Comparative Study of Complement Protein Activity of Blood Serum against *Shigella flexneri* in Urban and Slum population of Bangladesh

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DECLARATION

I hereby declare that the thesis project titled "**Comparative Study of Complement Protein Activity of Blood Serum against *Shigella flexneri* in Urban and Slum population of Bangladesh**" submitted by me has been carried out under the supervision of Fahareen-Binta-Mosharraf, Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is based on actual and original work carried out by me. Any reference to work done by any other person or institution or any material obtained from other sources have been duly cited and referenced.

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Dedicated to…

My Parents

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ABSTRACT

Complement are group of serum proteins which can be activated by antigen-antibody complexes or other substances, which may result in lysis of a microbial target, or a variety of other biological effects important in both innate and adaptive immunity. The last several years have seen an enormous expansion of parallel research on bacteria and the complement system and acquaint the role of complement proteins in biological phenomena. This study sought to find out the in vitro examination of bactericidal action complement proteins derived from blood sera of a defined group of urban and slum people against *Shigella flexneri* generating a critical problem in modern medical therapy for bacterial infections. This comparative study showed the susceptibility of clinical isolates of *Shigella flexneri* to bactericidal action of complement proteins of both urban and slum people blood serum. However the slum people indicated more effective complement mediated killing in comparison to urban people.

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LIST OF ABBREVIATIONS

LPS	Lipoplysaccharide
Stx	Shiga Toxin
HUS	Hemolytic Uremic Syndrome
T3SA	Type III Secretion Apparatus
CDPH	Chicago Department of Public Health
CDC	Centers for Disease Control and Prevention
OAg	O Antigen
T3SS	Type III Secretion System
Ipa	Invasion plasmid antigen
vir	Virulence
ics	Intracellular spread gene
IL-1β	Interleukin-1β
NK cells	Natural Killer cells
FAE	Follicular Associated Epithelium
IFN-γ	Gamma interferon
PMN	Polymorphonuclear cells
BGS	Buffered Glycerol Saline
TMP-SMX	Trimethoprim-Sulfamethoxazole
AAP	American Academy of Pediatrics

MDPH	Michigan Department of Public Health
icddr,b	International Centre for Diarrhoeal Disease Research, Bangladesh
NE	Neutrophil Elastase
CFUs	Colony Forming Units
XLD	Xylose-Lysine-Deoxycholate

1. Background

1.1 *Shigella spp.*

Shigella are gram-negative, non-motile, facultatively anaerobic, non-sporulating, rod shaped bacteria that cause the disease shigellosis, which is also known as bacillary dysentery. Organisms of the genus *Shigella* belong to the *Enterobacteriaceae* family [Hale & Keusch, 1996]. This group of bacteria was first described by and named after Japanese scientist Kiyoshi Shiga in 1898, after he isolated what he called *Bacillus dysenteriae* (now known to be *Shigella dysenteriae* serotype 1) from a patients' stool during a dysentery epidemic in Japan in 1897 [Lonnen, 2007, Thatcher, 2005]. *Shigella* infection is a major public health problem in developing countries with poor sanitation. Humans are the natural reservoir for this organism. Endogenous *Shigella* species are not present in any natural food products, but a wide variety of foods may be contaminated [Sureshbabu *et al.*, 2014]. Transmission of the bacteria occurs by the fecal–oral route. *Shigella* species have a very low infective dose, as low as 10 to 100 organisms. Once ingested these survive gastric acidity and invade the colonic mucosa, resulting in mucosal abscesses and ulceration [Weir, 2002]. Destruction of the epithelial layer causes symptoms like watery diarrhoea, severe abdominal pain and cramping, eventuating in the bloody mucoid stool characteristic of bacillary dysentery. If needed treatment is not provided then shigellosis patients may develop secondary complications such as septicaemia and haemolytic uremic syndrome [Jennison & Verma, 2004].

1.1.1 The Four Species of *Shigella spp.*

One of the major diarrheal diseases in Bangladesh, especially among children, as well as in several other developing countries is shigellosis [Haider *et al.*, 1989]. It is caused by any one of the four species or groups of *Shigella*, namely, *S.dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei* [Talukdar *et al.*, 2001]. Each of these species, with the exception of *S.sonnei*, is further subdivided into serotypes. Serotypes are defined based on the structure of exposed terminal O polysaccharides that form part of their outer membrane lipopolysaccharides (LPS) [Zaidi & Estrada-Garcia, 2014].

(a) ***Shigella dysenteriae***

The species *Shigella dysenteriae* is also referred to as Group A. It has 13 serotypes [Sureshbabu *et al.*, 2014]. *S.dysenteriae* serotype 1 is known as Shiga's bacillus. It is indole negative and is the only member of the family that is always catalase negative [Parija, 2009]. It is the agent of epidemic shigellosis and is responsible for large-scale outbreaks in Central Africa, Southeast Asia, and the Indian subcontinent. In endemic areas it is isolated from up to 30% diarrheal patients [Hale, 1991]. *Shigella dysenteriae* serotype 1 is unique among *Shigella* species because it produces a potent toxin known as the Shiga toxin (Stx). Stx is a bipartite molecule composed of a single enzymatic A subunit and a pentamer of receptor-binding B subunits. The toxin binds to a glycolipid receptor found in target cells, globotriaosylceramide. It is then endocytosed preferentially by the clathrin-coated pathway. The A subunit is proteolytically cleaved and reduced, generating an A1 and an A2 peptide. The A1 peptide inhibits mammalian protein synthesis by cleaving the N-glycosidic bond at adenine residue 4324 in the 28S RNA of the 60S host cell ribosome. The importance of this toxin is that infections with Stx-producing bacteria may lead to hemolytic uremic syndrome (HUS), an often fatal kidney failure condition, particularly in children [Torres, 2004].

(b) ***Shigella flexneri***

Shigella flexneri is named after Flexner (1900), who described the first of the mannitol fermenting shigellae from Phillipines [Parija, 2009]. The species is also referred to as Group B and has 6 serotypes. It causes shigellosis or bacillary dysentery. *S. flexneri* is widespread in developing countries and is responsible for the worldwide endemic form of this disease [Sureshbabu *et al.*, 2014]. Among the four species of *Shigella*, *S. flexneri* is the most prevalent species in Bangladesh [Haider *et al.*, 1989]. This human-specific pathogen is transmitted via the fecal–oral route. It targets the large intestine, resulting in acute inflammation, tissue edema, and erosion of the colonic epithelium. The infection strategy of *S. flexneri* is based on:

(i) The transfer of bacterial proteins, termed "effectors," into targeted host cells through the type III secretion apparatus (T3SA), which induces the uptake of the bacteria and perturbs host cellular processes

(ii) The capacity of the intracellular bacteria to spread from cell to cell using actin microfilament-mediated cytoplasmic movement and reactivation of the T3SA [Arena *et al.*, 2015]

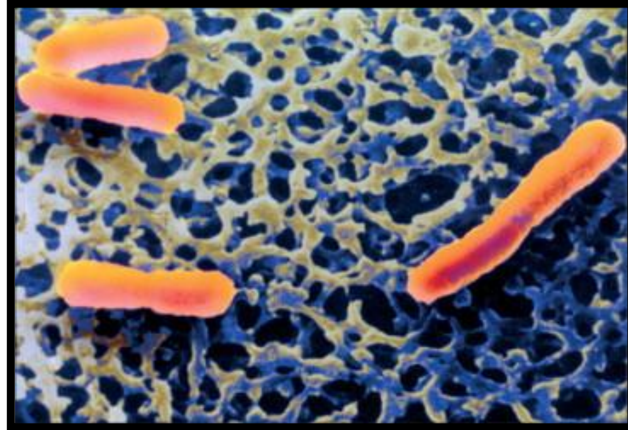


Figure 1.1: Colored scanning electron micrograph showing *Shigella flexneri* [CDC, 2011]

(c) *Shigella boydii*

Shigella boydii is named after Boyd, who first described these strains from India (1931) [Parija, 2009]. The species is also referred to as Group C and has 18 serotypes [Sureshbabu *et al.*, 2014]. In comparison with other *Shigella* serogroups this species has been less often reported worldwide. *S.boydii* is typically related with people who have travelled to endemic areas but it is relatively rare in developed countries. Isolation rate of this species is less than 1–2% of the total *Shigella* isolates, except in the Indian subcontinent [Ranjbar *et al.*, 2008].

Shigella of any species can cause serious illness among people with compromised immune systems. Shigellosis is characterized by watery, bloody, or mucoid diarrhea; fever; stomach cramps; and nausea [Bowen, n.d.]. There are many reports of outbreaks of shigellosis in which different kinds of foods are involved as the vehicle of transmission of *Shigella* spp. In fact, data based on public health reports indicate that food-borne *Shigella* infections are more common than waterborne infections in the United States and other industrialized countries [Islam *et al.*, 1993]. In 1998, an outbreak of foodborne illness occurred at Chicago due to *Shigella boydii*

CDPH (Chicago Department of Public Health) serotype 18. The suspected food vehicles were parsley and cilantro imported from Mexico used to prepare bean salad [Chan & Blaschek, 2005].

(d) *Shigella sonnei*

Shigella sonnei is named after Sonne, who first described these strains from Denmark (1915) [Parija, 2009]. The species is also referred to as Group D and has 1 serotype [Sureshbabu *et al.*, 2014]. *Shigella sonnei* normally causes mild self-limiting infection. In recent years it has become the most usual *Shigella* species in the developed world. The spread of *S.sonnei* generally occurs in institutional or crowded settings, such as day-care centers and prisons and in military field settings. Shigellosis is the third leading bacterial gastrointestinal disease in the United States, with 25,000 cases reported in 1998 and 18,000 cases reported in 1999. Approximately 900 cases of *S.sonnei* infection are reported annually in the United Kingdom, and 15 cases of *S.sonnei* infection were reported to the National Disease Surveillance Centre in Ireland in 2001 [DeLappe *et al.*, 2003].

In most of the patients infected with *S.sonnei*, watery diarrhea occurs as a prodrome, or as the only clinical manifestation [Hale & Keusch, 1996]. However, some patients with *Shigella* infection- especially those with *S.sonnei*-never progress to the dysenteric phase, whereas others may develop dysentery without a prodrome [Bergelson *et al.*, 2008].

An outbreak of *S.sonnei* occurred in the Los Angeles County Department of Public Health (Los Angeles County, CA), in May 2012. It infected 43 people. Four representative isolates were submitted to the Centers for Disease Control and Prevention (CDC). Two of these isolates were obtained from asymptomatic, male employees of a bridge club and two from women in the same club. One of the male employees was a food handler [Karlsson *et al.*, 2013].

1.2 *Shigella flexneri*

1.2.1 Taxonomic Classification of *S. flexneri*

S. flexneri is classified as shown in the following table:

Table 1.1: Taxonomic Classification of *Shigella flexneri*

Domain	Bacteria
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gamma proteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Shigella*
Species	*Shigella flexneri*

1.2.2 Characteristics and Morphology of *S. flexneri*

S. flexneri, is a gram-negative bacilli, under the genus *Shigella*, belonging to the family Enterobacteriaceae. It has a cell wall containing a lipopolysaccharide (LPS) structure. LPS is composed of three covalently-linked domains. These are:

(i) Lipid A, which is embedded in the outer membrane

(ii) The central oligosaccharide core

(iii) The O polysaccharide or O antigen (OAg), which is exposed to the bacterial surface

In *S. flexneri*, the O antigen has two preferred chain lengths, a short OAg (S-OAg) of 11 to 17 repeat units and a very long OAg (VL-OAg) of about 90 repeat units. The O antigen also has additional glucose residues which changes its conformation making it more compact and short [Martinić *et al.*, 2011].

S. flexneri, like all other *Shigella* species is a short, rod-shaped bacterium, about 0.5 X 1-3µm in size. It is non-motile, nonsporing and noncapsulated. It grows at a temperature range of 10-40ºC, with an optimum temperature of 37ºC and pH of 7.4 [Parija, 2009].

The colonies of *S. flexneri* on nutrient agar are small, circular, convex, smooth and translucent [Parija, 2009]. They show moderate to heavy growth and appear to be grayish-white in color [Beckton, Dickinson and Company, 2006].

1.3 Virulence of *Shigella flexneri*

1.3.1 Genetic Basis of Virulence

Shigella flexneri, like all *Shigella* species, possesses a 213-kb virulence plasmid [Phalipon & Sansonneti, 2007]. This plasmid (pVir) contains the majority of genes involved in *Shigella* virulence. A 31-kb section of this plasmid encodes the type III secretion system (T3SS) and many of the secreted effector proteins [Marman *et al.*, 2014]. The genes within this region have been broadly identified [Torres, 2004].

1.3.2 Virulence Factors

Shigella flexneri, like all *Shigella* species, contains certain virulence factors. These are:

a) **The type III secretion system (T3SS):** The T3SS is a needle-like structure that causes the translocation of *Shigella* effector proteins from the bacterial cytoplasm to the membrane and cytoplasm of the host cell. It assembles in a structure spanning both the inner and outer bacterial membranes and extends a 60-nm needle into the external environment. Contact of bacteria with host cells results in a secretion signal, upon which a rapid burst of protein secretion occurs [Phalipon & Sansonetti, 2007].

