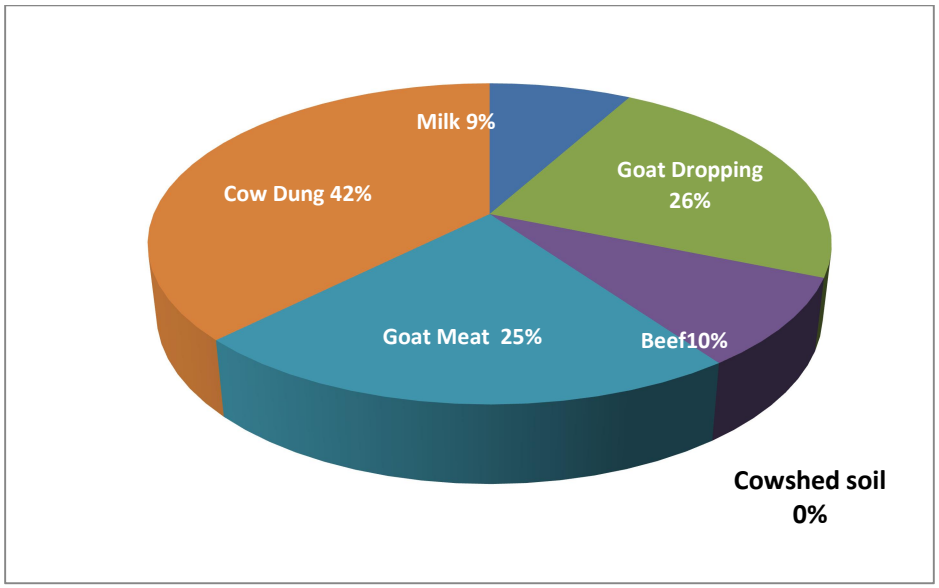

Fig. 19 Prevalence of STEC in cattle related sources

Likewise, 10% of beef samples were carrying STEC of which only one carried *E. coli* O157:H7. Therefore, 2% of the total beef sample carried *E. coli* O157:H7. On the other hand, in case of milk, 9% of the total tested samples were harboring STEC as they were positive for eaeA and one of the *stx* genes. From milk, no *E. coli* O157:H7 could be isolated. No STEC or *E. coli* O157:H7 was found from soil of cowshed.

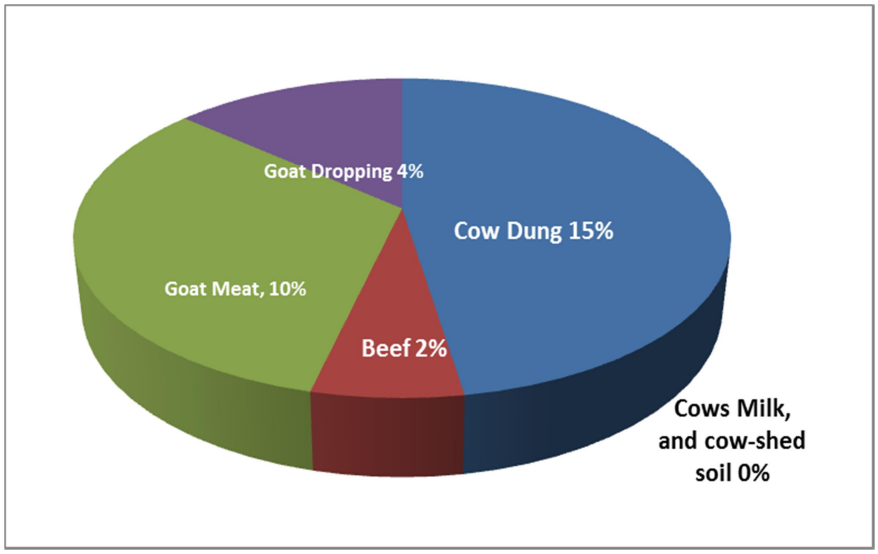

Fig. 20 Rate of isolation of *E. coli* O157:H7 from different cattle related sources

Table 22. Overall occurrence of *E. coli* O157:H7 and STEC in different samples

Types and No. of samples	No. of isolates positive for Genes					*E. coli* O157:H7	% of *E. coli* O157 :H7	% of STEC sample
	eae A	*stx*-1	*stx*-2	*rfb*E	*fli*C			
Cow dung (33)	25	13	7	10	10	10	6/33=18	4/33=42
Beef (50)	19	5	2	1	1	1	1/50=2	5/50 =10
Cows' milk (43)	47	3	1	-	-	-	-	4/43=9
Goat Meat (48)	18	7	12	12	11	9	5/48=10	12/48=25
Goat dropping (23)	10	4	2	4	1	1	1/23=4	6/23=26
Cow shed soil (22)	1	--		--	--	--	--	--

+ = positive, - = negative control/ or negative results.
Numbers in parenthesis denotes the number of samples studied

