Comparative Study of Complement Protein Activity of Blood Serum against *Escherichia coli* in Urban and Slum Population of Bangladesh

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DECLARATION

I hereby solemnly declare that the thesis project titled "Comparative Study of Complement Protein Activity of Blood Serum against *Escherichia coli* in Urban and Slum Population of Bangladesh" submitted by the undersigned has been carried out under the supervision of Fahareen Binta Mosharraf, Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University.

The presented dissertation is based on original research work carried out by myself and has not been submitted to any other institution for any degree or diploma. Any reference to work done by any other person or institution or any material obtained from other sources have been accordingly cited and referenced.

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DEDICATION

To My Parents,

Two people who encouraged and tolerated me this whole time.

ABSTRACT

The complex of serum proteins known as complement plays key roles in the lytic and inflammatory properties of the immune system, including both innate and adaptive immunity. The last several years have seen an enormous expansion in our understanding of the details of complement biochemistry and in our appreciation of the role of complement proteins in biological phenomena. This observation particularly calls for the in vitro examination of complement proteins activity derived from blood serum of a defined group of urban and slum people against *E. coli*, a major cause of large scale epidemics and thousands of sporadic cases of gastrointestinal illness. In this exertion we attempt to make a comparative analysis on complement mediated killing of clinical isolates of *E. coli*, derived from serum collected from urban and slum population. However the slum population showed more effective complement mediated killing in comparison to urban population.

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LIST OF ABBREVIATIONS

EMB	**Eosin Methylene Blue Agar**
NA	**Nutrient Agar**
NB	**Nutrient Broth**
NS	**Normal Saline**
IMViC	**Indole, Methyl red, Voges-Proskauer, Citrate**
MR-VP	**Methyl red-Voges-Proskauer**
STEC	**Shiga Toxin Producing *E. coli***
ml	**Milliliter**
µl	**Micro liter**
g	**Gram**
rpm	**Revolutions per Minute**
e.g.	**Example**
et al.	**And others**
pH	**Negative logarithm of hydrogen ion concentration**

1. Introduction and Literature Review

1.1 Background

Escherichia coli or *E. coli* is a widely known species of bacteria from the family of gram negative bacteria- Enterobacteriaceae and genus- *Escherichia*. The genus was named after, German bacteriologist Theodor Escherich who discovered the organism from human colon in 1885 [Feng *et al*., 2002]. Escherich was also the first to show that this organism has the ability to cause certain diseases like infant diarrhea, gastroenteritis etc. The organism was initially named Bacterium coli but later was changed into *Escherichia coli* to honor its discoverer [Feng *et al*., 2002]. It is a gram-negative, facultatively anaerobic, rod shaped bacterium found most commonly in lower intestines of warm blooded animals [Tortora *et al*., 2012]. The "O" and "H" antigens on the bacteria and their flagella form different serotypes. There are now more than 700 serotypes of *E. coli* present [Griffin & Tauxe, 1991]. Most *E. coli* strains are harmless, even known as an essential organism to humans as they inhabit the gut area as normal flora to aid the hosts by producing vitamin K_2 [Bentley & Meganathan, 1982].The beneficial normal flora also protects the intestine of their host by preventing invasion by other pathogenic organisms. *E. coli* and other facultative anaerobes constitute about 0.1% of gut flora [Eckburg *et al*., 2005]. So fecal matter from a healthy individual contains a large amount of *E. coli* but the number reduces slowly. Though most kinds of *E. coli* do not cause disease in humans, some serotypes have the potential to cause infections of the gastrointestinal tract, urinary tract etc. of their hosts [Eisenstein & Zaleznik, 2000]. *E. coli* is the major cause of some mild and severe diarrhea [Bower *et al.,* 1999]. The minor infections and severe diseases occur when the pathogenic strain of the bacterium (for e.g. *E. coli*O157:H7) enters host through fecal-oral route. *E. coli* O157:H7 has proved to be one of the most dangerous strains that have a high prevalence of illnesses throughout the world. This strain produces a toxin called shiga toxin that is similar to the toxin produced by *Shigella dysenteriae*. The toxin has the ability to inhibit protein synthesis which leads to the death of cells. This killing of cells leads to a breakdown of the lining and to hemorrhage. The first response is commonly a bloody diarrhea. The toxin also disrupts small blood vessels, mainly the glomerulus which leads to kidney failure and the development of the often hemolytic uremic syndrome. The toxin has effect on lungs and as well as the nervous system. Shiga toxin–producing *E. coli* (STEC) cause approximately 100,000 illnesses, 3,000 hospitalizations, and 90 deaths annually in the United States [Mead *et al.*, 1999 & CDC, 2009]. *E. coli* cells are able to survive outside the body for a limited amount of time, which

makes them potential indicator organisms to test environmental samples for fecal contamination [Feng *et al*., 2002]. But persistent *E. coli* has demonstrated that they can survive in the environment, outside of a host for extended periods [Thompson, 2007].

1.1.2 Character & Morphology

E. coli is a Gram-negative, facultative anaerobic, typically rod shaped bacterium which does not sporulate. Cells are about 2.0 micrometers (μm) long and 0.25–1.0 μm in diameter. Motile strains possess flagella in a peritrichous arrangement.

Table 1: *Escherichia coli* Classification

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

Domain and Kingdom: *Escherichia coli* fits into the domain and kingdom of Bacteriabecause members of this group are unicellular microorganisms [Moder, 2008].

Phylum: *Escherichia coli* fits into the phylum Proteobacteria because members of this group are Gram-negative bacterium with an outer membrane composed primarily of lipopolysaccharides [Moder, 2008].

Class: *Escherichia coli* fits into the class Gamma Proteobacteria because members of this group are facultatively anaerobic Gram-negative bacterium [Moder, 2008].

Order: *Escherichia coli* fits into the order Enterobacteriales because members of this group are rod-shaped facultatively anaerobic Gram-negative bacterium [Moder, 2008].

Family: *Escherichia coli* fits into the family Enterobacteriaceae because members of this group are motile via peritrichous flagella that grows well at 37^{o}C, is oxidase negative, catalase positive and reduces nitrates [Moder, 2008].

Genus: *Escherichia coli* fits into the genus *Escherichia* because members of this group are mostly opportunistic floras that are enteric (colonize in the intestinal tract of mammals) [Moder, 2008].

Species: *Escherichia coli* is one of the five species, recognized under the genus *Escherichia*. What makes *E. coli* unique is by these biochemical activities: ferments lactose, possesses lysine decarboxylase, is Vogus-Proskauer negative, produces indole, does not grow on nitrate, and doesnot produce H_2S [Moder, 2008].

1.1.3 Metabolism

E. coli is a heterotrophic organism which means the bacterium can live on a wide variety of substrates and uses mixed-acid fermentation in anaerobic conditions. The necessary carbon of the bacterium comes mainly from the host glucose which is broken down into carbon by means of central metabolism, a procedure consisting of three steps:

1. Embden-Meyerhof-Parnas (EMP) Pathway
2. Tricarboxylic Acid (TCA) cycle
3. Pentose Phosphate Cycle (PPP)

E. coli can synthesize carbon from also gluconate with the help of enzymes of the Entner-Doudoroff Pathway but the bacterium does not use the pathway directly. This alternative pathway differs from the EMP in that the pyruvate from gluconate is converted into carbon-dioxide andacetaldehyde. The acetaldehyde is then converted into ethanol. The pyruvate from EMP can be converted into ATP by *E. coli*, since sufficient amount of ATP is required to carry out metabolic processes [Gottschalk, 1979]. During these metabolic processes, the bacterium produces lactate, succinate, ethanol, acetate, and carbon dioxide. Since many pathways in mixed-acid fermentation produce hydrogen gas, these pathways require the levels of hydrogen to be low, as is the case when *E. coli* lives together with hydrogen-consuming organisms, such as methanogens or sulphate-reducing bacteria [Madigan & Martinko, 2006].

1.1.4 Habitat

Escherichia coli cycles between two principal habitats. The bacteria start its life in the intestines of warm-blooded animals and spend most of the time there. Then after excreted into the environment, they spend the rest of their life in water, sediment, and soil-that are

shown to be quite distinct with respect to physical conditions and the spectrum and level of available nutrients. The next stage of the cycle could be death or with a small probability, colonizing a new host [Savageau, 1983].The gram-negative species *Escherichia coli* spend a good part of their lives as residents of animal hosts they are most commonly known as a commensal of the lower intestine of mammals, although pathogenic variants also exist [Puente & Finlay, 2001]. The animal host is believed to be the primary habitat of this enteric species, which are genetically endowed to do well in this environment [Savageau, 1998]. The *E. coli* genome encodes proteins that mediate resistance to acid pH as well as growth on lactose, which is critical for a commensal of mammals [Blattner *et al*., 1997 and Lawrence & Ochman, 1998]. Once excreted from an animal host *E. coli* starts battling for survival, facing limited nutrient availability, osmotic stress, large variations in temperature and pH, and predation. From this, it can be predicted that *E. coli* will have at least two phenotypically distinct cell types and that these will have dual molecular control mechanisms of opposite type for the regulation of certain functions [Savageau, 1983].

1.2 Types of *E. coli*

A huge part of the bacterial population is of *E. coli* since it is diverse both genetically and phenotypically. The diversity can be proved by the fact that only 20% genes of the typical *E. coli* genome are shared among all the strains [Lukjancenko, 2010]. The differences at molecular level have the ability to make changes at characteristic level such as, lifecycle or physiology.

Serotype is a subdivision system of bacteria based on major surface antigens. In case of *E. coli*, the antigens are:

- O antigen: The outer membrane of an *E. coli* cell contains millions of lipopolysaccharide (LPS) molecules, which consists of –

1. O antigen - a polymer of immunogenic repeating oligosaccharides (1–40 units)
2. Core region - phosphorylated nonrepeating oligosaccharides
3. Lipid A (endotoxin)

O antigen is encoded by the rfb gene cluster.

- K antigen: A thick, mucous-like, layer of polysaccharide that surrounds some pathogen *E. coli*, known as acidic capsular polysaccharide (CPS). There are two separate groups of K antigen, I and II. Group I is associated with capsular polysaccharides and II with extraintestinal diseases [Brenner *et al*., 2005].
- H antigen: The H antigen is flagellin, of the flagella that allow *E. coli* to move. The H antigen group starts from H1 to H56 with some exceptions. These are encoded by the fliC gene [Abbadi & Strockbine, 2007].

E. coli needs only to acquire a combination of mobile genetic elements to become a highly adapted pathogen capable of causing a range of diseases, from gastroenteritis to extraintestinal infections of the urinary tract, bloodstream and central nervous system. Hundreds of millions of people are affected annually with these diseases. Eight *E. coli* pathovars have been well characterized, and each uses varieties of virulence factors to weaken host immunity [Croxen & Finlay, 2013]. These pathovars are grouped into two large groups; diarrhoeagenic *E. coli* and extraintestinal *E. coli*. Enteropathogenic *E. coli* (EPEC), entero-haemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC; including *Shigella*), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) are diarrhoeagenic. And the other two pathovars uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) are extraintestinal *E. coli* (ExPEC) [Croxen, & Finlay, 2013].

i) Enteropathogenic *E. coli* (EPEC): The first pathotype described was EPEC which is primarily associated with causing diarrhea in children in developing countries. Pathogenesis of EPEC involves a plasmid-encoded protein known as EPEC adherence factor (EAF). This factor enables localized adherence of bacteria to intestinal cells. A non fimbrial adhesion called intimin, which is an outer membrane protein, mediates the final stages of adherence. The pathotype does not produce ST or LT toxins. Adherence procedure is very complicated and has dramatic effects in the structure of the cells which results in rearrangements of actin. The phenomenon is sometimes called "attachment and effacing" of cells. EPEC strains are not as invasive as *Shigella,* and unlike ETEC or EAEC, they cause an inflammatory response. The diarrhea and other symptoms of EPEC infections probably are caused by bacterial invasion of host cells and interference with normal cellular signal transduction, rather than by production of toxins [Todar, 2008].

ii) Entero-haemorrhagic *E. coli* (EHEC): This pathotype was first recognized in 1982 which can cause bloody diarrhea. The main reservoir is known to be cow intestine. The primary cause of hemorrhagic colitis (HC) (bloody diarrhea) which can progress to potentially fatal hemolytic uremic syndrome (HUS) is EHEC. The bacteria are transmitted to humans through contaminated foods like undercooked ground beef, cold sandwiches, vegetables, water and other unpasteurized drinks like raw milk, apple juice. The pathogen colonizes the intestinal area including the distal ileum and large bowels of human body. EHEC are characterized by the production of verotoxin or Shiga toxins (Stx) [Todar, 2008]. There are many serotypes of Stx-producing *E. coli* but only those that have been clinically associated with HC are designated as EHEC. Of these, O157:H7 is the prototypic EHEC and most often causes illness worldwide [Todar, 2008]. EHEC are considered to be "moderately invasive". Nothing is known about the colonization antigens of EHEC but fimbriae are presumed to be involved. The bacteria do not invade mucosal cells as readily as Shigella, but EHEC strains produce a toxin that is virtually identical to the Shiga toxin. The toxin plays a role in the intense inflammatory response produced by EHEC strains and may explain the ability of EHEC strains to cause HUS. The toxin is phage encoded and its production is enhanced by iron deficiency [Griffin & Tauxe, 1995; Todar, 2008].

iii) Enterotoxigenic *E. coli* (ETEC): ETEC is the disease commonly referred to as traveler's diarrhea. ETEC causes diarrhea in children in developing countries and is the main cause of diarrhea in travelers to developing countries. Transmission occurs through water and foods like soft cheese, raw vegetables etc. The diseases vary from minor discomfort to a severe cholera-like syndrome. The disease requires colonization and elaboration of one or more enterotoxins. Both traits are plasmid-encoded. ETEC may produce a heat-labile enterotoxin (LT) that is similar in molecular size, sequence, antigenicity, and function to the cholera toxin (Ctx). 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. ETEC may also produce a heat stable toxin (ST) that is of low molecular size and resistant to boiling for 30 minutes. Their small size explains why they are not inactivated by heat.

