Vaccine Efficacy of a 45 kDa Outer Membrane Protein of *Escherichia coli* O157:H7 in Mice Models

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOTECHNOLOGY

Submitted by

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Dedicated

To

My Precious Parents and Adorable Sister

DECLARATION

I hereby solemnly declare that the research work embodying the results reported in this thesis entitled **"Vaccine Efficacy of 45 kDa Outer Membrane Protein of *Escherichia coli* O157:H7 in Mice Models"** submitted by the undersigned, has been carried out under joint supervision of Professor Chowdhury Rafiqul Ahsan, Department of Microbiology, University of Dhaka and Professor Naiyyum Choudhury, former Coordinator of Biotechnology and Microbiology programmes, BRAC University. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree of diploma.

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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
pmol - Picomole
mm - Millimeter
cm - Centimeter
µm - Micrometer
nm - Nanometer
mA - Milliampere
V - Volt
PBS - Phosphate buffer solution
e.g. - For example
et al. - And others
pH - Negative logarithm of hydrogen ion concentration
bp - Base pair
rpm - Rotation per minute
UV - Ultra violet
spp. – Species

psi - Pounds per square inch

SD - Standard deviation

Fig - Figure

Abstract

Escherichia coli O157:H7 is an extremely pathogenic organism that produces a potent Shiga toxin which can cause a number of brutal diseases like Hemorrhagic Colitis (bloody diarrhoea), renal failure causing Hemolytic Uremic Syndrome (HUS), and fatal neurological defects. Cattle and other ruminant animals are the main reservoirs of Shiga-toxin producing *E. coli* (STEC) organisms. CD-17 is the bovine isolate of this strain, whose antigenic structural parts had been employed in this study to be used as a vaccine against these virulent strains. In previous studies, despite undergoing several trials and strategies to produce an effective vaccine against STEC, no successful treatment could be launched. However in recent years, a 45kDa antigen was found to be present in normal healthy human serum against *E. coli* O157:H7. The main objective of this study was to determine the vaccine efficacy of the 45kDa outer membrane protein (OMP) of *E. coli* O157:H7 (CD-17 isolate) in mice models (in vivo) to develop and establish an efficient vaccine that can be used against these pathogens in the target regions of the world. For this, the outer membrane protein (OMP) of *E. coli* O157:H7 (CD-17 isolate) was extracted by the TSE (Tris-Sucrose-EDTA) extraction method, followed by its SDS-PAGE analysis and Western Blotting. The distinct band of 45kDa that appeared in the membrane after Western Blot was sonicated into a suspension and injected into only one set of experimental mice, keeping another set as control. After immunization, another western blotting was done, which confirmed the presence of 45kDa protein bands in the immunized set of mice and their babies (First Generation F-1, who were not injected 45kDa). When all the three sets of mice (experimental/immunized, F-1 and control/non-immunized) were challenged with the lethal dose of 10^{10} cells/ml of *E.coli* O157:H7 pathogen, it was observed that within a time period of 11 days, 85.7% of control mice have died whereas even after 60 days, 100% of the immunized mice were as healthy as before. Moreover, 100% of the non-injected babies (F-1) that were produced from mating of the immunized mice had also survived brilliantly like their parents. Therefore, this study demonstrates that the 45kDa protein vaccine will serve as an efficient treatment against the highly virulent *E.coli* O157:H7 strain, and will also sustain its immunogenicity in their next generation. This vaccine targets the developed countries where this lethal strain of *E.coli* O157:H7 still imposes a huge life-threat to the community leading to death. The world-wide problem of traveler's diarrhoea of foreigners can also be expectantly solved by this vaccine. However, further characterization of the 45kDa surface protein is necessary to confirm other functions of this protein.

Chapter 1

INTRODUCTION

&

LITERATURE REVIEW

1. Introduction and Literature Review

1.1. Background

Escherichia coli is a gram-negative, facultatively anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia,* as named after the German scientist Theodor Escherich who isolated the type species of the genus. It is a normal inhabitant of the large intestinal tracts of warm-blooded organisms (endotherms) and usually occurs singly or in pairs. Since 1885, *E. coli* has been recognized as both a harmless commensal and a versatile pathogen (Bower, 1999). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts, give rise to many common bacterial infections, including cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI), traveler's diarrhoea and also clinical infections such as neonatal meningitis (Bower, 1999). *E. coli* and other facultative anaerobes constitute about 0.1% of gut flora, and fecal-oral transmission is the major route through which pathogenic strains of the bacterium cause disease.

Shiga toxin producing *E. coli* (STEC) are pathogenic strains of the bacterium *E. coli* that produce a potent toxin called Shiga toxin (Stx). Shiga toxin causes premature destruction of the red blood cells and damage in blood vessels, which then clog the body's filtering system, the kidneys, causing Hemolytic Uremic Syndrome (HUS), and also plays a key role in other events that result in hemorrhagic colitis (bloody diarrhoea). HUS is a life-threatening complication of EHEC infection that appears to involve between 4 and 8% of all children who develop outbreak associated hemorrhagic colitis. The disease most commonly involves three clinical features, namely, acute renal failure, thrombocytopenia, and micro-angiopathic hemolytic anemia, a fatality rate of between 5 and 10% is usually associated with this syndrome (Abbott *et al.,* 1994).

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 and has been linked to the consumption of undercooked beef, particularly in fast-food restaurants (Abbott *et al.,* 1994). The shiga toxin producing *E. coli* (STEC), *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. In addition, this strain causes the buildup of fluid (since the

kidneys do not work), leading to edema around the lungs, legs and arms. This increase in fluid buildup especially around the lungs impedes the functioning of the heart, causing an increase in blood pressure. It is the number one cause of acute kidney failure in children and is also responsible for causing severe abdominal cramps, bloody diarrhoea and vomiting in human beings. Unlike several other groups of enteropathogenic *E. coli* O157:H7 is associated with two unusual biochemical markers: the inability to produce the enzyme, ß-glucuronidase and delayed D-sorbitol fermentation.

Human outbreaks of STEC-related disease occur through consumption of contaminated food or water, through direct contact with infected animals or environments contaminated by their feces, or by direct human-to-human contact with infected persons (i.e. secondary infections). Cattle and other ruminant animals such as deer are natural reservoirs of STEC, which can lead to contamination of meat and milk during harvest and processing. Other foods such as fresh produce can become contaminated with STEC from these animals due to water contamination, run-off from animal production units, or dust and insects from animal production facilities. Due to a low infectious dose and severity of disease manifestations for most STEC, their presence in raw and processed foods poses an important human health risk. Thus, the food industry, its regulators and the public health authorities are focusing substantial resources and considerable efforts towards investigation and control of STEC infection, and elimination of STEC from the food supply.

Medical diagnostic and public health laboratories should be encouraged to use techniques that detect *stx* genes or toxins in clinically and epidemiologically appropriate specimens. However, the search should not be restricted to looking for O157:H7 only. Other EHEC strains, which also have major implications in terms of diagnosis, food industry and human health, should be focused for further characterizations and health complications caused by them should also be determined.

1.2. Literature Review

1.2.1. Wide uses of *E. coli*

E. coli is the most widely studied prokaryotic model organism, and an important species in the fields of biotechnology and microbiology, where it has served as the host organism for the majority of work with recombinant DNA. In scientific research, *E. coli* is a standard vector for rapid mass production of biological building blocks, from DNA to protein. *E. coli* is a common bacterium that is being studied intensively by geneticists because of its small genome size, normal lack of pathogenicity and ease of growth in the laboratory. They are needed by the body to aid in the production of several vitamins, such as Vitamin K and the B-Vitamins.

1.2.2. Classification of *E.coli*

Table: 1.1. Classification of *E.coli*

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

Domain and Kingdom: Bacteria, because they are unicellular microorganisms

Phylum: Proteobacteria, because they are Gram-negative (G-) bacteria with an outer membrane composed primarily of lipopolysaccharides.

Class: Gamma Proteobacteria, because they are facultatively anaerobic G- bacterium.

Order: Enterobacteriales, because they are rod-shaped facultatively anaerobic G- bacterium.

Family: Enterobacteriaceae, because they are motile via peritrichous flagella that grow well at 37ºC, is oxidase negative, catalase positive, and reduces nitrates.

Genus: Escherichia, because they 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. *E. coli* is unique by its biochemical activities such as: lactose fermentation, containing lysine decarboxylase,

being Vogus-Proskauer negative, indole production, no growth in nitrate, and no H_2S gas production.

1.2.3. Types of *E. coli*

A certain group of *E. coli* isolates have implicated a wide range of diseases in animals and humans worldwide. Till now, eight pathogenic variants (pathovars) of *E. coli* 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) (Sangwan, 2016).

Six pathovars of diarrhoeagenic *E. coli* are: 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). The two extraintestinal *E. coli* (ExPEC) pathovars are: Uropathogenic *E. coli* (UPEC) and Neonatal meningitis *E. coli* (NMEC). Other pathovars have been identified, but their mechanisms of pathogenesis are not well defined (Croxen and Finlay, 2010).

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

EPEC is a major cause of potentially fatal diarrhoea in infants in developing countries (Kaper *et al*., 2004). They are an important cause of traveler's diarrhoea in Mexico and in North Africa (Uttly *et al*., 1998). They do not contain any colonization factors or produce heat-stable (ST) or heat-labile (LT) toxins but they may produce an enterotoxin or has an array of virulence factors similar to that of *Shigella*. They produce a non fimbrial adhesin known as intimin, an outer membrane protein that mediates the final stages of adherence (Wellington and vanElsas, 1992). The phenomenon of EPEC strains adherence to the host intestinal mucosa is a very complicated process called "attaching and effacing" of cells, which produces remarkable effects in the ultra structure of the cells by deformation, resulting in rearrangements of actin in the vicinity of adherent bacteria. They cause the loss of microvilli and prevent the entry of bacteria into the mucosa. 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. Diarrhoea and other symptoms of EPEC infections might be caused by bacterial invasion of host cells and interference with normal cellular signal transduction, rather than by production of toxins.

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

EHEC is a highly infectious pathogen that colonizes the distal ileum and large bowel in humans by attachment and effacement and is often the causative agent of severe gastroenteritis outbreaks in developed countries. Cattle are a key reservoir for EHEC, which is also found in humans and goats. Transmission of EHEC strains usually occurs through contaminated food and water in human. In North America, Japan and parts of Europe, most outbreaks are due to EHEC serotype O157:H7, the most infamous member of this pathotype, whereas other serotypes are important health concerns in other developed countries. Adults and children infected with EHEC suffer from haemorrhagic colitis (bloody diarrhoea) 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". It uses bacterial fimbriae for attachment (*E. coli* common pilus, ECP). The EHEC strains do not invade mucosal cells as readily as *Shigella* but produce a toxin which is phage encoded, and is virtually identical to the Shiga toxin. The production of this toxin is enhanced by iron deficiency and it plays a role to elicit an intense inflammatory response and may explain the ability of EHEC strains to cause HUS (Griffin, 1995; Samuel *et al.*, 1988).

(iii). Enterotoxigenic *E. coli* (ETEC)

ETEC is the leading bacterial cause of diarrhoea in infants and travelers in underdeveloped countries or regions of poor sanitation. Each year, ETEC causes more than 200 million cases of diarrhoea and 380,000 deaths, mostly in children in developing countries. ETEC strains are noninvasive, and they do not leave the intestinal lumen. ETEC is acquired by ingestion of contaminated food and water and adults in endemic areas evidently develop immunity. ETEC possess colonization factors (pili, K antigen) to enhance their virulence. The disease requires colonization and elaboration of one or more enterotoxins. Both traits are plasmid-encoded. ETEC adhesins are fimbriae which are species-specific, and help to bind enterocyte cells in the small intestine. 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. ETEC can produce two proteinaceous enterotoxins, which 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 (larger in size) is very similar to cholera toxin in both structure and function. 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 vanElsas, 1992). The smaller protein, ST enterotoxin causes cGMP accumulation in the target cells and a subsequent secretion of fluid and electrolytes into the intestinal lumen.

(iv). Enteroinvasive *E. coli* (EIEC)

EIEC are invasive organisms. They closely resemble *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. EIEC infection causes a clinical syndrome that is identical to shigellosis, with profuse diarrhoea and high fever. EIEC apparently lacks fimbrial adhesions but posses a specific adhesion like *Shigella,* which is thought to be an outer membrane protein. They do not produce LT, ST toxin or the *Shigella* toxin (Wellington and van Elsas, 1992). Though EIEC do not produce enterotoxin, they invade the intestinal mucosa like dysentery bacilli. They cause kerato-conjunctivitis on instillation into the eyes of guinea pig (Sereny test) which is a diagnostic method for EIEC. Another diagnostic method is their invasion of HeLa cells in tissue culture. EIEC is late lactose fermenter and may be anaerogenic. They have antigenic relationship with shigella.

(v). Enteroaggregative *E. coli* (EAEC)

EAEC are noninvasive organisms and their significance in human disease is controversial. The distinctive feature of EAEC strains is their ability to aggregate tissue culture cells by fimbrae. These strains are associated with persistent diarrhoea in young children. The bacteria adhere to the intestinal mucosa and cause non-bloody, watery diarrhoea without causing inflammation or fever. They produce ST enterotoxin and a hemolysin similar to that of ETEC. Recently, a distinctive heat labile plasmid encoded toxin has been isolated from these strains, called the EAST (EnteroAggregative ST) toxin, whose role in virulence is not proved yet (Wellington and van Elsas, 1992).

