Immune response to Shiga toxin producing *Escherichia coli* : Detection of antibodies against outer membrane proteins of the bacteria in healthy population of Bangladesh

A Dissertation Submitted to the Department of Mathematics and Natural Sciences, BRAC University in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biotechnology

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Declaration

I hereby solemnly declare that the research work embodying the results reported in this thesis entitled "**Immune response to Shiga toxin producing *Escherichia coli* : Detection of antibodies against outer membrane proteins of the bacteria in healthy population of Bangladesh**" submitted by the undersigned, has been carried out under joint supervision of Professor Naiyyum Choudhury, Coordinator of Biotechnology Program, Department of Mathematics and Natural Science, BRAC University, Professor Chowdhury Rafiqul Ahsan, Department of Microbiology, University of Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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Dedication

To
My Beloved
Family

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Abstract

Escherichia coli O157:H7, a serotype of Shiga toxin producing *E. coli* (STEC) is responsible for numerous food and waterborne outbreaks more reported in industrialized countries than from developing countries. Until recently, no outbreak has been reported in Bangladesh nevertheless of their prevalence in the environment. Therefore, the hypothesis of possible immunity present in the healthy population in Bangladesh need to be investigated. As outer membrane proteins (omp) of *E. coli* O157:H7 act as important components to stimulate the immune response during infection, the present study has been focused on investigating immune response in healthy human sera against outer membrane proteins of *E. coli* O157:H7 isolated and characterized for stx 1 and stx 2 positive from local bovine feces and comparing with reference clinical strain of *E. coli* O157:H7 as well. Previously isolated and characterized strains are re confirmed using simple biochemical tests for *E. coli.* Crude protein extraction is carried out using simple sds-lysis and extraction of outer membrane proteins are performed by using Tris-sucrose-EDTA (TSE) buffer. For both crude extract and omps, proteins are characterized by SDS-PAGE assay where a prominent band of molecular weight 45 kDa is obtained for both vegetables and bovine isolates. Western blot experiment showed similar band pattern of 45 kDa in 48 randomly collected healthy human sera accompanied by ELISA with different dilutions and one positive and negative control. This prominent band of molecular weight 45 kDa has been obtained indicating its role as an immunogen. Further, ELISA was performed on 336 randomly collected healthy human sera where it clearly indicates the high level of immunogenic response against outer membrane proteins is present in all tested human sera indicating the presence of acquired immunity in this particular population against the outer membrane proteins. All these data strongly suggest the immunogenic role of ~45 kDa outer membrane protein of *E. coli* O157:H7 and the evidence that immunity against this protein is present in human sera of Bangladesh. This study could explore a new insight in the field of immunology and vaccine development and highly demands for further study in vivo.

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List of Abbreviations

MW	:	Molecular weight
kDa	:	Kilodalton
mg	:	Milligram
gm	:	Gram
kg	:	Kilogram
L	:	Liter
ml	:	Milliliter
µl	:	Microliter
mM	:	Millimolar
M	:	Molar
mm	:	Millimeter
µm	:	Micrometer
mA	:	Milliampere
V	:	Volt
PBS	:	Phosphate buffer solution
e.g.	:	For example
et al.	:	And others
pH	:	Negative logarithm of hydrogen ion concentration
rpm	:	Rotation per minute
BCIP	:	5-bromo- 4-choro-indolyl phosphate
NBT	:	Nitroblue tetrazolium

1. Introduction and Literature Review

1.1 Background

Escherichia coli has its primary niche in the large intestine and lower part of the small intestine of mammals, larger birds and reptiles and has been particularly well studied in humans and domestic animals. It is a diverse species with both commensal forms and pathogenic forms [Zhou *et. al*., 2010].

Normally, *E. coli* bacteria live in the intestines of healthy people and animals. Most varieties of *E. coli* are not dangerous or cause short-lived diarrhea but certain strains, such as *E. coli* O157:H7 can cause severe abdominal cramps, bloody diarrhea and vomiting. *E. coli* is a Gram-negative, facultative anaerobic, rod-shaped bacterium of the genus Escherichia that is commonly found in the lower intestine of warm-blooded organisms [Tortora *et al*., 2012].

The genus *E. coli* is named after Theodor Escherich who isolated the type species of the genus. It is a gram-negative bacillus occurring singly or in pairs. *E. coli* is facultative anaerobe with both a fermentative and respiratory type of metabolism. It is either nonmotile or motile by peritrichous flagella. *E. coli* is a major facultative inhabitant of the large intestine and ubiquitous in the human environment. It is one of the most frequent causes of the many common bacterial infections, including cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI) and traveler's diarrhea and other clinical infections such as neonatal meningitis. Since 1885, *E. coli* has been recognized as both a harmless commensal and a versatile pathogen [Bower.,1999].

E. coli is a gram negative, rod shaped bacterium that is often used in laboratory studies and is usually harmless, residing in the human digestive tract as part of the normal flora. *E. coli* O157:H7 is a pathogenic strain of *E. coli* that was first identified as a cause of disease in the United States in 1982, during an investigation into an outbreak of gastrointestinal illness. The organism is also called enterohemorrhagic *E. coli* (EHEC) because it causes abdominal pain with diarrhea that may contain blood. It lives in the intestines of healthy cattle and can contaminate meat during slaughter. It is believed that the widespread use of antibiotics by American farmers has promoted the horizontal gene transfer of antibiotic resistance genes and pathogenicity islands that has led to the creation of new pathogenic strains such as *E. coli* O157:H7.

In a previous study in Bangladesh, a total of 452 children with diarrhoea and 602 matched control children without diarrhoea were investigated for the presence of diarrhoeagenic *E. coli* [Albert et al., 1995]. In children (up to 5 years of age) with diarrhoea, enteropathogenic *E. coli* (EPEC) was the most prevalent (15.5 %), followed by enterotoxigenic *E. coli* (12 %), enteroaggregative *E. coli* (9.5 %) and diffuse adherent *E. coli* (8.2 %). Enterohaemorrhagic *E. coli* possessing a stx gene was not detected in any of the children with diarrhoea but was detected in five children without diarrhoea. No further characterization of these five isolates was performed. Therefore, the specific role of STEC in causing diarrhoeal illness in this area is difficult to estimate. The reasons for the low prevalence of STEC-associated diarrhoea among hospitalized and community patients are still not clear. However, protective immunity against STEC could be an explanation, and was addressed by most of the studies done in developing countries [Gianantonio et al., 1964; Lopez et al., 1989; Navarro et al., 2003; Seriwatana et al., 1988]. This could be associated with the repeated antigenic stimulation in a contaminated environment where diarrhoeal diseases are considered endemic [Navarro et al., 2003]. It has also been suggested [Beutin et al., 1998] that EPEC infections in early childhood confer cross-reacting protective immunity against STEC types that share common antigens (such as LPS and intimin) with classical EPEC strains.

However, a detailed study of the immune status of patients with diarrhoea as well as healthy controls in areas where enteric pathogens are considered to be endemic should be carried out in order to explain these phenomena [Islam *et al.,* 2006].

1.2 Literature Review

1.2.1 *Escherichia coli*

E. coli is a common bacterium that has been studied intensively by geneticists because of its small genome size, normal lack of pathogenicity and ease of growth in the laboratory. The bacterium *E. coli*, originally known as Bacterium coli commune, belongs to the Family *Enterobacteriaceae* (Table 1.1) and was first isolated and characterized in 1885 by the German scientist and pediatrician Theodore Escherich. The bacteria naturally and harmlessly exists in the intestines of all warm-blooded animals, hence the Greek-related root of its Family name, *Enterikos*, meaning “intestine”. It has been stated that the average *E. coli* population in an adult’s

intestine is approximately 0.1 percent of the total bacteria. The bacteria are needed by the body to aid in the production of several vitamins, such as Vitamin K and the B-Vitamins. *E. coli* is the major component of normal healthy stool. Some species of *E. coli* co-exist with us nicely; some species cause serious disease. Most of the *E. coli* causes no trouble to humans. Problems arise either if *E. coli* gets somewhere they shouldn't be (such as the urinary tract) or if people are infected by one of the dangerous strains, such as *E. coli* O157:H7. *E. coli* is the major cause of diarrhea (some mild and some severe) [Bower *et al.*,, 1999]. It is also a common friendly bacterial inhabitant of the intestinal tract, aiding in the digestion of food. In scientific research, *E. coli* is a standard vector for rapid mass production of biological building blocks, from DNA to protein. Rare would be a biological researcher who has not used *E. coli*. The strain found in healthy intestines and used in the laboratory is different from the dangerous strain being reported in the news. *E. coli* O157:H7 can be lethal. Its extra genes enable it to stick to the walls of the intestine and produce the virulent Shiga toxin, which injures cells in the intestinal walls as well as blood vessels in the intestine, leading to bleeding. In the bloodstream, these toxins damage other blood vessels, particularly in the kidneys, leading to renal failure.

1.2.2 Morphological features:

Table 1.1: Classification of *E. coli*

Specific classification	
Domain	Bacteria
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gamma Proteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Escherichia*
Species	*Escherichia coli (E. coli)*

- **Domain and Kingdom:** *E. coli* fits into the domain and kingdom of Bacteria because members of this group are unicellular microorganisms.

- **Phylum:** *E. coli* fits into the phylum Proteobacteria because members of this group are Gram-negative (G-) bacterium with an outer membrane composed primarily of lipopolysaccharides.
- **Class**: *E. coli* fits into the class Gamma Proteobacteria because members of this group are facultatively anaerobic G- bacterium.
- **Order:** *E. coli* fits into the order Enterobacteriales because members of this group are rod-shaped facultatively anaerobic G- bacterium.
- **Family**: *E. coli* fits into the family Enterobacteriaceae because members of this group are motile via peritrichous flagella that grows well at 37 ^{o}C, is Oxidase negative, Catalase positive, and reduces nitrates.
- **Genus**: *E. coli* fits into the Genus Escherichia because members of this group are mostly opportunistic flora that is enteric (colonize in the intestinal tract of mammals).
- **Species:** *E. coli* is one of five species recognized under the Genus Escherichia. What makes *E. coli* unique is by these biochemical activities: ferments lactose, possesses lysine decarboxylase, is Vogus-Proskauer negative, produces indole, doesn't grow on nitrate, and doesn't produce H_2S.

1.2.3 Biochemistry of *E. coli*

E. coli, is a prokaryotic organism without a nuclear membrane, is a representative living material often used in laboratories and class rooms. *E. coli* reproduces rapidly (under optimal situation 0.5 hr/generation) such that results for a number of experiments can be quickly obtained. Certain mutants of *E. coli* have been defined that cannot express certain proteins at saturation growth and therefore die. *E. coli* was also the organism used to elucidate the regulation of the *lac* operon in genetics. Its ability to take up exogenous genetic material under the procedure known as DNA-mediated cell transformation has also made it a popular model for studies using recombinant DNA. Using recombinant DNA techniques, *E. coli* can be manipulated in research laboratories and in the classroom to produce any DNA, RNA or protein of interest. Also, it is easy to manipulate *E. coli* both genetically and biochemically. Most importantly, it shares fundamental characteristics, such as DNA and messenger RNA; with all other organisms. The value of *E. coli* in recombinant DNA makes it a good model organism to study the genetic material. An *E. coli* genome contains between 4200 and 5500 genes, with

<2000 genes conserved among all strains of the species (the core genome). The bacterium's pan-genome (genetic repertoire of a given species) consists of almost 20,000 genes [Mora *et al*., 2011]. Continuous gene flux occurs during *E. coli* divergence, mainly as a result of horizontal gene transfers and deletions. This genetic plasticity accelerates the adaptation of *E. coli* to varied environments and lifestyles, as it allows multiple gene combinations that result in phenotypic diversification and the emergence of new hypervirulent (STEC and EAEC O104:H4-B1-ST678) and successful (ExPEC O25b:H4-B2-ST131) strains that combine both resistance and virulence genes, which in classical pathogenic *E. coli* strains traditionally have been mutually exclusive [Mora *et al*., 2011].