   The transcription and expression of this *S. flexneri* T3SS is induced by the VirF/VirB system when the temperature is shifted from 30°C to 37°C [Zurawski *et al.*, 2006].

b) **Effector proteins:**

The effector proteins are secreted by the type III secretion system (T3SS) into host cells. They allow the bacterium to invade, multiply within the intestinal epithelium, and subvert cellular and immune functions during bacterial internalization [Ashida *et al.*, 2015].

The Ipa (Invasion plasmid antigen) proteins: These proteins are necessary for entry of *S. flexneri* into epithelial cell. IpaB and IpaD regulate the entry of proteins through the secretion system. IpaB and IpaC form a complex interacting with the epithelial cell membrane, which forms a pore. It is assumed that the other Ipa proteins then travel into the host cytoplasm through this pore. IpaC and IpaA appear to organize and manage the cytoskeletal rearrangements needed for direct uptake of the organism into the normally nonphagocytic epithelial cell. Once the *Shigella* containing vacuole is formed within the infected cell, IpaB mediates lysis of the vacuole and the bacterium is then free in the cytosol [Philpott *et al.*, 2000].

IpaB also causes macrophage cell death. It binds to caspase-1 in order to begin apoptosis and to promote the cleavage of IL-1β and IL-18 into biological active forms [Faruque, 2012].

IcsA/VirG outer membrane protein: This protein allows the spreading of bacteria within the cytoplasm and dissemination into adjacent cells [Cersini *et al.*, 1998].

Effector proteins with unknown functions: The functions of these bacterial virulence effectors are yet to be explained [Phalipon & Sansonetti, 2007].

c) Toxin:

S. flexneri produces one type of enterotoxin (ShET1), while 80% of other *Shigella* produces another type of enterotoxin (ShET2) [Hui *et al.*, 2003]. ShET1 is thought to cause the watery diarrhea that occurs prior to dysentery. The enterotoxin should be considered as an important virulence factor, since watery diarrhea occurs in all patients but dysentery does not [Mcvey *et al.*, 2013].

d) **Lipopolysaccharide (LPS):**

The LPS of *Shigella* is composed of the hydrophobic domain (Lipid A), a core oligosaccharide and an O polysaccharide or O antigen. The LPS is considered as an important virulence factor of the bacteria due to its O antigen and Lipid A part. The O antigen is involved in various aspects of *Shigella* virulence such as serum resistance, invasion and intra/intercellular spread. The Lipid A part, of the LPS, induces pro-inflammatory cytokines and chemokines and plays a part in the inflammatory damage and destruction of intestinal epithelium [Tang *et al.*, 2014].

e) **Acid Tolerance:**

S. flexneri has to survive in the acidic environment of the stomach and LPS plays an important role in its acid resistance. LPS contains O antigen. The presence of a polymerized S-OAg is needed for the acid resistance of *S. flexneri*. This property is enhanced if the O antigen is glucosylated. Other than that, the addition of phosphoethanolamine to the 1′ phosphate of lipid A increases resistance of *S. flexneri* to extreme acid conditions, provided that O antigen is produced [Martinić *et al.*, 2011].

1.4 Pathogenesis of *Shigella flexneri*

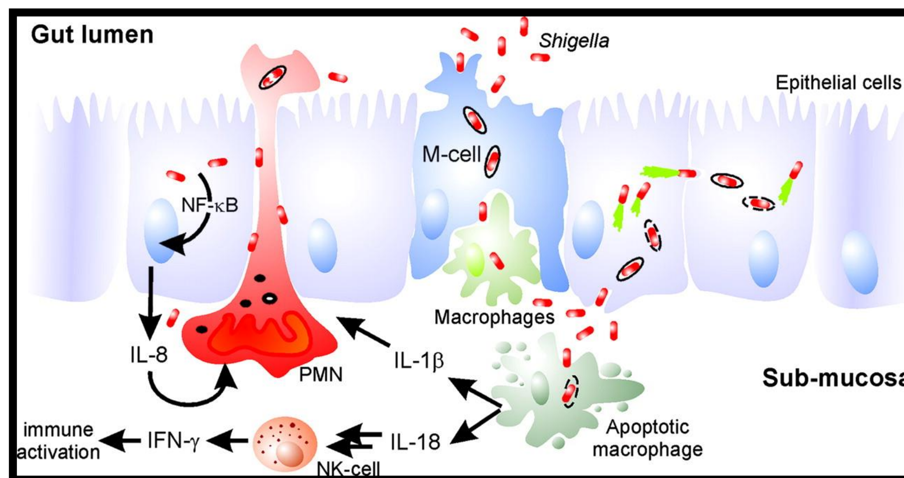


Figure 1.2: Cellular pathogenesis of *Shigella* spp. [Schroeder & Hilbi, 2008]

The bacterium *Shigella flexneri* is a causative agent of shigellosis, which is a severe infection of the colonic epithelium. It is primarily transmitted between hosts via the fecal-oral route to its infective site in the colon [Gore & Payne, 2010]. The organism enters the colonic epithelium by using the M cells, which are specialized epithelial cells in the follicular associated epithelium (FAE) that overlie lymphoid tissue [Wassef *et al.*, 1989]. M cells allow the intact organism to traverse into the underlying subepithelial pocket, inhabited by macrophages. Macrophages engulf *Shigella*, but instead of successfully destroying it in the phagosome, the macrophage succumbs to apoptotic death [Philpott *et al.*, 2000]. This macrophage cell death is accompanied by the release of the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18. IL-1β signaling sets off the strong intestinal inflammation characteristic of shigellosis. IL-18, on the other hand, is involved in the generation of an effective antibacterial response. It activates natural killer (NK) cells and promotes the production of gamma interferon (IFN-γ), thus amplifying innate immune responses [Schroeder & Hilbi, 2008].

After crossing of the intestinal barrier via M cells and escape from macrophage killing, *S. flexneri* makes contact with the basolateral membrane of the epithelial cells [Gillespie & Hawkey, 2006]. The organism then initiates a sequence of steps to invade these cells [Strelkauskas *et al.*, 2015]. It enters the cells by a micropinocytic process, which requires polymerization of actin at the site of entry. The bacteria lyse the phagocytic vacuole, soon after its entry, and travel into the cytoplasm where they replicate [Schaechter & Lederberg, 2003]. This access to the cytoplasm allows the invading pathogen to interact with host cell microfilaments [Mounier *et al.*, 1992]. *Shigella* uses these actin microfilaments for making a polymerization actin tail behind the bacterium and propelling *S. flexneri* through the cytoplasm until it contacts the cytoplasmic membrane. The force of the contact creates a protrusion in the neighbouring epithelial cell, causing lysis of both membranes and release of *S. flexneri* into the neighbouring cell [Gillespie & Hawkey, 2006].

The invasion of epithelial cells by *Shigella* activates the transcription and secretion of IL-8. IL-8 is chemotactic for PMN cells and it assembles these cells to the infected subepithelial area, where they transmigrate through the epithelial lining to reach lumenal bacteria. The arrival of

these cells across the epithelial layer in response to *Shigella* disrupts the integrity of the epithelium allowing lumenal bacteria to cross into the sub-mucosa without the need of M cells [Jennison & Verma, 2004].

All these processes consisting of macrophage killing, destruction of the epithelial layer and the massive influx of PMN cells, worsen the bacterial infection and tissue lesion. The severe tissue destruction caused by *Shigella* spp. results in an impaired adsorption of water, nutrients, and solutes, which might cause the watery diarrhea as well as the blood and mucus in stools characteristic of shigellosis [Schroeder & Hilbi, 2008].

1.5 *Shigella flexneri* and Shigellosis

Diarrheal diseases claim the lives of at least five million children per year in developing countries and shigellosis or bacillary dysentery is responsible for approximately 10% of these deaths [Hale, 1991]. *Shigella flexneri* is responsible for the worldwide endemic form of this disease.

1.5.1 Reservoir and Means of Transmission

The main reservoir of *Shigella* species are human intestines [Thatcher, 2005]. Transmission of the organism occurs by the fecal-oral route. The 3 major ways of contracting shigellosis are:

(i) Eating contaminated food. This mainly refers to food washed in fecally contaminated water or handled by a person with poor hygiene.
(ii) Drinking contaminated water.
(iii) Person-to-person contact [Weir, 2002].

Flies can act as carriers of *Shigella* species from feces to foods left uncovered. *S. flexneri* can survive in feces for 12 days at 25ºC, and can be transferred by flies, resulting in contamination of uncontaminated food [Islam *et al.*, 1993].

Other than flies, fingers, feces, food and fomites, i.e., any contaminated object such as a cutting board, door handle or toys, can be involved in the transfer of the pathogen [Weir, 2002]. In endemic areas of Bangladesh, e.g., Matlab, Teknaf, and urban Dhaka, food-borne transmission of shigellosis could be a mode of transmission within homes and in small communities where hygienic food practices and facilities for refrigerating food are lacking [Islam *et al.*, 1993].

1.5.2 Symptoms

Shigellosis is a severe form of bloody diarrhea which is endemic in developing countries. The symptoms occur within 24-48 hours of ingestion of the etiologic agent and may persist in untreated adults for up to 7 days. The organism may be cultivated from stools for 30 days or longer [Hale & Keusch, 1996]. The symptoms of shigellosis are:

- **a)** Mild diarrhea
- **b)** Initial copious watery diarrhea
- **c)** Nausea
- **d)** Vomiting [Nygren *et al.*, 2013]
- **e)** Painful abdominal cramps
- **f)** Tenesmus [Zychlinsky *et al.*, 1996]
- **g)** Dysentry with frequent, small, painful, mucoid, bloody stools [Nygren *et al.*, 2013]

Shigellosis may cause some rare complications. One of these complications is bacterimia, which is mainly caused by *S. flexneri*, the most frequently encountered species in endemic zones and travelers [Grondin *et al.*, 2012]. It may also result in neurologic complications, especially seizures, which are common in young patients. Encephalopathy can develop as well, particularly during infection with *S. flexneri* [Paradise *et al.*, 1996]. Reactive arthritis or Reiter syndrome can occur too, with *S. flexneri* infections [Hale, 1991].

1.5.3 Diagnosis

Shigellosis is diagnosed by conducting a stool culture and other laboratory tests. These are mentioned below:

a) **Stool culture:** Stool culture is the most effective method for confirming the presence of infecting organism, in a shigellosis patient. Freshly passed stool specimens, with blood-tinged plugs of mucus, is obtained during acute phase of the disease. Rectal swabs may also be used in order to culture shigellae [Hale & Keusch, 1996], although culture from a stool sample may give a better yield [Agha & Goldberg, 2006]. These specimens are placed in buffered glycerol saline (BGS) or Cary-Blair medium [Seidlin *et al.*, 2006], if transport is required. Otherwise, since *Shigella* spp. are fastidious organism, they should be handled promptly and inoculated optimally onto agar at bedside [Agha & Goldberg, 2006]. The swabs are normally inoculated in MacConkey agar and *Salmonella*-*Shigella* agar to differentiate lactose fermenter and non-lactose fermenter [Seidlin *et al.*, 2006]. After overnight incubation at 37°C, colorless, non-lactose-fermenting colonies are inoculated into tubed slants of Kligler's Iron Agar or Triple Sugar Iron Agar. In these differential media, *Shigella* species produce an alkaline slant and an acid butt with no bubbles of gas in the agar. This reaction gives a presumptive identification, and slide agglutination tests with antisera for serogroup and serotype confirm the identification [Hale & Keusch, 1996].

b) **Fecal leukocytes:** Raid and easy determination of presence or absence of leukocytes in the stool can be done by microscopic examination. In a study that examined the usefulness of fecal leukocytes in predicting the etiology of diarrhea, the presence of fecal leukocytes was related with a bacterial cause of acute diarrhea in 89% of cases. Patients infected with *Shigella* had fecal polymorphonuclear leukocytes in 70 to 100% of samples tested, with at least 10-25 cells/hpf in most of the patients [Agha & Goldberg, 2006].

c) **PCR:** PCR can be used to amplify the gene coding for the invasion plasmid antigen H (*ipaH*), a gene nearly exclusively derived from the four *Shigella* spp. in Asia. Studies using *ipaH*-based PCR have been published from several Asian countries, including Thailand, Bangladesh, and more recently, India. These studies suggest that *ipaH* can be detected in a large percentage of patients with diarrhoea who are culture-negative for *Shigella* [Seidlin *et al.*, 2006].