ETEC have species specific fimbriaes which act as adhesins. These fimbrial adhesins adhere to specific receptors on enterocytes of the proximal small intestine. Symptoms of infection include diarrhea without fever. The bacteria colonize the GI tract by means of a fimbrial adhesion and are noninvasive, but produce either the LT or ST toxin [Todar, 2008].

iv) Enteroinvasive *E. coli* (EIEC): There are no known animal reservoirs of EIEC. So the primary source for EIEC appears to be infected humans. EIEC are non-motile. Pathogenicity is primarily due the ability to invade and destroy colonic tissue. EIEC closely resemble Shigella in their pathogenic mechanisms and the kind of clinical illness they produce. EIEC penetrate and multiply within epithelial cells of the colon causing widespread cell destruction. The clinical syndrome is identical to Shigella dysentery and includes a dysentery-like diarrhea with fever. EIEC apparently lack fimbrial adhesins but do possess a specific adhesion outer membrane protein. EIEC are invasive organisms. They do not produce LT or ST toxin [Wellington & Van Elsas, 1992; Todar, 2008].

v) Enteroaggregative *E. coli* (EAEC): EAEC strains have the ability to attach to tissue culture cells in an aggregative manner. These strains are associated with persistent diarrhea in young children. They resemble ETEC strains in that the bacteria adhere to the intestinal mucosa and cause non-bloody diarrhea without invading or causing inflammation. This suggests that the organisms produce an enterotoxin of some sort. Recently, a distinctive heat-labile plasmid-encoded toxin has been isolated from these strains, called the EAST (Entero Aggregative ST) toxin. They also produce a hemolysin. The role of the toxin and the hemolysin in virulence has not been proven. The significance of EAEC strains in human disease is controversial [Wellington & Van Elsas, 1992; Todar, 2008].

vi) Diffusely adherent *E. coli* (DAEC): Some types of EPEC are referred to as diffusely adherent *E. coli* (DAEC), based on specific patterns of adherence. They are an important cause of traveler's diarrhea in Mexico and in North Africa [Todar, 2008]. DAEC strains are significantly associated with diarrhea in children and urinary tract infections in adults [Servin *et al.,* 2005]. The strains are heterogeneous with respect to plasmid content and serotype [Girón *et al.*, 1991]. They generate a diffuse adherence pattern on HeLa and HEp-2 cells. This pattern is mediated by proteins encoded by a family of related operons, which includes both fimbrial and afimbrial adhesins, collectively designated Afa–Dradhesions. [Servin *et al.,* 2005]

vii) Uropathogenic *E. coli* (UPEC): Strains of UPEC are the primary cause of urinary tract infections, including both cystitis and pyelonephritis. These bacteria have evolved a multitude of virulence factors and strategies that aid in bacterial growth and persistence within the environment of host's urinary tract. Expression of adhesins like type 1 and P pili allow UPEC to bind and invade host cells and tissues within the urinary tract. Hemolysin and

cytotoxic necrotizing factor 1 help UPEC cause extensive tissue damage, bacterial dissemination as well as releasing host nutrients and disabling immune effector cells. These toxins also have the capacity to alter inflammatory responses, host cell survival, and cytoskeletal dynamics [Wiles *et al*., 2008].

viii) Neonatal meningitis *E. coli* (NMEC): Most cases of *E. coli* meningitis occur in newborns. It may also occur in people who have a CSF shunt or have head injuries and head surgeries. In a study, 84% of *E. coli* from the cerebrospinal fluid of neonates with meningitis had capsular (K1) polysaccharide. The K1 capsular antigen has been shown to be immunochemically identical to the meningococcal Group B polysaccharide. The high prevalence of the K1 antigen in neonatal meningitis suggests that this capsular polysaccharide is related to *E. coli* invasiveness in the newborn [Robbins *et al*., 1974].

1.3 Pathogenesis of *E. coli*

Since there are over 700 serotypes of *E. coli*, the probability of a strain being pathogenic is high. There are several highly adapted *E. coli* strains that have acquired specific virulence attributes. These strains are able to adapt to new niches which allows them to cause a broad spectrum of disease. These virulence attributes are frequently encoded on genetic elements [Kaper *et al.,* 2004] and generally associated with the three antigens of the bacterium; the O antigen derived from the cell wall, H antigen derived from flagella and the secreting K antigen derived from a polysaccharide capsule. Three general clinical syndromes can result from infection with the pathogenic *E. coli*: enteric/diarrheal disease, urinary tract infections (UTIs) and sepsis/meningitis [Kaper *et al*., 2004]. Pathogenic *E. coli* strains follow a multi-step process to infect a host body. Generally the steps that are followed are similar to that of other mucosal pathogens: colonization of a mucosal site, evasion of host defenses, multiplication and host damage.

1.3.1 Enteric/Diarrheal Diseases

E. coli typically colonizes the gastrointestinal tract of human to coexist in good health. Since the bacteria are adapted to survive in the gastrointestinal tract, the pathogens are able to infect the intestines easily, resulting in diarrhea. The types of diarrhea are further categorized based on the six subgroups, ETEC, EIEC, EHEC, EPEC, EHEC and DAEC.

The six recognized categories of diarrhoeagenic *E. coli* each have unique features in their interaction with eukaryotic cells.

- EPEC adhere to small bowel enterocytes, but destroy the normal microvillar architecture, inducing the characteristic attaching and effacing lesion. Cytoskeletal derangements are accompanied by an inflammatory response and diarrhoea [Kaper *et al*., 2004]. A recent study showed that EPEC can disrupt cell polarity [Muza-Moons *et al.,* 2003].
- EHEC also induce the attaching and effacing lesion, but in the colon. The distinguishing feature of EHEC is the elaboration of Shiga toxin (Stx), systemic absorption of which leads to potentially life-threatening complications [Kaper *et al*., 2004].
- ETEC adhere to small bowel enterocytes and induce watery diarrhoea by the secretion of heat-labile (LT) and/or heat-stable (ST) enterotoxins [Kaper *et al*., 2004].
- EAEC adheres to small and large bowel epithelia in a thick biofilm and involves secretory enterotoxins and cytotoxins [Kaper *et al*., 2004].
- EIEC invades the colonic epithelial cell, lyses the phagosome and moves through the cell by nucleating actin microfilaments. The bacteria either move laterally through the epithelium by direct cell-to-cell spread or exit and re-enter the plasma membrane [Kaper *et al.,* 2004].
- DAEC elicits a characteristic signal transduction effect in small bowel enterocytes that manifests as the growth of long finger-like cellular projections, which wrap around the bacteria [Kaper *et al*., 2004].

1.3.2 Urinary Tract Infections

90% of UTI cases are caused by Uropathogenic *E. coli* [Moder, 2008]. This results from the pathogenic *E. coli* strains having specific adherence factors which aid them in adhering and colonizing sites that *E. coli* generally does not colonize. UPEC causes urinary tract infections upon infecting the urethra, an unlikely region to be infected with the organism. The pathogen has the ability to adhere and colonize the urethra and bladder with the help of adhesins like fimbriae. Finally inflammation of the bladder results in infection [Moder, 2008].

1.3.3 Sepsis/ Meningitis

This type of *E. coli* is the most common cause of Gram negative neonatal meningitis, with a case fatality rate of 15–40% and severe neurological defects in many of the survivors [Unhanand *et al*., 1993; Dawson *et al*., 1999]. *E. coli* that cause meningitis are spread

haematogenously. The bacteria enter through the gastrointestinal tract or nasopharynx into blood. Blood then carries the bacteria to the central nervous system without any damage to the blood brain barrier. If not treated, this infection may result into death [Moder, 2008].

1.4 Virulence Factors

The diseases caused by a particular strain of *E. coli* depend on distribution and expression of an array of virulence determinants, including adhesins, invasins, toxins, and abilities to withstand host defenses.

1.4.1 Adhesins

Adhesins allow the pathogenic *E. coli* to adhere to sites that they do not normally inhabit, like small intestine and urethra. Most frequently these adhesins form distinct morphological structures called fimbriae (also called pili) or fibrillae, which can belong to one of several different classes. Fimbriae are rod-like structures of 5–10 nm in diameter that are distinct from flagella. Fibrillae are 2–4 nm in diameter and are either long and wiry or curly andflexible [Cassels & Wolf, 1995]. The Afa adhesins that are produced by many diarrhoeagenic and uropathogenic *E. coli* are described as a fimbrial adhesins but in fact seem to have a fine fibrillar structure that is difficult to visualize [Keller *et al*., 2002]. Adhesins of pathogenic *E. coli* can also include outer-membrane proteins, such as intimin of UPEC and EHEC, or other non-fimbrial proteins. Some surface structures trigger signal transduction pathways or cytoskeletal rearrangements that can lead to disease. Surface structures that are present on commensal *E. coli* strains can induce signalling cascades if the organism encounters the appropriate receptor. The LPS of *E. coli* and other Gram-negative bacteria binds to Toll-like receptor 4 (TLR4), triggering a potent cytokine cascade that can lead to septic shock and death [Tapping *et al*., 2000]. Flagellin, the main component of flagella, can bind to TLR5, thereby activating interleukin (IL)-8 expression and an inflammatory response [Hayashi *et al*., 2001].

1.4.2 Invasins

Invasins help the pathogens to invade the host immunity system thus eliminating the barrier that is keeping them from infecting the host. Hemolysin is such an invasin. Shigella like invasins are utilized for intracellular invasion and spreading [Todar, 2008].

1.4.3 Toxins

Secreted toxins and other effector proteins are present in greater numbers which trigger signal transduction pathways and affect variety of eukaryotic processes. Different strains of EPEC produce 3 different toxins, the heat-labile enterotoxin (LT), heat-stable enterotoxin a (STa) and heat-stable enterotoxin b (STb) which helps in ion secretion [Kaper *et al*., 2004]. The Shiga toxin (Stx) of EHEC cleaves ribosomal RNA, thereby disrupting protein synthesis and killing the intoxicated epithelial or endothelial cells [Melton-Celsa & O'Brien, 1998]. The cytolethal distending toxin (CDT) has the ability to block cell division. [De Rycke & Oswald, 2001] Another toxin that blocks cell division is called Cif (cycle-inhibiting factor) [Marches *et al*., 2003]. The cytotoxic nectrotizing factors (CNF 1 and CNF 2) deaminate a crucial glutamine residue of RhoA, Cdc42 and Rac, thereby locking these important signalling molecules in the 'on' position and leading to marked cytoskeletal alterations, multinucleation with cellular enlargement, and necrosis [Lerm *et al.*, 1999]. The Map protein of EPEC and EHEC stimulates filopodia formation and targets mitochondria to disrupt membrane potential in these organelles [Kenny *et al*., 2002].

1.4.4 Plasmids

There are now numerous examples of plasmids that encode crucial virulence factors of pathogenic *E. coli*, including plasmids in EAEC that encode fimbriae and toxins, plasmids in EIEC/Shigella that encode a type III secretion system and invasion factors. There are also R factors and drug resistance plasmids. Although many of these plasmids are self-transmissible, some lack conjugation genes and can only be transferred with a conjugative plasmid. For ETEC, the genes that encode both LT and ST are found on plasmids, but some estA genes encoding STa are on transposons that can be inserted into either plasmids or the chromosome [McVeigh *et al*., 2000].

1.4.5 Others

K antigen, LPS and capsules have other properties like, antiphagocytic, defense mechanism against antibactericidal reactions and defense against immune responses. Flagella aid in motility and chemotaxis [Todar, 2008].

1.5 Transmission

Most intestinal infections are caused by contaminated food or water, when a person eats food, or drinks water or ice contaminated with the pathogen. Human or animal wastes (e.g., feces) are the ultimate source of contamination. Animals, mostly cattle play as reservoirs of the pathogens. The high prevalence of *E. coli* O157 and non-O157 STEC in some cattle populations, combined with the lack of effective on-farm control strategies to reduce carriage, represents a significant risk of contamination of the food supply and the environment. Non-O157 STEC are also harbored in other ruminants, including swine [Fratamico, 2007]. Pathogenic *E. coli* are believed to mostly live in the intestines of cattle, but these bacteria have also been found in the intestines of chickens, deer, sheep etc. If the infected meat is not cooked to 160°F (71°C), the bacteria can survive and infect after consumption of the contaminated meat. This is the most common way people in the United States become infected with *E. coli*. Any food that has been in contact with raw meat can also become infected. Other food products that can be contaminated with *E. coli* includes, raw milk or other dairy products which comes in contact with the pathogen from cow's udder. And also raw fruits and vegetables such as lettuce, alfalfa sprouts, or unpasteurized apple cider or other unpasteurized juices that have come in contact with infected animal feces [WebMD, 2014].

When water supply of a city/town is contaminated with human or animal feces, people get infected if not properly treated with chlorine. *E. coli* also spread from one person to another, when an infected person does not maintain hygiene, after a bowel movement. *E. coli* can spread from an infected person's hands to other people or through fomites [WebMD, 2014]

1.6 *E. coli* related Diseases

Escherichia coli is one of the most frequent causes of many common bacterial infections, including urinary tract infection (UTI), traveler's diarrhea, cholecystitis, bacteremia, cholangitis and other clinical infections such as neonatal meningitis and pneumonia [Madappa, 2015].

1.6.1 Enteric infections

Six different pathotypes of *E. coli* cause enteric infections by following six different mechanisms. ETEC is a cause of traveler's diarrhea resulting in watery diarrhea and abdominal cramping. EPEC causes childhood diarrhea. EIEC causes a *Shigella* -like dysentery. EHEC causes hemorrhagic colitis or hemolytic-uremic syndrome which is a type

of kidney failure. EAEC is primarily associated with persistent diarrhea in children in developing countries, and DAEC is a cause of childhood diarrhea and traveler's diarrhea in Mexico and North Africa. ETEC, EPEC, EAEC, and DAEC colonize the small bowel, and EIEC and EHEC preferentially colonize the large bowel prior to causing diarrhea [Madappa, 2015].

Shiga toxin–producing *E. coli* (STEC) is among the most common causes of food borne diseases. This organism is responsible for several gastrointestinal tract illnesses, including non bloody and bloody diarrhea. Patients with these diseases, especially children, may be affected by neurologic and renal complications, including HUS. Strains of STEC serotype O157-H7 have caused numerous outbreaks and sporadic cases of bloody diarrhea and HUS [Madappa, 2015]. In addition to *E. coli* O157, many other non-O157 STECs also cause disease. Some non-O157 STECs cause disease that is often severe, resulting in bloody diarrhea and sometimes HUS [CDC, 2014]. People with weakened immune systems, pregnant women, young children, and older adults are at increased risk for developing these complications.

1.6.1.1 Symptoms of Enteric Infections

Symptoms of intestinal infection generally begin between one and five days after being exposed to *E. coli* and can last from a few days to more than a week. Symptoms include:

- Abdominal cramping
- Sudden, severe watery diarrhea that may change to bloody stools
- Gas
- Loss of appetite/nausea
- Vomiting (uncommon)
- Fatigue
- Fever [Pietrangelo, 2015].

Symptoms of a severe *E. coli* infection may include:

- Bloody urine
- Decreased urine output
- Pale skin
- Bruising

- Dehydration [Pietrangelo, 2015].

1.6.2 Urinary tract infections

The urinary tract is the most common site of *E coli* infection, and more than 90% of all uncomplicated UTIs are caused by *E. coli* infection. The recurrence rate after a first *E. coli* infection is 44% over 12 months. *E coli* UTIs are caused by uropathogenic strains of *E. coli*. *E. coli* causes a wide range of UTIs, including uncomplicated urethritis/cystitis, symptomatic cystitis, pyelonephritis, acute prostatitis, prostatic abscess, and urosepsis. Uncomplicated cystitis occurs primarily in females who are sexually active and are colonized by auropathogenic strain of *E. coli*. Subsequently, the periurethral region is colonized from contamination of the colon, and the organism reaches the bladder during sexual intercourse [Madappa, 2015].

Complicated UTI and pyelonephritis are observed in elderly patients with structural abnormalities or obstruction such as prostatic hypertrophy orneurogenic bladders or in patients with urinary catheters. *E. coli* bacteremia is usually associated with UTIs, especially in cases of urinary tract obstruction of any cause. The systemic reaction to endotoxin (cytokines) or lipopolysaccharides can lead to disseminated intravascular coagulation and death. *E. coli* is a leading cause of nosocomial bacteremia from a GI or genitourinary source [Madappa, 2015].

1.6.2.1 Symptoms of UTI

Urinary tract infections do not always cause signs and symptoms, but when they do they may include:

- A strong, persistent urge to urinate
- A burning sensation when urinating
- Passing frequent, small amounts of urine
- Unusual-smelling, cloudy, or bloody urine
- Fever or chills
- Pelvic pain in women, rectal pain in men [Mayo Foundation for Medical Education and Research, 2015].