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

DAEC are able to invade intestinal epithelial cells and replicate intracellularly. It is a heterogenous group that generates a diffused 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 adhesions. These bacteria colonize the small bowel and have been implicated in diarrhoea in children between the ages of 18 months and 5 years, as well as in recurring urinary tract infections (UTIs) in adults (Servin, 2005). It is likely that DAEC are able to proliferate more effectively in hosts with defective innate immunity. They are associated with the ileal mucosa in Crohn's disease.

(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, multiple iron acquisition systems and a polysaccharide capsule (Wiles *et al*., 2008).

(viii). Neonatal meningitis *E. coli* (NMEC)

NMEC, a common inhabitant of the gastrointestinal tract, is the most frequent cause of Gram-negative associated meningitis in newborns. Fatality rates can approach 40 % (Kaper *et al*., 2004) and survivors are usually burdened with severe neurological disorders. The pathogenesis of NMEC is complex, as the bacteria must enter the bloodstream through the intestine and ultimately cross the blood–brain barrier into the central nervous system, which leads to meningeal inflammation and pleocytosis of the cerebrospinal fluid. Recently, a lambdoid phage that encodes O acetyltransferase was discovered, which acetylates the O antigen to provide phase

variation and diversity to the capsule and may therefore hide the bacteria from host defenses (Deszo *et al*., 2005).

1.2.4. Morphology and antigenic structure of *E.coli*

Bacterial cells are protected by a complex, multilayered structure called the cell envelope. In Gram-negative bacteria, the cell envelope is composed of an inner cytoplasmic membrane, a thin peptidoglycan cell wall, and a lipopolysaccharide-containing outer membrane that surrounds the peptidoglycan layer. The inner and outer membranes delimit an aqueous space termed the periplasm (Quan *et al.,* 2013). *E. coli* is Gram-negative, straight rod shaped, 1-3µm long and 0.3-1µm in diameter, arranged singly or in pairs. *E. coli* stains Gram-negative because its cell wall is composed of a thin peptidoglycan layer and an outer membrane. During the staining process, *E. coli* picks up the color of the counterstain safranin and stains pink. The outer membrane surrounding the cell wall provides a barrier to certain antibiotics such that *E. coli* is not damaged by penicillin. It is motile by peritrichous flagellae, though some strains are non-motile. They do not produce spores. Capsules and fimbriae are found in some strains (Sangwan, 2016).

E. coli has three antigens: O somatic/ part of lipopolysaccharide layer (without flagella), H flagella (with flagella) and K (Kapsular) proteins. K antigen is an envelope antigen, which encloses the O antigen, renders the strain inagglutinable by the O antiserum and contributes to virulence by inhibiting phagocytosis. The fimbrial antigen (F) has no significance in antigenic classification of *E. coli*. Type I fimbriae mediates adhesion of bacterium to human and animal cells. Such adhesion enhances bacterial pathogenicity e.g. urinary tract infection in which type I fimbriae has some possible role to play. Several fibrin structures resembling fimbriae have been demonstrated. They, most probably, play a very important role in pathogenesis of diarrhoeal diseases and urinary tract infection (Sangwan, 2016).

1.2.5. Biochemistry of *E.coli*

Being a facultatively anaerobic bacterium, it can undergo both a fermentative and respiratory type of metabolism. The optimum growth temperature is 37°C. On Nutrient agar, colonies are large, thick, greyish white, moist, smooth, opaque or translucent discs. Some strains may form mucoid colonies. On MacConkey agar medium, colonies are bright pink due to lactose

fermentation. Glucose, lactose, mannitol, maltose are fermented with acid and gas production, but sucrose is not fermented by typical strain of E. coli. In Triple sugar iron (TSI), acid and gas are produced.

The four biochemical tests widely used for entero-bacteriaceae classification are Indole (I), Methyl Red (MR), Voges Proskauer (VP) and Citrate (C) utilisation which are referred to as IMViC. *E. coli* gives positive results for Indole and MR tests, negative results for VP and citrate tests, H_2 S is not formed and urea is not hydrolysed.

1.3. Pathogenicity of *E.coli*

Gram-negative bacteria's cell-surface structure consists of three essential layers: cytoplasmic or inner membrane (IM), outer membrane (OM) and periplasmic space between the inner membrane and outer membrane. The outer membrane 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 outer membrane are phospholipids, LPS and proteins which help it to serve as a physical barrier between the bacterial body and its surroundings and protect the organism against bile salts, antibiotics, proteolytic enzymes and other hostile factors and also facilitate the uptake of nutrients.

1.4. Virulence Factors

1.4.1. Toxins

Secreted toxins and other effector proteins trigger signal transduction pathways in an astonishing variety of fundamental eukaryotic processes more numerously than the surface structures. 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). Besides the endotoxin associated with O antigen, some *E. coli* strains produce two types of exotoxin: enterotoxin and haemolysin. Enterotoxins responsible for diarrhoea are of two types: heat labile (LT) and heat stable (ST). LT is similar to cholera enterotoxin antigenically and its mechanism of action is conducted by stimulating the adenyl cyclase, cyclic adenosine monophosphate

(cAMP) system to produce fluid accumulation in the intestinal lumen. ST appears to stimulate fluid secretion into the gut through the mediation of cyclic guanosine monophosphate (cGMP) resulting into dehydration.

Three types of haemolysins produced by *E. coli* are not related to pathogenesis. *E. coli* forms a part of normal intestinal flora of man and animal and the commensal strains belong to several O groups. There are many strains of *E. coli* which include commensal strains as well as strains with virulence determinants that cause a wide variety of infections of all age groups of men and animals. The virulent strains of *E. coli* are specific pathogens in the gut (enteritis) and of extra-intestinal sites (urinary tract infection, wound infection). 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.

1.4.2. Adhesion or colonization

Specific adherence factors are present in pathogenic *E. coli* strains that help them to colonize sites of the host, such as the small intestine and the urethra. These adhesins form distinct morphological structures called fimbriae (also called pili) or fibrillae. Fimbriae are rod-like structures of 5–10 nm diameters that are distinct from flagella whereas fibrillae are 2–4 nm in diameter and are either long and wiry or curly and flexible (Cassels and Wolf, 1995). Some surface structures trigger signal transduction pathways or cytoskeletal rearrangements that can lead to disease. For example, adhesins 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). Surface structures present on commensal *E. coli* strains can also 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). Outer-membrane proteins (OMPs) can also serve as adhesins of pathogenic *E. coli*, such as intimin of UPEC and EHEC, or other non-fimbrial proteins (Tieng *et al*., 2002).

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

1.5.1. MacConkey Agar

This is the most commonly used medium for *E. coli* isolation which works as a selective media that inhibits the growth of other Gram-positive Enterobacteriaceae. It is composed of lactose, peptone, sodium chloride, bile salt, crystal violet (dye) and neutral red (dye) 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. Nitrogen and vitamin sources in MacConkey agar are made up from the enzymatic digest of gelatin, casein, and animal tissue. Lactose is the fermentable carbohydrate. During lactose fermentation, pH drop around the colony causes a color change in the pH indicator, neutral red dye and bile precipitation. Bile salts mixture and crystal violet dye are the selective agents, inhibiting Gram-positive cocci and allowing Gram-negative organisms to grow. Sodium Chloride maintains the osmotic environment. Agar is the solidifying agent (Holt and Krieg, 1994).

1.5.2. Eosin Methylene Blue (EMB) agar

It is a selective stain for gram-negative bacteria. EMB contains dyes that are toxic to gram-positive bacteria and hence, this medium has been specifically designed to discourage the growth of gram positive bacteria. EMB is the selective and differential medium for coliforms. It is a blend of two stains: eosin and methylene blue in the ratio of 6:1. EMB medium is composed of peptone, lactose, dipotassium phosphate, eosin (dye), methylene blue (dye), and agar. It provides a color indicator that helps to distinguish organisms that ferment lactose (e.g., *E. coli*) and those that do not (e.g., *Salmonella*, *Shigella*). If *E. coli* is grown in EMB agar medium, it will give distinctive metallic green sheen (due to the metachromatic properties of the dyes) which is enhanced by the bacterial movement using flagella, and strong acid end-products of fermentation.

1.6. Pathogenesis and clinical manifestations of *E. coli* O157:H7

Enterohaemorrhagic *E. coli* (EHEC) serotype O157:H7 is an important deadly pathogenic strain of *E.coli* that is responsible for major outbreaks of gastroenteritis worldwide and is of global concern to public health. Extrarenal complications such as rhabdomylosis, myocardial and

central nervous system (CNS) damage are associated with increased mortality in *E.coli* O157:H7 infections. *E.coli* O157:H7 belongs to the predominant category of Shiga toxin producing *E.coli* (STEC) (Armstrong *et al.,* 1995; Besser *et al.,* 1999), in which the 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.,* 2012). The pathogenicity of STEC O157:H7 is associated with various virulence factors, such as Shiga toxins 1 and 2 (Stx1 and Stx2), that are encoded by *stx*1 and *stx*2 genes, respectively. Stx1 is antigenically similar to Shiga enterotoxin produced by *Shigella dysenteriae* type 1. Stx2 is heterogeneous (Stx2c, Stx2d, Stx2e and Stx2f) and immunologically different from Stx1.

Bovine and domestic ruminants are the main reservoirs for EHEC, especially serotype O157:H7 that is transmitted to humans primarily through consumption of contaminated foods like unpasteurized dairy products, undercooked contaminated ground beef and other meats, contaminated fruits and vegetables. Fecal 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. EHEC colonize the gastrointestinal tract and after an incubation period of 2-3 days cause diarrhoea and abdominal pain. In about 80% of cases after a 2-4 day interval there is progression into hemorrhagic colitis (HC) or bloody diarrhoea. Within 3-13 days after the beginning of diarrhoea 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.

1.7. Outbreaks of *E.coli* infections

In 1996, the largest outbreak in Japan took place in school children, which was associated with radish sprouts (Mohawk and Brien, 2010). Out of 174 patients being examined, 101 cases of diarrhoea, 20 cases of HUS, and 53 patients symptom-free were found. EHEC O157:H7 was isolated from 21 (20.8%) of diarrhoeal paients and 70% from HUS patients (Akashi *et al*., 1994). Chapman and Siddons reported a study designed to determine the value of specific methods for isolation of *E. coli* O157:H7 from faeces (Goldwater *et al.,* 1996).

In 2011, Germany was reported as one of the largest food-borne EHEC outbreaks of gastroenteritis and HUS. The latest update indicate that as of July 22, 2011, out of a total of 4075

outbreak cases, 23 % involved HUS and 3.7% fatal cases 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, which 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.,* 2012).

The most common STEC serotype reported in Australia between 2001 and 2009 was *E.coli* O157 (58%) followed by O111 (14%) and O26 (11%). Most Shiga toxin producing *E. coli* cases notified in Australia are sporadic infections. Infected individuals usually suffer from bloody diarrhoea and some experience kidney failure due to HUS. HUS carries a 12% risk of death or end stage renal disease with 25% of survivors suffering long-term renal consequences. Australia's largest outbreak of STEC infection occurred in Queensland during August 2013 associated with a petting zoo at the RNA.

Diarrhoeal diseases are highly prevalent in Bangladesh. In a previous study of 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* 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* (EHEC) 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 and hence, the specific role of STEC in causing diarrhoeal illness in this area became difficult to estimate.

However in this case, protective immunity against STEC could be an explanation, and was addressed by most of the studies done in developing countries. 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 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 (Beutin *et al*., 2004). However, a detailed study of the immune status of patients with diarrhoea as well as healthy

controls in areas where enteric pathogens are considered endemic should be carried out in order to explain these phenomena (Islam *et al.,* 2007).

1.8. Treatment

Bacterial infections are usually treated with antibiotics. However, the antibiotic sensitivities of different strains of *E. coli* vary widely. The mainstay of treatment is the assessment of dehydration and replacement of fluid and electrolytes. Administration of antibiotics has been shown to shorten the course of illness and duration of excretion of enterotoxigenic *E. coli* (ETEC) in adults in endemic areas and in traveler's diarrhoea. However, the rate of resistance to commonly used antibiotics is increasing and they are generally not recommended. Antibiotic treatment methods pose problems, such as side-effects, drug resistance, drug sensitivity and allergies. The antibiotic used depends upon susceptibility patterns in the particular geographical region.

Antibiotic resistance is a growing problem. Some of this is due to overuse of antibiotics in humans, but some of it is probably due to the use of antibiotics as growth promoters in animal feeds. *E. coli* bacteria often carry multiple drug-resistance plasmids, and under stress, readily transfer those plasmids to other species through a process called horizontal gene transfer. Mixing of species in the intestines allows *E. coli* to accept and transfer plasmids from and to other bacteria. Thus, *E. coli* and the other enterobacteria are important reservoirs of transferable antibiotic resistance.