1.5.4 Treatment

The treatment of shigellosis can be done by using oral rehydration therapy or appropriate antibiotics. The use of oral rehydration therapy is an effective and safe measure for the treatment of acute diarrhea. However, if the patient is vomiting or is in shock from severe dehydration, then intravenous fluid replacement is required until initial fluid and electrolyte loss are corrected [Hale & Keusch, 1996]. Shigellosis is generally a self-limited disease and does not need antibiotic therapy, except in patients with severe colitis, who are benefitted by it [Kabir *et al.*, 1986]. *Shigella* species demonstrate increased resistance to both ampicillin and trimethoprim-sulfamethoxazole (TMP-SMX), as shown by a recent US surveillance data, which is why cefixime and ceftriaxone is recommended as alternative antibiotics in treatment of infections, caused by the bacteria [Erdman *et al.*, 2008].

Azithromycin can be used for the treatment of *Shigella* infections in pediatric patients, as suggested by AAP (American Academy of Pediatrics). Fluoroquinolones are at present contraindicated in children due to potential safety concerns, but they are recommended as a potential alternative therapy by the AAP for the treatment of *Shigella* infections in pediatric patients [Erdman *et al.*, 2008].

1.5.5 Prevention

The best way to prevent shigellosis is following proper hand washing and hygiene practices. Foodborne transmission can be reduced by ensuring proper hygiene and sanitation during harvesting, production, distribution and preparation of food. Infected food workers should be discouraged from working while ill [Morris & Potter, 2013].

Waterborne outbreaks of shigellosis can be prevented by making sure that safe drinking water is present. Drinking water should be protected and kept well away from toilet facilities as *shigella* can survive in tap or sterilized water for as long as 4-6 weeks [Gillespie & Hawkey, 2006].

The transmission of shigellosis in community settings, such as daycare centers, can be prevented by providing children and child care staff with fully stocked and operational hand washing

stations. The children should be supervised and assisted during hand washing. Other security measures include elimination of water play areas and exclusion of child care workers handling diapers from preparing food in child care centers or in restaurants [Morris & Potter, 2013].

1.6 Outbreaks

Shigellosis is an important public health problem and outbreaks have been reported both in developed and developing countries. The major modes of transmission are contaminated food as well as contaminated fresh produce, contaminated water and person-to-person contact.

1.6.1 *S. flexneri* outbreaks in USA

1.6.1.1 *S. flexneri* outbreak in Michigan, 1992

An outbreak of *S. flexneri*, which is a less common *Shigella* species in USA, occurred in 1992 in Michigan. The Michigan Department of Public Health (MDPH) was notified of a group of persons with *S. flexneri* infections, all of whom had eaten at different outlets of a single restaurant chain. This chain prepared many foods at a central kitchen and the infections were associated with eating tossed salad, prepared from raw vegetables, at this kitchen. Among the 46 patients identified, 44 had eaten this tossed salad. The contamination of the salad most likely occurred due to handling of salad components by infected salad preparers. The use of improper salad preparation techniques may also be a reason for this contamination [Dunn *et al.*, 1995].

1.6.1.2 *S. flexneri* outbreak in California, 1998

A restaurant associated outbreak occurred in 1998, in California, involving an unusual *S. flexneri* strain. The source of the epidemic may have been two asymptomatic culture-positive employees. The severity of the illness in this outbreak was significant. A previously healthy 21-year-old male and two women, ages 45 and 73 years, respectively, were hospitalized at a cost of more than $61,000. Reactive arthritis was a likely symptom among three non-hospitalized case patients who reported subsequent joint symptoms [Trevejo *et al.*, 1999].

The inspection of this restaurant revealed several hygiene violations, including a lack of hand washing between tasks and inadequate hand-washing facilities [Trevejo *et al.*, 1999]. This may have been a major reason resulting in contamination of the food.

1.6.2 *S. flexneri* outbreak in Taiwan, 1996

An outbreak of shigellosis occurred in a mountainous township of Nantou County in central Taiwan, during August through October in 1996. The infections continued to the end of the year. The cases, all of which identified as infections with *S. flexneri* serotype 2a, were widely distributed in villages of the township as well as two neighboring townships [Chiou *et al.*, 2001].

The outbreak occurred in the Renai Township. During July and August 1996, two typhoons hit central Taiwan in succession and destroyed many water supply facilities in Renai Township. The repairing of these facilities took two months. It was therefore suggested in the study that the *Shigella* strains had been dormant in the community and that the deterioration of sanitary and hygiene conditions favored the spread of the organism, resulting in the outbreak [Chiou *et al.*, 2001].

1.7 Antimicrobial Resistance of *Shigella flexneri*:

Antimicrobial resistance is a global problem. Antimicrobials are the most commonly prescribed group of drugs in general practice and in hospitals. However, the unregulated dispensing and production of these antimicrobials, inadequate access to effective drugs and sometimes drugs of questionable quality seem to be the major factors contributing to antimicrobial resistance [Faiz & Basher, 2011].

Shigellosis or bacillary dysentery is a leading cause of morbidity and mortality worldwide. The use of appropriate antimicrobial therapy for shigellosis may reduce symptom severity and illness duration, and may also prevent potentially lethal complications and further disease transmission [Chang *et al.*, 2011]. However, high rate of resistance to many of the first-line antimicrobial agents among the strains of *S. flexneri* have been reported from many parts of the world in recent years [Khaghani *et al.*, 2014]. Tetracycline, ampicillin and co-trimoxazole, once used as first-line antimicrobials, are no longer effective [“Antibiotics in the management of Shigellosis”, 2004].

The increased resistance to antimicrobials in the organism occurs due to the acquisition and dissemination of antimicrobial resistance genes by horizontal transfer. Mobile genetic elements such as plasmids, transposons, integrons and gene cassettes often contain these antimicrobial resistance genes. [Chang *et al.*, 2011].

1.7.1 Emerging Trends in Resistance among *S. flexneri*

The emergence and spread of antimicrobial resistance has created difficulty in the management of diseases like shigellosis. Over the decades, *Shigella* isolates resistant to multiple agents, such as sulphonamides, tetracycline, ampicillin, trimethoprim-sulphamethoxazole, and nalidixic acid have been reported from many countries, including Bangladesh [Rahman *et al.*, 2007]. In 1973, *S. flexneri* isolates were universally susceptible to ampicillin; however, by 1979 susceptibility decreased to 79% in urban Bangladesh. Similarly, the susceptibility of *S. flexneri* to tetracycline dropped from 79% in 1973 to 15% in 1979. In a recent study, at least 25% of *S. flexneri* isolates were resistant to three commonly used antibiotics such as ampicillin, co-trimoxazole and nalidixic acid [Faiz & Basher, 2011]. Another study conducted in Bangladesh in between 2001 and 2002 showed that the rates of resistance of *Shigella* isolates to ampicillin, trimethoprim-sulphamethoxazole, and nalidixic acid have increased to more than 50%, when compared with the resistance patterns in between 1991 and 1992 [Rahman *et al.*, 2007].

A majority of *Shigella* isolates from around the world, including USA, demonstrate some resistance to commonly used antibiotics, such as ampicillin, chloramphenicol, TMP-SMX and tetracycline. In developing countries, like India and China, resistance to traditional antimicrobials as well as fluoroquinolones (ciprofloxacin) is also increasing rapidly. In Kolkata, for example, resistance to fluoroquinolones, has increased up to 25% in *S. flexneri* isolates [Barrett & Stanberry, 2009].

1.7.2 Antimicrobial Therapy

The choice of antimicrobials for treating shigellosis has become very limited, due to the growing trend of antimicrobial resistance among *Shigella* isolates worldwide. This is the reason why newer antimicrobials are being tested and provided for treating this disease. In severe form of dysentery due to *S. flexneri*, where shigellae have penetrated intestinal epithelial cells in large

number [Gillespie & Hawkey, 2006], pivmecillinam (oral form of mecillinam), fluoroquinolones, azithromycin, and third-generation cephalosporins {cefixime [Rahman *et al.*, 2007] and ceftriaxone [Kabir *et al.*, 1986]} are prescribed for treatment.

Pivmecillinam is a synthetic form of penicillin used orally. It is the pivaloyloxymethyl ester of mecillinam, which is absorbed readily from the gastrointestinal tract. After absorption, it undergoes enzymatic hydrolysis by the action of non-specific esterases with liberation of mecillinam, which is the antimicrobially active form of the drug. The precise mode of action of mecillinam has not been completely explained, but it has been shown that mecillinam interferes with the bacterial cell wall [Dewar *et al.*, 2013]. Pivmecillinam is currently used as an empirical antimicrobial therapy for shigellosis in Bangladesh with caution since resistance to it is emerging [Rahman *et al.*, 2007].

Fluoroquinolones are antimicrobials, which work by inhibiting bacterial replication by blocking their DNA replication pathway [Mehta, 2011]. Fluoroquinolones, such as ciprofloxacin, are used for treating shigellosis, although resistance against them is increasing. In a study conducted at icddr,b, between January 2004 and December 2010, it was demonstrated that, the frequency of resistance to ciprofloxacin, among *S. flexneri* strains, increased from 0.7% in 2005 to 45.5% in 2010 [Rahman *et al.*, 2007].

Azithromycin is a macrolide antibiotic which inhibits bacterial protein synthesis, quorum-sensing and reduces the formation of biofilm [Panham *et al.*, 2014]. It is also used in the treatment of shigellosis, although not that commonly in Bangladesh [Rahman *et al.*, 2007].

Cephalosporins, such as cefixime, work by interfering with bacterial cell-wall synthesis and division. They bind to the cell wall and cause cell death [“Cefixime”, n.d.]. Ceftriaxone, which is another member of this family, also works in a similar way by inhibiting bacterial cell wall synthesis [“Ceftriaxone”, n.d.]. Cefixime and ceftriaxone both are used in the treatment of shigellosis. In a recent study, cefixime was found to be clinically effective in 78% of children with shigellosis, predominantly caused by *S. flexneri* [Rahman *et al.*, 2007]. In another study conducted in Bangladesh, to evaluate the clinical efficacy of ceftriaxone, its effect in reducing

stool frequency during 6 days was found to be significant in patients infected with *S. flexneri* [Kabir *et al.*, 1986].

1.8 Immunologic responses against *Shigella flexneri*

Immunologic response against *S. flexneri* is provided by both the innate and the adaptive immune systems. However, *Shigella*, like many enteric bacterial pathogens have the ability to evade these host immune systems and efficiently colonize the intestinal epithelium. The innate immune system triggers inflammation accompanied by inflammatory cytokine secretion, neutrophil recruitment and massive tissue destruction, in response to *Shigella* invasion and replication within host cells. The interactions between *Shigella* and adaptive immune system, such as T and B lymphocytes, have not been thoroughly investigated due to the lack of appropriate animal infection models that mimic human intestinal infection [Ashida *et al.*, 2015].

Shigella are gram-negative bacteria. Human serum is considered an important host defence mechanism against invasive diseases caused by gram negative bacteria. It has been established that besides natural antibodies in the serum of apparently normal human beings, there are other factors such as antitoxins, antiviral antibodies, opsonins and antilysins, which play a role in the bactericidal activity of human serum [Igumbor & Osayande, 2000]. The complement system, which exists in the blood, is a vital component of the immune system. The system consists of at least 30 proteins that orchestrate attack on pathogenic agents [Bloch *et al.*, 2011]. It has been reported that complement protein is essential for killing susceptible gram-negative bacteria [Igumbor & Osayande, 2000]. The functions of complement are numerous but it is most well known for its capacity to kill pathogens by creating pores in their surface membranes. Complement also participates in inflammatory reactions by attracting phagocytic cells to the site of injury. By opsonising pathogens, complement proteins can stimulate phagocytosis, a process that is mediated by complement receptors on the surface of phagocytic cells [Claire *et al.*, 2002].

The main barriers that control *Shigella* infection are neutrophils and monocytes. In response to inflammatory stimuli, neutrophils migrate from the circulating blood to infected tissues, where they efficiently bind, engulf and inactivate bacteria. In 2002, Zychlinsky and his group demonstrated that, in contrast to other cells, such as macrophages, neutrophils prevent the escape

of *Shigella* from phagocytic vacuoles in which the bacteria are killed. Human neutrophil elastase (NE) was identified as a key host defense protein, which degrades *Shigella* virulence factors at a 1000-fold lower concentration than that needed to degrade other bacterial proteins [Phalipon & Sansonetti, 2007].

Monocytes arrive to the site of infection within a few hours of *Shigella* infection. It is known that efficient bacterial phagocytosis by monocytes is opsonin dependent. Complement-dependent opsonization of *S. flexneri* with human serum resulted in efficient uptake, following which bacteria did not escape from the phagocytic vacuole and were rapidly killed. Complement-dependent uptake of *Shigella* by both monocytes and neutrophils is therefore likely to be important during the early stages of infection, prior to the production of specific antibody [Hathaway *et al.*, 2002].

1.9 Objectives of the Study

Shigellosis occurs as an endemic disease in Bangladesh, with the predominant species being *S. flexneri*. Shigellosis is transmitted via the fecal-oral route through ingestion of contaminated food or water or by person to person contact. The symptoms of shigellosis can range from mild diarrhea to severe dysentery with the passage of frequent bloody, mucoid stools, fever and abdominal cramps.