1.6.3 Acute Bacterial Meningitis

The vast majority of neonatal meningitis cases are caused by *E. coli* and group B streptococcal infections (28.5% and 34.1% overall, respectively). Pregnant women are at a higher risk of colonization with the K1 capsular antigen strain of *E coli*. This strain is also commonly observed in neonatal sepsis, which carries a mortality rate of 8%; most survivors have subsequent neurologic or developmental abnormalities. Low birth weight and a positive cerebrospinal fluid (CSF) culture result foretell a poor outcome. In adults, *E. coli* meningitis is rare but may occur following neurosurgical trauma or procedures [Madappa, 2015].

1.6.3.1 Symptoms of Bacterial Meningitis

A strain of *E. coli* called K1 causes about 20 percent of all cases of neonatal meningitis, according the Meningitis Research Foundation. Newborns may become infected with *E. coli* K1 during birth, or from bacteria later acquired in the hospital or home. Symptoms include:

- Irritability
- Breathing trouble
- Diarrhea
- Unusually cold or warm skin
- Fussy feeding
- Lethargy or inactivity
- Bulging soft spot at the top of the head (the fontanelle)
- Nausea [Everyday Health, 2014].

1.6.5 Intra-Abdominal Infections

E. coli intra-abdominal infections often result from a perforated viscus (eg, appendix) or may be associated with intra-abdominal abscess, cholecystitis, and ascending cholangitis. Patients with diabetes mellitus are also at high risk of developing pylephlebitis of the portal vein and liver abscesses. Intra-abdominal abscesses are usually polymicrobial, *E. coli* is one of the more common gram-negative bacilli observed together with anaerobes. Abscesses can be

caused by GI tract perforation or after anastomotic disruption with spillage of colon contents [Madappa, 2015].

Cholecystitis and cholangitis result from obstruction of the biliary system from biliary stone or sludge. This leads to stagnation and bacterial growth from circulation. When bile flow is obstructed, colonic organisms, including *E. coli*, colonize the jejunum and duodenum [Madappa, 2015].

1.6.6 Other Infections

Other *E. coli* infections include septic arthritis, endophthalmitis, suppurative thyroiditis, sinusitis, osteomyelitis, endocarditis, and skin and soft-tissue infections (especially in patients with diabetes) [Madappa, 2015].

1.7 Immune Response to *E. coli*

The innate immune system triggers inflammation accompanied by inflammatory cytokine secretion, neutrophil recruitment and massive tissue destruction in reponse to *E. coli* invasion and replication within host cells [Ashida *et al*., 2015]. Apart from white blood cells which act as the first defenders of the immune system and the other members of the phagocyte family, the complement system plays an important role in host defense against Gram-negative bacterial infection, including *E. coli* infection. The system consists of at least 30 proteins that orchestrate attack on pathogenic agents. Although the susceptibility of Gram-negative bacteria to complement attack has been under investigation for over 100 years, the mechanism(s) by which the complement directly kills Gram-negative bacteria (*E. coli*) is not understood [Bloch *et al*., 2011].

The complement system consists of three pathways: Classical, Alternative and Lectin pathway, each having their own unique method of activation. Activation of complement by gram-negative bacteria including *E. coli* can occur via the classical or the alternative pathway; the former usually requires for its activation recognition of bacterial surface antigens by certain antibody classes, whereas activation of the latter can be initiated and amplified, in the absence of antigen-antibody interactions, by poorly understood structural or conformational characteristics of the cell surface [Taylor, 1983]. Activation of each pathway results in the production of a macro molecular structure called the membrane attack complex (MAC) which consists of terminal or late acting complement proteins (C5b-9 complexes). The C5b-9 complexes are needed for direct killing of Gram-negative bacteria. However, the

exact mechanism(s) by which inner membrane damage to Gram-negative bacteria by C5b-9 complexes leads to cell death is not known [Bloch *et al*., 2011]. Opsonization is another immune process that targets *E. coli* for destruction, using phagocytes and opsonins which enhance the phagocytosis.

In addition to complement, lysozyme, calcium (Ca^{2+}) and magnesium (Mg^{2+}) are important components of extracellular fluids that are required for killing Gram-negative bacteria. Calcium and magnesium ions are essential for initiation of the classical complement sequence. Other factors such as antitoxins, antiviral antibodies, opsonins (C3b/ antibodies) and antilysins, play a role in the bactericidal activity of human serum [Igumbor & Osayande, 2000].

Scientists at the National Institute of Allergy and Infectious Diseases (NIAID), part of the National Institutes of Health, have shown how the O157:H7 strain of *Escherichia coli* causes infection and thrives by manipulating the host immune response. The bacterium secretes a protein called NleH1 that directs the host immune enzyme IKK-beta to alter specific immune responses. This process not only helps the bacterium evade elimination by the immune system, it also works to prolong the survival of the infected host, enabling the bacterium to persist and ultimately spread to unaffected individuals [Wan *et al*., 2011]. Although the systemic and local immune response to the O antigen of *Escherichia coli* has been well characterized, little information is available on the immune response to K antigen. while the K antigen of *Escherichia coli* functions as a virulence factor in upper urinary tract infections, this antigen does not elicit a significant immune response, whereas the O antigen does induce a significant antibody response which could be of protective or diagnostic benefit [Smith & Kaijser, 1976].

1.8 Outbreaks

1.8.1 Jack in the Box *E. coli* outbreak, 1993

The outbreak was in the western U.S. that began in late 1992, and lasted into 1993. It was linked to the consumption of hamburgers served by the Jack in the Box Restaurant chain. 732 people were infected with the *Escherichia coli* O157:H7 bacterium [Nestle, 2010; South Wales Echo, (Cardiff), 2008; Schlosser, 2001]. Investigation revealed that restaurant outlets were serving contaminated beef and were not cooking the hamburgers thoroughly. Seventy-three Jack in the Box restaurants were ultimately identified as part of the outbreak. Cases

were reported from Washington (602 cases/144 hospitalizations/3 deaths), Idaho (14 cases/4 hospitalizations/no deaths), California (34 cases/14 hospitalizations/1 death), and Nevada (58 cases/9 hospitalizations/no deaths) [MarlerClark, 2015]. The outbreak has been described as "far and away the most infamous food poison outbreak in contemporary history [Golan *et al*., 2004; Hanlon, 2001; Denn, 2011]. The majority of the victims were children aged under 10-years old [Hunter, 2009; Schlosser & Wilson, 2006]. *Four* children died and 178 other victims were left with permanent injury including kidney and brain damage [Rogers, 1995; Sylvester, 1995].

1.8.2 Germany *E. coli* O104:H4 outbreak, 2011

A devastating *E. coli* O104:H4 outbreak in Northern Germany occurred in May through June in 2011. It was eventually attributed to the consumption of fenugreek sprouts grown at a farm in Germany. Primary cases were associated with sprout consumption; secondary transmission was also documented. The *E. coli* outbreak resulted in more than 3950 cases, an unknown number of hospitalizations and 53 deaths [European Food Safety Authority, 2012]. The illness was characterized by bloody diarrhea, with a high frequency of serious complications, including hemolytic-uremic syndrome (HUS). Cases were reported throughout the European Union and the United States [MarlerClark, 2015]. Epidemiological fieldwork suggested fresh vegetables were the source of infection. An organic farm in Bienenbüttel, Lower Saxony, Germany which produces a variety of sprouted foods was identified as the likely source of the *E. coli* outbreak. The farm was shut down [CNN, 2010]. On 30 June 2011, the German Bundesinstitut für Risikobewertung (BfR) (Federal Institute for Risk Assessment), an institute of the German Federal Ministry of Food, Agriculture and Consumer Protection, announced that seeds of fenugreek imported from Egypt were likely the source of the outbreak [Bundesinstitut für Risikobewertung (BfR), 2011].

1.9 *E. coli* in Bangladesh

1.9.1 Overall Condition

Acute infectious diarrhea (AID) is a major cause of morbidity and mortality worldwide, and it remains a major public health challenge especially in developing countries where it is a leading cause of death [Kabir, 2011]. Every year nearly 1.4 billion episodes of AID occur in children less than 5 years of age in developing countries [Kosek *et al*., 2003; Parashar *et al.,* 2003] diarrheal illness account for an estimated 12600 deaths each day in children under 5

years of age in Asia, Africa and Latin America [Nguyen *et al*., 2005]. Numerous studies performed in different countries have reported diarrheagenic *Escherichia coli* pathotypes as being the most frequent and important among bacterial pathogens associated with AID in developing countries. However, the frequencies of these pathogens vary with geographic region and depend on the socioeconomic/sanitary conditions achieved [Black, 2010; O'Ryan *et al*., 2010]. In Bangladesh, the AID remains one of the most important health problems surpassed only by the respiratory diseases. One third of the total child death burden is due to diarrhea. Every year, a rural child suffers on average from 4.6 episodes of diarrhea, from which about 230,000 children die [Piechulek *et al*., 2003]. Diarrheagenic *Escherichia coli* has been reported to be responsible for 34% of diarrheal episodes in Bangladesh [ICDDR, B 2002].

1.9.2 *E. coli* Related Studies in Bangladesh

1.9.2.1 Controlled study of *Escherichia coli* diarrheal infections in Bangladeshi children

The relative contribution of diarrheagenic *Escherichia coli* organisms to diarrhea in Bangladeshi populations is not known. Albert and his group analyzed fecal *E. coli* from 451 children up to 5 years of age with acute diarrhea seeking treatment at a Dhaka hospital and from 602 matched control children without diarrhea from July 1991 to May 1992.The results showed that ETEC was significantly associated with diarrhea in the diarrheal children as a whole and especially in the age groups of 0 to 24 months and 37 to 48 months. EPEC was significantly associated with diarrhea in the diarrhea group as a whole and particularly in infants up to 1 year of age. Their overall data thus suggest that EPEC and ETEC are important causes of acute diarrhea in children in this setting [Albert *et al*., 1995].

1.10 Antimicrobial Agents for *E. coli*

Antimicrobial drugs have played an indispensable role in decreasing illness and death associated with infectious diseases in animals and humans [Aarestrup *et al*., 2008]. A wide range of antimicrobial agents effectively inhibit the growth of *E. coli*. The β-lactams, fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole are often used to treat community and hospital infections due to *E. coli* [Pitout, 2012]. β-lactams disrupt cell wall synthesis by binding to and inhibiting the penicillin-binding proteins essential for transpeptidation and carboxypeptidation reactions in cell wall peptidoglycan synthesis. Fluoroquinolones interfere with DNA supercoiling and promote DNA gyrase-mediated

double-stranded DNA. The aminoglycosides bind irreversibly to the 50S subunit of the 70S bacterial ribosomes. Sulfonamides and trimethoprim interfere with bacterial folic acid synthesis by inhibiting tetrahydropteric acid syntheses and dihydrofolatereductase, respectively [Allen *et al.,* 1998]. Silver nanoparticles were shown to be an effective bacteriocide. The particles accumulate in the bacterial membrane, forming pits in the cell wall. This increases the permeability, resulting into cell death [Sondi *et al*., 2003].

1.10.1 Antimicrobial Drug Resistance in *Escherichia coli*

Selective pressure exerted by antimicrobial drug use has been the major driving force behind the emergence and spread of drug-resistance traits among pathogenic and commensal bacteria [Daniel *et al*., 2012]. Among *E. coli*, b-lactamase production remains the most important contributing factor to b-lactam resistance. b-lactamases are bacterial enzymes that inactivate b-lactam antibiotics by hydrolysis, which results in ineffective compounds. The b-lactam antibiotics, especially the cephalosporins and b-lactam-b-lactamases inhibitor combinations, are major drug classes used to treat community-onset or hospital-acquired infections caused by *E. coli,* especially due to the ExPEC pathotype. Resistance to aminopenicillins (e.g. ampicillin) and early-generation cephalosporins (e.g. cefazolin) among *E. coli* is often mediated by the production of narrow-spectrum b-lactamases such as TEM-1, TEM-2 and to a lesser extent SHV-1 enzyme. Most importantly among *E. coli,* is the increasing recognition of isolates producing the so-called "newer b-lactamases" that causes resistance to the expanded-spectrum cephalosporins and/or the carbapenems. Plasmid-mediated resistance mechanisms can reduce fluoroquinolone susceptibilities in *E. coli.* Resistance to aminoglycosides may develop because of impaired uptake and aminoglycoside phosphorylation. Trimethoprim-sulfamethoxazole resistance results from alterations of different substrate enzymes or their overproduction, loss of bacterial drug-binding capacity, and decreased cell permeability [Allen *et al.,* 1998].

1.11Treatment

Medical care of *E coli* infection is based on the site and severity of infection [Madappa, 2015]. Home care is all that is required to treat an *E. coli* infection, in most cases. Drinking plenty of water for replenishing the lost water from diarrhea and vomiting, resting is having adequate amount of food to prevent malnutrition are very important. But for more severe symptoms, visiting a doctor is required. Antibiotics are not recommended because they have the ability to increase the risk of developing hemolytic uremic syndrome (HUS) [Wong *et al*.

2000]. Most individuals who do not develop HUS recover within two weeks [Siegler, 1995]. However, antibiotics and antimotility agents may be useful for other types of *E. coli*, such as enterotoxigenic *E. coli*, which causes traveler's diarrhea. In the absence of severe symptoms, such as bloody diarrhea or intense abdominal pain, some doctors believe it's acceptable to use antimotility medication [Benington-Castro, 2014]. In general, monotherapy with trimethoprim-sulfamethoxazole, aminoglycoside, cephalosporin, or a fluoroquinolones is recommended as the treatment of choice for most known infections with *E. coli*, although many broad spectrum agents remain highly active [Allen *et al.,* 1998].

In case of HUS, if left untreated it can lead to various severe complications like skin problems, decreased urination and sometimes seizures. So HUS requires prompt medical treatment which includes,

- IV fluid and electrolyte replacement
- Red blood cell transfusion
- Platelet transfusion (to help the blood clot normally)
- Kidney dialysis

If kidneys become permanently damaged, switching to a low-protein diet and medications such as angiotensin-converting enzyme (ACE) inhibitors, which lower the blood pressure, are treatments to help prevent further kidney damage. Long-term dialysis or a kidney transplant may also be needed. Doctors typically treat UTIs with a wide range of different antibiotics, such as ciprofloxacin (Cipro) and trimethoprim-sulfamethoxazole (Bactrim). However, some strains of *E. coli*, called extended-spectrum beta-lactamase (ESBL) *E. coli*, are resistant to most antibiotic treatments. There are now only a few classes of oral antibiotics that remain effective at treating UTIs from ESBL *E. coli*, such as fosfomycin (Monural) and nitrofurantoin (Macrobid) [Benington-Castro, 2014]. *E. coli* meningitis requires antibiotics, such as third-generation cephalosporins (eg, ceftriaxone). *E. coli* pneumonia requires respiratory support, adequate oxygenation, and antibiotics, such as third-generation cephalosporins or fluoroquinolones [Madappa, 2015].

1.12 Prevention

E. coli related infections cannot be prevented with vaccines or medications. The best ways to reduce the chance of being exposed to the organism are to avoid risky foods and watching out for cross-contamination.

- Washing fruits and vegetables thoroughly
- Avoiding cross-contamination by using clean utensils, pans, and serving platters
- Keeping raw meats away from other foods and away from other clean items
- Not defrosting meat on the counter
- Always defrosting meat in the refrigerator or microwave
- Refrigerating leftovers immediately
- Drinking only pasteurized milk products (avoid raw milk)
- Not preparing food, not swimming and bathing with others, if one has diarrhea
- Meat should be cooked properly. The U.S. Department of Agriculture provides guidelines for cooking meat and poultry to proper temperatures to make sure all bacteria are killed.