Although EHEC strains are usually susceptible to a variety of antibiotic drugs, there are no prospective studies showing conclusively that antibiotics are effective in altering the course of diseases. Instead, antibiotics sometimes may aggravate kidney complications, and contribute to antibiotic resistance. In a prospective study, Proulx demonstrated a trend towards a lower incidence of HUS in those receiving antibiotics (Proulx *et al.*, 1992). Another study suggested that patients who received antibiotics may be at greater risk of developing HUS (Nataro and Kaper, 1998); since these were not prospective, randomized trials, it could be that the patients who were most severely ill were more likely to receive antibiotics. The use of antibiotics may be harmful for two potential reasons: (i) lysis of bacteria by some antibiotics leads to increased

secretion of toxin in vitro; (ii) antibiotic therapy could kill other intracolonic bacteria, thereby increasing the systemic absorption of toxin (Cimolai *et al.*, 1994).

Treatment of renal disease due to EHEC is primarily supportive, except for some experimental therapies currently being evaluated in clinical trials. In the case of HUS, dialysis is required to cleanse the body of uremic toxins and to maintain fluid and electrolyte balance. Current treatment regimens include dialysis, hemofiltration, transfusion of packed erythrocytes, platelet infusions and other interventions as clinically indicated. Severe disease may require renal transplant. A compound named Synsorb-Pk, consisting of a chemically synthesized analog of Gb3 (the receptor for Shiga toxin coupled to diatomaceous earth), if ingested by patients with bloody diarrhoea could help to absorb toxin from the intestine and prevent the development of HUS. Initial phase I trials have been promising and phase III trials to assess efficacy are in progress (Armstrong *et al.*, 1995).

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. For example, 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. 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 (Edberg *et al.,* 2000).

The combination of cephalosporins and β-lactam - β -lactamases inhibitor play the important role of a major drug class used to treat community onset or hospital acquired infections caused by *E. coli,* especially due to the ExPEC pathotype. However, β -lactamases are bacterial enzymes that inactivate β -lactam antibiotics by hydrolysis, which results in ineffective compounds (Edberg *et al.,* 2000). In 1990s, ExPEC were relatively susceptible to 1st line antibiotics. However several surveillance studies 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 during 2000. Resistance to these agents is causing delays in appropriate therapy with subsequent increased morbidity and mortality (Edberg *et al.,* 2000).

1.9. Future Treatment/ Solution: Vaccines

The human immune system naturally protects us from infectious diseases by eliminating the invading pathogen. During this process a lifelong memory response is established to the pathogen. This memory response then protects us from a subsequent infection with the same pathogen. To avoid the costs, side-effects and drug resistance problems of antibiotic treatments, permanent treatment against pathogenic E. coli or STEC might be by the synthesis of vaccines. A vaccine for cattle is not practical because the bacteria do not cause illness in cattle and therefore do not stimulate their immune response (Albert *et al.,* 1995).

Vaccine is a biological preparation that provides active acquired immunity to a particular disease. It contains an agent that resembles a disease-causing microorganism and is often made from weakened or killed forms of the microbe, its toxins or one of its surface proteins. The agent stimulates the body's immune system to recognize the agent as a threat, resulting into its destruction, and finally keep a record (memory) of it so that the immune system can more easily recognize and destroy any of these microorganisms that it later encounters. Vaccines are designed to preemptively induce protective immunity in advance of infection, so that it can protect the host body from the first exposure to the disease-causing agent.

The immune system typically responds to foreign proteins within live invading organisms (i.e. viruses, bacteria). The primary reason for this is that we have two interacting immune systems: a non-specific innate immune system and a specific adaptive immune system. The innate system responds non-specifically to live organisms, alerting the adaptive immune system to respond rapidly and strongly with a specific immune response to the invading organism. All vaccines contain an active component (the antigen) which generates the protective immune response. Vaccines may also contain additional components. There are six main types of vaccines: attenuated (live) vaccines, inactivated vaccines, toxoid vaccines, subunit vaccines, conjugate and recombinant vaccines.

- Live, attenuated vaccines fight viruses and contain a weakened version of the living virus (e.g., measles-mumps-rubella and varicella vaccine). In the body of a vaccinated individual, the weakened vaccine strain cannot grow to the extent necessary to cause disease symptoms, but does grow sufficiently well to induce both a non-specific innate response and the specific adaptive response that results in protective immunity.

- Inactivated or Killed vaccines also fight viruses and contain the killed virus (e.g., polio vaccines). The virulent virus is grown in large quantity and then chemically inactivated so that it cannot grow in the body at all. However, when a relatively large amount of the killed preparation is administered, immunity can be induced.
- Toxoid vaccines prevent diseases caused by bacteria that produce toxins in the body and contain weakened toxins (e.g., diphtheria and tetanus vaccine).
- Subunit vaccines include only the essential antigens of the virus or bacteria (e.g. whooping cough vaccine). Only a part or component of the pathogen is manufactured, purified and used as a vaccine. Subunits of the pathogen might be the outer membrane protein, flagella, cell wall, DNA, etc.
- Conjugate vaccines fight a different type of bacteria which have antigens with an outer coating of sugar-like substances (polysaccharides) that "hide" the antigen from the child's immature immune system; the vaccine connects (conjugates) the polysaccharides to antigens, so the immune system can react.
- Recombinant vaccines: The gene segment (for a protein) from the disease-causing organism that stimulates a protective immune response (protein of interest) is inserted into the gene of the host/target cell, such as a yeast cell.

1.10. Vaccines against *E. coli*: current scenario

Many laws have been put into play and improvements have been done in suppressing the *E. coli* outbreaks. Now the next step in preventing *E. coli* is through vaccinations. There are no currently available vaccines to prevent disease due to EHEC, but a number of experimental approaches to develop safe, effective vaccines are being investigated in animals. Clearly there is some time to go before human trials are reported but the numerous and frequent outbreaks of EHEC disease constantly reminds of the urgent need to protect the population against these emerging and often devastating diseases. In 2013, a study on the vaccines estimated that their use could reduce *E. coli* infections in humans by up to 83 percent (Andrews, 2015).

Many different vaccine strategies have been applied with variable success in a number of animal models. The strategies have involved the use of recombinant virulence proteins such as Shiga

toxin (Stx), intimin, *E. coli* secreted protein A (EspA) or peptides, fusion proteins of A and B subunits of Stx 2 and Stx1 such as Stx2Am-Stx1B and avirulent ghost cells of EHEC O157:H7. A crucial antigen in any potential vaccine of *E.coli* is the Shiga toxin (Stx). Parenteral Stx toxoid vaccines have shown protective effects in rabbits (Bielaszewsha *et al.*, 1994) and pigs (Bosworth *et al.*, 1996). Attenuated *Vibrio cholerae* (Nataro and Kaper, 1998) and *Salmonella typhimurium* vaccine strains that express StxB have been constructed. A parenteral vaccine specific for O157 EHEC has been developed based on O157 polysaccharide conjugated to protein carriers (Konadu *et al.*, 1994).

DNA vaccines are also working as a recent development in EHEC prevention, providing hopeful results in a mouse model. The mode of administration (intramuscular, intranasal, oral, intragastric, etc) of these vaccines not only affects immunogenicity but also provides protective effect under challenge. Vaccination with a plant-based oral vaccine protected mice models against lethal systemic intoxication with Stx2. In March 2006, a vaccine eliciting an immune response against the *E. coli* O157:H7 O-specific polysaccharide conjugated to recombinant exotoxin A of *Pseudomonas aeruginosa* (O157-rEPA) was reported to be safe in children and adults. A phase III clinical trial to verify the large-scale efficacy of the treatment is planned. In January 2007, the Canadian biopharmaceutical company Bioniche announced it has developed a cattle vaccine which reduces the number of O157:H7 shed in manure by a factor of 1000, to about 1000 pathogenic bacteria per gram of manure.

1.11. Prevention of infections

As the saying goes, prevention is better than cure. Good sanitation and hygiene are essential to preventing *E. coli* infections. Washing hands thoroughly after using the toilet, handling animals, animal bedding, or any material contaminated with animal feces. Also, meat should be thoroughly cooked at temperatures above 70°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.

1.12. Summary

Even after huge progress in the understanding of pathogenesis and management, diarrhoea continue to be one of the most common and important causes of morbidity and mortality among infants and children in developing countries. Determination of the etiological agents of diarrhoea is important in developing rational therapy and in implementing control measures (Albert *et al.,* 1995). Targeting PMNs carrying Stx could be a productive strategy for future research, as could possible gene therapy.

Illnesses are outcomes of infections, with pathogens employing ingenious mechanisms to establish disease. In the developed countries, a disruption of normal bacterial-epithelial cross-talk and impaired maturation of the gut's immune system results an increase in immune-mediated gut disorders. The basis of management of gastroenteritis is oral rehydration therapies whose composition is continuously modified.

Drugs are usually of little use to treat these infections and health hazards, except for certain indications. Probiotics and other new agents that target mechanisms of secretory diarrhoea show promise to encounter such adverse conditions. However, on a global scale, preventive strategies to diminish the burden of diarrhoeal disease ultimately hold the greatest potential. These strategies include production of vaccines and, most importantly, approach to address the existing inequalities between the developed and developing countries in fields of nutrition, sanitation, and drinking water safety.

1.13. Objective of the study

In a recent study performed in Bangladesh, immune response to *E. coli* O157:H7 had been identified in detection of antibodies in healthy human sera against outer membrane proteins causing infection in Bangladesh. It had been shown that 45kDa protein present in the outer membrane of *E.coli* O157:H7 can work as an immunogen and might be most commonly responsible for the immunogenicity of most Bangladeshi people. This hypothesis of possible immunity present in the healthy population in Bangladesh needs to be further investigated *in vivo*.

The aim of the present work was to determine the immune response and vaccine efficacy of the 45kDa outer membrane protein of *E. coli* O157:H7 (CD-17) in mice models (*in vivo*). Since immunity against these bacteria has been already shown to have developed naturally by adaptive/acquired immune system in most Bangladeshi people, this vaccine would be most suitably applicable for the people of developed countries and the travelers coming to developing countries, where *E.coli* O157:H7 is still a big hazard and an important life-threatening pathogen.

Chapter 2

MATERIALS & METHODS

2. Materials and Methods

2.1. Working Place

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

2.2 Bacterial Strain Collection

A bovine isolate, STEC O157:H7 (CD-17) and a reference clinical strain *E. coli* O157:H7 NCTC 12079 was obtained from stock culture of Department of Microbiology, University of Dhaka, Bangladesh. The bovine strain was isolated from the fresh feces of 18 healthy cattle from six different dairy farms around Dhaka city, Bangladesh (Rabbi *et al.*, 2014). Biochemical tests according to the standard procedure had been performed to reconfirm the strain.

2.3. Confirmation of *E.coli* isolates

2.3.1. Observation in MacConkey Agar Medium

MacConkey agar is used for the isolation of gram-negative enteric bacteria and the differentiation of lactose fermenting from the lactose non-fermenting gram-negative bacteria. The morphological features of colonies developed after incubation on MacConkey agar plate were observed and studied. As *E. coli* can ferment lactose, it gives pink color colony on MacConkey agar.

2.3.2. Observation in EMB medium

Eosin Methylene Blue (EMB) agar medium contains lactose and eosin and methylene blue dyes that permit differentiation between enteric lactose fermenters and non-fermenter. *E. coli* form black colonies with a metallic green sheen caused by the production of acid that precipitates out the dyes onto the growth's surface (Cappuccino and Sherman, 2011).

2.4 Biochemical Tests for Reconfirmation

2.4.1. Indole Test

The indole test is a biochemical test performed on bacterial species to determine the ability of the organism to convert tryptophan into the indole. The amino acid tryptophan is a component of most of the proteins and so is available to microorganisms after protein breakdown. Some bacteria are able to produce an intracellular enzyme called tryptophanase that catalyzes the elimination of the indole residue from tryptophan. Indole accumulates in the culture media while the rest of the tryptophan molecule (pyruvate and ammonia) is used to satisfy nutritional needs. This can be detected by growing them in a tryptophan rich medium where the accumulation of indole can be revealed by adding Kovac's reagent. This reagent reacts with indole and gives a water-insoluble bright red compound on the surface of the medium (Cappuccino and Sherman, 2011).

2.4.2. Citrate Utilization Test

Simmons' citrate agar is used for differentiating gram-negative bacteria on the basis of citrate utilization. It is useful for selecting organisms that use citrate as its main carbon and energy source. It is a defined, selective and differential medium that tests for an organism's ability to use citrate as a sole carbon source and ammonium ions as the sole nitrogen source. The medium contains citrate, ammonium ions, and other inorganic ions needed for growth. It also contains bromothymol blue as a pH indicator. Bromothymol blue is green at pH below 6.9, and then turns blue at a pH of 7.6 or greater. When microorganisms utilize citrate, they remove the acid from the medium, which raises the pH and turns the pH indicator from green to blue. The color change in the medium from green to blue indicates that the microorganisms tested can utilize citrate as its only carbon source, which had been detected in these strains (Cappuccino and Sherman, 2011).