The population of Bangladesh, which consists of different groups due to their different lifestyle, food habit, health condition, may all be exposed to this pathogen, *Shigella flexneri*, at one time or other. Human blood serum from these various groups has the ability of exhibiting complement activity against this organism. Serum is the first body defense met by pathogen when it breaks through and enters the skin or epithelial lining of the organs. It contains complement, which can exhibit bactericidal activity through the classical or alternative pathway.

The objective of this study is to compare and investigate the complement activity of human blood serum, collected from urban and slum based population, against *Shigella flexneri*. The lifestyle of people living in urban areas and slum areas are quite different. They do not have similar facilities and hygiene conditions. The population living in slum areas often lacks these

and is therefore expected to be more exposed to the pathogen, *S. flexneri*. This comparative study will help to analyze whether the susceptibility of *S. flexneri*, to complement activity of human blood serum, is affected by this lifestyle difference in the urban and slum based population of Bangladesh. This type of work is rare in Bangladesh and therefore it will improve our understanding about the exposure of various population groups to this microorganism and their response against it.

2 Materials and Methods

2.1 Place of Study

The research study was carried out in the Microbiology Specialized Research Laboratory of the Department of Mathematics and Natural Sciences, BRAC University, Bangladesh.

2.2 Bacterial Strain

A strain of *Shigella flexneri* was obtained from the Microbiology Specialized Research Laboratory of the Department of Mathematics and Natural Sciences, BRAC University, Bangladesh.

2.3 Identification of *S.flexneri*

In order to reconfirm that the bacterial strain obtained from the Microbiology Specialized Research Laboratory was *S. flexneri*, some identification test were performed.

2.3.1 Identification on XLD (Xylose-Lysine-Deoxycholate) Agar

XLD agar is a selective medium for *Shigella*. *Shigella* colonies on XLD agar are transparent pink or smooth red with a diameter of 1 to 2 mm [“Isolation and identification of *Shigella*”, n.d.]. XLD contains sodium deoxycholate, which inhibits the growth of gram-positive microorganisms. It also contains xylose, which is fermented by practically all enterics except for the shigellae and this property enables the differentiation of *Shigella* species [Beckton, Dickinson and Company, 2007].

The procedure followed for identification on XLD agar is given below:

1) Colonies, assumed to be of *S. flexneri*, were obtained from Nutrient agar plate and streaked onto XLD agar plate for confirmation.

2) The inoculated XLD agar plate was incubated overnight at 37°C.

3) The result was observed (Figure: 3.2).

2.3.2 Biochemical Identification

Biochemical identification of *S. flexneri* was done according to the methods described in Microbiology: A Laboratory Manual [Cappuccino and Sherman, 2005]. The biochemical tests performed were indole production test, methyl-red test, Voges-Proskauer test and citrate utilization test.

2.3.2.1 Indole Production Test

The indole production test is based on the ability of some bacteria, which contain the enzyme tryptophanase, to hydrolyze tryptophan with the production of indole, pyruvic acid and ammonia. The test requires a medium rich in tryptophan and a reagent known as Kovac's reagent, which contains butanol, HCl and p-dimethylaminobenzaldehyde. If the addition of Kovac's reagent to the inoculated and incubated tryptophan rich medium, results in the formation of a cherry red color on its surface, then a positive test is indicated [Capuccino & Sherman, 2005]. However, if it results in the formation of a yellow color on the surface of the medium, then a negative test is indicated [MacWilliams, 2009].

The procedure followed for the indole production test is given below:

1) The organism to be tested was inoculated into peptone water and incubated overnight at 37°C.
2) 10 drops of Kovac's reagent was added to the inoculated medium following incubation.
3) The result was observed and recorded (Figure: 3.3 (C)).

2.3.2.2 Methyl-Red Test

The methyl-red test is used to identify enteric bacteria based on their ability to perform mixed-acid fermentation. If the inoculated and incubated MR-VP medium contains bacteria that ferment glucose by the mixed acid pathway and produce acids, such as lactic, acetic and formic acids, then the pH of the medium will be lowered to about 4. The pH indicator methyl-red will turn red color at this pH, indicating a positive test. Nevertheless, if the inoculated bacteria ferment

glucose to produce non-acidic end products such as 2,3-butanediol and acetoin, then the pH of the medium will be increased to about 6.0. The pH indicator methyl-red will turn yellow color at this pH, indicating a negative test [Capuccino & Sherman, 2005].

The procedure followed for the methyl-red test is given below:

1) The organism to be tested was inoculated into MR-VP medium and incubated overnight at 37°C.
2) 5 drops of methyl-red indicator was added to the inoculated medium following incubation.
3) The result was observed and recorded (Figure: 3.4 (C)).

2.3.2.3 Voges-Proskauer Test

The voges-proskauer test is majorly used to determine the ability of some organisms to produce neutral or non-acidic end products, like acetoin, from organic acids that result from glucose metabolism. The reagent used for this test is the Barritt's reagent, consisting of a mixture of alcoholic a-napthol and 40% potassium hydroxide solution. The acetoin (acetyl methyl carbinol) is oxidized to a diacetyl compound in the presence of a-napthol catalyst and a guanidine group, which is present in the peptone of the MR-VP medium. This reaction results in the formation of a pink complex that gives the medium a rose color. If a deep rose color is developed in the culture 15 minutes after addition of Barritt's reagent, then the presence of acetoin is ensured and a positive result is indicated. If no color change occurs then the result is considered to be negative [Capuccino & Sherman, 2005].

The procedure followed for the voges-proskauer test is given below:

1) The organism to be tested was inoculated into MR-VP medium and incubated overnight at 37°C.
2) After incubation, 10 drops of Barriitt's reagent A was added to the inoculated medium and the medium was shaken.
3) Immediately 10 drops of Barritt's reagent B was added to the medium and the medium was shaken.

4) The result was observed and recorded 15 minutes after the addition of Barritt's reagent (Figure: 3.5 (C)).

2.3.2.4 Citrate Utilization Test

The citrate utilization test is used to differentiate enteric bacteria based on their ability to produce the enzyme citrase and use citrate as the only source of carbon. Simmon's citrate agar is used for this test. Citrate is the sole source of carbon in this medium while inorganic ammonium salt is the sole source of nitrogen [MacWilliams, 2009]. Citrate utilizing bacteria use the citrase enzyme and convert citrate to oxaloacetic acid and acetate. These products are further enzymatically converted to produce pyruvic acid and carbon dioxide. The medium becomes alkaline during this reaction, since the carbon dioxide reacts with sodium and water to form sodium carbonate, which is an alkaline product [Capuccino & Sherman, 2005]. In addition, ammonium hydroxide is produced when the ammonium salts in the medium are used as the only nitrogen source. The pH indicator present in Simmons citrate agar is bromthymol blue, which is green at neutral pH. The production of these alkaline products increases the pH of the medium to above 7.6, resulting in color change of bromthymol blue from green to blue, which indicates a positive test [MacWilliams, 2009].

The procedure followed for the citrate utilization test is given below:

1) The organism to be tested was inoculated into Simmons citrate agar slants and incubated overnight at 37°C.
2) The result was observed and recorded (Figure: 3.6 (C)).

2.4 Preservation of *S.flexneri*

T_1N_1 agar medium was used for the preservation of *S. flexneri* strain. The method followed for preparing the stock sample is given below:

1) 3ml T_1N_1 agar medium was prepared and taken in a small vial.
2) The vial was autoclaved at 121°C for 15 minutes and then allowed to solidify.

3) The T_1N_1 agar butt was inoculated by stabbing bacterial growth of *S. flexneri* from nutrient agar plate and then incubated at 37°C overnight.
4) 200µl of sterile glycerol was added and the vial was sealed with parafilm and stored at room temperature.

2.5 Serum Sample Collection

2.5.1 Site of Collection

The serum samples used in the study were collected from two different locations:

(i) 50 serum samples were collected from BRAC University, Mohakhali, Dhaka.

(ii) 50 serum samples were collected from TNT slum, Mohakhali, Dhaka.

2.5.2 Procedure for Collection

Blood samples were collected from individuals present at the two study locations, with the help of a trained nurse. Each individual was required to fill up a questionnaire. It contained useful information essential for conducting the study.

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 2.1: The Questionnaire Form

Each serum sample was collected following the steps given below:

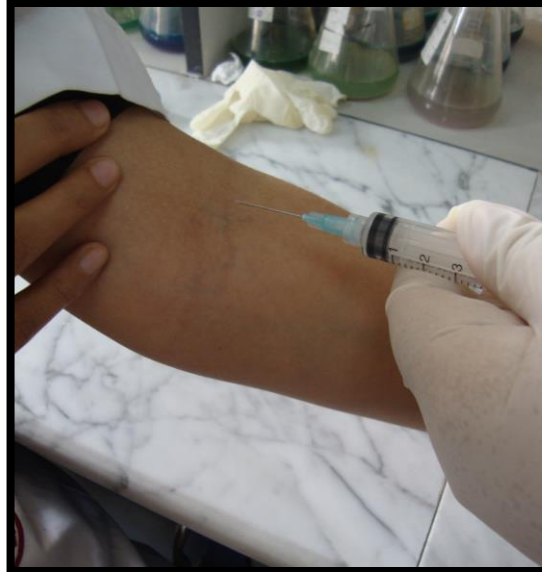


Figure 2.2: Collection of Blood sample

1) Blood sample, about 5ml, was collected by venipuncture procedure, with a sterile disposable syringe and placed into a sterile test tube.

2) The tube was kept in a slant position, undisturbed, in an incubator and incubated at 37°C for 1 hour to allow blood clotting.

3) After completion of incubation, the tube was kept at 4°C overnight in a standing position.

4) Serum was collected from the tube using a micropipette and placed into an appropriately labeled sterile microcentrifuge tube.

5) The serum containing microcentrifuge tube was centrifuged at 3000rpm for 10 minutes, in order to obtain pure serum.

6) After centrifugation, the clear supernatant, which is the serum, was collected into an appropriately labeled sterile microcentrifuge tube.

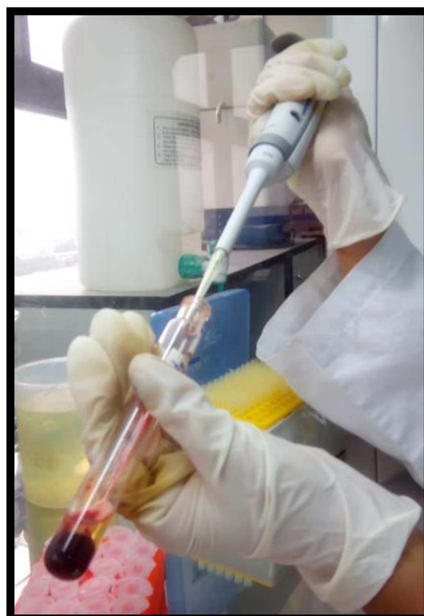


Figure 2.3: Collection of serum sample after blood clotting

2.5.3 Preservation of Collected Serum

The collected serum samples were stored at -20°C until further use. Repeated freezing and thawing of the samples were avoided in order to ensure better preservation.

2.6 Bactericidal Assay of Human Serum against *S. flexneri*

The bactericidal assay of human serum against *S. flexneri* was carried out by modifying and following some steps of the method used by Bugla-Ploskonska *et al.* in the 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". The steps followed were:

1) The strains were grown overnight in YP medium and then 50µl of the bacterial culture was transferred to 3 ml of fresh YP medium and incubated at 37°C for 1h in a water bath

2) After incubation, the bacterial cells were centrifuged (2500Xg for 20min at 4°C) and suspended in physiological saline to obtain a six-fold dilution.

3) The bacteria with serum were incubated in a water bath at 37°C.

4) After 0 (T0) and 180 (T3) minutes, the samples were collected, diluted and cultured on nutrient agar plates for 18 h at 37°C.

5) The microorganisms were distributed by L-shaped glass after appropriate dilution prior to overnight incubation in 37°C.

The complete procedure followed for bactericidal assay of human serum against *S. flexneri* is as follows:

1) A loop full of *S. flexneri* from nutrient agar plate was inoculated in 3ml of nutrient broth and incubated overnight at 37°C.

2) 25µl of the overnight bacterial culture was then transferred to 1.5ml nutrient broth, kept in a labeled sterile microcentrifuge tube.

3) The culture was incubated for 1 hour at 37°C.

4) After incubation, the bacterial cells were centrifuged at 2500Xg for 20min at 4°C [Bugla-Ploskonska *et al.*, 2009].

5) After centrifugation, the cells were suspended in physiological saline to obtain a six-fold dilution and vortexed.

6) 100µl of serum was taken in a labeled sterile microcentrifuge tube and diluted with 100µl of physiological saline. The diuted serum was then vortexed.

7) 150µl of the diluted serum was transferred into a labeled sterile microcentrifuge tube and mixed with 150µl of the diluted bacterial culture and vortexed.

8) The bacteria with serum were incubated at 37°C [Bugla-Ploskonska *et al.*, 2009].

9) At 0 minute and 180 minutes of incubation, the bacteria and serum sample was collected and cultured on nutrient agar plates using the spread plate method. The plates were incubated overnight at 37°C [Bugla-Ploskonska *et al.*, 2009].

