Identification and Characterization of Neurotoxic Factor(s) present in *Shigella*

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PhD Thesis
November 2014

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Identification and Characterization of Neurotoxic Factor(s) present in *Shigella*

A dissertation submitted to
the Faculty of Biological Science
of The University of Dhaka

in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

Submitted By
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Registration No 213
Session: 2006-2007
November 2014

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Dedicated to

My daughter Alishba
My son Adyan
&
My mother Mrs. Laila Azam

Acknowledgements

I would like to express my wholehearted appreciation to all those who made it possible for me to complete this PhD thesis. I am deeply indebted to my supervisor, Dr. Kaisar Ali Talukder, Senior Scientist and Head, Enteric and Food Microbiology Laboratory of icddr,b without whose able guidance this thesis work would not have been possible. I would like to express my special gratitude to him for sharing his vast knowledge, experience and important evaluations of the research works, giving me encouragement, offering me opportunities of exposure to international research groups and seminars, continuous guidance throughout the thesis work and training needed to become an independent researcher. I am really thankful to him.

I wish to express my best and profound gratitude to my supervisor, Dr. Chowdhury Rafiqul Ahsan, Professor, Department of Microbiology, University of Dhaka, for his unreserved commitment to bring me to completion of this thesis. I count myself very fortunate to have been his student and be benefited from his critical guidance on improving my scientific view.

I express my heartfelt gratitude, indebtedness and sincere appreciation to my respected supervisor, Dr. Alam Nur-E-Kamal, Professor, City University New York, USA for his constant supervision, constructive criticism and never-ending inspiration throughout the entire period of study.

I am very grateful to Prof. Dr Mahmuda Yasmin, Chairman, Department of Microbiology, University of Dhaka, for her generous support of this study. It is my pleasure to express my complements to all teachers of The Department of Microbiology, University of Dhaka, for their encouragement.

I sincerely thank Dr. Enamul Kabir, Assistant Professor, Sir Salimullah Medical College for helping me in histopathological experiments in animal model, Ms. Zabeen Kamal, City University of New York, USA, for her help with western blotting experiments and analyses, Mr. Ijaz Ahmed for his continuous help with neuron culture experiment at the time of my stay at University of Medicine and Dentistry of New Jersey, USA.

I am also grateful to Dr. Munirul Alam for permitting me to use the fluorescence microscope in his lab, Dr. Marzia for her great help with Immunostaining experiments, Mr. Safi Ahmed for providing invaluable advice on HeLa cell culture and Mr. K. M. Nasirul Islam for his help to carry out the animal experiment in his facilities in the Animal resources branch of icddr,b.

I sincerely thank my colleagues: Mr. D. K. Dutta, Dr. Amin, Dr. Zhahir, Dr. Shamim, Mr. Nurul Amin Bhuiyan, Mr. Aslam, Dr. Salam, Ms. Mahmuda, Ms. Trisheeta, Ms. Halima, Mr. Atanu, Mr. Shawkat, Mr. Shahnaij, Ms. Sharmin Luna, Mr. Hasan, Mr. Nayeem, Mr. Shawpan, Mr. Preetom, Mr. Hossain and Mr. David for their precious assistance and support throughout my research work. My special thanks to Kohinur Apa, Sabir Vai and Pavel and Fatema for their constant help during my study. My special thanks to Yasmin Apa for her inspiration in all times.

It is hard to say enough about the patient love of my husband Ahtesham and my kids Alishba and Adyan, and my parents and in-laws whose support enabled me to complete my PhD research. My special thanks to Ahtesham for helping me to preparing this thesis book. My family has given a lot of love and care to me and especially my mother whose unending courage was amazing, my sisters and brother and all other family members always wished me success in all my endeavors. My special thanks to my father for his persisting encouragement.

I am obliged to many people whose names do not appear here, but I do acknowledge their direct or indirect help in this thesis. Finally, I bow down my head to the Almighty Allah, who showered blessings and provided me strengths and courage to overcome all challenges and accomplish my research work.

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Abbreviations

µg	Microgram
µl	Micro litre
AIDS	Acute immune deficiency syndrome
AMP	Adenosine mono phosphate
ATCC	American type culture collection
ATM	Ataxia telangiectasia mutated
bp	Base pair
BSA	Bovine serum albumin
Cdks/CDKs	Cycline dependent kinases
cfu	Colony forming unit
dATP	Deoxy adenosine triphosphate
dCTP	Deoxy cytidine triphosphate
dGTP	Deoxy guanosine triphosphate
DMEM	Dulbucco's modified eagles medium
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
dTTP	Deoxy thymidine triphosphate
EDTA	Ethylene diamine tetra acetic acid
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
FBS	Fetal bovine serum
Gb3	Globotriaosyceramide
Gb4	Globotetraosyceramide
gm	Gram
HC	Hemorrhagic colitis
HeLa cell	Human cervical epithelial cell
HEp-2	Human laryngeal epithelial cell

Abbreviations (cont.)

HUS	Hemolytic uremic syndrome
Icddr,b	International Center for Diarrhoeal Disease Research, Bangladesh.
IP	Intra-peritoneal
kbp	Kilo base pair
kDa	Kilo dalton
l	Litre
LD50	Lethal dose 50%
mcg	Microgram
MDa	Mega Dalton
min	Minute
No	Number
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pH	Negative logarithm of hydrogen ion concentration
PI	Propidium iodide
RIP	Ribosome inactivating protein
RNA	Ribonucleic acid
Spp.	Species
STEC	Shiga toxin-producing *Escherichia coli*
Stx	Shiga toxin
TSB	Trypticase soy broth
TTP	Thrombotic thrombocytopenic purpura
UV	Ultra violet

Technical Terms

stx$_1$	gene for Shiga toxin 1
stx$_2$	gene for Shiga toxin 2
set	gene for *Shigella* Enterotoxin 1(ShET-1)
sen	gene for *Shigella* Enterotoxin 1(ShET-2)
*ipa*H	gene for *Shigella* invasive plasmid antigen
ial	gene for *Shigella* invasive associated locus

Abstract

Infection by *Shigella* is associated with a significant number of human deaths worldwide. Complications from Shigellosis include leukemoid reaction, hemolytic uremic syndrome (HUS), seizure, encephalopathy, abdominal cramps, rectal pain and septicemia, which significantly increase morbidity and mortality. Abnormalities in the central nervous system (CNS) and HUS are more common in children under 5 years of age. The majority of *Shigella* associated neurological findings were reported in patients with *S. sonnei and S. flexneri.* But both the species do not usually produce shiga toxin, as both are lacking the structural gene encoding shiga toxin production. However, neurotoxic principle(s) in *S. flexneri* has not yet been discovered. In this study an attempt was taken to identify neurotoxic factor(s) present in *Shigella* to understand the molecular basis of toxin induced mammalian cell death.

A total of 385 clinical isolates of different serotypes of *Shigella* isolated between 1997 and 2013 from diarrhoeal patients attending the Dhaka treatment centre of icddr,b were included in this study. Of these, 238 were *S. flexneri,* 80 *S. dysenteriae,* 42 *S. boydii* and 25 *S. sonnei.* All these strains were characterized following standard Microbiological and Biochemical methods. These strains were screened for the presence of known toxin and virulence genes such as *ipa*H, *ial*, *ipa*BCD, *set*, *sen* and *stx* by PCR and plasmid profiling. Cytotoxic and neurotoxic activity of the toxin were determined using HeLa cells and cerebellar granule neurons from rat pups respectively. Fluid accumulation in Rabbit ileum and histopathological changes of the rabbit loop segments was studied for enterotoxic activity. DNA fragmentation and chromatin condensation assay were used as markers of apoptosis. The biological status of cells at different phases of cell cycle was analyzed by FACS at different time intervals. The toxin(s) preparations involved 40%, 60% and 80% saturation of the culture supernatant with ammonium sulphate. Toxin(s) containing fraction was prepared by column chromatography using sephadex G-75.

Of 385 isolates, PCR and plasmid analysis showed that almost all serotype of *Shigella* contain the 140MDa plasmid and *sen* gene except 17 *S. flexneri* 1c strains. All *S. dysenteriae* 1 contained stx_1 gene whereas the *set* gene is only present in *S. flexneri* 2a and rarely in *S. flexneri* 2b. Seventeen 140 MDa lacking *S. flexneri* 1c strains were also negative for the common toxin gene. These 17 strains were noninvasive by sereny and Congo red binding test. Therefore, these 17 *S. flexneri* 1c strains were extensively used for comparative analysis. *S. flexneri* 1c (K-314 and K-915) were used as a representative of these 17 strains for partially purified toxin fractions and source of novel toxic factor(s) in this study. The degree of cytotoxicity differs in different species of *Shigella.* Around 72% (172/238) strains of *S. flexneri* possessed the cytotoxic activity. Of *S. flexneri,* serotype 1c had severe effect (more than 85% cell death) on HeLa cell. Serotype 2a, 2b, X variant and type 4 had almost similar cytotoxic effect (>70% cell death) while serotype

3a, Y variant, 4X, 6a and 6b showed less cytotoxic effect (40-45% cell death). In case of *S. dysenteriae* (n=80), only serotype 1 and some strains of serotype 4 showed cytotoxic effect (>90% cell death). In contrast, *S. boydii* (n=42) supernatant caused elongation of the HeLa cells whereas *S. sonnei* (n=25) caused the rounding of the cell with less frequency. It is interesting to note that the 17 *S. flexneri* 1c strains lacking 140 MDa plasmid and toxin gene showed strong cytotoxic effect (>90% cell death) on HeLa cells in dose dependant manner. Further analysis showed that toxic factors in these *S. flexneri* 1c exhibited biochemical properties similar to those of protein. Heat treatment significantly reduced cytotoxic activity of *S flexneri* 1c (K-314). DNase and RNase treatment did not affect cytotoxic activity while protease treatment significantly reduced its cytotoxic activity. HeLa cells were also treated with 40%, 60% and 80% fractions of *S. flexneri* 1c K-314 strain. However, only 60% fraction showed strong cytotoxic activity. This fraction contains an active protein component molecular mass of 100–125kDa which is sensitive to heat and trypsin. In the rabbit ileal loops assay, *S. flexneri* 1c (K-314) was unable to accumulate fluid. But the histo-pathological report of the loop segments showed moderate inflammatory infiltrate in lamina propria, submucosa of the wall of the intestine and sheared off of tips villi in some places with Grade-3 inflammation. However, lysates *S. dysenteriae* 1 caused fluid accumulation and enterocyte necrosis and shearing of tip of villi with Grade-4 inflammation. In this report, the culture supernatant of *S. flexneri* exhibited not only the cytotoxic activity, but also impaired neurological function in mice. The LD50 dose was found to be 1 µg. The sign of toxic effect appeared after 30h and finally hind limb paralysis and rapid breathing were observed and all mice died within 36h. In primary rat brain (cerebellar) neurons culture model it was found that these strains showed strong neurotoxic activity. The neurite length (at least 100 neurons from random fields) was determined from both toxin treated and untreated neurons. Reduction of neurite length indicated the presence of neurotoxic factor. *S. flexneri* induced apoptosis in HeLa cells. The toxin fraction(s) has been shown to induce apoptosis as determined by morphological changes (rounding) inhibiting polymerization of actin filament, chromatin condensation, and chromosomal DNA fragmentation in HeLa cells. Phosphorylation of the H2AX and release of cytochrome C indicated that toxin from *S. flexneri* might activate additional pathways leading to apoptosis. The progression of HeLa cell was arrested at G_0/G_1 phase as the percentage of cells in that phase increased gradually from 17.25 to 51.52 with time.

This study represents the first attempt for identification and characterization of neurotoxic factor present in *S. flexneri*. The toxin produced by *S. flexneri* 1c showed cytotoxic, enterotoxic and neurotoxic activity. The above evidences suggested that toxic factors in *S. flexneri* are different from that of *stx*, *set*, and *sen*. Identification and characterization of toxic factors in *S. flexneri* may provide critical information in understanding the molecular basis of *Shigella*-induced neurotoxic effect on different types of human cells.

1.0 Introduction

1.1 Overview

Shigella **s**pp. are the causative agents of bacillary dysentery, causing an estimated 165 million cases per year and over 1 million deaths (Talukder and Azmi, 2012). Most of the infections and deaths occur in under five children in developing countries where *Shigella* are endemic. However, *Shigella* are also frequently isolated enteric pathogens in the U.S., causing an estimated disease burden of over 400,000 cases per year, primarily due to ingestion of contaminated food or water (Mead *et al*.,1999). Outbreaks of shigellosis can also occur in industrialized settings with suboptimal hygiene, such as day care centers (Gupta *et al*., 2004). *Shigella* has one of the lowest infectious doses among pathogenic bacteria, requiring as few as 10-100 organisms to cause disease in humans (Dupont *et al.,*1989). This low infectious dose in contrast to the 4 to 6 logs required for infection by *Salmonella* or *Vibrio cholerae*, largely accounts for the person-to-person transmission of *Shigella*. Four 'species' within the *Shigella* "genus" cause significant disease in humans following ingestion of contaminated food or water: *S. dysenteriae, S. flexneri, S. sonnei*, and *S. boydii*. Each serogroup of *Shigella* contains multiple serotypes, and at least 54 serotypes are currently recognized (Talukder and Azmi,2012). Typical bacillary dysentery, characterized by fever, abdominal cramps, and diarrhea containing blood and mucous results from the death and sloughing of contiguously invaded epithelial cells and the induction of inflammatory responses by the bacteria (Sansonetti *et al.*, 2001) *S. dysenteriae* is unique because it produces Shiga toxin and causes the most severe symptoms, including bloody diarrhea and the hemolytic uremic syndrome (HUS). *S. flexneri* and *S. sonnei* are the most frequently isolated species, causing dysentery and non-bloody diarrhea. Diagnosis of shigellosis is essentially clinical. Laboratory diagnosis includes stool culture and polymerase chain reaction (PCR). Treatment includes use of an effective antibiotic, rehydration therapy (if there is dehydration) and appropriate feeding during and after an episode of shigellosis. Indiscriminate uses of the drugs and horizontal gene transfer have led *Shigella* species to become resistant to commonly used antibiotics. Moreover to develop a new effective vaccine, it is important to know the most frequent serotypes and their changes around the world because immunity to *Shigella* is serotype specific. But the genetic variability between serotypes and emergence of atypical strains accentuate the problems for the development of an effective vaccine. Infection of *Shigella* results various types of complication especially in

young children. Complications in Shigellosis include leukemoid reaction, and haemolytic uraemic syndrome, seizure, encephalopathy, abdominal cramps, rectal pain and septicemia, which significantly increase morbidity and mortality (Proulx *et al*., 2001; Siegler, 1992). These manifestations are difficult to explain solely on the basis of cell invasion leading to the hypothesis that *Shigella* produce toxin(s), which elicits host cell death and fluid secretion (O'Brien *et al*., 1992; Sanosonetti, 1998). Several virulence factors (e.g. Ipa and Vir) have been found to be associated with the development of colonization and invasion property (Hale, 1991). Invasion-associated locus and invasion plasmid antigen (*Ipa*H) genes located either in plasmid or chromosome are responsible for invasiveness in *Shigella* spp. (Frankel *et al*., 1989; Venkatesan *et al*., 1989). Cytotoxic and/or neurotoxic principles are likely to be associated with the development of complications associated with Shigellosis (Cherla *et al*., 2003). A clear understanding of the role of toxin in Shigellosis has remained to be studied.

Development of antibiotic resistance in *S. dysenteriae* type 1 has been found to cause periodic emergence of epidemic in the world. In Bangladesh, three such epidemics have been reported since 1973 (Talukder *et al*, 2003). Each of these epidemics caused high rate of mortality particularly in children under age of 5 years (Talukder *et al*. 2003; Bennish and Wojtyniak , 1991). Various strains of *S. dysenteriae* 1 produce Shiga toxin (Stx) having strong cytotoxic activity to mammalian cells (Hale and Formal, 1980; Hughes *et al*, 2000). Multiple types of toxins and their corresponding protein product have been characterized (Nakao and Takeda, 2000). Two subgroups of Stx (Stx1 and Stx2) have recently been identified. A large number of variants of Stx1 and Stx2 have been isolated illustrating diversity of Stx family (Gannon and Gyles 1990; Ito *et al*., 1990; Lin *et al*., 1993). *S. flexneri* is one of the most prevalent serotype found in Shigellosis (Ferreccio, *et al*., 1991; Henry, 1991). In recent studies in Bangladesh, it has been shown that *S. flexneri* is linked to more than 50% of Shigellosis (Khan *et al*., 2004; Talukder and Azmi, 2012). *S. flexneri* bears invasive genes including ipa and vir (Sasakawa *et al*., 1992). In addition, two enterotoxins have been identified in *S. flexneri*: *Shigella* enterotoxin 1 (ShET-1) encoded by the set1 chromosome gene and *Shigella* enterotoxin 2 (ShET-2) encoded by the sen gene (140 MDa plasmid) (Vargas *et al*., 1999). Cytotoxic activity of enterotoxins remained to be studied. Abnormalities in CNS are more common during shigellosis than with other childhood infections. It has been shown that seizures in shigellosis is more frequent in children 5 years of age or less and

reported an altered consciousness. Among *Shigella* species, Shiga toxin is produced in substantial quantities by *S. dysenteriae* type 1. Incidence of HUS and convulsion has been shown to be more in *S. dysenteriae* type 1. Moreover, Shiga toxin producing strains of *Shigella* and *E. coli* have been shown to be associated with disease involving central nervous system in children and adults. These observations suggest a possible role of toxin(s) in developing convulsion and HUS in shigellosis. The majority of *Shigella* associated neurological findings were reported in patients with *S. sonnei and S. flexneri* (Khan *et al*., 1999). Both species do not usually produce shiga toxin, as both are lacking the structural gene encoding shiga toxin production. However, neurotoxic principle(s) in *S. flexneri* has not yet been discovered.

In order to study the involvement of toxin(s) in developing clinical complication, several *Shigella* strains were screened to identify known toxin genes by PCR. It was found that some of the strains carry one or more known toxin genes. Interestingly, a group of *Shigella* was found that lacks the 140MDa plasmid and was also negative for known toxin genes (*set, sen, stx*1) commonly present in *Shigella*. These strains showed strong cytotoxic effect on HeLa cells and have effect on neuronal outgrowth on primary neuron culture model as well thereby are consider as neurotoxic. These results indicate that some *Shigella* strains may have additional cytotoxic factor(s). In this study an attempt was taken to identify neurotoxic factor(s) present in *S. flexneri*. This study will expand current understanding of the development of clinical manifestations in shigellosis and may lead to the discovery of novel inhibitors affecting the function of nervous system.

1.2 Literature Review

1.2.1 Shigellosis

Shigellosis, also known as acute bacillary dysentery, is characterized by the passage of loose stools mixed with blood and mucus and accompanied by fever, abdominal cramps and tenesmus. It may be associated with a number of complications of which haemolytic uraemic syndrome is the most serious. The disease is one of the major diarrheal diseases in many developed and developing countries including Bangladesh and is caused by any one of the four species/group of *Shigella.* It is endemic throughout the world, and is one of the major causes of morbidity and mortality, especially among children <5 years of age (Wen X., *et al*., 2012; Bardhan P., *et al*., 2010). Infection and outbreaks associated

with this organism are prominent in developing countries and are strongly associated with poverty, poor sanitation, personal hygiene and poor water supply. Shigellosis can occur in sporadic, epidemic and pandemic forms (Talukder and Azmi, 2012).

1.2.2 The Organism: *Shigella*

Shigella is a genus of gamma proteobacteria in the family *Enterobacteriaceae.* They are Gram-negative, nonmotile, non-spore forming, rod-shaped bacteria, appearing singly, in pairs and in chains, facultatively anaerobic, oxidase negative, and ferment glucose and other carbohydrates without producing gas. By definition, all *Shigella spp.* are lysine decarboxylase negative. In additionally, the *Shigella* are Voges-Proskauer negative and methyl-red positive, do not utilize Simmons citrate, nor produce H_2S and are arginine dihydrolase and urease negative very closely related to *Escherichia coli. Shigellae*, with the exception of *S. dysenteriae* Type 1, are catalase positive. The optimum temperature of growth is 37ºC.

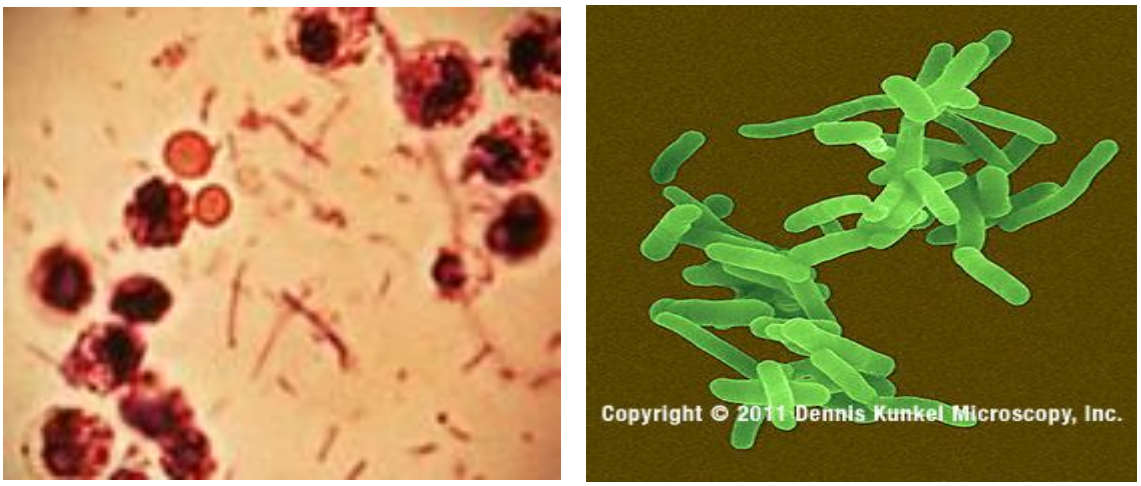


Figure 1.1 A. Photomicrograph of *Shigella* spp. in stool specimen B. *Shigella* photograph, Microscopic view

1.2.3 Serotype

Shigellosis is caused by one of four pathogenic species of the genus *Shigella*. The species are classified according to serogroups and so the species are often referred to as serogroups. *Shigella dysenteriae, S. flexneri*, *S. boydii*, and *S. Sonnei* are the four species that can cause disease in humans, which correspond to the serogroups A, B, C, and D, respectively. This pathogen can be further divided into serotypes based on the structure of the O-antigen component of LPS present on the outer membrane of the cell wall (Talukder and Azmi, 2012). *S. dysenteriae* (group A) has 16 serotypes (Bopp *et al*.,

2003); *S. flexneri* (group B) has 17 classical serotypes and subserotypes (Bopp *et al*., 2003), *S. boydii* (group C) has 20 serotypes and *S. sonnie* has a single serotype (Levine *et al*., 2007).

Table 1.1 Serotypes of *Shigella* spp.

Group	Species	Serotypes	Provisional Serotypes	Severity of disease
Group A Mannitol non-fermenter.	*Shigella dysenteriae*	13 serotypes Type 1 – 13	*S. dysenteriae*14 *S. dysenteriae*15 KIVI-162 (*S. dysenteriae*16)	Most severe.
Group B Mannitol fermenter, serologically non-distinct.	*Shigella flexneri*	6 serotypes Types 1 - 6	*S. flexneri* 1c *S. flexneri* Type-4 (4d) *S. flexneri* 4X (Z var.)	Mild/ severe.
Group C Mannitol fermenter, serologically distinct	*Shigella boydii*	18 serotypes	*S. boydii* 19 *S. boydii* 20	Mild/ severe.
Group D Late lactose fermenter, mannitol fermenter, serologically distinct.	*Shigella sonnei*	1 serotypes Phase I and Phase II	_	Mild.

(Adopted from Rowe *et al.*, 1984; Farmer *et al.*, 1991; Ansar *et al.*, 1995; Talukder and Azmi, 2012; and Grimont *et al.*, 2007).

1. 2.4 Global Distribution and Epidemiology of *Shigella* infection

Although *Shigella* is endemic worldwide, it affects certain populations more than others. In developing countries, high rates of morbidity and mortality are known to occur among displaced populations (Kotloff *et al*., 1999). In general, *S. sonnei* is more common in developed countries and *S. flexneri* and *S. dysenteriae* are more prevalent in developing countries. The proportions of each species vary from country to country. *S. dysenteriae* serotype 1 was the dominant species after its discovery in 1918, but was replaced by *S. flexneri* after World War I and virtually disappeared. Following World War II, *S. sonnei* gradually became the dominant species in the industrialized countries, while *S. flexneri* has persistent in developing countries especially in Asian countries. There are few exceptions such as Thailand, where *S. sonnei* has also replaced *S. flexneri* as the most common isolate (von Seidlein *et al*., 2006). *S. boydii* is present primarily in the Indian

subcontinent whereas it was the second most common species accounting 23% in Bangladesh. In all these countries 4% of isolates were of *S. dysenteriae* spp., but none was *S. dysenteriae* type 1 (von Seidlein *et al*., 2006).

1.2.5 Shigellosis in Bangladesh

In a recent study conducted by Talukder and Azmi in 2012 showed that of 15,990 strains of *Shigella* species isolated at the clinical microbiology laboratory of icddr,b between 1997 and 2013, *S. flexneri* was found to be the most predominant species (61%) followed by *S. boydii* (17%), *S. sonnei* (10%), *S. dysenteriae* (8%), and *Shigella*-like organism (SLO) which did not agglutinate with any antisera of the established *Shigella* serovars (3%) (Figure 1.2). During this study period, 23.4% of *S. flexneri*, 8% of *S. dysenteriae*, and 23% of *S. boydii* isolates were identified as atypical (Talukder and Azmi, 2012) since these isolates did not react with either type or group antigen-specific known antisera. Over the last 35 years, *S. flexneri* was always dominant in Bangladesh except during 1993-95 when it was replaced by *S. dysenterae*. Within *S. flexneri*, *S. flexneri* 2a was the predominant serotype.

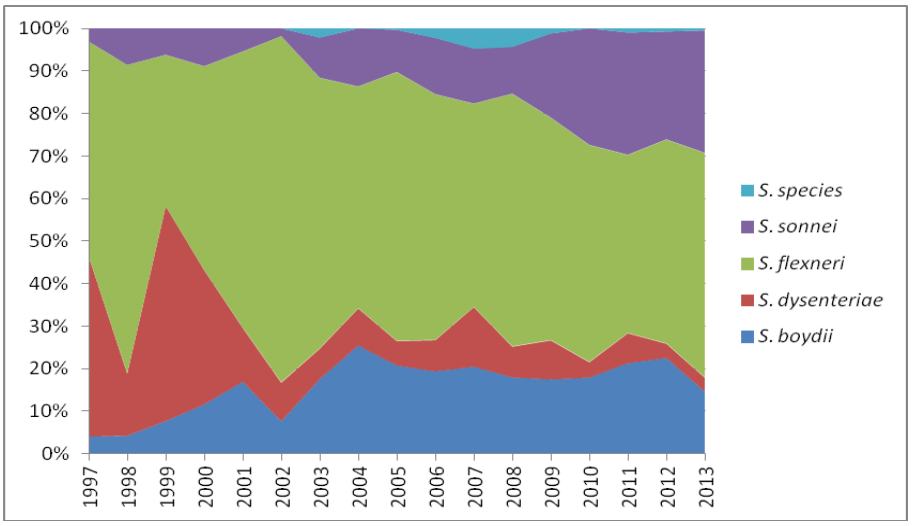


Figure 1.2 Prevalence of *Shigella* spp isolated from diarrhoeal patients in Dhaka, Bangladesh from 1997 to 2013 (n=15,990).

The prevalence of *S. sonnei* has surprisingly increased (27% in 2010 from 8% in 2000) and the prevalence of *S. dysenteriae* (3%) has drastically reduced in 2013 (Figure 1.1). It is interesting to note that since 2004, *S. dysenteriae* type 1 was not found at all in Bangladesh. This indicates that improved sanitation system might have an impact on the epidemiology of *Shigella* spp.

1.2.6 Clinical feature of *Shigella* infection

After *Shigella* infection symptoms appear abruptly after an incubation period of 12 hours to approximately 2 days and include high fever, generalized toxicity, anorexia, nausea, crampy abdominal pain, and diarrhea. (Shai Ashkenazi, 2004). Typically the diarrhea initially consists of high-volume watery stools, which may be followed by frequent, small volume, bloody mucus stools associated with urgency and painful defecation. 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. However, the exact mechanism underlying the onset of diarrhea during shigellosis is still poorly defined. Notably, *Shigella* enterotoxin 1 (ShET1) and ShET2, which are produced by several *Shigella* strains, were found to induce fluid secretion into the intestine, thus accounting for the watery phase of diarrhea (Fasano *et al.*,1997, Nataro *et al.*,1995). Moreover, Shiga toxin, which is produced only by *S. dysenteriae* serotype 1, is cytotoxic for a variety of cell types and is responsible for the development of vascular lesions in the colon, the kidney, and the central nervous system (Cherla *et al.*,2003). Due to the high toxicity of Shiga toxin, infections with *S. dysenteriae* serotype 1 are frequently associated with life-threatening complications (Cherla *et al.*,2003; O'Loughlin *et al.*,2001). It is becoming increasingly clear that *S. flexneri* skillfully exploits and evades the potentially harmful responses of the immune system (Phalipon *et al.*,2007).

1.2.7 Complications Associated with *Shigella* infection

Infection of *Shigella* results various types of complication include both intestinal and extra-intestinal manifestations (Bennish *et al*., 1991) especially in young children.

1.2.7.1 Extra Intestinal Complications of *Shigella* infection

1.2. 7.1.1 Bacteremia due to *Shigella* infection

Although usually confined to the colonic mucosa, bacteremia or septicemia rarely occurs during shigellosis and has been reported mainly in developing countries (Strulens *et al*. 1985). For example, according to one study from Bangladesh, 4 percent of patients with shigellosis had *Shigella* bacteremia (Strulens *et al*. 1985). Bacteremia occurred more frequently in malnourished young infants and in patients infected with *S. dysenteriae* serotype 1, and it was associated with a significant mortality rate.

1.2. 7.1.2 Surgical Complications associated with *Shigella*

Miron and coworkers (Miron *et al*., 2000) reviewed reports published during a 40-year period of 57 children with surgical complications of shigellosis.

1.2. 7.1.3 Urogenital System

Although rare occurrences, vulvovaginitis and urinary tract infections caused by *Shigella* spp. are well-documented.(Edward, 1999; Anatoliotaki *et al.*,2003) Recent publications from developed countries highlight the problems in establishing the diagnosis and treating this infection (Anatoliotaki *et al.*,2003).

1.2. 7.1.4 Neonatal Shigellosis

A recent publication emphasized the unique problem of shigellosis in the neonatal period (Viner *et al*., 2001). Newborn infections account for only 0.6 percent of all cases of shigellosis in the 0- to 10-year age group. Only 1.6 percent of all infants exposed to the infection become ill (Prado *et al*., 1999; Viner *et al*., 2001) This finding is explained by the presence of maternal protective factors that pass through the placenta or are transferred during breastfeeding (Edward, 1999; Gomez *et al*., 2003). Neonates with shigellosis may have only low-grade fever with mild diarrhea, often not bloody. Complications, however, occur more commonly than in older children and include septicemia, meningitis, dehydration, colonic perforation, and toxic megacolon (Viner *et al*., 2001) The mortality rate also is twice as high as in older children, reaching 30 to 40 percent in developing countries, although less than 1 percent in developed regions.

1.2. 7.2 *Shigella* associated neurological manifestation

Hemolytic uremic syndrome and central nervous system (CNS) complications are among the most common extra-intestinal manifestations of shigellosis. Seizures and acute transient encephalopathy state (manifested by headache, delirium, lethargy, hallucinations, confusion, and depressed sensorium) are the most commonly reported neurological manifestations in pediatric population with shigellosis (Ozuah *et al*., 1998; Hiranrattana *et al*., 2005). These may accompany or even precede the development of intestinal symptoms. The case may be misdiagnosed as a primary CNS disease, if the neurological symptoms appeared first (Somech *et al*., 2001). Both seizures and *Shigella* associated encephalopathy are usually benign, and are rarely followed by neurological sequalae (Zvalunov *et al*., 1990).

The neurologic manifestations of shigellosis usually are associated with a favorable outcome, although a study from Bangladesh noted a 29 percent mortality rate in affected children compared with 6 percent in those without neurologic symptoms (Khan *et al*., 1999). Seizures are the most common neurologic manifestation, followed by lethargy and disorientation or coma, which often is referred to as encephalopathy (Ozuah, 1998; Khan *et al*., 1999; Perles *et al*., 1995). In the minority of patients, the encephalopathy may be severe, unresponsive to antibiotic therapy, and fatal, even in developed countries (Plotz *et al*., 1999) The pathogenesis has not been elucidated. In developed countries, where hypoglycemia and electrolyte abnormalities do not play a significant pathogenic role, hyponatremia and early development of brain edema have been reported (Khan *et al*., 1999; Perles *et al*., 1995; Goren *et al*., 1992). In a retrospective study between 1999 and 2010 in icddr,b Bangladesh it was found that among the major bacterial pathogens isolated from patients with convulsion (n=70) *V. cholerae* is the predominant pathogen followed by *S. flexneri* (Figure 1.3). (Talukder unpublished data)

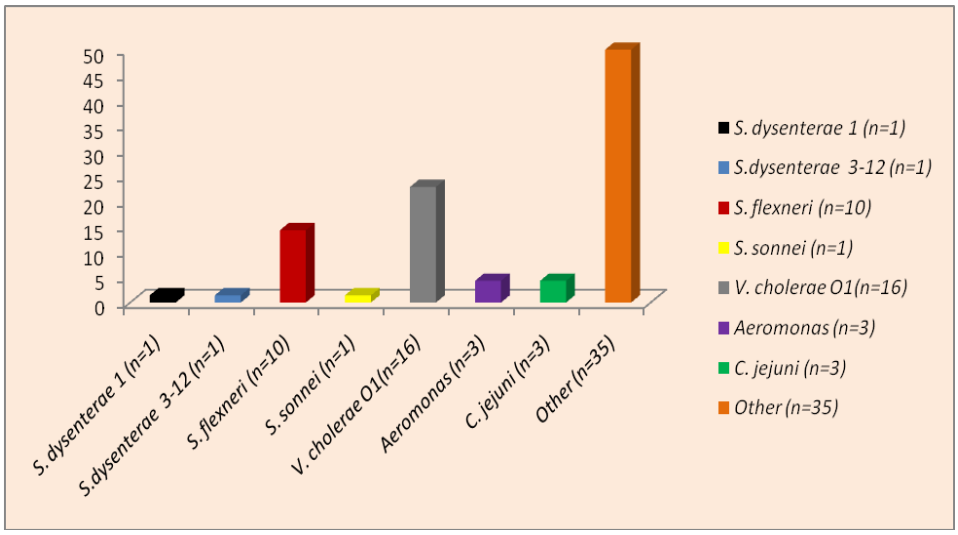


Figure 1.3 Major bacterial pathogens isolated from diarrhoeal patients with the history of convulsion (n=70) between 1999 and 2010

A particularly fulminant form of acute *Shigella* -associated encephalopathy known as Ekiri syndrome (Japanese: epidemic diarrhea) was first descried in Japanese patients in the early 1900's (before and immediately after the Second World War). The major clinical abnormalities were rapidly developing seizures and coma in patients with high fever and few dysenteric symptoms (Kobayashi *et al*., 1986). Only few cases of fulminant *Shigella* encephalopathy (Ekiri syndrome) have been described in the second half of the last century. The largest series of this rare complication of shigellosis was

reported by Goren *et al* (Goren *et al*., 1992), who studied 15 cases of fatal *Shigella* encephalopathy during the years 1980 to 1990. Having ruled out all other causes, acute fulminating encephalopathy (Ekiri syndrome) secondary to *S. sonnei* infection was a logical explanation for the sudden and severe neurological deterioration. Cerebral edema is a common finding reported in most patients with *Shigella*-associated encephalopathy, either by CT brain or at autopsy (Bennish *et al*., 1991; Goren *et al*., 1992; Perlez *et al*., 1995). Focal or diffuse areas of low signal intensity were also reported in CT brain in cases of fulminating *Shigella* encephalopathy (Akl *et al*., 1989). The pathogenesis of *Shigella* associated neurological dysfunction is not well understood. Shiga toxin, which is produced in appreciable amount by *S. dysentriae*, was proved to be a neurotoxin in animal models. It acts on the neurons indirectly by inducing vascular endothelial damage in the brain and spinal cord with secondary neurological dysfunction. It also has cytotoxic activity, probably related to its ability to inhibit protein synthesis in mammalian cells (O'Brien *et al*., 1987). The majority of *Shigella* associated neurological findings were reported in patients with *S. sonnei and S. flexeneri* (Khan *et al*., 1999). Both species do not usually produce shiga toxin, as both are lacking the structural gene encoding shiga toxin production (Ashkenazi *et al*., 1990; Bartlett *et al*., 1986). Balter *et al* (Bartlett *et al*., 1986) studied the role of nitric oxide (NO) in *Shigella* - related seizure in an animal models. NO is an important neurotransmitter in both peripheral and central nervous system. Overproduction of NO has been linked to neurotoxicity during ischemia. It also has a role in some form of neurodegenerative brain disease and in seizures induction (Garthwaite *et al*., 1991). In their study, Balter *et al* (Bartlett *et al*., 1986) found that *S. dysenteriae* infection elevate serum NO level, and this lowered the threshold of induced convulsions in mice. They hypothesized that NO may have a role in induction of neurological manifestation of human shigellosis by acting as a mediator to cytotoxins produced by different *Shigella* species and possibly by other enteric infections.