2.4.3. Triple Sugar Iron (TSI) Agar Test

The Triple Sugar Iron (TSI) agar test differentiates among the different groups of the Enterobacteriaceae and distinguishes it from other Gram-negative intestinal organisms. This difference is made on the basis of variable carbohydrate fermentation patterns and hydrogen sulfide production. TSI agar medium contains concentrations of 1% lactose, 1% sucrose and 0.1% glucose. For easy observation of 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: Interpretations of various results in TSI test

Results (slant/butt)	Symbol	Interpretation
Red/yellow	K/A	Glucose fermentation only; Peptone catabolized
Yellow/yellow	A/A	Glucose and lactose and/or sucrose fermentation
Red/red	K/K	No fermentation; Peptone catabolized
Red/no color change	K/NC	No fermentation; Peptone used aerobically
Yellow/yellow with bubbles	A/A,G	Glucose and lactose and/or sucrose fermentation; Gas produced
Red/yellow with bubbles	K/A,G	Glucose fermentation only; Gas produced
Red/yellow with bubbles and black precipitate	K/A,G, H_2S	Glucose fermentation only; Gas produced; H_2S produced
Red/yellow with black precipitate	K/A, H_2S	Glucose fermentation only; H_2S produced
Yellow/yellow with black precipitate	A/A, H_2S	Glucose and lactose and/or sucrose fermentation; H_2S produced
No change/no change	NC/NC	No fermentation
A=acid production; K=alkaline reaction; G=gas production; H_2S=sulfur reduction		

(http://www.austincc.edu/microbugz/triple_sugar_iron_agar.php)

2.5. Methods

2.5.1. Extraction of Outer Membrane Proteins (OMPs)

Gram-negative bacteria (e.g. *E.coli)* contain an outer membrane whose composition is distinct from the inner cytoplasmic cell membrane and provides protection against a harsh environment by interfacing the cell with the environment. 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 profoundly contaminated with soluble cytoplasmic proteins. These contaminants make 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, it was found that a modified version of the method of Oliver and Beckwith consistently produces the cleanest extract of periplasmic and outer membrane proteins. It is designated as the very simple method called TSE extraction because it uses a Tris-sucrose solution supplemented with EDTA. Cytoplasmic and inner membrane protein contaminants are not evident on SDS polyacrylamide gels and contribute to less than 6% of total signal in very sensitive mass spectrometry analysis. This straightforward method is therefore ideal for analyzing specific proteomic changes in the cell envelope (Quan *et al.*, 2013).

Cell lysis disturbs the carefully controlled cellular environment, allowing endogenous proteases and phosphatases to become unregulated. As a result extracted proteins become degraded or 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.

2.5.1.1. TSE extraction method

Composition of Tris-Sucrose-EDTA (TSE) buffer includes: 200 mM Tris–HCl, pH 8.0, 500 mM sucrose, 1 mM EDTA. This buffer was stored at 4ºC and aliquoted into an appropriate amount. Protease inhibitor cocktail was freshly added to the TSE buffer immediately before use. In this

study, AMRESCO Protease Inhibitor (100X) had been used by diluting into 10X concentration, which was then added to the TSE buffer (1 mL Protease Inhibitor added to 9 mL TSE buffer).

Freshly sub-cultured bacterial colony of 24 hours had been inoculated into 5 mL LB (Luria-Bertani) broth in a test tube and incubated for 3 hours. 1ml from this bacterial suspension had been taken into the 100 mL fresh LB broth in a conical flask and incubated for 20-22 hours. The protocols were performed at an optical density of 1.0 at 600 nm. All the solutions in this protocol were prepared using distilled de-ionized water and reagent grade chemicals, followed by proper filtration and storage at 4°C. The procedures described below had been carried out on ice unless otherwise specified.

1) Bacterial cells had been harvested from the 100 mL suspension by centrifugation at 3,000×*g* (RCF) for 20 min at 4°C.
2) The supernatant was discarded and the last few drops of liquid were carefully removed with a pipette.
3) The pellet had been gently resuspended (using a wireloop) into 2 mL of the mixed solution of TSE buffer and Protease Inhibitor Cocktail (10X).
4) The cells were then incubated in TSE buffer on ice for 30 min.
5) The cell suspension was transferred to a microcentrifuge tube and centrifuged at 16,000×*g* (maximal speed) for 30 min at 4°C.
6) The supernatant was then transferred to a new microcentrifuge tube. This supernatant constitutes the envelope extract.
7) Since the separation of outer membrane proteins from periplasmic proteins is desired, this supernatant is centrifuged at 26,000×*g* for 1 hour at 4°C. The pellet from this step contains the outer membrane proteins; the supernatant contains the periplasmic proteins.
8) The supernatant and pellet is kept in two different micro centrifuge tubes and stored at -20°C. The pellet was used as the loading sample in SDS-PAGE run.

2.5.2. Estimation of protein concentration

For this study, NanoDrop method was used to determine the protein concentration. Thermo Scientific NanoDrop 2000c apparatus was used in this process. These were the first dual-mode

UV-Vis spectrophotometers and fluorospectrometers designed specifically for the life science market and have fundamentally changed DNA, RNA and protein analysis with microvolume and cuvette capability for protein quantification.

When researchers work with protein, they need instrumentation that can accommodate both purified protein as well as a variety of protein assays. NanoDrop instruments have specific protocols and modules designed to facilitate the protein quantification process.

It has a wide spectral range (190-840nm) for measuring a variety of sample types. Purified protein is measured at an absorbance of 280nm (A280). In this technique, no dilution is required even for highly concentrated samples as patented sample retention system automatically optimizes path length to accommodate low and high concentrations. Only around 2 μL of the protein sample is required. It calculates the sample purity ratios at 260/280 nm.

While the most logical blanking solution is generally the buffer/solvent at the same pH and ionic strength as that of the unknown protein samples, it is important that the blanking solution have minimal absorbance at the wavelengths of interest. Although the operating software displays a flat baseline when a buffer blank is measured, the spectrometer still detects the absorbance of the buffer solution when measuring an unknown sample.

2.5.3. Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE): Analysis of the Outer Membrane Proteins of *E.coli* CD-17 isolate

Polyacrylamide Gel Electrophoresis (PAGE) is used for separating proteins ranging in size from 5 to 2,000 kDa and other biological macromolecules (nucleic acids) according to their electrophoretic mobility, due to the uniform pore size provided by the polyacrylamide gel. Pore size is controlled by the concentrations of acrylamide and bis-acrylamide powder used in creating a gel. Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides. 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.

Preparation requires casting two different layers of acrylamide between glass plates. The lower layer (separating, or resolving gel) is responsible for actually separating polypeptides by size. Separating gels are usually made in 6%, 8%, 10%, 12% or 15%. The upper layer (stacking gel) includes the sample wells. It is designed to sweep up proteins in a sample between two moving boundaries so that they are compressed (stacked) into micrometer thin layers when they reach the separating gel. 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 needs to be identified or probed in the sample. The smaller the known weight the higher the percentage should be used.

To determine the protein profile of the outer/surface materials from Shiga-Toxin producing *E.coli* bovine isolate (CD-17), they were subjected to SDS-PAGE analysis, followed by Coomassie Blue Staining.

2.5.3.1. Preparation of the Separating/ Resolving/ Running Gel

1) Fresh glass plates were assembled in the gel casting chamber of the Bio-Rad Mini-Protean II Electrophoresis System 125BR apparatus of SDS-PAGE.
2) A 12.5% separating gel was made by gently mixing distilled water, Tris HCl (pH 8.8) along with 10% SDS and 30% acrylamide bis acrylamide solution according to the table given below. These mixtures were mixed well and degassed.

Table 2.2: Preparation of the separating gel (12.5%)

Composition	Amount
Distilled water	1.60 mL
1.5M Tris HCl (pH 8.8) (lower gel buffer)	1.25 mL
30% Acrylamide-bis acrylamide solution	2.10 mL
10% SDS	0.10 mL
10% Ammonium per sulfate (APS)	30 μL
TEMED (lastly added)	8.5 μL

3) This step was followed by rapid addition of the 10% freshly prepared Ammonium per sulfate (APS) and N, N, N', N' Tetramethyl- ethyledene amine (TEMED).
4) As soon as APS and TEMED were added to the mixture, the freshly mixed solution was carefully poured into the glass plate chamber with a pipette. The gel mixture was poured to a level of about 3 cm below the top edge of the glass plates. Precaution was taken during pouring the gel mixture to avoid bubble formation (Jahan *et al.,* 2014).
5) It was then carefully over layered by water saturated iso-butanol to avoid gel drying as it may result in cracking and uneven surface of the gel.
6) It was then left undisturbed for sometime to allow polymerization of the gel. A very sharp liquid-gel interface was visible with naked eye when the polymerization was completed.
7) The butanol overlying the gel was poured out and any unpolymerized acrylamide were rinsed from the gel plates and extra moisture was removed with a piece of 1 mm Whatman filter paper.

2.5.3.2. Preparation of the Stacking Gel

After polymerization of the separating gel, a 5% stacking gel mixture was prepared.

1) Just like the separating gel, stacking gel mixture was also prepared by primarily mixing the distilled water, Tris HCl (pH 6.8) along with 10% SDS and acrylamide bis acrylamide solution according to the table given below.

Table 2.3: Preparation of the stacking gel (5%)

Composition	Amount
Distilled water	2.137 mL
0.5M Tris HCl (pH 6.8) (upper gel buffer)	937 µL
30% Acrylamide-bis acrylamide solution	625 µL
10% SDS	37 µL
10% Ammonium per sulfate (APS)	22 µL
TEMED	8.5 µL

2) After this step 10% ammonium per sulfate and TEMED was added promptly.
3) The gel mixture was then rapidly poured over the previously made separating gel.
4) A 10 well comb was then immediately pressed between the two glass plates carefully so that no bubbles form inside the comb channels.
5) The gel was then allowed to sit undisturbed for polymerization.

2.5.3.3. Sample Preparation

1) The bacterial protein sample (which was previously extracted) needs to be thawed just before loading.
2) The protein sample was mixed with 2X Sample Buffer at a ratio of 1:1 and was then heated in boiled water bath (100ºC) for 3 mins. During boiling, precaution was taken so that the sample does not spill out. 2X Sample Buffer compositions includes: 0.5M Tris HCl (pH 6.8), 10% SDS, 2-mercaptoethanol, glycerol and distilled water, which is normally stored at 4°C.

3) 5 μl tracking dye (0.1% Bromophenol Blue, BPB) was then added to the boiled mixture. 0.1% BPB dye composition includes: 50% glycerol, BPB dye powder and distilled water, which is stored at 4°C.

2.5.3.4. Sample Loading

1) After polymerization of the stacking gel, it is ready for sample loading.
2) Before loading the sample, the comb was removed from the glass chamber very gently so that the well dividers do not crack.
3) The glass chamber was then fixed in the electrophoresis unit and was placed in the buffer reservoir.
4) The wells were filled with the TGS running buffer by pouring running buffer inside two glass chambers of the electrophoresis unit up to the top mark of the apparatus, which is $1/3^{rd}$ of the height of the reservoir. Composition of TGS Running Buffer includes: Tris base, glycine, 10% SDS, and distilled water. pH of this buffer needs to be adjusted to 8.3 (8.1-8.5), which is stored at 4°C and not used more than 8 times.
5) 40 μL of sample mixture was then loaded to each well using a micropipette.
6) The first column from left was loaded with 8 μL of Molecular weight standard (PageRuler™ Plus Prestained Protein Ladder, ThermoFisher Scientific, Catalogue number# 26619) protein marker.

2.5.3.5. Running the Gel

1) After loading the sample(s) in the wells, the apparatus was connected to the Power Pack (NanoPAC-300) adjusting at 18 mA current supply, keeping the voltage supply free.
2) The power supply was kept on for around 1hour 20 mins.
3) As soon as the tracking dye reached the bottom level of the gel, the power supply was turned off. Gel run is considered to be complete at this stage.

2.5.3.6. Staining and Destaining of the Gel

1) After gel run had been completed, the gel was carefully released from the glass plates and immersed in a fresh staining solution (0.1% Coommassie Brilliant blue R250). Coomassie blue stain strongly binds to all proteins. The amount of bound dye is proportional to protein content.
2) The gel was then incubated in the water bath at 55ºC for 1 hour.
3) The gel was taken out of the staining solution and poured into a container containing destaining solution (7% Acetic acid in dH_2O) and again placed in the water bath at 55ºC for 30 mins.
4) When the gel background became transparent, it was taken out of the destaining solution and immersed in distilled water.

Hence, the protein bands were stained successfully and became visible at this stage to confirm the presence of desired protein bands of the sample (Jahan *et al.,* 2014).

2.5.4. Determination of the Molecular Weight of the Protein

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

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

1) The negative values of Log-10 (R_f) value were plotted (y-axis) against the known molecular weight (x-axis) in a graph paper.
2) A calibration curve was prepared by using the molecular weight standards.
3) The molecular weights of the immunogenic proteins were estimated from linear calibration curve.

2.5.5. Western Blot Analysis to determine Immunogenicity

Western blotting, also known as immunoblotting or protein blotting is a core technique in cell and molecular biology, which is used to separate and detect the presence of a specific protein in a complex mixture extracted from cells. In this technique, a mixture of proteins is separated based on molecular weight and type through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with labeled antibodies which are specific to the protein of interest. The Western blotting procedure relies upon three key elements to accomplish this task: (i) the separation of protein mixtures by size using gel electrophoresis (SDS-PAGE); (ii) the efficient transfer of separated proteins to a solid support/membrane (Blotting); (iii) the specific detection of a target protein by appropriately matched primary and secondary antibodies (Mahmood *et al*., 2012).