10) After completion of incubation, both the plates were observed and the results were recorded.

3 Results

3.1 Bacterial Strain

The strain of *Shigella flexneri* that was obtained from the Microbiology Specialized Research Laboratory of the Department of Mathematics and Natural Sciences, BRAC University, Bangladesh, was streaked on nutrient agar.

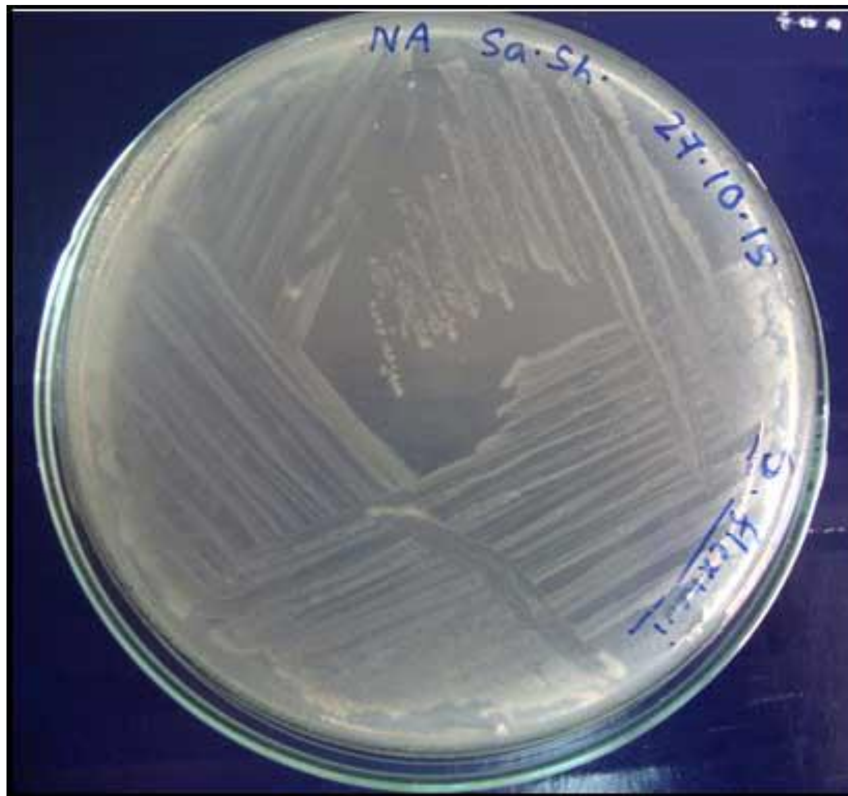


Figure 3.1: Nutrient agar plate showing typical grayish-white colonies of *S.flexneri*

3.2 Identification of *S. flexneri*

3.2.1 Identification on XLD agar

The assumed *S. flexneri* colonies from nutrient agar were streaked onto XLD agar. After incubation, the XLD agar showed typical red-pink colonies of *S. flexneri*.

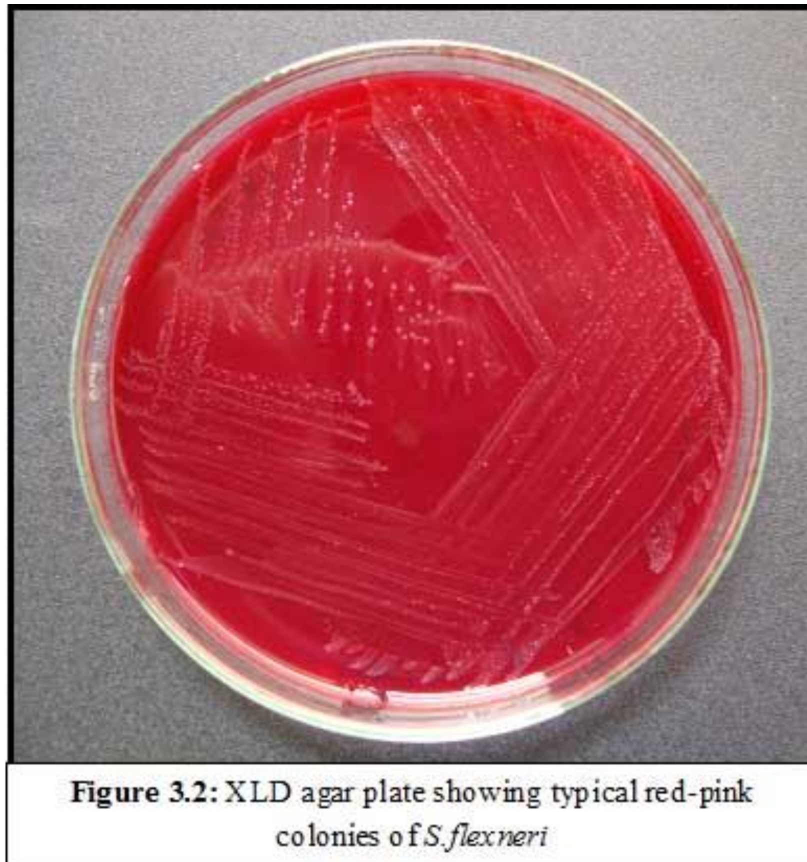


Figure 3.2: XLD agar plate showing typical red-pink colonies of *S.flexneri*

3.2.2 Biochemical Identification

S. flexneri colonies, from nutrient agar plate, were subjected to different biochemical tests. The results observed and recorded were typical to that of *S. flexneri*, as shown in the table:

Table 3.1: Typical Results of Biochemical Tests of *S. flexneri*

Strain	Indole Production	Methyl Red (MR)	Voges-Proskauer (VP)	Citrate Utilization
S.flexneri	Negative (-)	Positive (+)	Negative (-)	Negative (-)

3.2.2.1 Indole Production Test

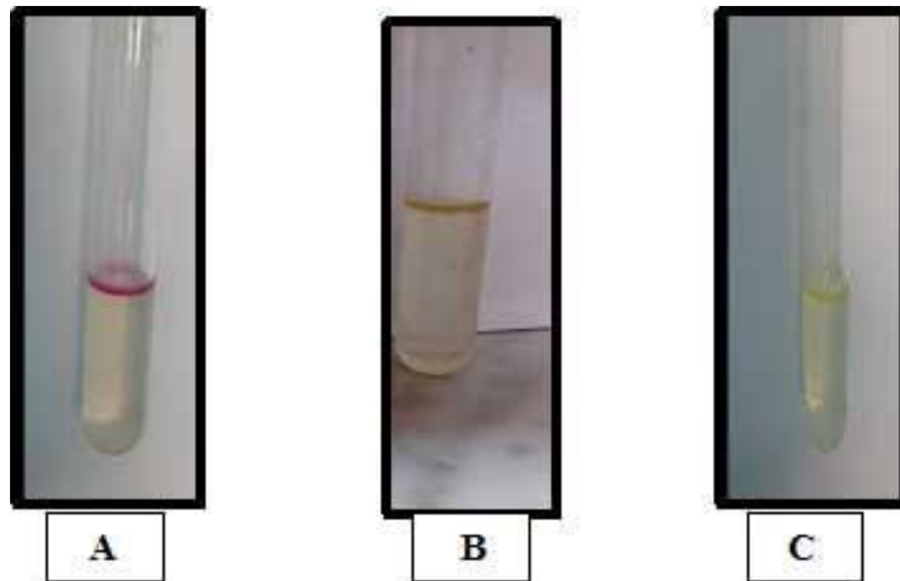


Figure 3.3: Test tubes showing results for Indole Production Test: (A) Positive Control (B) Negative Control (C) *S. flexneri* negative on Indole Production Test

3.2.2.2 Methyl-Red Test

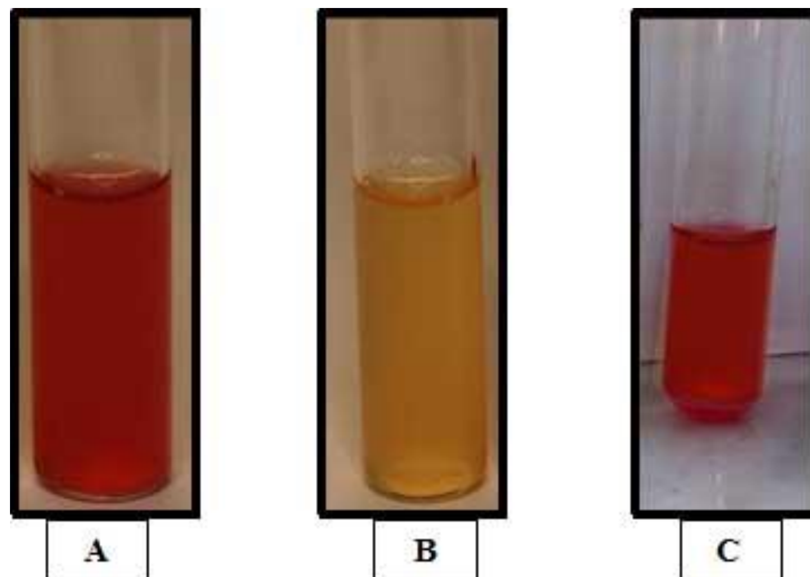


Figure 3.4: Test tubes showing results for Methyl Red Test: (A) Positive Control [Acharya, 2014] (B) Negative Control [Acharya, 2014] (C) *S. flexneri* positive on Methyl Red Test

3.2.2.3 Voges-Proskauer Test

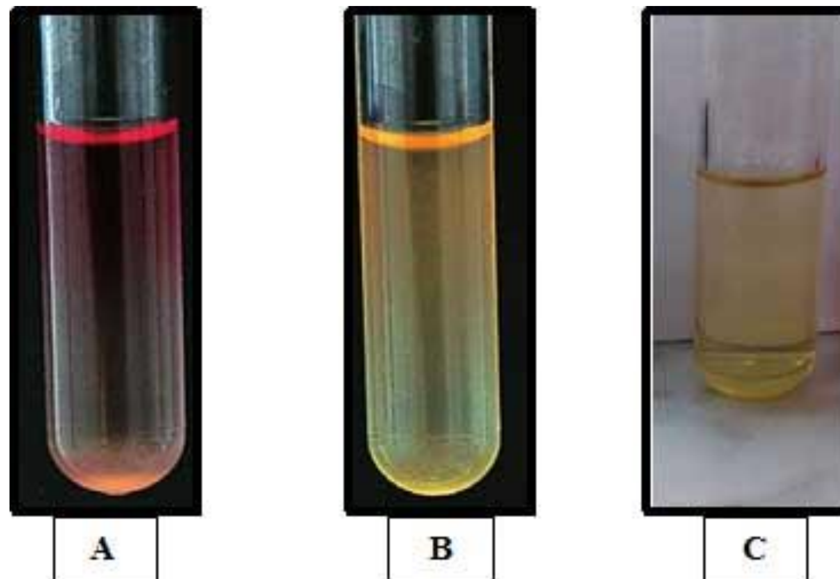


Figure 3.5: Test tubes showing results for Voges-Proskauer Test: (A) Positive Control [Aryal, 2015] (B) Negative Control [Aryal, 2015] (C) *S. flexneri* negative on Voges-Proskauer Test

3.2.2.4 Citrate Utilization Test

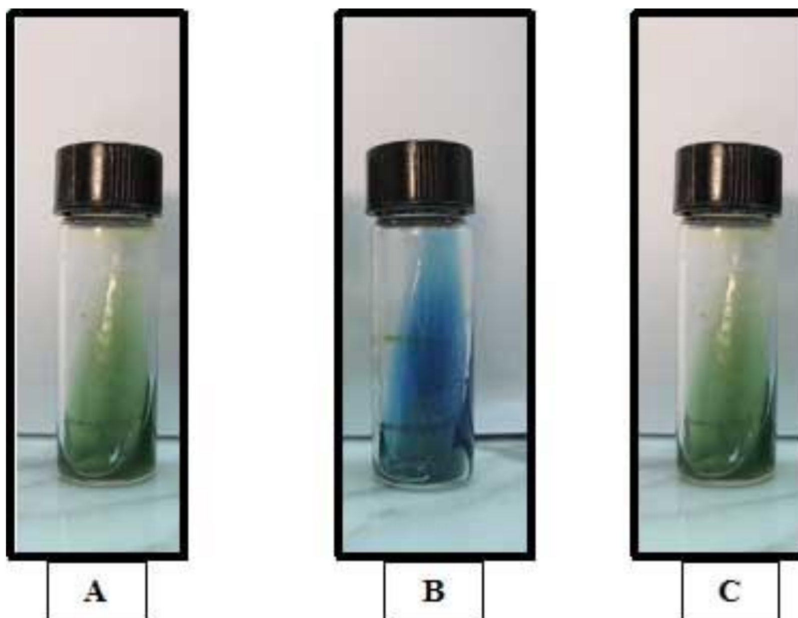


Figure 3.6: Vials showing results for Citrate Utilization Test: (A) Positive Control (B) Negative Control (C) *S. flexneri* negative on Citrate Utilization Test