Khan *et al* (Khan *et al*., 1999) in a large study discussed the prognostic factors that affect the outcome in children fulminating *Shigella* Encephalopathy (Ekiri Syndrome): A Case Report December 2007, with shigellosis who presented with neurological manifestation. They found that patients who were unconscious and with documented seizures at presentation were at great risk of fatal outcome in *Shigella* associated encephalopathy. Although the mechanism that underlies neurological dysfunction in some cases of

Shigella enteritis is unclear, yet the syndrome exists as a well recognized clinical entity. It needs to be considered by pediatricians as a differential diagnosis in children presenting with acute encephalopathy of obscure origin. Stool cultures are recommended in those patients with or without intestinal symptoms. Early recognition and prompt and intensive measures to prevent or treat brain edema may improve the outcome.

1.2.8 Clinical management of shigellosis

The typical symptoms of shigellosis include diarrhoea/ dysentery with frequent mucoid bloody stool, abdominal cramps and tenesmus. The major clinical intervention of diarrhoea consists of fluid and electrolyte rectification. Lactoferrin is an iron-binding protein present in mammalian milk and have antimicrobial, anti-inflammatory and immunomodulatory functions. Several *in vitro* and *in vivo* studies have shown the influence of lactoferrin on enteric pathogens including *Shigella* by inhibiting the growth and impairing the function of surface expressed virulence factors (Ochoa and Cleary, 2009). Mother's milk may protect infants from many gastrointestinal infections and hence feeding during and after shigellosis is emphasized.

1.2.9 Pathogenesis of *Shigella* infection: Molecular and Cellular basis

The key determinant of *Shigella* pathogenesis is the ability to invade and colonize the intestinal epithelium (Sansonetti, 2001). However, the pathogenesis of shigellosis is a multistep process and subtle combination, particularly at the early stage of the disease, 1) the ability of the bacteria to cross the epithelium in selected areas corresponding to M cells 2) the intrinsic invasive properties of the bacteria for epithelial cells and 3) the inflammatory response achieved by the cellular components of the intestinal barrier that disrupt the barrier and facilitate bacterial invasion (Zychlinsky *et al.,* 1997). Expression of the *Shigella* invasive phenotype in the presence of the various cell populations that constitute the intestinal barrier (Figure 1.4), particularly M cells, epithelial cells, resident macrophages, and polymorphonuclear leukocytes (PMNs), engages variable interactions whose result constitutes the overall process leading to rupture, invasion, and inflammatory destruction of the intestinal barrier.

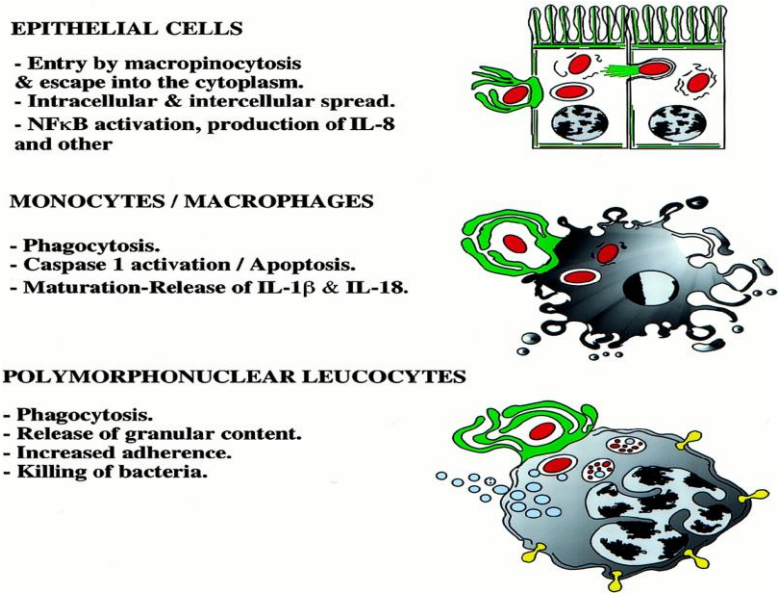


Figure 1.4 Differential expression of the *Shigella* invasive phenotype depending on the cellular target (Sansonetti, 2001)

1.2.10 *Shigella* Invasive Phenotype: the genetic approaches

All *Shigella* share a basic feature of pathogenesis (Figure 1.5), invading the M cells overlying the Peyer's patches and transcytosing to the submucosal side of the epithelial barrier. *Shigella*s are then taken up by macrophages where they induce expression of cytokines including IL-lβ and IL-18, initiating the inflammatory response. *Shigella* quickly kills the macrophages and escape to invade epithelial cells via the basolateral membrane. Once inside epithelial cells, Shigella escape the vacuole, multiply in the cytoplasm, and use actin polymerization to propel themselves to adjacent cells, allowing spread from cell to cell (reviewed in (Sansonetti *et al*., 2001)). A critical element of pathogenesis is a Type Three Secretion System (T3SS) encoded by a virulence plasmid, which translocates *Shigella* effectors directly into host target cells where they seize normal cell function to promote bacterial invasion and the subsequent inflammatory response (Hilbi *et al*., 2008). More than 25 T3SS effectors have been identified in *Shigella*, the majority of which are primarily involved in inducing acting cytoskeleton rearrangement to promote bacterial invasion, and in modulating cell signaling pathways and the innate immune response.

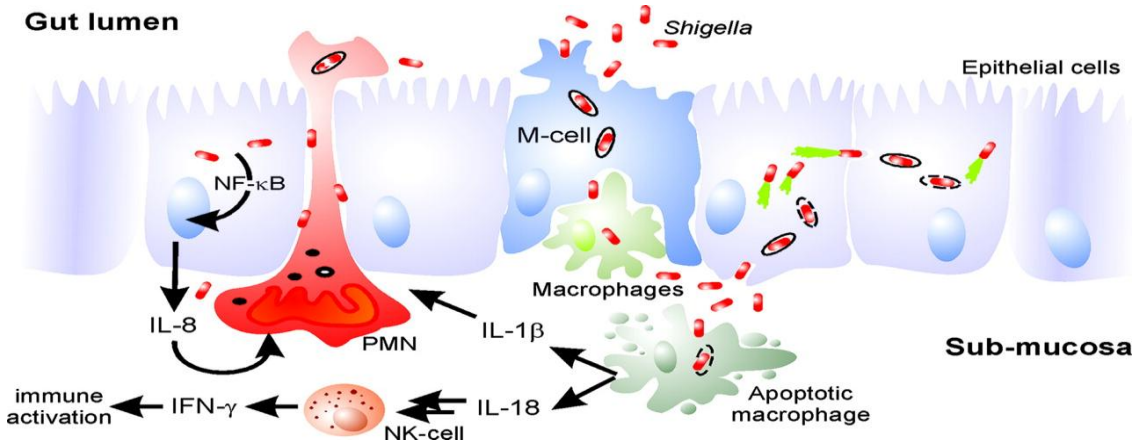


Figure 1.5 Cellular pathogenesis of *Shigella* spp. *S. flexneri* passes the EC barrier by transcytosis through M cells and encounters resident macrophages. The bacteria evade degradation in macrophages by inducing an apoptosis-like cell death, which is accompanied by proinflammatory signaling. Proinflammatory signaling by macrophages and EC further activates the innate immune response involving NK cells and attracts PMN. (Schroeder G N , and Hilbi H Clin. Microbiol. Rev. 2008; 21:134-156)

1.2.11 Molecular determinants of *Shigella* pathogenesis

1.2.11.1 The *Shigella* Virulence Plasmid

The cellular pathogenesis and clinical presentation of shigellosis are the sum of the complex action of a large number of bacterial virulence factors. The essential molecular machinery required for bacterial invasion and intracellular survival is encoded on the large virulence plasmid of *Shigella* (Sansonetti *et al.*,2001; Sasakawa *et al.*,1986). Sequencing of virulence plasmids from different *Shigella* strains revealed that these plasmids of approximately 200 kb contain a mosaic of around 100 genes and a comparable number of IS (Buchrieser *et al.*,2000; Venkatesan *et al.*,2001, Yang *et al.*,2005). The core of the plasmid is the conserved 31-kb entry region, which was shown to be necessary and sufficient for EC invasion and macrophage killing (Maurelli *et al.*,1985). The PAI-like region consists of 34 genes that are organized into two clusters transcribed in opposite directions (Figure 1.6). Based on their functions, these genes can be divided into four different groups.

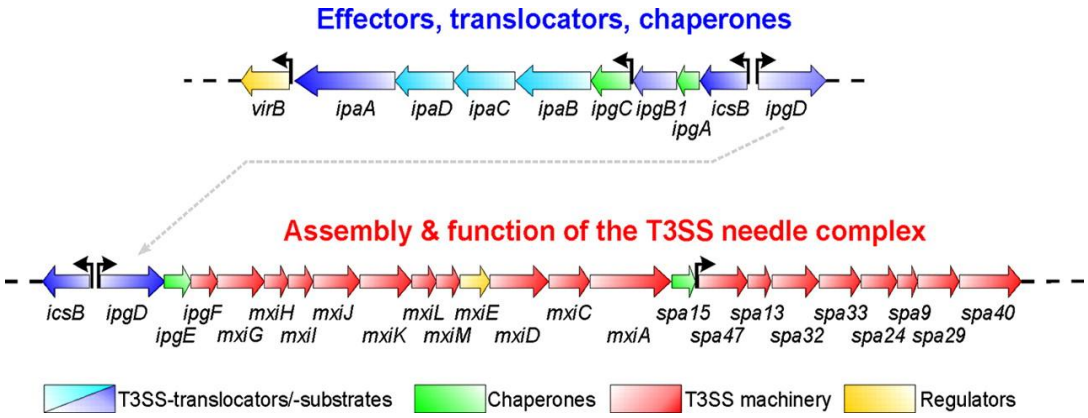


Figure 1.6 Map of the 31-kb "entry region" on the *S. flexneri* virulence plasmid pWR100. The genes indicated encode structural components of the Mxi-Spa T3SS, secreted translocator and effector proteins, chaperones, and regulatory proteins. The region shown is essential and sufficient to invade EC and induce macrophage cell death. (Schroeder G N , and Hilbi H Clin. Microbiol. Rev. 2008; 21:134-156)

Two independent groups have recently reported the complete sequence analysis of the large virulence plasmid in *S. flexneri* serotype 5a (Buchrieser *et al*., 2000, Venkatesan *et al*., 2001). The DNA sequence indicated that the genes necessary for entry of bacteria into epithelial cells are clustered within a 31-kb region of the virulence plasmid. The genes within this region have been extensively characterized. (Philpott *et al*., 2000) The entry region is a pathogenicity island-like cluster that contains: a) the *mxi* and *spa* genes encoding components of a type III secretion apparatus; b) the *ipa* B, C and D and *ipg*D genes encoding proteins secreted by this machinery; c) the *ipg*C and *ipg*E genes encoding cytoplasmic chaperones required for stability of IpaB and IpaC, and IpgD, respectively; d) the *vir*B gene encoding a protein required for transcription of the *mxi*, *spa* and *ipa* genes; and e) additional genes of unknown function. (see Table 1.2) .Outside of the entry region, other genes associated with virulence have been identified. They include: a) the *icsA* (*virG*) gene encoding an outer membrane protein that is directly responsible for the ability of the bacteria to move within the cytoplasm of infected cells; b) the *virF* gene encoding a transcriptional activator that controls expression of *icsA* and *virB*; and c) the *sepA* gene, which encodes a secreted serine protease of the autotransporter family.

In addition, the virulence plasmid contains two copies of the *shet2* gene encoding a putative enterotoxin, and genes encoding several secreted proteins, which include *virA*, *ipaH4.5*, *ipaH7.8*, *ipaH9.8* and six uncharacterized genes designated (outer *Shigella* proteins): *ospB*, *ospC1*, *ospD1*, *ospE1*, *ospF* and *ospG*. The proteins encoded in this plasmid are directly involved in the entry into epithelial cells and invasive phenotypes observed in the pathogenesis of *Shigella* strains.

1.2.11.2 Chromosomal Genes

Several chromosomal loci participate in the pathogenic process by complementing the core virulence of the invasion plasmid genes and are, therefore, needed for full virulence. They can be categorized into two groups:

1. Genes regulating the expression of the plasmid virulence genes. The virR gene encodes a histone-like molecule controlling the temperature-dependent expression of the Ipa and Mxi-Spa proteins, and the keratoconjunctivitis provocation (kcpA) gene positively regulates virG (Sansonetti *et al*., 2001, Maurelli and Sansonetti, 1988)

2. Genes needed for bacterial survival in the intestine and for resisting host defense mechanisms, such as those encoding lipopolysaccharides (LPS) and siderophores. *Shigella*e lacking complete LPS (“rough colonies”) are avirulent (Edwards BH, 1999).

Table 1.2 Virulence factors encoded on the *S. flexneri* virulence plasmid

Effector	Biochemical activity	Host cell target(s)	Virulence function and/or phenotype
IpaA	Vinculin activation	Vinculin, β_1-integrins, Rho signaling	Efficient invasion, actin cytoskeleton rearrangements, disassembly of cell-matrix adherence
IpaB	Membrane fusion	Cholesterol, CD44, caspase-1	Control of type III secretion, translocon formation, phagosome escape, macrophage apoptosis
IpaC	Actin polymerization	Actin, β-catenin	Translocon formation, filopodium formation, phagosome escape, disruption of EC tight junctions
IpaD			Control of type III secretion, membrane insertion of translocon
IpaH7.8			Efficient phagosome escape
IpaH9.8	E3 ubiquitin ligase	Splicing factor U2AF, MAPK kinase	Host cell transcriptome modulation, reduction of inflammation
IcsA*a*(VirG)		N-WASP, vinculin	Recruitment of actin-nucleating complex required for actin-based motility and intercellular spread
IcsB			Camouflage of IcsA to prevent autophagic recognition
IcsP*a*	Serine protease		Cleavage of IcsA, modulation of actin-based motility
IpgB1	RhoG mimicry	ELMO protein	Induction of Rac1-dependent membrane ruffling
IpgB2	RhoA mimicry	RhoA ligands	Induction of actin stress fiber-dependent membrane ruffling
IpgD	Phosphoinositide 4-phosphatase	Phosphatidylinositol 4,5-bisphosphate	Facilitation of entry, promotion of host cell survival
OspB			T3SS substrate, unknown function
OspC1		Nucleus and cytoplasm	Induction of PMN migration
OspD1			T3SS substrate, unknown function in host cells, antiactivator of MxiE
OspE2		Focal contacts	Maintenance of EC morphology, efficient intercellular spread
OspF	Phosphothreonine lyase	MAPKs Erk and p38	Inhibition of histone phosphorylation and NF-κB-dependent gene expression, reduction of PMN recruitment
OspG	Protein kinase, ubiquitination inhibitor	Ubiquitin-conjugating enzymes	Downregulation of NF-κB activation, reduction of inflammation
PhoN2*a*	Apyrase		Unipolar localization of IcsA
SepA*a*	Serine protease		Promotion of intestinal tissue invasion and destruction
VirA	Cysteine protease	α-Tubulin	Facilitation of entry and intracellular motility by degradation of microtubules

(Adopted from Schroeder G N , and Hilbi H Clin. Microbiol. Rev. 2008;21:134-156)

1.2.12 Toxins of *Shigella*

Toxins are perhaps the best characterized virulence factors, because they are most often the easiest to purify by concentrating the supernatant, and because they have distinct phenotypes. However as virulence factors, they often have essential roles in altering and often killing host cells, usually by an enzymatic process (Schihavo and van Der Goot, 2001, Steele-Mortimer *et al.,* 2000). *Shigella* generally produces two type of toxin *Shigella* Enterotoxin and Shiga toxin are the example of *Shigella* exotoxin. Exotoxins are usually proteins that are secreted by bacteria out into the supernatant. Endotoxin is lipopolysaccharide (LPS), which is principally carbohydrate, not protein, non enzymatic and not normally secreted. Because LPS is critical component of *Shigella* generally it is not considered as virulence factor, although it stimulates toxic effects in the host by activating immune responses and cytokine production.

1.2.12.1 *Shigella* Enterotoxin

Enteroinvasive E. coli and *Shigella* infection are often manifested by watery diarrhoea. In contrast, surprisingly little is known of the precise mechanism by which *Shigella* cause watery diarrhoea. It has previously been hypothesized, that *Shigella* produces an enterotoxin. However, except for the cytotoxin /neurotoxin /enterotoxin elaborated by *S. dysenteriae* (Fontaine *et al*.**,** 1988); little convincing proof has been generated to substantiate the contention that these organisms in fact produce enterotoxins in *S. flexneri*. Recently, two novel enterotoxins elaborated by *Shigella* species have been reported that may be responsible for the watery phase of shigellosis (Fasano *et al*.**,** 1995; Nataro *et al*., 1995).

1.2.12.1.1 *Shigella* Enterotoxin 1 (ShET-1)

ShET-1 is a chromosomally encoded, 55 kDa complex protein that is universally elaborated by *S. flexneri* 2a strains but only rarely other serotypes (Noriega *et al*., 1995). Sequencing analysis of the genes encoding this toxin disclosed the presence of two contiguous open reading frame (ORFs) of 534 (*set1A*) and 186 (s*et1B*) bp respectively, governed by the same promoter and separated by only 3 bp, *set1* gene product having a size of ~20 kDa and another gene product being 7 kDa in size. The ShET-1 holotoxin has an apparent size of 55 kDa, it is tantalizing to speculate that A1–B5 configuration of one 20 kDa subunit (typical of the size of A subunits, other enterotoxins) bonded to five B subunits, each 7 kDa in size (typical of B subunits of other enterotoxin). ShET-1 was

found in 45% of S. *flexneri* strain but was not in either *S. sonnei* or *S. dysenteriae* (Vargas *et al*., 1999).

1.2.12.1.2 *Shigella* Enterotoxin 2 (ShET-2)

ShET-2 is a 62.8 kDa single protein that was originally detected in enteroinvasive *Escherichia coli* (EIEC) and referred to as EIEC enterotoxin is encoded in the *sen* gene (Fasano *et al*., 1991). The gene encoding this toxin is located on the 140 MDa invasive plasmid of *Shigella* and seems to be present in more than 80% of a wide array of *Shigella* serotype examined (Narato *et al*., 1995). Narato and co-worker found that the *sen* gene in 73% of *S. flexneri* strains using a DNA probe and Vargas and co-worker found ShET-2 together with ShET-1 in 36% of *S. flexneri* strains, 57% of *S. sonnei* strains and *S. dysenteriae* strains. Gene sequence analysis showed that both ShET-1 and ShET-2 are genetically unrelated to shiga toxin elaborated by *S. dysenteriae* 1. Furthermore, the three toxins are also seem to be immunologically unrelated (Fasano *et al*., 1997)

1.2.12.2 Shiga Toxins (Stxs)

S. dysenteriae serotype 1 is unique among *Shigella* species in the production of a potent toxin known as the Shiga toxin (Stx). Shiga toxin (Stx) belongs to the large family of ribosome-inactivating proteins (RIPs). There are main two subgroups of Shiga toxin: Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). Each Shiga toxin molecule 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 (Gb3: Galα1-4-Galβ1-4-glucosylceramide), and it is 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. Inhibition of protein synthesis by RIPs has been proposed to be responsible for induction of apoptosis in mammalian cells (Obrig *et al.*, 1987; Endo *et al*., 1988a; Endo *et al*., 1988b). However, cytotoxic effect due to inhibition of protein synthesis inhibitor has been controversial (Sandvig and vanDeurs, 1992; Sakamoto *et al.,* 2003). It is possible that RIPs activates yet an unidentified signaling pathway(s) leading to cell death.

S. dysenteriae serotype 1 and Shiga toxin-producing *E. coli* (STEC) cause bloody diarrheal diseases that may progress to life-threatening extraintestinal complications.

They produce one or more Shiga toxins (Stxs). Following the ingestion of the organisms, the expression of Stxs is critical for the development of vascular lesions in the colon, kidneys and central nervous system. While the capacity of Shiga toxins to inhibit protein synthesis by catalytic inactivation of eukaryotic ribosomes has been well described, it is also apparent that Shiga toxins trigger apoptosis in many cell types. It was evidenced that Shiga toxins induce apoptosis of epithelial, endothelial, leukocytic, lymphoid and neuronal cells. However, recent studies by Talukder and coworker have shown that Stxs trigger programmed cell death by activation of p53/ATM dependant DNA damage signaling pathway in mammalian cells (Talukder *et al*., 2012). The mechanisms of apoptosis induction by these toxins are newly emerging, and the data published to date suggest that the toxins may signal apoptosis in different cell types via multiple mechanisms (Cherla, 2003). Shiga toxin genes in both *E. coli* and *S. dysenteriae* are generally phage-borne (Unkmeir and Schmidt, 2000). Infections with bacteria producing Shiga toxins are responsible for widespread disease and for the death of a large number of people (Takeda *et al*., 1993; Kaper, 1998; Paton and Paton, 1998 Uchida *et al*., 1999; Bower, 1999; Paton *et al*., 2000; Kitov *et al*., 2000). Bacteria producing Shiga-toxins seem to be the most common cause of hemolytic-uremic syndrome (HUS), characterized by thrombocytopenia, microangiopathic hemolytic anemia and renal failure. In some cases, even neurological symptoms are seen (Paton and Paton, 1998).

1.2.13 *Shigella* and neurotoxicity

1.2.13.1 Neurotoxin

Neurotoxins are an extensive class of exogenous chemical which can adversely affect function in both developing and mature nervous tissue (Olney *et al*., 2002). The term can also be used to classify endogenous compounds which when abnormally concentrated can prove neurologically toxic (Spencer *et al*., 2000). Though neurotoxins are often neurologically destructive, their ability to specifically target neural components is important in the study of nervous systems (Kiernan *et al*., 2005). Common examples of neurotoxins include lead, ethanol, glutamate, nitric oxide (NO), botulinum toxin, tetanus toxin, and tetrodotoxin. Some substances such as nitric oxide and glutamate are in fact essential for proper function of the body and only exert neurotoxic effects at excessive concentrations. Neurotixin activity can be characterized by the ability to inhibit neuron control verion concentrations across the cell membrane, or communication between

neurons across a synapse. Local pathology of neurotoxin exposure often includes neuron excitotoxicity or apoptosis but can also include glial cell damage, Macroscopic manifestations of neurotoxin exposure can include widespread central nervous system damage such as intellectual disability, persistent memory impairments, epilepsy, and dementia. Additionally, neurotixin-mediated peripheral nervous system damage such as neuropathy or myopathy is common. Support has been shown for a number of treatments aimed at attenuating neurotoxin-mediated injury, such as antioxidant, and antitoxin administration.

1.2.13.2 Mechanism of activity

Neurotoxins are potent molecules used by various bacteria to interact with a host organism, specifically act on neuronal cells (clostridial neurotoxins) leading to characteristics neurological affections. But many other toxins are multifunctional and recognize a wider range of cell types including neuronal cells. As neurotoxins are compounds which adversely affect the nervous system, a number of mechanisms through which they function are through the inhibition of neuron cellular processes. These inhibited processes can range from membrane depolarization mechanisms to inter-neuron communication. By inhibiting the ability for neurons to perform their expected intracellular functions, or pass a signal to a neighboring cell, neurotoxins can induce systemic nervous system arrest as in the case of botulinum toxin (Arnor *et al*., 2001), or even nervous tissue death(Brocardo *et al*., 2011). The time required for the onset of symptoms upon neurotoxin exposure can vary between different toxins, being on the order of hours for botulinum toxin (Thyagarajan *et al*., 2009) and years for lead (Lewendon *et al*.,2001).

Table 1.3 Classification of neurotoxin

Neurotoxin classification	Neurotoxins
Na channel inhibitors	Tetrodotoxin
K channel inhibitors	Tetraethylammonium
Cl channel inhibitors	Chlorotoxin,
Ca channel inhibitors	Conotoxin
Inhibitors of synaptic vesicle release	Botulinum toxin, tetanus toxin
Receptor inhibitors	Bungarotoxin, Curare
Receptor agonists	25I-NBOMe,JWH-018
Blood brain barrier inhibitors	Aluminium, mercury
Cytoskeleton interference	Arsenic, ammonia
Ca-mediated cytotoxicity	Lead
Multiple effects	Ethanol
Endogenous neurotoxin sources	Nitric oxide, glutamate

Various enterotoxins interact with the enteric nervous system, for example by stimulating afferent neurons or inducing neurotransmitter release from enterochromaffin cells which result either in vomiting, in amplification of the diarrhea, or in intestinal inflammation process. Other toxins can pass the blood brain barrier and directly act on specific neurons.

According to the nature of the target and the type of modification, intracellular active toxins cause a dramatic alteration of cellular functions such as protein synthesis, cell homeostasis, cell cycle progression, vesicular traffic, and actin cytoskeletal rearrangements. The nervous system which is diffused through all the organism is one of the main target of bacterial toxins. If neurotoxins, like clostridial neurotoxins, exclusively interact with neuronal cells from the central or peripheral nervous system inducing specific neurological symptoms, other toxins recognize a broader range of cell types including neuronal cells. For example, cytotoxins can trigger necrosis or apoptosis in various cell types as well as in neuronal cells. Thereby various toxins, in addition to their specific activity on some cell types, affect certain neuronal cells, directly or indirectly, leading to neurological symptoms associated with typical clinical signs resulting from the other affected cells.

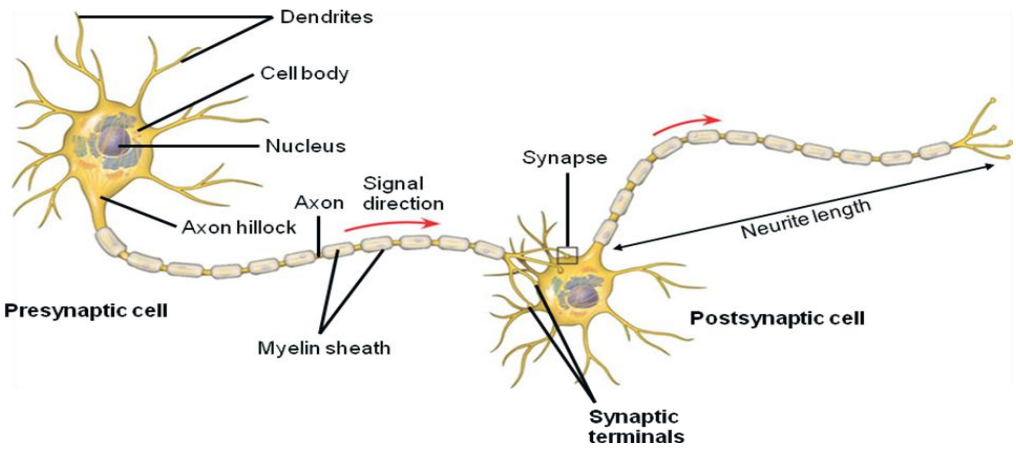


Figure 1.7 Development of nervous system is absolutely dependent on targeted growth of axons and dendrites. Nerve cells (neurons) use chemicals called neurotransmitters to send messages. The message (neural signal) travels in a specific direction from one cell to the next across a connecting synapse (SIN-aps), often transmitted from the axon terminal of one cell (the presynaptic neuron) across the synapse to the dendrites of the next (the postsynaptic neuron). Neurotoxin inhibits the ability for neurons to perform their expected intracellular functions, or pass a signal to a neighboring cell.

1.2.13.3 Shiga toxin induced neurotoxicity

Neurological (seizure, convulsion) and renal (HUS) complications have been reported to be associated with infection of certain *Shigella Spp*. and *E coli*. It has also been indicated that toxin producing ability of these bacteria might be responsible for these complication. Shiga toxin has been demonstrated to possess glycosidase activity leading to elimination bases from rRNA and destabilizes rRNA. RNA fragments produced by shiga toxin play a major role in developing neurological manifestations in shigellosis. In our preliminary results, we have provided convincing evidence that RNA/shiga toxin activate DNA damage signaling pathway as evidenced by activation of ATM kinase, induction of apoptosis (Talukder *et al*., 2012), inhibition of neurite outgrowth of cerebellar granule neurons. These results indicate that shiga toxin-induced activation of DNA damage signaling plays a major role in neurological abnormalities. In current application, we intend to establish the involvement of toxin(s) in developing neurological abnormalities in shigellosis and to understand the molecular mechanism of *Shigella* toxin-induced activation of DNA damage signaling pathway(s). Understanding the molecular mechanism of shiga toxin-induced activation of DNA damage signaling pathways will be useful in blocking signal(s) that lead to the damage of nervous system in shigellosis.

1.2.14 Apoptosis

Apoptosis (programmed cell death) and necrosis are currently defined as the two major modes of cell death. In some instances, necrosis is a passive process characterized by the loss of plasma membrane integrity, cell swelling and inflammation due to the release of cellular contents into the periphery. In contrast, apoptosis is an active process generally characterized by morphological cell changes including cell shrinkage associated with cytoplasmic condensation and vacuolation, membrane blebbing, apoptotic body formation, chromatin condensation, nuclear fragmentation, and loss of cell adhesion (Zimmermann *et al*., 2001). However, as has been recently noted, when describing apoptotic versus necrotic changes, it is extremely important to discriminate between antemortem biochemical changes versus postmortem morphological changes in cells (Kanduc *et al*., 2002). Apoptosis is initiated in response to stimuli which activate genetically programmed signaling cascades. Cytoplasmic constituents are not spilled into the extracellular milieu so that inflammation is not a characteristic of apoptotic cell death. Apoptotic bodies are generally internalized and processed by phagocytic cells.

A major biochemical feature of apoptosis is the internucleosomal fragmentation of genomic DNA, although it should be noted that DNA fragmentation is not a universal characteristic of apoptosis (Nagata, 2000). DNA fragmentation appears to be a multi-step process with DNA first cleaved into large fragments (50-200 kb) and subsequently degraded to nucleosomal units of multiples of 180-200 bp. The presence of fragmented DNA (DNA laddering) is readily assessed using agarose gel electrophoresis. DNA fragmentation also forms the basis of the TUNEL staining assay in which the fragments are used as substrates for the enzyme terminal deoxyribonucleotidyl transferase. Although many stimuli may activate programmed cell death, most stimuli appear to signal through a common, tightly regulated pathway involving the sequential activation of proteases called caspases. Caspases are cysteine-dependent aspartate-specific proteases that exist as inactive precursors or pro-caspases. In general, caspase activation requires a single proteolytic cleavage of a latent single chain zymogen to produce a heterodimer. The caspase heterodimers then self pair to form the active tetrameric molecule (Stennicke *et al*., 2000).

Programmed cell death takes place through two pathways categorized on whether apoptotic signals originate outside of or within cells. The extrinsic or extracellular pathway of programmed cell death generally requires the ligation of receptors of the tumor necrosis factor receptor (TNFR) superfamily containing cytoplasmic death domains.

1.2.14.1 *Shigella* mediated Apoptosis in Human cell lines

Apoptosis is involved in the interaction between host cells and bacterial pathogens as a number of bacterial pathogens appear to be capable of manipulating host cells apoptotic pathways (Gao *et al.,* 2000). *Shigella* is able to induce host cell apoptosis by effector proteins into the host cytoplasm via type III secretion system, which involve direct binding and activation of caspase-I (Hilbi *et al*., 1998, Mersh *et al*., 1999). *Shigella* induces apoptosis in macrophages but not often other cell types (Zychlinsky *et al*., 1996). In vivo induction of apoptosis in *Shigella* was demonstrated in animal models (Zychlinsky *et al*., 1996) and dysenteric patients (Islam *et al*., 1997). *Shigella* kills its host macrophages by releasing IpaB. IpaB is sufficient to induce apoptosis in macrophage (Chen *et al*., 1996). IpaB directly bind and activate caspase-I and thus

thought to be the key key molecule in the induction of caspase-I dependent apoptosis by *Shigella* infection (Hilbi *et al*., 1998, Chen *et al*., 1996).

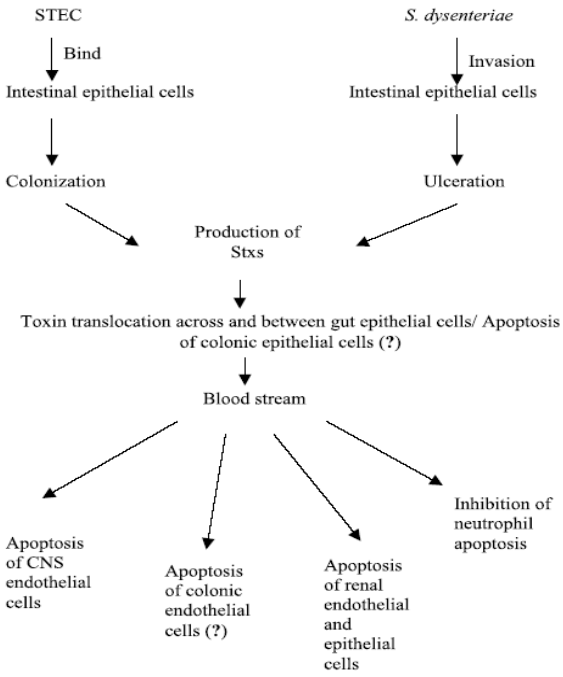


Figure 1.8 Model of disease progression of bacillary dysentery/hemorrhagic colitis to extraintestinal complications.