  ⇨poultry: 165 degrees Fahrenheit
  ⇨ground meat, eggs: 160 degrees
  ⇨steaks, pork chops, roasts, fish, shellfish: 145 degrees

- Practicing good hygiene and following food safety guidelines can go a long way to prevent an *E. coli* infection. Washing hands before handling, serving, or eating food, and especially after touching animals, working in animal environments, or using the bathroom should be done regularly [Pietrangelo, 2015].

1.14 Objective of the Study

For being a small yet populated country, people of Bangladesh come from different background with different lifestyle, food habit and health condition. These factors may affect the immune responses of the population thus presenting varying degree of susceptibility to microbe mediated diseases.

Escherichia coli are mostly harmless and an important part of a healthy human intestinal tract. However, some *E. coli* are pathogenic, causing illnesses like diarrhea, urinary tract infections, meningitis etc. *E. coli* related enteric infections have a greater prevalence in Bangladesh than most diseases. 34% of the diarrhea related infections are caused by the diarrheal *E. coli* in Bangladesh. People from every background are exposed to this organism by means of environment since *E. coli* is also a beneficial organism inhabiting human and animal guts which occasionally comes in contact with the environment through fecal contamination. But a certain infectious dose is required to develop an infection. When an individual is exposed to the bacteria, different components of blood come forward to play their roles. Complement, as a vital part of the body's immune system, provides a highly effective means for the destruction of the invading bacteria and for immune complex elimination.

As a developing country, Bangladesh still has a large portion of her people living in slums, below the poverty line. These people have a completely different life style from people living in the urban area. Their living pattern, food habit, hygienity, vaccination etc. differs from those who are well off. The objective of this study is to observe and compare the complement mediated inhibition capability of human blood serum collected from these two different population groups of two different standards of living against *E. coli*. Comparative data tables and charts according to the result would demonstrate the comparison which would be the basis of the observation from experiment where the factors will be analyzed and comparative complement mediated inhibition activity of blood of the two different population of Bangladesh would be documented. Detailed comparative studies like comparing the susceptibility of the people living in urban area to people living in slum, against a specific potential pathogen microorganism has not been done yet in Bangladesh. So this study has the potential to demonstrate if parameters based on life style, affect the complement activity of serum.

2. Materials and Methods

2.1 Working place

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

2.2 Bacterial Strain

Escherichia coli strain used for this particular research work was collected from BRAC University Microbiology Laboratory. Subculture was prepared from the laboratory culture for using in research purpose. To keep the bacteria viable and to prevent contamination from other microorganisms, subculture was done every week. The freshly cultured colonies were used in the procedure.

2.3 Identification of *E. coli*

Though the bacterial strain provided by the laboratory was of *E. coli*, some identification tests were done for reconfirmation.

2.3.1 Selective Media: One of the tests was streaking on Eosin Methylene Blue (EMB) to ensure the presence of *E. coli* as EMB acts as a selective media for this gram negative bacillus.

1. A freshly prepared EMB plate was taken.
2. A loop was taken and flamed before picking up a colony from subculture NA plate.
3. Streak was done on the EMB plate.
4. EMB plate was incubated overnight at 37ºC.
5. Green sheen colonies should be seen the next day.

The green sheen colonies are shown in figure 3.1 in the result section.

2.3.2 Biochemical Tests: Biochemical tests were performed according to the procedures written in Microbiology Laboratory Manual [Cappuccino & Sherman, 2005]. The biochemical tests carried out were IMViC tests: indole production test, methyl-red test, Voges-Proskauer test and citrate utilization test.

a) Indole Production Test

Some bacteria characteristically produce an enzyme called tryptophanase which converts tryptophan into indole and other metabolic products. When a media containing tryptophan as substrate is used, the conversion to indole can be detected by Kovac's reagent. The reagent reacts with the converted indole and produces a cherry red color. Red reagent layer at the top determines the presence of indole producing culture, from tryptophan. Absence of red color indicates an indole negative reaction [Cappuccino & Sherman, 2005].

1. Using sterile technique, a colony taken from NA was inoculated into an Indole tube, containing peptone broth by means of stab inoculation.
2. The tube was inoculated overnight at 37ºC.
3. After incubation, 10 drops of Kovac's reagent were added and agitated gently.

The result is shown in figure 3.2 in the result section.

b) Methyl Red Test

Some bacteria oxidize glucose as a major substrate to produce large quantities of acidic end products. This acidic property of the media is detected by a pH indicator, methyl red. The indicator shows red color when at pH 4 (positive reaction) and yellow at pH 6 (negative reaction) [Cappuccino & Sherman, 2005].

1. Using sterile technique, a colony taken from NA was inoculated into a tube, containing peptone, dextrose and potassium phosphate by means of loop inoculation.
2. The tube was inoculated overnight at 37ºC.
3. After incubation, 5 drops of Methyl Red reagent were added.

The result is shown in figure 3.3 in the result section.

c) Voges-Proskauer's Test

Utilizing the organic acids that result from glucose metabolism, some bacteria are able to nonacidic or neutral end products. Detection of the end product requires oxidation of it into a diacetyl compound. This reaction occurs in the presence of α-naphthol catalyst and a guanidine group that is present in the peptone of the MR-VP medium.The α-naphthol catalyst is found in Barritt's reagent with a combination of 40% potassium hydroxide. The reaction

changes into a deep red color within 15 minutes of adding the reagent. The absence of color formation demonstrates a negative result [Cappuccino & Sherman, 2005].

1. Using sterile technique, a colony taken from NA was inoculated into a tube, containing peptone, dextrose and potassium phosphate by means of loop inoculation.
2. The tube was inoculated overnight at 37ºC.
3. After incubation, 10 drops of Barritt's A reagent were added, immediately followed by 10 more drops of Barritt's B reagent. The mixture was shaken well every 3 to 4 minutes. Result was recorded after 15 minutes.

The result is shown in figure 3.4 in the result section.

d) Citrate Utilization Test

Some microorganisms are able to utilize citrate with the help of citrate permease, an enzyme that facilitates the transport of citrate in the cell when other major substrates are scarce. Once the citrate is inside the cell, it is acted on by another enzyme, citrase. After a series of reactions, carbon dioxide is produced which changes the nature of the medium with the help of sodium and water. The production of sodium carbonate changes the bromothymol blue indicator incorporated into the medium from green to deep Prussian blue [Cappuccino & Sherman, 2005].

1. Using sterile technique, a colony taken from NA was inoculated into a vial, containing a slant of Simmon's Citrate agar by means of streak inoculation.
2. The vial was inoculated overnight at 37ºC.
3. Result observed after incubation.

The result is shown in figure 3.5 in the result section.

2.4 Preservation

Preservation was done to protect the organism from contaminations during interruptions of laboratory works due to sample collection, holidays etc. For short term preservation, T_1N_1 media was used.

2.4.1 Stock preparation

1. T_1N_1 media was prepared and 1.5 ml was taken into vials.
2. The vials were then autoclaved at 121ºC for 15 minutes. After autoclaving, they were kept at room temperature over night to gelatinize.
3. The next day, two *E. coli* colonies were taken from the NA subculture plate with the help of a sterile loop to inoculate the gelatinized T_1N_1 media, by means of stab inoculation. Two vials were inoculated in this method.
4. The vials were then incubated at 37ºC, overnight.
5. After incubation, the top of the media inside the vials were layered with 200 µl sterile glycerol. The vials were sealed with parafilm and kept at room temperature.

The growth of *E. coli* in T_1N_1 media is shown in figure 3.6 in the result section.

2.5 Serum Collection

Serum is the clear, yellow colored aqueous layer that can be isolated from clotted blood. It contains proteins of different types which participate in the defense mechanism of the body, called complement system. Serum is an environment in which bacterial cells should not exist. The serum complement system provides innate defense against microbial infections. It consists of at least 35 proteins, mostly in pre-activated enzymatic forms [Bugla-Ploskońska *et al*., 2009].

2.5.1 Location and Sample Number

Blood samples were collected mainly from two locations:

1. BRAC University (Urban area): 50 sample
2. T&T Slum (Slum area): 50 sample

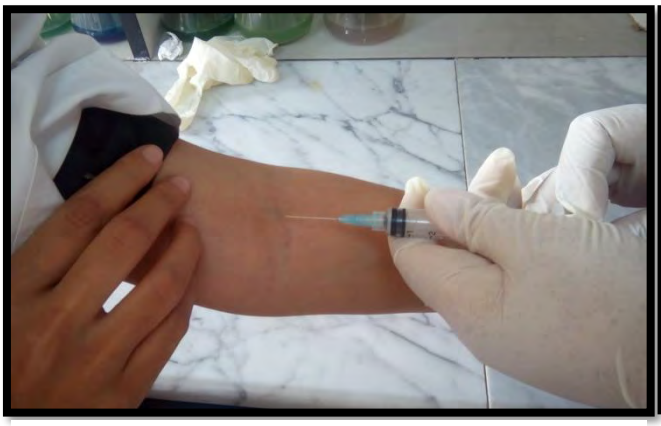


Figure 2.1: Collecting serum samples from BRAC University (Urban area)

Figure 2.2: Collecting serum samples from T&T Slum (Slum area)

N

During the sample collection procedure the area concerning safety of the volunteers were taken seriously since blood drawing is an invasive process. Each step was performed with extra caution. Below are some of the precautions taken during this procedure:

- Blood was collected with the help of an experienced nurse.
- New gloves were worn.
- Newly bought, sealed packet syringes were used to draw blood.
- Hexisol and fresh cotton were used to disinfect the area from where blood was later drawn.
- After drawing blood band aids were provided to the volunteers.
- Blood samples were stored in autoclaved test tubes at appropriate temperature.
- The used syringes were safely discarded so that other individuals as well as the environment do not come in contact with the potent dangers that might accompany bodily fluids.

2.5.2 Procedure

Serum isolation from the collected blood samples is one of the most important steps of the total procedure as this whole research work is based on serum activity. Serum collection procedure is elaborately described below:

1. The 5 ml blood was kept in a slanted position for 1 hour at 37ºC, just after the collection.
2. After the incubation the blood was kept at 4ºC overnight in a standing position.
3. The next day, the blood was split into 2 layers, one of clotted blood (blood cells) and the other of serum which is a clear, yellowish, aqueous layer.
4. 2 sterile micro centrifuge tubes were labeled according to the name of the individual to whom the blood sample belongs.

Figure 2.3: Serum collection

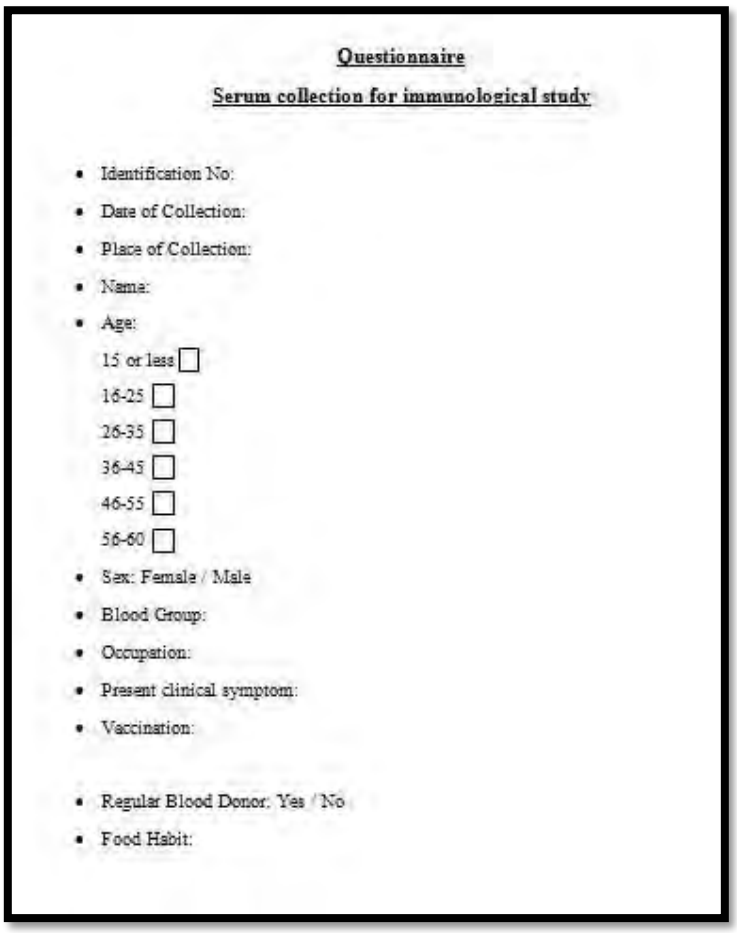
5. The yellowish clear serum layer was transferred into one of the micro centrifuge tubes. This step was repeated until the whole layer had been transferred. The transferring was only stopped when blood cells started to contaminate the drawn out serum layer.
6. To avoid the accidental blood cell in the isolated serum which may interfere with the serum activity, centrifugation at 3000 rpm for 10 minutes was done. This helps the remaining blood cells to form a precipitate, leaving a clear serum supernatant.
7. After centrifugation, the serum supernatant was transferred into the other sterile, labeled micro centrifuge tube.

The before and after serum isolation figures (figure number 3.7 and 3.8) are juxtaposed in the result section.

Each serum sample was isolated following these steps. All these steps were performed inside a laminar flow cabinet to avoid any and all kinds of contamination. All the equipments like test tubes, micro pipette tips, micro centrifuge tubes etc were previously autoclaved. Each and every work was done with extra caution to eliminate unwanted contamination and errors.

2.5.3 Survey

Before collecting blood, each of the volunteers was asked to fill out a questionnaire. A completed questionnaire would provide information about name, age, sex, blood group, occupation, present clinical symptoms, vaccination, donor ship and food habit about the volunteer and also other information such as place and date of collection.



Questionnaire

Serum collection for immunological study

- Identification No:
- Date of Collection:
- Place of Collection:
- Name:
- Age:

  15 or less ☐
  16-25 ☐
  26-35 ☐
  36-45 ☐
  46-55 ☐
  56-60 ☐
- Sex: Female / Male
- Blood Group:
- Occupation:
- Present clinical symptom:
- Vaccination:

- Regular Blood Donor: Yes / No
- Food Habit:

Figure 2.4: Questionnaire sample

This survey was done to help understand the body condition of the volunteer and its effect on the individual's serum activity. For e.g. if a volunteer is exposed to any diseases, if yes, then is the disease causing the serum to act differently than that of a healthy individual. It was kept in mind that each of the information can act as a factor that may or may not affect the serum activity. The place of collection is the most important piece of information which helped, in comparing the two different population (urban and slum area) based on their life style. Overall, this survey serves the purpose of aiding, strengthening and supporting the research procedure and thus establishing an organized, data based result.

2.5.4 Preservation

The serum samples were stored at -20ºC.

2.6 Bactericidal activity of human serum against *E. coli*

The serum activity testing procedure was divided into 3 parts, taking 3 days including observation of results. Below is the elaborate description of the procedure that had been followed for each individual serum.