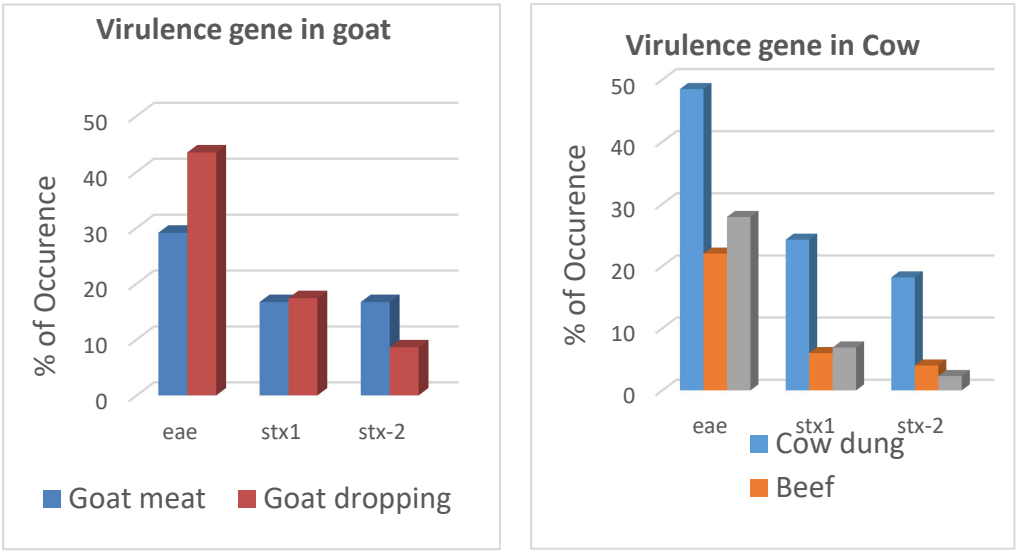

Fig 21. Occurrence of Virulence genes in goat and cow samples

In case of goat, 25% of the meat samples were found to have *eae*A, *stx*-1 or *stx*-2 genes or both. So, these samples were identified as STEC. Among them 5 samples carried *E. coli* O157:H7 as the isolates were *eae*A*, stx, rfb*E and *fli*C gene positive and also showed positive reaction for serological tests. So about 10% of the goat meat samples were found to be reservoir of *E. coli* O157:H7. Likewise, 26% of goat dropping samples harboring STEC and *E. coli* O157:H7 was found in 4% of the total samples (table 22).

DISCUSSION

World Health Organization reported that about eleven million children under five years old were died of gastroenteritis owing to *E. coli* (WHO 2005). Among various serotypes Shiga toxin producing *E. coli* (STEC) is the main causal organism of food borne disease worldwide (Kaufman *et al*. 2006). STEC is able to cause mild diarrhea to more sever hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Brett *et al.* 2003).

Out of 200 serotypes of STEC, around 160 have been recovered from humans, of which *E. coli* O157:H7 causes majority of serious human infections such as hemorrhagic colitis (HC), hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (TTP). Serogroup O157:H7 together with O111 are responsible for many of the serious cases (Paton and Paton 1998). Serogroup O113 has been recognized as major STEC associated with cases of HUS in Australia (Paton and Paton 1999). The other important serotypes are O26:H11, O111:H-, O145:H-, O45:H2 and O4:H-. Different studies have been reported substantial morbidity and mortality associated with outbreaks of gastro-intestinal disease caused by STEC. These studies have highlighted this group of pathogen as threat to human health (Beutin *et al.* 1999; Ahmed *et al*. 1997; Brotman *et al*.1994).

The major reservoir of *E. coli* O157: H7 and many other STEC are food producing animals, cattle in particular. Most disease outbreaks have been found to be involved with foods of cattle origin such as beef, mutton and raw milk that become contaminated with cattle feces at slaughterhouse or dairy farms. However, these bacteria have also been reported from other domestic animals and wild life, such as sheep, goats, deer, dogs, horses, swine, cats, sea-gulls and rats (Elder *et al*. 2000). The findings of the present investigation supported these as reservoirs of *E. coli* O157: H7 and many other STEC. In recent years, there has been an increase in the numbers of outbreaks linked with the consumption of fruit juice, vegetables and sprouts which were somehow associated with reservoirs of *E. coli* O157: H7 and / or STEC. Houseflies which are commonly developed

in animal manure if contained *E. coli* O157: H7 and/or STEC may also contribute in the contamination of food and drinks (Alam *et al.* 2006).

Prevalence of *E. coli* O157: H7 in cattle feces increases during warmer months of the year which correlates with seasonal occurrence of human diseases. The contribution of *E. coli* O157:H7 in cattle feces ranges from 10^2 to 10^5 cfu/g. The quantity of *E. coli* O157:H7 in the manure is influenced by the higher temperature of summer and consequently affect the potential of pollution of the environment (Alam *et al*. 2006).

The present study was aimed to isolate *E. coli* O157:H7 and STEC from different cattle related sources like cow-dung, beef, raw milk, soils of cow-shed, goat meat and goat droppings and characterize them. For this purpose, samples were collected randomly from different market areas and cattle farms around Dhaka city, as their presence in cattle based sources were reported from our neighboring countries like India (Pal *et al.* 1999; Dutta 2000; Chattopadhya 2001), Thailand (Suthienkul *et al.*1990), Vietnam (Vu-Khac and Cornick 2008), Malayasia (Son *et al.* 1996, 1998; Radu *et al*. 1998). There are limited studies on the prevalence of *E. coli* O157:H7 and STEC in Bangladesh. Previous studies showed that STEC serotypes are present in cattle, calves and children (Nazir *et al*. 2005, 2007; Munshi *et al.* 2012; Talukder *et al*. 2013). Studies have also been found where *E. coli* O157:H7 had isolated from chicken (Mamun *et al.* 2016). Therefore, meat, raw milk and water sources can be continually contaminated by feces of animals, persons handling meat and some other factors. A very low infectious dose of this pathogen could be a serious threat to public health if proper precautions are not taken.