Western Blot technique (Towbin *et al*., 1979) was applied in this case study to check the immunological profile of the extracted protein sample/ antigen (outer membrane protein) from the working bacterial strain (STEC bovine isolate CD-17) against the previously collected healthy human sera (of Bangladeshi people) serving as the primary antibodies. Confirming the presence of this specific 45 kDa protein band as an immunogen through Western Blotting, this protein was isolated and used to serve as the immunogenic dose to animal models (mice), which was further challenged to prove its vaccine efficacy.

2.5.5.1. Relocation/ Transfer of the Protein from Gel to Nitrocellulose Membrane

1) The polyacrylamide gel was prepared according to the procedure described in the previous section (SDS-PAGE analysis, Section 2.5.2). In this case, a one-well comb was used during gel preparation in order to obtain a long band of the same protein in the gel, which would be transferred to the membrane.
2) Now instead of staining-destaining the gel, it was carefully taken into a container and washed with distilled water.

3) Each component/material of the transferring unit was soaked in the transfer buffer (Towbin buffer) before assembly in the Bio-Rad Trans-Blot Electrophoretic Transfer Cell apparatus. The sandwich components were assembled in the following order:

Red side of the cassette (facing positive electrode) → Sponge (smooth surface inside) → 3 pieces of Whatman blotting paper → Nitrocellulose membrane → Gel → 3 pieces of Whatman blottion paper → Sponge (smooth surface in side) → Black side of the cassette (facing negative electrode).

4) The cassette was then arranged and placed in the proper orientation and the apparatus was filled with the transfer buffer (Towbin buffer) to a height that covered the electrode panels. Composition of Towbin buffer (transfer buffer) includes: 20mM Tris-base, 150mM glycine, 15% methanol and 0.02% SDS. pH of this buffer needs to be within 8.1-8.5 and stored at 4ºC.
5) The power supply was then connected with the power pack and adjusted at a constant 25 Volt, 400mA overnight (20-22 hours).

2.5.5.2. Immuno Detection of Antigens

1) After overnight transfer, the Nitrocellulose membrane was removed from the cassette by using a clean forcep and was stained in the Ponceau stain for 3 mins with moderate shaking in the hand. Composition of Ponceau stain (0.1%) includes: Ponceau stain, acetic acid and distilled water. This staining solution is stored at 4°C inside a bottle wrapped with aluminium foil.
2) After rinsing with a little deionized water, sharp bands of protein were revealed due to a successful protein transfer. The most clear and distinct band came in between the ladder's 36kDa and 55kDa, which is the desired band 45kDa.
3) The whole nitrocellulose membrane was then cut into three sections, with two thin strips at two extremes of the membrane (in order to carry on with the steps of Western Blotting) and a large section at the middle (in order to match with the specific 45kDa protein band at the end of Western Blotting).

4) The molecular weight (45kDa) of the distinct protein band was labeled in the large middle section of the membrane and was kept undisturbed for the calibration of the molecular weight of the proteins that are immunogenic.

5) The two thin strips were marked and transferred to a plastic box and incubated for at least 1 hour in 2% skim milk (blocking solution) on a rotary shaker for blocking the protein.

6) The strips were then shaken at room temperature for 1.5 hours in a solution of primary (1°) antibody (previously collected healthy human sera) diluted at 1:80 in 2% skim milk on a rotary shaker.

7) After that, the strips were washed three times with 0.1% Tween-20 in PBS for 5 min each, followed by washing with PBS only for 1 min.

8) Both the strips were then incubated in a solution of secondary (2°) antibody (anti-human antibody/ Anti-human polyvalent immunoglobulin, Alkaline Phosphatase conjugate Sigma A-3313). The antibody (AP-conjugate) was diluted at 1:5000 in 2% skim milk and was shaken at room temperature for 1.5 hours.

9) Then the strips were washed once more as the way it was previously done at step **7).**

10) Before the last wash, the corresponding specific substrate of the enzyme conjugate (2° AP-conjugate Antibody) was prepared. Substrate composition includes: Fast Red TR, Naphthol AS-MX Phosphate and 50mM Tris-HCl (pH: 9.14).

11) The strips were added to the substrate solution and shaken gently in a dark area/surrounding for the appearance of the antigenic (immunogenic) bands on the membrane (red colored sharp bands).

12) The strips were then dried and compared to the labeled large middle section of the nitrocellulose membrane. This membrane strips can be stored at -20ºC.

2.5.6. Separation of the 45-kDa Surface Protein from the Nitrocellulose membrane

A 12.5% separating (resolving) gel was prepared with the combination of a 5% stacking gel **(Section 2.5.3.1 and 2.5.3.2**). Approximately 400µL of antigen (protein sample) was loaded to each well in the gel along with 8µL Protein Marker to run the SDS-PAGE since a one-well comb was used in this case to obtain a long band of the same protein in the gel, which would be transferred to the membrane. The separated proteins (bands) in the gel were then transferred to

Nitrocellulose membrane (**Section 2.5.5.1**). This process was repeated for about 20 times. Protein bands in the membrane were visualized by Ponceau staining. By cutting the membrane into three sections, two thin strips were undergone the entire process of Western Blotting to detect and confirm the immunogenic protein band (45kDa). The large middle section of the membrane that was carefully labeled specifically at 45kDa was matched and calibrated with the protein marker and the two thin strips containing the visible red band (indicating immunogenicity). This band of interest (45kDa), as determined by the molecular weight marker, was spliced out/ excised carefully with a sharp end scissor into a long horizontal strip. The strip was then cut into smaller pieces and placed in a micro centrifuge tube containing 0.2-0.5 mL of elution buffer or Phosphate Buffer Saline (PBS). This was finally stored at -20ºC and later, sonicated into a powdered form (appropriate as injectables).

2.5.7. Sonication

Sonication is the act of applying sound waves and energy to agitate particles in a sample, for various purposes like for mixing solutions, speeding the dissolution of a solid into a liquid by shearing it into smaller fragments, etc. Ultrasonic frequencies (>20 kHz) are usually used, leading to the process also known as ultrasonication. These vibrations can disrupt molecular interactions, break clumps of particles apart and lead to mixing. In the laboratory, it is usually applied using an ultrasonic bath or an ultrasonic probe, known as a sonicator.

In this study, Omni-Ruptor 4000 Sonicator had been used to sonicate the spliced out small pieces of protein-bound nitrocellulose membranes into a powdery solution with PBS. This liquid solution (suspension) of the desired protein (45kDa) serves as appropriate vaccinating injectable into the mice models.

2.5.8. Collection of Mice (Animal Model)

In the present study, mice model system was used to investigate the immunogenicity and protective efficacy of the 45kDa bacterial outer membrane/surface protein. Twenty-eight Swiss Albino mice (4-5weeks old) were collected from the animal resources of International Center for Diarrhoeal Diseases Research, Bangladesh (ICDDR, B).

2.5.9. Immunization of Mice with Isolated Protein

Twenty-eight Swiss Albino mice had been divided into two groups, each containing fourteen mice. One group had been named "Control", which was not immunized by the 45kDa protein, but only injected with the fresh sonicated nitrocellulose membrane suspension (in PBS). The other group had been named "Experimental", which was immunized with the protein suspension (45kDa) isolated through the previous sections of this study. To demonstrate the protective efficacy of the 45kDa (outer membrane protein) in mice, they were immunized three times through Intra-muscular (IM) injection on Day 0, Day 21, and Day 35. Intra-muscular injection had been applied alternatively in the left thigh of all mice in the first dose, then right thigh in the second dose and again left thigh in the final (third) dose. Concentration of injected protein had been increased in each dose, but a volume of 20µL injectable (for each mouse) was kept constant in all the doses.

In the previous studies, different investigators have demonstrated good immunization with a protein dose of 50µg/kg body weight of the animal model. In this case study, each mouse weighed 20gm and so, 1µg of protein had been injected in the first dose (1.5µL protein suspension+18.5µL PBS). In the second dose 1.5µg protein (2.25µL protein suspension+17.75µL PBS) and finally in the third dose 3.35µg protein (5µL protein suspension+15µL PBS) had been injected in each mouse. Three weeks after the completion of these three doses, a confirmatory western blotting analysis had been performed with mice sera to test the development of the 45kDa protein immunization in the experimental mice.

2.5.10. Confirmation of vaccine development in animal models

A further SDS-PAGE protocol (as discussed in **Section 2.5.3**) had been undertaken with the sample protein (OMP of STEC CD-17 strain) by executing a one-well gel run, which was transferred to an irreversible nitrocellulose membrane by overnight blotting. Western Blotting (as discussed in **Section 2.5.5**) was then finally performed to confirm the presence of 45kDa protein immunization in the "Experimental" group of mice models.

Blood had been collected from both the group of mice (four mice from each group) through cardiac puncture (Shimizu, 2004). Serum (from mice blood) was isolated by allowing the blood to clot for 1-2 hours at room temperature and then separated by centrifugation into a

fresh micro centrifuge tube. This mice serum had been used as the primary (1°) antibody (instead of human sera as previously done) in a dilution ratio of 1:50 and the rest of the reagents, secondary antibody, substrates and the entire protocol were followed likewise to **Section 2.5.5** in this study.

2.5.11. Challenging the mice with live *E.coli* O157:H7 strain

A week after it is confirmed that the 45kDa protein immunization has been successfully developed in the "Experimental" group of mice, both the "Experimental" and "Control" groups (10+10 mice respectively) were challenged with the Shiga Toxin producing *E. coli* O157:H7 bacterial strain, which is a highly virulent and pathogenic serotype. The bacterial challenging dose had been optimized by several trial runs and was established by comparing with the McFarland Standards to determine the lethal dose. The value of Lethal Dose (LD_{50}) for a substance is the dose required to kill half the members of a tested population after specified test duration. LD_{50} figures are used as a general indicator of a substance's acute toxicity.

In this case study, the efficient Lethal Dose (LD_{50}) was found to be 10^{10} cells/ml of *E.coli* O157:H7. A volume of 100µL of 10^{10} cells/ml was gently injected intra-peritoneally (IP) into the peritoneal/abdominal cavity of each mice. The consequences of these mice were observed for 60 days.

In this study, some mice were mated after their final (third) dose immunization and they produced 12 babies (First Generation mice, F-1). This step was executed with the objective of determining whether the 45kDa protein vaccine is efficient in its next generation as well. Those babies were not injected/ actively immunized by the 45kDa protein and were kept undisturbed in a different cage. Their blood was also collected through cardiac puncture (similarly to "Experimental" and "Control" groups), serum isolated and a confirmatory Western Blot was performed to determine any presence of immunogen (that might be inherited through passive immunity from their immunized mothers) exactly in the same way and conditions as the "Experimental" group of mice. When they were 4-5 weeks old, they had also been challenged with the same Lethal Dose (100µL of 10^{10} cells/ml) and were observed for 60 days as well.

Chapter 3

RESULTS

3. Results

3.1. Collection and Confirmation of *E.coli* isolate

In this study, STEC O157:H7 (CD-17), a bovine isolate was collected from Microbiology Laboratory of Dhaka University, which was isolated (in a previous study) from bovine feces. The isolates were further confirmed phenotypically by appropriate enrichment, selective plating in MacConkey and EMB medium, and biochemical tests in indole, citrate and TSI agar medium.

In MacConkey agar plates, colonies showed typical characteristics of *E.coli*. The round shaped colonies had a moderate size of 1-2mm with raised elevations, opacity and the characteristic deep pink color showing the lactose fermenting nature of *E.coli* [Fig. 3.1(a)]. In EMB plates, the isolates showed green metallic sheen growths [Fig. 3.1(b)]. Hence, the suspected isolates were confirmed to be *E.coli*.

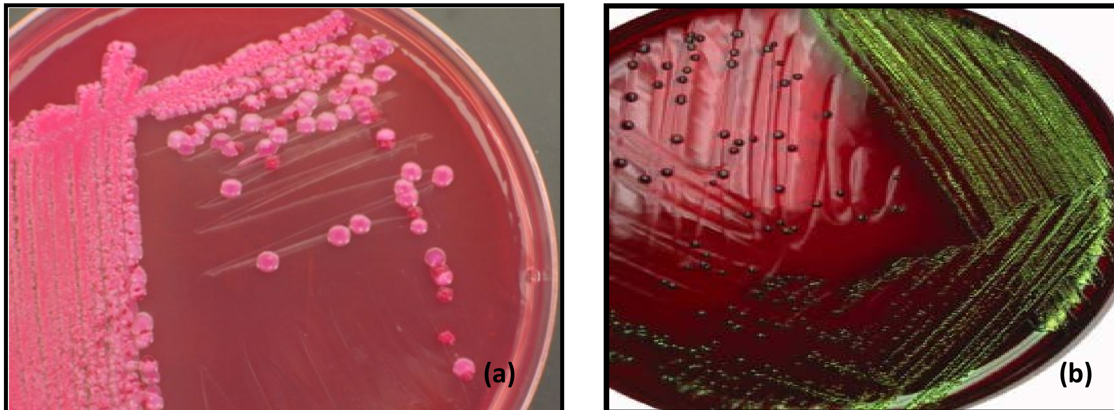


Fig. 3.1: (a) *E.coli* pink lactose fermenting colony growth in MacConkey agar plate. **(b)** *E.coli* metallic green sheen in EMB agar plate.