1.2.4 Types of *E. coli*

Certain isolates of *E. coli* have been implicated in a wide range of diseases that affect either animals or humans worldwide. To date, eight pathovars and their mechanisms of disease have been extensively studied. These pathovars can be broadly classified as either diarrhoeagenic *E. coli* or extraintestinal *E. coli* (ExPEC). Six pathovars-enteropathogenic *E. coli* (EPEC), entero-haemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC; including *Shigella*), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) are diarrhoeagenic and two pathovars-uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) are the most common ExPEC isolates. Other pathovars have been identified, but their mechanisms of pathogenesis are not as well defined [Croxen and Finlay, 2010].

i. **Enteropathogenic *E. coli* (EPEC)**

EPEC is a major cause of potentially fatal diarrhea in infants in developing countries [Kaper *et al*., 2004]. They do not possess any colonization factors and do not produce ST or LT toxins. They produce a non fimbrial adhesin designated intimin, an outer membrane protein that mediates the final stages of adherence. Although they do not produce LT or ST toxins, there are reports that they produce an enterotoxin similar to that of *Shigella*. Other virulence factors may be related to those in *Shigella* [Wellington and vanElsas., 1992]. Adherence of EPEC strains to the intestinal mucosa is a very complicated process and produces dramatic effects in the ultra structure of the cells resulting in rearrangements of actin in the vicinity of adherent bacteria. The phenomenon is sometimes called "attaching and effacing" of cells. EPEC strains are said to be

"moderately-invasive" meaning they are not as invasive as *Shigella*, and unlike ETEC or EAEC, they cause an inflammatory response. The diarrhea and other symptoms of EPEC infections probably are caused by bacterial invasion of host cells and interference with normal cellular signal transduction, rather than by production of toxins. They are an important cause of traveler's diarrhea in Mexico and in North Africa [Uttly *et al*., 1998].

ii. **Enterohemorrhagic *E. coli* (EHEC)**

Cattle are a key reservoir for EHEC, which is a highly infectious A/E (human attaching and effacing) pathogen that colonizes the distal ileum and large bowel in humans and is often the causative agent of outbreaks of severe gastroenteritis in developed countries. Transmission to humans usually occurs through contaminated food and water. In North America, Japan and parts of Europe, most outbreaks are due to EHEC serotype O157:H7, whereas other serotypes are important health concerns in other developed countries. Adults and children infected with EHEC suffer from haemorrhagic colitis (bloody diarrhea) and further complications can lead to the potentially fatal haemolytic uraemic syndrome (HUS) [Kaper *et al*., 2004]. EHEC are also considered to be "moderately invasive". Nothing is known about the colonization antigens of EHEC but fimbriae are presumed to be involved. The bacteria do not invade mucosal cells as readily as *Shigella* but EHEC strains produce a toxin that is virtually identical to the Shiga toxin. The toxin plays a role in the intense inflammatory response produced by EHEC strains and may explain the ability of EHEC strains to cause HUS. The toxin is phage encoded and its production is enhanced by iron deficiency [Griffin *et al.*, 1995; Samuel *et al.*, 1988]

iii. Enterotoxigenic *E. coli* (ETEC)

ETEC is an important cause of diarrhea in infants and travelers in underdeveloped countries or regions of poor sanitation. The disease varies from minor discomfort to a severe cholera-like syndrome. ETEC is acquired by ingestion of contaminated food and water and adults in endemic areas evidently develop immunity. The disease requires colonization and elaboration of one or more enterotoxins. Both traits are plasmid-encoded. ETEC adhesins are fimbriae which are species-specific. For example, the K-88 fimbrial Ag is found on strains from piglets; K-99 Ag is found on strains from calves and lambs; CFA I and CFA II are found on strains from humans. These fimbrial adhesins adhere to specific receptors on enterocytes of the proximal small

intestine. Enterotoxins produced by ETEC include the LT (heat-labile) toxin and/or the ST (heat-stable) toxin, the genes for which may occur on the same or separate plasmids. The LT enterotoxin is very similar to cholera toxin in both structure and mode of action. It is an 86 kDa protein composed of an enzymatically active (A) subunit surrounded by 5 identical binding (B) subunits. It binds to the same identical ganglioside receptors that are recognized by the cholera toxin (i.e., GM1) and its enzymatic activity is identical to that of the cholera toxin [Wellington and van Elsas., 1992].

iv. Enteroinvasive *E. coli* (EIEC)

EIEC closely resembles *Shigella* in its pathogenic mechanisms and the kind of clinical illness they produce. EIEC penetrates and multiplies within epithelial cells of the colon causing widespread cell destruction. The clinical syndrome is identical to *Shigella* dysentery and includes a dysentery-like diarrhea with fever. EIEC apparently lacks fimbrial adhesions but does possess a specific adhesion that as in *Shigella* and is thought to be an outer membrane protein. Also, like *Shigella*, EIEC strains are invasive organisms. They do not produce LT or ST toxin and unlike *Shigella*, they do not produce the *Shigella* toxin [Wellington and van Elsas., 1992].

v. Enteroaggregative *E. coli* (EAEC)

The distinguishing feature of EAEC strains is their ability to attach to tissue culture cells in an aggregative manner. These strains are associated with persistent diarrhea in young children. They resemble ETEC strains in that the bacteria adhere to the intestinal mucosa and cause non-bloody diarrhea without invading or causing inflammation. This suggests that the organisms produce a toxin of some sort. Recently, a distinctive heat labile plasmid encoded toxin has been isolated from these strains, called the EAST (EnteroAggregative ST) toxin. They also produce a hemolysin related to the hemolysin produced by *E. coli* strains involved in urinary tract infections. The role of the toxin and the hemolysin in virulence has not been proven. The significance of EAEC strains in human disease is controversial [Wellington and van Elsas., 1992].

vi. Diffusely adherent *E. coli* (DAEC)

DAEC is a heterogenous group that generates a diffuse adherence pattern on HeLa and HEp-2 cells. This pattern is mediated by proteins encoded by a family of related operons, which includes both fimbrial (for example, Dr and F1845) and afimbrial (Afa) adhesins, collectively designated Afa–Dr adhesins [Servin *et al.*, 2005]. DAEC isolates that express any of the Afa–Dr adhesins (which are referred to as Afa–Dr DAEC) colonize the small bowel and have been implicated in diarrhea in children between the ages of 18 months and 5 years, as well as in recurring urinary tract infections (UTIs) in adults [Servin *et al.*, 2005].

vii. Uropathogenic *E. coli* (UPEC)

UPEC infections account for roughly 80% of all UTIs, causing cystitis in the bladder and acute pyelonephritis in the kidneys. UPEC has the challenge of moving from the intestinal tract to establish an infection in the urinary tract, where it uses peptides and amino acids as the primary carbon source for fitness [Alteri *et al*., 2009]. The ability to ascend the urinary tract from the urethra to the bladder and kidneys reflects exceptional mechanisms for organ tropism, evading innate immunity and avoiding clearance by micturition. Several highly regulated virulence factors contribute to this complex pathogenesis, including multiple pili, secreted toxins (for example Sat and vacuolating autotransporter toxin (Vat)), multiple iron acquisition systems and a polysaccharide capsule [Wiles *et al*., 2008].

1.3 Pathogenicity of *E.coli:*

The cell-surface structure of Gram-negative bacteria consists of three essential layers: the cytoplasmic or inner membrane (IM), the outer membrane (OM) and the periplasmic space between the IM and OM. The OM of *E. coli* has a highly specialized structure and is usually associated firmly with the underlying peptidoglycan layer predominantly through lipoprotein/matrix protein and linked with cell-surface lipopolysaccharides (LPS). The major components of the OM are phospholipids, LPS and proteins which help it to serve as a physical barrier between the bacterial body and its surroundings and make the organism resistant to protects the cell against bile salts, antibiotics, proteolytic enzymes and other hostile factors and also facilities the uptake of nutrients. The aim of the present work was to detect the diversity of *E. coli* collected from different sources estimated by outer membrane protein (OMP).

1.3.1 Detection of *E. coli* : Culture-Based Methods

1.3.1.1 Tryptic Soy Broth

Tryptic Soy Broth is used for the cultivation of a wide variety of microorganisms. Enzymatic Digest of Casein and Enzymatic Digest of Soybean Meal are nitrogen sources in TSB. Dextrose is the carbon energy source that facilitates organism growth. Sodium Chloride maintains osmotic balance; Dipotassium Phosphate is a buffering agent. [Cunnif *et al.*,1995]

1.3.1.2 MacConkey Agar

The agar medium most commonly used for the isolation of *E. coli* is MacConkey agar. It is a selective media which contains lactose as sugar, peptone, sodium chloride, bile salt; inhibits the growth of other gram positive Enterobacteriaceae, crystal violet and neutral red is also used to understand the nature of fermentation.

MacConkey Agar is recommended for the detection and isolation of Gram-negative organisms from clinical, dairy; food, water, pharmaceutical and industrial sources. Enzymatic digest of gelatin, enzymatic digest of casein, and enzymatic digest of animal tissue are the nitrogen and vitamin sources in MacConkey Agar. Lactose is the fermentable carbohydrate. During Lactose fermentation a local pH drop around the colony causes a color change in the pH indicator, Neutral Red and bile precipitation. Bile salts mixture and Crystal Violet are the selective agents, inhibiting Gram-positive cocci and allowing Gram-negative organisms to grow [Holt and Krieg, 1994]. Sodium Chloride maintains the osmotic environment. Agar is the solidifying agent.

1.4 Virulence Factors:

1.4.1 Adhesion/colonization Pathogenic *E. coli* strains possess specific adherence factors that allow them to colonize sites that *E. coli* does not normally inhabit, such as the small intestine and the urethra.. Most frequently these adhesins form distinct morphological structures called fimbriae (also called pili) or fibrillae, which can belong to one of several different classes. Fimbriae are rod-like structures of 5–10 nm diameters that are distinct from flagella. Fibrillae are 2–4 nm in diameter and are either long and wiry or curly and flexible [Cassels and Wolf., 1995]. The Afa adhesins that are produced by many diarrhoeagenic and uropathogenic *E. coli* are described as afimbrial adhesins, but in fact seem to have a fine fibrillar structure that is difficult

to visualize [Keller *et al*., 2002]. Adhesins of pathogenic *E. coli* can also include outer-membrane proteins, such as intimin of UPEC and EHEC, or other non-fimbrial proteins. Some surface structures trigger signal transduction pathways or cytoskeletal rearrangements that can lead to disease. For example, the members of the Dr family of adhesins that are expressed by DAEC and UPEC bind to the Decay-Accelerating Factor (DAF also known as CD55), which results in activation of phosphatidylinositol 3-kinase (PI-3-kinase) and cell-surface expression of the major histocompatibility complex (MHC) class I-related molecule MICA [Tieng *et al*., 2002]. The IcsA protein of EIEC nucleates actin filaments at one pole of the bacterium, which allows it to move within the cytoplasm and into adjacent epithelial cells on a 'tail' of polymerized actin [Golderg *et al*., 1995]. Even surface structures that are present on commensal *E. coli* strains can induce signaling cascades if the organism encounters the appropriate receptor. The LPS of *E. coli* and other Gram-negative bacteria binds to Toll-like receptor 4 (TLR4), triggering a potent cytokine cascade that can lead to septic shock and death [Tapping *et al*., 2000]. Flagellin, the main component of flagella, can bind to TLR5, thereby activating interleukin (IL)-8 expressions and an inflammatory response [Hayashi *et al*., 2001].

1.4.2 Toxins

More numerous than surface structures that trigger signal transduction pathways are secreted toxins and other effector proteins that affect an astonishing variety of fundamental eukaryotic processes . Concentrations of important intracellular messengers, such as cyclic AMP, cyclic GMP and Ca^{2+}, can be increased, which leads to ion secretion by the actions of the heat-labile enterotoxin (LT), heat-stable enterotoxin a (STa) and heat-stable enterotoxin b (STb), respectively, all of which are produced by different strains of ETEC [Sears and Kaper,1996]. The Shiga toxin (Stx) of EHEC cleaves ribosomal RNA, thereby disrupting protein synthesis and killing the intoxicated epithelial or endothelial cells [Melton-Celsa and O'Brien, 1998]. The cytolethal distending toxin (CDT) has DNaseI activity that ultimately blocks cell division in the G2/M phase of the cell cycle [De Rycke and Oswald., 2001]. Another toxin that blocks cell division in the same phase, called Cif (cycle-inhibiting factor), does not possess DNaseI activity, but might act by inhibition of Cdk1 kinase activity [Marches *et al*., 2003]. The cytotoxic nectrotizing factors (CNF 1 and CNF 2) deaminate a crucial glutamine residue of RhoA, Cdc42 and Rac, thereby locking these important signaling molecules in the 'on' position and leading to

marked cytoskeletal alterations, multi nucleation with cellular enlargement, and necrosis [Lerm *et al*.,1999]. The Map protein of EPEC and EHEC has at least two independent activities stimulating Cdc42-dependent filopodia formation and targeting mitochondria to disrupt membrane potential in these organelles [Kenny *et al*., 2002]. The various toxins are transported from the bacterial cytoplasm to the host cells by several mechanisms. LT is a classic A–B subunit toxin that is secreted to the extracellular milieu by a type II secretion system [Tauschek *et al*., 2002]. Several toxins, such as Sat, Pet and EspC are called autotransporters because part of these proteins forms a β-barrel pore in the outer membrane that allows the other part of the protein extracellular access [Henderson *et al*., 1998]. The SPATEs (serine protease auto transporters of enterobacteriaceae) are a subfamily of serine protease autotransporters that are produced by diarrhoeagenic and uropathogenic *E. coli* and *Shigella* strains. EPEC, EHEC and EIEC contain type III secretion systems, which are complex structures of more than 20 proteins forming a 'needle and syringe' apparatus that allows effector proteins, such as Tir and IpaB to be injected directly into the host cell [Hueck *et al*., 1998]. The UPEC haemolysin is the prototype of the type I secretion mechanism that uses TolC for export from the cell [Balakrishnan *et al*., 2001]. No type IV secretion systems have been described for pathogenic *E. coli*, with the exception of the type IV-like systems that are involved in conjugal transfer of some plasmids.