Among the caspase, caspase-1 appeases to be unique since it induces apoptosis and activates two potent proinflammatory cytokines, IL-1β and IL-18 (Ghayur *et al*., 1997, Guy *et al*., 1997). Although casp-1 induces apoptosis when over expressed in tissue culture cells (Miura *et al*., 1993) the role of this enzyme in apoptosis has remained enigmatic. Casp-1 in processed shortly after *Shigella* infection suggesting that 1paB can promote this process. Cleavage of Pro II-1β indicates that the mature casp-1 in activate during infection. The proapoptotic function of Casp-1in *Shigella* induce apoptosis appears to be independent of mature of IL-1β. Pretreatment of macrophage with IL-1β, does not present *Shigella* induced apoptosis. Casp-1 induced apoptosis does not function through IL-1β.

Casp-1 is then activated and Cleves pro-IL-1β as well as apoptosis effector protein. Direct binding of IpaB to casp-1 bypassed the need for a signal transduction pathway and capase upstream of casp-1 (like casp-2). cas-1 mutant mice cannot convert/ activate IL-1β/IL-18, thus unable to induce apoptosis. Although Casp-3 is implicated in many

different apoptotic protease in a caspase cascade (Thonnberry, 1997), it is not necessary for *Shigella* killing (Hilbi *et al*., 1998). However, in case of *S. dysenteriae* type 1 the scenario is little different due to the presence of *stx*1 gene.

1.2.14.2 Shiga Toxin and Apoptosis

Numerous studies have examined the mechanism of cell death mediated by purified Stxs. Cultured cells or cell lines used in these studies include epithelial cells, endothelial cells, B-lymphoma cell lines, astrocytoma cells, monocytic cell lines, neutrophils and amniotic cell lines. These cell types respond differently to Stxs. For example, T84 cells, a human intestinal epithelial cell line, lack the toxin glycolipid receptors and are not killed by Stxs *in vitro*, yet are capable of toxin translocation from apical to basolateral surfaces (Hurley *et al*., 1999). Bovine crypt intestinal epithelial cells, in contrast, express Gb3 but escape killing by routing the toxins to lysosomes rather than to the ER (Hoey *et al*., 2003). Purified Stx2 has been shown to inhibit cell death of neutrophils, cells that normally undergo spontaneous apoptosis, suggesting that the toxins may increase the life span of cells capable of mediating extensive vascular damage (Liu *et al*., 1999). While Stxs may not induce apoptosis in all cell types, there is ample evidence suggesting that apoptosis is critical in the development of vascular lesions and tissue damage following translocation of the toxins into the bloodstream. Based on the current available literature, a model for the progression of disease is shown in Figure 1.8 In contrast to the studies using T84 cells (Smith *et al*., 2003) treated the human intestinal epithelial cell line, HCT8, with Stx1 and demonstrated the activation of the stressactivated kinase cascades JNK/SAPK and p38, and the activation of caspase 3 leading to apoptosis with DNA fragmentation. Toxin enzymatic activity was required for the activation of stress-activated kinases and cytotoxicity.

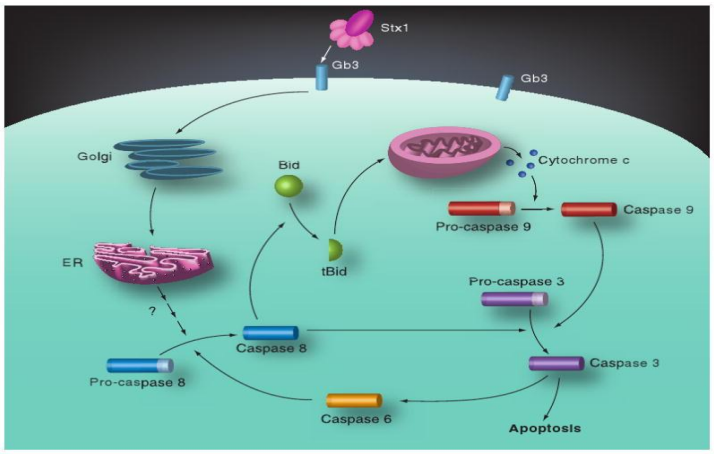


Figure 1.9 Putative mechanism of Shiga toxin-induced apoptotic signaling pathways in monocytic THP-1 cells. Following toxin internalization, caspase 8 is rapidly activated, which in turn triggers caspase-dependent and mitochondrial-dependent apoptotic signaling cascades. Caspase 8 may directly activate caspase 3, which in turn may activate caspase 6 to create an amplication loop for activation of the executioner caspase. Caspase 8 also cleaves Bid. tBid molecules translocate to mitochondria and facilitate the release of cytochrome c. With dATP and Apaf-1, cytochrome c forms the apoptosome, leading to sequential activation of caspases 9 and 3. Apoptotic signaling requires the retrograde transport of holotoxin to the ER.

Many studies have documented that purified Stxs induce apoptosis in cultured primary epithelial cells, including human renal proximal tubule and renal cortical epithelial cells (Kodama *et al*., 1999); Karpman *et al*., 1998). Apoptosis of renal cortical cells *in vivo* was demonstrated in biopsies taken from three children with HUS (Karpman *et al*., 1998). The biopsies showed extensive cortical necrosis and thrombotic microangiopathy, and TUNEL staining revealed apoptotic nuclei in tubules and glomeruli. A similar pattern of TUNEL staining was noted in the kidney tissues of mice fed Stx2-producing *E. coli*. The mechanisms of Stx-induced apoptosis have been extensively studied using the human laryngeal epithelial cell line HEp-2and the human cervical epithelial cell line HeLa. In HEp-2cells, Stx1, Stx2 and Stx2c induce apoptosis by activating caspases 8, 9 and 3 (Ching *et al*., 2002).

It has become increasingly clear that Stxs induce apoptosis in some, but not all, cell types. The capacity of the toxins to trigger programmed cell death pathways may contribute to the development of bloody diarrhea and extraintestinal complications. An overall model of apoptosis induction mechanisms by Stxs derived from recently described studies is shown in Figure. 1.2. Apoptosis induction is complex, and may involve extracellular and intracellular signaling pathways. Although Stx-mediated activation of the upstream caspase, caspase 8, has been reported, it is not clear whether caspase 8 is activated by the extrinsic pathway acting through engagement of membrane-

associated receptors, or by intrinsic signaling proteins through the ribotoxic stress response. Receptor-ligand complexes directly interacting with caspase 8 following treatment of cells with toxins or purified B-subunits remain to be identified. If apoptosis induction requires retrograde transport, the precise points in the trafficking process initiating apoptotic signals are currently unknown. Studies using HeLa and HMEC-1 cells have suggested that toxin enzymatic activity is necessary to induce apoptosis through a mechanism completely independent of caspase activation. Such a mechanism may involve protein synthesis inhibition and proteasome-mediated degradation of anti-apoptotic proteins.

Finally, it should be noted that Stxs may not trigger a uniform apoptotic signaling cascade in all cell types. Thus, the differential signaling events caused by the toxins in different cell types await further clarification. A better understanding of the mechanisms of apoptosis induction by Stxs may lead to the development of effective therapeutic strategies to interrupt the progression of disease from the bloody diarrheal phase to life-threatening complications.

1.2.14.3 Apoptosis: molecular approaches

Increases in cytosolic Ca^{2+} levels due to activation of ion channel-linked receptors, such as that for the excitatory amino acid neurotransmitter glutamic acid, can induce permeability transition (PT) of the mitochondrial membrane. PT constitutes the first rate-limiting event of the common pathway of apoptosis. Upon PT, apoptogenic factors leak into the cytoplasm from the mitochondrial intermembrane space. Two such factors, cytochrome C and apoptosis inducing factor (AIF), begin a cascade of proteolytic activity that ultimately leads to nuclear damage (DNA fragmentation, DNA mutations) and cell death. Release of cytochrome C from mitochondria is a major event during apoptosis. Released cytochrome C has been shown to activate caspase-dependent apoptotic signals. Parallel to nuclear accumulation of cytochrome C, acetylated histone H2A, but not unmodified H2A, was released from the nucleus to the cytoplasm. It was also found that cytochrome C induce chromatin condensation. The nuclear accumulation of cytochrome C may be directly involved in the remodeling of chromatin.

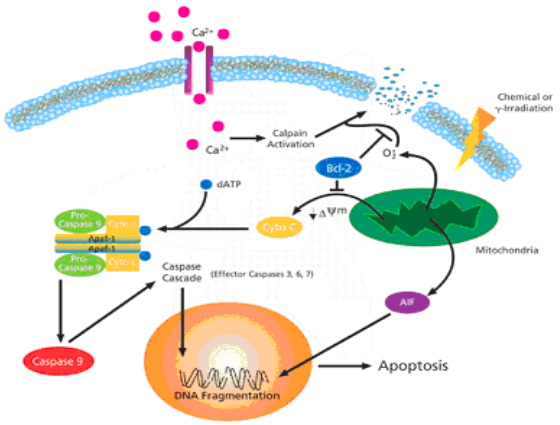


Figure 1.10 Nuclear translocation of cytochrome C during apoptosis. Upon permeability transition (PT), apoptogenic factors leak into the cytoplasm from the mitochondrial inter membrane space. Two such factors, cytochrome C and apoptosis inducing factor (AIF), begin a cascade of proteolytic activity that ultimately leads to nuclear damage (DNA fragmentation, DNA mutations) and cell death. Release of cytochrome C from mitochondria is a major event during apoptosis.

1.2.15 Activation of DNA damage signaling

Cells respond to DNA damage by activating DNA repair and DNA damage signaling pathways. While DNA repair proteins directly interact with DNA lesions, activation of DNA damage signaling pathways may be triggered by the effect the DNA lesions have on replication, transcription or chromatin topology (Ljungman, 2005). In addition to DNA repair, DNA damage-induced signal transduction pathways have evolved to add an extra layer of defense against induction of mutagenesis in eukaryotes (Fig. 1). By activating cell cycle checkpoints these pathways provide extended time for DNA repair enzymes to complete repair before entering the critical cell cycle stages of S-phase and mitosis. Furthermore, in multicellular organisms the activation of these signal transduction pathways may lead to the induction of apoptosis, which is an effective means to avoid mutagenesis by removing cells containing unrepairable DNA damage. In contrast to DNA repair enzymes, which by necessity need to directly interact with DNA lesions, signal transduction pathways may be activated by factors monitoring only parts of the genome for DNA damage or some other cellular "readout" of DNA damage. Three such potential cellular readouts of DNA damage are namely (i) blockage of replication, (ii) blockage of transcription, and (iii) alterations in chromatin topology.

1. 2.15. 1 The replication stress response

The precise duplication of the genome during S-phase is one of the most fundamental and important processes for any organism or population of cells (Bell and Dutta, 2002). Not only does the fidelity of the replication process itself need to be extremely high, the genome needs to be replicated exactly once (not more not less) or genetic instability will result. Furthermore, after the cell has initiated a round of replication there is no turning back. Any roadblock that the replication machinery may encounter during elongation must be dealt with efficiently and swiftly or the cell is doomed.

The replication machinery is well situated to be involved in damage sensing. During the replication process the chromatin structure is unraveled and the entire genome is effectively "scanned". It could be envisioned that DNA damage sensors may either scan the DNA ahead of the replication fork for damage or monitor the elongation process itself. The latter scenario is more likely to take place in cells since it is thought that DNA structures generated at sites of a stalled replication fork trigger the activation of the replication stress response.

1. 2. 15.2 The transcription stress response

While replication involves the entire genome, it is confined to only one phase of the cell cycle. Transcription on the other hand, operates throughout the cell cycle except during mitosis. It has been shown that lesions specifically in the transcribed strand of active genes trigger the induction of both p53 (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996; Ljungman *et al.*, 1999; Ljungman and Lane, 2004; Ljungman, 2000) and apoptosis (Ljungman and Zhang, 1996) following UV-irradiation. In fact, many other

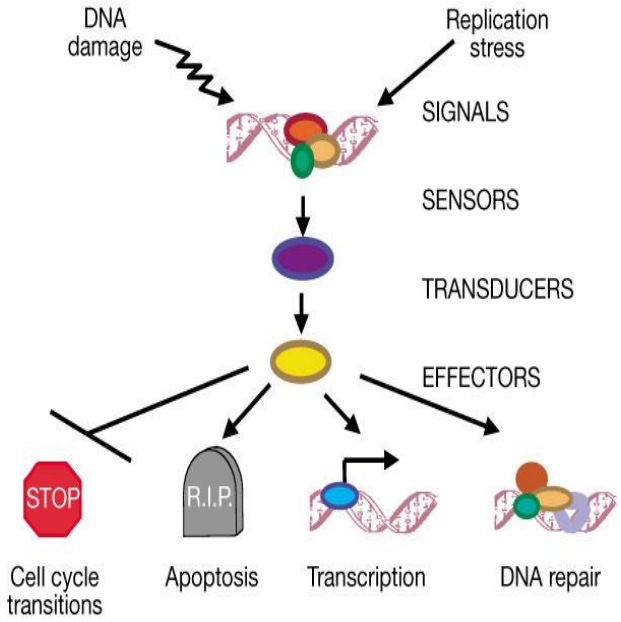


Figure 1.11 A contemporary view of the general outline of the DNA damage. Arrowheads represent activating events and perpendicular ends represent inhibitory events. Cell-cycle arrest is depicted with a stop sign, apoptosis with a tombstone. The DNA helix with an arrow represents damage-induced transcription, while the DNA helix with several oval-shaped subunits represents damage-induced repair. For the purpose of simplicity, the networks of interacting pathways are depicted as a linear pathway consisting of signals, sensors, transducers and effectors. (Adopted from Bing Bing S *et al*., 2000).

transcription-blocking drugs have been shown to induce p53 and apoptosis at doses that inhibit transcription by 50% or greater (Ljungman *et al*., 1999; Ljungman and Paulsen, 2001; Ljungman *et al*., 2001). Moreover, p53 can become induced following blockage of transcription even in the absence of DNA damage suggesting that cells have mechanisms to sense and respond to perturbations in transcription in addition to DNA strand breakage (Ljungman *et al*., 1999; Ljungman and Paulsen, 2001, Ljungman *et al*., 2001).

1.2.15.3 The chromatin alteration stress response

The superhelicity present in cellular DNA is largely constrained by the spooling of DNA around nucleosomes. However, some localized unconstrained torsional tension exists in the genome of eukaryotic cells (Ljungman and Hanawalt, 1992 and 1995; Jupe *et al*., 1993; Jupe *et al*., 1995). Specifically, this tension appears to be localized to the promoter regions of genes poised for transcription (Ljungman and Hanawalt, 1995). Importantly, radiation-induced single strand breaks induced at a distance of more than 50 kbp apart are sufficient to abolish the tension in the promoter region (Ljungman and Hanawalt, 1995). This sets up an interesting possibility that induction of DNA strand breaks may be monitored at a distance by proteins sensing the loss of DNA topology.

1.2.16 DNA Damage Checkpoints at Cell cycle

The cell cycle proceeds by a defined sequence of events where late events depend upon completion of early events (Hartwell *et al.*, 1989). The aim of the dependency of events is to distribute complete and accurate replicas of the genome to daughter cells (Russell *et al.*,1998). To monitor this dependency, cells are equipped with the checkpoints that are set at various stages of the cell cycle. When cells have DNA damages that have to be repaired, cells activate DNA damage checkpoint that arrests cell cycle. According to the cell cycle stages, DNA damage checkpoints are classified into at least 3 checkpoints: G1/S (G1) checkpoint, intra-S phase checkpoint, and G2/M checkpoint. The cell cycle includes an impressive system of checkpoints that, more or less, "scan" the DNA passing through the cycle for mutations and acts as a natural prevention against the duplication of cancerous cells.

Checkpoints along the cell cycle assess DNA passing them for damage or mutation. The cell cycle consists of four primary stages, G1 (GAP 1), S (Synthesis), G2 (GAP 2) and M (Mitosis). Each stage contributes to the successful replication of a cell in its own unique way. But in order for each of the stages to have participation in the cycle, DNA must clear all the checkpoints which it encounters along the way. In other words, there is a vital checkpoint that the DNA must pass upon entering the cycle called the G1 or restriction point and if DNA is unsuccessful in clearing this point, the cycle will never persist long enough to reach the S, G2, or M phases. Checkpoints along the cycle not only assess the DNA for damage but can actually act upon it in efforts to correct any mutation which is hindering its advancement in the cycle. Mechanisms within the checkpoints can delay the cycle until mutations are corrected. If mutations are irreversible, they can tag a cell for self-destruction (apoptosis) and thereby eliminating the chance that mutated DNA will be replicated. However, as we all know, this process is not always flawless, causing the spread of mutation filled, cancerous cells.

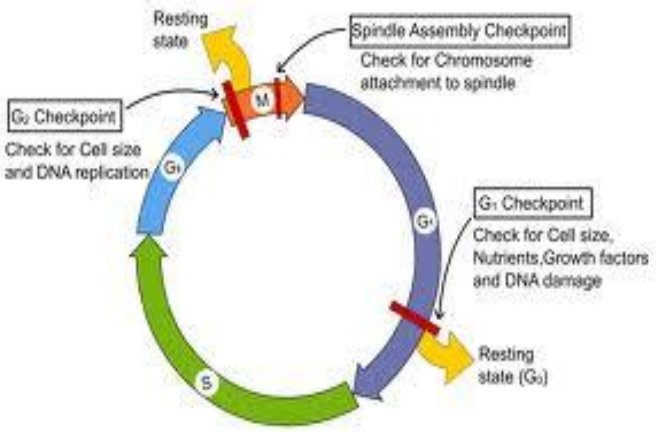


Figure 1.12 DNA damage check point in the cell cycle. Checkpoints along the cycle not only assess the DNA for damage but can actually act upon it in efforts to correct any mutation which is hindering its advancement in the cycle.

The first checkpoint which DNA must comply with as it begins the cycle is named G1 restriction point. This point is located right after completion of G1 phase but before S phase begins. This is a critical checkpoint as the onset of S phase brings with it the synthesis of DNA which the daughter cells will obtain. If the G1 checkpoint deems the DNA unsuitable for progression it can stop or delay the process sending it into an optional resting phase know as G0. A special protein referred to as P53 is essential in the function of the G1 restriction point as P53 has the ability to detect mutations in the genes which pass through the checkpoint. The G2 checkpoint is another checkpoint which DNA must overcome to complete a successful cycle. After completing S and G2 phases, the cell's DNA must pass the G2 checkpoint before advancing to the M or mitosis phase. A successful transition through this checkpoint will trigger the start of mitosis. Often times damage can occur to the DNA before it reaches this checkpoint and therefore, in efforts to stop the transmission of mutated genes to daughter cells, it is likely that the cycle will be inhibited at this point. Without DNA checkpoints throughout the process of cell division and replication, the transferring of mutated genes would be more likely. Viable checkpoints are necessary to ensure that DNA being replicated is mutation free. Cancer may spread with more amplification and at a must quicker rate if it weren't for the detection of checkpoints in the process of cell division.

1.2.16.1 Cell Cycle Arrest at Different Checkpoint

1.2.16.1.1 G_1/ S Checkpoint

The G_1/S cell cycle checkpoint controls the passage of eukaryotic cells from the first "gap" phase (G_1) into the DNA synthesis phase (S). Two cell cycle kinases, CDK4/6-cyclin D and CDK2-cyclin E, and the transcription complex that includes Rb and E2F are pivotal in controlling this checkpoint. During G_1-phase, the Rb-HDAC repressor complex binds to the E2F-DP1 transcription factors, inhibiting downstream transcription. Phosphorylation of Rb by CDK4/6 and CDK2 dissociates the Rb-repressor complex, permitting transcription of S-phase-promoting genes including some that are required for DNA replication. Many different stimuli exert checkpoint control including TGFβ, DNA damage, contact inhibition, replicative senescence and growth factor withdrawal. The first four act by inducing members of the INK4 or Kip/Cip families of cyclin dependent kinase inhibitors (CKIs). TGFβ also inhibits the transcription of cdc25A, a phosphatase required for CDK activation. In response to DNA damage-induced activation of the

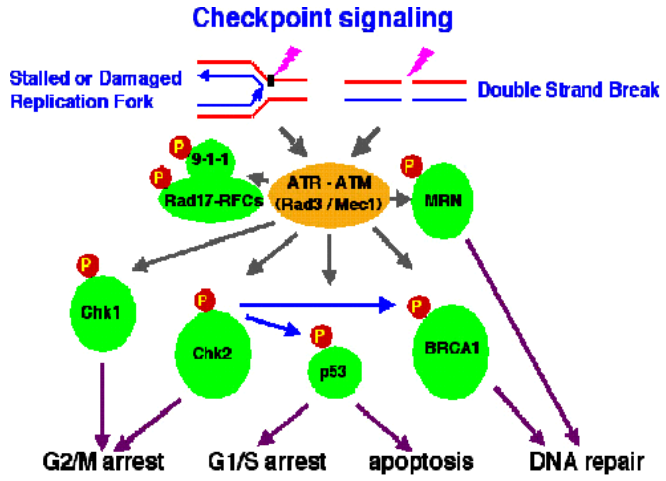


Figure 1.13 DNA Damage Signaling, DNA Repair, Cell Cycle Arrest and Apoptosis ATM/ATR/Chk1/2 pathway, cdc25A is ubiquitinated and targeted for degradation via the SCF ubiquitin ligase complex. Targeted degradation of cdc25A in mitosis via the APC ubiquitin ligase complex allows progression through mitosis. Growth factor withdrawal activates GSK-3β, which phosphorylates cyclin D, leading to its rapid ubiquitination and proteosomal degradation. Ubiquitin/proteasome-dependent degradation and nuclear export are mechanisms commonly used to rapidly reduce the concentration of cell cycle control proteins.

1.2.16.1.2 G_2/M DNA Damage Check Point

The G_2/M DNA damage checkpoint prevents the cell from entering mitosis (M-phase) if the genome is damaged. The cdc2-cyclin B complex is pivotal in regulating this transition. During G_2-phase, cdc2 is maintained in an inactive state by the kinases Wee1 and Myt1. As cells approach M phase, the phosphatase cdc25 is activated by phosphorylation. Cdc25 then activates cdc2, establishing a feedback amplification loop that efficiently drives the cell into mitosis. DNA damage activates the DNA-PK/ATM/ATR kinases, initiating two parallel cascades that inactivate cdc2-cyclin B. The first cascade rapidly inhibits progression into mitosis: the Chk kinases phosphorylate and inactivate cdc25, which can no longer activate cdc2. The second cascade is slower. Phosphorylation of p53 dissociates it from MDM2, activating its DNA binding activity. Acetylation by p300/PCAF further activates its transcriptional activity. The genes that are turned on by p53 constitute effectors of this second cascade. They include 14-3-3, which binds to the phosphorylated cdc2-cyclin B complex and exports it from the nucleus; GADD45, which apparently binds to and dissociates the cdc2-cyclin B complex; and p21/Cip1, an inhibitor of a subset of the cyclin-dependent kinases including cdc2 (CDK1).

1.3 Aims and Objectives

Shigellosis is one of the major diarrheal diseases in both developed and developing countries. *Shigella* infection causes serious complications including CNS manifestation (seizures, and coma) and haemolytic uremic syndrome (HUS). Abnormalities in CNS are more common during shigellosis than with other childhood infections. These observations suggest a possible role of toxin(s) in developing neurological menifestation in shigellosis. It is hypothesized that toxin plays a critical role in neuronal damage and HUS associated with *Shigella* infection. For identification and characterization of the neurotoxic factor(s) present in *Shigella* this study was conducted with the following specific aims and objectives:

- To detect the virulence and toxin producing gene(s) in *Shigella* spp by PCR assay.
- To characterize the toxin(s) of a representative strains using biochemical methods (Heat stability, pH, response to Protease and DNase).
- To purify the novel toxic factor(s) using ammonium sulphate precipitation and gel filtration (column chromatography).
- To investigate the activity of toxin(s) in cell culture and animal models.
- To investigate the enterotoxin activity by fluid accumulation in rabbit ileal loop assay.
- To investigate the cytotoxic activity in HeLa Cell line.
- To investigate the neurotoxic activity in neuron culture model.
- To demonstrate the toxin induced activation of DNA damage signaling in HeLa Cell line.

2.0 Materials and Methods

2.1 Bacterial Strains

A total of 385 clinical isolates of different serotypes of *Shigella* isolated from patients attending the Dhaka treatment center of icddr,b, Bangladesh during a period over 1997 to 2013 were used in this study. Of these 238 were *S. flexneri*, 80 *S. dysenteriae*, 42 *S. boydii* and 25 *S. sonnei*. These strains were isolated and characterized in the Clinical Microbiology Laboratory of icddr,b following standard Microbiological and Biochemical methods (World Health Organization, 1987). A single colony of confirmed *Shigella* strains was grown in Trypticase Soy Broth (TSB) with 0.3% yeast extract and stored at -70˚C after addition of 15% glycerol for further use. *S. flexneri* 2a, YSH 6000 strain (Sasakawa *et al.,* 1986) and *E. coli* (MC-1061) strain (lacking 140 MDa invasive plasmid, sensitive to all antibiotics) were used as positive and negative control respectively in the Sereny test, Congo red binding ability and for PCR for toxin and virulence genes. *E. coli* strains PDK-9, V-517, Sa, RP4 and R1 were used as plasmid molecular weight standard. *E. coli* MC-1061 and VTEC-3 (a Shiga toxin producing *E. coli*) were used as negative and positive control respectively in the cytotoxicity assay, DNA fragmentation, chromatin condensation and FACS assay. *V. cholera*e 569B strain used as positive control for the fluid accumulation assay. All these strains, which were used as standard, were collected from the Enteric and Food Microbiology Laboratory of icddr,b.

2.2 Serological Typing

All the strains were serologically confirmed using commercially available antisera kit (Denka Saiken, Co. Ltd. Japan) specific for all group factor antigens of *S. dysenteriae, S. boydii* and *S. sonnei* and in case of *S. flexneri* (i) all type and group-factor antigens and (ii) monoclonal antibody reagents specific for all *S. flexneri* type-and group-factor antigens (Reagensia AB, Stockholm, Sweden). Strains were subcultured on MacConkey agar (Difco, Becton Dickinson & Company Sparks, MD, USA) plates and after overnight incubation, serological reactions were performed by the glass slide agglutination test as described previously by El-Gendy *et al*., 1999.

2.3 Plasmid Analysis

2.3.1 Isolation of Plasmid DNA using Alkaline Lysis Method

Plasmid DNA was prepared according to the simplified alkaline lysis method of Kado and Liu (1981).

Reagents

i. Solution I : 40 mM tris-NaOAc, 2 mM EDTA, pH 7.4.
ii. Solution II (lysing solution) : 3% SDS, 50 mM Tris, pH 12.6.
iii. Solution III : Phenol: Chloroform: Isoamyl alcohol (25:24:1).

Procedure

An isolated colony of each strain was inoculated into 1.5 mL of TSB broth with 0.3% yeast extract (YE) and incubated overnight at 37˚C on a water bath shaker. Cells were collected in a polypropylene microcentrifuge tube by centrifuging the broth culture in an eppendorf centrifuge (Model No. 5415 C) at 13,000 rpm for 5 minutes. Supernatant was removed and the pellet was suspended in 100 μL of solution I by pipetting. Then 200 μL of solution II was added and was mixed gently by rapid inversion of the tube and was incubated at 55˚C for 1 hour in a water bath. After incubation, the tubes were taken out and an equal volume of solution III (300 μL) was added and mixed well by slowly inverting the tubes until a milky white suspension was formed. Then the tubes were centrifuged at room temperature for 6 minutes at 13,000 rpm which formed three layers, the upper layer was the plasmid solution, middle layer consisted of cell debris together with other proteinated fractions, and the lower layer was the phenol. Phenol layer was carefully discarded with micropipette. Then the tubes were centrifuged at room temperature for 9 minutes at 13,000 rpm. Using a micropipette and cut tips the plasmid solution was removed carefully and transferred into a new eppendorf tube.

2.3.2 Separation of Plasmid DNA by Agarose Gel Electrophoresis

Reagents

i. Agarose (Sigma Chemical Co., St. Louis, Mo).
ii. TBE (Tris-borate EDTA) buffer (GIBCO-BRL).
iii. Tracking dye (10x concentration).
iv. Ethidium bromoide (10 mg/ml).

Procedure

Plasmid DNA was separated by horizontal electrophoresis in 0.7% agarose slab gels in a Tris-borate EDTA (TBE) buffer at room temperature at 100 volt (50 mA) for 3 hours. Briefly, 30 μL of plasmid DNA solution was mixed with 3 μL of loading dye (Appendix II) and was loaded into the individual well of the gel electrophoresis for around 2.5hrs. After the electrophoresis the gel (5mm thick) was then stained with 0.5 μg/mL of ethidium bromide for 25 minutes and then destained with distilled water at room temperature. DNA bands were visualized and photograph was taken using Gel Doc^{TM} XRT with Image Lab^{TM} software. The molecular weight of the unknown plasmid DNA was determined on the basis of its mobility through agarose gel and was compared with the mobility of the known molecular weight plasmids (Haider *et al.,* 1989). Plasmids present in strains *Escherichia coli* PDK- 9 (140, 105, 2.7 and 2.1 MDa), R1 (62 MDa), RP_4 (36 MDa), Sa (23 MDa) and V517 (35.8, 4.8, 3.7, 3.4, 3.1, 2.0, 1.8 and 1.4 MDa) were used as molecular weight standard.

2.4 Detection of Virulence and Enterotoxin Genes by PCR Assay

Materials

i. 10X PCR buffer (GIBCO-BRL).
ii. 50 mM $MgCl_2$ (GIBCO-BRL).
iii. 2.5 mM dNTPs (GIBCO-BRL).
iv. *Taq* DNA polymerase (5U/μl, GIBCO-BRL).
v. Primers.
vi. Mineral oil (GIBCO-BRL).
vii. Filtered deionized water.
viii. Agarose.
ix. Ethidium bromide (10 mg/ml).

x. TBE (Tris-borate EDTA) buffer (GIBCO-BRL).

xi. 100 bp DNA size standard (Bio-Rad).

Procedure

The method, which was previously described by Vargas, *et al.*, 1999 was followed to detect the enterotoxin genes. Representative strains were grown on MacConkey agar for overnight. A single colony of each isolate was suspended in 25 µl of reaction mixer containing 2.5 µl of 10X PCR buffer, 1.5 µl of 50 mM $MgCl_2$, 2 µl of 2.5 mM dNTPs, 1 µl of primer (forward and reverse) together with 1 unit of *Taq* DNA polymerase (5 U/µl). Volume of the reaction mixture was adjusted by adding filtered deionized water. PCR assays were performed in a DNA thermal cycler (model 480; perkin-Elmer Cetus, Emeryville, Calif.) with the following program: 30 cycles at 95°C for 50 sec, 55°C for 1.5 min and 72°C for 2 min with a final extension at 72°C for 10 min for *set1, sen* genes. 30 cycles at 94°C for 60 s, 55°C for 1.0 min and 72°C for 1.0 min with a final extension at 72°C for 10 min for *stx* genes. Amplification products were subjected to horizontal gel electrophoresis in 1% agarose gel in TBE (tris-borate EDTA) buffer at room temperature at 100 volt (50 mA) for 1h. Briefly, 10 µl of amplified DNA for each sample was mixed with 1 µl of tracking dye and loaded into an individual well of the gel (5 mm thick). DNA bands were detected by staining the gel with ethidium bromide (0.5 µg/ml) for 30 minutes at room temperature and photographs were taken using Gel Doc™ XRT with Image Lab™ software.100 bp DNA size standard (Bio-Rad) was used as marker to measure the molecular size of the amplified products.

Table 2.1 Primers used for detection of virulence and enterotoxin genes

Gene encoding virulence factor	Oligonucleotide sequence (5′ to 3′)	Size of amplified product (bp)	Reference
*set*1A	TCACGCTACCATCAAAGA TATCCCCCTTTGGTGGTA	309	Fasano *et al*., 1995
*set*1B	GTGAACCTGCTGCCGAATTC ATTTGTGGATAAAAATGACG	147	Fasano *et al*., 1995
sen	ATGTGCCTGCTATTATTTAT CATAATAATAAGCGGTCAGC	799	Nataro *et al*., 1995
*stx*1	ATCTCATGCGACTACTTGAC ACCCTGTAACGAAGTTTGCG	140	Bonnet *et al*., 1998
*stx*2	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATTACACAGGAGC	540	Bonnet *et al*., 1998
*Ipa*H	TGGAAAAACTCAGTGCCTCT CCAGTCCGTAAATTCATTCT	423	Talukder *et al*., 2003
*Ipa*BCD	GCTATAGCAGTGACATGG ACGAGTTCGAAGCACTC	600	Talukder *et al*., 2003
ial	CTGGATGGTATGGTGAGG GGAGGCCAACAATTATTTCC	320	Talukder *et al*., 2003

2.5 Tests for Invasiveness

2.5.1 Keratoconjunctivitis Assay (Sereny Test)

Sereny test was performed according to the procedure described elsewhere (Sereny, 1957; Mackel *et al.*, 1961). Briefly, overnight culture of bacteria, suspended to a density of approximately 10^{10} viable cells in 20 μL of phosphate-buffered saline, was dropped into the conjunctival sac of the guinea pig. The other eye served as the control. *S. flexneri* 2a (YSH6000) carrying a 140 MDa invasive plasmid was used as a positive control. The guinea pigs were observed daily for 72 hours and their inflammatory responses were graded.

2.5.2 Determination of Congo Red Binding Ability

Trypiticase Soy broth (TSB) containing 0.3% yeast extract with 1.5% agar and 0.01% Congo red (Sigma chemical company ltd.) was used to study the pigment binding ability of the representative strains (Sakai, 1986, Sasakawa, 1986). A single colony from the overnight culture was streaked on the Congo red plate and incubated at 37C for 24h. *S. flexneri* 2a (YSH6000) carrying a 140 MDa invasive plasmid and *E. coli* ATCC-25922 lacking the 140 MDa invasive plasmid were used as a positive and negative control, respectively. After

incubation plates were visually observed for Congo red binding ability, colonies were differentiated as pigmented (Pcr^+), and non pigmented (Pcr^-).

2.5.3 Detection of TTSS genes

Materials

i. 10X PCR buffer (GIBCO-BRL).
ii. 50 mM $MgCl_2$ (GIBCO-BRL).
iii. 2.5 mM dNTPs (GIBCO-BRL).
iv. *Taq* DNA polymerase (5U/μl, GIBCO-BRL).
v. Primers.
vi. Mineral oil (GIBCO-BRL).
vii. Filtered deionized water.
viii. Agarose.
ix. Ethidium bromide (10 mg/ml).
x. TBE (Tris-borate EDTA) buffer (GIBCO-BRL).
xi. 100 bp DNA size standard (Bio-Rad).