3.2. Reconfirmation by Biochemical Tests

For further confirmation, the isolates were subjected to three biochemical tests: Indole, Citrate and TSI agar tests, with appropriate reagents and reaction conditions. The results are shown in the following table (Table 3.1) and figures (Fig 3.2). A positive result in indole test is shown by the presence of a red or reddish-violet color in the surface layer of the broth, as in case of *E.coli* [Fig. 3.2(a)]. An organism turns the citrate medium into blue color if it is citrate positive (can

utilize citrate), and remains in its natural green color if it is citrate negative (cannot utilize citrate) as seen in *E.coli* [Fig. 3.2(b)].

In case of TSI test, bacteria that ferment any of the three sugars in the medium will produce byproducts. These byproducts are usually acids, which changes the color of the red pH-sensitive dye (phenol red) to a yellow color. Since *E.coli* ferments both lactose and glucose, it resulted in a yellow/yellow tube (yellow in both butt and slant) and remained that way due to the large amount of acid production in the reaction. It used thio-sulphate as an electron acceptor and reduced it to hydrogen gas, which accumulated as bubbles along the slant (between the agar and the glass). Hydrogen production also lifted the agar from the butt of the tube and fractured the agar [Fig. 3.2 (c)].

Table 3.1: Typical results of biochemical tests of *E. coli* isolates

Strains	**Indole production**	**Citrate Utilization**	**Triple Sugar Iron (TSI)**
STEC 0157:H7	Yes	No	Acid butt, acid slant, Gas production
STEC CD-17	Yes	No	Acid butt, acid slant, Gas production

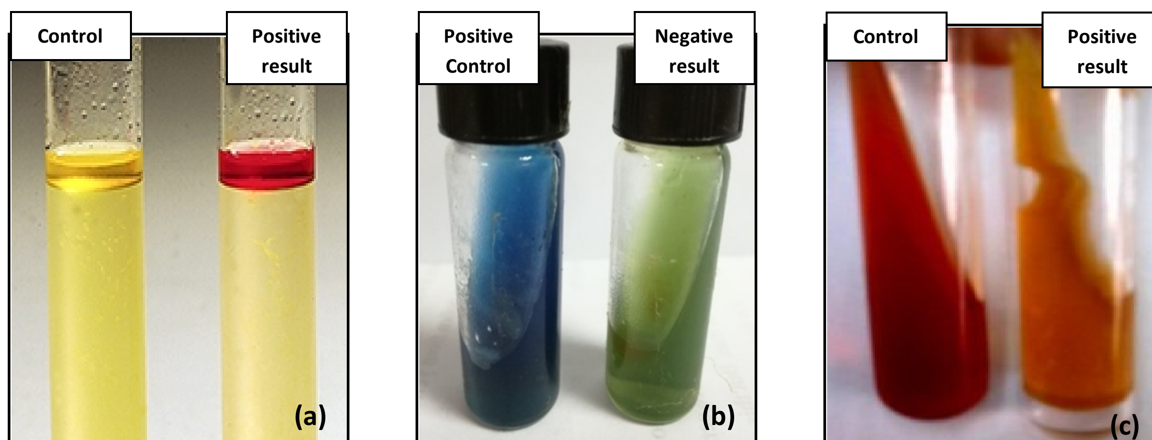


Fig. 3.2: **(a)** Indole test of *E.coli* showing positive result (at right side) and control (at left). **(b)** Citrate utilization test of *E.coli* showing negative result (at right) and positive control (at left). **(c)** TSI agar test of *E.coli* showing positive result by producing acid butt and slant, along with gas production that shifts the butt upward and cracks the medium (at right) and control (at left).

3.3. Extraction of Desired Protein (Outer Membrane Protein) by TSE method

In previous studies, it was found that other periplasm extraction methods (presumably optimized for maximal recovery of recombinant proteins) also release a substantial amount of cytoplasmic proteins. For various strains (including those that over-express various proteins), TSE method was found to generate the cleanest envelope pattern of all methods tested. TSE stands for Tris, Sucrose and EDTA, which are the components of the extraction buffer. Sucrose is a well-known protein stabilizer, protecting the released proteins. EDTA facilitates periplasmic extraction by chelating divalent ions, which normally stabilize the lipopolysaccharide (LPS) in the outer membrane, resulting in LPS release and increased permeability of the outer membrane. This method is a modified version of Oliver and Beckwith which consistently produces the cleanest extract of periplasmic and outer membrane proteins. Since envelope proteins constitute only about 4–16% of the proteome, a clean extract is expected to produce a distinctive band pattern with very little overlap with that generated by whole cell extraction (Quan *et al.,* 2013).

In this simple TSE extraction procedure, cells are first pelleted to remove the media, resuspended in a concentrated solution of sucrose in Tris buffer supplemented with EDTA, incubated for 30 min on ice, and then re-centrifuged. The supernatant is the TSE periplasm and pellet is the outer

membrane extract. The centrifugation steps act to efficiently separate the soluble envelope proteins from other cellular components. The outer membrane proteins can be isolated by an additional ultra-centrifugation step. This method is an effective way to extract soluble envelope proteins and additionally is less labor intensive than many other methods.

TSE method had been carried out several times (10-12 times) to obtain this isolated, purified protein of the working strain STEC O157:H7 (bovine isolate CD-17). The concentration of protein was then quantified by NanoDrop method at an absorbance of 280 nm (Table 3.2). The protein concentration of the sample protein (outer membrane protein of the bacterial strain) was determined to be 7.617 mg/ml.

Table 3.2: Protein quantification of STEC CD-17 outer membrane protein (mg/ ml)

Sample strain	Protein (mg/ml)	Absorbance(nm)	Purity ratio (260/280)	Sample Type
E.coli O157:H7 (CD-17 bovine isolate)	7.617 mg/ml	280nm	1.59	1Abs = 1mg/ml

3.4. SDS-PAGE Analysis of the Extracted Protein

The protein (OMP) profile of STEC O157:H7 (CD-17 bovine isolate) was determined by SDS-PAGE analysis. Distinct protein-bands that were desired, was obtained from the SDS-PAGE analysis, which were visible in the gel after appropriate staining-destaining steps as shown in Fig 3.3. The protein bands were measured by comparing with the corresponding known molecular weight of pre-stained protein marker (Fig. 3.4).

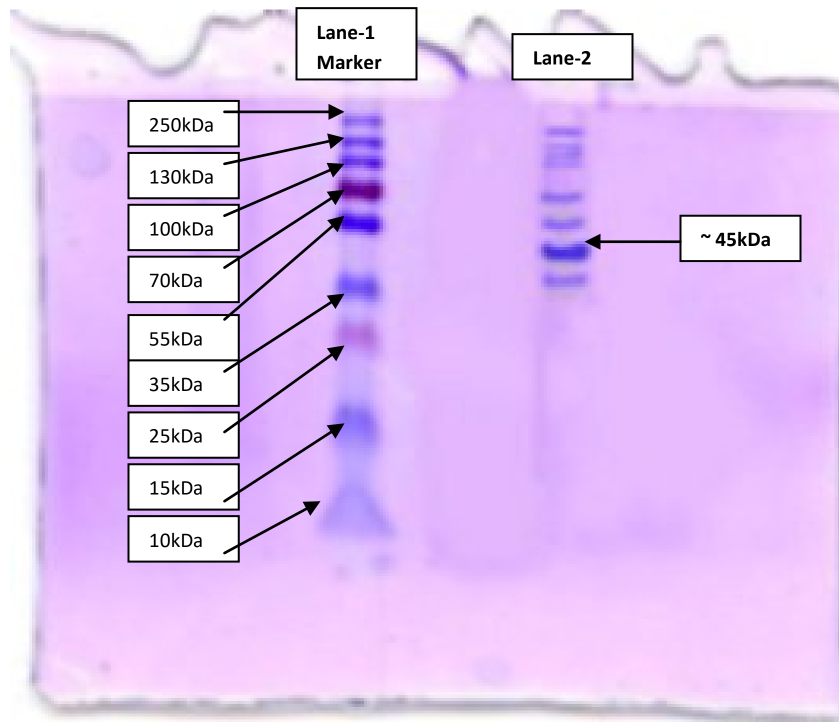


Fig. 3.3: Stained gel of SDS-PAGE analysis of *E.coli* outer membrane protein. Lane-1 is the Protein Ladder/Marker and Lane 2 is STEC CD-17 strain's OMP. The most distinctly sharp band was found at ~45kDa (between 55kDa and 35kDa bands of the Protein Marker).

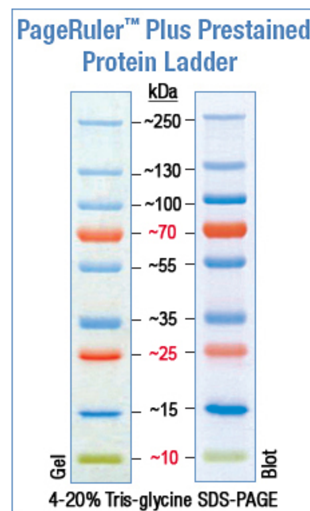


Fig. 3.4: Known molecular weight of Prestained Protein Marker.

3.5. Molecular Weight of the Desired Protein

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

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

Here, the most distinct band was seen to have formed at a distance of 42mm (from initial point) out of a total distance migration of 171mm (complete gel run distance).

$$R_f = 42\text{mm}/\ 171\text{mm} = 0.2456$$

$$= 24.56$$

So, the linear calibrated curve that intersects the standard graph meets at ~45kDa in the x-axis. Hence, the molecular weight of the immunogenic protein band that was most distinct was determined from the scaled graph to be ~45kDa.

3.6. Western Blot Analysis proving Immunogenicity

Initially, western blotting was performed to confirm the presence of 45kDa proteins in the mass population of Bangladeshi peoples' healthy sera, as hypothetically concluded in a previous study (unpublished data). When it was determined, these protein bands on the irreversible nitrocellulose membrane (transferred/relocated by overnight blotting from the SDS-PAGE gel to the membrane) was excised and spliced out using a scissor. The long strips of membrane containing 45kDa protein bands were cut into smaller pieces and sonicated with PBS solution. This pure protein solution/suspension was used to immunize one set of mice (experimental group) in three doses. Similarly, a control group of mice had been injected with only fresh sonicated nitrocellulose membrane. After completion of these three immunization doses, some

mice from the set of experimental group were mated and mice babies were born, which were named as First Generation mice (F-1) group. The baby mice were not injected anything.

After three weeks of the final immunization dose, a confirmatory western blotting analysis had been performed using mice sera of the experimental, control and F-1 group of mice. It was done to check the development of the 45kDa protein immunization in the experimental mice and also whether 45kDa is present in their F-1 (Generation-1) group of mice (even though these mice babies were not actively immunized). A prominent antigenic band was obtained on the nitrocellulose membrane that had been treated with mice sera of the experimental group (immunized by 45kDa OMP for 5 weeks) and the F-1 group (First Generation mice). Molecular weight of the distinct antigenic band was found to be 45kDa, as confirmed by comparing with the molecular weight standards (protein ladder).

Presence of 45kDa protein band indicated the production of adequate amounts of anti-45 kDa antibodies in both the sets of mice (experimental and F-1), as shown in Fig. 3.5. However, the control sera collected from mice injected with only sonicated nitrocellulose membrane showed no antibodies against the 45 kDa OMP, as demonstrated by the absence of any protein bands in the western blotting result (as shown in Fig. 3.5).

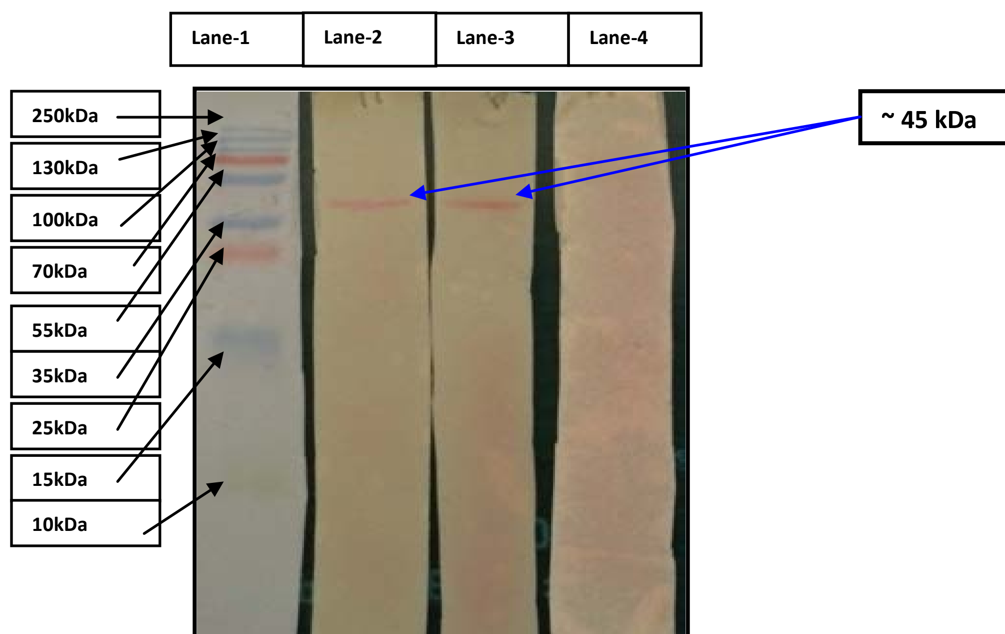


Fig. 3.5: Western Blotting analysis showing distinct bands at ~45kDa in both the nitrocellulose membrane strips (Lane-2 and Lane-3) of F-1 (First Generation) mice sera and experimental mice sera respectively. No bands were observed in the membrane strip of control mice sera (Lane-4). Lane-1 shows the Protein Marker bands with known molecular weights.