The versatility of the *E. coli* genome is conferred mainly by two genetic configurations: virulence related plasmids and chromosomal pathogenicity islands. All six categories of diarrheagenic *E. coli* have been shown to carry at least one virulence related property upon a plasmid. EIEC, EHEC, EAEC and EPEC strains typically harbor highly conserved plasmid families, each encoding multiple virulence factors [Hales *et al*., 1992; Nataro *et al*., 1987; Wood *et al*., 1986]. McDaniel and Kaper have shown recently that the chromosomal virulence genes of EPEC and EHEC are organized as a cluster referred to as a pathogenicity island [McDaniel *et al*., 1995; McDaniel and Kaper., 1997]. Such islands have been described for uropathogenic *E. coli* strains [Donnenberg and Wech, 1996] and systemic *E. coli* strains [Bloch and Rode**,** 1996] as well and may represent a common way in which the genomes of pathogenic and nonpathogenic *E. coli* strains diverge genetically. Plasmids and pathogenicity islands carry clusters of virulence traits, yet individual traits may be transposon encoded (such as ST) [So and McCarthy, 1980] or phage encoded (such as Shiga toxin) [O'Brien *et al*., 1992].

1.5 EHEC : A deadly pathogenic strain of *E.coli*

1.5.1 Virulence factors associated with EHEC

Enterohaemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 is an important pathogen that can cause a variety of clinical symptoms ranging from mild to severe bloody diarrhoea. The main virulence factors of EHEC are Shiga toxins (Stx), responsible for the hemorrhagic syndrome of the infection such as hemolytic uremic syndrome (HUS). EHEC is widely distributed in domestic ruminants, an important route for transmission to humans. Bovine are the main reservoir for EHEC, especially serotype O157:H7 that is transmitted to humans primarily through consumption of contaminated foods. Faecal contamination of water and other foods may also lead to infection. EHEC can survive and persist in different ecological habitats such as soil, manure and aquatic environment.

1.5.2 Pathogenesis and clinical manifestations

EHEC colonize the gastrointestinal tract and after an incubation period of 2-3 days cause diarrhea and abdominal pain. In about 80% of cases after a 2-4 day interval there is progression into hemorrhagic colitis (HC) or bloody diarrhea 6. Within 3-13 days after the beginning of diarrhea about 10-15% of patients with HC go on to develop a life-threatening condition known as hemolytic uremic syndrome (HUS) which includes the triad of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. It is the most frequent cause of acute renal failure and mortality in children *E.Coli* O157:H7 is the predominant serotype that has caused major outbreaks of gastroenteritis worldwide and is of global concern to public health. Extrarenal complications such as rhabdomylosis, myocardial and CNS damage are associated with increased mortality in *E.coli* O157:H7 infections. *E.coli* O157:H7 belongs to the larger category of Shiga toxin producing *E.coli* (STEC). Shiga toxins (Stxs) produced by EHEC are the major virulence factors that are thought to be responsible for the pathogenesis of HUS [Biju *et al.*, 2014].

1.5.2.1 EHEC and Human Disease

HUS and other devastating manifestations of EHEC infection are caused by Stxs, extremely potent cytotoxins that enter host cells expressing toxin receptors and block protein synthesis by irreversibly damaging ribosomal RNA [Endo *et al*., 1988].

EHEC are a subgroup of Stx-producing *E. coli* (STEC) that together comprise hundreds of O:H serotypes and are commonly carried by healthy wild and domesticated ruminant animals [Beutin *et al.,* 1993].

While the attention devoted to EHEC O157:H7 is justified by the pathogenicity, low infectious dose, and ability of the bacteria to survive in extra-intestinal environments, a number of non-O157:H7 EHEC cause severe human disease and are often implicated in HUS and their animal reservoirs and modes of transmission are not well understood [Karch et al., 2005]. Non-O157:H7 EHEC lack the biochemical characteristics differentiating them from nonpathogenic E. coli, and thus present a special detection challenge and are perhaps insufficiently investigated [Witold *et al.,* 2011].

1.5.2.2 Identification and Detection of EHEC O157:H7

1.5.2.2.1 Symptoms

An EHEC infection can make a person very ill. Symptoms usually begin two to five days after ingesting contaminated foods or liquids, and may last for up to eight days or more. The following are some of the most common symptoms associated with EHEC. However, each person may experience symptoms differently:

- Abdominal cramps
- Severe bloody diarrhea
- Non-bloody diarrhea
- Little to no fever
- Fatigue
- Nausea
- Hemolytic uremic syndrome (HUS), a serious complication that can lead to kidney failure and death

Symptoms may range from none to HUS. In HUS, an individual's red blood cells (oxygen-carrying cells in the bloodstream) are destroyed and the kidneys stop working. Approximately 5 to 10 percent of infections can result in this syndrome. Children and the elderly may be more prone to develop this complication, which may be life-threatening.

1.5.2.2.2 Diagnosis

EHEC can be confirmed with a special stool culture. Stool samples are tested to compare with the source or contaminated food that has caused an outbreak. Almost all EHEC O157 display delayed (negative) fermentation of D-sorbitol with >99% of the sorbitol-negative H7 strains of the O157 serotype [al-Saigh *et al*., 2004] and resistant to several wide-spectrumantibiotics and antimicrobial agents [Ratnam *et al*., 1988]. The bacteria are commonly identified by plating on sorbitol MacConkey agar supplemented with cefixime and potassiumtellurite (CT-SMAC) [Chapman *et al*., 1991] and confirmed serologically and biochemically as pale colonies positive for O157 and H7. The probability of detectioncan is greatly increased by nonselective enrichment in broth (e.g., buffered peptone water, or McConkey broth) without antimicrobials other than novobiocin as fully viable bacteria of some strains may not grow in selective media. Low prevalence or EHEC O157 may thus reflect scarcity of the bacteria, but can also result from subjecting samples to exacting storage and culture conditions [Witold *et al.,* 2011].

1.5.3 Sources

EHEC of the O157:H7 serotype are worldwide zoonotic pathogens responsible for the majority of severe cases of human EHEC disease. EHEC O157:H7 strains are carried primarily by healthy cattle and other ruminants, but most of the bovine strains are not transmitted to people, and do not exhibit virulence factors associated with human disease. Prevalence of EHEC O157:H7 is probably underestimated. Carriage of EHEC O157:H7 by individual animals is typically short lived, but pen and farm prevalence of specific isolates may extend for months or years and some carriers, designated as super shedders, may harbor high intestinal numbers of the pathogen for extended periods. Strains of EHEC O157:H7 exhibit high genetic variability but typically a small number of genetic types predominate in groups of cattle and a farm environment. Transmission to people occurs primarily via ingestion of inadequately processed contaminated food or water and less frequently through contact with manure, animals, or infected people [Witold *et al.,* 2011].

1.5.3.1 Sources of Human Infection

1.5.3.1.1 Undercooked contaminated ground beef and other meats

Ground beef is a particularly efficient transmission vehicle of EHEC due to the ease of cross-contamination, dispersion of the bacteria throughout the substrate, and poor efficiency of dry heat as a sterilizing agent, whereas bacteria contaminating the surface of a meat slab are unlikely to survive heat exposure [Witold *et al.*, 2011].

1.5.3.1.2 Unpasteurized dairy products

Uncooked/unpasteurized cow and goat milk was a vehicle of infection in several clusters of HUS caused by *E. coli* O157:H7 [Wells *et al*., 1991; Bielaszewska *et al*., 1997] and EHEC O157:H7 were found in sheep dairy products. [Caro *et al*., 2007]

1.5.3.1.3 Contaminated fresh fruits and vegetables

Produce provides a variety of vehicles for transmission of EHEC as the bacteria can attach to intact or processed fruits and vegetables as well as survive in fruit juice. *E. coli* O157:H7 survived for 20 days in apple cider [Besser *et al*., 1993] and outbreaks were traced to this vehicle [Hilborn *et al*., 2000]. The bacteria can grow in apple tissue and defeat decontamination procedures [Janes *et al*., 2005], although pasteurization is protective, as are some additives [Uljas and Ingham., 1999; Comes and Beelman., 2002; Knight and McKellar., 2007]. The ability of EHEC O157:H7 to survive and grow in produce is highly dependent on the plant species and specific conditions; a cocktail of four strains grew on cut mangoes and papayas at 238 ^{o}C, but only on papayas at 128 ^{o}C; the bacteria survived for at least 180 days on both substrates [Strawn and Danyluk., 2009].

1.5.4 Outbreaks

1.5.4.1 EHEC outbreak in Japan, 1994

An outbreak in a nursery school in Japan in late summer to autumn was described by [Akashi et al., 1994] *o*f 174 patients examined, there were 101 cases of diarrhoea and 20 cases of HUS; 53 patients were symptom-free. From 101 diarrhoea cases, EHEC 0157:H7 was isolated from 21 (20.8%). From 20 cases of HUS, EHEC 0157:H7 was isolated from 14 cases (70%), and it was also isolated from seven (13.2%) of 53 symptomless patients. Chapman and Siddons reported a

study designed to determine the value of specific methods for isolation of *E. coli* 0157:H7 from faeces. [Goldwater *et al.*,1998]

1.5.4.2 EHEC outbreak in Germany, May to June 2011

This year Germany was the starting point of one of the largest ever reported food-borne EHEC outbreaks of gastroenteritis and HUS. The latest update indicate that as of July 22, 2011, a total of 4075 outbreak cases, 908 of which (23 %) involved HUS, including 34 fatal cases (3.7%) have been confirmed. Germany accounted for over 95% of the STEC cases. Other than Germany and other EU states, few cases have also been reported from United States of America and Canada and can be linked to travel-related exposures in north Germany shortly before becoming ill.

In the past decade the other reported cases of HUS associated with this strain include two isolates from patients with HUS in Germany in 2001, one in France in 2004, another single case in 2006 from Korea,two cases of HUS in the Republic of Georgia in 2009, and one case from Finland in 2010 [Biju *et al.*, 2014].

1.5.5 Transmission of EHEC O157:H7

Outbreaks of *E. coli* 0157:H7 are almost invariably traced to foods of bovine origin. Other foods that have been implicated in the spread of E. coli 0157:H7 include apple juice and cider, milk, cheese, yogurt, soy beans, lettuce, tomatoes, other fruits and vegetables, and basically anything grown on or near herds of cattle. Contaminated water sources such as lakes, ponds and reservoirs have also been linked to outbreaks. Person to person transmission is an important source of infection by children. This occurs indirectly by the fecal-oral route. Because cattle on farms typically live for about 2 years before being slaughtered, long term treatment of cattle would be necessary to prevent transmission.

A conceptual distinction should be made between mere retention of viability by the bacteria in non-supportive or hostile environments that may provide a conduit for transmission, versus the ability of the bacteria to multiply and persist for extended periods in habitats that may form environmental reservoirs. EHEC survive well and multiply in raw manure and bedding, but do

not survive proper composting or waste treatment. *E. coli* O157:H7 inoculated in moist bovine manure initially declined, but subsequently grew to a maximum in 5 days at 378 ^{o}C [Delazari *et al*., 1998], EHEC O157:H7 human strain 932 survived best at 228 and 378 ^{o}C for up to 70 days [Wang *et al*., 1996], and *E. coli* O157 survived up to 18 weeks at 158 ^{o}C, and 14 weeks at 258 ^{o}C; the best survival occurred with high concentrations of inocula [Fukushima *et al.*, 1999].

EHEC O157:H7 can be transmitted to humans by contaminated undercooked meat and dairy products, produce and fruit juice, drinking and surface water, and, to lesser extent, by contact with animals or manure, and person-to-person contact. The bacteria can colonize plants by mechanisms distinct from those mediating colonization of mammalian tissues, survive for extended time in water and soil, and multiply in manure and other substrates. Survival and transmission can be aided by formation of biofilm, and extraintestinal survival can be influenced by virulence characteristics [Witold *et al.*, 2011].

1.5.6 Treatment and prevention

1.5.6.1 Infection

EHEC are usually transmitted to humans through the consumption of contaminated food or water. EHEC are highly contagious. A low number of pathogen is enough.

1.5.6.2 Clinical course

After the infection with EHEC bacteria, clinical symptoms such as bloody or bloodless diarrhea in combination with abdominal pain and vomiting usually develop after two to ten days. HUS is a serious complication that occurs in 5 to 10% of symptomatic EHEC infections and is the most common cause of acute kidney failure in young children.

1.5.6.3 Therapy

Although EHEC are usually susceptible to antibiotic drugs, an antibacterial treatment is not recommended since it may lead to increased toxin secretion. Thus, treatment of EHEC is mainly supportive. In the case of HUS, dialysis is required to cleanse the body of uremic toxins and to maintain fluid and electrolyte balance.