Table 2.2 Primers for the detection of TTSS-genes of *Shigella* species

Target Gene	Oligonucleotide sequence (5′ to 3′)	Size of amplified product (bp)	Annealing temperature	Reference
*ipa*C	CCCCCGGGGGAAATTCAAAACACAAAACCAACC CCAAGCTTCGCACGAATATTACCCGCAATCTGACT	400	55	Faruque *et al.*, 2002
*ipa*D	ATGAATATAACAACTCTGACT ATGGACAAAAAGTTTATCTGT	450	55	Faruque *et al.*, 2002
*vir*A	CTGCATTCTGGCAATCTCTTCACATC TGAGAGCTAACTTCGTAAGCCCTCC	650	65	Kumao *et al.*, 2002
*mxi*C	GTAGGTGATGTATGCTTG GATCACTTTCATCTCCTG	1068	47	Ruiting *et al.*, 2001
*mxi*A1	GAGATGAAAGTGATCCAG AAATGTACCAGTATAGCC	2061	48	Ruiting *et al.*, 2001
*mxi*A2	ATTATAAAAAGGTCGTAG AGCATACGATATAACGAG	1983	48	Ruiting *et al.*, 2001
*ipa*BCD	GCTATAGCAGTGACATGG ACGAGTTCGAAGCACTC	500	55	Faruque *et al.*, 2002

Procedure

Representative strains were grown on MacConkey agar for overnight. A single colony of each isolate was suspended in 25 µl of reaction mixer containing 2.5 µl of 10X PCR buffer, 1.5 µl of 50 mM $MgCl_2$, 2 µl of 2.5 mM dNTPs, 1 µl of primer (Table 2.2) together with 1 unit of *Taq* DNA polymerase (5 U/µl). Volume of the reaction mixture was adjusted by adding filtered deionized water. PCR assays were performed in a DNA thermal cycler (model 480; perkin-Elmer Cetus, Emeryville, Calif). Amplification products were subjected to horizontal gel electrophoresis in 1% agarose gel in TBE (tris-borate EDTA) buffer at room temperature at 100 volt (50 mA) for 1h. Briefly, 10 µl of amplified DNA for each sample was mixed with 1 µl of tracking dye and loaded into an individual well of the gel (5 mm thick). DNA bands were detected by staining the gel with ethidium bromide (0.5 µg/ml) for 30 minutes at room temperature and photographs were taken using Gel Doc™ XRT with Image Lab™ software.100 bp DNA size standard (Bio-Rad) was used as marker to measure the molecular size of the amplified products.

2.6 Toxin Preparation and Concentration

2.6.1 Preparation of Culture Filtrate

Materials

i. Trypticase soy broth (TSB)
ii. Sucrose
iii. PBS (pH: 7.2)
iv. Dialysis bags
v. 0.22 µm Millex filter (Millipore corp., USA)
vi. EDTA

Procedure

Culture filtrates of all the strains were prepared following the method described elsewhere (Sanyal *et al*., 1980) and concentrated by the method described by Nur-E-Kamal *et al*., 1993. Briefly, the organisms were grown at 37°C for 4 hr in 5 ml TSB medium to develop inoculum. The primary inoculums was then added to 40 ml of sterile TSB medium and grown overnight. Then the cells were pelleted by centrifugation (SORVALL RC-5B

refrigerated centrifuge) at 12,000 g for 20 minutes at 4^{o}C. After centrifugation the supernatant was collected in EDTA treated dialysis bag followed by concentration of the diluted supernatant as describes earlier (Nur-E-Kamal *et al*., 1993). Briefly, the dialysis bag containing crude supernatant was placed in fresh and dried sugar at 4^{o}C. After 1 hr of concentration, the proteins were collected out from the dialysis bag and filtered through 0.22 µm Millipore filter and stored at –20^{o}C for future use.

2. 6. 2 Preparation of Cell Lysate

Materials

Trypticase soy broth (TSB)

i. PBS (pH: 7.2)
ii. 0.22 µm Millex filter (Millipore corp., USA)
iii. Sonicator

Procedure

The cell pellet of Shigella strains were washed with PBS (pellet was suspended in 5 ml normal saline and centrifuged at 12,000xg for 10 minutes) and repeated for three times. The washed pellet was then re-suspended in 5 ml of PBS. The cells were placed in an ice cool chamber and were sonicated by ultrasonic vibration (30 sec x 15 times). Sonicated samples were then centrifuged at 12,000xg for 20 min at 10°C and supernatant was then filtered through the 0.22 mm Millipore membrane filter and was stored at -20°C by alliquoting in eppendrof tubes.

2.6.3 Partial Purification of *Shigella* Novel Toxin from Representative *S. flexneri* (K-314) Strain

Materials

i. Trypticase soy broth (TSB)
ii. PBS (pH: 7.2)
iii. 0.02M Tris buffer
iv. Ammonium sulphate
v. Dialysis bags
vi. 0.22 µm Millex filter (Millipore corp., USA)

Procedure

One litre of culture supernatant of *S. flexneri* (K-314) strain was saturated to 40%, 60% and 80% with continuous addition of ammonium sulphate maintaining the pH at 7.2 with addition of dilute ammonium hydroxide solution. The saturated suspensions were held overnight at 4ºC and centrifuged at 22,000xg for 20 min at 4ºC. The precipitate was dissolved in 10 ml PBS buffer at pH 7.2 and dialysed against the 0.02M Tris buffer for 24-36 hours at 4ºC with 6-8 changes of buffer for complete removal of ammonium sulphate. The dialysed sample was passed through a membrane filter of 0.22 µm average pore diameter and stored at -20°C by alliquoting in eppendorf tubes. The filtrate was treated as partially purified toxin fractions. All the fractions were studied for biological activities and the 60% fraction showed strong enterotoxic and cytotoxic activities. Therefore, 60% fraction was further purified by column chromatography technique.

2.6.4 Further Purification of Biologically Active Toxin Fraction (60%) of *S. flexneri* (K-314) by Sephadex G-75 Gel Chromatography

Materials

i. Sephadex G-75
ii. PBS (pH: 7.2)
iii. Sterile deionized water
iv. Syringe
v. Glass wool
vi. 0.22 µm Millex filter (Millipore corp., USA)
vii. EDTA

Procedure

The active fraction (60% fraction) was further purified by column chromatography technique using sephadex G-75 (Pharmacia). Sephadex G-75 was washed three times with sterile deionized water and swelled for overnight by same water. Syringe column was plugged with glass wool at the bottom and the column was filled with Sephadex G-75 slurry very slowly and carefully without any bubble inside. PBS was passed through column for three times and the gel was settled for 2 hours in column. One ml sample was applied on top of the gel and four fractions (F1, F2, F3 and F4) consisting of 1 ml each, were collected by centrifugation of the column at 1,000xg for 3 min with addition of PBS. Fractions were then filtered

through the 0.22 µm Millipore membrane filter and were stored at -20°C by alliquoting in eppendrof tubes. All the fractions were studied for biological activities.

2.7 Bio Rad Protein Assay

Materials

i. Bio-Rad dye reagent
ii. Bovine serum albumin (BSA)
iii. Whatman filter paper # 1
iv. Filtered distilled deionized water

Procedure

Protein concentration of the all the samples were estimated by Bio-Rad protein assay, which is based on the Bradford method. It involved the addition of an acidic dye solution, and subsequent measurement at 595 nm with a spectrophotometer (Shimadzu, Japan). Dye reagent was diluted so that 1 part dye reagent was mixed with 4 parts deionized water. The diluted dye reagent was then filtered through Whatman #1 filter (or equivalent) to remove particulates. Five dilutions of a protein standard (BSA) were prepared for the preparation of standard curve against average absorbance values. The linear range of the assay for BSA is 0.2 to 0.9 mg/ml. 100 µl of each standard along with sample solution was pipetted into a clean, dry test tube. Protein solutions are assayed in duplicate. 5.0 ml of diluted dye reagent was added to each tube and was mixed vigorously by vortexing. The mixture was incubated at room temperature for at least 5 minutes. Absorbance was measured at 595 nm. It should be noted that absorbance would increase over time so that samples should incubate at room temperature for no more than 1 hour. Concentrated culture supernatant, cell lysate and different partially purified fractions were determined from the standard curve.

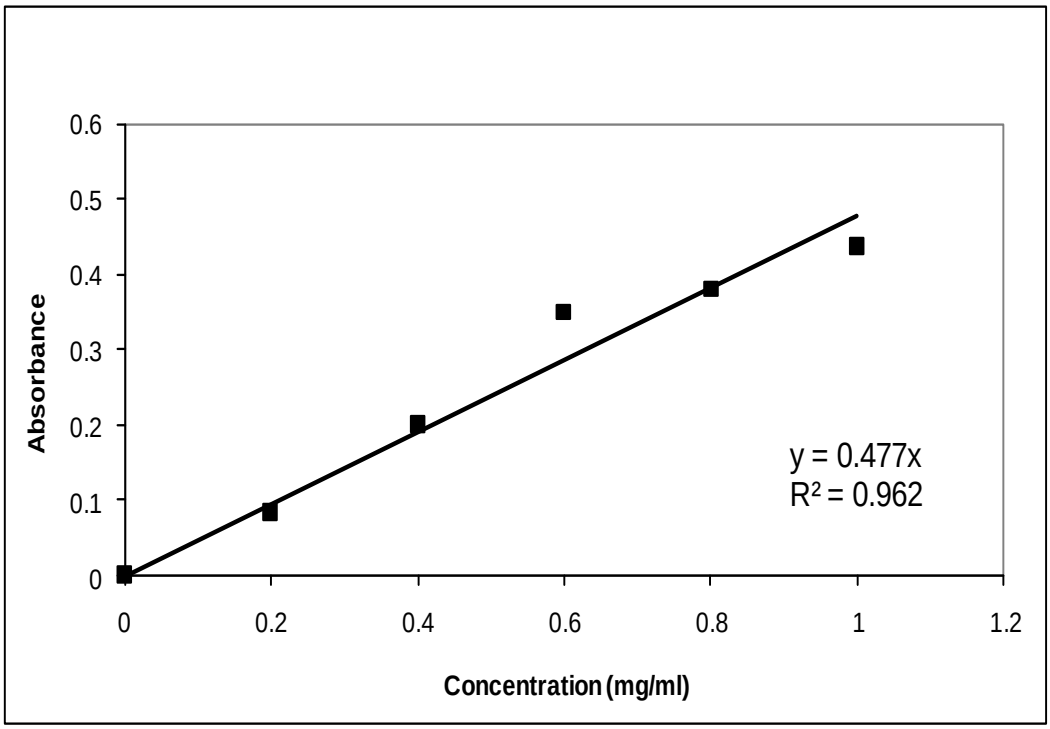


Figure 2.1 Standard curve of protein (BSA)

2.8 Biochemical Properties of Different Toxic Fractions of *S. flexneri* 1c (K-314 and K-915)

2.8.1 Heat Treatment

Procedure

Lysates from *S. flexneri* (K314 and K915) were heated for 1hr at 100°C and then centrifuged for 3 min. The filtrate was then applied into the HeLa cells and studied its cytotoxic activity.

2.8.2 Enzyme Treatment with Protenase K, DNase, and RNase

Materials

i. Proteinase K QIAGEN (Cat. No. 19131)
ii. DNase I (Invitrogen, Catalogue number 18047019),
iii. RNase QIAGEN (Cat. No.19101)

Procedure

Protease K was purchased from QIAGEN (Cat. No. 19131) and digestion was performed according to supplier information. Briefly, proteinase K solution (5 mAU) was added to 100µl of the *S. flexneri* 1c (K-314 and K-915) lysate (1mg/ml total protein) in a buffer

solution. The resulting mixture was incubated at 37°C for 15 min and used for cytotoxic activity. RNase was purchased from QIAGEN (Cat. No.19101) and 10µl was added to 100µl (1 mg/ml total protein) of the *S. flexneri* 1c (K-314 and K-915) lysate and incubated for 30 min at 25°C. The RNase treated sample was assayed for cytotoxic activity. With regard to DNase (Invitrogen, Catalogue number 18047019), 100µl of *S. flexneri* 1c (K-314 and K-915) lysate was mixed with 10µl (1 mg/ml) of DNase I. The solution was incubated at 37°C for 60 min. The DNase I treated samples were used for cytotoxic activity testing. Enzyme-treated bacterial cell lysates were studied for cytotoxic activity against HeLa cells as described above.

2.9 Cytotoxicity Assay in HeLa Cell Line

2.9.1 HeLa Cell Monolayer Preparation from Stock

Materials

i. Delbucco's Modified Eagles Medium (DMEM) ,
ii. Fetal Bovine Serum
iii. Penicillin, Streptomycin, Glutamine
iv. HeLa cell line culture medium (DMEM+ 10% FBS+ 1% (penicillin, streptomycin and glutamine)

Procedure

HeLa cell line was prepared from stock vial stored previously at liquid N_2 (-80°C). The stock vial was thawed very rapidly at 37°C water bath. After thawing the cells were taken into a V-bottomed falcon tube and added with 1 ml of fresh medium (only DMEM). Cells were pelleted by centrifugation for 5 minute at 1500 g. Supernatant was discarded and the cells were re-suspended in 3 ml of fresh medium by mixing well with sterile Pasteur pipette. The whole suspensions were then distributed into two 25 cm^2 flat bottomed flask containing 10 ml of cell line culture medium. The cells were then propagated in 25 cm^2 culture flask at 37°C in 5% CO_2 /95% air atmosphere.

2.9.2 HeLa Cell Counting

Materials

i) 0.4% Trypan Blue solution

ii) Hemocytometer

Procedure

Cell suspension were taken and diluted 1: 2 (or higher, as needed to obtain a minimum count of 150 cells / 5 squares) into 0.4% Trypan Blue solution. Twelve µl were applied to one side of a hemocytometer. Hemocytometer holds 10 µl on each side. Cells were counted in five squares. Number of cells per milliliter and total number of cells were calculated.

Cell count x 1000 (factor converting µl to ml) x 2 (Trypan blue dilution) x 2 (two halves of hemocytometer) = cell count / ml

2.9.3 Cytotoxicity Analysis of *Shigella* toxin (ShET-1, ShET-2 and STX) with different Dose in HeLa cell Line

Procedure

The cytotoxic activity of Shigella toxin in HeLa cell line was determined by a previously described method of Blanco *et al*., 1990. Briefly, HeLa cell monolayers were obtained by seeding 10^4-10^5 cells in 96 well tissue culture plates with ensuring similar distribution of cells at each well. After 48 hrs of incubation filtered culture supernatant of each strain were treated to the cell monolayer at different dilution (neat, 1:4, 1:10, and 1:40) and incubated for overnight at 37^oC in 5% CO_2 /95% air atmosphere and the cytotoxic effects were observed under inverted microscope for each dilution.

2.9.4 Cytotoxicity Assay of Concentrated Culture Supernatant, Cell Lysate and Different Fractions of *S. flexneri* 1c (K-314)

Procedure

The cytotoxic activity of concentrated culture supernatant and cell lysate in HeLa cell line were determined by a previously described method of Blanco *et al*., 1990. Briefly, HeLa

cells monolayer was prepared by seeding 10^4-10^5 cells in 96 well tissue culture plates containing 10^5cells/ml in DMEM (Sigma) with 10% fetal bovine serum and incubated overnight in an atmosphere of 5% CO_2 ensuring similar distribution of cells at each well. 20 µl of concentrated culture supernatant and cell lysate were applied at each well and the plate was gently shaken for equal distribution of applied sample in a well. The plate was incubated for overnight at 37°C in 5% CO_2 atmosphere. Concentrated culture supernatant of VTEC and PBS were used as positive and negative controls respectively. Cytotoxic activity was determined by studying the morphological changes (rounding) under an inverted microscope after 48 hours of incubation. Same experiment was also conducted to check the morphological changes up to 48 hours for 40%, 60% and 80% fractions of *S. flexneri* 1c (K-314). Four sephadex G-75 fractions (F1, F2, F3 and F4) obtained from 60%, were also checked for the morphological changes up to 24 hours.

2.10 Cell Viability Assay (MTT Assay)

Materials

i. MTT (4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)
ii. DMEM
iii. DMSO (dimethyl sulfoxide)

Procedure

Cell viability was evaluated using a modification of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay as described previously by Mosmann, 1983. Briefly, HeLa, MEF, and Caco-2 cells were cultured in DMEM containing 10% Fetal bovine serum to semiconfluency. Stx1 was added to a final concentration of 5, 25 or 100 ng/ml. Cells were cultured for 24 hours. Cell viability was evaluated using a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Briefly, a sterile solution of 0.1 mg/mL MTT in DMEM was added to each well in a multi-well plate, and incubated at 37°C for 4 hrs. The MTT color complex was then solubilized in 100 µl dimethyl sulfoxide (DMSO) to each well, the solution transferred to a 96-well plate and transmission evaluated at 570 nm by microplate reader. Data are expressed as percent of control (no oligo and no Cellfectin).

2.11 Enterotoxicity Assay

Enterotoxicity assay of toxin from representative *Shigella* strains were performed using animal model: rabbit ileal loop assay.

2.11.1 Rabbit Ileal Loop Assay

Reagents

I. Pentobarbital Sodium (Sigma)
II. PBS (pH: 7.2)

Procedure

Rabbit Ileal Loop Assay was performed according to the method described earlier (Singh and Sanayl, 1978). Briefly, adult albino rabbits (New Zealand) of 9-10 weeks age of nearly 2 kg of body weight were starved for 48 hours allowing water *ad libutum.* After proper anesthesia with lower dose of Pentobarbital Sodium (0.5 ml/kg body weight, intravenous), the intestine were exposed and loops of 5-7 cm in length with 3-5 cm intervals between each were tied beginning near the ileocaecal junction. One ml culture supernatant of each strain was inoculated in each loop and usually 6-7 loops were made in one rabbit. The animals were sacrificed after 18 hours with excess of Pentobarbital Sodium. The length of each loop and the volume of fluid accumulated were measured to determine the amount of fluid accumulated per unit length of gut. Each test was done in two rabbits. Results were considered valid only if the positive and negative controls gave appropriate responses in each animal.

2.11.1.1 Histopathological Examination of the Loop Segments

Materials

i. 10% Formalin
ii. Ethanol
iii. Xylene
iv. Paraffin wax
v. Harris Haematoxylin solution
vi. 1% eosin solution

Procedure

At the end of rabbit ileal loop assay the animals were sacrificed and the full-thick segments of the ligated ileam were preserved in 10% buffered formalin immediately for fixation. Tissue sections were transferred through baths of progressively more concentrated ethanol to remove the water. Then the tissue blocks were cleared by xylene to remove the alcohol and impregnated by molten paraffin wax at 50^{o}C-60^{o}C, which replaced the xylene. After the tissues have been dehydrated and infiltrated, the tissue samples were embedded using metallic moulds. These moulds were first lubricated with liquid paraffin and then the melted paraffin was poured into it. The tissues were carefully embedded in proper plane at the bottom of the mould. The respective number of the tissue was inserted in the paraffin wax by the side of mould. The melted paraffin was allowed to harden at room temperature. After hardening, the mould was removed and blocks were trimmed properly to mount on block holder. Then the blocks were kept in ice chamber of a refrigerator before cutting sections.

Each block of tissues was mounted on the holder and fitted in the microtome machine. Sections were cut at 5 micron thickness. Ribbons of good sections were selected and floated on luke-warm water in the water bath. The sections were then taken on albumenized glass slides. Then the slides were kept in inclined position to drain off the excess water at room temperature.

The slides were stained by routine haematoxylin and eosin staining method (H & E). After deparaffinisation using standard technique, hydration of tissue section was done by descending of ethanol to water. Harris Haematoxylin solution was applied on tissue section for 2-6 min and washed well in running tap water for 5-10 min until section became blue. 1% acid alcohol was added for 1 min for differentiation and then slide was dipped into alkaline solution (Ammonia water) for 1 min and washed by running tap water till the sections became pale-blue (5 min). Finally 1% eosin solution was added for 1-2 min for counter staining and then washed in running tap water for 1-5 min. Then after dehydrating through graded alcohol and cleaning by xylene, mounting was done with DPX using no 2 cover slip. Finally, pathological changes of tissues were examined under light microscope and photograph was taken by digital camera. The histological changes were graded from 0 to 4+, with 0 (normal) being no change and 4+ equaling severe enterocyte necrosis (Fernandez, *et al*., 1984).

2.11.2 Enterotoxic Activity by Removable Intestinal Tie Adult Rabbit Diarrhoea ((RITARD) Model Test

Reagents

i. Pentobarbital Sodium (Sigma)
ii. PBS (pH: 7.2)

Procedure

The adult rabbit intestinal assay was performed essentially according to Spira *et al*., (1981) employing adult albino rabbits, New Zealand strain (Animal Lab. division, icddr,b) averaging 2.0 kg in weight. Rabbits were starved for 24 hours but given water *ad libitum*. After proper anaesthesia with a lower dose of sodium pentobarbital solution (0.5 ml/kg body weight, intravenous), the intestine was exposed. The cecum was brought out through a midline incision and ligated with no. 11 umbilical tape as close to the ileocecal junction as possible without compromising the blood supply to the area. The small intestine was then brought out and a length of umbilical tape was tied in a slip knot around it to close it in the vicinity of the mesoappendix. Ten ml of bacterial suspension in PBS (3×10^8/ml) was injected into the lumen of the anterior jejunum. Usually, the desired dose was administered in an arbitrary chosen volume of 10 ml. After the sample was injected, the intestine and cecum were returned to the peritoneal cavity, and the incision was closed. The loose ends of the slip knot were brought through the incision in the muscle layers and held while the incision was sutured around them. The loose ends were left in the space between the muscle and skin layers and clipped to the skin by means of a surgical clip. The rest of the skin was sutured and the animal was kept in a holding box, and its temporary tie was removed 2 hours after the challenge had been introduced. After the surgical clip was removed, the slip knot was opened and the umbilical tape was pulled gently from the intestine of the animal. The unclosed portion of the skin incision was sutured. The animal was then returned to its cage and provided with food and water. Each test was done in duplicate. Ten ml PBS injected in duplicate rabbits were used as negative controls.

For monitoring the disease, rabbits were observed for overt diarrhoea, signs of weakness and for death. Rectal swabs were taken daily and plated onto MacConkey agar to identify shedding of the challenge organisms. Observations were made at 24 hours intervals for 5

days. Rabbits alive after 5 days were considered to have survived the infection. Each rabbit was autopsied and the appearance of the gut was recorded.

2.12 Paralytic Lethal Activity Assay

Procedure

Five groups (six mice per group) of 6 to 8 weeks old adult Swiss albino mice (Animal Lab. division, icddr,b) were injected intra-peritoneally (IP) with four different doses (0.1µg, 1µg, 10µg, 50µg per mice) of Toxin fractions of *S. flexneri* 1c (K-314). *E. coli* MC-1061 and PBS were used as negative control. Deaths were monitored twice daily to determine the 50% lethal dose (LD50). Again six mice per group were injected (IP) with 4xLD50 of toxin fractions. Mice were observed for signs of toxic activity until death occurred

2.13 Neurotoxicity Assay

2.13.1 Isolation of Neuron from Rat Cerebellum (Neuron culture)

Materials

i. Basal media (BME)
ii. Neurobasal media
iii. HEPES
iv. 0.25% Trypsine EDTA
v. Trypsin Inhibitor (TypeII-S)
vi. DNase I
vii. B-27 Suppliment
viii. Penicillin-Streptomycin Solution
ix. Glutamine L-200mM
x. Poly L Lysine Hydrobromide (PLL)
xi. 70% Alcohol

Procedure

Cerebellar granule neuronal cultures were prepared as described by Meiners *et al*., 1999. Neuronal cultures were cultivated from postnatal day 8 (P8) rat pups. Brains were removed into a Petri dish containing 5 ml of BMEM with 2 M HEPES buffer (BMEMHEPES).

Cerebella were removed, and meninges and blood vessels were peeled off and discarded to ensure minimal contamination from endothelial cells. Cerebella were then minced into fine pieces (0.5 mm) with dissecting knives and incubated in BMEM-HEPES containing 0.025% trypsin for 10 min at 37°C. After incubation, the trypsinization was halted by adding 1 ml of BMEM containing 0.025% soybean trypsin inhibitor and 0.05% DNase I. The tissue was then gently triturated through a fire-polished Pasteur pipette until it was dispersed into a homogeneous suspension. The suspension was transferred into a fresh tube. DMEM–25 mM KCl–10% heat-inactivated FCS (3–4 ml) was added to any remaining tissue clumps, and the trituration was repeated. Cells were then filtered through an ethanol-sterilized 40 mm mesh and centrifuged for 10 min at 1500 rpm. The pellet of cerebellar granule neurons was resuspended in DMEM–25 mM KCl–10% FCS. About 30, 000 neurons/well were seeded on poly-L-lysine (PLL)-coated glass coverslips and used for neurite outgrowth assays as described below.

2.13.2 Neuronal Outgrowth Assay

Materials

i. Para formaldehyde
ii. Triton X
iii. PBST (0.5% triton X in PBS)
iv. Goal serum (Blocking antibody)
v. MSX Tubulin Beta III isoform (Primary antibody)
vi. Cy3-conjugated goat anti-mouse (Secondary antibodies)
vii. Fluoromount-G mounting solution

Procedure

Cerebellar granule neurons were plated onto the coverslips at a density of 60,000 neurons per well and cultured for 48 hr in DMEM–25 mM KCl–10% FCS. Bacterial lysates and culture supernatant (as described above) were added to the neuron culture and incubated for another 24 hrs. After overnight incubation neurons were fixed in the coverslips with 4% paraformaldehyde and the extent of neurite outgrowth was determined by staining neurofilaments with monoclonal anti-neurofilament antibodies. Coverslips were removed from the 24 well plate and rinsed in PBS to remove the blocking solution (10% goat serum in PBST) and incubated for 30 min at 4°C with the appropriate primary antibody against the

neurofilaments (MSX Tubulin Beta III isoform) diluted 1:100 in PBS. The coverslips were washed in PBS and incubated with Cy3-conjugated goat anti-mouse secondary antibodies diluted 1:500 in PBS-serum and incubated for 30 min at 4°C. Coverslips were rinsed in PBS, followed by ddH_2O, and then mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL) on microscope slides. Images of the cultures were captured using a Macintosh Quadra 700 and analyzed with NIH Image software (available at http://rsb.info.nih.gov/). The average total neurite length per neuron and its branches was determined. The average length per neuron was determined from a sample of at least 100 neurons from random fields. The total neurite length was calculated as the sum of the lengths of individual neurites. Reduction of the neurite length indicates the presence of neurotoxic factor.

2.14 Molecular Mechanism of Toxin Induced Activation of DNA Damage Signalling in HeLa cells

2.14.1 Morphological Alteration Study by Staining of the Actin Filaments

Materials

i. Fluorescein isothiocyanate (FITC)–conjugated phalloidin
ii. PBS (pH: 7.2)
iii. PBS + 2% FBS
iv. Triton-X 100

Procedure

Morphological alteration of the Hela cell was determined by staining the actin filaments with fluorescein isothiocyanate (FITC)–conjugated phalloidin according to the method described earlier (Aragon *et al*., 1997). HeLa cells were grown on cover-slips placed in the 24 well plate containing $5x10^4$ cells in DMEM (Sigma) with 10% fetal bovine serum and incubated over night in an atmosphere of 5% CO_2. After confluence growth of monolayer, the cells were then treated with *S. flexneri* 1c fraction. The plate was incubated for 24 hours. Media was removed and toxin treated cells were washed with 1 ml PBS. Cells were fixed by adding 0.5 ml of 4% paraformaldehyde for 15 min and then washed with 1 ml PBS. Then 0.5 ml of 0.5% Triton X solution was added to each well, waited for 5 min and washed with 1 ml of PBS. Sixty µl of phalloidin in PBS (1:100) was applied on each cover-slip and incubated at

room temperature for 1 hour. After the incubation, the cover-slip was washed for six times in fresh PBS and the excess liquid from the slide was removed by gently blotting around the sample with an absorbent tissue. Finally, the cover-slip was mounted on a glass slide and slide was kept at 4^{o}C. Cells were observed under an epifluorescence microscope (Axioskop, Carl Zeiss, Inc., Germany) and images were recorded through a digital camera (AxioCam MRc, Carl Zeiss).

2.14.2 Chromatin Condensation Assay (by propidium iodide staining)

Materials

I. Propidium iodide 4% Para-formaldehyde
II. PBS (pH: 7.2)
III. PBS + 2% FBS
IV. Triton-X 100

Procedure

Propidium iodide (PI) staining was performed following standard procedures. Breifly, HeLa cells were grown on a cover-slip placed in the 24 well plate containing $5x10^4$ cells in DMEM with 10% fetal bovine serum and incubated overnight in an atmosphere of 5% CO_2. After confluence growth of a monolayer, the cells were treated with *S. flexneri* 1c fraction and incubated for 24 hours. Culture medium was removed and toxin treated cells were washed with 1 ml PBS. Cells were then fixed with 4% paraformaldehyde for 15 min and washed with 1 ml PBS.

2.14.3 DNA Fragmentation Assay by Agarose Gel Electrophoresis

Materials

i. Tris-HCl
ii. EDTA
iii. Triton-X100
iv. Phenol: Chloroform: Isoamylalcohol
v. 3M NaCl
vi. Ethanol
vii. TE buffer

viii. RNase A stock solution (10 mg/ml)

Procedure

DNA fragmentation assay by agarose gel electrophoresis was determined by the method described previously by Tayeb and William, 1999. Briefly, HeLa cells were grown in six wells (corning) plate in an amount so that each of the well contains $3x10^6$ cells. The plates were grown into CO_2 injected special incubator for 24 - 48 hrs. After confluence growth of monolayer, lysate from *S. flexneri* 1c was added to a final concentration of 25 or 100 ng/ml. The media containing toxin was removed by using Pasteur pipette with negative pressure produced by suction pump. 4 ml of PBS (pH: 7.2) was added to each well and shaken gently. After that the PBS was also removed by Pasteur pipette. PBS (pH: 7.2) wash was repeated for one more time. When the washing was completed the wells was added with 400 µl of lysis buffer followed by 30 minutes incubation on ice. Lysed cells were then collected in 1.5 ml eppendorf tube and the cellular debris were separated from cellular cytoplasmic contents by centrifuging at 10,000 g for 10 min. The DNA fragments were then extracted from the supernatant with phenol: chloroform: isoamylalcohol (25:24:1). Same volume of phenol: chloroform: isoamylalcohol was added to the supernatant collected from the previous step. Then the solution was mixed vigorously by inverting the eppendorf tubes repeatedly for 30 minutes. As soon as the milky white color of the solution develops the mixture was centrifuged at 13,000 g for 10 minutes. After centrifugation the mixture was separated into 3 distinct layers, the upper layer containing desired fragmented DNA, the middle containing protein complex and the bottom layer containing phenol. Thus the upper layer was taken very carefully by using cut-tips. DNA was precipitated by adding 1/10 volume of 5M NaCl and 2 volume of absolute ethanol. Following addition of both the salt and alcohol the mixture was incubated overnight at –20°C. In the next day the DNA was pelleted by centrifugation at 10,000 g for 20 min. The pelleted DNA was then rinsed with 70 % ethanol followed by resuspending in TE (Tris- EDTA) buffer containing 100 µg/ml RNase A. After 2 hrs of incubation at 37°C, DNA samples were run in 1.5% agarose gel by simple gel electrophoresis as described method. After 2 hr run the gel was then stained with 0.02% ethidium bromide, and visualized under UV light.

2.15 Flow Cytometry (FACS analysis)

Materials

i. Cell suspension buffer: PBS (pH: 7.2) containing 2% FBS
ii. DNA Extraction buffer: 192 ml 0.2 M Na_2HPO_4, 8 ml 0.1 M citric acid: P^H : 7.8
iii. Propidium iodide Staining Solution: 3.8 mM Na-citrate, 50 µg/ ml PI (Sigma) in PBS
iv. RNase A Stock Solution: 10 mg/ml RNase A (Sigma)

Procedure

Flow cytometric analysis for cell cycle distribution was performed accordingly to the suppliers protocol (Becton Dickinson, CA, USA) with ModFit LT^{TM} Software (Becton Dickinson, CA, USA) using FACScaliber (Becton Dickinson, CA, USA). Briefly, HeLa cells were grown in six wells (corning) plate in an amount so that each of the well contains $3x10^6$ cells. The plates were grown into CO_2 injected special incubator for 24-48 hrs. After confluence growth of monolayer, the cells were then treated with culture supernatant of different each strain. For each well 400 µl of culture supernatant was applied. After application the plate was gently shaken for the equal distribution of toxin so that the toxin equally affects all the cells. Cells were cultured for 2, 4, 6, 8 and 24 hours. In each case the cells were harvested by trypsinization. Briefly, cells in each well were treated with 400 µl of Trypsin-EDTA followed by incubation in 37^{o}C for 1-2 minutes. As soon as the incubation is completed Trypsin is immediately neutralize by adding 500 µl of cell suspension buffer PBS containing 2% FBS to each well. The trypsinized cells were then collected into the v-bottomed falcon tube followed by 2 times washing with PBS. Therefore, the $1\text{-}2\times10^6$ cells were re-suspended in 1 ml PBS. After harvesting $1\text{-}2\times10^6$ cells were fixed with ice-cold absolute ethanol. Briefly, 3 ml of absolute ethanol was added drop wise to 1 ml of cell suspension with vigorous agitation so that the cells were not allowed to form clump. Then the cells were incubated for 1 hr at 4^{o}C. After incubation the fixed cells were pelleted at little higher speed (2000g for 5min) as the cells became flocculent. Fixed cells were washed twice with PBS followed by re-suspending in 500 µl of PBS. 0.2-1.0 ml of DNA extraction buffer was added to the cell suspension followed by incubation in room temperature for 5 min. After centrifugation the DNA was stained with 1 ml of Propidium iodide (Sigma) staining solution. The DNA materials were then added with 50 µl RNase A. Finally the stained DNA materials were kept in 4^{o}C until analyzed by Flow cytometer (FACSciliber).

2.15.1 Cell Acquisition by FACScaliber

DNA materials were analyzed by FACScaliber with the help of Cell Quest software (Becton Dickinson) by taking FL2-A in the Y axis and FL2-W in the X axis.

2.15.2 Cell Distribution Analysis by ModFit LT^{TM} Software

Percentage of cell population in different phase was analyzed by ModFitLT software. The synchronization assay was done to check whether the cells are in G_0/G_1, S or G_2M phase.

2.16 Subcellular Fractionation and Western Blotting

2.16.1 Subcellular Fractionation

Materials

i. 2X Lammelli SDS sample buffer
ii. hsp60 (a mitochondrial protein)
iii. Topoisomerase I (a nuclear protein)

Procedure

HeLa cells were grown in six-well plate in an amount so that each of the well contains $3x10^6$ cells. Measured amount of cells were plated in each well. Equal distribution of cells through out the well was confirmed by gentle shake clockwise and anti-clockwise. The plates were grown into CO_2 injected special incubator for 24-48 hr. After confluence growth of monolayer, the cells were then treated with toxin (25 ng/ml) or BSA (25 ng/ml). After 6 hours of treatment, the cells were collected and processed. (a) A Nuclear fraction was prepared as described earlier (Nur-E-Kamal *et al*., 2004). Briefly, proteins in nuclear fraction were dissolved in SDS Lammelli sample buffer and separated by SDS-PAGE (12%). (b) Cells were lysed in SDS Lammelli sample buffer to prepare total cellular protein extract. Proteins in total cellular extract were separated by SDS-PAGE (12%). The cytoplasmic fraction was separated from mitochondrial and nuclear fraction by centrifugation at 12,000 g for 18 min at 4^oC. Equal volume of 2X Laemmli SDS sample buffer was added to nuclear, mitochondrial, and cytoplasmic fractions. Fractionation of the mitochondrial and nuclear proteins was confirmed by probing the membrane for hsp60 (a mitochondrial protein) or topoisomerase I (a nuclear protein) using their specific antibodies.

2.16.2 Resolving Proteins by SDS-PAGE

Materials

i. Separating Gel: 12.5%
ii. Stacking Gel: 5%
iii. RUNNING Buffer pH 8.3
iv. 10% SDS
v. 30% Acryl amide and Bis Acryl amide
vi. 10% APS
vii. 0.1% BMB (Tracking dye)
viii. Commassie Stain
ix. TEMED
x. Sample Loading Buffer

Procedure

Prepare polyacrylamide gel according to standard protocol. Load samples and run gel at 25 mA (2 gels run at 50 mA) in 1X SDS Running Buffer. [At this point, the gel can either be transferred to a membrane (see Western protocol) or stained with Coomassie (see below)].