Very few number of *E. coli* O157:H7 and STEC are present in environment and food samples Moreover, environmental samples contain many other bacteria in huge number besides the target bacteria for which the desired bacteria may be lost by direct plating. Therefore, enrichment medium such as Trypticase Soy Broth (TSB) supplemented with Novobiocin (20mg/L) and bile salt (1.5% w/v) was used prior to isolation on selective

CT-SMAC agar plate in this study. Nataro *et al.* (1998) also used this enrichment and CT-SMAC media for isolation of *E. coli* O157:H7 and STEC. Novobiocin in the medium allows the growth of *E. coli* O157:H7 and STEC but inhibits Gram negative bacterial growth present in the samples. Bile salt inhibits the growth of Gram-positive and other non-enteric bacteria. The *E. coli* O157:H7 and STEC usually cannot ferment sorbitol in CT-SMAC medium within 24 hrs of inoculation thereby forming colorless colonies on CT-SMAC medium. The CT-SMAC was also used in other studies for all types of STEC isolation (Pal *et al.*1999; Wells *et al.* 1991).

Screening by cultural study

In the present study thirty three (33) cow-dung, fifty (50) beef, forty three (43) raw milk and twenty two (22) cow-shed soil samples were investigated. In the preliminary screening on CT-SMAC, a total of 1210 isolates were obtained from cow-dung, 252 isolates from beef, 503 isolates from raw milk, and 53 isolates from cow-shed soil as suspected *E. coli* O157:H7 and STEC. Likewise, 1523 isolates from 45 goat meat and 131 isolates from 23 goat dropping samples were found and selected as suspected STEC and *E. coli* O157:H7 by primary screening. Then, further selection was done on EMB medium. The isolates which produce green metallic sheen on dark growth were selected for MUG test. This distinguishes *E. coli* O157:H7 and STEC from other *E. coli*. MUG negative isolates, and were subjected to biochemical tests such as citrate utilization test, TSI, MR-VP reaction, motility and indole production.

Prelimenary identification by biochemical tests

All the biochemical analyses generated 29 isolates from cow-dung, 20 from beef, 50 from milk, 2 isolates from cow-shed soil, which gave reaction pattern similar to that of *E. coli* O157:H7 and STEC. On the other hand 19 isolates from goat meat and 15 isolates from goat dropping showed identical reaction to that of *E. coli* O157:H7 and / or STEC. It is to be noted that *E. coli* O157:H7 and STEC are not differentiable by biochemical test.

Therefore, these isolates were characterized further by molecular markers and serological properties.

Identification by PCR of marker genes

In order to determine the potential virulence genes and other marker genes of *E. coli* O157:H7 and STEC isolated from different cattle related sources were subjected to virulence genotyping. Genetic approaches are used worldwide due to their higher discriminatory power and availability of global data. In the study, nucleic acid based method was used to detect some distinctive genes of STEC and *E. coli* O157:H7. The use of PCR to amplify *eae*A serves as a highly specific and sensitive method to detect *E. coli* O157:H7 which has been using widely (Kawasaki *et al.* 2005). The *eae*A gene amplification positive isolates were then subjected to amplification of *stx*-1, *stx*-2, *rfb*E and *fli*C genes. The genes *stx*-1 and *stx*2 are responsible for production of shiga toxins whereas *rfb*E and *fli*C genes are responsible for O157 antigen and flagellar antigens of *E. coli* O157:H7, respectively. The later two genes are markers for *E. coli* O157:H7. In this study, the *eae*A gene negative samples were not further investigated, as presence of *eae*A gene serve as a highly specific and sensitive method to detect *E. coli* O157:H7 (Paton and Patton 1998).

The molecular technique applied for the identification of STEC and serotype *E. coli* O157:H7, the isolates selected on the basis of their biochemical properties. The genotypic characterization was carried out by amplification of *stx*-1, *stx*-2 and *eae*A genes which are responsible for virulence of STEC and *E. coli* O157:H7 and *rfb*E gene for serogroup O157 and *fli*C gene for flagella of O157:H7. Out of 33 samples of cow-dung, 25 isolates were positive for *eae*A gene, among which 13 were positive for *stx*1 gene, 7 isolates were positive for *stx*2 gene, altogether, these were from 14 samples. Therefore, in this study, 42% of the cow dung samples could be characterized as STEC. Similar finding was presented by Vu-Khack *et al.* (2008) of Vietnam where they found 23% fecal samples of cattle contained STEC. Wells of USA (1991) isolated STEC (SLTEC) from 8.4% adult

cow and 19% of heifer and calves from their rectal content. Similar result was also found from a Canadian study of a randomly selected cattle at slaughter where STEC was recovered from 10.5% beef cattle, 19.5% of dairy cows and 3.5% of veal cow (Clarke *et al.* 1988). STEC was also recovered from dung of 17% dairy cows and 9.4% bulls in Germany (Montenegro *et al.* 1999). Fecal content of 10.5% cattle and 9.8% calves found to contain STEC in India (Pal *et al.* 1999) and 11-84% from cattle in Thailand (Suthienkul *et al*. 1990). In Bangladesh, similar study showed that STEC was isolated from rectal content of 37.9% buffalo, 20% cow samples (Islam *et al.* 2008), which is very much similar to the present findings. Presence of higher percentage of STEC in cattle dung, which are ultimately used as green manure can contaminate irrigation water and ultimately carried to different types of fresh produce, in particular lettuce, cabbages, and tomatoes, the vegetable frequently taken as green salad.