1.5.6.4 Outcome and Prevention

Without complications, the illness is self limiting and most patients fully recover after about 10 days. Antibiotics are contraindicated because they have not been shown to be effective in altering the course of the illness, may aggravate kidney complications, and contribute to antibiotic resistance. Patients with HUS often require blood transfusions and kidney dialysis. Almost 40% of patients who survive HUS will have some form of long term damage to their kidneys, nervous system or other areas such as the heart, lungs, and pancreas. Bloody diarrhea almost always precedes HUS and an early diagnosis is essential for effective treatment. Good sanitation and hygiene are essential to preventing *E. coli* infections. Also, meat should be thoroughly cooked at temperatures above 70 ^{o}C in order to kill the bacteria and fruits and vegetables should be washed before consumption. Proper slaughtering procedures should be followed at farms to prevent fecal contamination, although there is currently not enough data to recommend ways to prevent contamination of EHEC during the growth, slaughter, processing of beef.

1.6. Bangladesh perspective

1.6.1 Overall pictures of Bangladesh: Spread of infection

The presence of Shiga toxin 1 or 2 genes, typically acquired by a bacteriophage, qualifies an *E. coli* as a Shiga toxin producing *E. coli* (STEC). These isolates are also referred to as verotoxin-producing *E. coli* (VTEC). Enterohaemorrhagic *E. coli* (EHEC) is a subset of STEC that was originally described by its association with hemorrhagic colitis. EHEC is often LEE positive and forms similar attachment and effacing lesions as EPEC. The most common EHEC serogroup is O157:H7 and this serogroup had been responsible for various world-wide outbreaks of infection. Of interest, STEC O104:H4, that was recently responsible for major outbreaks of hemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) in Europe (especially in Germany), can be considered as a hybrid of EHEC and enteraggregative *E. coli* (EAEC). Infections with STEC can range from mild watery diarrhea to bloody diarrhea (hemorrhagic colitis) and the risk of HUS. The first symptom is usually watery diarrhea followed by fever, abdominal cramping and vomiting. Both O157 and non-O157 STEC can have similar clinical presentations; however, O157 has higher rates of complications such as HC and HUS. Most O157:H7 are unable to ferment sorbitol within a 24 hr period and sorbitol MacConkey and

chromogenic media had been used to detect this serogroup. Shiga toxins can be detected with enzyme immunoassay (EIA) and PCR techniques; these tests can also be directly performed on stool specimens that will ensure the additional detection of non-O157 isolates. The course of infection is usually self-limiting and resolves within 7 days. Treatment of infection is mostly supportive and the use of antibiotics is not recommended. Currently there is no way to prevent the development of HUS following STEC infection. Alternative treatment modalities such as the use of monoclonal antibodies are ongoing [Albert *et al.*,1995].

1.6.2 Outbreaks

Diarrheal diseases are highly prevalent in Bangladesh. However, the relative contribution of diarrheagenic Escherichia coli organisms--those that are enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive, enterohemorrhagic, enteroaggregative, and diffuse adherent--to diarrhea in Bangladeshi populations is not known. With DNA probes specific for these diarrheagenic *E. coli* strains, we analyzed fecal *E. coli* from 451 children up to 5 years of age with acute diarrhea seeking treatment at a Dhaka hospital and from 602 matched control children without diarrhea from July 1991 to May 1992. Enteroinvasive *E. coli* was not isolated from any children; enterohemorrhagic *E. coli* was not isolated from any diarrheal children [Albert *et al.*,1995].

1.6.3 Treatment and prevention available in Bangladesh

Certain precautions can be followed in prevention of EHEC which are given below:

- Cooking all ground beef, pork, lamb, or sausage thoroughly.
- Washing all vegetables and fruits with water, especially if they are not for cooking.
- Consuming only pasteurized milk and milk products and avoiding raw milk.
- Consuming only pasteurized juices and ciders.
- Assuring that infected people, especially children, wash their hands carefully and frequently with soap to reduce the risk of spreading the infection.
- Avoiding swallowing lake or pool water while swimming.
- Washing hands thoroughly after using the toilet.
- Washing hands thoroughly after handling animals, animal bedding, or any material contaminated with animal feces.

- People with diarrhea should not:
    - Swim in public pools or lakes
    - Bathe with others
    - Prepare food for others

A vaccine for cattle is not practical because the bacteria do not cause illness in cattle and therefore do not stimulate their immune response. It is recommended that physicians have any patient with bloody diarrhea tested for EHEC. There are a variety of ways to detect it in the laboratory. It is possible to screen for EHEC by inoculating stool specimens onto MacConkey medium containing sorbitol instead of lactose [Albert *et al.*,1995].

1.6.4 Current treatment strategies: a multi-targeted approach

HUS comprises acute renal failure and its consequential perturbation of fluid and electrolyte balance, hemolysis, disruption of the clotting cascade with thrombocytopenia, with the risk of stroke. This syndrome, together with the further effects of toxin, and complement complex formation, must be managed and addressed urgently using a multitargeted approach. This involves the institution of general supportive measures, antiplatelet and thrombolytic agents and thrombin inhibitors, selective use of antimicrobials, probiotics, toxin neutralizers (synthetic and natural binders, antibodies, and so on); and antibodies against key pathogenetic pathway elements to interrupt pathological processes (for example, inhibition of terminal complement complex formation). Targeting PMNs carrying Stx could be a productive strategy for future research, as could possible gene therapy. The management of D+HUS is complex by virtue of the nature of the condition and the variety of pathways affected [Goldwater *et al.*,2012].

1.7 Antimicrobial resistance

A wide range of antimicrobial agents effectively inhibit the growth of *E. coli*. The β-lactams, fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole are often used to treat community and hospital infections due to *E. coli* β-lactams disrupt cell wall synthesis by binding to and inhibiting the penicillin-binding proteins essential for transpeptidation and carboxypeptidation reactions in cell wall peptidoglycan synthesis. Fluoroquinolones interfere with DNA supercoiling and promote DNA gyrase-mediated double-stranded DNA. The aminoglycosides bind irreversibly to the 50S subunit of the 70S bacterial ribosomes.

Sulfonamides and trimethoprim interfere with bacterial folic acid synthesis by inhibiting tetrahydropteric acid syntheses and dihydrofolate reductase, respectively [Allen *et al.,* 1998].

1.7.1 Resistance to antimicrobial agents

The b-lactam antibiotics, especially the cephalosporins and b-lactam-b-lactamases inhibitor combinations, are major drug classes used to treat community-onset or hospital-acquired infections caused by *E. coli*, especially due to the ExPEC pathotype. Among *E coli*, b-lactamase production remains the most important contributing factor to b-lactam resistance. b-lactamases are bacterial enzymes that inactivate b-lactam antibiotics by hydrolysis, which results in ineffective compounds.

Resistance to aminopenicillins (e.g. ampicillin) and early-generation cephalosporins (e.g. cefazolin) among *E. coli* is often mediated by the production of narrow-spectrum b-lactamases such as TEM-1, TEM-2 and to a lesser extent SHV-1 enzyme. Most importantly among *E. coli,* is the increasing recognition of isolates producing the so-called "newer b-lactamases" that causes resistance to the expanded-spectrum cephalosporins and/or the carbapenems. These enzymes consist of the plasmid-mediated AmpC b-lactamases (e.g. CMY types), extended-spectrum b-lactamases (e.g. TEM, SHV, CTX-M types), and carbapenemases (KPC types, metallo-b-lactamases (MBLs) and OXA-types). CMY, CTX-M, and NDM types of b-lactamase are mostly responsible for the emerging resistance to the β-lactam antibiotics among *E. coli*. The VIM, IPM, KPC and OXA-48 β-lactamases had been described in various members of the Enterobacteriaceae (especially *Klebsiella* spp.) and are not yet commonly encountered among *E. coli* [Allen *et al.,* 1998].

1.7.2 Emerging Trends in Resistance among *E. coli*

E. coli, especially the ExPEC pathotype, is an important cause of community and nosocomial-acquired infections, especially of urinary tract infections, bloodstream infections, surgical site infections, pneumonia and sepsis. The cephalosporins, fluoroquinolones, and trimethoprim-sulfamethoxazole are considered as 1st line agents and often used to treat community and hospital infections caused by *E. coli*. The management of infections caused by ExPEC has been complicated by the emergence of antimicrobial resistance to first line antibiotics. Until the late

1990s, ExPEC were relatively susceptible to 1st line antibiotics, however severalsurveillance studies during the 2000's across Europe, North and South America, have shown that between 20 – 55% of ExPEC are resistant to 1st line antibiotics including the cephalosporins, fluoroquinolones, and trimethoprim-sulfamethoxazole. Resistance to these agents is causing delays in appropriate therapy with subsequent increased morbidity and mortality. [Allen *et al.,* 1998]

1.7.3 Plasmid-mediated AmpC-b-lactamases

E .coli possess a chromosomal gene that encodes for an AmpC β-lactamase. Usually, low amounts of these β-lactamases are produced because the AmpC gene is regulated by a weak promoter and a strong attenuator system. Occasionally, cephamycin and/or cephalosporin-resistant *E. coli* are encountered that produce plasmid-mediated β-lactamases, derived from bacteria with chromosomally encoded AmpC-cephalosporinases. *E. coli* that produce plasmid-mediated or imported AmpC b-lactamases were first reported in the 1980's. These enzymes (e.g. CMY, ACT, FOX, ACT, and DHA types) are derivatives of the chromosomally encoded AmpC cephalosporinases of bacteria such as *Enterobacter* spp., *C. freundii, M. morganii, Aeromonas spp. and Hafnia alvei* and are not inhibited by the "classical" b-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. However, different types of inhibitors such as boronic acid and cloxacillin have the ability to inhibit chromosomal and plasmid-mediated AmpC b-lactamases. Resistance to the fourth generation cephalosporins (e.g. cefepime) are caused by point mutations in AmpC β-lactamases and is called extended-spectrum cephalosporinases. The genes are typically encoded on large plasmids containing additional antibiotic resistance genes that are responsible for multi-resistant phenotype, leaving few therapeutic options [Allen *et al.,* 1998].

1.7.4 Metallo-β-lactamases (e.g. NDM-1)

Recently, a new type of metallo-β-lactamase (MBL), named NDM, was described in *K. pneumoniae* and *E. coli* recovered from a Swedish patient who was hospitalized in New Delhi, India. MBLs have the ability to hydrolyse a wide variety of b-lactams, including the penicillins, cephalosporins and carbapenems, but not the monobactams (i.e. aztreonam), and are inhibited by metal chelators such as EDTA. The majority of NDM-1-producing bacteria are broadly resistant

to various drug classes and also carry a diversity of other resistance mechanisms (e.g. to aminoglycosides and fluoroquinolones), which leaves limited treatment options [Allen *et al.*, 1998].

1.7.5 Antimicrobial therapy: Drugs of Choice

Despite the concerning trends in antimicrobial resistance among *E. coli* isolates worldwide, a growing armamentarium of antimicrobial agents provides multiple options for treating *E. coli* infections. Ironically, these newer agents are more readily available and affordable in developed nations where *E. coli* resistance is less of a problem, compared to the developing world. As with other Enterobacteriaceae, where and when available, antimicrobial testing of the infecting strain should direct therapy. In other situations, knowledge of recent local susceptibility patterns is useful for guiding treatment. In general, monotherapy with trimethoprim-sulfamethoxazole, aminoglycoside, cephalosporin, or a fluoroquinolones is recommended as the treatment of choice for most known infections with *E. coli*, although many broad spectrum agents (such as ß-lactam/ß-lactamase inhibitor combinations and the carbapenems) remain highly active [Allen *et al.*, 1998].

1.7.6 Treatment of infections due to multi-resistant *E. coli*

The presence of ESBLs and AmpC b-lactamases complicates antibiotic selection especially in patients with serious infections such as bacteraemia. The reason for this is that these bacteria are often multiresistant to various antibiotics and an interesting feature of CTX-M-producing isolates is the co-resistance to the fluoroquinolones. Antibiotics that are regularly used for empiric therapy of serious community-onset infections, such as the third generation cephalosporins or fluoroquinolones are often not effective against ESBL and or AmpC-producing bacteria. This multiple drug resistance has major implications for selection of adequate empiric therapy regimens. Empiric therapy is prescribed at the time when an infection is clinically diagnosed while awaiting the results of cultures and anti-microbial susceptibility profiles. Multiple studies in a wide range of settings, clinical syndromes, and organisms have shown that failure or delay in adequate therapy results in an adverse mortality outcome. This is also true of infections caused by ESBL-producing bacteria [Allen *et al.*, 1998].

1.8 EHEC epidemiology: developed versus developing countries

Diarrhea continues to be one of the most common causes of morbidity and mortality among infants and children in developing countries Determination of the etiological agents of diarrhea is important in developing rational therapy and in implementing control measures [Albert *et al.*,1995].