2.16.3 Transfer to Nitrocellulose Membrane

Materials

i. 50 mM sodium phosphate buffer (pH:7.5)
ii. Whatman no. 1 filter paper
iii. Scotch-Brite pad and
iv. Cathode grid

Procedure

Proteins resolved by SDS-PAGE were electrophoretically transferred to an NCM by using a modification of the procedure (Towbin et al.) for SDS-containing gels. A sandwich was constructed consisting sequentially of anode grid, Scotch-Brite pad, sheet of Whatman no. 1 filter paper, NCM, polyacrylamide slab gel, sheet of Whatman no. 1 filter paper, Scotch-Brite pad, and cathode grid. The sandwich was placed in an electrophoric destainer (E-C Apparatus Corp., St. Petersburg, Fla.) filled with 50 mM sodium phosphate buffer (pH 7.5)

that had been deaerated under reduced pressure for 12 h. Electrophoresis was at 6 V/cm for 2 hr at ambient room temperature.

2.16.4 Western Blotting

Materials

i. Transfer Buffer
ii. 1X SDS Running Buffer in 20% Methanol
iii. 1X PBS/0.1% Tween 20
iv. Blotting Buffer, stored at $4^{o}C$
v. 5% milk in 1X PBS/0.1% Tween 20

Procedure

Western blotting was performed accordingly to the ECL protocol provided by the suppliers (Amersham Biosciences) using specific antibodies. Briefly, Protein samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline containing 0.05% (w/v) Tween 20. After O/N blocking the blot was washed 3 times with distilled water for 10 minutes. Ten ml of the primary antibody solution (anti-cytochrome C and anti-$H2AX^{P}$) was then added to the membrane and kept in a $4^{o}C$ Shaker for O/N. After O/N incubation the blot was washed 3 times (10min/wash) with 1X TBST. Then 10 ml of secondary antibody solution was added to each blot and incubated at RT on a shaker. After that the blot was washed again for 3 times with 1X TBST (10 min/wash). Proteins were visualized using the ECL system (Amersham).

2.17 Role of Small Plasmid in the Pathogenesis of 140MDa Lacking *S. flexneri* 1c

2.17.1 Curing of 140MDa Plasmid of *S. flexneri* 1c (K-212)

Materials

i. Acridine orange (Sigma, St Louis, MO)
ii. Trypticase soy agar (TSA) (Difco) plate
iii. 37°C incubator
iv. API 20E

Procedure

The minimal inhibitory concentration (MIC) and subinhibitory concentration of acridine orange were determined by the agar dilution method (Nagshetty *et al*., 2010). Plasmid curing was performed as described previously (Miller, 1972). *S. flexneri* 1c (K-212) strain was grown in TSB in the presence of acridine orange (Sigma, St Louis, MO) at different concentrations (20, 40, 60, 80, 100 and 120µg/mL) for 24 h at 37°C and then plated on trypticase soy agar (TSA) (Difco). Physical loss of the plasmid in the cured derivative was confirmed by agarose gel electrophoresis following extraction of the plasmid DNA from the respective cultures. The percentage curing efficiency was expressed as the number of colonies with cured phenotype per 100 colonies tested. Plasmid-cured strains were confirmed by the API 20E system and serology. Plasmid cured strains were tested for cytotoxicity in HeLa cell monolayer.

2.17.2 Transformation of Small Plasmid from *S. flexneri* 1c (K-314) into *E. coli* Bl-21

2.17.2.1 Competent Cell Preparation

Reagents

i 0.01 M $CaCl_2$

ii Ampicillin

Procedure

An isolated colony of *E. coli* BL21 was inoculated into 4 ml of LB broth and incubated at 37˚C for overnight. After that 100ml fresh LB medium was inoculated with 1ml of saturated overnight culture on a water bath for 2 to 3 hours. Then the culture was placed in an ice bath for 10 minutes. 2ml culture was taken into polypropylene microcentrifuge tube then centrifuge at 5000rpm for 5 min at 4˚C. Supernatant was decanted and pellet was suspended in 1 ml 0.1M $CaCl_2$ and was incubated on ice for 40 min. Then centrifugation was carried out at 5000 rpm for 5 min and the supernatant were removed and the pellet was resuspended in 1 ml 0.1M $CaCl_2$ solution. 200 µl of this solution was aliqouted in Eppendorf tube and kept on -20˚C for further use.

2.17.2.2 Isolation of Plasmid DNA for bacterial transformation

Reagents

i. 50mM glucose
ii. EDTA
iii. Tris-HCl (pH 8.0)
iv. 0.2 N NaOH
v. 1% sodium dodecyl sulfate (SDS)
vi. 3M NaOAc (pH 4.8)

Procedure

Small scale preparation of *E. coli* plasmid DNA was performing using a modification of the alkaline extraction method of Birn-boim and Dolly (1979). A 1.5ml aliqoute of culture grown to approximately 10^9 cells was collected and suspended in 100µl of a solution containing 50mM glucose, 10mM EDTA, 25mM Tris-HCl (pH 8.0) and 4mg/ml lysozyme, and placed on ice for 2-3 min. two hundered µl of a second solution containing 0.2 N NaOH and 1% sodium dodecyl sulfate (SDS) were added, mixed gently and kept on ice for 5 min. immediately after 5 min, 150µl of 3M NaOAc (pH 4.8) was added, mixed gently and kept on ice for 5 min. The chromosomal DNA with insoluble materials were pelleted down by centrifugation at 15,000 rpm for 5 min at room temperature, the clear supernatant was collected and the plasmid DNA was precipitated by adding 1 ml of 95%(v/v) ethanol. After centrifugation (at 15,000 rpm for 3 min) the precipitate was suspended in 250 µl of washing buffer [(50mM Tris-HCl (pH 8.0), 100mM NaOAc(pH 4.8)] and again precipitated with 1ml of ethanol. The plasmid DNA collected by centrifugation was rinsed with 70% ethanol and dried. The dried sample was suspended in 40 µl of TE [(10mM Tris-HCl(pH 8.0), 1mM EDTA(pH 8.0)] and kept at 4˚C.

2.17.2.3 Transformation

Materials

i. Luria Broth (LB) at 37ºC
ii. Selective LB plates containing 50 µg of ampicillin/ml
iii. Marker plasmid (pUC 18)

Procedure

200 µl of competent cell suspension was taken into the two different eppendorf tubes then 20µl of plasmid DNA (K-314) was added in one tube and 1µl of plasmid DNA (pUC 18) in another tube. Tubes were kept on ice for 30 min with mild mixing then heat shock was given at 42˚C for 45 seconds. Tubes were then kept in room temperature for 10 minutes. 1 mL LB broth was then added and incubated the cells at 37ºC with mild shaking for 1 hour min for the expression of antibiotic resistance maker (β-lactamase) encoded by the plasmid. Then 25µl and 100 µl of above suspension were spread on LB plates containing ampicillin (50µg/ml). The plates were incubated overnight at 37ºC.

Table 2.3 Amount of Plasmid DNA used for transformation assay

Tube	Competent cell	Plasmid (K-314)	pUC 18 Marker Plasmid
1	200µl	20 µl	0 µl
2	200µl	0 µl	20 µl
3	200µl	0 µl	0 µl

2.17.2.4 Co-Transfection

Materials

i. Competent cells
ii. PUC 18 marker plasmid
iii. K-314 plasmids
iv. LA+Amp plate
v. LB plate
vi. Ice
vii. 42˚C water bath
viii. 100% ethanol

Procedure

DNA (20 µl K-314 plasmid + 1µl pUC 18, 20µl K-314 + 10 µl pUC 18, 20 µl K-314 + 0µl pUC 18 and 0 µl K-314 plasmid + 1µl pUC 18) was added in five different tubes containing competent cell separately and one control was used having only the competent cell. Then tubes were kept on ice for 30 minutes after that heat shock was given at 42˚C for 30 seconds. Tubes were then kept at room temperature for 10 minutes. 1 mL LB was then added and

incubated for 1 hour at 37˚C with shacking. Centrifugation was carried out at 7000 rpm for 5 minutes, then 1 mL supernatant was removed and the pellet was dissolved in the remaining. Selective plating was done (25µl and 100 µl in LB + Amp plate).

Table 2.4 Amount of Plasmid DNA used for co-transfection assay

Tube	Competent cell	Plasmid (K-314)	pUC 18 Marker Plasmid
1	200µl	20 µl	0 µl
2	200µl	20 µl	1 µl
3	200µl	20 µl	10 µl
4	200µl	0 µl	1 µl
5	200µl	0 µl	0 µl

2.17.3 Restriction Digestion of the Transformed Plasmid

Reagents

i. 100X BSA
ii. Restriction endonuclease Hind III
iii. Agarose
iv. TBE buffer

Procedure

Restriction endonuclease analysis was performed according to the method described by Maniatis *et al*., 1982. Plasmid DNA (500-1000 ng) was suspended in 50 µl of reaction mixer containing 5 µl of 10X buffer 2, 0.5 µl of 100X BSA, and 1 µl of Hind III. Volume of the reaction mixture was adjusted by adding filtered de-ionized water (43.5µl). After 4hr incubation at 37˚C waterbath, the sample were loaded onto a horizontal agarose slab gel (1%) in a TBE buffer, and electrophoresed at 100mA for 5-6h.

2.18 Characterization of the 140MDa lacking *S. flexneri* 1c strains

2.18.1 Keratoconjunctivitis assay (Sereny test)

The Sereny test was performed by a procedure described elsewhere (Sereny *et al*., 1957; Mackel *et al*., 1961). Briefly, an overnight culture of bacteria, suspended to a density of

approximately 10^{10} viable cells in 20 µl of phosphatebuffered saline, was dropped into the conjunctival sacs of guinea pigs. One eye served as the control. The guinea pigs were observed daily for 72 h, and their inflammatory responses were graded.

2.18.2 Determination of Congo red binding ability

TSBY with 1.5% agar and 0.01% Congo red (Sigma Chemical Co. Ltd.) was used to study the pigment binding abilities of the test strains by previously described procedures (Sakai *et al*., 1986; Sasakawa *et al*., 1986).

2.18.3 Isolation of plasmid DNA

Plasmid DNA was prepared by the alkaline lysis method of Kado and Liu (Kado and Liu, 1981), with some modifications as indicated previously (Talukder *et al*., 2002). The molecular weight of the unknown plasmid DNA was assessed by comparing with the mobilities of the plasmids of known molecular weights (Haider *et al*., 1989). The plasmids present in previously described strains *E. coli* PDK-9, R1, RP4, Sa, and V-517 (Talukder *et al*., 2002) were used as molecular weight standards.

2.18.4 Pulsed -Field Gel Electrophoresis

Intact agarose-embedded chromosomal DNA from *Shigella* was prepared and PFGE was performed using the contour-clamped homogeneous electric field (CHEF-Mapper) apparatus from Bio-Rad Laboratories (Richmond, CA, USA) according to the procedures described elsewhere (Pichel *et al*., 2012). Genomic DNA was digested with *Xba*I restriction enzyme (Gibco-BRL). The restriction fragments were separated by using CHEF-mapper system apparatus in 1% pulsed-field certified agarose in 0.5 × TBE buffer. The DNA size standards used was the *Salmonella enterica* serovar Braenderup (H9812) ranging from 20.5 to 1,135 kb (Hunter *et al*., 2005). Banding patterns were analyzed according to the established criteria reported elsewhere (Tenover *et al*., 1995) Analysis of the TIFF images was carried out by the BioNumerics software package (Applied Maths, Belgium) using the Dice coefficient and unweighted pair group method with arithmetic means (UPGMA) to generate dendrograms with 1.5% tolerance values.

2.19 Statistical analysis

Results are presented as Standard Error Mean (SEM). Student's t-test was used to test the null hypothesis that there is no significant difference between each individual parameter measured in the control and treatment groups over time. Difference was considered to be significant if $p \leq 0.05$. Statistical calculations were performed using GraphPad Software (SanDiego, CA).

3.0 Result

3.1 Plasmid Profile Analysis

Analysis of plasmid DNA of 385 *Shigella* spp. showed in Table 3.1 revealed that all the strains irrespective of serotype contained a heterogeneous population of plasmids ranging between 140 and 0.8 MDa. The 140-MDa plasmid carrying the virulence marker was present in all the strains. Based on molecular weight, the pattern of different plasmids at the serotype level was also very diverse (Table 3.1). A number of small plasmids were also found to be present universally all the strains of a particular serotypes of *S. dysenteriae* (Table 3.1). Plasmids size of 140 MDa, 2.7 MDa, 1.8 MDa and 1.4 MDa were present in all strains of serotype 3, 6, 9, 11 and 12 whereas serotype 2 and 4 contained 140 MDa, 4 MDa and 1.6 MDa plasmids. All *S. dysenteriae* 1 strains contained the 140 MDa, 6 MDa and 2 MDa plasmids. Except for *S. boydii* 6, 7, 16 and 17 most of the serotypes revealed multiple plasmids with one or more pattern each, whose sizes ranging from 140 MDa to 1 MDa is shown in Table 3.1. Almost all the strains contained 140 MDa invasive plasmid and the correlation between serotypes and plasmid patterns for the different *S. boydii* were shown in Table 3.1. In case of *S. sonnei* heterogeneous plasmid patterns ranging in size from approximately 120 to 1.0 MDa was found by analyzing plasmid DNA. Forty-six percent of *S. sonnei* strains harboured the 120 MDa invasive plasmid. A number of small plasmids of ~5, ~1.8 and ~1.4 MDa in size were also found in more than 92% of the strains and were considered to be the core plasmids of *S. sonnei*. Plasmid analysis showed that almost all serotype of *S. flexneri*, except certain strains of *S. flexneri* 1c (n=17) contained the 140MDa plasmid (Figure 3.1). The small plasmid of size between 2.8MDa and 2.6MDa was present in all *S. flexneri* serotypes. The plasmid pattern of the *S. flexneri* 1c showed that 88% of the serotype 1c strains harbored the 140, 2.8, 2.1, 1.8, and 1.0 MDa plasmid. Most of the serotype 1c strains (72%) contained the 1.6MDa plasmid. Similarly, 22% of serotype 1c strains contained the 4MDa plasmid. Beside these small plasmids, a middle-range plasmid (90 to 35 MDa) was found in 20% of serotype 1c strains. The representative plasmid pattern of *S. flexneri* 1c showed in the Figure 3.1.

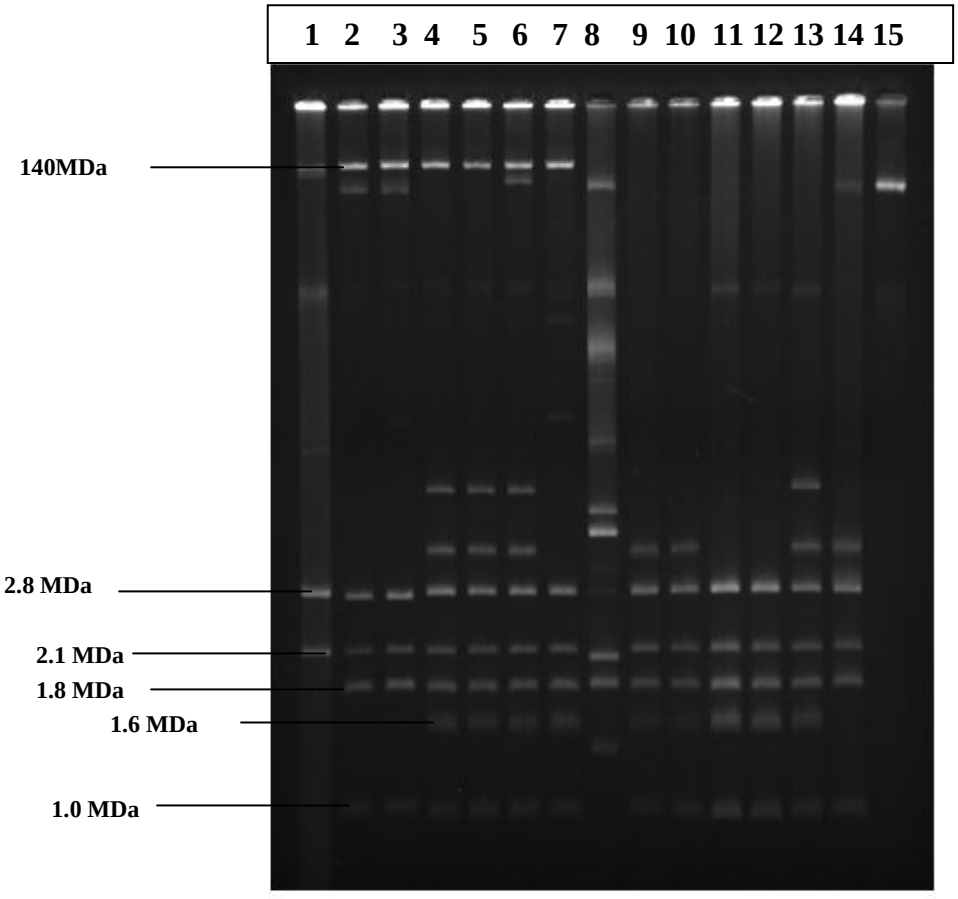


Figure 3.1 Plasmid analysis of *S. flexneri* 1c strains; Plasmid DNA was isolated from different strains of *S. flexneri* 1c strains. Plasmid DNA was characterized by agarose gel electrophoresis. Lane 1, *E. coli* PDK-9; Lane 2, *S. flexneri* 1c K-911; Lane 3, *S. flexneri* 1c K-212; Lane 4, *S. flexneri* 1c K-1362; Lane 5, *S. flexneri* 1c K-1622; Lane 6, *S. flexneri* 1c K-1614; Lane 7, *S. flexneri* 1c K-923; Lane 8, *E. coli* V-517; Lane 9, *S. flexneri* 1c K-314; Lane 10, *S. flexneri* 1c K-1387; Lane 11, *S. flexneri* 1c K-1776; Lane 12, *S. flexneri* 1c K-915; Lane 13, *S. flexneri* 1c K-961; Lane 14, *S. flexneri* 1c K-9878; Lane 15, *E. coli* R_1. Expected position of 140 MDa, 2.7MDa, 2.1MDa, 1.8MDa, 1.6MDa and 1.0MDa plasmid DNA is shown by an arrow.

3.2 Detection of *Shigella E*nterotoxin (*set, sen, stx*1 *and stx*2) and Virulence (*ipa*H, *ial* and *ipa*BCD) Ggenes by PCR Assays

The randomly selected 385 *Shigella* strains were analyzed for the presence of virulance and toxin genes such as *ipa*H, *ial*, *ipa*BCD *set*, *sen*, stx_1 and stx_2 commonly present in *Shigella* by PCR. PCR analysis showed that all serotypes of *Shigella* contain the *ipa*H gene. *Shigella* enterotoxin 2 (*sen*), *ial*, *ipa*BCD genes (located in140 MDa plasmid) were present in all strains except 17 *S. flexneri* 1c strains lacking the 140MDa plasmid. All *S. dysenteriae* 1 contained stx_1 gene whereas the *Shigella* enterotoxin 1 gene (*set*) was only present mostly in *S. flexneri* 2a and rarely in other serotypes (Figure 3.2). The *Shigella* enterotoxin 1 (*set*1) gene was not present in the strains of *Shigella sonnei* tested, whereas the *Shigella* enterotoxin 2 gene (*sen*) was present in all strains containing a 120 MDa plasmid. Seventeen 140 MDa lacking *S. flexneri* 1c strains were also negative for the common toxin and virulence genes. Therefore, these *S. flexneri* 1c strains (devoid of all toxin genes) were extensively used for comparative analysis and partially purified toxin fractions from the representative strains of *S. flexneri* 1c (K-314 and K-915) were used as a source of novel toxic factor(s) in this study.

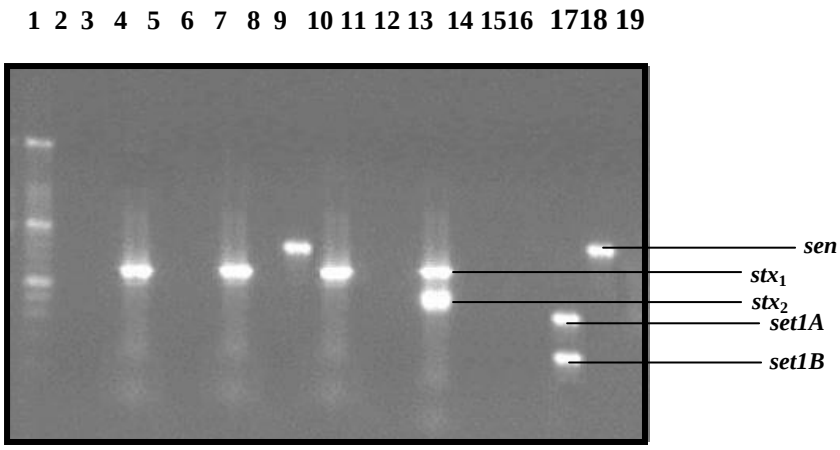


Figure 3.2 Identification of bacterial toxin genes by PCR. Expected positions of PCR product of *set1A, set1B, sen, stx*1 and *stx*2, are shown by arrows. Lane1, Marker 100 bp ladder; Lane 2, *S flexneri* 1c (K-314;140 MDa plasmid less); Lane 3, *S flexneri* 1c (K-899;140 MDa plasmid less); Lane 4, *S. dysenteriae* 1 (KO-220;140 MDa plasmid less); Lane 5, *S flexneri* 1c (K-915;140 MDa plasmid less); Lane 6, *S flexneri* 1c (K-211;140 MDa plasmid less); Lane7, *S. dysenteriae* 1 (KO-234;140 MDa plasmid less); Lane 8, *S flexneri* 1c (K-1036;140 MDa plasmid less); Lane 9, *S. flexneri* 1c (K-212;140 MDa plasmid containing); Lane10, *S. dysenteriae* 1 (K-573; 140 MDa plasmid containing); Lane 11,12&13; VTEC-3, Lane 14,15 &16, MC 1061; Lane 17,18 &19: YSH-6000 *S. flexneri* 2a.

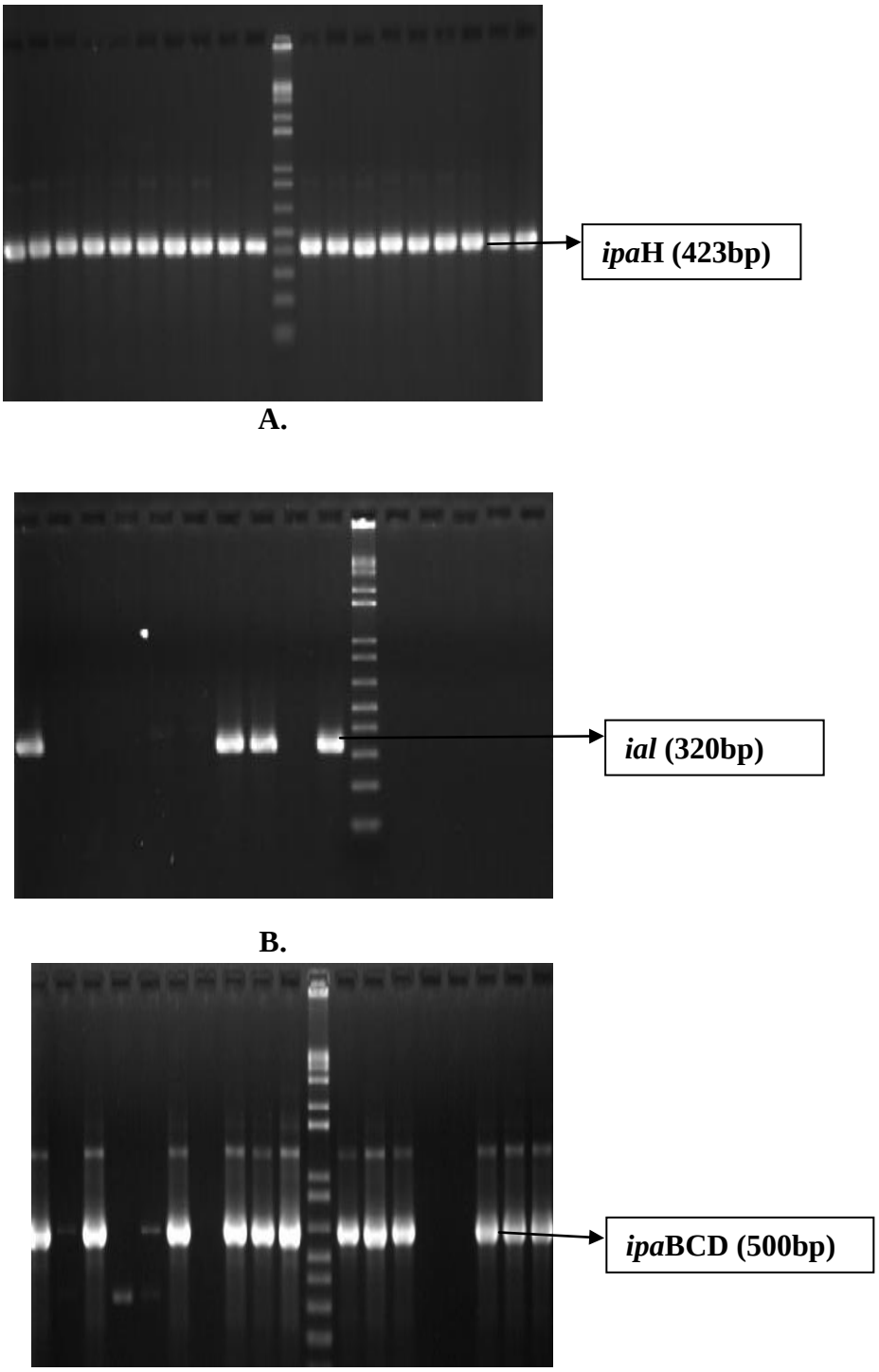


Figure 3.3 Gel electrophoresis of *ipa*H, *ial* and *ipa*BCD PCR products of representative *Shigella* strains. A. Gel electrophoresis of *ipa*H, **B.** Gel electrophoresis of *ial* and **C.** Gel electrophoresis of *ipa*BCD, PCR products. Expected positions of PCR product of *ipa*H, *ial* and *ipa*BCD shown by arrows.

Table 3.1 Summary of Plasmid and PCR Based Identification of Virulence and Toxin genes in *Shigella* spp (n=385)

Shigella serotype	Number of strain tested	Plasmid pattern	Plasmid	Presence of virulence and toxin genes							Cytotoxic effect (% cell death)
				*ipa*H	*ial*	*ipa*BCD	*set*	*sen*	*stx*1	*stx*2	
***S. dysenteriae* 1**	15	P1(n=6)	140, 6, 2	+	+	+	-	+	+	-	95
		P2(n=5)	140, 35-90, 6, 2								
		P3(n=2)	140, 6, 4, 2								
		P4(n=2)	140, 35-90, 6, 4, 2								
S. dysenteriae* 1	5	P1(n=2)	6, 2	-	-	+	-	-	+	-	95
		P2(n=2)	6, 4, 2								
		P3(n=1)	35-90, 6, 2								
***S. dysenteriae* 2**	10	P1(n=3)	140, 4, 1.6	+	+	+	-	+	-	-	45
		P2(n=3)	140, 35-90, 4, 1.6								
		P3(n=2)	140, 1.6								
		P4(n=1)	140, 35-90, 1.6								
		P5(n=1)	140, 35-90, 4, 3.2, 1.6								
***S. dysenteriae* 3**	4	P1(n=2)	140, 2.7, 1.8, 1.4	+	+	+	-	+	-	-	40
		P2(n=2)	140, 35-90, 2.7, 1.8, 1.4								
***S. dysenteriae* 4**	15	P1(n=9)	140, 35-90, 4, 1.6	+	+	+	-	+	-	-	90
		P2(n=6)	140, 4, 1.6								
***S. dysenteriae* 6**	3	P1(n=1)	140, 2.7, 1.8, 1.4	+	+	+	-	+	-	-	35
		P2(n=1)	140, 35-90, 2.7, 1.8, 1.4								
		P3(n=1)	140, 2.7, 1.4								
***S. dysenteriae* 9**	7	P1(n=4)	140, 2.7, 1.8, 1.4, 1.2	+	+	+	-	+	-	-	35
		P2(n=3)	140, 35-90, 2.7, 1.8, 1.4, 1.2								
***S. dysenteriae* 11**	6	P1(n=3)	140, 4, 2.7, 1.8, 1.4, 1.0	+	+	+	-	+	-	-	40
		P2(n=2)	140, 3-90, 4, 2.7, 1.8, 1.4, 1.0								
		P3(n=1)	140, 4, 2.7, 1.4, 1.0								
***S. dysenteriae* 12**	5	P1(n=2)	140, 2.7, 1.8, 1.4	+	+	+	-	+	-	-	45
		P2(n=2)	140, 35-90, 2.7, 1.8, 1.4								
		P3(n=1)	140, 2.7, 1.4								
Novel *S. dysenteriae* (KIVI-162)	10	P1(n=10)	140, 2.7, 2.0	+	+	+	-	+	-	-	40

Result

Shigella serotype	Number of strain tested	Plasmid pattern	Plasmid	Presence of virulence and toxin genes							Cytotoxic effect (% cell death)
				*ipa*H	*ial*	*ipa*BCD	*set*	*sen*	*stx*1	*stx*2	
***S. flexneri* 1a**	7	P1(n=4)	140, 3.4, 2.8, 2.1, 1.8, 1.0	+	+	+	-	+	-	-	85
		P2(n=3)	140, 35-90, 2.8, 2.1, 1.8, 1.0								
***S. flexneri* 1b**	15	P1(n=9)	140, 35-90, 3.4, 2.8, 2.1, 1.8, 1.0	+	+	+	-	+	-	-	85
		P2(n=6)	140, 35-90, 2.8, 2.1, 1.8, 1.0								
***S. flexneri* 1c**	56	P1(n=22)	140, 35-90, 3.4, 2.8, 2.1, 1.8, 1.0	+	+	+	-	+	-	-	90
		P2(n=12)	140, 35-90, 2.8, 2.1, 1.8, 1.0								
		P3(n=9)	140, 3.4, 2.8, 2.1, 1.8, 1.0								
		P4(n=6)	140, 4, 3.4, 2.8, 2.1, 1.8, 1.6, 1.0								
		P5(n=4)	140, 35-90, 2.8, 2.1, 1.8, 1.0								
		P6(n=3)	140, 2.8, 2.1, 1.8, 1.6, 1.0								
S. flexneri* 1c	17	P1(n=5)	35-90, 3.4, 2.8, 2.1, 1.8, 1.0	+	-	-	-	-	-	-	90
		P2(n=3)	35-90, 2.8, 2.1, 1.8, 1.0								
		P3(n=3)	3.4, 2.8, 2.1, 1.8, 1.0								
		P4(n=3)	2.8, 2.1, 1.8, 1.0								
		P5(n=2)	3.4, 2.8, 2.1, 1.8, 1.0								
		P6(n=1)	3.4, 2.8, 2.1, 1.8, 1.6, 1.0								
***S. flexneri* 2a**	35	P1(n=27)	140, 2.7, 2.1	+	+	+	+	+	-	-	80
		P2(n=8)	140, 35-90, 2.7, 2.1								
***S. flexneri* 2b**	21	P1(n=13)	140, 2.7, 2.1	+	+	+	+/-	+	-	-	75
		P2(n=8)	140, 35-90, 2.7, 2.1								
***S. flexneri* 3a**	12	P1(n=7)	140, 3.4, 2.6, 2.0	+	+	+	-	+	-	-	55
		P2(n=5)	140, 35-90, 3.4, 2.6, 2.0								
***S. flexneri* Type 4**	14	P1(n=7)	140, 5.9, 4, 2.7	+	+	+	-	+	-	-	75
		P2(n=4)	140, 35-62, 5.9, 4, 2.7								
		P3(n=3)	140, 5.9, 4, 2.7, 0.7								

Result

Shigella serotype	Number of strain tested	Plasmid pattern	Plasmid	Presence of virulence and toxin genes							Cytotoxic effect (% cell death)
				*ipa*H	*ial*	*ipa*BCD	*set*	*sen*	*stx*1	*stx*2	
***S. flexneri* 4X**	15	P1(n=11)	140, 35-90, 4.8, 2.6, 1.8, 1.6	+	+	+	-	+	-	-	50
		P2(n=4)	140, 35-90, 4.8, 2.6, 1.6								
***S. flexneri* X var**	7	P1(n=7)	140, 3.4, 2.7, 2.1	+	+	+	-	+	-	-	75
***S. flexneri* Y var**	16	P1(n=11)	140, 2.6, 1.8, 1.6	+	+	+	-	+	-	-	40
		P2(n=5)	140, 35-90, 2.6, 1.8, 1.6								
***S. flexneri* 6a**	13	P1(n=4)	140,2.6,1.8	+	+	+	-	+	-	-	45
		P2(n=4)	140,35-90, 2.6,1.8								
		P3(n=3)	140, 2.6,1.8, 1.6								
		P4(n=2)	140,35-90, 2.6,1.8, 1.6								
***S. flexneri* 6b**	10	P1(n=4)	140, 2.6, 1.6	+	+	+	-	+	-	-	50
		P2(n=2)	140, 35-90, 2.6, 1.6								
***S. boydii* 1**	3	P1(n=2)	140, 3.4,2.7, 1.4	+	+	+	-	+	-	-	50
		P2(n=1)	140, 35-90, 3.4,2.7, 1.4								
***S. boydii* 2**	2	P1(n=1)	140, 3.6, 2.7, 1.8	+	+	+	-	+	-	-	50
		P2(n=1)	140, 35-90, 3.6, 2.7, 1.8								
***S. boydii* 3**	2	P1(n=2)	140, 4, 3.1, 1.4, 1.0	+	+	+	-	+	-	-	30
***S. boydii* 4**	3	P1(n=2)	140, 4, 2.7, 1.8	+	+	+	-	+	-	-	45
		P2(n=1)	140, 35-90, 4, 2.7, 1.8								
***S. boydii* 5**	2	P1(n=2)	140, 6, 1	+	+	+	-	+	-	-	40
***S. boydii* 8**	3	P1(n=1)	140, 3.4, 2.7	+	+	+	-	+	-	-	55
		P2(n=1)	140, 35-90, 3.4, 2.7								
		P3(n=1)	140, 35-90, 2.7								
***S. boydii* 9**	2	P1(n=2)	140, 4.8, 4.0, 3.4, 2.5, 1.4	+	+	+	-	+	-	-	30
***S. boydii* 10**	2	P1(n=1)	140, 5, 2.7, 1.8, 1.4	+	+	+	-	+	-	-	50
		P2(n=1)	140, 35-90, 5, 2.7, 1.8, 1.4								
***S. boydii* 11**	3	P1(n=1)	140, 4.8, 2.7	+	+	+	-	+	-	-	45
		P2(n=1)	140, 35-90, 4.8, 2.7								
		P3(n=1)	140, 35-90, 2.7								

Shigella serotype	Number of strain tested	Plasmid pattern	Plasmid	Presence of virulence and toxin genes							Cytotoxic effect (% cell death)
				*ipa*H	*ial*	*ipa*BCD	*set*	*sen*	*stx*1	*stx*2	
***S. boydii* 12**	4	P1(n=2)	140, 2.7, 2.1, 1.8	+	+	+	-	+	-	-	50
		P2(n=1)	140, 35-90, 2.7, 2.1, 1.8								
		P3(n=1)	140, 2.7, 2.1,								
***S. boydii* 13**	2	P1(n=1)	140, 3.6, 3.4, 1.4, 1	+	+	+	-	+	-	-	45
		P2(n=1)	140, 35-90, 3.6, 3.4, 1.4, 1								
***S. boydii* 14**	3	P1(n=2)	140, 4, 3.4, 1.6,1.4	+	+	+	-	+	-	-	45
		P2(n=1)	140, 35-90, 4, 3.4, 1.6,1.4								
***S. boydii* 15**	2	P1(n=1)	140, 1.8	+	+	+	-	+	-	-	30
		P2(n=1)	140,35-90, 1.8								
***S. boydii* 18**	2	P1(n=2)	140, 3.7, 3.4, 2.7, 1.7	+	+	+	-	+	-	-	50
***S. boydii* 20**	7	P1(n=7)	140, 3.4, 2.7, 2.1, 1.8	+	+	+	-	+	-	-	50
S. sonnei	15	P1(n=15)	120, 5, 1.8, 1.4	+	+	+	-	+	-	-	35
S. sonnei	10	P2(n=10)	5, 1.8, 1.4	+	-	+	-	-	-	-	35

Note: *S. flexneri* 1c* strains (devoid of all toxin genes) were extensively used for comparative analysis and partially purified toxin fractions from the representative strains of *S. flexneri* 1c (K-314 and K-915) were used as a source of novel toxic factor(s) in this study.