Considering the presence of *E. coli* O157:H7 in the cow dung samples, 10 isolates were positive for both *rfb*E and *fli*C genes along with *eae*A gene. These isolates were also positive for *stx*-1 or *stx*-2 or both. Therefore, in the present study, out of 33 cow-dung samples these 10 isolates were confirmed as shiga toxin producing *E. coli* O157:H7. These 10 isolates were recovered from 6 samples. So, in the present findings, 18.18% cow dung were contaminated with *E. coli* O157:H7. This rate is much higher than the report of Nakasone (2005) where he found *E. coli* O157:H7 in 2% of cow dung and of Perera (2015) who found 4% of dung samples contaminated with *E. coli* O157:H7. In India, STEC was isolated from 21.90% of fecal samples of goyal (Bos frontalis) which belonged to 14 sero-group, but no *E. coli* O157:H7 was reported (Rajkhowa *et al*. 2010). In Thailand, *E. coli* O157 was found in 1.54% of bovine feces (Verapan *et al*. 2000). In Bangladesh Islam *et al.* (2008) isolated STEC O157 from rectal content of 7.2% cow whereas, Fazley *et al*. 2014 recovered *E. coli* O157:H7 from 8% samples of cow dung.

According to Zhao *et al.* (1998) and Oldfield (2001), undercooked beef is a major cause of food borne outbreaks. In our study 5 out of 50 beef samples were found positive for

stx-1 or *stx*-2 genes along with *eae*A genes. Therefore, prevalence of STEC in beef was 10%. This result is supported by the findings of Islam *et al*. (2008) who isolated STEC from 8.2% of beef samples. Sukhumungoon *et al*. (2011) reported that 23% cattle meat were contaminated with STEC in Thailand. In India, 1.7% raw beef samples were found STEC positive (Chattopadhaya *et al.* 2001). STEC O157 was isolated from 9% of the minced beef and from raw beef surface swab, and STEC O157 were present in 3.7% samples (Manna 2006). Verappan *et al*. (2000) reported 4.2% of retail beef samples were *E. coli* O157 positive in his study.

In the present study, only one isolate was found which is positive for *eae*A, *stx*-1, *rfb*E and *fli*C. This isolate was confirmed as *E. coli* O157:H7. So, out of 50 samples, only one (1) *E. coli* O157:H7 was isolated which is 2% of total number of samples. This result is in agreement with other reports like 1% in beef of Iran (Jamshidi *et al.* 2008), 2.2% of ground beef in USA (Doyle *et al*. 1997), 2.8% meat and meat product in South Africa (Abong`o BO and Momba 2009). But the rate of isolation in the present study was lower than prevalence rate reported from Ethiopia (8%) (Adem *et al*. 2008), Netharlands (10.4%) (Heuvelink *et al*. 1999), England (13.4%) (Chapman *et al.* 1997), Malaysia (36%) (Radu *et al*. 1998) and 89.50% (Premarathne *et al.* 2017**).**

Raw milk is a medium that sprouts the development of several microbes with resultant spoilage of the product or infections/ intoxications in consumers (Murinda *et al.* 2004; Oliver *et al.* 2005). In the present study, 43 milk samples were included from cow, which were collected from around the Dhaka city. It was found that 19 isolates were positive for *eae*A gene of which, only 3 were *stx*-1 positive and 1 isolate was *stx-2* positive. Therefore, a total of 4 isolates were identified as STEC in milk. So, in case of milk, although in a lesser number then cow dung, but 9.3% of samples were contaminated with STEC. The probable source of contamination could be the cow itself, may be during milking process. Similar results were found in Spain (16%) goat milk (Cortes *et al.* 2005), 40% in fresh milk in Nigeria (Waziri *et al.* 2010), 33.5% raw milk in Malaysia

(Chy *et al.* 2004) and 19.6% milk samples in Denmark (Boel and Jensen 2012) and 36% camel milk in Nairobi, Kenya (Njage *et al.* 2012) were found to be contaminated with STEC. In the present study, no *E. coli* O157:H7 was found in milk samples as none were positive for *fli*C or *rfb*E genes but there are many reports about the presence of *E. coli* O157:H7 in milk such as 6.4% in Denmark (Boel and Jensen 2012), 3.5% in Ethiopia (Bedesh *et al*. 2018). In the present study, all the STEC were accompanied with *eae*A genes which indicated that this milk could be an important vehicle for transmission of STEC to humans and can cause serious human infection.