Despite much progress in the understanding of pathogenesis and of management, diarrhoeal illnesses remain one of the most important causes of global childhood mortality and morbidity. Infections account for most illnesses, with pathogens employing ingenious mechanisms to establish disease. In the developed world, an upsurge in immune-mediated gut disorders might have resulted from a disruption of normal bacterial-epithelial cross-talk and impaired maturation of the gut's immune system. Oral rehydration therapies are the mainstay of management of gastroenteritis, and their composition continues to improve. Malnutrition remains the major adverse prognostic indicator for diarrhoea-related mortality, emphasising the importance of nutrition in early management. Drugs are of little use, except for specific indications although new agents that target mechanisms of secretory diarrhoea show promise, as do probiotics. However, preventive strategies on a global scale might ultimately hold the greatest potential to reduce the burden of diarrhoeal disease. These strategies include vaccines and, most importantly, policies to address persisting inequalities between the developed and developing worlds with respect to nutrition, sanitation, and access to safe drinking water [Nikhil *et al.*, 2004].

1.9 Vaccine development: Current pictures

Several vaccine strategies have been used with variable success in a number of animal models. The strategies have involved the use of recombinant virulence proteins such as Stx, intimin and *E. coli* secreted protein A (EspA) or peptides or fusion proteins of A and B subunits of Stx 2 and Stx1 such as Stx2Am-Stx1B or avirulent ghost cells of EHEC O157:H7. The application of live attenuated bacteria such as *Salmonella* as a carrier for vaccine proteins against mucosal pathogens including EHEC have obvious advantages.

Antibodies produced in humans with HUS and in rabbits immunized with type III secreted proteins (T3SPs) from four STEC serotypes, and experimentally infected cattle revealed proteins

common to several HUS serotypes. These were highly immunogenic in vaccinated and naturally infected subjects and represent future candidates for a STEC vaccine.

As well as protein-based vaccines, DNA vaccines are a recent development in EHEC prevention, providing encouraging results in a mouse model. The mode of administration (intramuscular, intranasal, oral, intragastric, and so on) for a number of these vaccines not only affects immunogenicity but also protective effect under challenge. Vaccination with a plant-based oral vaccine protected mice against lethal systemic intoxication with Stx2. This is seen as encouraging. Clearly there is some time to go before human trials are reported but the numerous and frequent outbreaks of EHEC disease constantly remind us of the urgent need to protect the population against these emerging and often devastating zoonoses [Goldwater *et al.*,2012].

1.10 Objective of the study:

Enterohemorrhagic *E. coli* (EHEC) strains are a class of pathogenic microorganisms responsible for numerous food- and waterborne outbreaks, and can cause illness ranging from nonbloody diarrhea to copious bloody discharge in humans. In some individuals, the disease progress to a more serious stage, and about 2% to 7% of the diarrheal cases (particularly in the pediatric population) can be fatal due to acute kidney failure (hemolytic-uremic syndrome [HUS]).

The first outbreak of STEC O157:H7 was recorded in the United States in 1982 and other outbreaks occurred later in United Kingdom, continental Europe, Africa, New Zealand and Japan over the next decade. In fact, most of the outbreaks were often reported from the industrialized countries than from developing countries because of the advanced surveillance and reporting systems in the industrialized countries. There are few reports of the prevalence of the Shiga-toxin producing *E.coli* (STEC) in Bangladesh those included the isolation and molecular characterizations of these organisms from the diarrheal patients, slaughtered animals, raw meat and other food samples. Domestic ruminants are considered the main reservoirs of these organisms are highly prevalent in animal reservoirs and in the human food-chain. But no detail studies were reported describing the enteropathigenicity or virulence properties of the locally isolated STEC O157:H7 from different sources. Also there is no comparative study on the bovine and clinical isolates as no STEC O157:H7 has been isolated from patients in the recent

past in Bangladesh. The aim of the present work was to identify the immune response to *Escherichia coli* O157:H7 in detection of antibodies in healthy human sera against outer membrane proteins causing infection in Bangladesh.

2. Materials and Methods:

2.1 Working place:

The experiments of this research study were carried out in the laboratory of the Department of Mathematics and Natural Sciences, BRAC University and Department of Microbiology, University of Dhaka, Bangladesh.

2.2 Bacterial strains:

A reference clinical strain *E. coli* O157:H7 NCTC 12079 and a negative control strain *E. coli* K-12 were obtained from the Department of Microbiology, University of Dhaka, Bangladesh.
Two bovine STEC O157:H7 (CD 11 and CD 17) were isolated from the fresh feces of 18 healthy cattle from six different dairy farms around Dhaka city, Bangladesh and were used in the present study [Rabbi *et. Al*., 2013].

Eight *E.coli* strains [ASI(7), ASI(8), UFL(14), UFL(17), MBCV2(12), MBCV2(16), ML2(3), ML2(7)] isolated from different raw vegetables around Dhaka city.

2.3 Isolation of *E. coli* strains:

2.3.1 Isolation of *E. coli* from vegetable samples

Twenty five gram of selected raw vegetable was measured and finely chopped aseptically. This was added to 225 ml of enrichment broth and homogenized and incubated at 37 ^{o}C for 16-18 hr. Then 1 ml of enriched broth was subjected to 10-fold dilution series from 10^{-1} to 10^{-4} in 9 ml of PBS. From each 10^{-1} to 10^{-3} dilution tubes, 0.1 ml of suspension was spread plated in duplicate onto MacConkey agar plates. The plates were then incubated at 37 ^{o}C for 16-18 hr.

2.3.2 Isolation of *E. coli* from bovine samples

A reference clinical strain *E. coli* O157:H7 NCTC 12079 and a negative control strain *E. coli* K-12 were obtained from the Department of Microbiology, University of Dhaka, Bangladesh. Two bovine STEC O157:H7 (CD 11 and CD 17) were isolated from the fresh feces of 18 healthy cattle from six different dairy farms around Dhaka city, Bangladesh and were included in the present study. The isolation and detection procedures of the bovine strains are described below: One gram of each bovine feces sample was placed in 9 mL of trypticase soy broth (TSB)

supplemented with 20 mg/mL novobiocin (Wako, Japan), and incubated at 37 °C for 16-18 h. The enriched samples were streaked onto sorbitol MacConkey agar (Oxoid, England) plates supplemented with 0.5 mg/L cefixime and 1.5 mg/L potassium tellurite (Sigma, Germany) and incubated as above. After incubation, non-sorbitol fermenting colonies were streaked onto eosine methylene blue (EMB) agar (Oxoid, England) and 4-methyl-umbelliferyl-β-D-glucuronide (MUG) agar (Difco, USA) and incubated at 37 °C for 18-22 hr. Colonies showing green metallic sheen on EMB agar and no fluorescence on MUG agar, were characterized by indole production, citrate utilization, methyl red, Voges-Proskauer, triple sugar iron and oxidase tests as described by [Cappuccino and Sherman., 2011]. Presence of the O157 and H7 antigens in biochemically positive colonies were investigated by latex agglutination test kit (WellcolexTM, Remel, USA) and the two STEC O157:H7 strains (CD 11 and CD 17) were re-confirmed by PCR targeting the *rfb*O157 and *flic*H7 genes [Rabbi *et. al*., 2013].

2.4 Confirmation of *E. coli* Isolates:

2.4.1 Phenotypic Properties on MacConkey Agar Plate:

The morphological features of colonies (size, shape, elevation, form, pigmentation and opacity) developed after incubation on MacConkey agar plate were carefully studied and recorded. One or two *E. coli* colonies were picked up from the plate and subcultured onto fresh MacConkey plate to get pure culture.

2.4.2 Phenotypic Properties on EMB (Eosine methylene blue) Plate:

Assumed *E. coli* isolates from the fresh MacConkey plate were streaked onto eosin methylene blue (EMB) agar plate for confirmation as *E. coli*. EMB agar medium contains lactose and the dyes eosin and methylene blue that permit differentiation between enteric lactose fermenters and non-fermenter. The *E. coli* colonies are black colonies with a metallic green sheen caused by the large quantities of acid that is produced and that precipitates out the dyes onto the growth's surface. [Cappuccino and Sherman., 2011].

2.4.3 Biochemical Identification:

Biochemical tests were performed with green metallic sheen producing isolates according to the methods described in Microbiology Laboratory Manual [Cappuccino and Sherman., 2011]. The

biochemical tests performed were indole production test, methyl-red test, Voges-Proskauer test, citrate utilization test, triple sugar iron agar test and carbohydrate fermentation (lactose, dextrose, sucrose) test.

2.4.3.1 Indole Production Test

The amino acid tryptophan is a component of nearly all proteins and is therefore available to microorganisms as a result of protein breakdown. Some bacteria are able to produce an enzyme called tryptophanase that catalyzes the removal of the indole residue from tryptophan. Indole accumulates in the culture media while the rest of the tryptophan molecule (pyruvate and NH_3) is used to satisfy nutritional needs. The production of indole from tryptophan by microorganisms can be detected by growing them in a medium rich tryptophan. The accumulation of indole in the medium can be detected by adding Kovac's reagent, which reacts with indole, giving a water-insoluble bright red compound on the surface of the medium. [Cappuccino and Sherman., 2011].

2.4.3.2 Methyl Red Test

The methyl red test is used to detect a specific type of fermentation called mixed acid fermentation. Some bacteria ferment glucose and produce large quantities of acidic end products that lower the pH of the medium below 5.0. The addition of pH indicator methyl red is used to detect this acidity (methyl red is red at pH 4.4 or below and yellow at pH 6.2 or above). A mixed acid fermentation is detected by growing bacteria in MR-VP medium and then after an incubation period, adding some methyl red reagent to the culture medium. If mixed acid fermentation occurred, a yellow color will develop. [Cappuccino and Sherman., 2011].

2.4.3.3 Voges-Proskaure Test

The Voges-Proskaure test is used to detect a specific organism that carry out 2, 3-butanediol fermentation. When bacteria ferment sugars producing 2, 3-butanediol as a major end product, they accumulate this compound in the medium. The addition of 40% KOH and a 5% solution of alpha-naphthol in absolute ethanol will reveal the presence of acetoin (acetyl methyl carbinol), a precursor in the synthesis of 2, 3-butanediol. The acetoin, in the presence of KOH, will develop a pink color imparting a rose color to the medium. The reaction will occur in the presence of alpha-naphthol catalyst and a guanidine group that is present in the peptone of the MR-VP

medium. Aeration increases the rate of oxidation of 2, 3-butanedio to acetoin. [Cappuccino and Sherman., 2011].

2.4.3.4 Citrate Utilization Test

The citrate test determines the ability of microorganisms to use citrate as the sole source of carbon and energy. Simmon's citrate agar, a chemically defined medium with sodium citrate as the carbon source, NH_4^+ as a nitrogen source and the pH indicator bromophenol blue, is commonly used for this test. When microorganisms utilize citrate, they remove the acid from the medium, which raises the pH and turns the pH indicator from green to blue. A color change in the medium from green to blue indicates that the microorganisms tested can utilize citrate as its only carbon source. [Cappuccino and Sherman., 2011].

2.4.3.5 Triple Sugar-Iron (TSI) Agar Test

The triple sugar-iron (TSI) agar test is designated to differentiate among the different groups or genera of the Enterobacteriaceae and to distinguish the Enterobacteriaceae from other Gram-negative intestinal-bacilli. This difference is made on the basis of difference in carbohydrate fermentation patterns and hydrogen sulfide production by the various groups of intestinal organisms. TSI agar medium contains lactose and sucrose in 1% concentrations and glucose in a concentration of 0.1%. To facilitate observation carbohydrate utilization patterns, TSI agar medium is made with slant and butt. TSI agar medium also contains sodium thiosulfate and ferrous sulfate for detection of hydrogen sulfide production, which is indicated by blackening of medium. [Cappuccino and Sherman., 2011].

Table 2.1: Types of results observed in TSI test

Reactions	Results
(a) Alkaline slant (red) and acid butt (yellow) with and 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

Reactions	Results
(b) Acid slant (yellow) and acid butt (yellow) with and without gas production	Large amount of acid production from lactose and/or sucrose fermentation has caused acid reaction on the slant surface.
(c) 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.

2.5 Storage of confirmed strains:

Preparation of stock:

For short-term preservation, T_1N_1 agar medium was used.

2.6 Preparation of Crude Extract: Cell Lysis and Protein Extraction

Historically, physical lysis was the method of choice for cell disruption and extraction of cellular contents; however, it often requires expensive, cumbersome equipment and involves protocols that can be difficult to repeat due to variability in the apparatus (such as loose-fitting compared with tight-fitting homogenization pestles). Also, traditional physical disruption methods are not conducive for high throughput and smaller volumes typical of modern laboratory research. In recent years, detergent-based lysis methods have become the norm. Through empirical testing by trial and error, different detergent-based solutions composed of particular types and concentrations of detergents, buffers, salts and reducing agents have been developed to provide the best possible results for particular species and types of cells. Detergents have both lysing and solubilizing effects.

2.6.1 Cell lysis

Cell lysis disturbs the carefully controlled cellular environment, allowing endogenous proteases and phosphatases to become unregulated. As a result extracted proteins become degraded or artifactually modified by the activities of these molecules. To prevent these effects and obtain the best possible protein yield in cell lysis, protease and phosphatase inhibitors are added to the lysis reagents. Numerous compounds have been identified and used to inactivate or block the activities of proteases and phosphatases by reversibly or irreversibly binding to them.