3.3 Tests for Invasiveness

With the exception of 17 *S. flexneri* 1c strains, all strains harbored the 140-MDa invasive plasmid, had the ability to bind to Congo red and showed keratoconjunctivitis in guinea pig eyes (Figure 3.4) by representative strains, attesting to their invasive trait. Representative strains of *S. sonnei* containing a 120 MDa invasive plasmid were positive for keratoconjunctivitis in the guinea pig eye. The macroscopic changes in guinea pig's eye for the characteristic of keratoconjunctivitis were observed in all the strains, same as the positive control (*S. flexneri* 2a YSH 6000), while the strain *E. coli* (MC-1061) without 140 MDa showed negative result (Figure 3.4). Redness and swelling of pulpebra, hyperemic bulber conjunctiva, and bulbopulpebra adhesion were commonly observed in the positive samples. The inflammatory changes were culminated at 18 to 24 h after inoculation for all the strains as with the positive control and disappeared within 7 days. Seventeen *S. flexneri* serotype 1c strains were noninvasive by the Sereny test and did not contain the 140 MDa plasmid, nor did they bind to Congo red.

(A) Keratoconjunctivitis negative **(B) Keratoconjunctivitis positive**

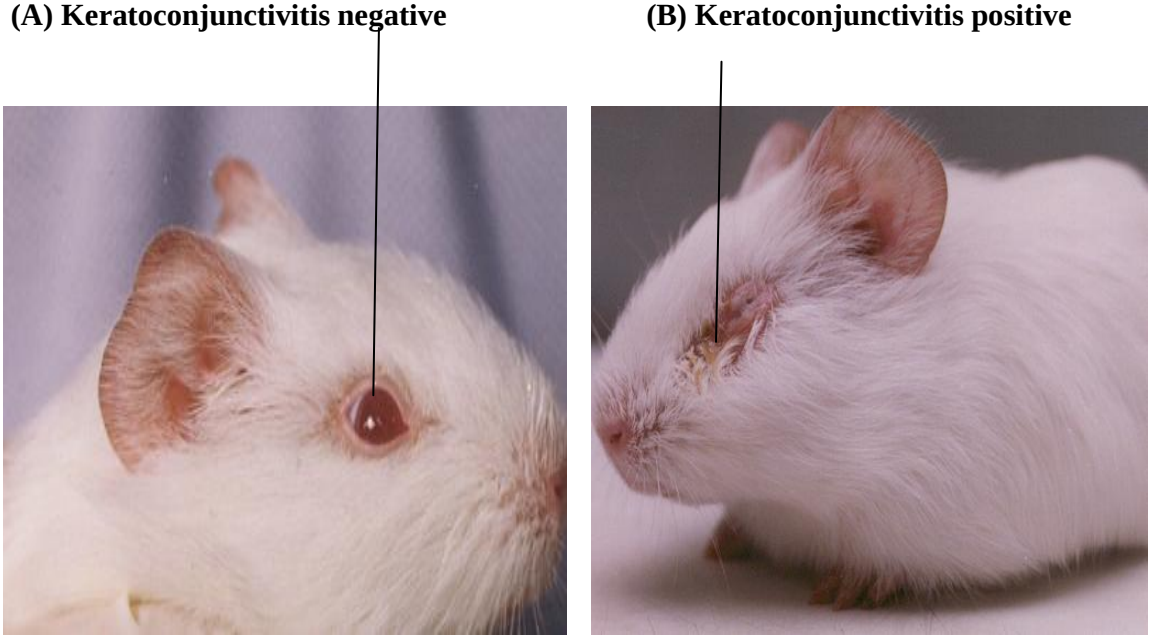


Figure 3.4 Guinea pig eyes showing the characteristics of keratoconjunctivitis. The Sereny test was performed by a procedure described elsewhere (Sasakawa, 1986). Briefly, an overnight culture of bacteria, suspended to a density of approximately 10^{10} viable cells in 20 µl of phosphate-buffered saline, was dropped into the conjunctival sacs of guinea pigs. One eye served as the control. The guinea pigs were observed daily for 72 h, and their inflammatory responses were graded.

3.4 Detection of Genes for TTSS

Seven different virulence genes (*mxi*C, *mxi*A1, *mxi*A2, *ipa*BCD, *ipa*C, *ipa*D, *vir*A) responsible for TTSS in *Shigella* Spp were detected by PCR analysis (Figure 3.5).Of these, most prevalent gene was *vir*A (79%) followed by *ipa*BCD (78%), *mxi*A2 (49%), *mxi*A1 (66%), *mxi*C (59%), *ipa*C (59%) and *ipa*D (59%). The essential genes for TTSS were not found in the 140 MDa plasmid lacking *S. flexneri* 1c (n=17) strains.

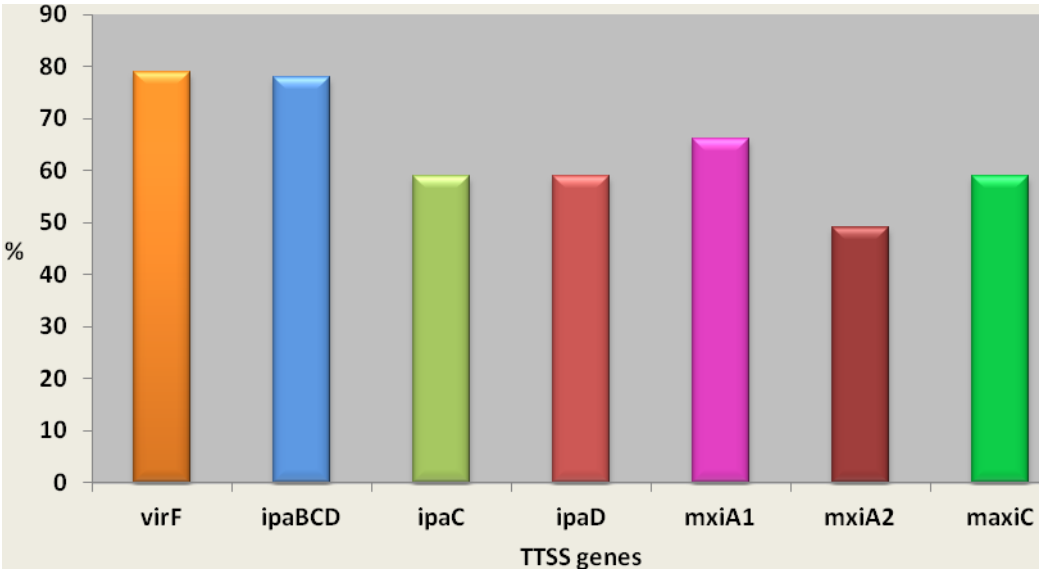


Figure 3.5 Prevalence of different TTSS genes among representative *Shigella* strains. These essential genes responsible for *Shigella* pathogenesis were present in all *Shigella* strains containing 140MDa plasmid whereas negative in 140MDa lacking 17 *S. flexneri* 1c strains.

3.5 Cytotoxic Activity in HeLa Cells

Concentrated culture supernatant and cell lysate of different serotypes of *Shigella* strains were assayed in HeLa cells for the cellular cytotoxic potential. The degree of cytotoxicity differed in different species of *Shigella*. Around 72% (172/238) strains of *S. flexneri* possessed the cytotoxic activity whether they contain the *set*, *sen* or *stx* genes or not. (Figure3.6). Of *S. flexneri*, serotype 1c had severe effect (more than 85% cell death) on HeLa cell followed by serotype 1b and 1a (>85% cell death). Serotype 2a, 2b, X variant and type 4 had almost similar cytotoxic effect (>70% cell death). The culture supernatant from serotype 3a, Y variant, 4X, 6a and 6b showed less cytotoxic effect (40-45% cell death). In case of *S. dysenteriae* (n=80), only serotype 1 and some strains of serotype 4 showed cytotoxic effect (>90% cell death). In contrast, *S. boydii* (n=42) supernatant caused elongation of the HeLa cells whereas *S. sonnei* (n=25) caused the rounding of the cell with less frequency. Cytotoxic activity was determined by studying the morphological changes (rounding) under an inverted microscope and and later determined by nuclear condensation assay. It is interesting to note that the 17 *S. flexneri* 1c strains those were negative for the 140 MDa plasmid and toxin gene showed strong cytotoxic effect (>90%) on HeLa cells (Figure3.6). Each experiment was repeated more than three times showing similar results. Results indicated significant difference of control and treatment group $p \leq 0.0005$.

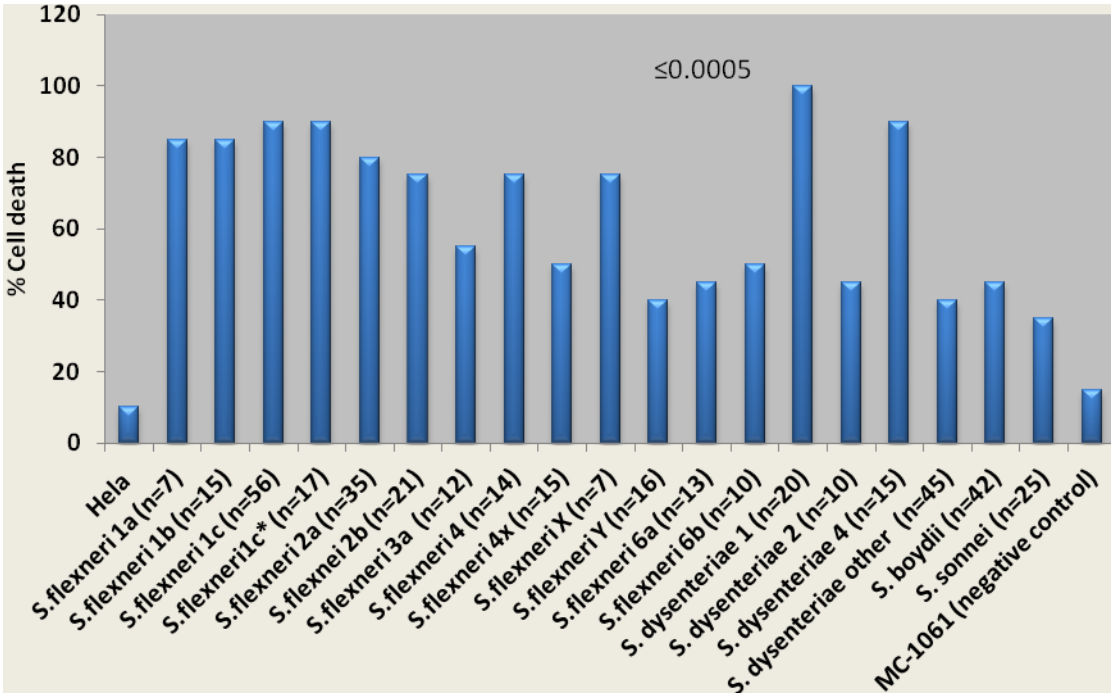


Figure 3.6 Comparative cytotoxic effects of different serotypes of *Shigella* on HeLa cells. Cytotoxic activity was determined by studying the morphological changes (rounding, shrinkage and elongation) of the HeLa cells under an inverted microscope after 48h incubation and later determined by nuclear condensation assay. Results indicate significant difference of control and treatment group $p \leq 0.0005$.

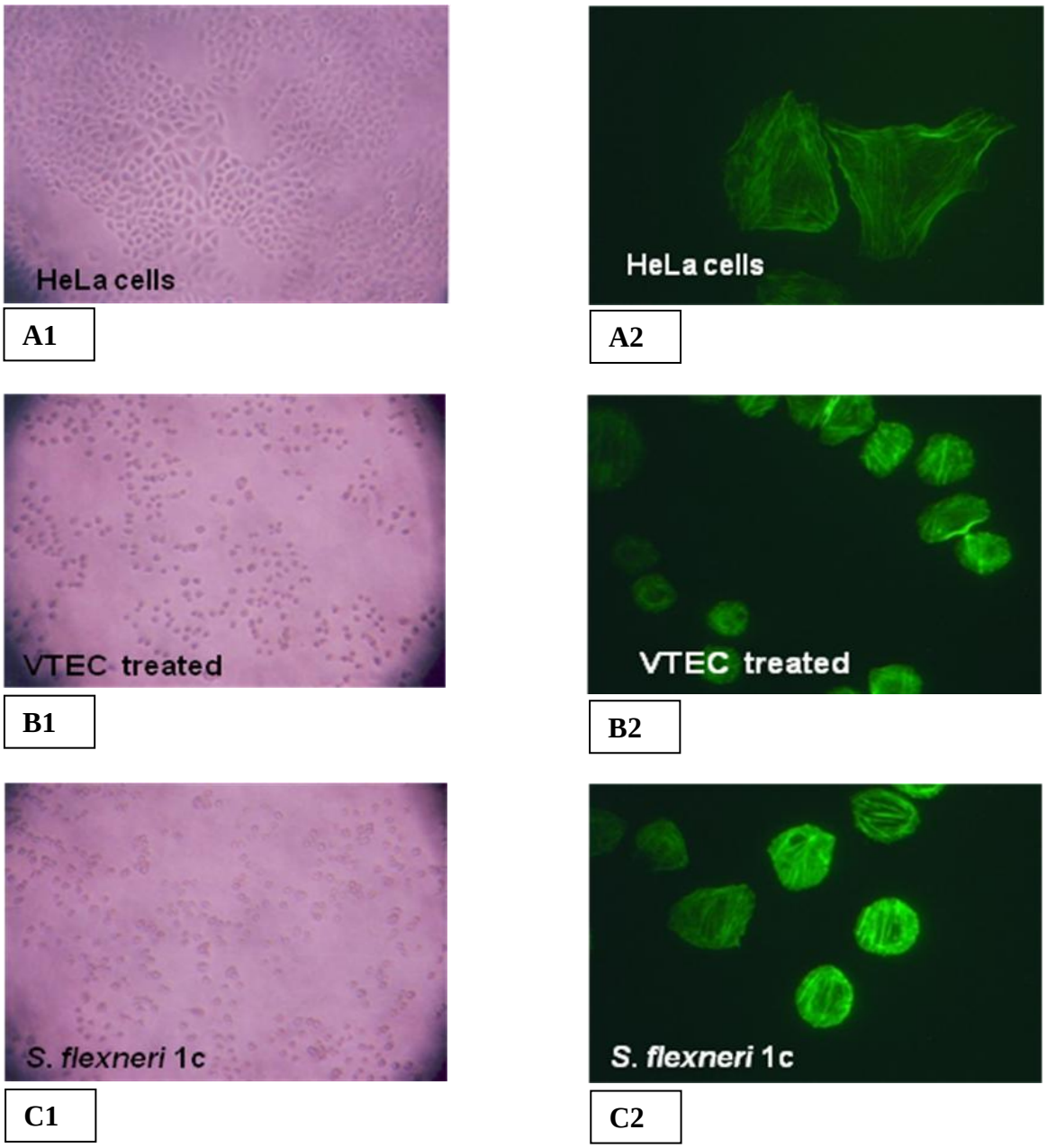


Figure 3.7 Cytotoxic effect of bacterial cell lysate on HeLa cells. HeLa cells were seeded on culture plates and incubated overnight. Cells were treated with culture supernatants from *E. coli* MC-1061, (A1x100 & A2x400); VTEC-3, (B1x100 & B2x400); and *S. flexneri* 1c K-314, (C1x100 & C2x400) for 24 hrs. HeLa cells were fixed and images were captured. The morphological changes of the HeLa cells with respected culture supernatant were confirmed by staining actin filament with FITC conjugated Phalloidin (A2, B2 & C2).

Table 3.2 Characterization of 140MDa plasmid negative *S. flexneri* 1c (n=17)

Strain ID	Serotype	140 MDa plasmid	Congo red binding ability	Test for invasiveness (sereny test)	Presence of virulence and toxin genes							TTSS-genes							Cytotoxic effect
					*ipa*H	*ial*	*ipa*BCD	*set*	*sen*	*stx*1	*stx*2	*ipa*B	*ipa*C	*ipa*D	*Mxi* C	*MxiA*1	*MxiA*2	*VirA*	
K-211	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
K-240	*S. flexneri* 1c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K-314	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
K-899	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
K-915	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
K-961	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
K-1362	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
K-1383	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
K-1387	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
K-1776	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
K-9295	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
K-9717	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
KP-15	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
KP-19	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
KP-41	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
KP-44	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
KP-55	*S. flexneri* 1c	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+

3.6 Partially purified toxin fraction of *S. flexneri* 1c induce cytotoxic Activity in HeLa Cells

Both concentrated culture supernatant and cell lysate of *S. flexneri* 1c (K-314 and K-915) strains showed positive cytotoxic effect in dose dependant manner. After 24 hours, viability of cells was determined by MTT assay (Figure 3.8). HeLa cells were also treated with 40%, 60% and 80% fractions of *S. flexneri* 1c K-314 strain. However, only 60% fraction showed strong cytotoxic activity (Figure 3.9). The 60% toxin fraction was applied in HeLa cells with different doses and time interval for determining its cytotoxic activity. The results showed that percent cell death increased with increasing doses and times. Each experiment was repeated more than three times showing similar results, p ≤0.0005 (Figure 3.8). Data indicated significant difference between control and treated HeLa cells exposed to different concentration of toxin.The 60% toxin fraction was further purified and the fractions (F1, F2, F3 and F4) were also studied for cytotoxic activities. The F3 fraction was found to have strong cytotoxic activity and this fraction was considered as the active fraction. This fraction contains a active protein component molecular mass of 100–125kDa which was sensitive to heat and trypsin.

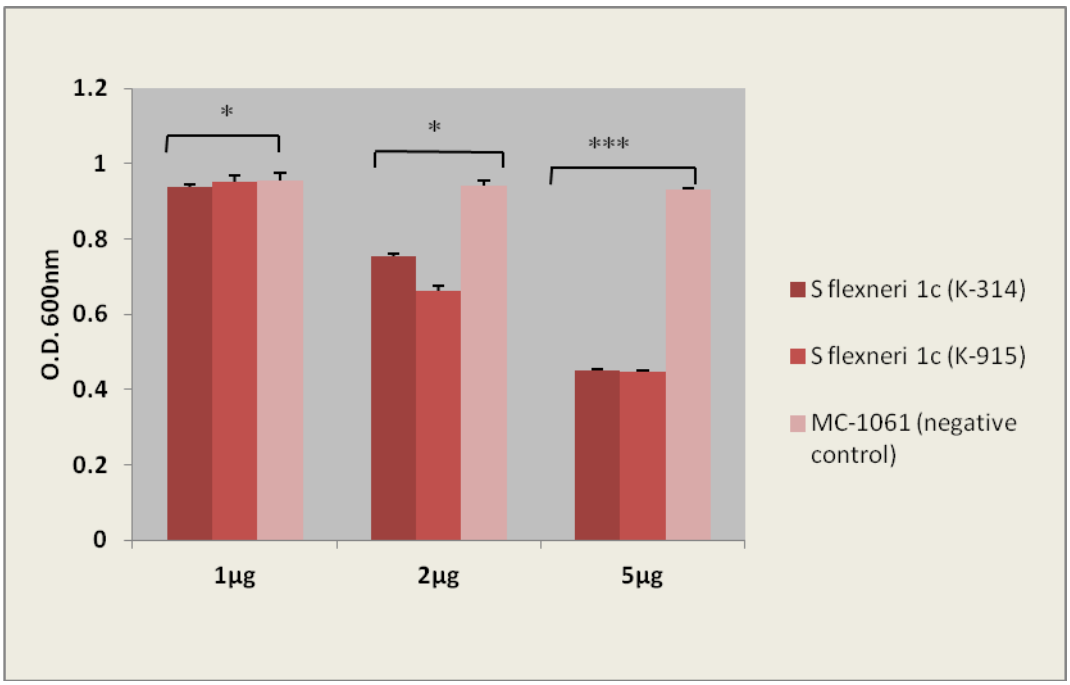
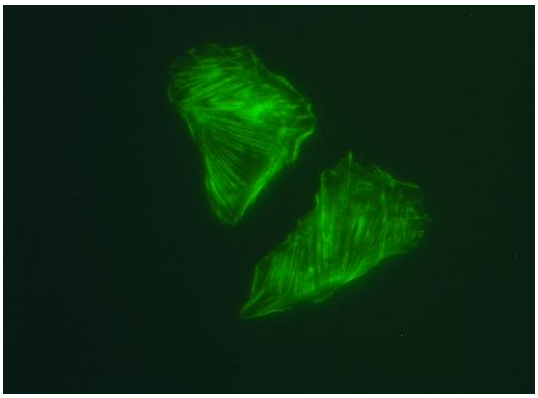
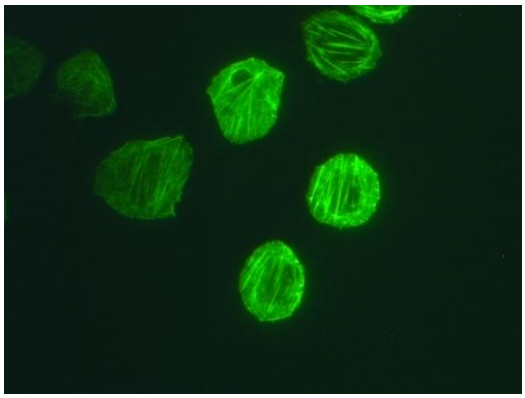


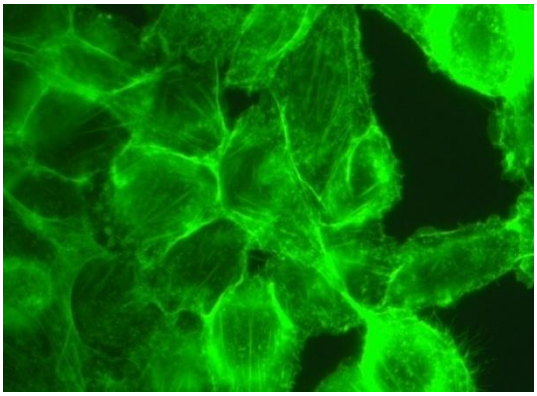
Figure 3.8 Cytotoxic effect of *S. flexneri* 1c lacking 140MDa plasmid on HeLa cells. Cell lysate of *S. flexneri* 1c (K-314 and K-915) strain showed positive cytotoxic activity on HeLa cells in dose dependant manner. After 24 hours, viability of cell was determined by MTT assay. Each experiment was repeated more than three times showing similar results. Data are presented as mean ± SEM. Asterisks (*) indicate significant difference between control and treated HeLa cells exposed to different concentration of toxin, (*) p≤0.05, (***) p ≤0.0005.



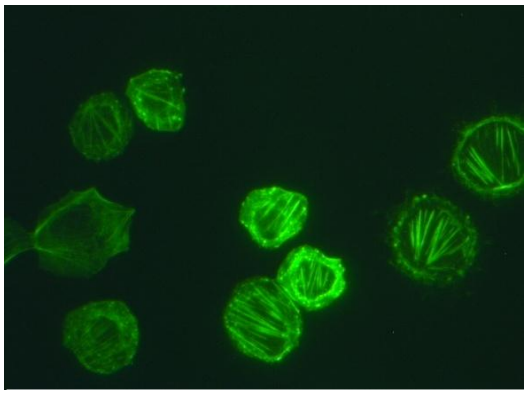
A. *E. coli* MC-1061 treated HeLa cells



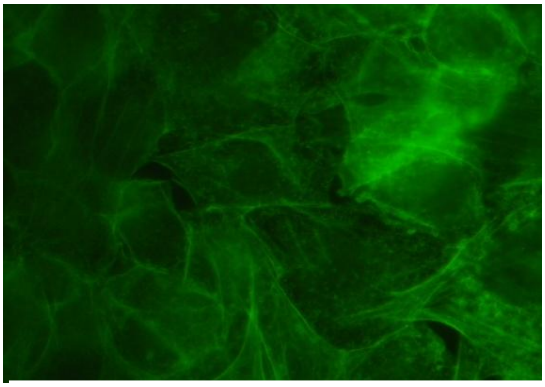
B. VTEC treated HeLa cells



C. *S. flexneri* 1c K-314 (40%) treated HeLa cells



D. *S. flexneri* 1c K-314 (60%) treated HeLa cells



E. *S. flexneri* 1c K-314 (80%) treated HeLa cells

Figure 3.9 Partially purified toxin fractions of *S. flexneri* 1c (K-314) induced cytotoxic effect on HeLa cells. HeLa cells were treated with *E. coli* MC-1061, (Ax400); VTEC-3, (Bx400) partially purified toxin fraction of *S. flexneri* 1c (K-314) 40%, (Cx400); 60%, (Dx400) and 80%, (Ex400) for 24 hrs. Cells were fixed and images were captured. The morphological changes of the HeLa cells with respected culture supernatant were confirmed by staining actin filament with FITC conjugated Phalloidin.

3.7 Toxic factor(s) in *S. flexneri* 1c exhibited biochemical properties of protein

Lysates from *S. flexneri* 1c (K-314 and K-915) were heated and studied its cytotoxic activity in HeLa cells. It was found that heat treatment significantly reduced cytotoxic activity of *S flexneri* 1c (Figure 3.10). *S flexneri* 1c (K-314 and K-915) lysates were also treated with DNase, RNase, and protease. Treated lysates were studied for cytotoxic activity against HeLa cells. As shown in Figure 3.11, DNase and RNase treatment did not affect cytotoxic activity of *S flexneri* 1c (K-314) lysate while protease treatment significantly reduced its cytotoxic activity. Similar results was found in case of lysate from *S flexneri* 1c K-915 (data not shown). These results suggest that cytotoxic factor(s) in *S flexneri* 1c is possibly protein in nature. Cell survival was determined by MTT assay. Data indicated significant difference between control and treated HeLa cells exposed to heat, (Figure 3.10); DNase, RNase and Protease (Figure 3.11), $p \leq 0.0005$.

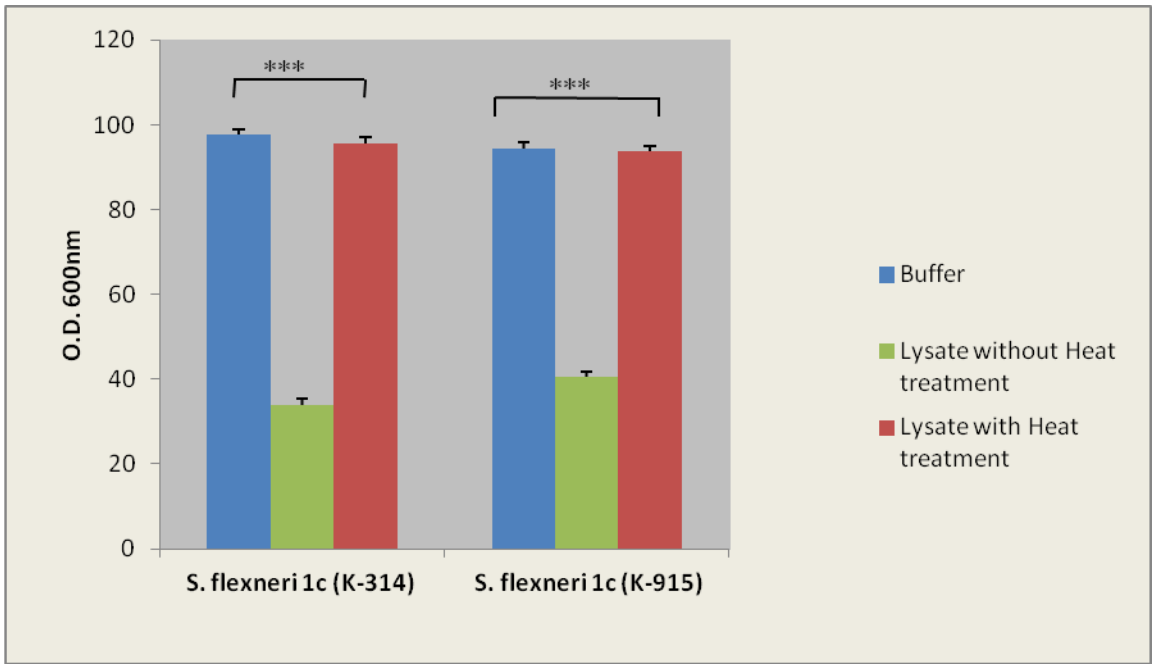


Figure 3.10 Effect of heat treatment on cytotoxic activity of *S. flexneri* 1c cell lysate. HeLa cells were treated with PBS (buffer), *S flexneri* 1c (K-314 and K-915) cell lysates with (lysate with heat treatment) or without (lysate without heat treatment) heat treatment overnight. Cell survival was determined by MTT assay. Data are presented as mean ± SEM. Asterisks (*) indicate significant difference between control and treated HeLa cells exposed to heat, (*) $p \leq 0.05$, (***) $p \leq 0.0005$.

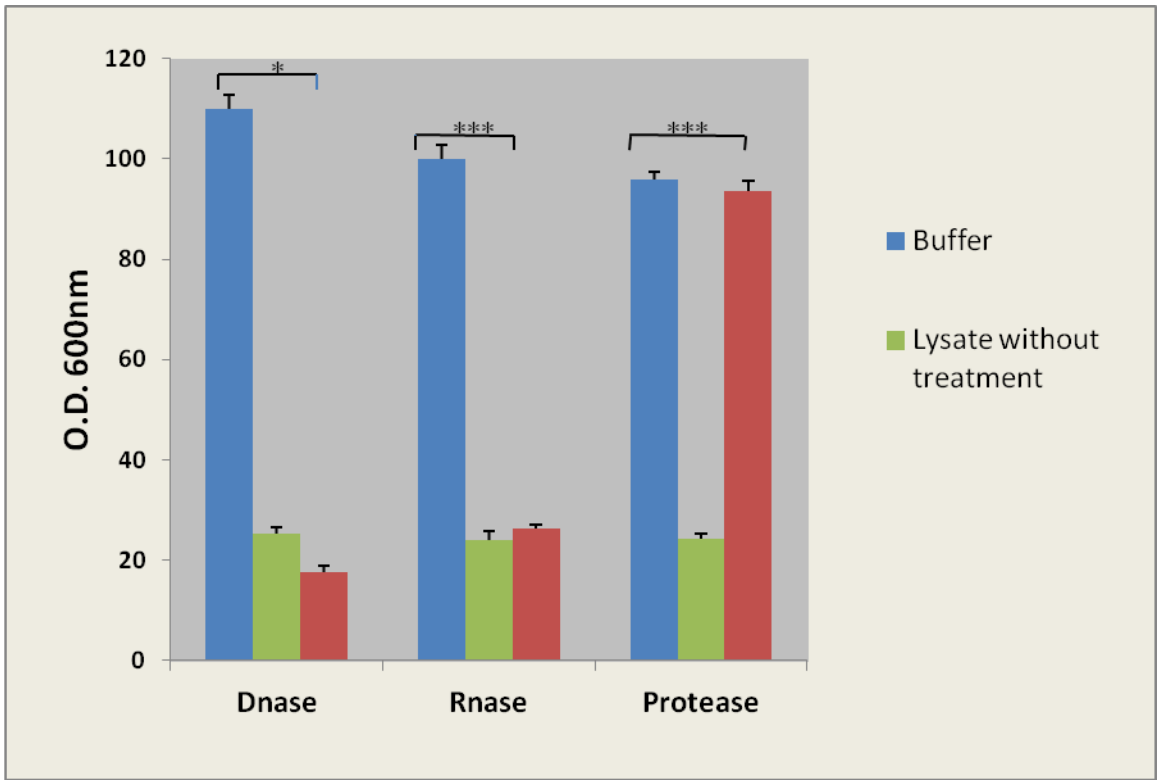
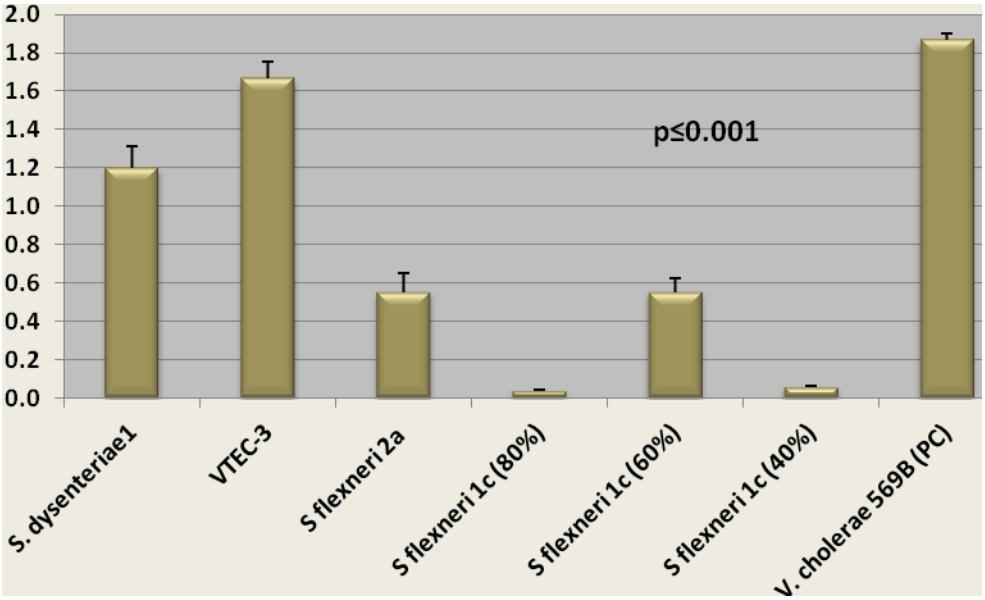


Figure 3.11 Effect of DNase, RNase, and protease on cytotoxic activity of *S. flexneri* 1c cell lysate. *S. flexneri* 1c (K-314) lysate was treated with DNase, RNase, or protease as described in "Materials and Method". HeLa cells were treated with PBS (buffer), K-314 lysate treated (lysate with treatment) or untreated (lysate without treatment) with DNase, RNase, or protease for overnight. Cell survival was determined by MTT assay. Each experiment was repeated more than three times showing similar results. Data are presented as mean ± SEM. Asterisks (*) indicate significant difference between control and treated HeLa cells exposed to DNase, RNase and protease, (*) $p \leq 0.05$, (***) $p \leq 0.0005$.