The soil of cow-shed is supposed to be contaminated with cow-dung and may harbor STEC. From this stand point 22 samples of cow-shed soil were investigated. But none of the samples were found to contain either STEC or *E. coli* O157:H7. This indicates that cattle firms are practicing proper cleanliness and maintaining good hygiene.

In the present study, 48 goat meat samples were included, of which 12 isolates were *stx*-1 positive and 8 isolates were *stx*-2 positive. All of these isolates were *eae*A positive and belonged to 12 samples. Therefore, in this study 25% (12 out of 48) of goat meat samples were STEC positive. This result is close to the result of Kironmoayi *et al*. (2011) where they detected STEC in 40% of mutton and 48% of mutton swabs in Hyderabad, India. Sukhumungoon *et al*. (2011) reported that 38.5% of goat meat were contaminated with STEC.

In the present study, *E. coli* O157 was found in 16.86% samples (8 of 48) isolated from goat meat. This result is much higher than reports from other countries like Iran. It was around 1.7% (Rahimi *et al.* 2012). In the present investigation, prevalence of *E. coli* O157:H7 in goat meat was 10.4% (5 of 48) of the total samples. This result is higher than report from Ethiopia (2%) by Hiko *et al*. (2008) and from USA reported by Jacob (2013) who found 2.7% of carcases swab of goat samples at slaughter.

The feces of goat or goat dropping was also investigated for prevalence of STEC and *E. coli* O157:H7 because cow dung, goat dropping might be a source of *E. coli* O157:H7. In the present study STEC was found in 26% (6 in 22) of the samples. This rate is much higher than most of the previous studies, which is 11.1% in fecal content of goat (Jacob *et al*. 2013) and 10% in Bangladesh (Islam *et al*. 2008); but in agreement with the result of Vu-Khack *et al*.(2008) of Vietnam who found STEC in 38.5% goat feces samples. In the present study, 4 out of 22 samples were positive for *rfb*E gene but only one of them was positive for *fli*C gene; i.e. these 03 are supposed to be *E. coli* O157, but not H7. Therefore, prevalence of *E. coli* O157 is 18.5%. This rate is higher than that reported in Bangladesh (9.1%) by Islam *et al*.(2008). Only one (1) isolate in the present study can be confirmed as *E. coli* O157: H7 as it was positive for *eae*A, *stx*-2, *rfb*E and *fli*C genes (4.5%).This rate is also higher than other reports which was found to be 1.4% in Riyadh, Saudi Arabia (Josef *et al.* 2015). However, higher prevalence rate of *E. coli* O157:H7 in fecal sample of goat was reported in England (9.9%) by Josef *et al.*(2015), in France (95%) by Bastian *et al.* (1999) and in Australia (40%) by Fagan *et al.* (1999).

Potential of multiplex PCR

Multiplex PCR is a modern aspect of detection procedure of any microorganisms. In this technique, multiple genes can be amplified at a time in a single reaction mixture. In this case time consuming biochemical, serological and cyto-toxicity tests could be avoided. Therefore, multiplex PCR gene amplification is a very efficient procedure in terms of cost per test and time of detection. For detection of STEC and *E. coli* O157:H7 a multiplex PCR can be a new dimension. Sahilah *et al*. (2010) of Malaysia used multiplex PCR technique to detect *stx-1* and *stx-2* in *E. coli* O157:H7. Fratamico *et al*. (2000) designed a multiplex PCR assay to simplify detection of *E. coli* O157:H7. They engaged simultaneous amplification of five genes, such as *fliC, stx-1 stx-2, eaeA* and *hlyA* evaluated this technique as it reduce the time required for confirmation of isolates by up to 3 to 4 days. In the present study, multiplex PCR method was experimented for the detection of *eae*A, *stx*-1 and *stx*-2 genes of *E. coli* O157:H7 and STEC. Primer pair

selection is critical in the multiplex PCR assay for simultaneous detection of *eae*A, *stx*-1 and *stx*-2 genes of *E. coli* to ensure specificity, sensitivity and to avoid cross reactivity. With the purified genomic DNA of suspected STEC and *E. coli* O157:H7, optimization of the multiplex PCR was done to have similar amount of specific PCR product. However, high concentration of PCR primers or templates can creates interference in the amplification process. So, concentration of primer pairs which gave vigorous products in individual PCR was kept low. PCR condition was set considering annealing temperature of all primer pair.

Identification by serological test

Various tests can be used to detect STEC and *E. coli* O157:H7, for example direct cultivation, series of biochemical tests, molecular analysis etc. However, benefits of combining conventional method along with sero-diagnostic testing of STEC and *E. coli* O157:H7 added additional value. Serological identification is a critical and credible step in the diagnosis. Sometime this technique is solely used to detect *E. coli* O157:H7 from both environmental, food or human sources. Adem (2008) isolated *E. coli* O157:H7 from goat meat in Ethiopia with this technique. In the present study, the commercially available Wellcolex latex agglutination kit (Ramel, USA) was used to detect the presence of O157 and H7 antigens in the isolates. Culturally and biochemically positive isolates which are *eae*A and stx-1/stx-2 positive were subjected to serological tests. In this study, 9 isolates from cow-dung, 1 from beef were found to show agglutination reaction with anti-O157 sensitize latex and 8 isolates from cow-dung and 1 from beef showed positive reaction against H7 antisera. No isolates from raw-milk and soil of cow shed was found to be positive for agglutination test.