Some steps of the method used in a research paper titled, "Killing of Gram-Negative Bacteria with Normal Human Serum and Normal Bovine Serum: Use of Lysozyme and Complement Proteins in the Death of *Salmonella* Strains O48" in 2009 by G. Bugla-Ploskońska and his team, were modified and followed in this procedure. The steps are,

1. The strains were grown overnight in YP medium and then 50 µl of the bacterial cultures was transferred to 3 ml of fresh YP medium and incubated at 37ºC for 1 h in a water bath [Bugla-Ploskońska *et al*., 2009].
2. After incubation the bacterial cells were centrifuged (2500xg for 20 min at 4°C) and suspended in physiological saline to obtain a six-fold dilution [Bugla-Ploskońska *et al*., 2009].
3. Then, the bacteria were mixed with NHS (Normal Human Serum). The serum was diluted with 0.1 M NaCl [Bugla-Ploskońska *et al*., 2009].
4. The bacteria with serum were incubated in a water bath at 37ºC. [Bugla-Ploskońska *et al*., 2009].
5. After 0 (T0) and 180 (T3) min, the samples were collected, diluted and cultured on nutrient agar plates for 18 h at 37ºC. The bacteria were cultured in solidified agar medium in Petri dishes [Bugla-Ploskońska *et al*., 2009].
6. The average number of colonies was estimated from the plates. [Bugla-Ploskońska *et al*., 2009].

2.6.1 Part 1 (Day 1):

1. 5 ml of sterile nutrient broth was taken. 1.5 ml was transferred into a sterile 2 ml microcentrifuge tube and 0.5 ml was discarded.
2. The rest of the 3 ml NB was inoculated with *E. coli* taken from the subculture with the help of a sterile loop.
3. The inoculated test tube was kept in an incubator at 37ºC for 24 hours.
4. The 2 ml microcentrifuge tube containing 1.5 ml NB was kept at 4ºC overnight.

2.6.2 Part 2 (Day 2):

1. The next day after incubation, 25 µl of the *E. coli* culture was transferred to the previously stored fresh 1.5 ml NB containing microcentrifuge tube.
2. The inoculated NB was incubated at 37ºC for 1 hour.
3. After the 1 hour incubation, the bacterial cells were centrifuged at (2500xg for 20 min at 4°C) and suspended in physiological saline to obtain a six-fold dilution
4. A new sterile microcentrifuge tube was taken for the preparation of serum-saline mixture. In this tube, 100 µl of serum and another 100 µl of sterile saline were taken. Thus a diluted serum mixture was prepared for the next steps.
5. In another sterile microcentrifuge tube, 150 µl of the serum-saline mixture and 150 µl of the *E. coli*-saline mixture were taken. The mixture was mixed well.
6. After 0 minute, 100 µl of the serum, saline & *E. coli* mixture was cultured on freshly prepared NA plate by means of spreading.
7. The microcentrifuge tube containing rest of the serum, saline & *E. coli* mixture was incubated for 180 minutes.
8. After 180 minutes incubation, 100 µl from the mixture was cultured on a new NA plate through spreading. The plates were then incubated at 37ºC overnight.

2.6.3 Part 3 (Day 3):

1. The next day, the 0 minute and 180 minutes plates were observed and results were recorded.

The 0 minute and 180 minutes results of 5 serum samples from each area are shown in figure 3.9 and 3.10 consecutively.

All these steps were performed inside a laminar flow cabinet to avoid all kinds of contamination. All the mediums and equipments like NA, NB, NS, Petri plates, test tubes, micro pipette tips, micro centrifuge tubes etc were previously autoclaved and sterile. Each and every work was done with extra caution to eliminate unwanted contamination and errors.

3. Results

3.1 Bacterial Strain

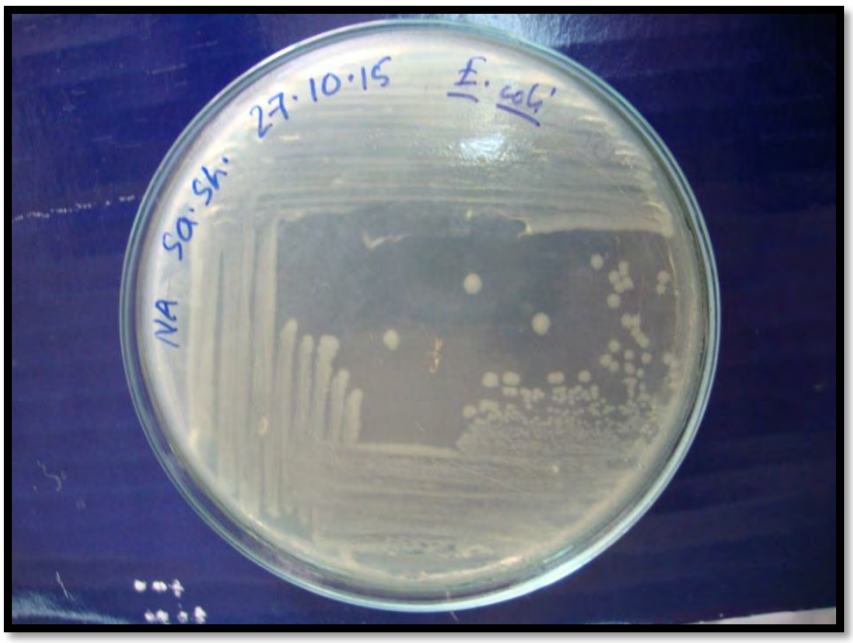


Figure 3.1: *E. coli* colonies on Nutrient Agar

E. coli give colorless colonies on Nutrient Agar. To keep the bacteria viable and free from contamination, subculture was done on NA weekly, via the streaking procedure.

3.2 Identification

Identification of the bacteria was done for reconfirmation. *E. coli* was reconfirmed by streaking on a selective media and by performing biochemical tests.

3.2.1 Selective Media

E. coli strain provided by the laboratory was streaked on EMB agar, a selective media for *E. coli* to confirm the presence of the bacteria. After overnight incubation at 37ºC, the growth was observed. The characteristic green sheen colonies of *E. coli* on EMB are shown in the figure given below.

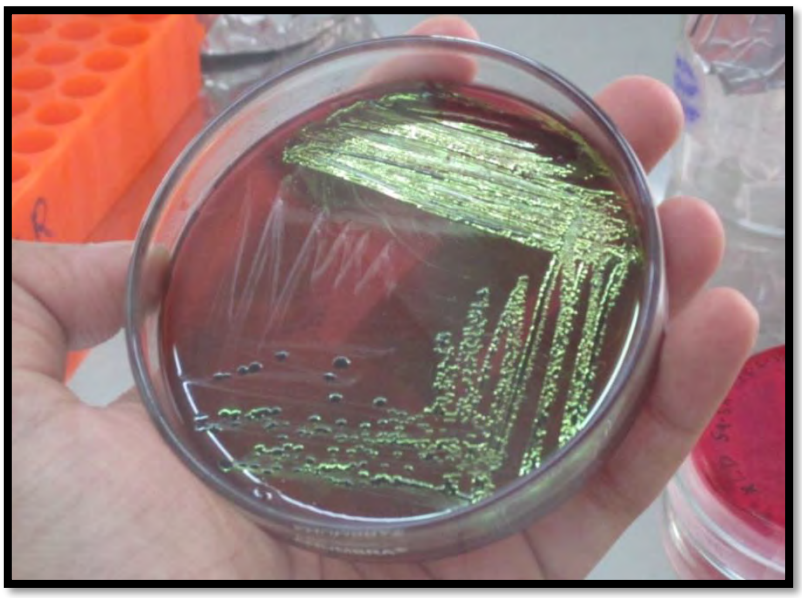


Figure 3.2: Green sheen *E. coli* colonies on EMB

As *E. coli* gives characteristic green sheen colonies on EMB, the presence of *E. coli* had been confirmed with this experiment.

3.2.2 Biochemical Tests

Isolate which gave metallic green sheen was subjected to different biochemical tests. The biochemical test results that the isolate showed were typical for *E. coli*.

a) Indole Production Test

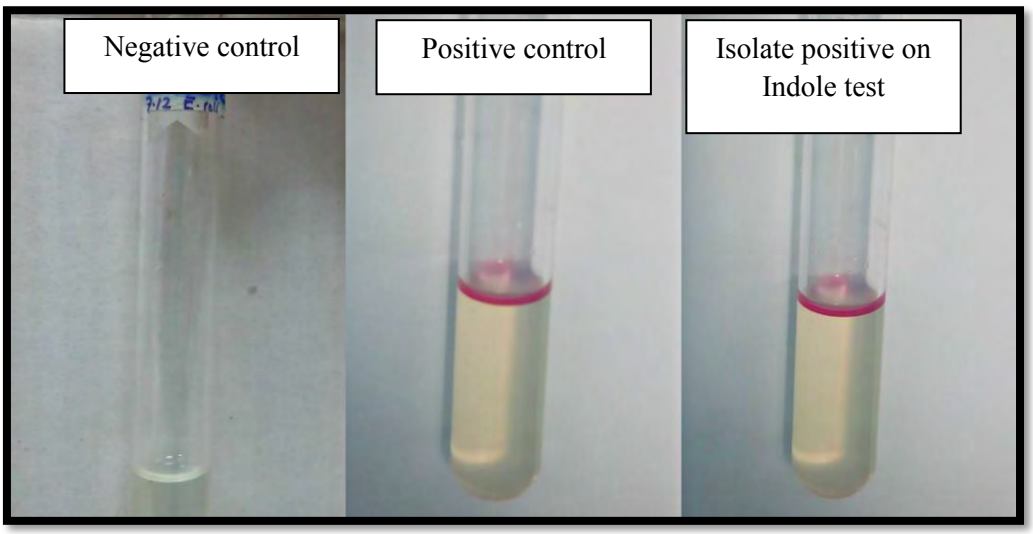


Figure 3.3: Test tubes showing result for Indole production (negative control, positive control, isolate)

b) Methyl Red Test

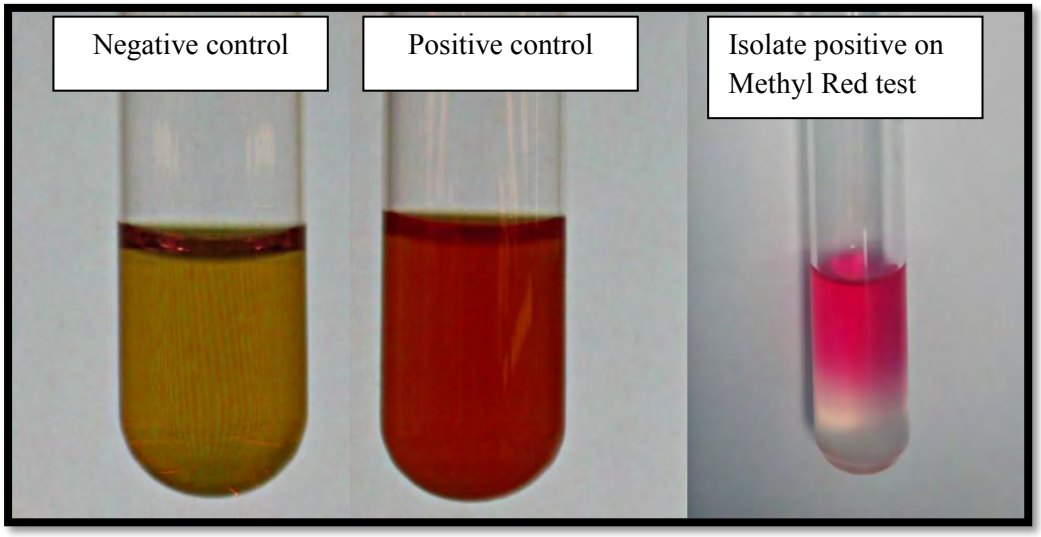


Figure 3.4: Test tubes showing result for Methyl Red test (negative control, positive control, isolate)

c) Voges-Proskauer's Test

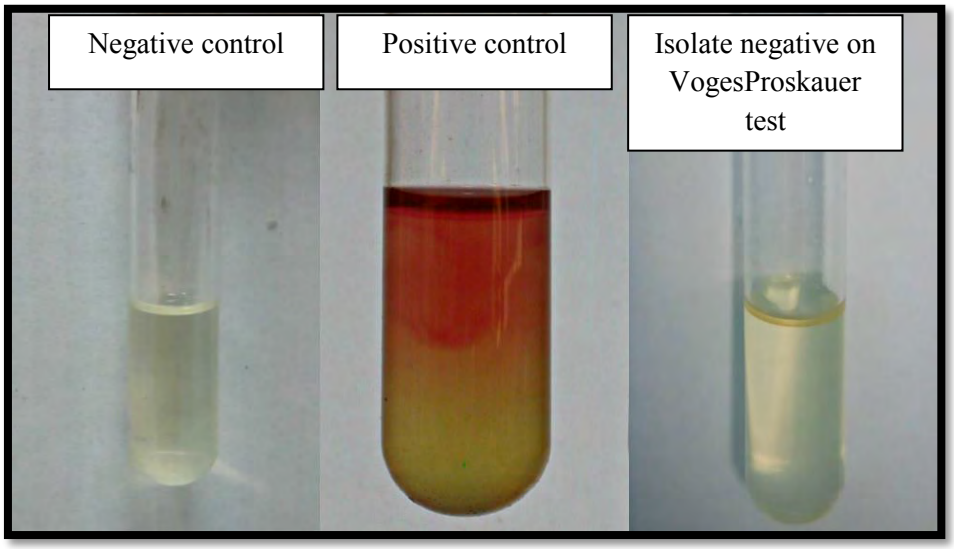


Figure 3.5: Test tubes showing result for Voges-Proskauer test (negative control, positive control, isolate)

d) Citrate Utilization Test

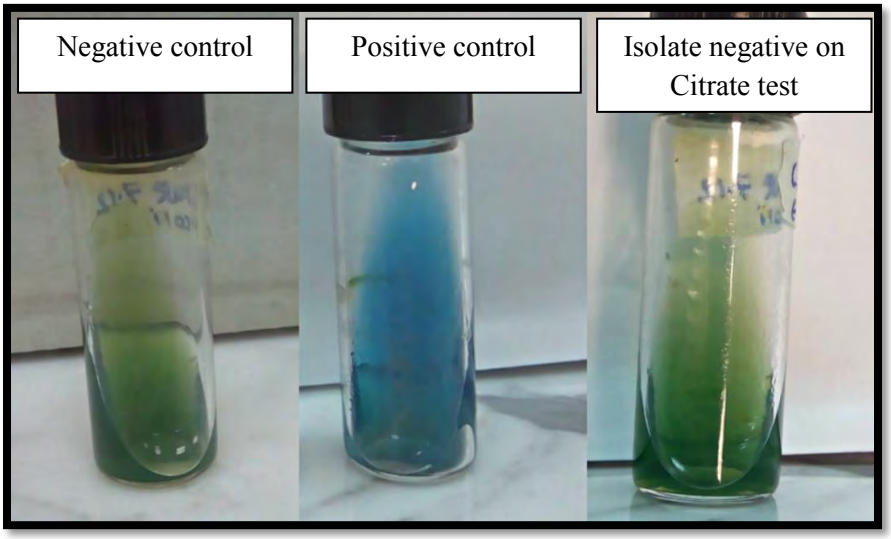


Figure 3.6: Test tubes showing result for Citrate test (negative control, positive control, isolate)

The selective media and biochemical test results ensure that the isolate tested here is of *Escherichia coli*.

3.3 Preservation

T_1N_1 media Preservation: T_1N_1 media was inoculated with *E. coli* for long term preservation. The freshly prepared media was inoculated, incubated at 37ºC overnight. After incubation, the media turned turbid in places where it had been stabbed with inoculated loop thus ensuring the growth of the bacteria.