3.8 Induction of fluid accumulation in Rabbit Ileal Loop

3.8.1. Rabbit Ileal Loop Assay

The concentrated culture supernatant, and cell lysates of different serotypes of *Shigella* strains were studied for their ability to cause fluid accumulation in rabbit ileal loop. It was observed that different serotype showed fluid accumulation in different ranges in the ligated loops. It was found that *S. dysenteriae* 1 (contains s*tx*$_1$ gene) and VTEC-3 (a verotoxin producing *E coli*, contains both *stx*$_1$ and *stx*$_2$ gene) induced fluid accumulation in rabbit ileal loops (Table. 3.3). *S. flexneri* 2a (containing se*t* and *sen* genes) accumulate small amount of fluid as compared to *S. dysenteriae* 1. Concentrated culture supernatant and different toxin fractions (40%, 60% and 80%) of *S flexneri* 1c (K-314, devoiding of known toxin genes) did not cause fluid accumulation in the loop. (Figure 3.12). Concentrated culture supernatant of *V. cholerae* 569B was used as a positive control and was found to cause accumulation of 1.7 ml/cm fluid, and MC-1061 (negative control) did not induce any accumulation of fluid (Figure 3.12)



B

Figure 3.12 *Shigella* toxin induced fluid accumulation in rabbit ileal loop. A (inset), fluid accumulation in rabbit ileal loop, Loop 1, positive control (569B *V. cholerae*); loop: 2, *S. dysenteriae* 1 (K-571, fluid accumulation); loop 3 with VTEC-3(fluid accumulation); loops 4, 5 and 6, 40%, 60% and 80% toxin fraction (no fluid accumulation) of *S. flexneri* 1c (K-314); and loop 7, *E. coli* MC-1061 (no fluid accumulation). **B**. Bar graph showing mean fluid accumulation (ml/cm) in rabbit Ileal loop after inoculation of 0.5 ml of 40%, 60%, 80% of toxin fractions *S. flexneri* 1c (K-314), *S. dysenteriae* 1 and concentrated culture supernatant of 569B *V. cholerae* used as positive controls and caused different volumes of fluid accumulation. *E. coli* MC-1061 was used as negative control and did not produce fluid. Each experiment was repeated more than three times showing similar results. Data are presented as mean ± SEM. Data indicate significant difference in fluid accumulation between control and test strains $p \leq 0.0005$.

3. 8. 2. Histopathological Study

The histopathological changes of the ligated loop segments were examined by light microscopy. Ileal segments exposed to toxin showed different degree of inflammation in mucosa, sub-mucosa and also sometimes in muscle layer. The histological changes were graded from 0 to 4+, with 0 (normal) being no change and 4+ equaling severe enterocyte necrosis. It also revealed that loop segments treated with concentrated culture supernatant of *S. flexneri* 1c showed moderate inflammatory infiltrate in lamina propria and in submucosa of the wall of the intestine. It also showed sheared off of tips villi in some places with Grade-3 inflammation (Figure 3.13, A1 & A2). Loop segment treated with *S. dysenteriae* 1 revealed moderate collection of inflammatory infiltrate in lamina propria and in submucosa and also in muscle layer in some places. It also showed congested blood vessels in submucosa of the wall of intestine with Grade-4 inflammation and sheared off of tips of villi in some places with prescence of necrosed material (Figure 3.13, B1 & B2). The section exposed to enterotoxin(s) of *V. cholerae* 569B (positive control) revealed extensive villi destruction in most of the places with dense collection of inflammatory infiltrate in all the layers including muscle layers. It also reveals extensive areas of enterocyte necrosis with .Grade-4 inflammation and villi integrity was hampered (Figure 3.13, C1 & C2). No pathological change except mild inflammation in *E. coli* MC-1061 treated loop was observed (Figure 3.13 D1 & D2).

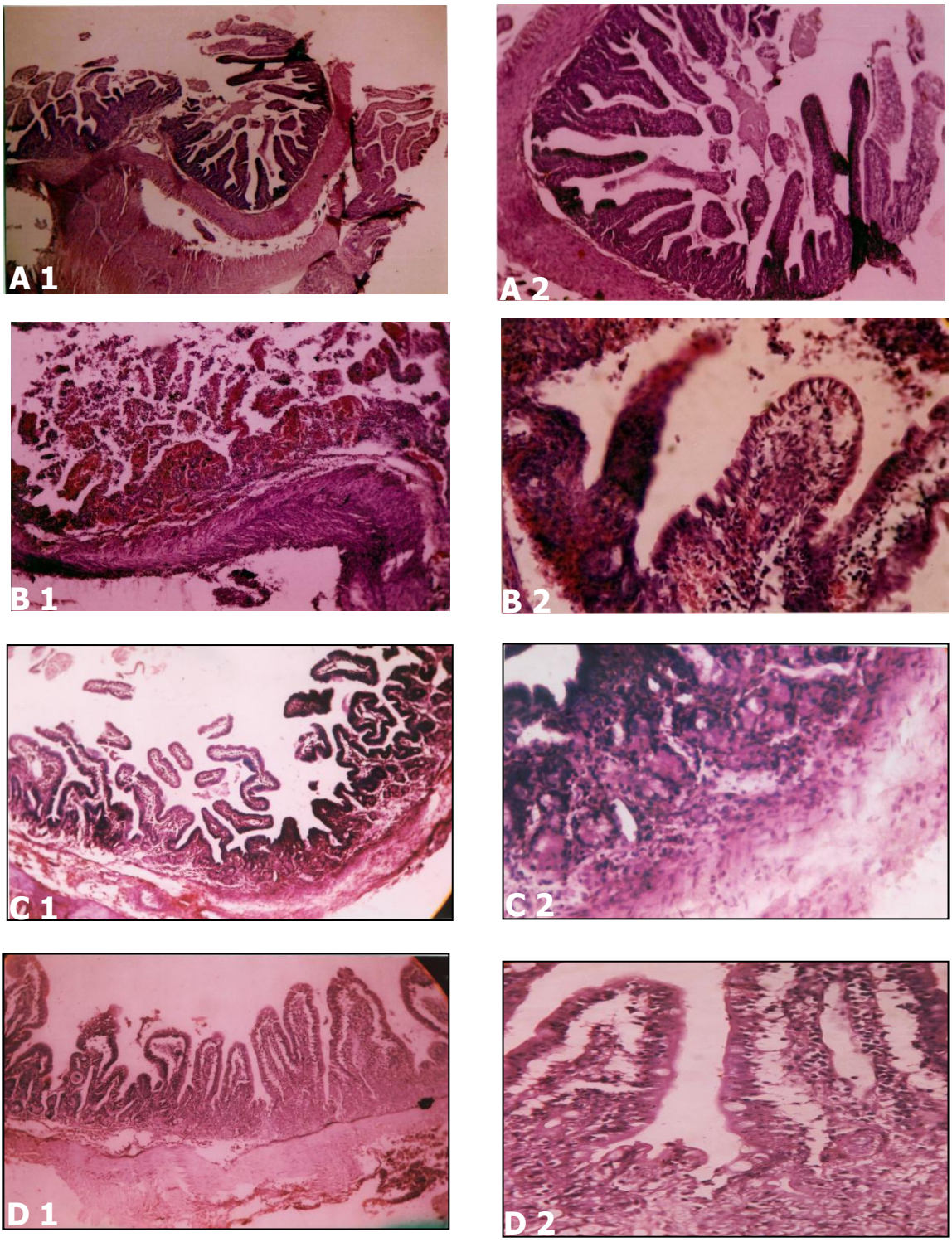


Figure 3.13 Effect of toxin in rabbit ileal loop tissue. Photomicrograph H & E stained section from an ileal loop segment: *S. flexneri* 1c treated loop (A1x100 and A2x400) revealed moderate inflammation in mucosa, submucosa and Villi integrity was more or less maintained (Grade-3 inflammation). Enterocyte necrosis and shearing of tips of villi also sometime in muscle layers, with hampered villi integrity (Grade-4 inflammation) was observed in *S. dysenteriae* 1 and *V. cholerae* 569B treated loop (B1x100 and B2x400) and(C1x100 and C2x400); *E. coli* MC-1061 as a negative control treated loop (D1x100 and D2x400) exhibited normal ileal loop with mild inflammation (Grade-1 inflammation).

3. 8. 3. *S. flexneri* 1c strain induced diarrhoea by RITARD Model

The adult rabbit intestinal assay was performed to inoculate 10 ml (3 x 10^8/ ml) of *S. flexneri* 1c (K-314) strain suspension into the lumen of the anterior jejunum. The signs and symptoms of rabbits were observed after 24 hour. Five days observations were recorded and shown in Table 3.3. Rabbit produced watery stools with mucus after 48 hour and lasted for 2 days. After five days, at autopsy the small intestine of rabbits appeared normal. Rectal swabs were taken daily and identified as *S. flexneri* 1c by serology. *E. coli* MC-1061 injected rabbits served as negative controls and did not produce diarrheal signs and symptoms.

Table 3.3 Sign and symptoms produced in rabbit after challenged with *S. flexneri* 1c (K-314)

Day	Sign and symptoms	
	Rabbit 1	Rabbit 2
Day 1	Mild amount of mucous and normal appearance	Stool with mucous, normal appearance
Day 2	Stool with mucous watery, Diarrhoea, weak	Stool with mucous, Diarrhoea, weak
Day 3	Stool with moderate amount of mucous and normal appearance	Stool with moderate amount of mucous and normal appearance
Day 4	Mild amount of mucous and normal appearance	Stool with mucous and normal appearance
Day 5	Stool with mucous and normal appearance	Stool with mucous and normal appearance

3.9 *S. flexneri* 1c (K-314) induced paralytic lethal effect in mice

S. flexneri 1c (K-314) was administered intra-peritoneally at different doses (0.1 µg, 1 µg, 5 µg, 10 µg and 50 µg per mice) in mice and LD50 dose was found to be 1 µg. The sign of toxin effect appeared within 36 hours which included ruffled fur, huddling and disinclination to move, when effective dose (4xLD50) was given. Hind limb paralysis and rapid breathing were observed as sign of severe illness and all mice died within 48 hours (Figure.3.14). When PBS and culture supernatant of *E. coli* MC-1061 were injected in mice no signs of toxic effect was observed.

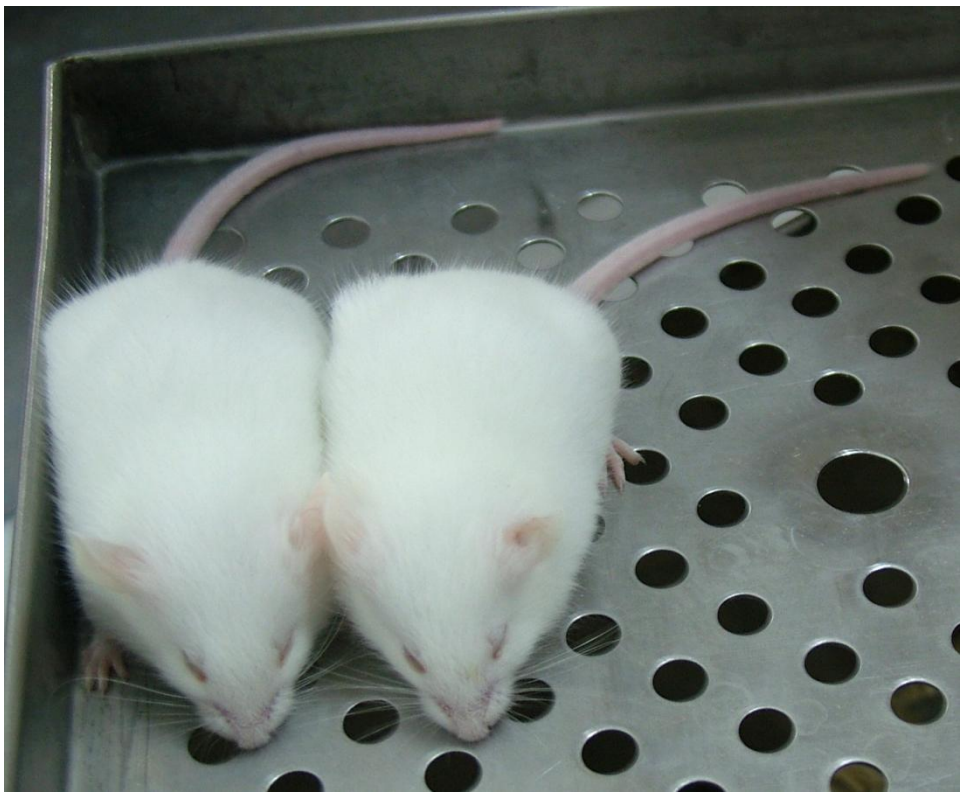
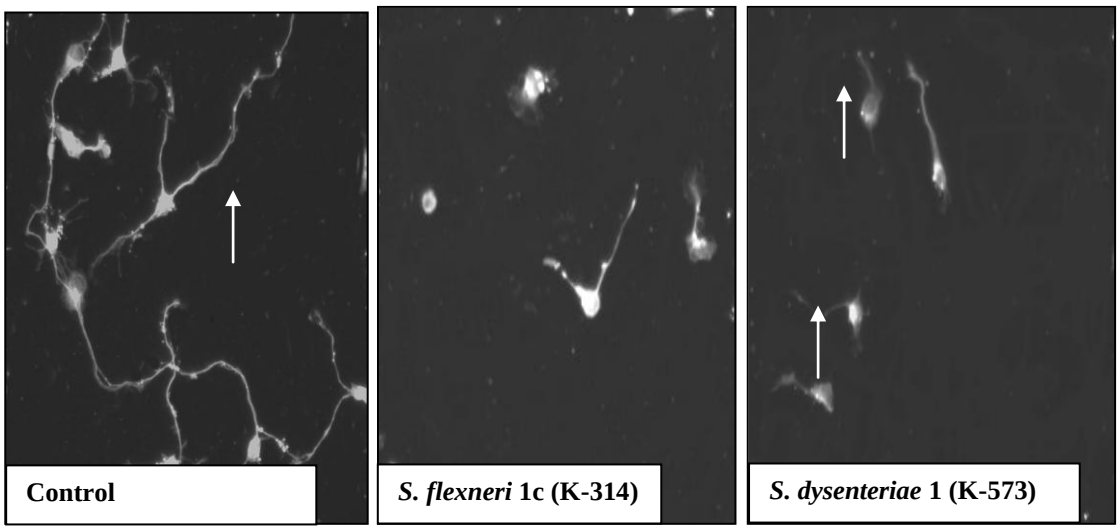


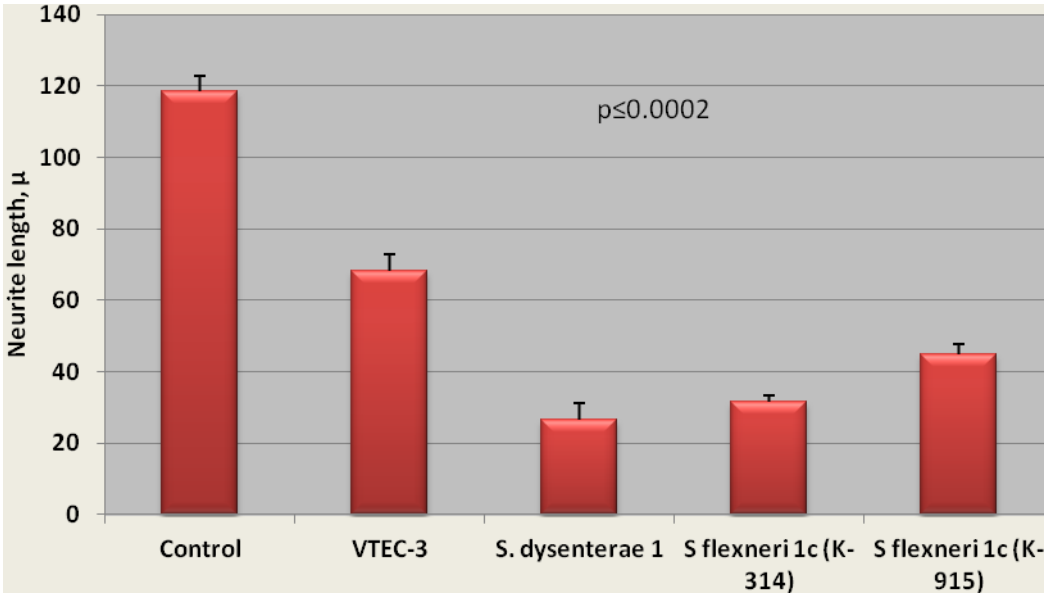
Figure 3.14 Effect of *S. flexneri* 1c (K-314) after intra-peritoneal injection in mice. Hind limb paralysis and rapid breathing were observed as signs of severe illness and all mice died within 48 hours.

3.10 Lysate of *S. flexneri* contain a neurotoxic factor

Lysate from different serotype of *S. flexneri* were studied for their neurotoxic activity. The average total neurite length/neuron was determined from more than 100 neurons isolated. Neurite length of neurons showed significant difference. Isolation of neurons and neurite outgrowth assays were done in a double-blinded manner. In the primary neuron culture model it was found that lysate of *S. flexneri* 1c K-314 and K-915 (free of all toxin gene) reduce the neurite length (Figure 3.15). Lysate from *S. dysenteriae* 1 containing stx1 also have neurotoxic effect on the neuron. Data indicated significant difference between control and toxin treated cerebellar granule neuron $p \leq 0.0002$.



A



B

Figure 3.15 Cell lysates from *S. flexneri* 1c inhibits neurite outgrowth of cerebellar granule neurons. **A.** Cortical neurons isolated from rat brains fail to extend neurites in culture. Cortical neurons (CNs) were isolated from 8-day-old rat and cultured on PLL coated glass coverslips. Neurons were incubated for 24 h and were then fixed and stained for neurofilaments. Images were captured using a fluorescent microscope. **B.** The average total neurite length/neuron was determined from more than 100 neurons isolated. Neurite length of neurons showed significant difference. Isolation of neurons and neurite outgrowth assays were done in a double-blinded manner. Data are presented as mean ± SEM and indicate significant difference between control and toxin treated cerebellar granule neuron $p \leq 0.0005$.

3. 11 *S. flexneri* 1c strain induce DNA damage and Apoptosis in mammalian cell

3. 11. 1 Morphological changes in HeLa cells

Cytotoxic activity was determined by studying the morphological changes (rounding, elongation) in HeLa cells under a microscope after treatment with *S. flexneri* 1c for 24 hours and morphological changes were assessed by staining actin filaments with fluorescein isothiocyanate (FITC)–conjugated phalloidin. *E. coli* MC-1061 treated HeLa cells (control) showed normal morphology of cell with actin filament (Figure 3.16 A1& A2). However, the actin filaments were destroyed and all the cells become rounded when treated with *S. flexneri* 1c (Figure 3.16 C1& C2) and become elongated when treated with culture supernatant of *S. boydii* (Figure 3.16 B1 & B2).

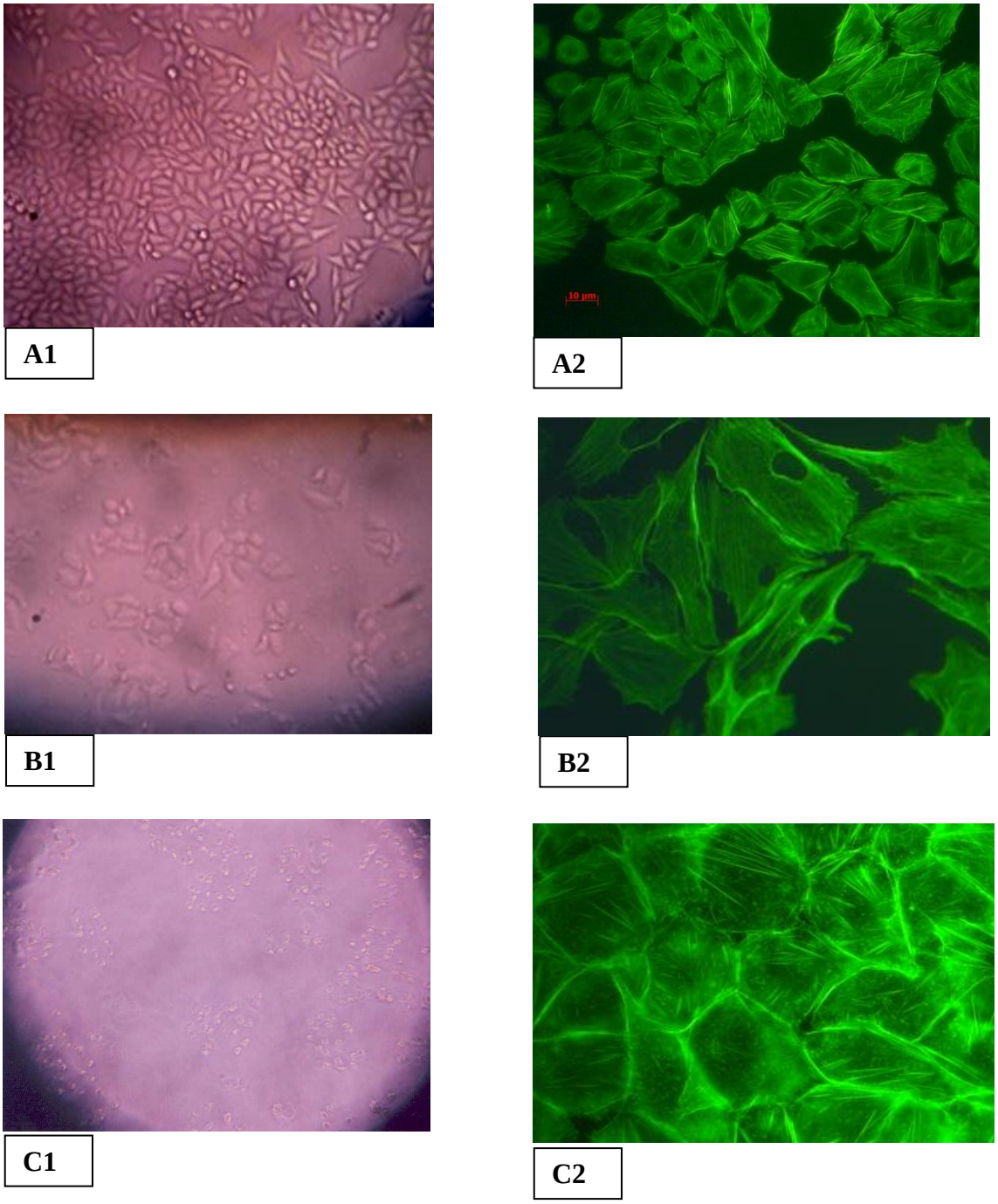
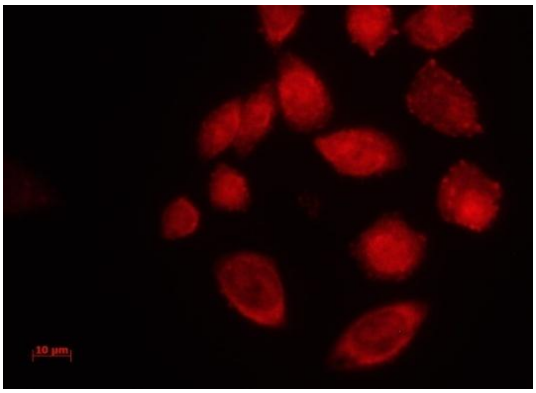


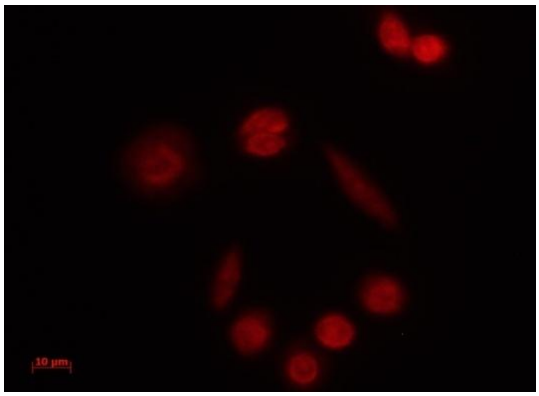
Figure 3.16 Morphological alterations of HeLa cells by *S. flexneri* 1c (K-314). HeLa cells were seeded on culture plates and incubated overnight. Cells were treated with *E. coli* MC-1061 (A1x100 & A2x400), culture supernatants from *S. boydii* (B1x100 & B2x400) and *S. flexneri* 1c K-314, (C1x100 & C2x400) for 24 hrs. HeLa cells were fixed and images were captured. The morphological changes of the HeLa cells with respected culture supernatant were confirmed by staining actin filament with FITC conjugated Phalloidin (A2, B2 & C2).

3.11.2 Chromatin condensation in HeLa cells

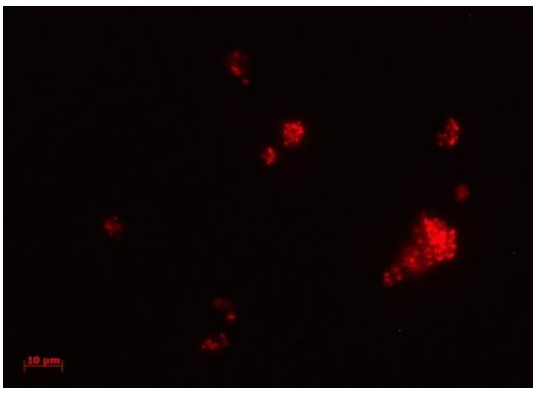
Chromatin condensation effect of HeLa cell was exhibited by PI staining after treatment with different toxin fractions of *S. flexneri* 1c (K-314). The PI is a fluorescent vital dye that stained apoptic nuclei (condensed chromatin). *E. coli* MC-1061 treated HeLa cells showed normal appearance of HeLa cells (Figure 3.17) while toxin treated cells condensed chromatin within the HeLa cells. It was observed that the 60% toxin fraction had severe neuclear condensation.



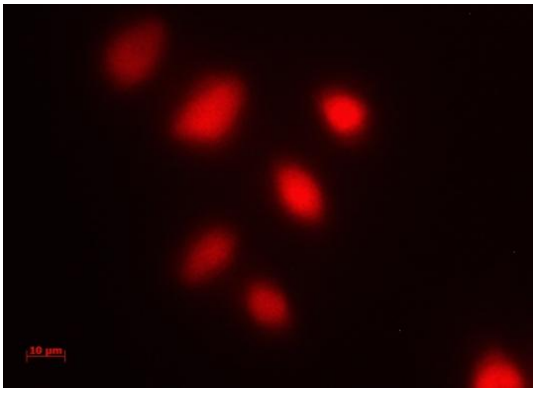
HeLa cells



***S. flexneri* 1c K-314 (40%)**



***S. flexneri* 1c K-314 (60%)**



***S. flexneri* 1c K-314 (80%)**

Figure 3.17 Induction of chromatin condensation by *S. flexneri* 1c (K-314) in HeLa cells. HeLa cells were treated with different toxin fraction (40%, 60% and 80%) of *S. flexneri* 1c (K-314) for 24 hours. HeLa cells were fixed with paraformaldihyde and stained with Propidium Iodide as described in Materials and Methods.

3.11.3 DNA fragmentation of *S. flexneri* 1c (K-314) treated HeLa cells by agarose gel electrophoresis

One of the later steps in apoptosis is DNA fragmentation, a process which results from the activation of endonucleases during the apoptotic program. These nucleases degrade the higher order chromatin structure into fragments of 50 to 300 kb and subsequently into smaller DNA pieces of about 200 bp length. Fragmentation of chromosomal DNA was studied in HeLa cells treated with *S. flexneri* 1c (K-314). After 24 hours of treatment HeLa cells were lysed and DNA fragmentation analysis was performed. It was found that *S. flexneri* 1c (K-314) induced fragmentation of chromosomal DNA in HeLa cells and a clear laddering of DNA was found in dose dependant manner (Figure 3.18). *E. coli* MC-1061 treated cells were used as negative controls (C) which did not produce fragmentation of DNA. Cycloheximide (CHX), a potent inhibitor for protein synthesis did not cause any significant fragmentation of chromosomal DNA (Figure 3.18) in HeLa cells.

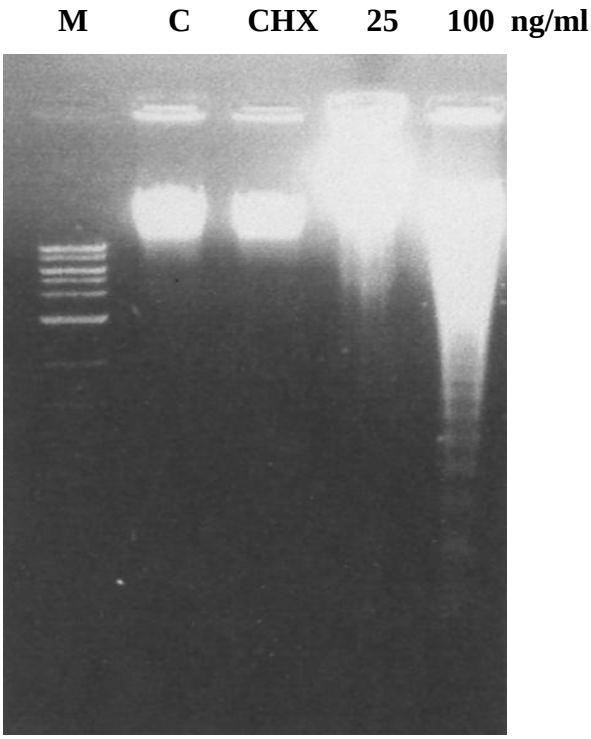


Figure 3.18 *S. flexneri* 1c (K-314) induces chromosomal DNA fragmentation in HeLa cells. HeLa cells were treated with C: *E. coli* MC-1061, CHX: cycloheximide and *S. flexneri* 1c (K-314) (25 and 100ng/ml). DNA fragments were separated by agarose gel electrophoresis (1.5%) and DNA visualized by Ethidium bromide staining.

3.11.4 Induction of Cytochrome C Release from the Mitochondria by *S. flexneri* 1c (K-314)

Release of cytochrome C from the mitochondria as one of the markers of apoptosis was studied. HeLa cells were grown in six-well plate in an amount so that each of the well contains $3x10^6$ cells. After confluence growth of monolayer, the cells were then treated with *S. flexneri* 1c (K-314) (5, 10, 25 and 100ng/ml). After 24 hours of treatment, the cells were harvested. Total cellular proteins were extracted, resolved by SDS-PAGE and western blotting was performed using anti-cytochrome C. It was also found that *S. flexneri* 1c (K-314) induced cytochrome C release from the mitochondria (Figure 3.19). Equal loading of protein extract was confirmed by blotting the membrane with antibodies against topoisomerase I.

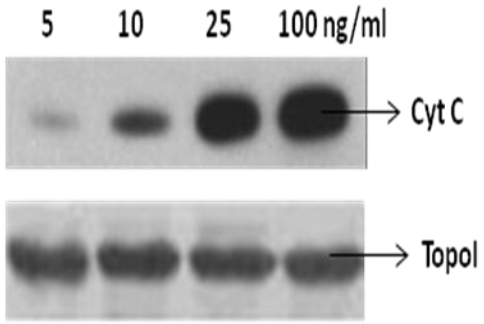


Figure 3.19 Treatment of HeLa cells with *S. flexneri* 1c (K-314) induced cytochrome C release from the mictochondria. HeLa cells were cultured to semiconfluency. *S. flexneri* 1c (K-314) (5, 10, 25 or 100 ng/ml) or equal volume of culture medium (0) was added to cells. After 4 hours of treatment, cells were collected. Nuclear fraction was prepared as described previously (Nur-E-Kamal *et al.*, 2004). Proteins present in nuclear fraction were dissolved in SDS Laemmli sample buffer. Western blotting was performed using cytochrome C. Equal loading of protein extract was confirmed by blotting the membrane with actin antibody (TopoI).

3.12 Activation of DNA Damage Signaling Pathway by *S. flexneri* 1c (K-314)

Markers of apoptosis such as chromatin condensation, fragmentation of chromosomal DNA, and nuclear translocation of cytochrome C were studied. It was found that *S. flexneri* 1c (K-314) caused morphological alteration of HeLa cells (Figure 3.16), induced chromatin condensation (Figure 3.17) and fragmentation of chromosomal DNA (Figure 3.18) in HeLa cells. It was also found that *S. flexneri* 1c (K-314) induced cytochrome C released from the mitochondria (Figure 3.19). *S. flexneri* 1c (K-314) activates DNA damage signaling pathway was studied. *S. flexneri* 1c (K-314) treated cells were studied for activation of PARP cleavage and phosphorylation of H2AX. As shown in Figure 3.20, *S. flexneri* 1c (K-314) induced phosphorylation of H2AX and activated PARP cleavage within 8 hours in HeLa cells. These results indicated that activation of DNA damage signaling due to *S. flexneri* 1c (K-314) may have an important role in inducing apoptosis in mammalian cells. Equal loading of protein extract was confirmed by blotting the membrane with antibodies against actin (total cell extract).

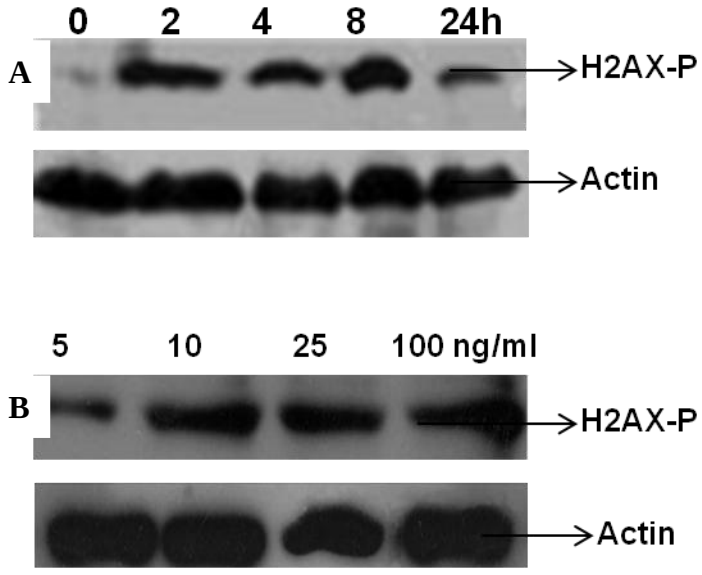
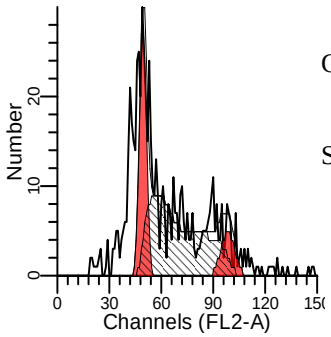


Figure 3.20 Induction of H2AX phosphorylation and PARP cleavage by *S. flexneri* 1c (K-314) in HeLa cells. Hela cells were cultured in DMEM containing 10% fetal calf serum in standard cell culture condition and incubated to reach to semiconfluent. **A.** HeLa cells were treated with *S. flexneri* 1c (K-314) to a final concentration of 100 ng/ml and incubated for 0, 4, 8, and 24 hrs. Cells were treated with *S. flexneri* 1c (K-314) at indicated concentrations for 12 hours. Cells were lysed in SDS Laemmli buffer. Proteins were separated by SDS-PAGE (12%). Western blotting was performed according to the ECL protocol provided by the suppliers (Pierce, Rochford, IL) using antibodies against phospho-H2AX (Dr. D. Chen, Lawrence Berkeley National Laboratory). Equal loading of total proteins was confirmed by blotting the membrane with anibody against actin (Sigma-Aldrich). Arrows show the position of Phospho-H2AX and actin.