3.7. Final Challenge Result

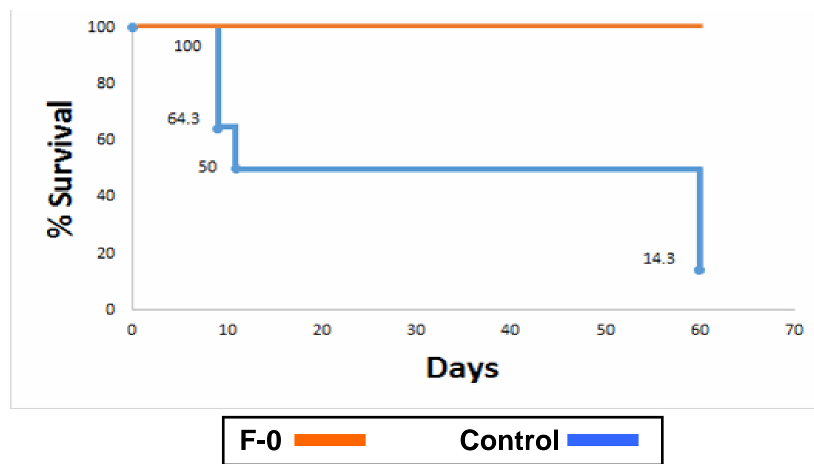
All the three sets of mice (control, experimental and F-1) were challenged by the most pathogenic and infective strain of *E. coli* O157:H7. 100µL of 10^{10} cells/ml was gently injected intra-peritoneally (IP) into the peritoneal/abdominal cavity of each mice.

Within 9 days, 35.7% and within 11 days, 50% mice of the control set successfully died. This refers to the fact that after 9 days, 64.3% and after 11 days, 50% mice of the control set were surviving respectively. Only 14.3% mice could survive till 60 days, with gradual development of weakness, slowed movement and paralysis. This clearly demonstrates how extremely infectious, virulent and lethal the challenging dose of *E.coli* O157:H7 was, which could efficiently kill 85.7% non-immunized mice of the control set.

100% mice of both the experimental set (F-0) and First Generation mice (F-1) set could survive till 60 days successfully. They had been observed to be fully healthy, in proper growth and

movement even after 60 days. The graphs below are [Fig. 3.6 (a) and Fig. 3.6 (b)] showing the percentage of survival of mice models against the time period, of experimental set (F-0) and first generation set (F-1) against the control set respectively. This result not only proves the 45kDa vaccine efficacy in the immunized set of mice, but also establishes the fact that the vaccine would be equally efficient even in its next generation, as it can be successfully inherited by the off-springs (F-1) from their mothers (F-0) through passive immunization. Hence, antibody response against the 45kDa protein was evaluated, that evidently indicates immunogenicity of the 45kDa *E.coli* O157:H7 outer membrane protein in mice model.

(a)



(b)

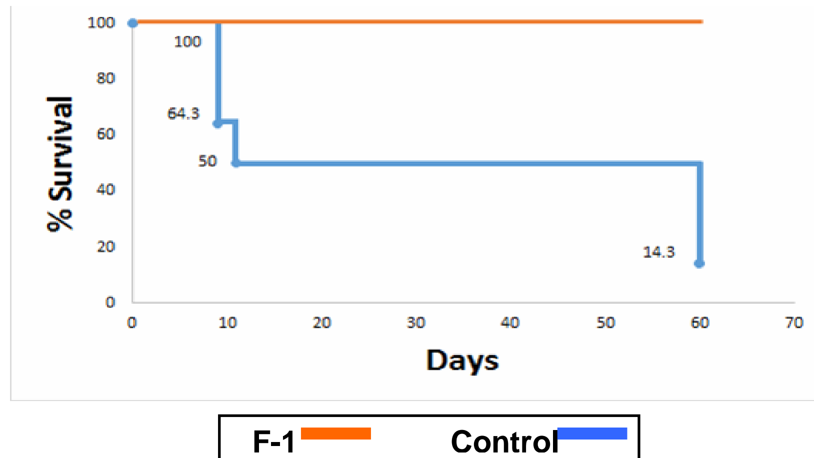


Fig. 3.6: The graphs are showing the percentage rates of survival of the mice models of **(a)** Experimental set (F-0) and Control set, and **(b)** First Generation set (F-1) and Control set, against the time period of 60 days after being challenged with the Lethal Dose of the most virulent pathogen, *E.coli* O157:H7.

The *E.coli* (STEC O157:H7 bovine isolate CD-17) 45 kDa OMP has been found to be a unique surface protein present in many of the predominant serotypes of the *E.coli* (unpublished data). The immunogenicity of the 45 kDa surface protein in mice model indicates a high titer antibody production in the experimental mice. These mice sera may serve as good source of antibodies to be used for immunological diagnosis of the *E.coli*, irrespective of the serotype distribution. However, further characterization of the 45 kDa surface protein is necessary to confirm other functions of this protein.

Since this 45kDa protein has been already confirmed to be existing in the healthy human sera of mass population of Bangladesh, this protein vaccine would serve its purpose more successfully in the developed countries where *E.coli* O157:H7 is still a life-threatening pathogen that has the ability to cause severe health hazards, leading to death. Traveler's diarrhoea, which is a very common problem world-wide that causes dangerous health issues to foreigners, can be hopefully mitigated by the use of this vaccine.

Several bacterial infections (including cholecystitis, urinary tract infection (UTI), bacteremia, cholangitis), neonatal meningitis, renal failure, Hemolytic Uremic Syndrome (HUS), edema, severe abdominal cramps, bloody diarrhoea and vomiting in human beings are mostly caused by the Shiga toxin producing *E.coli* O157:H7. These major health hazards can be hopefully resolved by the 45kDa protein vaccine (as discovered in this case study), which has proved to provide a successful immunogenicity against this lethal bacterial strain *E.coli* O157:H7 in the vaccinated models, that also continues to protect their babies (next generation) through genetic inheritance. Strategies to diminish burden of diarrhoeal disease and such severe diseases ultimately hold the greatest potential.

3.8. Conclusion

In this case study, mice models had been used, which were 4-5 weeks old. They had been injected with the 45kDa OMP of *E. coli* O157:H7 (CD-17 isolate) three times and were then finally challenged with the whole cell pathogenic strain of *E. coli* O157:H7 to see the vaccine

efficacy of 45kDa protein in the immunized mice. Successfully, all these mice could survive the infectious challenge, while 85.7% of non-immunized control mice died within 11 days due to toxic infection of the bacteria. This result doubtlessly proved the efficiency of this 45kDa OMP vaccine. However, the rest 14.3% of control set mice (non-immunized) that could survive through the infection raises a question. This unexpected survival might have been caused by the inaccuracy of dosing, injecting lesser amount of lethal dose that was insufficient to kill, or by mistakenly immunizing them along with the set of immunized mice while injecting 45kDa OMP doses. Although not certain, but they might have also been already exposed to *E. coli* O157:H7 beforehand (from the Animal House Farm) and hence developed immunity against it by themselves.

However, development of this 45kDa protein vaccine through this case study, builds a new insight in the field of immunology and vaccine development, which can be further studied for advanced modifications in order to combat deadly diseases by *E. coli* O157:H7 throughout the world.

Chapter 4

DISCUSSION

4. Discussion

Enterohemorrhagic *E. coli* (EHEC) are a group of zoonotic bacterial pathogens containing many serotypes that are responsible for outbreaks of bloody diarrhoea, leads to hazardous health conditions like Hemorrhagic Colitis (HC), and develop a life-threatening sequela of infection called Hemolytic Uremic Syndrome (HUS) in humans and neurological abnormalities which in severe cases can be fatal. These complications are attributed to Shiga toxins (Stx), shared with *Shigella dysenteriae* and acquired via horizontal gene transfer through phages. The most common cause of outbreaks and sporadic cases of bloody diarrhoea and HUS in the US are strains of the serotype O157:H7, which is a member of the larger category of Shiga toxin-producing *E. coli* (STEC). This group of *E. coli* is solely defined by its capacity to produce Shiga toxin type 1 (Stx1), Shiga toxin type 2 (Stx2), or both toxins (as well as variants of these) (Mohawk and Brien, 2010). The recent outbreak of severe infections with Shiga toxin (Stx) producing *E. coli* (STEC) serotype O157:H7 highlights the need to understand horizontal gene transfer among *E. coli* strains, identify novel virulence factors and elucidate their pathogenesis (Zhang *et al*., 2007).

E. coli O157:H7 have been implicated in several human outbreaks since their being established as food-borne pathogens in 1982. However, *E. coli* O157:H7 infection (in people and animals) can be traced back as early as the 1970's. It is responsible for an estimated 73,480cases of illness, 2,168 hospitalizations, and 61 deaths annually in USA, according to data published by Mead *et al.* in 1999 and Mohawk and Brien, 2010. *E. coli* O157:H7 is one of the most notorious and harmful foodborne pathogens, infecting an estimated 265,000 people in the U.S. each year, according to the U.S. Centers for Disease Control and Prevention (Andrews, 2015). Human disease ranges from self-limiting watery diarrhoea to debilitating bloody diarrhoea that can advance into often fatal, extraintestinal, secondary sequelae in susceptible patients. Cattle are the primary reservoirs for O157, with their recto-anal junction (RAJ) serving as the colonization site in which these human foodborne pathogens persist (Kudva *et al*., 2014).

Although it has been nearly 30 years since the discovery of *E. coli* O157:H7 as an enteric pathogen and despite the recent increase in the rate of severe disease associated with infection by the organism, no treatment yet exists. In general, antibiotic therapy is contraindicated as it may promote toxin expression from the lysogenized phage that typically carries *stx* genes.

Additionally, antimotility agents are not recommended as they can promote the sustained presence, and consequent toxin expression, of EHEC in the gastrointestinal tract (Mohawk and Brien, 2010). Unfortunately, despite the best control measures, *E. coli* O157:H7 remains a serious health concern.

A variety of treatment and prevention strategies to protect against *E. coli* O157:H7 is currently in development, which include toxin receptor analogs, passive antibody therapy, and vaccines to protect humans against the systemic effects of the toxin (Mohawk and Brien, 2010). In 2013, a study on the vaccines estimated that their use could reduce *E. coli* infections in humans by up to 83 percent (Andrews, 2015). There have been some promises when testing different ETEC candidate vaccines for protection against diarrhoea in adult travelers. However, no *E. coli* O157:H7 vaccines have yet been developed and licensed for immunization of humans and none of them demonstrated effectiveness against the most important target group (young children and elderly people) in endemic areas. Against this background, intense efforts are in progress to try to improve the immunogenicity of different available candidate vaccines, as well as to develop new types of ETEC vaccines (Svennerholm and Tobias, 2008).

The present study was carried out to observe the long term systemic immunogenicity of the STEC O157:H7 (bovine isolate CD-17) strain's 45 kDa outer membrane protein in the mice models. For this, outer membrane proteins of the *E.coli* were extracted by the TSE extraction procedure and later, this 45kDa outer membrane protein was isolated by the SDS-PAGE and Western blotting method.

Powerful electrophoretic techniques have been developed to separate macromolecules on the basis of molecular weight. The mobility of a molecule in an electric field is inversely proportional to molecular friction which is the result of its molecular size and shape, and directly proportional to the voltage and the charge of the molecule. Proteins could be resolved electrophoretically in a semi-solid matrix strictly on the basis of molecular weight if, at a set voltage, a way could be found to charge these molecules to the same degree and to the same sign. Under these conditions, the mobility of the molecules would be simply inversely proportional to their size.

In PAGE, proteins charged negatively by the binding of the anionic detergent SDS (sodium dodecyl sulfate) separate within a matrix of polyacrylamide gel in an electric field according to their molecular weights. Polyacrylamide is formed by the polymerization of the monomer molecule-acrylamide crosslinked by N,N'-methylene-bis-acrylamide (abbreviated BIS). Free radicals generated by ammonium persulfate (APS) and a catalyst acting as an oxygen scavenger (-N,N,N',N'-tetramethylethylene diamine [TEMED]) are required to start the polymerization since acrylamide and BIS are nonreactive by themselves or when mixed together.