When the goal of cell lysis is to purify or test the function of a particular protein, special attention must be given to the effects of the lysis reagents on the stability and function of the protein(s) of interest. Certain detergents will inactivate the function of particular enzymes, and long-term stability of extracted/purified proteins often requires that they be removed from the initial lysis reagents and/or stabilized by addition of particular compounds.

2.6.2 Protein refolding

Some cell lysis and protein solubilization methods cause the denaturation of proteins. Functional tests with such proteins require that they be renatured. Many proteins spontaneously refold into their native, functional structures when the denaturing solubilization reagents are removed by dialysis. Other proteins, however, will fold into non-functional and even insoluble forms by this process. In such cases, specialized sets of buffer conditions must be tested to identify those that promote the highest possible yield of properly refolded protein.

2.7 Isolation of Bacteria Envelope Proteins

Outer membrane proteins (OMPs) of Gram-negative bacteria are key molecules that interface the cell with the environment. Traditional biochemical and genetic approaches have yielded a wealth of knowledge relating to the function of OMPs. Proteomic analysis on cell envelope proteins from Gram-negative bacteria requires specific isolation techniques. The conventional extraction methods such as osmotic shock cause extracts to be heavily contaminated with soluble cytoplasmic proteins. These cytoplasmic protein contaminants constitute the major signal in proteomic analysis and can overwhelm the signals coming from genuine envelope components.

> After extensive testing of various protocols for the preparation of envelope contents, a modi fi ed version of the method of Oliver and Beckwith consistently produces the cleanest extract of periplasmic and outer membrane proteins.

2.8 Gel electrophoresis

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge.It is used in clinical chemistry to separate proteins by charge and/or size (IEF agarose, essentially size independent) and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge.

Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving.

Gel electrophoresis uses a gel as an anticonvective medium and/or sieving medium during electrophoresis, the movement of a charged particle in an electrical field. Gels suppress the thermal convection caused by application of the electric field, and can also act as a sieving medium, retarding the passage of molecules; gels can also simply serve to maintain the finished separation, so that a post electrophoresis stain can be applied. DNA Gel electrophoresis is usually performed for analytical purposes, often after amplification of DNA via PCR, but may be used as a preparative technique prior to use of other methods such as mass spectrometry, RFLP, PCR, cloning, DNA sequencing, or Southern blotting for further characterization.

2.8.1 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) is used for separating proteins ranging in size from 5 to 2,000 kDa due to the uniform pore size provided by the polyacrylamide gel. Pore size is controlled by controlling the concentrations of acrylamide and bis-acrylamide powder used in creating a gel. Care must be used when creating this type of gel, as acrylamide is a potent neurotoxin in its liquid and powdered form.

Traditional DNA sequencing techniques such as Maxam-Gilbert or Sanger methods used polyacrylamide gels to separate DNA fragments differing by a single base-pair in length so the sequence could be read. Most modern DNA separation methods now use agarose gels, except for particularly small DNA fragments. It is currently most often used in the field of immunology and protein analysis, often used to separate different proteins or isoforms of the same protein into separate bands. These can be transferred onto a nitrocellulose or PVDF membrane to be probed with antibodies and corresponding markers, such as in a western blot.

Typically separating gels are made in 6%, 8%, 10%, 12% or 15%. Stacking gel (5%) is poured on top of the resolving gel and a gel comb (which forms the wells and defines the lanes where

proteins, sample buffer and ladders will be placed) is inserted. The percentage chosen depends on the size of the protein that one wishes to identify or probe in the sample. The smaller the known weight the higher the percentage should be used. Changes on the buffer system of the gel can help to further resolve proteins of very small sizes.

Proteins, unlike nucleic acids, can have varying charges and complex shapes, therefore they may not migrate into the polyacrylamide gel at similar rates, or at all, when placing a negative to positive EMF on the sample. Proteins therefore, are usually denatured in the presence of a detergent such as sodium dodecyl sulfate (SDS) that coats the proteins with a negative charge. Generally, the amount of SDS bound is relative to the size of the protein (usually 1.4 g SDS per gram of protein), so that the resulting denatured proteins have an overall negative charge, and all the proteins have a similar charge to mass ratio. Since denatured proteins act like long rods instead of having a complex tertiary shape, the rate at which the resulting SDS coated proteins migrate in the gel is relative only to its size and not its charge or shape. Proteins are usually analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), by native gel electrophoresis, by quantitative preparative native continuous polyacrylamide gel electrophoresis (QPNC-PAGE), or by 2-D electrophoresis.

Characterization through ligand interaction may be performed by electroblotting or by affinity electrophoresis in agarose or by capillary electrophoresis as for estimation of binding constants and determination of structural features like glycan content through lectin binding. Proteins are usually analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), by native gel electrophoresis was followed by coomassie blue staining [Laemmli *et al.*, 1970]. The procedure is given below:

2.9 Determination of the molecular weight of the protein

To determine the relative mobility (R_f) of the protein, migration distance of the protein was divided by the migration distance of the tracking dye.

$$\text{Relative mobility } (R_f) = \frac{\text{Distance of protein migration.}}{\text{Distance of the Tracking dye migration.}}$$

The negative values of (R_f) value were plotted (x-axis) against the known Log10 molecular weight (y-axis) in a graph paper.

i. A calibration curve was prepared by using the molecular weight standards.
ii. The molecular weights of the immunogenic proteins were estimated from linear calibration curve.

2.10 Estimation of protein concentration:

The most accurate method of determining protein concentration is probably acid hydrolysis followed by amino acid analysis. Most other methods are sensitive to the amino acid composition of the protein, and absolute concentrations cannot be obtained. The procedure of Lowry et al. is no exception, but its sensitivity is moderately constant from protein to protein, and it has been so widely used that Lowry protein estimations are a completely acceptable alternative to a rigorous absolute determination in almost all circumstances in which protein mixtures or crude extracts are involved. The reactions result in a strong blue color, which depends partly on the tyrosine and tryptophan content. The method is sensitive down to about 0.01 mg of protein/mL, and is best used on solutions with concentrations in the range 0.01–1.0 mg/mL of protein [Lowry *et al*., 1951].

2.11 Collection and separation of blood serum:

Blood consist of a fluid called plasma in which various groups of formed elements (erythrocytes, leukocytes and thrombocytes) are suspended. It is possible to demonstrate the nature of the suspension by centrifuging a sample of whole blood in a test tube for a short time at low speed. After treatment the heavier cells settle at the bottom and the plasma is visible as pale-yellow fluid above them. A thin layer of white cells and platelets are known as buffy coat. 336 in number of normal healthy human sera were collected randomly to establish the relationship between the heterogeneity and the virulence properties of different groups of strains present in healthy human sera.

336 in number of normal healthy human sera were collected randomly from Life and Care hospital to establish the relationship between the heterogeneity and the virulence properties of different groups of strains present in healthy human sera.

2.12 Relocation of the Protein from the Gel to Nitrocellulose Membrane: Western Blot

Western blot technique [Towbin *et al.*, 1979] was applied was applied to check the immunological profile of the prepared antigen against the human sera.

2.14 Immunological Methods:

Immunoassays, particularly ELISA, have played a vital role in screening of *E. coli* from food samples and stool specimens. For this, antibodies against particular *E. coli*, outer membrane antigens of that *E. coli* strain and Shiga toxins are often used [Paton and Paton., 1998]. Latex agglutination assay for specific *E. coli* antigens have greatly facilitated [Nataro and Kaper., 1998]. An immunomagnetic separation system has recently been developed to reduce the time and improve the sensitivity of detection of *E. coli*. Cytotoxic activity of *E. coli* present in stool on Vero cell line is also an extremely sensitive method to detect the current or recent presence of toxin producing *E. coli*, but this method is time consuming [Khan *et al.*, 2003].

3. Result

3.1 Isolation and Identification of *E.coli:*

E. coli strains used in this study were isolated from various environmental sources as raw vegetables and bovine feces. After appropriate enrichment, selective plating and biochemical tests the isolates were further confirmed phenotypically.

Sixty raw vegetable samples were collected from various local bazaar and super shops around Dhaka city. The samples were enriched at 37 ^{o}C for 18 hr in enrichment broth containing Bile Salt (Figure 3.1). After enrichment; the culture broth was subjected to ten fold dilution series. From appropriate dilutions, the broth cultures were spread plated onto MacConkey Agar plates and the plates were incubated at 37 ^{o}C for 18 hr. After incubation, different types of colonies were observed on MacConkey agar plates (Figure 3.2). Colonies showing typical colony

characteristics of *E. coli* (Table 3.1) were subcultured onto MacConkey plates. Over 65 isolates were taken from primary isolation plates and considered for further investigation.

Figure 3.1: Selective enrichment of various sources: (a) bovine, (b) vegetables.

Figure 3.2: (a) Nutrient agar plate showing typical yellow colonies of *E. coli*

(b) MacConkey agar plate showing typical pink lactose fermenting colonies of *E. coli*

Table 3.1: Colony characteristics of *E. coli* on MacConkey plate

Colony characteristics	MacConkey plate
Size	Moderate (1-2 mm)
Shape	Round
Elevation	Raised
Margin	Entire
Color	Deep pink Color
Opacity	Opaque

3.2 Identification of Suspected *E. coli* Isolates

3.2.1 Presumptive Identification on EMB Plate

From MacConkey plates, suspected *E. coli* isolates were picked up using needle and streaked onto EMB agar plates. After incubation, isolates showed green metallic sheen growths were selected for further identification (Figure 3.3) and others were discarded.

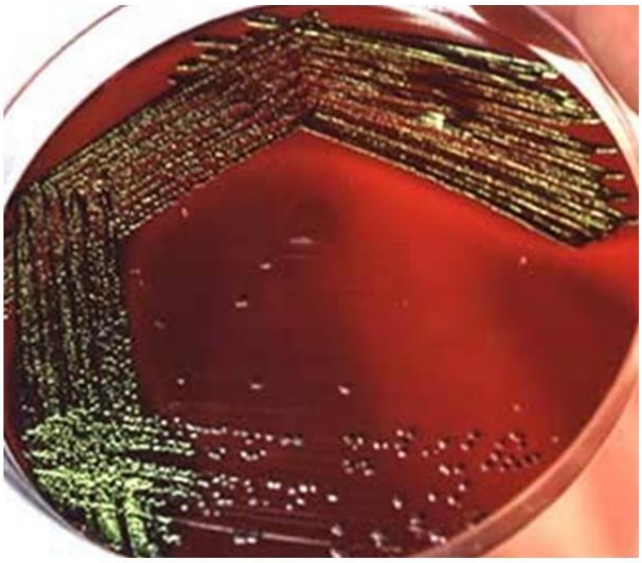

Figure: 3.3 EMB agar plate showing typical green metallic sheen growths of presumptive *E. coli* isolates.

3.2.2 Biochemical Identification

Presumptive isolates which gave metallic green sheen were subjected to different biochemical tests. Isolates showed pattern of biochemical reactions typical for *E. coli* as mentioned in (Table 3.2).