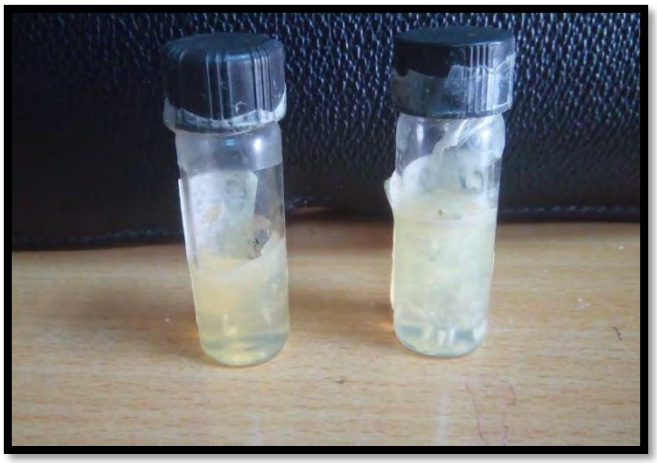


Figure 3.7: Inoculated T_1N_1 media

3.4 Serum collection

Figure 3.8: Blood and serum in separate layers after overnight refrigeration

Figure 3.9: Fresh serum after isolation

After collection, the sample blood was subjected to 1 hour incubation at 37ºC and overnight refrigeration at 4ºC. After 24 hours, the separated layer of serum was collected into sterile microcentrifuge tubes and centrifuged until a fresh serum sample free from blood cells was isolated.

3.5 Serum activity

The following table shows the activity of serums collected from both urban and slum area, against *E. coli* at two different times. The 0 minute result usually shows a high count due to the lack of time which the serum needs to act upon the bacteria. But with time as the serum starts to inhibit the growth of the bacteria, the 180 minutes result shows a low count.

Table 2: The ability of 25 representative serum samples from urban area (BRAC University), to inhibit the growth of *E. coli*

Sample Number	Urban Area (BRAC University)	
	No. of colonies at 0 minute	No. of colonies at 180 minutes
1	540	2
2	304	1
3	72	0
4	1516	0
5	10	0
6	276	2
7	3	0
8	352	4
9	568	3
10	468	26
11	328	1
12	296	0
13	45	0
14	96	0
15	45	0
16	124	0
17	58	0
18	10	0
19	204	0
20	67	0

21	206	0
22	390	0
23	172	0
24	620	0
25	350	5

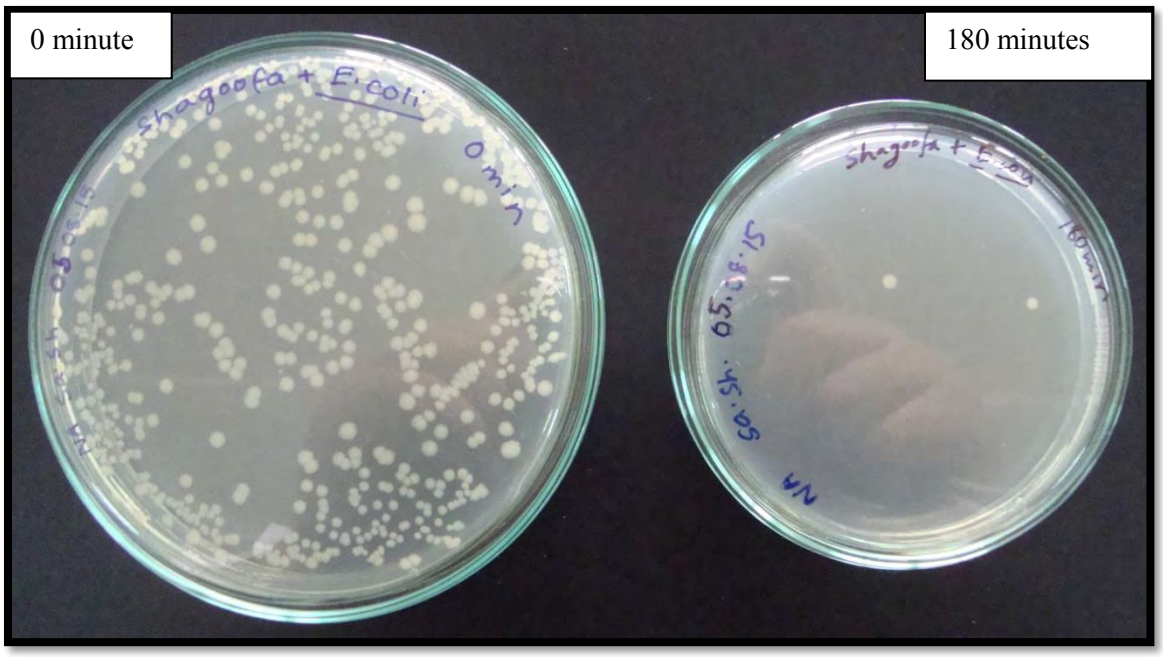
Table 3: The ability of 25 representative serum samples from slum area (T&T Slum), to inhibit the growth of *E. coli*

Sample Number	Slum Area (T&T Slum)	
	No. of colonies at 0 minute	No. of colonies at 180 minutes
1	2640	1
2	808	3
3	724	0
4	1044	0
5	1480	0
6	1350	33
7	1408	1
8	1040	0
9	232	1
10	253	2
11	1412	1
12	2048	4
13	736	1
14	1688	0

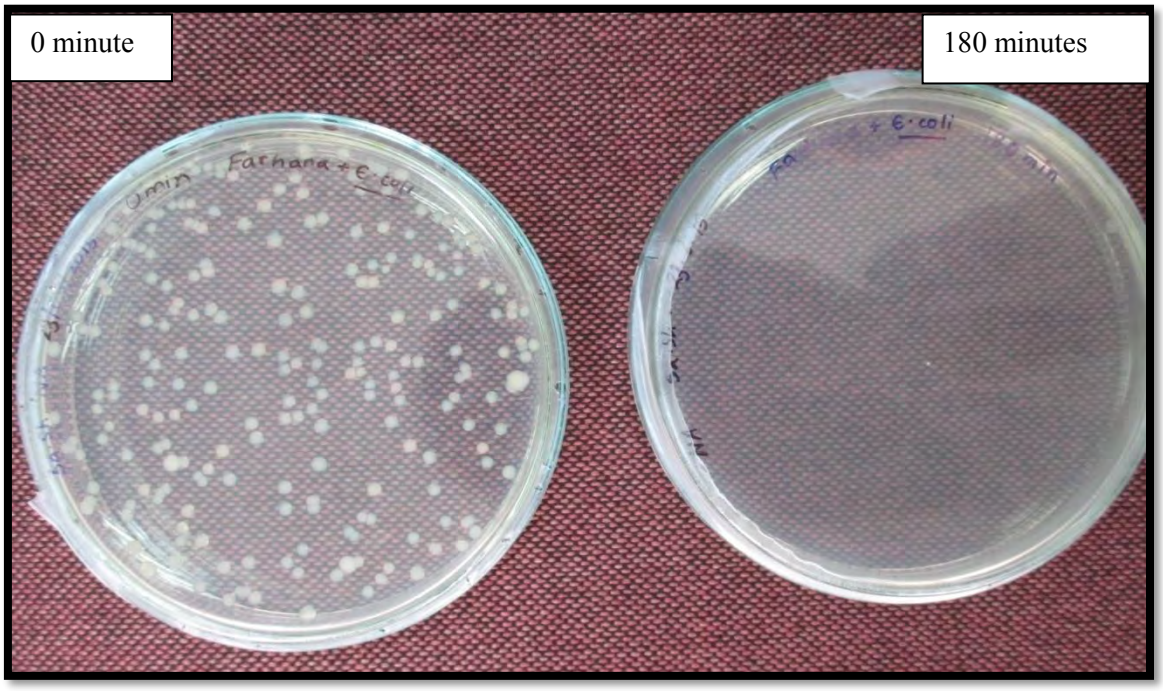
15	1100	2
16	320	0
17	736	9
18	261	0
19	126	0
20	128	0
21	72	0
22	142	0
23	272	1
24	128	0
25	310	4

Below are figures of serum complement activity of 5 serum samples from each area at 0 minute and 180 minutes.

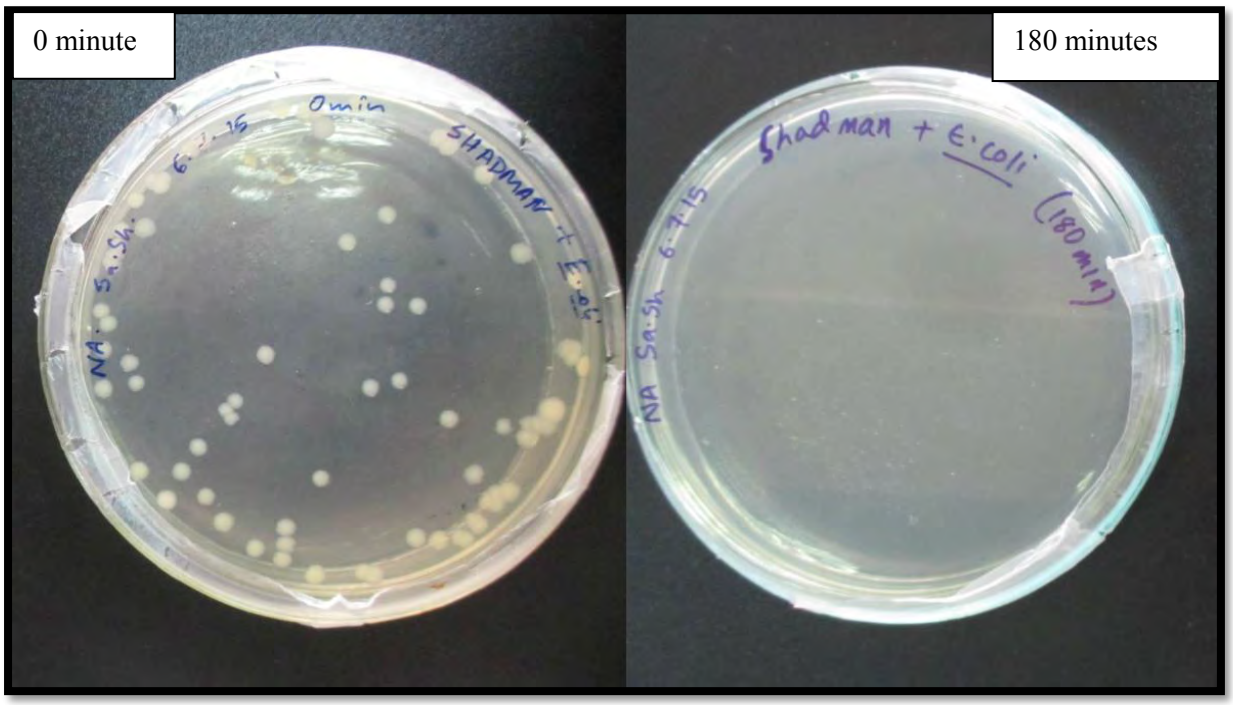
3.5.1 Five Serum Samples from Urban Area, BRAC University



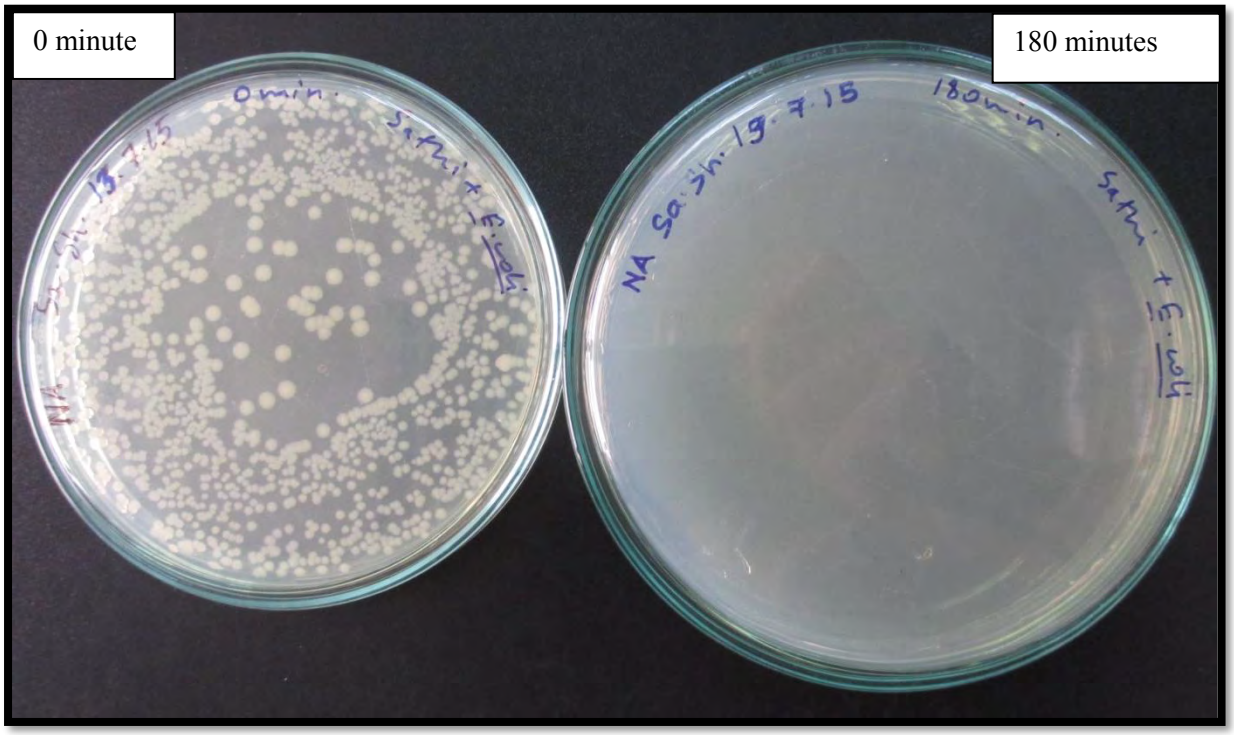
a)



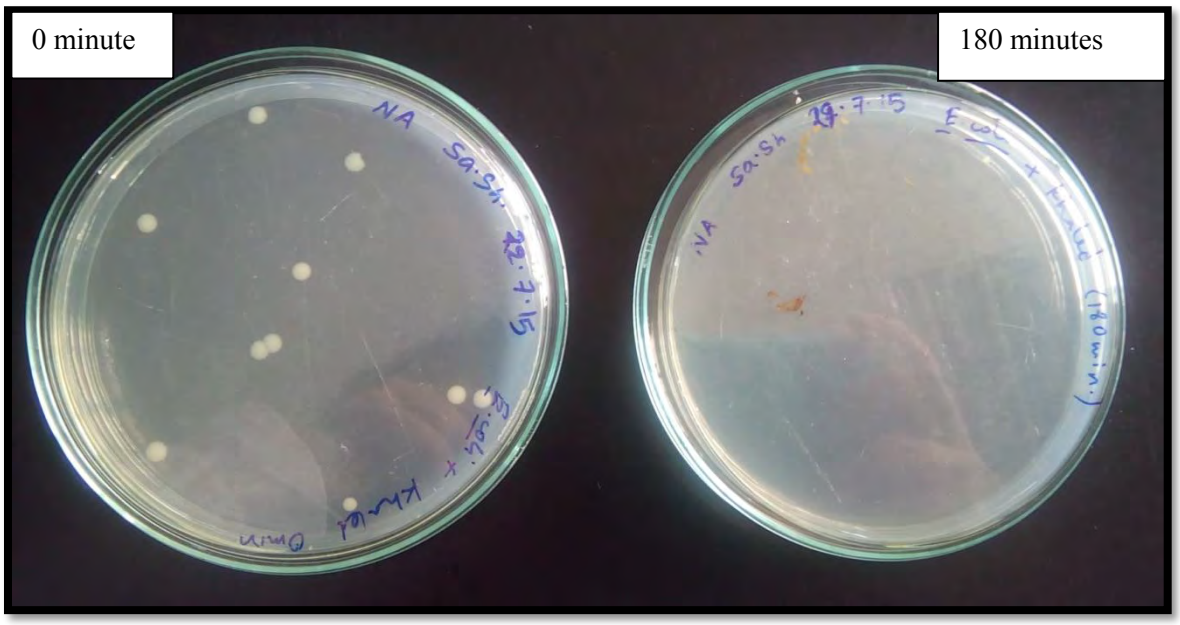
b)