In case of goat, 12 isolates from goat meat samples showed agglutination reaction against O157 antisera whereas 11 isolates showed positive reaction against H7 antisera. 9 isolates showed positive agglutination reaction against both O157 and H7 antisera. So, in total these 9 isolates were confirmed as *E. coli* O157:H7. These isolates were from 5 samples.

So prevalence of *E. coli* O157: H7 in goat meat was 10.4% (5 of 48). In case of goat dropping 5 isolates showed positive agglutination reaction against O157 anti-sera and only one (1) isolate showed agglutination against H7 anti-sera. Therefore, serologically 22.7% (5 of 22) goat dropping samples contained STEC O157:NM and only one (1) isolate was *E. coli* O157:H7 i.e., prevalence rate is 4.5%.

Confirmation of *E. coli* O157:H7 by 16S rRNA gene sequencing

The isolated *E. coli* O157:H7 isolates were subjected to16S rRNA sequencing to determine the specificity of the strain and confirm their molecular identification. The 16S rRNA sequencing was done as it is the most common house -keeping genetic marker which is highly conserved between different species of bacteria. In addition 16S rRNA gene contain hyper variable regions that can provide species-specific signature sequences useful for the identification of bacteria. In the present study 16S rDNA were extracted from *E. coli* O157:H7 isolates by boiling method. These 16S rDNA were amplified by PCR with universal primers 27F and 1492R. The PCR products were analyzed by gel electrophoresis and sequenced.

The raw sequenced data was edited using chromas 2.31 software (available at http://www technelysium.com.au./chromas.html). After BLAST searching (available at w.w.w.ncbi.nlm.nih.gov/blast) the edited sequencing data of the isolates showed more than 98% identity with 16S rDNA sequence of *E. coli* O157:H7 available in GenBank.

Phenotypic characterization of isolated *E. coli* O157:H7

Production of hemolysin is an important phenotypic characteristics of STEC as well as *E.coli* O157:H7. Herbert and Karch (1996) showed that 66% of the STEC were found to have hemolytic properties. In their study, 22 out of 36 strains were positive for the EHEC *hly*A gene of *E. coli* O157:H7, 20 showed entero-hemolytic phenotype in blood agar plate. Beatriz *et al*. (2002) also reported 2 out of 3 STEC isolates had hemolytic phenotype in blood agar plate. According to Mario *et al*. (2010) entero-hemolysin

production was observed in only 11 (29%) of the STEC strain and was not associated with specific biotypes or serotypes. In the present study, entero-hemolytic activity was performed on sheep blood agar plate. None of the isolates were able to produce hemolysis on blood agar plate as they produced no zone of hemolysis around the growth. However, according to Paton *et al.* (1998) nearly all *E. coli* O157:H7 which produced entero-hemolysin, are not hemolytic on standard blood agar plate.

To detect antibiotic resistance 16 *E. coli* O157:H7 isolates collected from different cattle related sources, such as cow-dung, beef, goat meat and goat dropping were subjected to antibiotic sensitivity test. A total of 7 antibiotics namely Ampicillin, Streptomycin, Kanamycin, Ciprofloxacin, Nalidixic acid, Chloramphenicol and Novobiocin were chosen for sensitivity test. 89% isolates were found resistant to Ampicillin. This result has a similarity to the work of Ahmed and Shimamoto (2015) who found 90.3% of *E. coli* O157:H7 isolates were resistant to Ampicillin. In the present study, Streptomycin resistance was found in 19% of the isolates. This result is in agreement with the report of Kim *et al.* (1994) where resistance to Streptomycin was 7.8%. On the other hand, this finding is in contrast with Willkerson *et al*. (2004) who found 66% isolates of *E. coli* O157:H7 were resistant to Streptomycin. Ahmad and Shimamoto (2014) found 87.1% resistance to Streptomycin.

Only 10% of the isolates were resistant to Kanamycin. In a study of Abraham *et al.* (2019) reported that 20% of isolates were resistant to Kanamycin which is very much similar to the present study. But Ahmad and Shimamoto (2015) reported 96.8% isolates were resistant to Kanamycin which is much higher than that of the present study. In the present study *E. coli* O157:H7 isolates showed 30% and 38% resistance to Chloramphenicol and Ciprofloxacin, respectively. Moreover, all the isolates showed 100% resistance to Novobiocin.

Ability to distinguish between pathogenic and non-pathogenic organism is an important parameter when research is dependent on monitoring virulence characteristics of bacteria in working culture. In this respect Congo-Red binding medium test has been used to see the invasiveness of *E. coli* (Harry *et al*. 1998). In the present study, Congo red medium was used to detect the invasiveness of *E. coli* O157:H7. All the isolates were found to be non-invasive because none of the isolates could bind Congo-red color. This result is in contrast with the findings of Gupta *et al*. (2013) where 11.11% of *E. coli* was found to bind with Congo red color.