3.3 Preservation of *S.flexneri*

S. flexneri preserved on T_1N_1 agar medium is shown below:

Figure 3.7: T_1N_1 agar showing growth of *S. flexneri* culture

3.4 Serum sample Collection

The pure serum, which is obtained after centrifugation and preserved at -20^oC, is shown below:

Figure 3.8: Pure serum obtained after centrifugation and preserved at -20^oC

3.5 Bactericidal Assay of Human Serum against *S. flexneri*

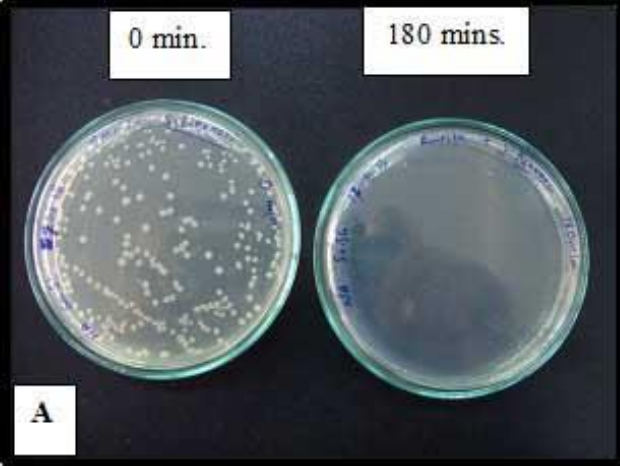
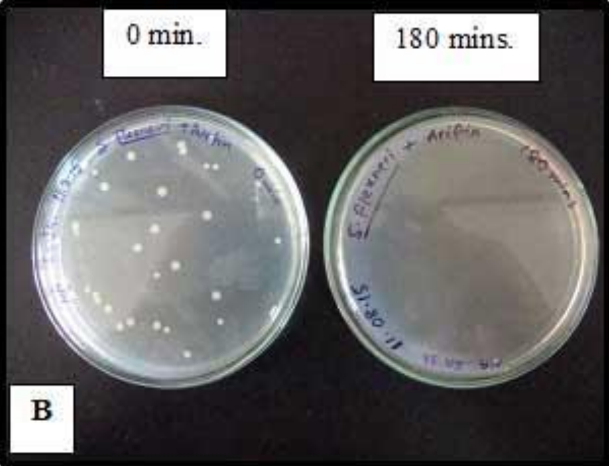
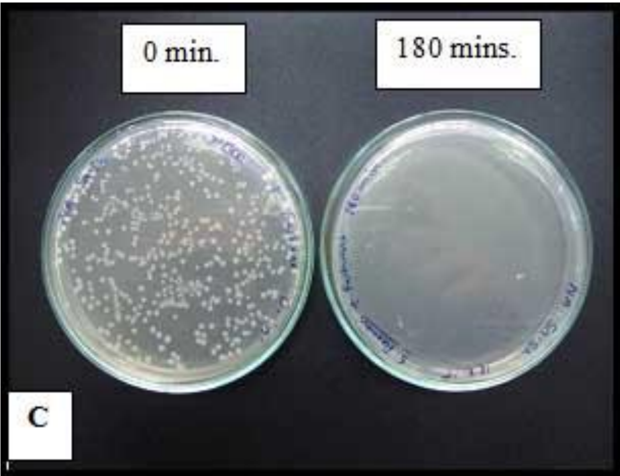
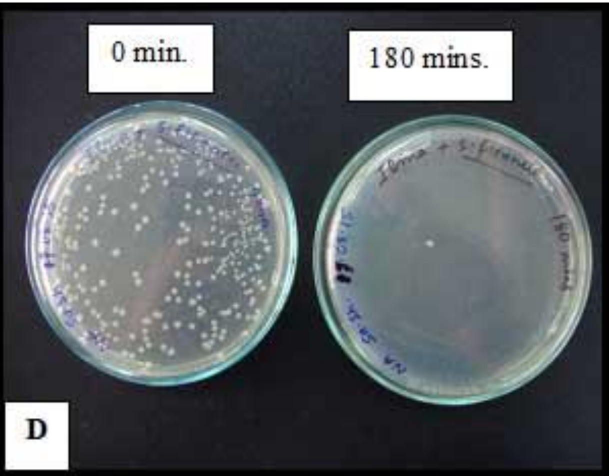
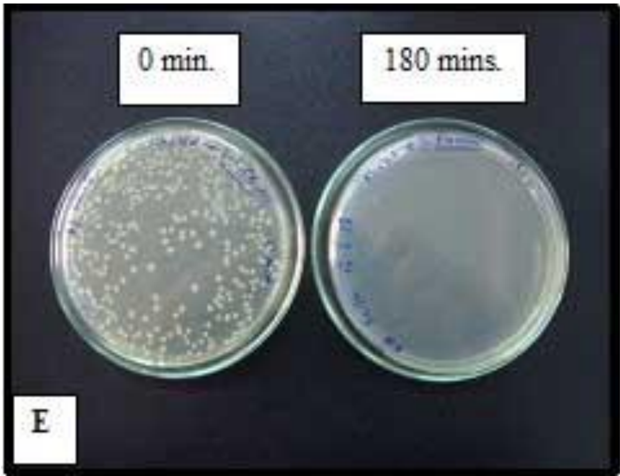
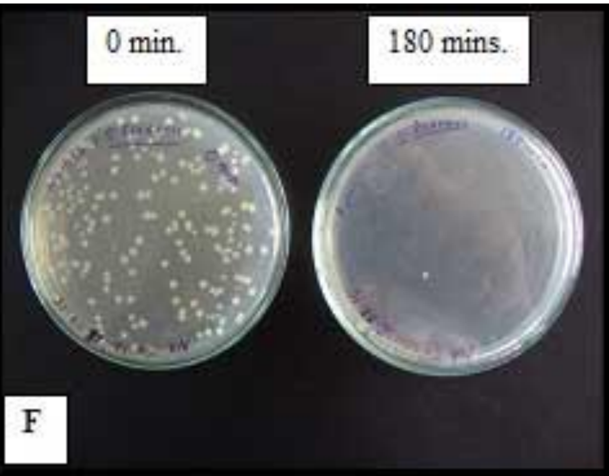
The ability of human serum, collected from urban population and slum population, to inhibit growth of *Shigella flexneri* was assessed by taking colony counts from nutrient agar plates at 0 minute and 180 minutes. The plates displayed growth of the bacteria immediately after mixing with serum, at 0 minute and 3 hours after incubation with serum, at 180 minutes. The results from 25 representative samples are depicted in Table 3.2 and Table 3.3.

Table 3.2: The ability of human serum collected from urban population to inhibit the growth of *S. flexneri* at 180 minutes in 25 representative samples

Sample No.	Serum Sample	Colonies of *S. flexneri*	
		0 minute	180 minutes
1	SU1	TNTC	0
2	SU2	33	0
3	SU3	TNTC	0
4	SU4	TNTC	1
5	SU5	TNTC	0
6	SU6	194	1
7	SU7	252	0
8	SU8	206	0
9	SU9	265	6
10	SU10	TNTC	0
11	SU11	TNTC	1

Sample No.	Serum Sample	Colonies of *S. flexneri*	
		0 minute	**180 minutes**
12	SU12	46	0
13	SU13	TNTC	0
14	SU14	51	1
15	SU15	TNTC	0
16	SU16	TNTC	0
17	SU17	TNTC	0
18	SU18	136	0
19	SU19	144	0
20	SU20	200	0
21	SU21	224	0
22	SU22	231	5
23	SU23	236	0
24	SU24	212	0
25	SU25	279	3

The following figures display the growth of *S. flexneri* at 0 minute and 180 minutes in 10 representative serum samples collected from urban area.


0 min.
180 mins.
A

0 min.
180 mins.
B

0 min.
180 mins.
C

0 min.
180 mins.
D

0 min.
180 mins.
E

0 min.
180 mins.
F

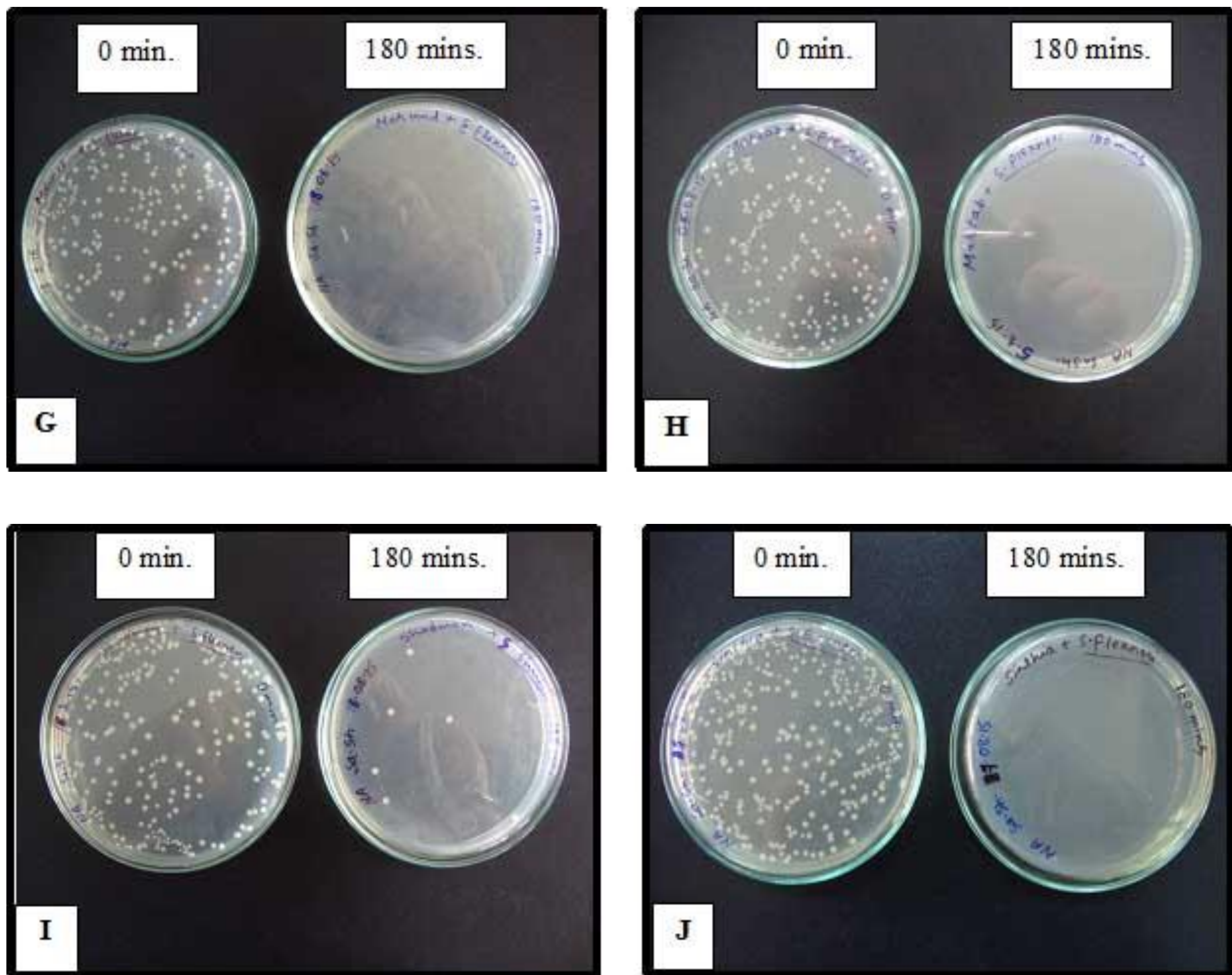


Figure 3.9: Colonies of *S. flexneri* on nutrient agar at 0 minute and 180 minutes with different serum samples collected from urban population: (A) SU1 (B) SU2(C) SU3 (D) SU4 (E) SU5 (F) SU6 (G) SU7 (H) SU8 (I) SU9 (J) SU10

The following figure is a graphical representation of serum activity against *S. flexneri* in 10 representative samples of the urban population.