0 minute
180 minutes
0min
SHADMAN + E.coli
NA Sa.Sh. 6.7.15
Shadman + E.coli (180 min)

c)


0 minute
180 minutes
0min
Sathi + E.coli
NA Sa.Sh. 13.7.15
180min
NA Sa.Sh. 19.7.15

d)



e)

Figure 3.10:

a) Urban serum sample 1 activity at 0 minute & at 180 minutes

b) Urban serum sample 2 activity at 0 minute & at 180 minutes

c) Urban serum sample 3 activity at 0 minute & at 180 minutes

d) Urban serum sample 4 activity at 0 minute & at 180 minutes

e) Urban serum sample 5 activity at 0 minute & at 180 minutes

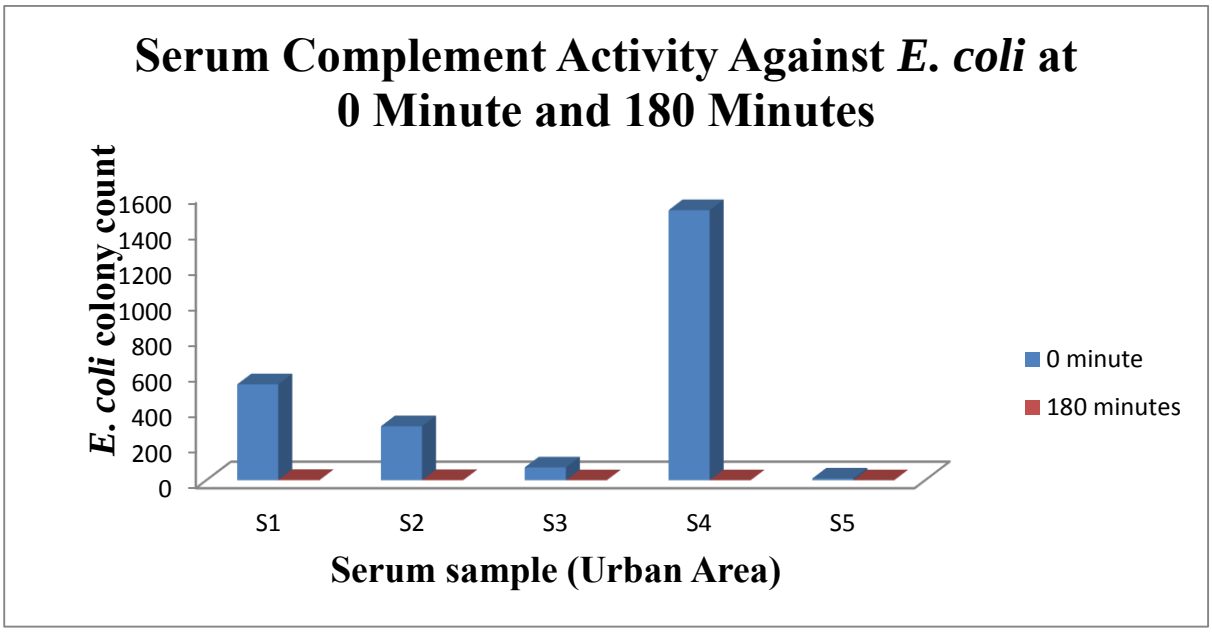
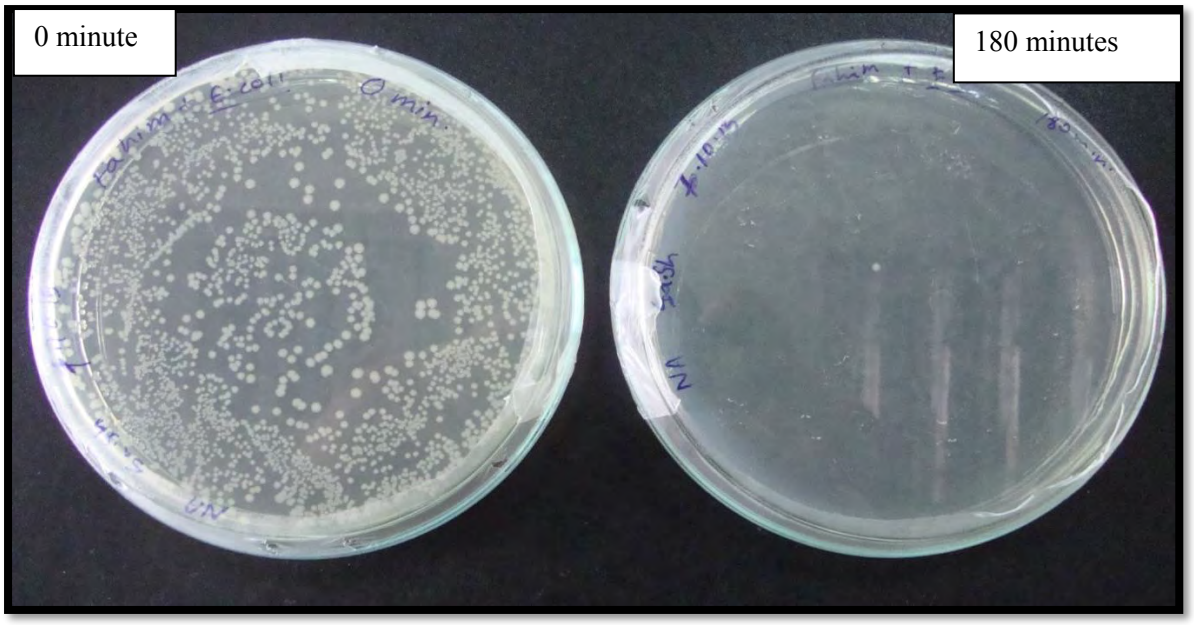
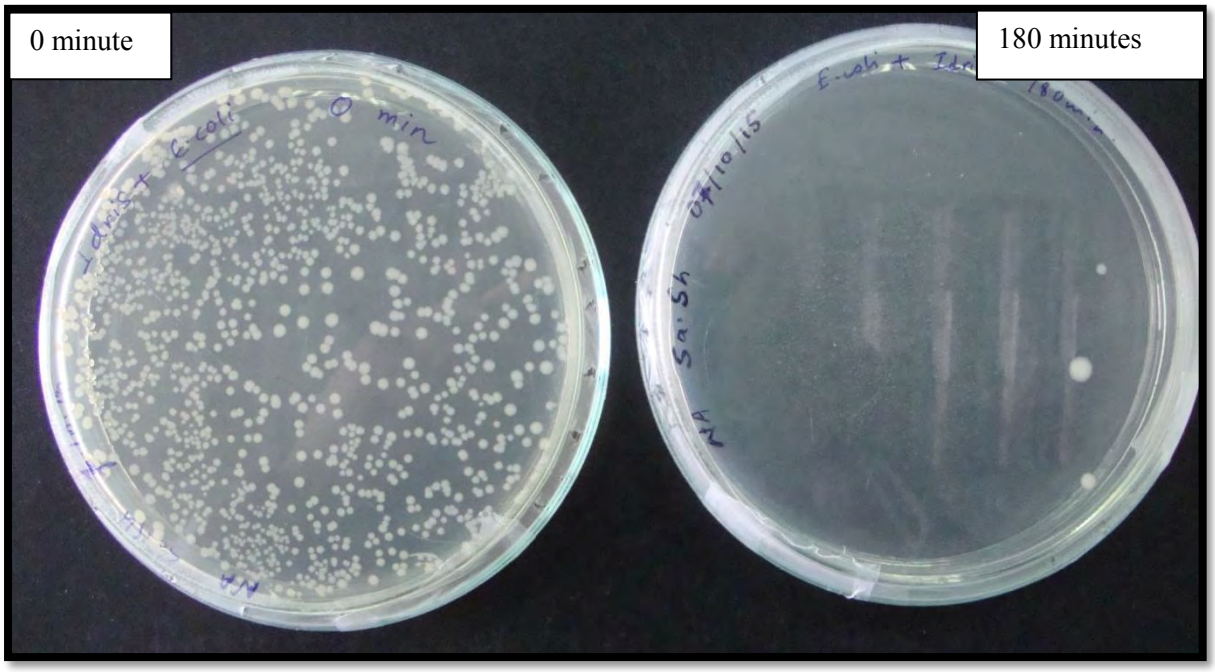


Figure 3.11: Graphical Representation of Complement Activity of Different Serum Samples (BRAC University) against *E. coli* at 0 Minute and 180 Minutes

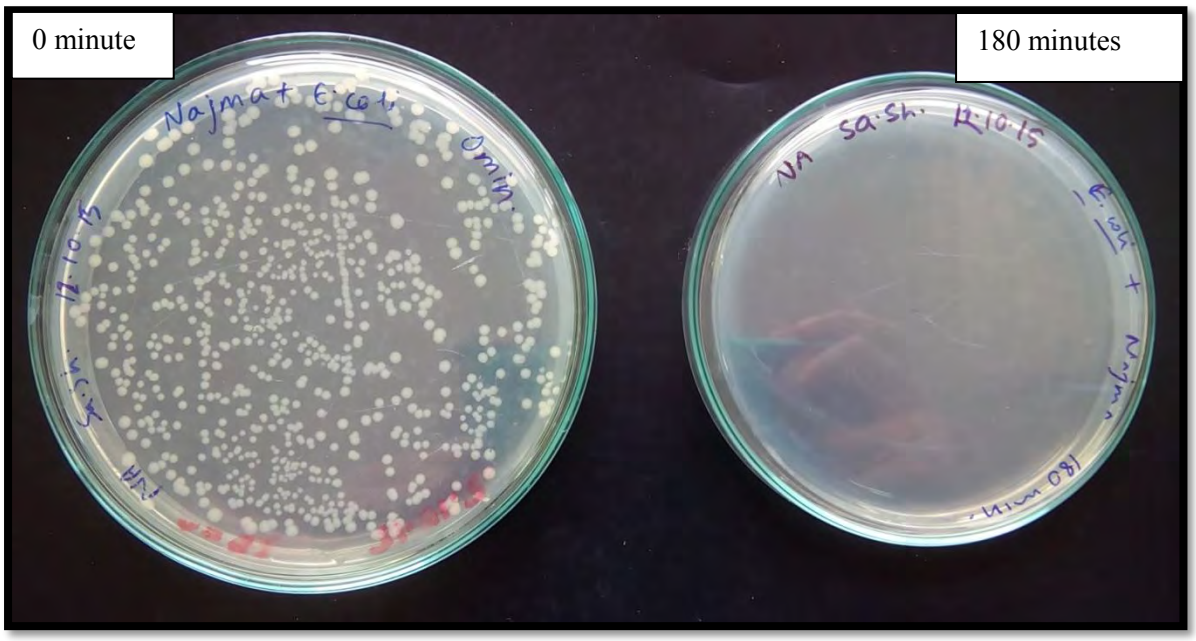
3.5.2Five Serum Samples from Slum Area, T&T Slum



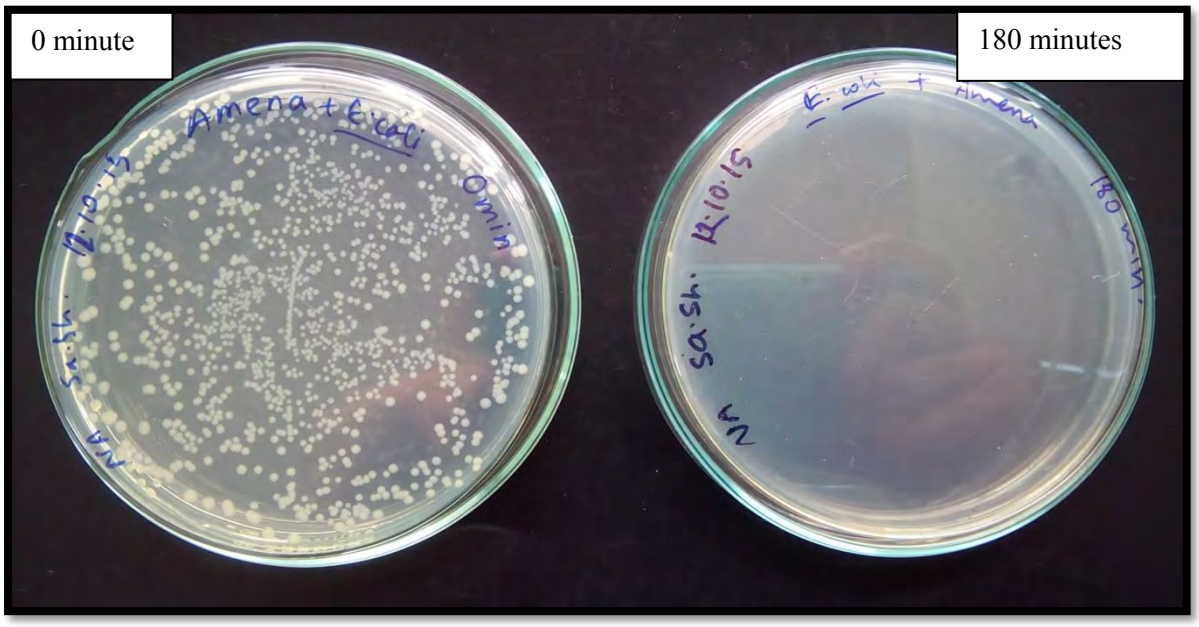
a)



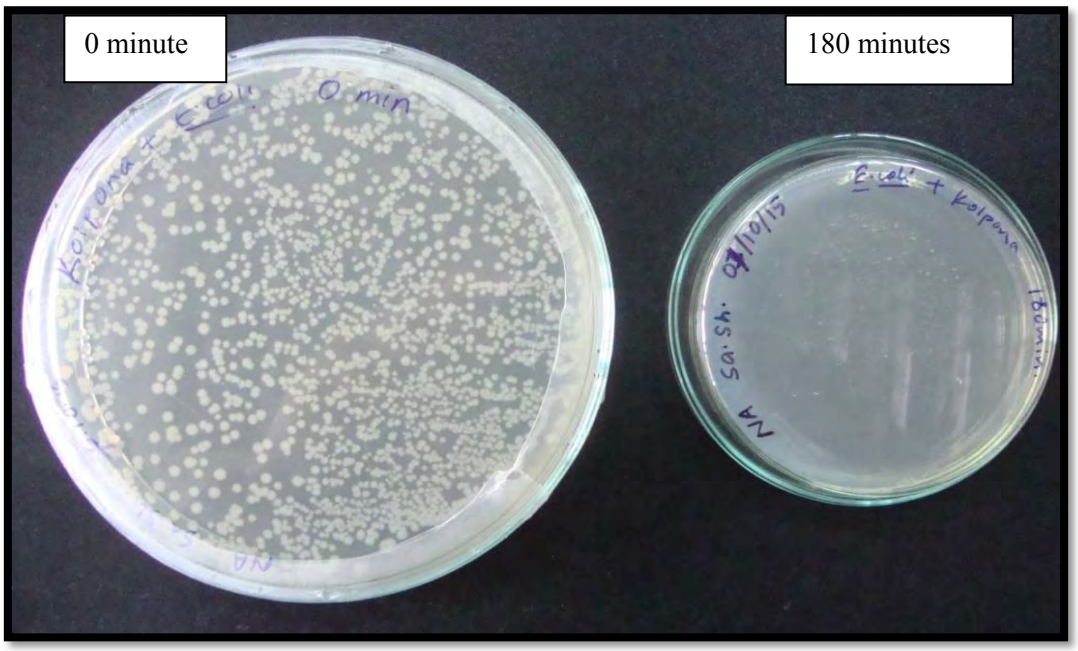
b)