Table 3.2: Typical results of biochemical tests of *E. coli* isolates

Strain ID	Indole production	Methyl Red (MR)	Voges Proskauer (VP)	Citrate utilization	Triple Sugar Iron (TSI)
STEC	+	+	-	-	Acid butt, acid slant, Gas
K12	+	+	-	-	Acid butt, acid slant, Gas
ASI(7)	+	+	-	-	Acid butt, acid slant, Gas
ASI(8)	+	+	-	-	Acid butt, acid slant, Gas
UFL(14)	+	+	-	-	Acid butt, acid slant, Gas
UFL(17)	+	+	-	-	Acid butt, acid slant, Gas
MBCV2(12)	+	+	-	-	Acid butt, acid slant, Gas
MBCV2(16)	+	+	-	-	Acid butt, acid slant, Gas
ML2(3)	+	+	-	-	Acid butt, acid slant, Gas
ML2(7)	+	+	-	-	Acid butt, acid slant, Gas
CD 17	+	+	-	-	Acid butt, acid slant, Gas
CD 11	+	+	-	-	Acid butt, acid slant, Gas

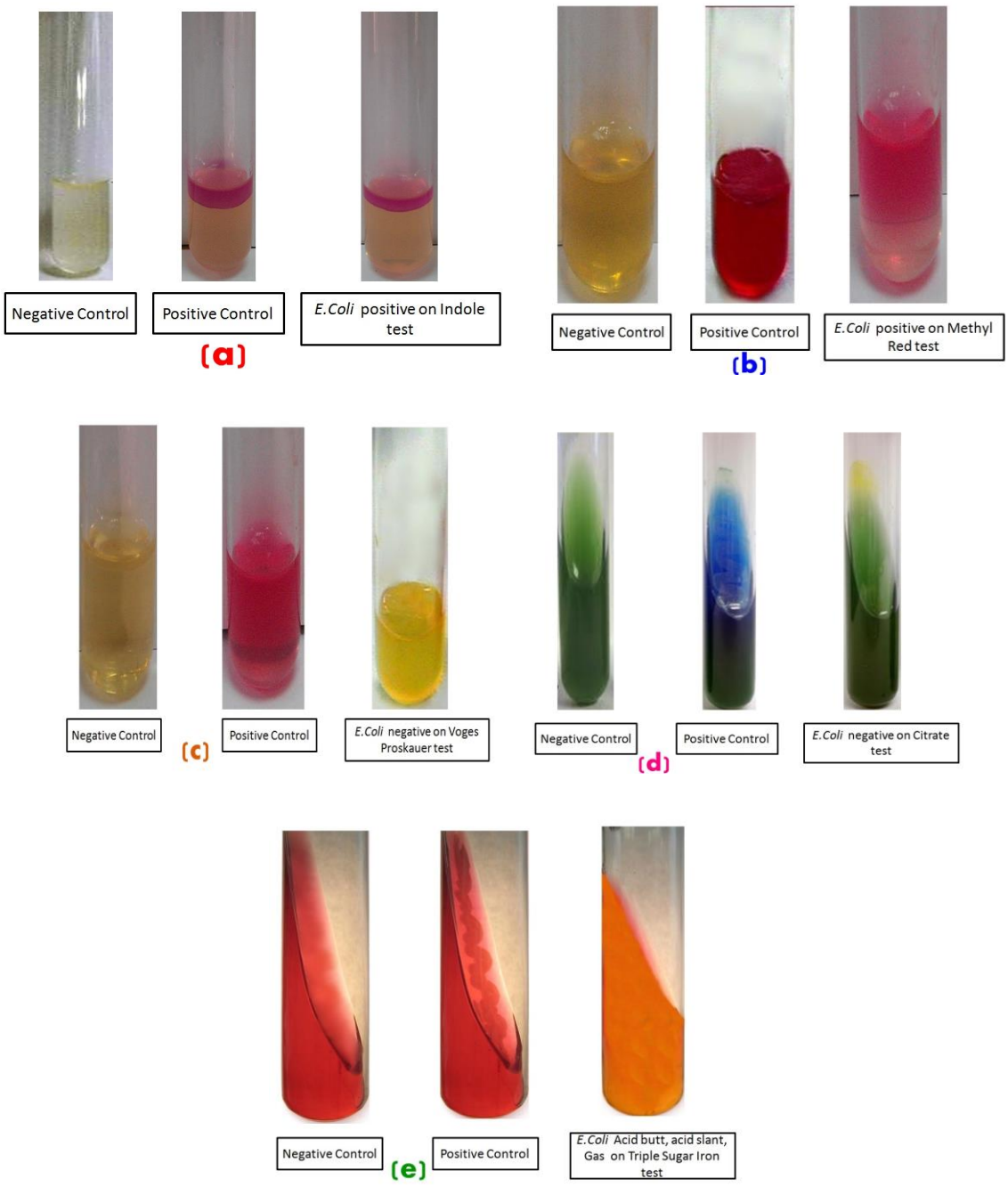

Figure 3.4: Test tubes showing biochemical tests for (a) Indole production, (b) Methyl red and (c) Voges -Proskauer test (Negative control, Positive control, Isolates)

Test tubes showing biochemical tests for (d) citrate utilization, (e) TSI

3.3 Cell lysis and Protein extraction:

3.3.1 Crude extract: Bacterial whole cell extract were prepared using simple detergent lysis procedure and the crude extract are used immediately or stored at -20 ^{o}C for further study.

3.3.1 Outer membrane protein extraction: OMPs are extracted according to previously describe methods and quantify the amount of total protein present in OMPs extract followed by SDS-PAGE analysis.

Table 3.3: Protein estimation outer membrane protein of strain CD 17 (µg/ µl)

Sample	OD	From Graph	Calc. of OMP (µg/ µl)
CD 17	0.1855	23.23	3.09

3.4 SDS-PAGE Analysis of the Extracted Protein

The *E.coli* surface proteins were extracted by the whole cell lysate method and Tris-sucrose-EDTA (TSE) extraction procedure. Bands obtained from SDS-PAGE analysis of crude extract are shown in (Table 3.5, Table 3.6 and Table 3.7) were measured by known molecular weight of prestained protein marker (Table 3.4).

Table 3.4: Known molecular weight of prestained protein marker

Proteins	Marker band (known MW) kDa	Rf	M.Log MW
Myosin	**120**	0.04	2.07
B-galactosidase	**90**	0.17	1.95
Bovine serum albumin	**50**	0.37	1.69
Carbonic anhydrase	**35**	0.55	1.54
Soybean tripsin inhibitor	**26**	0.66	1.41
Lysozyme	**20**	0.77	1.30

Table 3.5: Determination of molecular weight of protein in STEC, UFL (14), K12, MBCV2 (12) and MBCV2 (16) in kDa from crude extract.

STEC	UFL (14)	K12	MBCV2 (12)	MBCV2 (16)
105	105	105	102	102
102	102	102	91	91
91	91	91	82	82
82	82	73	73	73
73	70	70	66	66
70	66	66	59	62
66	59	59	53	53
62	56	53	45	45
56	53	45	40	40
45	45	40	36	36
40	40	36	34	34
36	36	34	32	32
34	34	32	31	31
32	32	31	29	29
31	31	29	27	27
29	29	27	26	26
27	27	26	24	24
26	26	22	22	23
24	22	21	21	21
22	21	20	20	20
21	20			
20				

Table 3.6: Determination of molecular weight of protein in UFL (17), ASI (7), ASI (8) , ML2 (2) and ML2 (3) in kDa from crude extract.

UFL (17)	ASI (7)	ASI (8)	ML2 (2)	ML2 (3)
105	105	105	105	105
102	102	102	102	102
91	91	91	91	91
82	82	82	82	82
70	73	73	73	73
66	66	66	70	59
59	62	62	66	53
56	56	56	62	45
53	45	45	56	40
45	40	40	45	36
40	38	38	40	32
36	34	34	36	31
34	31	31	34	29
32	27	27	32	27
31	24	24	31	26
29	22	22	29	22
27	21	21	27	20
26	20	20	26	
22			24	
21			22	
20			21	

Table 3.7: Determination of molecular weight of protein in CD 17 and CD 11 in kDa from crude extract.

CD 17	CD 11
105	105
102	102
91	91
82	82
73	73
70	70
66	66
62	59
56	56
45	45
40	40
36	36
34	34
32	32
31	31
29	29
27	27
26.3	26.3
24	24
22	22
21	21
20	20

3.4.1 Tris-sucrose-EDTA (TSE) extraction procedure: Outer membrane proteins of *E. coli* strains were extracted following TSE extraction method. The observed data are shown in the (Table 3.8) and in (Figure 3.5).

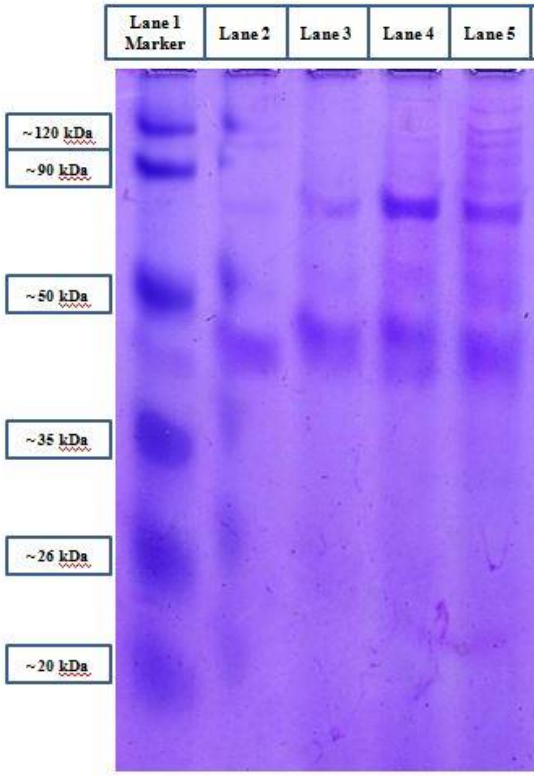

Figure 3.5: SDS-PAGE Analysis omp of *E. coli* strains : Lane 1 is Marker protein band and Lane 2 to lane 5 is STEC, K12, CD 17 and MBCV2 (12) respectively

Table 3.8: Determination of molecular weight of protein in STEC, K12, CD 17 and MBCV2 (12) kDa from TSE extraction procedure.

STEC	K12	CD 17	MBCV2 (12)
105	87	127	127
50	73	120	120
45	53	96	96
	45	87	87
		82	82
		78	78
		62	62
		53	53
		45	45

Comparative analysis of groups of SDS-PAGE results of outer membrane proteins among the whole cell lysate and the TSE extraction procedure reveals presence of protein bands at ~45kDa which gradually shows the similar band patterns in all the isolates from different sources.

3.5 Western Blot:

Western blot was performed on 48sera from 336 randomly collected healthy human sera. A representative figure is shown in (Figure 3.6). Bands obtaied in western blot analysis are shown in (Table 3.9).

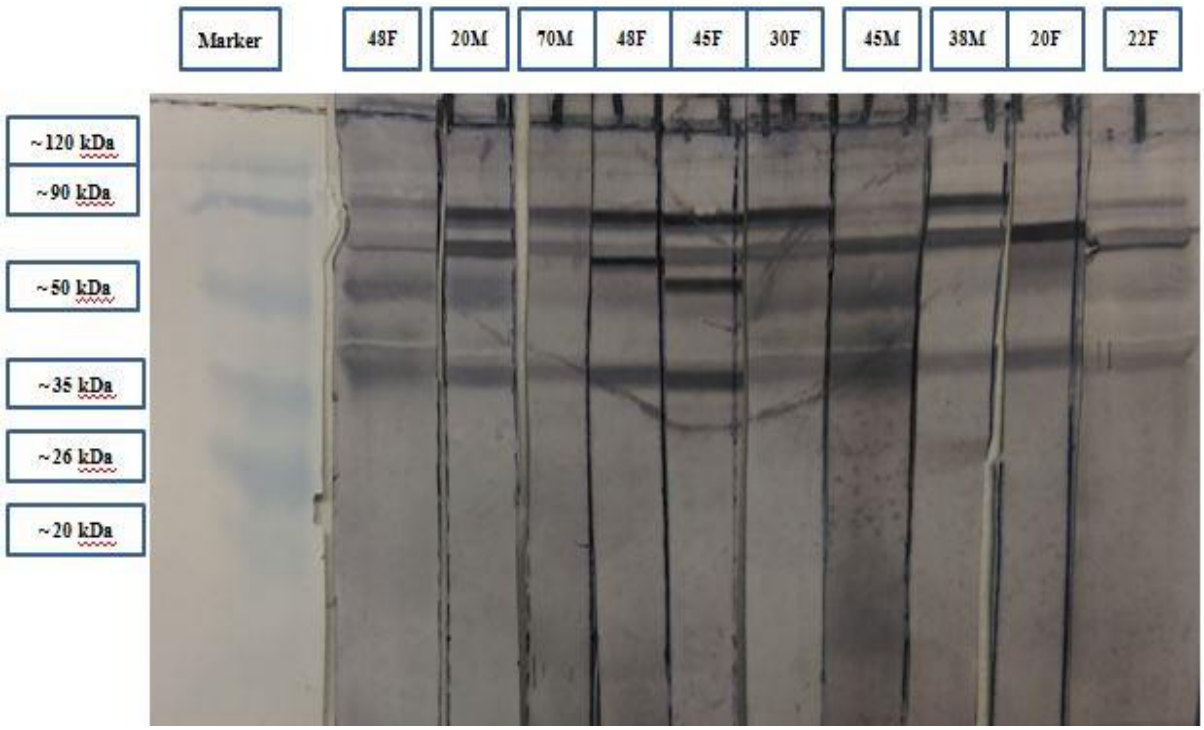

Figure 3.6: Western Blot analysis of *E.coli* outer membrane protein with Human sera. Lane 1 indicates the marker, lane 2 indicates a female of 48 years, lane 3 indicates a male of 20 years, lane 4 indicates a male of 70 years, lane 5 indicates a female of 48 years, lane 6 indicates a female of 45 years, lane 7 indicates a female of 30 years, lane 8 indicates a male of 45 years, lane 9 indicates a male of 38 years, lane 10 indicates a female of 20 years and lane 11 indicates a female of 22 years.

Table 3.9: Western Blot analysis of *E.coli* CD 17 outer membrane protein against human sera

Lane 1 (band in kDa)	Lane 2 (band in kDa)	Lane 3 (band in kDa)	Lane 4 (band in kDa)	Lane 5 (band in kDa)	Lane 6 (band in kDa)	Lane 7 (band in kDa)	Lane 8 (band in kDa)	Lane 9 (band in kDa)	Lane 10 (band in kDa)
84	84	84	84	84	84	84	84	84	84
68	68	68	68	68	68	68	68	68	68
55	55	45	63	59	55	63	45	63	63
45	45	38	45	45	45	51	38	45	45
38	38		38	38	38	45		38	38
						38			

On the basis of the western blot results the determinated *E. coli* outer membrane protein was found at the band of 45 kDa and 45 kDa. Western blot analysis of neonatal serum was performed as control (Figure 3.7). A comparative study is performed on western blot data of both crude extract and outer membrane proteins and the protein band of 45 kDa is shown to be prominent in both case (Table 3.10).