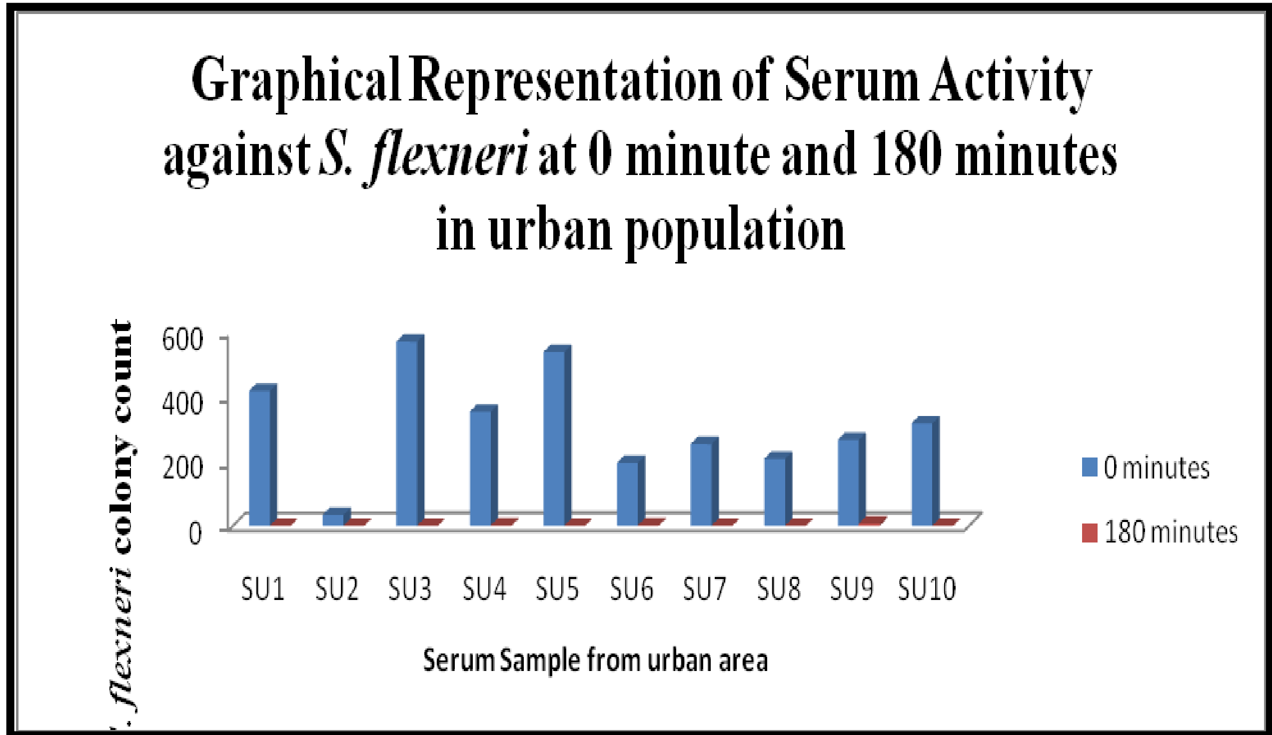


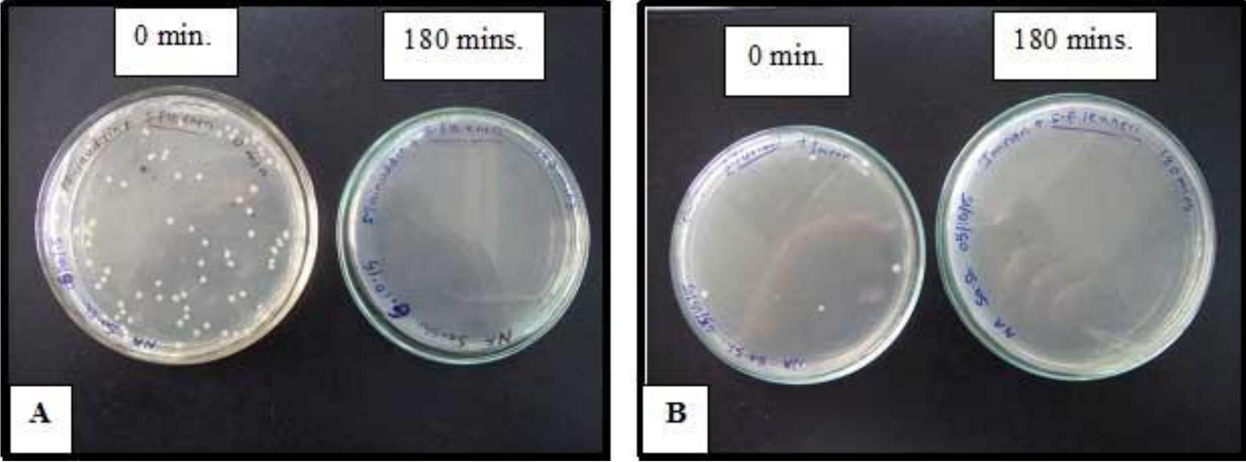
Figure 3.10: Graphical representation of serum activity against *S. flexneri* at 0 minute and 180 minutes in urban population

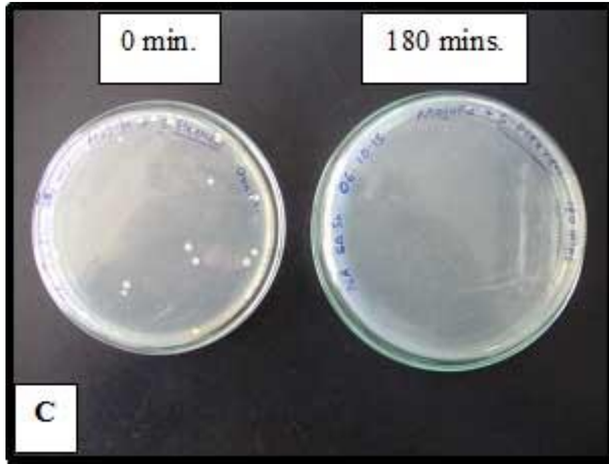
Table 3.3: The ability of human serum collected from slum population to inhibit the growth of *S. flexneri* at 180 minutes in 25 representative samples

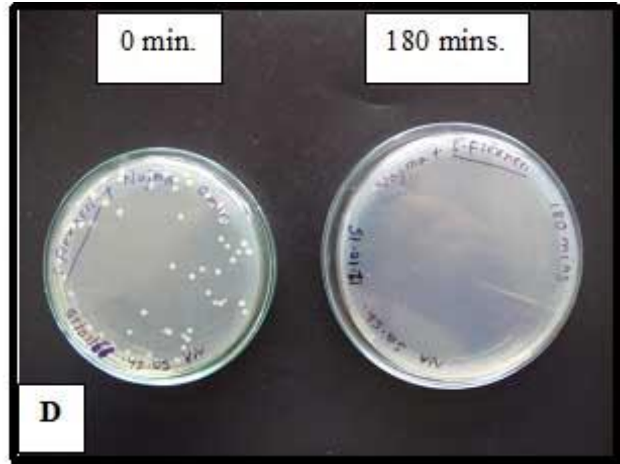
Sample No.	Serum Sample	Colonies of *S. flexneri*	
		0 minute	180 minutes
1	SS1	94	0
2	SS2	6	0
3	SS3	23	0
4	SS4	69	0
5	SS5	111	0
6	SS6	68	0
7	SS7	205	0
8	SS8	227	0
9	SS9	95	0
10	SS10	63	0
11	SS11	252	9
12	SS12	47	31
13	SS13	TNTC	0
14	SS14	1	0
15	SS15	46	1
16	SS16	39	0
17	SS17	130	27
18	SS18	44	0
19	SS19	10	0

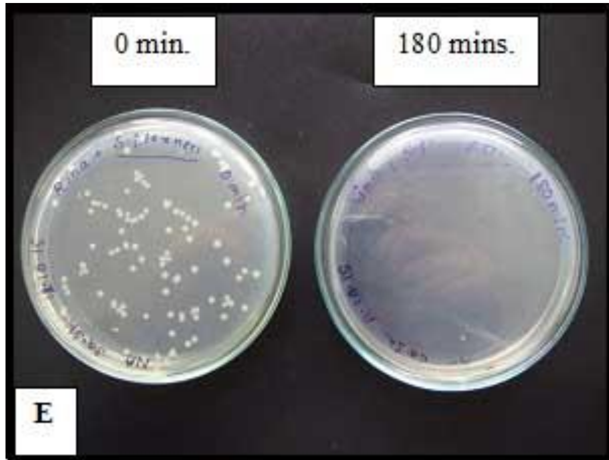
Sample No.	Sample Name	Colonies of *S. flexneri*	
		0 minute	180 minutes
20	SS20	85	0
21	SS21	1	0
22	SS22	138	0
23	SS23	173	0
24	SS24	5	0
25	SS25	193	0

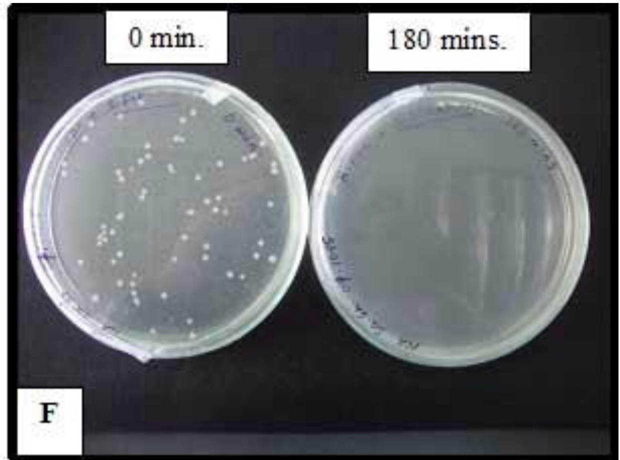
The following figures display the growth of *S. flexneri* at 0 minute and 180 minutes in 10 representative serum samples collected from slum area.

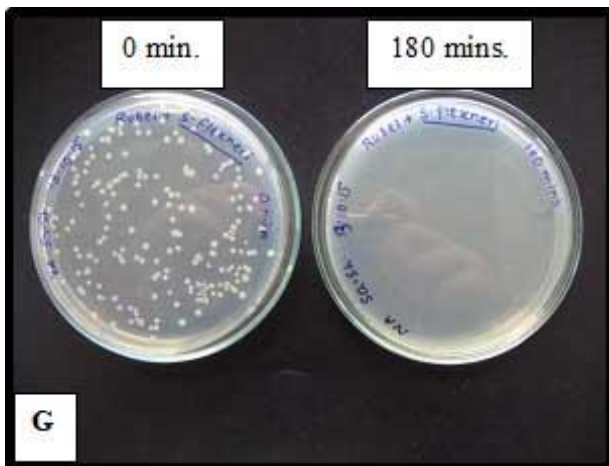


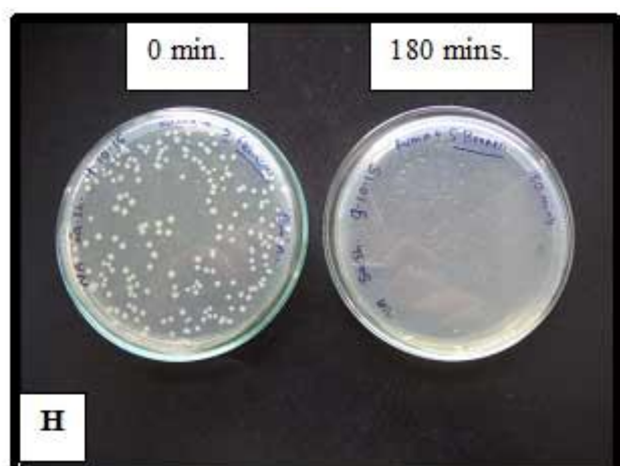

0 min.
180 mins.
C


0 min.
180 mins.
D


0 min.
180 mins.
E


0 min.
180 mins.
F


0 min.
180 mins.
G


0 min.
180 mins.
H

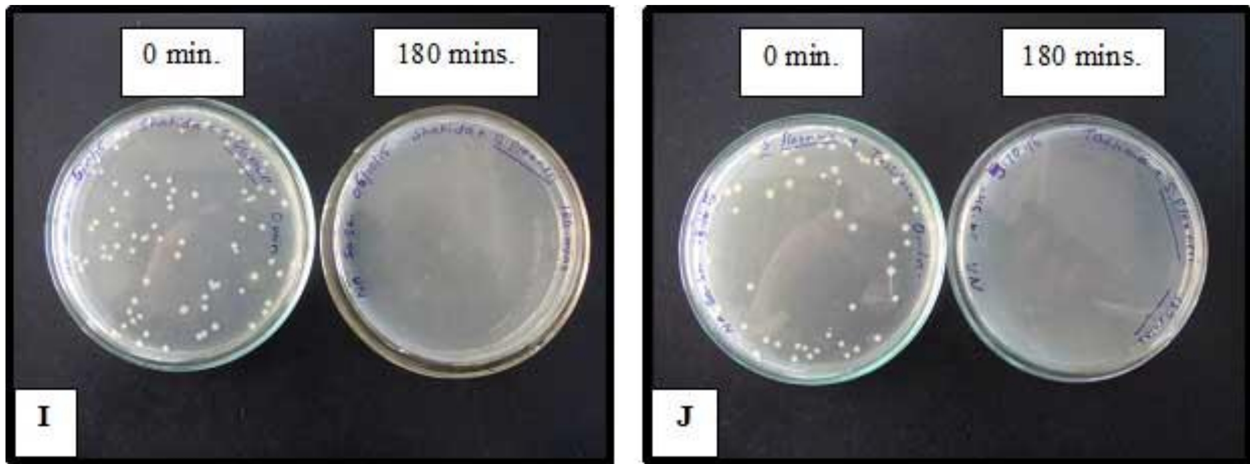


Figure 3.11: Colonies of *S. flexneri* on nutrient agar at 0 minute and 180 minutes with different serum samples collected from slum population: (A) SS1 (B) SS2 (C) SS3 (D) SS4 (E) SS5 (F) SS6(G) SS7 (H) SS8 (I) SS9 (J) SS10

The following figure is a graphical representation of serum activity against *S. flexneri* in 10 representative samples of the slum population.