The distinct advantage of acrylamide gel systems is that the initial concentrations of acrylamide and BIS control the hardness and degree of crosslinking of the gel. The hardness of a gel in turn controls the friction that macromolecules experience as they move through the gel in an electric field, and therefore affects the resolution of the components to be separated. Hard gels (12-20% acrylamide) retard the migration of large molecules more than they do small ones. In certain cases, high concentration acrylamide gels are so tight that they exclude large molecules from entering the gel but allow the migration and resolution of low molecular weight components of a complex mixture. Alternatively, in a loose gel (4-8% acrylamide), high molecular weight molecules migrate much farther down the gel and, in some instances, can move right out of the matrix. Care must be taken when creating this type of gel, as acrylamide is a potent neurotoxin in its liquid and powdered form.

Sodium dodecyl sulfate (SDS or sodium lauryl sulfate) is an anionic detergent which denatures proteins molecules without breaking peptide bonds. It binds strongly to all proteins and creates a very high and constant charge:mass ratio for all denatured proteins. After treatment with SDS, irrespective of their native charges, all proteins acquire a high negative charge.

Denaturation of proteins is performed by heating them in a buffer containing a soluble thiol reducing agent (e.g. 2-mercaptoethanol; dithiothreitol) and SDS. Mercaptoethanol reduces all disulfide bonds of cysteine residues to free sulfhydryl groups, and heating in SDS disrupts all intra- and intermolecular protein interactions. This treatment yields individual polypeptide chains which carry an excess negative charge induced by the binding of the detergent, and an identical charge:mass ratio. Thereafter, the denatured proteins can be resolved electrophoretically strictly on the basis of size in a buffered polyacrylamide gel which contains SDS and thiol

reducing agents. SDS-PAGE gel systems are exceedingly useful in analyzing and resolving complex protein mixtures. The mobility of polypeptides in SDS-PAGE gel systems is proportional to the inverse of the log of their molecular weights. This property makes it possible to measure the molecular weight of an unknown protein with an accuracy of +/- 5%, quickly, cheaply and reproducibly.

As current is applied, the proteins start to migrate downward through the stacking gel toward the positive pole, since they are negatively charged by the bound SDS. Since the stacking gel is very loose, low and average molecular weight proteins are not impeded in their migration and move much more quickly than in the running gel. The rapid migration of proteins through the stacking gel causes them to accumulate and stack as a very thin zone at the stacking gel/running gel boundary, and most importantly, since the 4-5% stacking gel affects the mobility of the large components only slightly, the stack is arranged in order of mobility of the proteins in the mixture. This stacking effect results in superior resolution within the running gel, where polypeptides enter and migrate much more slowly, according to their size and shape.

In all gel systems, a tracking dye (usually Bromophenol blue) is introduced with the protein sample to determine the time at which the operation should be stopped. Bromophenol blue is a small molecule which travels essentially unimpeded just behind the ion front moving down toward the bottom of the gel. Few protein molecules travel ahead of this tracking dye. When the dye front reaches the bottom of the running gel, the current is turned off to make sure that proteins do not electrophorese out of the gel into the buffer tank.

Nitrocellulose membranes are a popular matrix used in protein blotting because of their high protein-binding affinity, compatibility with a variety of detection methods (chemiluminescence, chromogenic, and fluorescence), and the ability to immobilize proteins, glycoproteins, or nucleic acids. Protein immobilizations are assumed to occur by hydrophobic interactions, and high salt and low methanol concentrations help improve protein immobilization to the membrane during electrophoretic transfer, especially for proteins with higher molecular weights. Nitrocellulose membranes are not optimal for electrophoretic transfer of nucleic acids, as the high salt concentrations that are required for efficient binding will effectively elute some or all of the charged nucleic acid fragments.

While both nitrocellulose and PVDF (Poly-Vinylidene Di-Fluoride) membranes are used for Western blotting and amino acid analysis, nitrocellulose is ideal in detecting low molecular weight proteins while PVDF is more suitable for detecting higher molecular weight proteins. Nitrocellulose membranes are chosen for fast binding and steady support. Nitrocellulose was one of the first membranes used for Western blotting and remains a popular choice, as protein binding to nitrocellulose is instantaneous, nearly irreversible and the membrane is easily hydrated. Nitrocellulose membranes are of high quality, 100% pure with high surface area and excellent uniformity. It provides superior binding capacity without background interference. They are convenient as they are available as ready-to-use pre-assembled membrane/filter paper sandwiches or several sizes of pre-cut sheets or economically priced rolls for cutting to any dimension. They provide high affinity protein binding, blocks easily, and exhibits very low background in chemiluminescent western blotting. They are compatible with all standard immunoblotting and nucleic acid detection methods.

Pure nitrocellulose membranes are the most frequently used membrane for the transfer of both protein and nucleic acids. It is a proven medium for western, northern, and southern blotting that produces excellent signal-to-noise results. A nitrocellulose membrane is easy to handle, wets readily with water-based solutions, and resists the brittleness usually associated with nitrocellulose. The 0.45um pore size is recommended for most analytical blotting, including protein, ssDNA, and RNA transfers.

In microbiology, McFarland standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range to standardize microbial testing. Original McFarland standards were mixing specified amounts of barium chloride and sulfuric acid together. Mixing the two compounds forms a barium sulfate precipitate, which causes turbidity in the solution. A 0.5 McFarland standard is prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2.2H_2O$), with 9.95 mL of 1% sulfuric acid (H_2SO_4). McFarland standards are prepared from suspensions of latex particles, which lengthen the shelf life and stability of the suspensions. The standard can be compared visually to a suspension of bacteria in sterile saline or nutrient broth. If the bacterial suspension is too turbid, it can be diluted with more diluent. If the suspension is not turbid enough, more bacteria can be added.

In this case study, McFarland Standard-1 had been used to compare the turbidity and prepare the bacterial suspension of challenging dose (Lethal Dose). McFarland Standard REF 70900 had been used, which interprets that the McFarland Standard-1 indicates the bacterial concentration of $3x10^8$ cells /ml, with the theoretical Optical Density of 0.25, at an absorbance of 550nm. Several trial runs had been performed on fresh mice models to evaluate which infectious dose serves as the most efficient and most sufficient lethal dose. Five trial sets of bacterial concentration (10^4, 10^5, 10^6, 10^7 and 10^8 cells/ml) had been injected into fresh mice models to determine the most effective lethal dose. It was observed that mice injected with $1x10^6$ cells/ml and above concentrations were dying earlier than those of lesser concentrations. The final dose of bacterial challenge had been $1x10^4$ times more concentrated than the trial run concentration.

One ml sized syringes (27GX½ in., 1.0ml, Terumo Company) had been used for the Intramuscular (IM) and Intraperitoneal (IP) injections. When a small volume (less than 1.0 ml) is administered, an insulin syringe is convenient. Because of the risk of embolism, air bubbles in fluid, syringe and needle must be purged out (Shimizu, 2004). IM injections were given at the right or left thigh muscles of the mice, while the IP injections were given at the lower left/right quadrant of the mice body.

The use of human subjects to investigate the steps required for *E. coli* O157:H7 vaccine to evoke intestinal pathology is considered unethical because of the possibility that a volunteer could develop HUS. Thus, numerous *in vitro* assays and animal models have been developed in an attempt to mimic various aspects of *E. coli* O157:H7 disease in humans. Many animal models have been developed to facilitate study of EHEC pathogenesis *in vivo* as well.

Mouse models in particular offer a number of benefits that includes low relative costs for purchase and maintenance, ease of care and handling, ready availability of numerous immunological reagents, variations in genetic backgrounds among inbred mouse strains as well as access to transgenic and recombinant inbred animals, and, very importantly, the feasibility of using sufficient numbers of animals in a single study to perform meaningful statistical analyses on the resultant data. In general, these animal models exist in two varieties: those solely focused on the effects of Stx (in the absence of bacteria) and those that explore *E. coli* O157:H7 infection. Models that evaluate toxicity rely on injection of Stx (with or without LPS) and often measure mortality as the endpoint of the investigation. Such *in vivo* assays have been used to explore

differences in relative toxicity among Stx toxin types to assess the protective capacity of some factor (Mohawk and Brien, 2010).

Successful development of the 45 kDa protein vaccine through this case study highlights the necessity of some further advanced researches to characterize this protein. The mechanism associated with the immunogenic effect of the 45 kDa protein can be investigated. There might be other functions of this protein which can be examined. In future researches, measures should be taken to conduct and maintain more purification steps after protein extraction to avoid any kind of contaminations in the protein sample. Since vaccines are delicate substances and are usually incorporated into the host organisms directly, proper aseptic conditions are obligatory to maintain. Storing, transporting and administering vaccines in proper conditions are some of the biggest logistical challenges which should be resolved. Larger sample size gives more significant graphs and more reliable results. So, in future investigations, analyses with a larger sample size can be done to obtain better results.

Chapter 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°C for 15 min.

1. Mac Conkey 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. Peptone water

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

6. T_1N_1soft agar

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

7. Luria Bertani broth

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

8. 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

Reagents and Buffers

1. Kovac's reagent

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

2. Methyl red reagent

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

3. Barritt's reagent

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

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

4. Phosphate buffered saline (PBS)

PBS was prepared by dissolving 8.0 gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na_2HPO4 and 2.0 gm of KH_2PO4 in 800 ml of distilled water. The pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 liter by distilled water. The solution was sterilized by autoclaving and was stored at room temperature.

5. 0.5 M EDTA

18.61 gm of $Na_2EDTA.2H_2O$ (di-sodium ethylene diamine tetra-acetic acid) was dissolved in 80 ml of distilled water and the pH was adjusted to 8.0 with pellets of NaOH. The final volume was made up to 100 ml with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

6. Gel loading buffer

10 x concentrated loading buffer consisted of 800µl of 20% Ficoll 400, 400µl of 0.1 M EDTA (pH 8.0), 10 µl of 0.25% bromophenol blue and 200µl of 1% SDS in 590 µl of distilled water. It was stored at 4°C in 1 ml aliquot.

7. 30% acrylamide (50ml)

14.5g acrylamide + 0.5g bis-acrylamide + dH_2O up to 50ml. Store at 4^oC. The powder is neurotoxic. Use mask during handling.

8. 0.5M Tris HCl pH 6.8 (100ml)

Take 6.57g [0.5xM.W.(121.14)/10] of Tris base in 70ml dH_2O, mix, bring the pH to 6.8 with concentrated HCl and then bring the total volume of the solution up to 100ml mark by adding dH_2O. Store at 4^oC.

9. 1.5M Tris HCl pH 8.8 (200ml)

Take 36.34g [1.5xM.W.(121.14)/20] of Tris base in 150ml dH_2O, mix, bring the pH to 8.8 with concentrated HCl and then bring the total volume of the solution up to 200ml mark by adding dH_2O. Store at 4^oC.

10. 10% SDS (50ml)

5g SDS + dH_2O up to 50ml mark. Stored at room temperature.

11. 10% Ammonium persulphate (10% APS-5ml)

Take 0.5g APS and add dH_2O up to 5ml mark and mix. Aliquot 0.5ml in each eppendorf tube and store at -20^oC.

12. TEMED

Readymade. Store at room temperature.

13. Saturated butanol

Take 50ml dH_2O in a beaker with a magnetic stirrer. Keep adding butanol to water provided the machine is turned on. Stop adding butanol when the solution gets saturated. Store at 4^0C.

14. Running buffer

- 3.0g Tris base
- 14.4g Glycine
- 10ml 10%SDS
- 1000ml dH_2O

The pH of the solution should be 8.3, which may range from 8.1 to 8.5. If unsuccessful, make the soln. again. The solution is to be stored at 4^oC. The bottle is to be marked after every usage. It should not be used more than 8-10 times.

15. Preparation of 0.1% BPB

At first, 2ml of 50% glycerol solution is to be made by mixing 1ml glycerol with 1ml dH_2O. Then weigh 2mg of Bromophenol blue (BPB) and add 50% glycerol up to 2ml mark. Store at 4^oC. 5µl of 0.1% BPB is to be added per sample.

16. 2X Sample buffer: (2ml - Store at 4^oC)

- 0.5M Tris HCl pH 6.8- 0.4ml
- 10% SDS- 0.4ml
- 2-mercaptoethanol- 0.04ml
- Glycerol- 0.4ml
- dH_2O- 0.76ml

Sample and 2X sample buffer are to be mixed in a proportion of 1:1, according to the volume of the wells. Sample-sample buffer mixture is to be boiled for 3 mins using floating tray for denaturing the proteins.

17. **Staining the gel after electrophoresis**

At first, 200ml 7% acetic acid solution is to be made by dissolving 14ml acetic acid in 186ml dH_2O. 100ml should be used as destaining solution and the rest should be used to prepare 0.1% Coomassie blue solution as the stainer of the gel. To prepare this solution, 100mg of the dye is to be dissolved by adding 7% acetic acid up to the 100ml mark. Solution is to be stored at 4^oC.

18. **Destaining of the gel**

Destaining of the gel should be done by 7% acetic acid.

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, Germany
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	Shanghai Ruosuaa, Technology companChina
Disposable micropippete tips	Eppendorf, Ireland
Western blot apparatus	Neido, Japan