Enterotoxicity test was done by Rabbit Ilial loop assay according to the procedure of Sanyal *et al.* (1975). Live cell culture of isolated *E. coli* O157:H7 was used. The inoculated intestine was exposed and volume of the fluid accumulated was measured. If the fluid accumulation was 0.4 ml per cm of loop, the loop was considered positive. In the present study fluid accumulation ranged from 0 - 2.7ml per cm. Out of 16 *E. coli* O157:H7 isolates 11 showed strong enterotoxic activity (1.0– 2.7 ml/cm. fluid accumulation). This result is in concurred with Benjamin and Yushau (2018) who reported all six (6) of their isolates showed strong enterotoxic fluid accumulation (1.1-1.4 ml/cm.).

CONCLUSION

The bacteria *Escherichia coli* has been the concern of scientists for its role as food borne pathogen. Among the vast number of serotypes shiga toxin producing *E. coli* (STEC) are the most prominent which causes severe bloody diarrhea to hemorrhagic uremic syndrome. *E. coli* O157:H7 is the most prominent and deadliest serotype of STEC that is responsible for many outbreaks worldwide. The cattle and other domestic animals are considered to be the natural host of this pathogen and cause infection to human through the animal.

Estimation states that diarrheal disease contribute for 4.1% of the total daily global burden of diseases and cause for the deaths of 1.8 million people every year (Islam *et al.* 2006). STEC non-O157 have also been proved to be an important pathogen. One report suggested that some non-O157:H7 STEC strain can cause human illness which accounts for 20-50% of total STEC infection. (Mead *et al.* 1999)

Our findings suggest that dairy cattle are an important reservoir of *E. coli* O157:H7 and other STEC that causes human disease. High prevalence of STEC and *E. coli* O157:H7 in cow-dung and goat dropping indicate that these pathogens are exposed to water body, beef and beef product which will lead to high infection in human. Presence of STEC in milk, beef, goat meat increase health risk in Bangladesh.

Our present study showed high prevalence of STEC and *E. coli* O157:H7 in animals (cow, goat). In Bangladesh previous studies also showed higher prevalence of STEC in different animals (cow, goat, and buffalo, chicken) and other food staffs (beef, meat juice water). These data indicate the possible route of transmission of STEC from animal reservoir to human population.

In present study isolated STEC were *eae*A positive. As *eae*A gene is responsible for efficacy which can make infection more severe, these STEC strains could be very

dangerous. On the other hand in previous study very low prevalence of STEC in stool sample of diarrheal patient indicates there must be a physiological mechanism of human body for which STEC cannot be found in human sample.

Further work to assess the potential burden of STEC and *E. coli* O157:H7 in Bangladesh is warranted. In Bangladesh the cooking style and consumption pattern of beef and goat meat is safe but increasing practice of fast food culture will increase the risk contamination.

Serological identification in combination with multiplex PCR technique may eases the work and reduce the time of identification where real-time PCR is not available.

In many studies prevalence rate is high because they use real-time PCR or other higher and sensitive techniques but their isolation rate is not as high as prevalence rate. So more intensive study will show the actual picture of prevalence of STEC and *E. coli* O157:H7 in Bangladesh.

Observation of antibiotic response of *E. coli* O157:H7 showed that they are resistant to Novobiocin (100%) and ampicillin (87.5%). Further study with antibiotic sensitivity will provide the indication on use of proper drug against *E. coli* infection. This data can give light to appropriate use of antibiotic and help both medicine and veterinary practitioners.

This study emphasizes that raw meat, milk, using unsanitary method in slaughter house and even the butchers are the main source of growth, proliferation and survival of STEC and *E. coli* O157:H7. Therefore several steps should be taken to eradicate or at least to lessen the rate or incident of infection. These steps may include:

- Vaccinating the animals
- Maintaining the proper sanitation and hygiene in slaughter house
- Improving method for meat marketing and packaging.

- Our traditional cooking system should be encouraged both in home and fast food shops.
- Intensive publicity is necessary to promote mass awareness in rural and farming people.
- Risk analysis should be done and introduced because it is a scientific method that evaluates, manage and communicate risk with the assistance of related stake holders. The interested parties and regulatory authorities can implement control measures to ensure safety on the basis of output of risk analysis (Signorini *et al*. 2009).

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APPENDIX-I

Media composition

The composition of the media used in the present study has been given below. Unless otherwise mentioned, all the media were autoclaved at 121°C for 15 min.

1. Sorbitol MacConkey agar (Oxoid, England)

Ingredients	Amount (g/L)
Peptone	20.0
Sorbitol	10.0
Bile salt no. 3	1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	15.0
pH	7.1 ± 0.2

2. Eosine methylene blue agar (Oxoid, England)

Ingredients	Amount (g/L)
Peptone	10.0
Lactose	10.0
Di-potassium hydrogen phosphate	2.0
Eosin Y	0.4
Methylene blue	0.06
Agar	15.0
Final pH	6.8 ± 0.2

3. Bacto EC medium with MUG (Difco, USA)

Ingredients	Amount (g/L)
Casein digest of peptone	20.0
Lactose	5.0
Sodium chloride	5.0
Bile salts mixture	1.5
Di-sodium phosphate	4.0
Monopotassium phosphate	1.5
4-methylumbelliferyl-β-D-glucuronide(MUG)	0.05
Final pH	6.9 ± 0.2