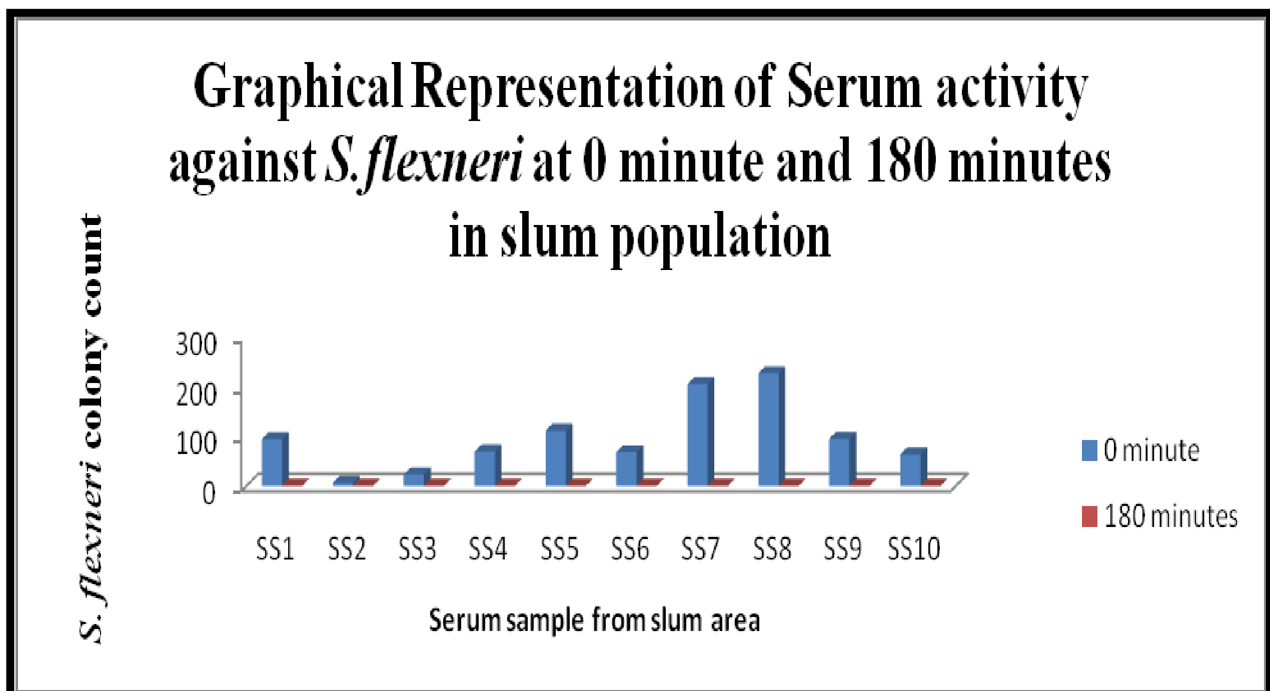


Figure 3.12: Graphical representation of serum activity against *S. flexneri* at 0 minute and 180 minutes in slum population

In order to compare the results, the average serum activity against *S. flexneri* in different areas were calculated at 0 minute and 180 minutes and depicted in a graph.

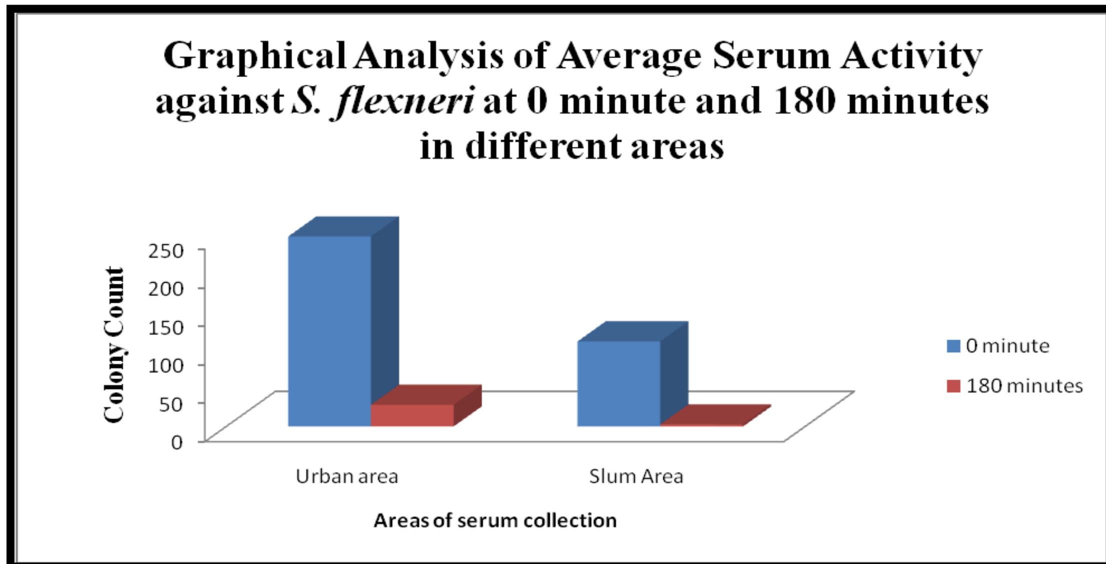


Figure 3.13: Graphical analysis of average serum activity against *S. flexneri* at 0 minute and 180 minutes in different areas

The percentage inhibition in growth of *S. flexneri* caused by serum samples collected from different areas was calculated for better understanding and comparison of the results.

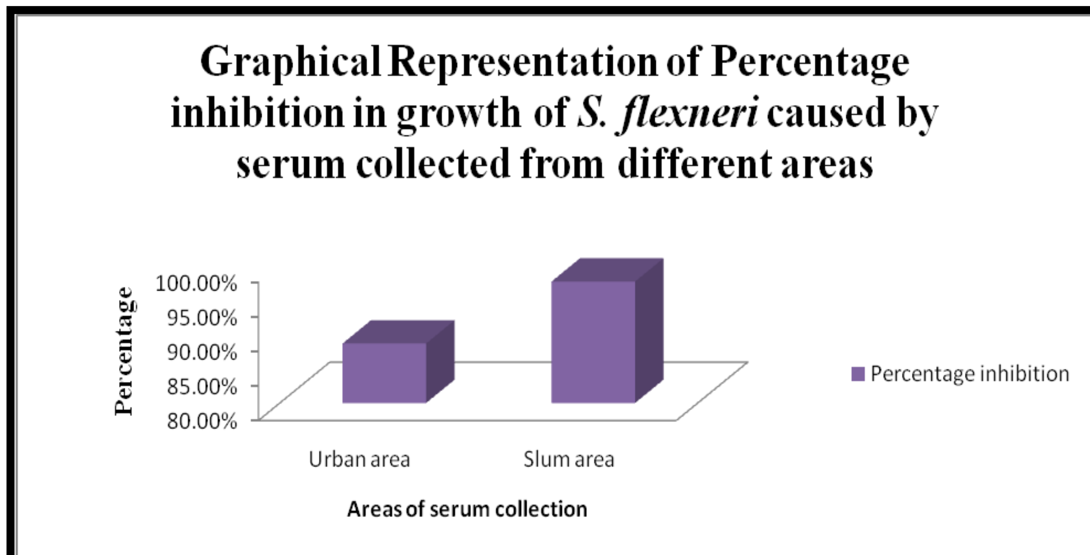


Figure 3.14: Graphical representation of percentage inhibition in growth of *S. flexneri* caused by serum collected from different areas

4. DISCUSSION AND CONCLUSION

Shigella flexneri is a Gram-negative bacterium and a human intestinal pathogen, causing the diarrhoeal disease shigellosis [Saeed *et al*., 2012]. Shigellosis is a disease which is recognized as a global problem with high morbidity and mortality, particularly in developing countries. It is highly endemic in Bangladesh, and occasionally flares into epidemics [Hossain *et al*., 1990]. *S. flexneri* is the predominant serotype of *Shigella* in Bangladesh [Islam *et al*., 1996]. *Shigella* is transmitted efficiently via the fecal-oral route [Weissman *et al.*, 1975] and through the contamination of food [Marler, 2013]. The symptoms of shigellosis can range from mild diarrhoea to severe dysentery with the passage of frequent bloody, mucoid stools; other symptoms include fever, intestinal cramps and convulsions [Lonnen, 2007].

Human serum is considered an important host defence mechanism against invasive diseases caused by gram negative bacteria [Igumbor & Osayande, 2000]. It provides the host with antibodies and complement [Okamura *et al.*, 1988]. The major role of the complement system is to recognize and promote the clearance of invading microorganisms [Bugla-Ploskonska *et al*., 2009]. This involves a number of mechanisms including cell-independent bactericidal activity with formation of membrane attack complex, and opsonization for uptake and killing by phagocytic cells [O'Shaughnessy *et al*., 2012]. The complement mediated activity of serum might differ in different population groups. Therefore it is important to further investigate this activity and learn more about it.

This study is reporting the comparative complement activity of blood serum against *Shigella flexneri* in urban and slum population. The two population groups are considered due to their significant differences in lifestyle. The slum based population live in overcrowded condition and often lack access to basic sanitation and safe drinking water. This might cause them to have a high exposure to the pathogen *S. flexneri* and exhibit higher resistance against it compared to the urban population.

The results obtained showed that serum samples collected from both urban and slum based population is able to decrease and inhibit the growth of *S. flexneri*. The serum activity against *S.flexneri* at 0 minutes and 180 minutes for 10 representative serum samples collected from

urban area and slum area shown in figures. Each sample, from both areas, demonstrates a significant decrease in growth of *S. flexneri* from 0 minute to 180 minutes.

In order to compare the complement activity of serum collected from the different population against *S.flexneri*, the average serum activity was determined. It was observed that the growth of *S. flexneri* at both 0 minute and 180 minutes was higher for serum samples collected from urban area compared to that collected from slum area. The percentage inhibition in growth of *S. flexneri* caused by serum samples collected from these different populations was then assessed. It clearly showed that serum collected from the slum population caused higher inhibition of *S. flexneri* in contrast to that collected from urban population.

The results of the study help us to draw the conclusion that the urban population has a better lifestyle in terms of living condition, sanitation, safe drinking water etc. They do not have high exposure to a pathogen like *S. flexneri*, which is not a normal microbial flora of the human body like *E. coli,* and the serum collected from them is unable to inhibit growth of *S. flexneri* as effectively as slum population. The slum population, on the other hand, has repeated exposure to this pathogen and their serum can inhibit its growth more efficiently and exhibit resistance against it.

Further studies are required using diverse population groups to compare the complement activity of serum against *S.flexneri* and find whether lifestyle changes cause any significant difference or not.

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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	Amounts (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	Amounts (g/L)
Lab-lemco Powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium Chloride	5.0

3. T_1N_1 Soft Agar

Ingredients	Amounts (g/L)
Tryptone	0.6
Sodium Chloride	0.3
Agar	0.42

4. Simmon's Citrate Agar (Oxoid, England)

Ingredients	Amounts (g/L)
Magnesium Sulfate	0.2
Ammonium dihydrogen phosphate	0.2
Ammonium phosphate	0.8
Sodium citrate	2.0
Sodium chloride	5.0
Agar	15.0
Bacto brom thymol blue	0.08

5. Peptone Water

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

6. MR-VP broth

Ingredients	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Potassium Phosphate	5.0

7. XLD (Xylose-Lysine-Deoxycholate) Agar (Himedia, India)

Ingredients	Amounts (g/L)
L-lysine	5.0
Lactose	7.50
Sucrose	7.50
Xylose	3.50
Sodium chloride	5.0
Sodium deoxycholate	2.50
Yeast extract	3.0

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 $4^{o}C$.

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 $4^{o}C$.

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 $4^{o}C$.

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 at $4^{o}C$.

APPENDIX-III

Instruments

The important equipments used through the study are listed below:

Name of Item	Specification
Autoclave	Model: HL-340, Gemmy Industrial cor., Taiwan
Balance	Radwag, WTB200
Centrifuge (High-speed refrigerated Micro centrifuge)	Model: GAM 1.5-2.4 Scanspeed 1730, Denmark
Hot Air Oven (Sterilizer)	Model: 02G Jero Tech, Korea
Incubator	Model: DSI500, Taiwan
Laminar airflow cabinet	SAARC
Micropipette (2-20µl)	Eppendorf, Germany
Micropipette (2-200µl)	Eppendorf, Germany
Micropipette (100-1000µl)	Eppendorf, Germany
Oven (Microwave Oven)	Model: MH6548SR, LG, China
Refrigerator	Model-0636, Samsung
Shaking Incubator	Model: WIS-20R, Daihan Scientific, Korea
Vortex Mixture	VM-2000, digisystem, Taiwan