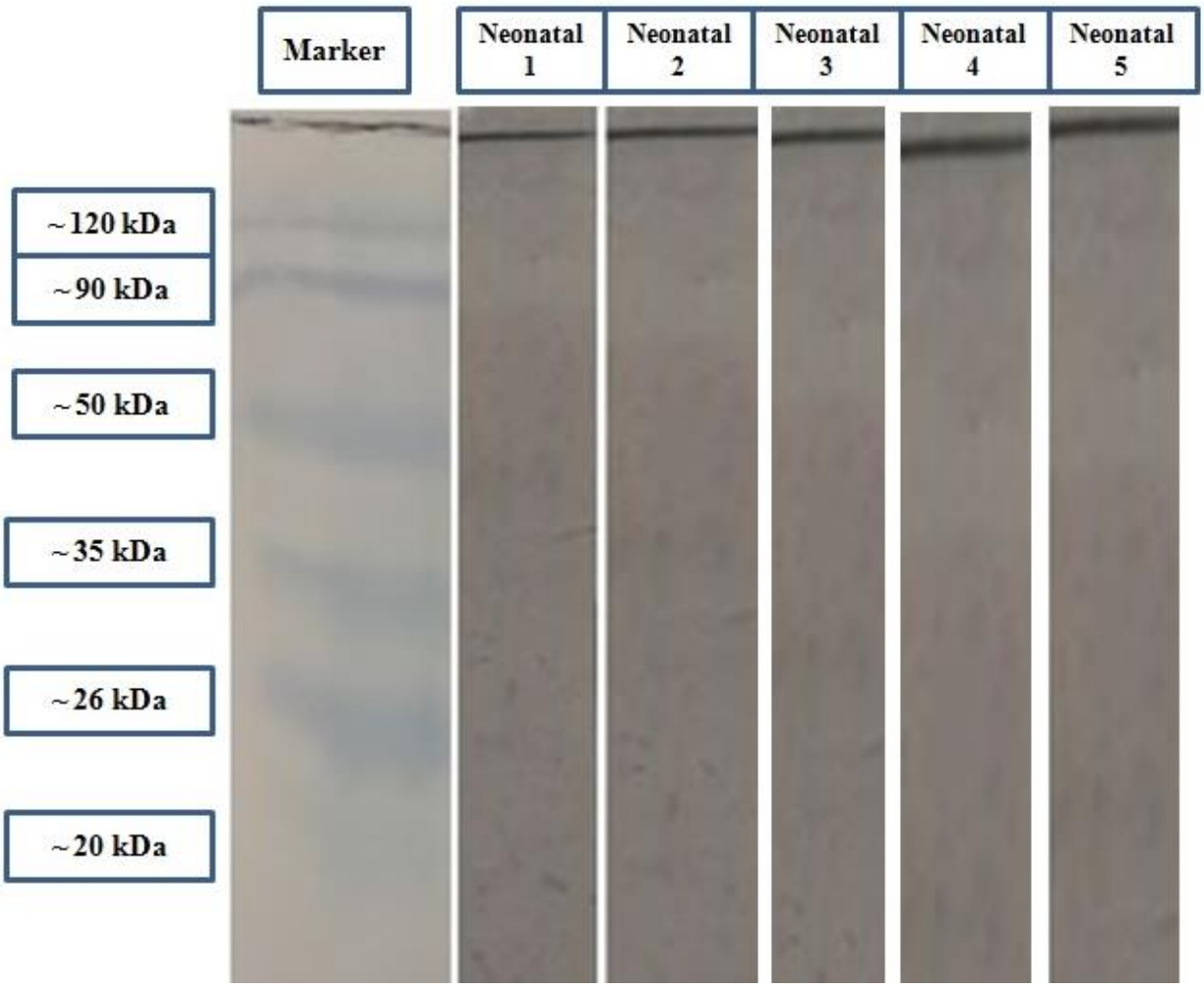

Figure 3.7: Western Blot analysis of *E. coli* outer membrane protein analysis with Human sera (Neonatal).

Table 3.10: A comparative analysis of the protein band (kDa) between the SDS-PAGE and Western blot of strain CD17

Strain	SDS-PAGE		Western blot
CD17	Crude extract	OMP	WB
	45	45	45

3.7 Determination of antibody responses using ELISA: Multiple dilutions of the human sera (1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200) were used in ELISA. The STEC positive *E. coli* infected serum was considered as positive control and healthy neonatal human sera as negative control (Table 3.11 and Figure 3.8).

Table 3.11: Detection of different primary antibodies against the particular antigen CD 17 using Human Sera by ELISA:

	1:100	1:200	1:400	1:800	1:1600	1:3200
A (negative ctrl)	0.062	0.047	0.033	0.025	0.013	0.005
B (Sera)	0.480	0.369	0.397	0.210	0.175	0.103
C (Sera)	0.359	0.284	0.206	0.157	0.116	0.094
D (Sera)	0.544	0.423	0.328	0.242	0.189	0.132
E (Sera)	0.439	0.367	0.269	0.188	0.132	0.101
F (Sera)	0.349	0.295	0.212	0.148	0.104	0.081
G (Sera)	0.317	0.286	0.227	0.159	0.1145	0.073
H (positive ctrl)	0.623	0.536	0.422	0.299	0.155	0.084

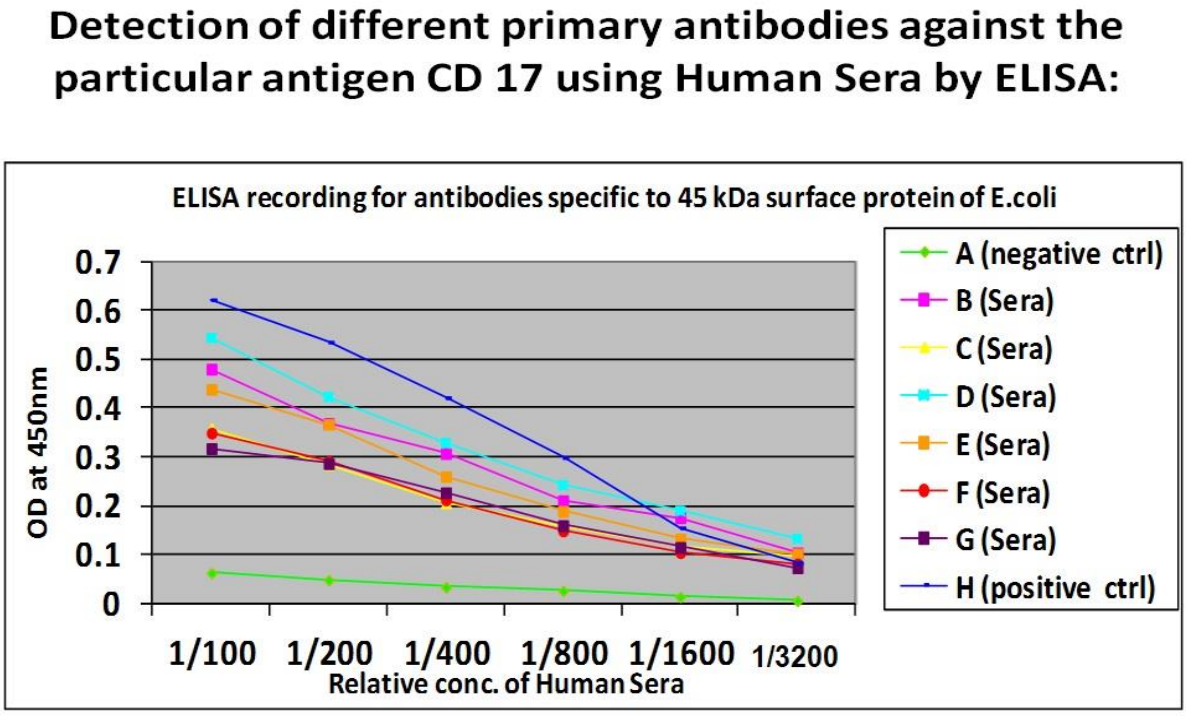

Figure 3.8: Immune response of human serum samples against CD 17 by ELISA using different dilutions.

4. Discussion

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a zoonotic enteric pathogen associated with hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans. Ruminants are the main reservoir of *E. coli* O157:H7 which usually colonizes the intestinal tract without causing clinical signs. Infected animals can shed the bacteria in their feces, so becoming direct or indirect sources of human infections via contaminated food or water. For this reason, EHEC O157:H7 control in ruminants merits more attention [Zhang *et al*., 2014].

Almost 15 years ago, outbreaks of disease was caused by enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7, this serotype has tended to dominate the world literature on EHEC. Evidence suggests that EHEC O157:H7 isolates are derived from one particularly successful clone of *E. coli* that has spread around the world. Long before the emergence of this strain, and ever since, there have been reports of human cases and outbreaks of disease caused by serotypes of *E. coli* other than O157:H7. On occasion, these may occur concomitantly with O157:H7 cases, which may lead to the false labeling of an outbreak as one caused by this serotype only [Goldwater *et al*., 1998].

Without diminishing the role of the O157:H7 clone, it is seen that the importance of recognizing that other serotypes are responsible for outbreaks as well as cases of sporadic human disease. It is also apparent from the literature that a wide variety of EHEC impinge on the human host from a wide range of food and non-food sources. Failure to identify the source is not uncommon. It is clear that non-O157:H7 are important (and probably underestimated) causes of disease. Clearly, other EHEC will be missed unless they are looked for specifically with a ‘broad brush’ approach (like, PCR detection of *stx* genes or direct detection of toxins in feces or faecal cultures). Medical diagnostic and public health laboratories should be encouraged to use techniques that detect *stx* genes or toxins in clinically and epidemiologically appropriate specimens, and should not restrict themselves to looking for O157:H7. The current focus on EHEC O157:H7, and seeming neglect of other EHEC, has major implications in terms of diagnosis, the food industry and human health. In addition, the practice of some authors of implying that EHEC O157:H7 is part of the definition of HUS should be discouraged. **5. References:**

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Appendices

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^{o}C for 15 min.

MacConkey agar (Oxoid, England)

Ingredients	Amount (g/L)
Peptone	20.0
Lactose	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. Simmon's citrate agar (Oxoid, England)

Ingredients	Amount (g/L)
Magnesium sulfate	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 thymol blue	0.08

4. Triple sugar iron agar

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

5. MR-VP broth

Ingredients	Amount (g/L)
Peptone	7 g
Dextrose	5 g
Di-potassium hydrogen phosphate	5 g
Final pH	6.9

6. Tryptic soy broth (Oxoid, England)

Ingredients	Amount (g/L)
Casein peptone	17.0
Soya peptone	3.0
Sodium chloride	5.0
Dipotassium phosphate	2.5
Dextrose	2.5
Final pH	7.3±0.2

Peptone water

Ingredients	Amount (g/L)
Peptone	10 g
Sodium chloride	5 g

T_1N_1soft agar

Ingredients	Amount (g/L)
Tryptone	10 g
Sodium chloride	10 g
Agar	6 g

Luria Bertani broth

Ingredients	Amount (g/L)
Tryptone	10 g
Yeast extract	5 g
Sodium chloride	10 g

Nutrient agar

Ingredients	Amount (g/L)
Peptone	5.0
NaCl	5.0
Beef extract	3.0
Agar	15
Distilled water	1L
pH	7.0

Appendix-II

Buffers and reagents

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^{o}C.

Methyl red reagent

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^{o}C.

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^{o}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 the final volume to 25 ml.This solution was covered with aluminum foil and stored at 4^{o}C.

Appendix-III

Instruments

The important equipment used through the study are listed below

Autoclave, Model no: WAC-47	Korea
Sterilizer, Model no: NDS-600D	Japan
Balance (Core series)	Adam, UK
Centrifuge, Model no (Code: 5433000.011)	Eppendorf, Germany
Digital Homogenizer (Wise Tis)	Korea
Freezer (-20°C)	Siemens, Germeny
Gel Documentation System	Major Science, Taiwan
Incubator	UK
Laminer air flow cabinet	UK
Micropipettes	Eppendorf, Germany
Oven(Universal drying oven)Model:LDO-060E	Labtech, Singapore
Thermal Cycler, Model no: 2720	Applied Biosystems,USA
Refrigerator,Model-0636	Samsung
Shaking Incubator (Model-WIS-20R)	Korea
Vortex Mixture	VWR International
Water bath	Korea
pH meter, Model: E-201-C	hanghai Ruosuaa, Technology companChina
Disposable micropippete tips	Eppendorf, Ireland
Western blot apparatus	Neido, Japan