0 minute
180 minutes
Najma + E.coli
0min.
NA
Sa.Sh.
12:10:15
E. coli + Najma
180 min

c)


0 minute
180 minutes
Amena + E.coli
0min
NA
Sa.Sh.
12:10:15
E. coli + Amena
180 min

d)



e)

Figure 3.12:

a) Slum serum sample 1 activity at 0 minute & at 180 minutes

b) Slum serum sample 2 activity at 0 minute & at 180 minutes

c) Slum serum sample 3 activity at 0 minute & at 180 minutes

d) Slum serum sample 4 activity at 0 minute & at 180 minutes

e) Slum serum sample 5 activity at 0 minute & at 180 minutes

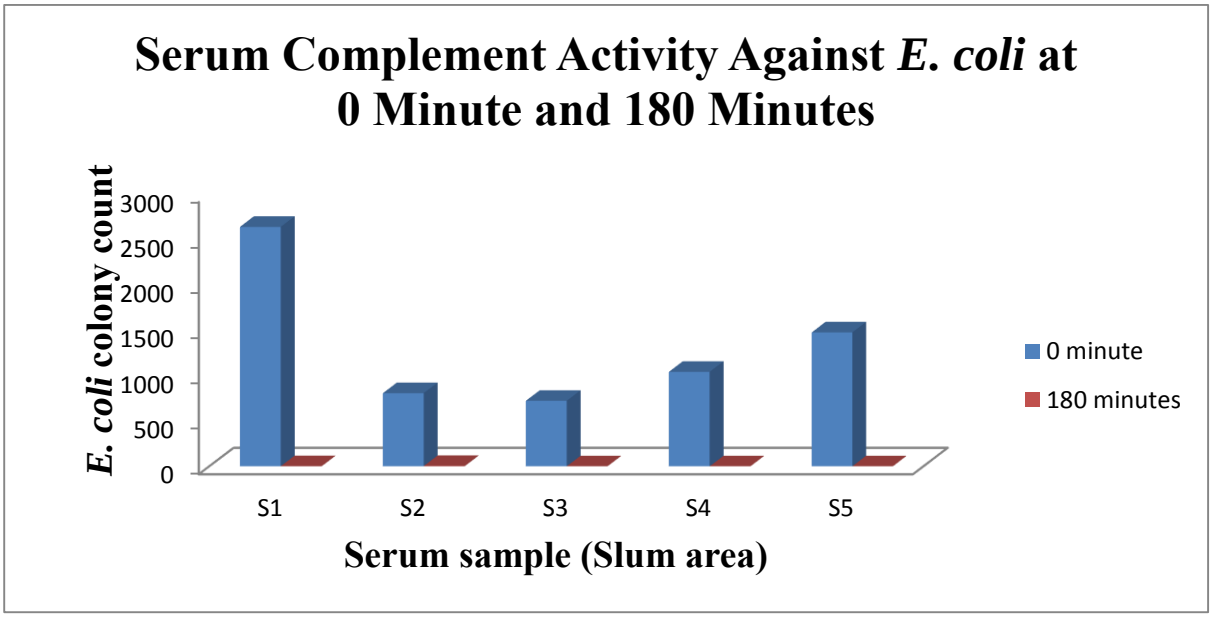


Figure 3.13: Graphical Representation of Complement Activity of Different Serum Samples (T&T Slum) against *E. coli* at 0 Minute and 180 Minutes

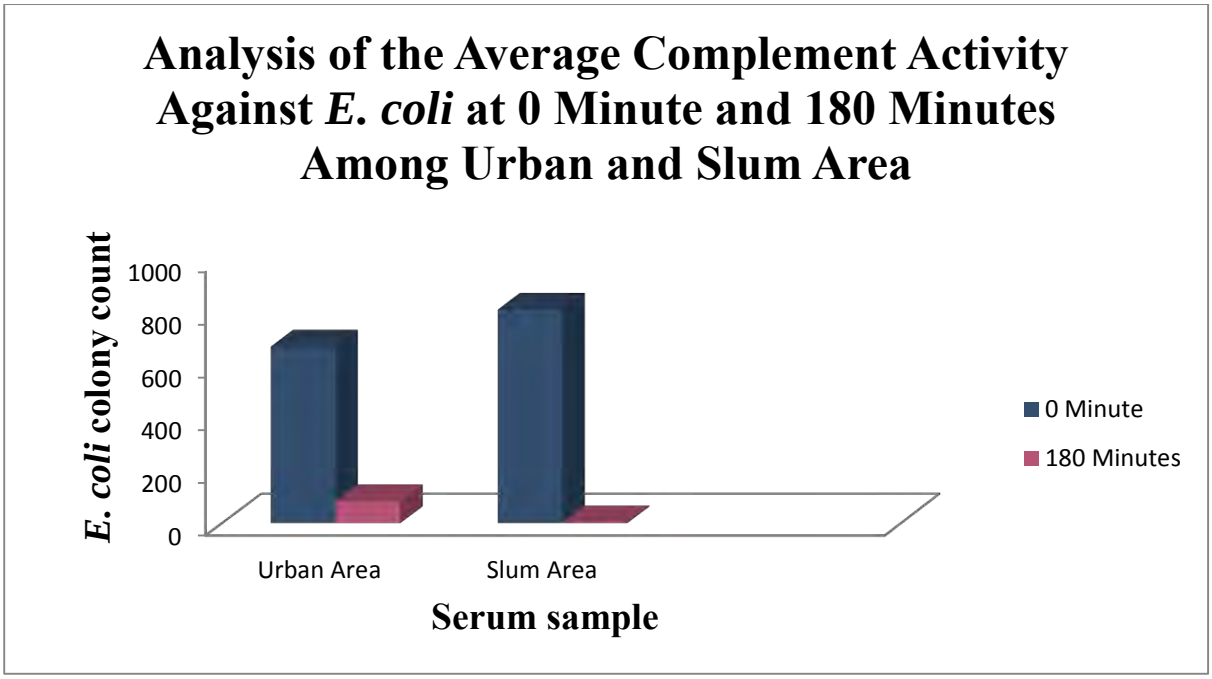


Figure 3.14: Graphical Representation of Analysis of the Average Serum Complement Activity against *E. coli* at 0 Minute and 180 Minutes of Both Urban and Slum Area Samples

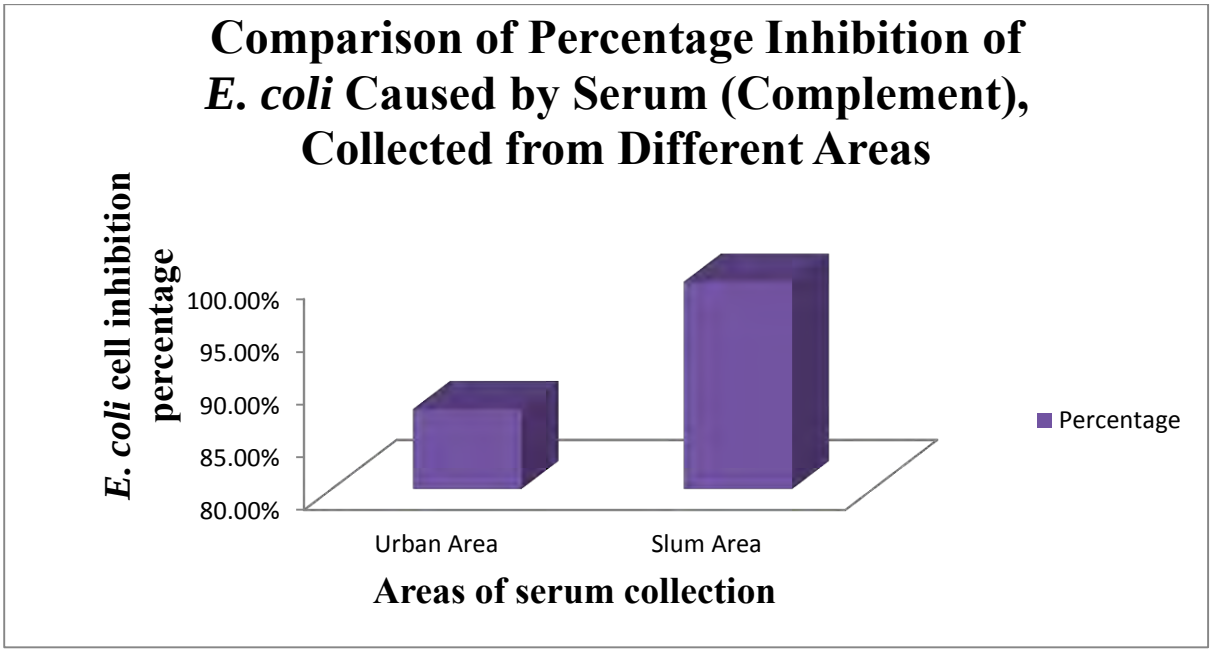


Figure 3.15: Graphical Representation of Comparison of Percentage Inhibition of *E. coli* Caused by Serum (Complement), Collected from Different Areas

4. Discussion and Conclusion

Despite of being a beneficial gut flora, *Escherichia coli* threatens humans with various diseases. *E. coli* has proved to be one of the major causes of mild and severe diarrhea [Bower *et al.,* 1999]. Since the organism is adapted to thrive in the intestinal environment, the incidence of intestinal diseases caused by the species is high than that of others. But with time *E. coli* has been able to expand its habitat in the human body and has been able to cause diseases like urinary tract infections, bacterimia, cholangitis, pneumonia, neonatal meningitis etc [Madappa, 2015]. There is now no reason to take this organism lightly since it has the potential to cause diseases that can be fatal.

As *E. coli* is a normal flora of the intestines of animals, the organism is found abundantly in the environment through feces. So each and every individual comes in contact with it. Intestinal diseases with higher prevalence than other *E. coli* related diseases, occur when the organism enters human body through fecal contaminated food, water etc. *E. coli* related enteric infections have a greater prevalence in Bangladesh than most diseases. 34% of the diarrhea related infections are caused by the diarrheal *E. coli* in Bangladesh. Bangladesh being a densely populated, small developing country, a large portion of the population is living below the poverty line. Naturally this part of the population is more exposed to the organism than those who are well off. It can be assumed that people who are living in slums would be more adapted to *E. coli* than those who do not. This study is reporting if the different lifestyle of the two sections of the population (one living in urban area, the other in slum area) of Bangladesh has any affect on the complement inhibition activity of the serums collected from both the places against *E. coli*. The comparative complement activity can be obtained from the column charts from the result section.

In "Analysis of the Average Complement Activity against *E. coli* at 0 Minute and 180 Minutes Among Urban and Slum Area" (fig. 3.13) chart, the average of the cell counts of 50 serum samples, both at 0 minute and 180 minutes was taken. This was done for both urban and slum area samples. The columns show that the complement inhibition of the slum area samples was much greater than that of the urban area samples. For better understanding another column chart was prepared, "Comparison of Percentage Inhibition of *E. coli* Caused by Serum (Complement), Collected from Different Areas" (fig. 3.14) from the previous chart where the complement mediated inhibition of *E. coli* cells of both the population was presented as percentages. This column chart clearly shows the difference between urban and

slum area complement mediated inhibition. Urban area serum samples were able to inhibit *E. coli* cells at approximately 87.59%. On the other hand slum area serum samples were able to inhibit *E. coli* cells at approximately 99.67%.

Both the charts reveal that the differences in living condition or life style do affect the complement mediated inhibition activity of human serum. Both the urban and slum population more or less has been exposed to *E. coli* but the slum population has adapted to the organism since they are more exposed to it through poor living condition. The complement system of their serum has been able to kill off more of the organism. Thus the slum population is able to show greater resistance than that of urban people against *E. coli*. The people living in slums can be more exposed to various potentially pathogen organisms due to a different lifestyle than people who live in the urban area but this exposure may actually aid the slum people in having a better immunity.

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APPENDIX-I

Media composition

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

1. Nutrient Agar (Himedia, India)

Ingredients	Amount (g/L)
Peptic digest of animal tissue	5.0
Beef Extract	1.50
Sodium chloride	5.0
Yeast extract	1.50
Agar	15.0

2. Nutrient Broth (Oxoid, England)

Ingredients	Amount(g/L)
Lab-lemcopowder	1.0
Yeastextract	2.0
Peptone	5.0
Sodiumchloride	5.0

3. T_1N_1 soft agar

Ingredients	Amount(g/L)
Tryptone	0.6 g
Sodiumchloride	0.3g
Agar	0.42 g

4. Simmon's citrate agar (Oxoid, England)

Ingredients	Amount(g/L)
Magnesiumsulfate	0.2
Ammonium dihydrogenphosphate	0.2
Ammoniumphosphate	0.8
Sodiumcitrate	2.0
Sodiumchloride	5.0
Agar	15.0
Bactobromthymolblue	0.08

5. MR-VP broth

Ingredients	Amount(g/L)
Peptone	7 g
Dextrose	5 g
Potassiumphosphate	5 g

6. Eosine methylene blue agar (Oxoid, England)

Ingredients	Amount(g/L)
Peptone	10.0
Sucrose	5.0
Lactose	5.0
Di-potassiumphosphate	2.0
EosinY	0.14
Methyleneblue	0.065
Agar	13.50

7. Peptone Water

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

APPENDIX-II

Buffers and Reagents

1. Kovac's reagent

5 g of para-dimethylaminobenzaldehyde was dissolved in 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 4ºC.

2. Methyl red reagent

0.1 g of methyl red was dissolved in 300 ml of 95% ethyl alcohol. Then distilled water was added to make the final volume 500 ml. This reagent was covered with aluminum foil and stored at 4ºC.

3. Barritt's reagent

Solution A

5 g of alpha-naphthol was dissolved in 95% ethanol. This solution was covered with aluminum foil and stored at 4ºC.

Solution B

40 g 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. This solution was covered with aluminum foil and stored at4ºC.

APPENDIX-III

Instruments

The important equipments used through the study are listed below:

Serial Number	Name of Item	Specification
1	Autoclave	Model: HL-340, Gemmy Industrial Cor., Taiwan
2	Freezer(-20°C)	Siemens, Germany
3	Incubator	Model: DSI500D, Taiwan
4	Micropipette(2-20 µl)	Eppendorf, Germany
5	Micropipette(20-200 µl)	Eppendorf, Germany
6	Micropipette(100-1000 µl)	Eppendorf, Germany
7	Oven (Microwave oven)	Model: MH6548SR, LG China
8	Refrigerator (4^oC)	Model: 0636, Samsung
9	Balance	Radwag, WTB200
10	Centrifuge (High speed refrigerated micro centrifuge)	Model: GAM 1.5-24 Scanspeed 1730, Denmark
11	Vortex Mixture	VM-2000, digisystem, Taiwan
12	Hot air oven (Sterilizer)	Model: 02G, Jero Tech, Korea
13	Laminar air flow cabinet	SAARC
14	Shaking incubator	Model: WIS-20R, Daihan Scientific, Korea
15	Disposable micropipette tips	Eppendorf, Ireland