4. Simmon's citrate agar (Difco, USA)

Ingredients	Amount (g/L)
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bacto brom thyol blue	0.08

5. MR-VP reagents

Ingredients	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Di-potassium hydrogen phosphate	5.0
pH	6.9

6. Triple sugar iron agar (BioMerieux, Franch)

Ingredients	Amount (g/L)
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Phenol red	0.0125
Agar	13.0
pH	7.3

7. Trypticase soy broth (Scharlau, Spain)

Ingredients	Amount (g/L)
Casein peptone	17.0
Soya peptone	3.0
Sodium chloride	5.0
Di-potassium phosphate	2.5
Dextrose	2.5
Agar	15.0
pH	7.3 ± 0.2

8. Peptone water

Ingredients	Amount (g/L)
Peptone	10.0
Sodium chloride	5.0

9. T_1N_1 soft agar

Ingredients	Amount (g/L)
Tryptone	10.0
Sodium chloride	10.0
Agar	6.0

10.Luria Bertani broth

Ingredients	Amount (g/L)
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	10.0

11.Blood agar

Ingredients	Amount (g/L)
Lab lemco powder	10.0
Peptone	10.0
NaCl	5.0
Agar	15.0
Sterile defibrinated blood	70 ml
pH	7.3 ± 0.2

APPENDIX-II

Buffers and reagents

1. 1M Tris-Cl (pH 8.0)

121.1 gm of Tris-base was dissolved in 800 ml of distilled water. The pH was adjusted to the desired value by adding concentrated HCl and the final volume was made up to 1 liter with distilled water. The solution was sterilized by autoclaving and was stored at room temperature.

2. Phosphate buffered saline (PBS)

PBS was prepared by dissolving 8.0 gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na_2HPO_4 and 2.0 gm of KH_2PO_4 in 800 ml of distilled water. The pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 liter by distilled water. The solution was sterilized by autoclaving and was stored at room temperature.

3. 0.5M EDTA

37.24 gm of EDTA was dissolved in 150 ml of distilled water and adjust the pH to 8.0 with pellets of NaOH. Adjust the volume up to 200 ml and autoclaved. 0.5M EDTA was stored at room temperature.

4. TE buffer

TE buffer (10 mM Tris-Cl/ 1mM EDTA, pH 8.0) was prepared by diluting concentrated stocks of 1 M Tris-Cl and 0.5 M EDTA in distilled water. The buffer was autoclaved and was stored at room temperature.

5. 10 X TBE (pH 8.3)

54.0 gm of Tris-base, 27.5 gm of boric acid and 20 ml of 0.5 M EDTA (pH 8.0) were taken and the pH was adjusted to 8.3. Then distilled water was added to the mixture to make 500 ml solution and the buffer solution was stored at room temperature.

6. 6 X Gel loading dye

3 ml glycerol, 25 mg bromo-phenol blue was taken and then distilled water was taken to make 10 ml solution. The gel loading dye was stored at room temperature.

7. Ethidium bromide solution

2.5 mg of ethidium bromide (Sigma, USA) was dissolved in 5 ml of distilled water at a concentration of 0.5 mg/ml. This solution was covered with aluminum foil and stored at room temperature.

8. Kovac's reagent

1.25 gm of para-dimethylaminobenzaldehyde was dissolved in 18.75 ml of amylalcohol. Then concentrated HCl was added to make the final volume 25 ml. This reagent was covered with aluminum foil and stored at 4°C.

9. Methyl red reagent

0.01 gm of methyl red was dissolved in 30 ml of 95% ethanol. Then distilled water was added to make the final volume 50 ml. This reagent was covered with aluminum foil and stored at 4°C.

10. Barritt's reagent

Solution A

1.25 gm of alpha-naphthol was dissolved in 95% ethanol with constant stirring to make 25 ml solution. This solution was covered with aluminum foil and stored at 4°C.

Solution B

10 gm of KOH was dissolved in distilled water. The solution became warm. After cooling to room temperature, creatine was dissolved by stirring. Distilled water was added to adjust final volume to 25 ml. This solution was covered with aluminum foil and stored at 4°C.

11. Oxidase reagent

100 mg of N,N,N^1,N^1-tetramethyl-p-phenylenediamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminum foil. Then the solution was stored at 4°C.

APPENDIX-III

Instruments

The important equipment used through the study are listed below:

- Autoclave, HL-42AE : Hirayama crop, Japan
- Class II microbiological safety cabinet : Labcaire, USA
- Stomacher, 400 CIRCULATOR : Seward, England
- Dual-intensity transluminator, TS-40 : USA
- Electric balance, Scout, SC4010 : USA
- Incubator : Japan
- Freezer (-30°C) : Liebherr, Germany
- Refrigerator (4°C) : Vest frost
- Gel documentation : Bio-Red, Germany
- Horizontal gel electrophoresis apparatus, H1-SET : UK
- Microcentrifuge, Mikro 20 : Germany
- Micropipettes : Eppendorf, Germany
- Microwave oven, D90N30 ATP : Butterfly, China
- Millipore filter, 045 µm : UK
- Automated thermocycler, 12137 : Bio-Rad, Japan
- Power pack : Biometra, Germany
- pH meter, HI 2211 : Hanna, China
- Centrifuge, 5804 : Eppendorf, Germany