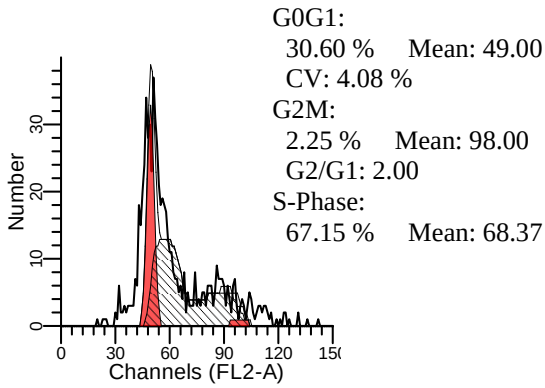
3.13 *S. flexneri* 1c (K-314) induced Cell cycle Arrest

3.13.1 Progression of HeLa cell (untreated) in different inter phase of Cell Cycle

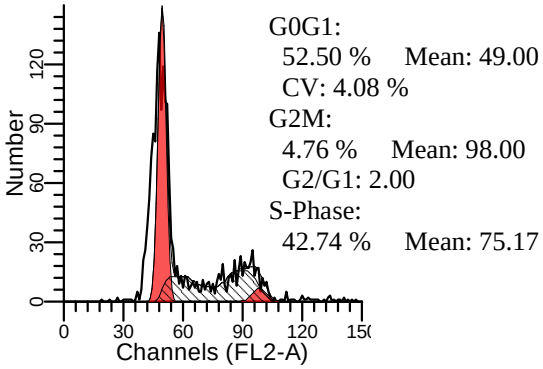
The cell cycle phase in which apoptosis has been triggered was analyzed. Percentage of cell population in different inter phases (G_1, S and G_2) of cell cycle at different time intervals (2hr, 4hr, 6hr, 8hr and 24hr) were measured by FACS analysis. It was found that untreated HeLa cells progressed through normal cell cycle (Figure 3.21 & 3.25).



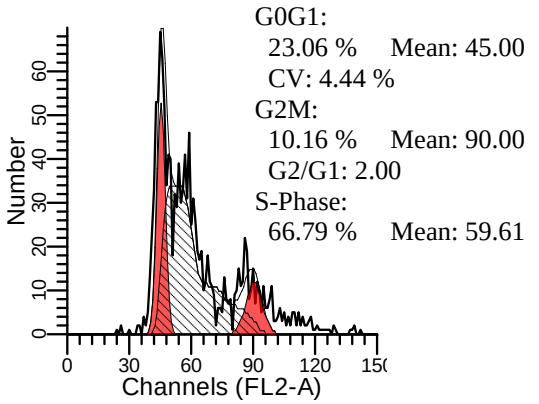
a) Distribution of cells in G1, S and G2 after 2hr



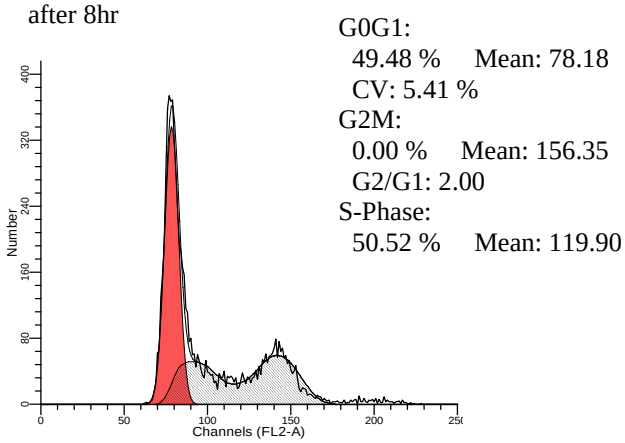
b) Distribution of cells in G1, S and G2 after 4hr



c) Distribution of cells in G1, S and G2 after 6hr



d) Distribution of cells in G1, S and G2 after 8hr

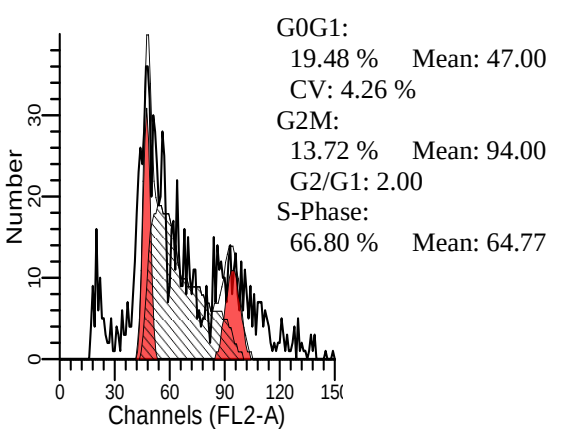


e) Distribution of cells in G1, S and G2 after 24hr

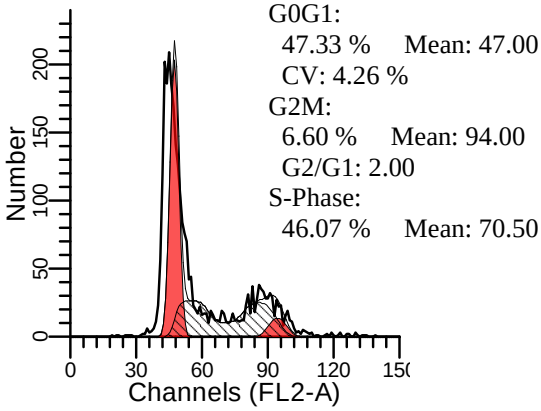
Figure 3.21 Distribution of cell population at different inter phases (G_1, S and G_2) of untreated HeLa cell populations after 2hr, 4hr, 6hr, 8hr and 24hr analyzed Modfit LT software.

3.13.2 Determination of the Percentages of MC-1061 Treated HeLa cell Population at Different Cell Cycle Phases

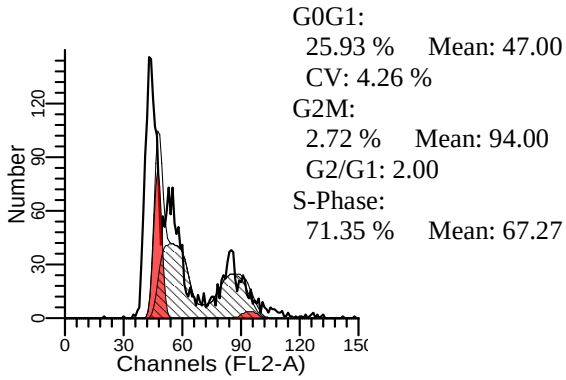
Percentage of cell population in different inter phases (G_1, S and G_2) of cell cycle of MC-1061 treated HeLa cell line at different time intervals (2hr, 4hr, 6 hr, 8 hr and 24 hr) were measured by FACS analysis. It was found that MC-1061 treated HeLa cells progressed through normal cell cycle like the untreated cells (Figure 3.22 & 3.25).



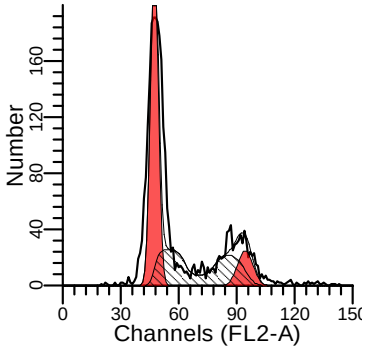
a) Distribution of cells in G1, S and G2 after 2hr



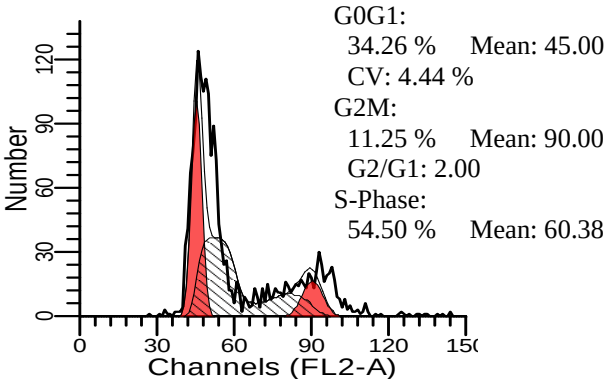
b) Distribution of cells in G1, S and G2 after 4hr



c) Distribution of cells in G1, S and G2 after 6hr



d) Distribution of cells after in G1, S and G2 after 8hr

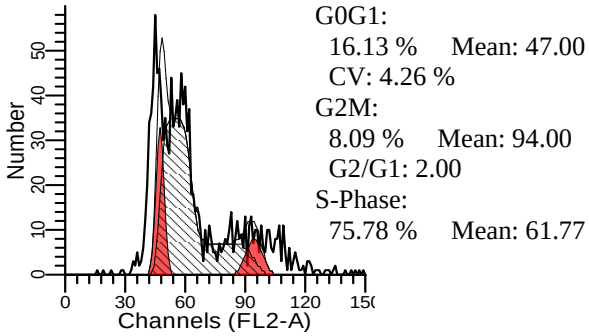


e) Distribution of cells in G1, S and G2 after 2rhr

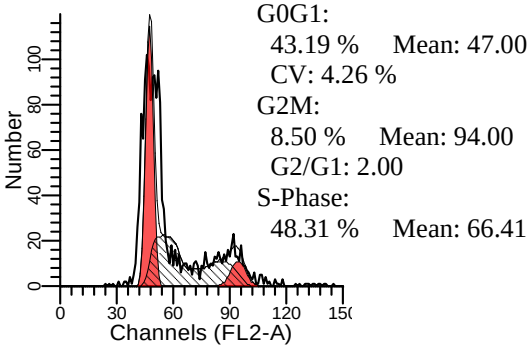
Figure 3.22 Distribution of cell population at different inter phases (G_1, S and G_2) of culture supernatant of MC-1061(Negative control) treated HeLa cell populations after 2hr, 4hr, 6hr, 8hr and 24hr analyzed Modfit LT software.

3.13.3 Determination of the Percentages of VTEC-3 Treated HeLa cell Population at Different Cell Cycle Phases

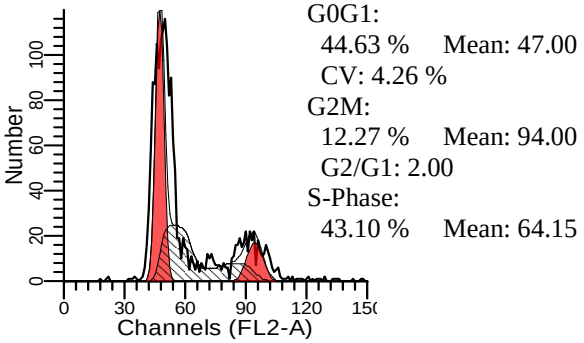
Percentage of cell population in different inter phases (G_1, S and G_2) of cell cycle of VTEC-3 treated HeLa cell line at different time intervals (2hr, 4hr, 6 hr, 8 hr and 24 hr) were measured by FACS analysis. The data from the flow cytometric (FACS) analysis indicated that VTEC-3 treated HeLa cells were arrested at G_0/G_1 phase and the cell percentage in that phase increased gradually from 16.13 to 59.77 (Figure 3.23 & 3.25).



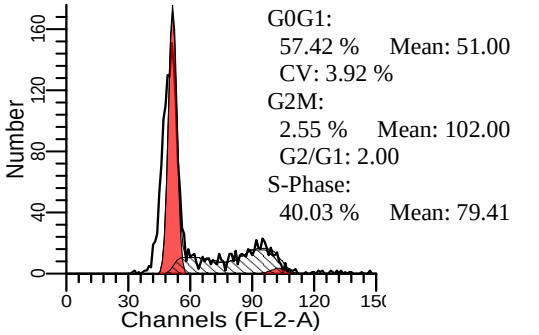
a) Distribution of cells in G1, S and G2 after 2hr



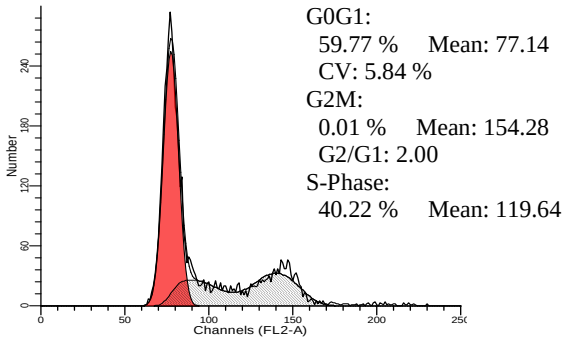
b) Distribution of cells in G1, S and G2 after 4hr



c) Distribution of cells in G1, S and G2 after 6hr



d) Distribution of cells in G1, S and G2 after 8hr

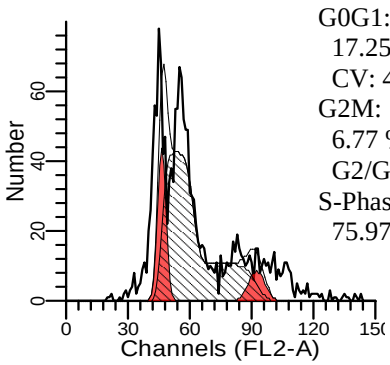


e) Distribution of cells in G1, S and G2 after 24hr

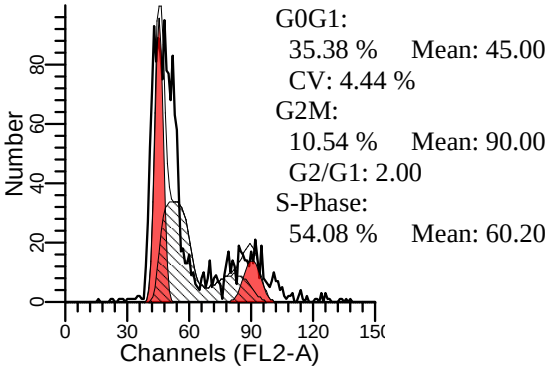
Figure 3.23 Distribution of HeLa cell populations at different inter phases (G_1, S and G_2) after 2hr, 4hr, 6hr, 8hr and 24hr analyzed Modfit LT software. HeLa cells were treated with culture supernatant of VTEC-3 (Positive control).

3.13.4 Determination of the Percentages of *S. flexneri* 1c (K-314) Treated HeLa cell Population at Different Cell Cycle Phases

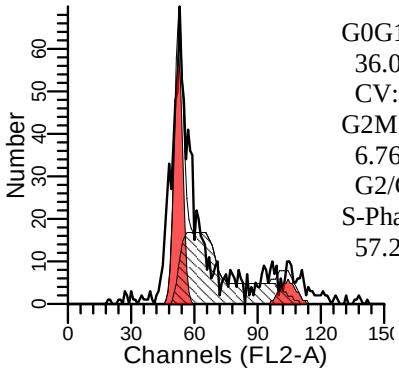
Percentage of cell population in different inter phases (G_1, S and G_2) of cell cycle of *S. flexneri* 1c (K-314) treated HeLa cell line at different time intervals (2hr, 4hr, 6hr, 8hr and 24hr) were measured by FACS analysis. The data from the flow cytometric (FACS) analysis indicated that *S. flexneri* 1c (K-314) treated HeLa cell were arrested at G_0/G_1 phase and the percentage of cells in that phase increased gradually from 17.25 to 51.52 (Figure 3.24 & 3.25).



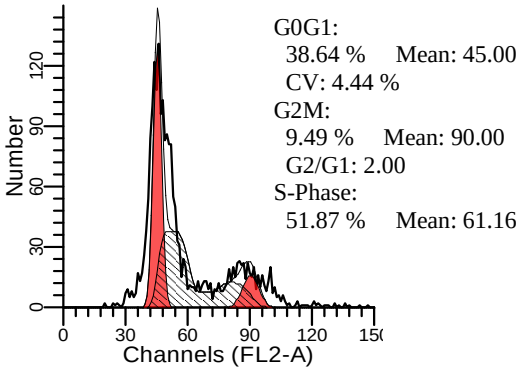
a) Distribution of cells in G1,S, G2 after 2hr



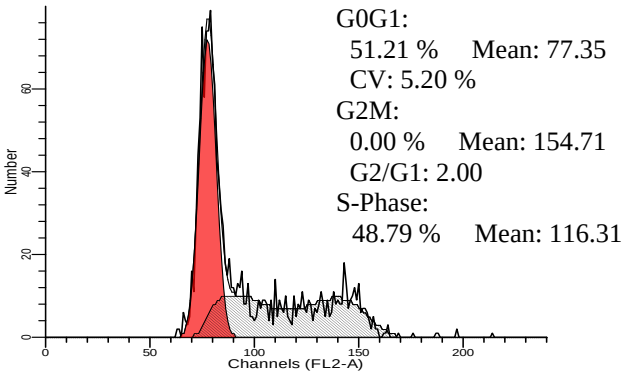
b) Distribution of cells inG1,S, G2 after 4hr



c) Distribution of cells in G1,S, G2 after 6hr



d) Distribution of cells inG1,S, G2 after 8hr



e) Distribution of cells in G1, S and G2 after 24hr

Figure 3.24 Distribution of HeLa cell populations at different inter phases (G_1, S and G_2) after 2hr, 4hr, 6hr, 8hr and 24hr analyzed Modfit LT software. HeLa cells were treated with *S. flexneri* 1c (K-314).

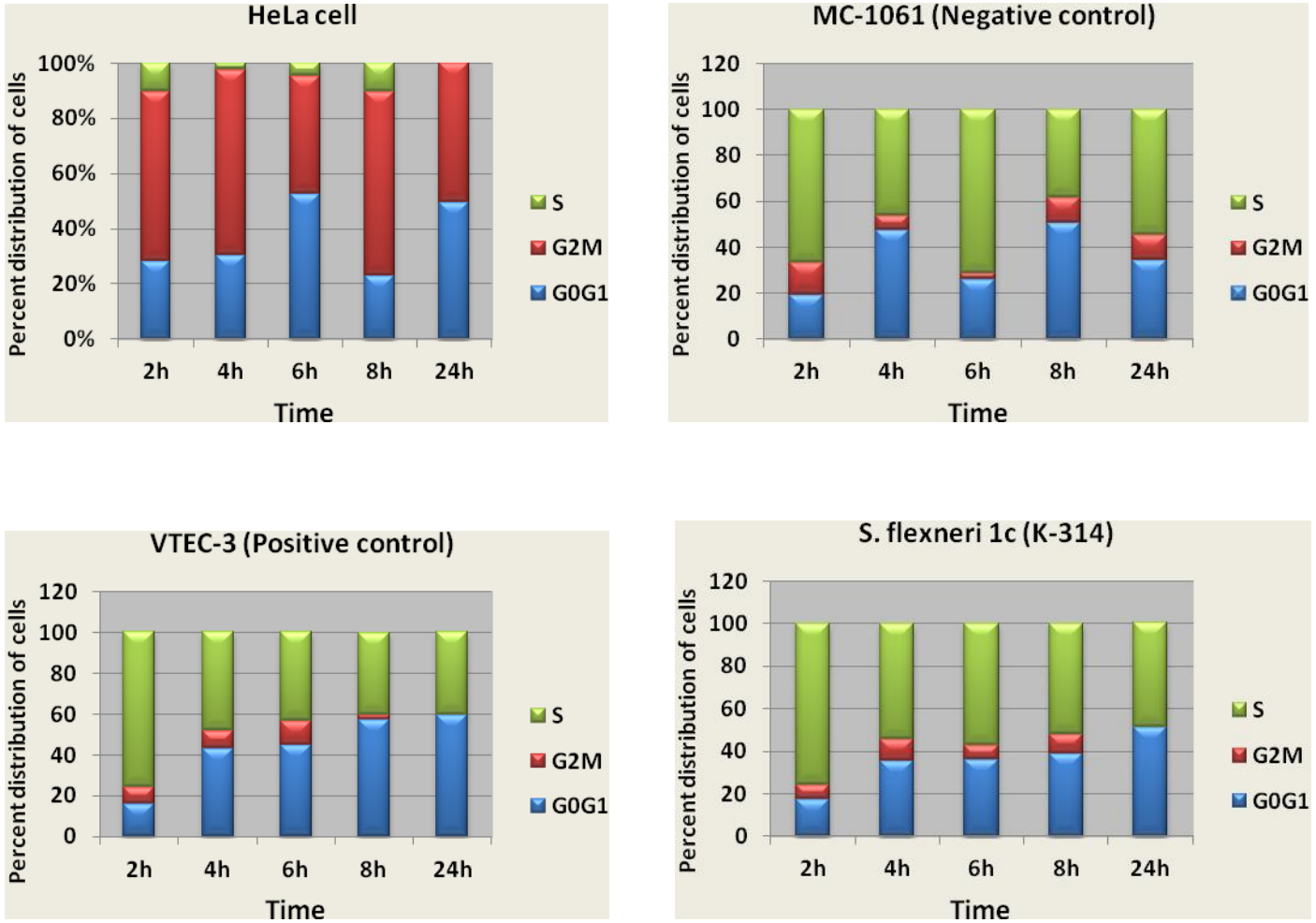


Figure 3.25 Graphical presentation of cell population in different inter phases (G_1, S and G_2) of cell cycle of HeLa cells (treated and untreated) at different time interval analyzed by Cell quest (BD) followed by Modfit LT software. Result indicates that *S. flexneri* treated HeLa cells were arrested at G_0/G_1 phase similar to VTEC-3.

3.14 Role of small plasmids in *S. flexneri* 1c induced cell cytotoxicity

Plasmid analysis of the *S. flexneri* 1c showed that about 88% strains harbored the 140MDa plasmid and 2.8, 2.1, 1.8, 1.6 and 1.0 MDa plasmid. The 140 MDa plasmid lacking strains (n=17) also contain these small plasmids. To elucidate the role of small plasmid in *S. flexneri* 1c induced cytotoxicity curing of large virulence plasmid (140MDa), transformation and co-transfection assay with the help of marker plasmid pUC 18was performed on representative *S. flexneri* 1c strains (K-212, 140MDa plasmid containing strain and K-314 140MDa plasmid lacking strain).

3.14.1 Curing of 140MDa large plasmid

Typical in vitro plasmid curing experiments involve exposure of plasmid containing cells to a drug throughout the growth cycle and subsequent assay of the population for the loss of plasmid specific traits. Curing activity was investigated at different concentrations of acridine orange ranging between 180 µg/mL and 260 µg/mL. The minimum inhibitory concentration (MIC) of acridine orange against clinical strains of *S. flexneri* 1c (K-212) was found to be 280 µg/ml indicating that acridine orange was not a potent antibacterial agent for the test strains. Acridine orange cured the 140 MDa and 90MDa plasmid in the clinical strains of *S. flexneri* 1c (K-212) (Figure 3.26). The curing efficiency was expressed as the number of colonies with cured phenotype per 100 colonies tested. The highest curing efficiency was 3.2% observed below the MIC at subinhibitory concentration 260 µg/mL plasmid cured strains were tested for the cytotoxic activity in the HeLa cells and it was found that it has strong cytotoxic effect (80% cell death) on HeLa cells. This result indicated that there is a possible role of small plasmid in their cytotoxicity.

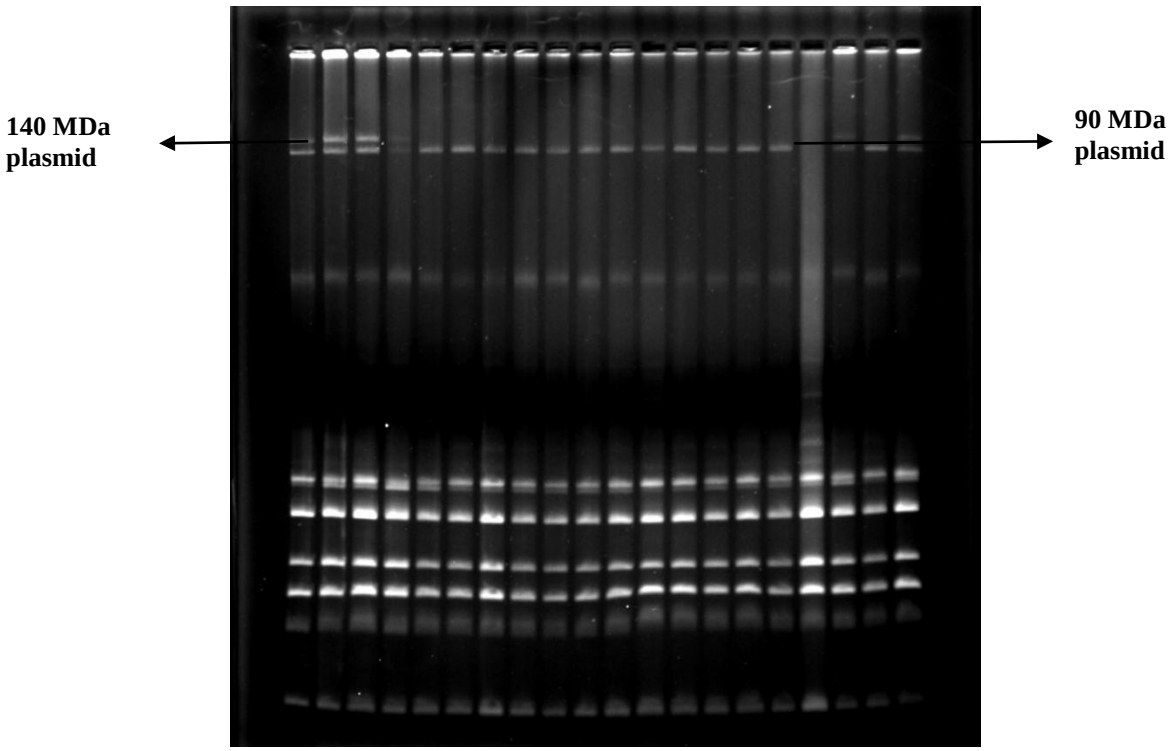


Figure 3.26 Plasmid analysis of *S. flexneri* 1c (K-212) strain after plasmid curing; Plasmid DNA was isolated after acridine orange treatment of *S. flexneri* 1c (K-212) and plasmid loss was evaluated by agarose gel electrophoresis. Lane 1-3, 18 and 20 *S. flexneri* 1c (K-212); Lane 4-16 and 19, 140MDa plasmid cured *S. flexneri* 1c (K-212); Lane 17, 140MDa and 90MDa cured *S. flexneri* 1c (K-212) strain. Expected position of 140 MDa, 90MDa 2.8MDa, 2.1MDa, 1.8MDa, 1.6MDa and 1.0MDa plasmid DNA is shown by an arrow.

3.14.2 Transformation of small plasmid into BL21

Through the process of transformation it was found that pUC18 was transformed independently into the recipient *E. coli* (BL21) strain. In case of representative K-314 strain, no plasmid was transformed into the recipient cell. This result was confirmed by plasmid analysis (Table-3.8)

Table 3.4 Transformation result

Tube	Competent cell	Plasmid		Growth on LB plate	Growth on LB+ Amp plate	Transformation
		K-314	pUC18			
1	200µL	20 µl	0 µl	+	-	-
2	200µL	0 µl	20 µl	+	+	+
3	200µL	0µl	0 µl	+	+	-

3.14.3 Co-transfection

Two plasmids 2.8 MDa and 2.1 MDa of *S. flexneri* 1c (K-314) were transferred into the recipient BL21 transformed cell T2 and T4 respectively (Figure 3.27, Lane 5 and 6). Initially pUC18 plasmid was transformed into the recipient strain BL21. Result summarized in Table-3.9.

Table 3.5 Co-transfection result

Tube	Competent cell	Plasmid		Growth on LB plate	Growth on LB+ Amp plate	Transformation
		K-314	pUC18			
1	200µL	20 µl	0 µl	+	-	-
2	200µL	20 µl	1 µl	+	+	+
3	200µL	20µl	10 µl	+	+	+
4	200µL	0 µl	1 µl	+	+	+
5	200µL	0 µl	0 µl	+	-	-

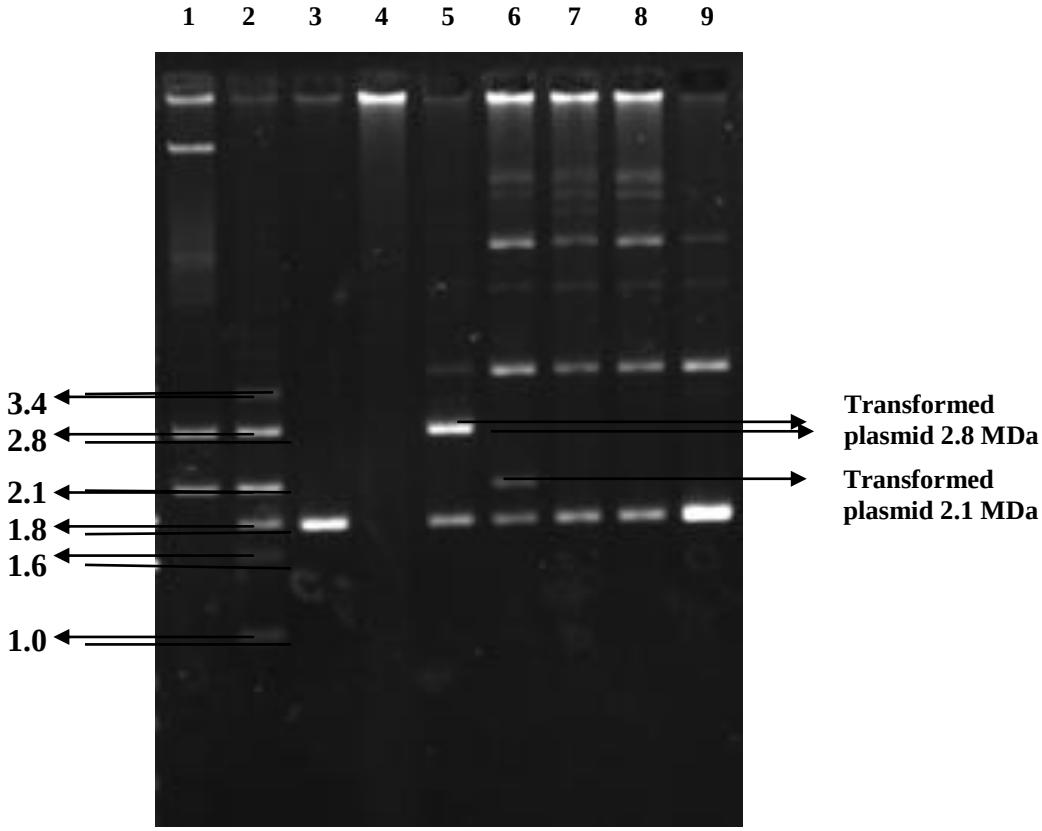


Figure 3.27 Agarose Gel electrophoresis of plasmid from transformed Cell. Plasmid DNA was isolated from transformed cells. Plasmid DNA was characterized by agarose gel electrophoresis. Lane-1, PDK-9; Lane-2, K-314 plasmid; Lane-3, pUC18 plasmid; Lane 4,BL21; Lane-5, T2; Lane-6, T4; Lane-7, T1; Lane-8, T3; Lane-9, T5. Expected position of 140 MDa, 2.8MDa, 2.1MDa, 1.8MDa, 1.6MDa and 1.0MDa plasmid DNA is shown by an arrow.

T= Transformed Cell

3.14.4 Restriction digestion of the Transformed Plasmid

Plasmid DNA was prepared from the transformed cell and digested with the restriction enzyme HindIII. Restriction endonuclease digestion of the transformed plasmid showed single plasmid of 2.8 MDa size was transformed into BL21.

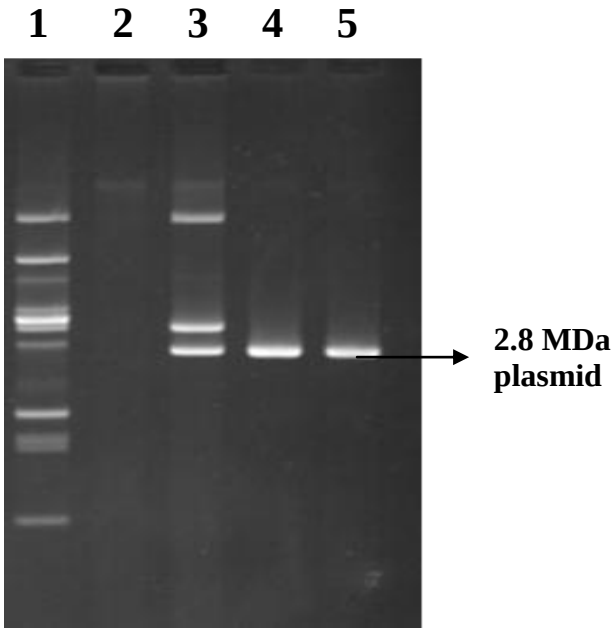


Figure 3.28 Agarose gel electrophoresis of Hind III restriction enzyme digested transformed plasmid. Plasmid DNA was isolated and digested with Hind III restriction enzyme. Lane-1, digested K-314 plasmid; Lane-2 digested BL21; Lane-3, digested plasmid of T1; Lane-4, digested plasmid of T2; Lane-5 digested plasmid of T4 Expected position of 2.8 MDa plasmid DNA is shown by an arrow.

3.14.5 Cytotoxicity assay of transformed bacteria on HeLa cells

Cell lysate of the transformed bacteria was prepared and tested on the HeLa cells for their cytotoxicity potential. It was observed that the transformed cell T2, containing the 2.8 MDa plasmid showed strong cytotoxic effect (80% cell death) on HeLa cells (Figure 3.29) indicating cytotoxic factor(s) may be located in this small plasmid. Further extensive study will be needed to conclude this finding.

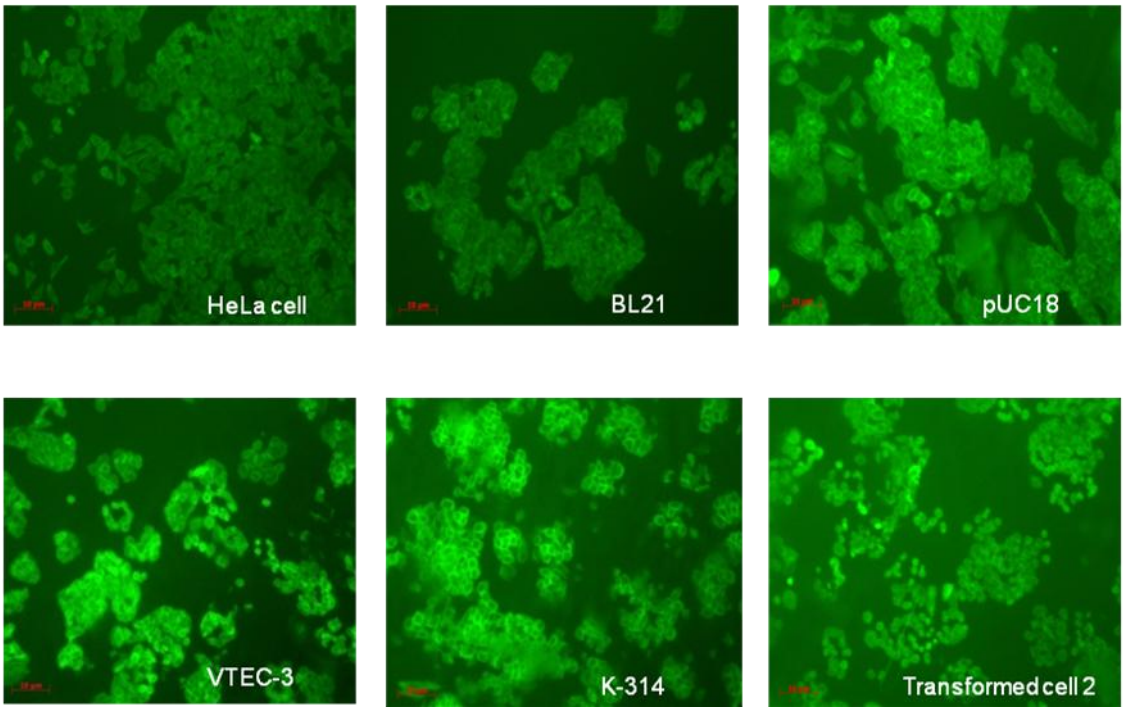


Figure 3.29 Cytotoxic effect of transformed bacteria into HeLa cells. HeLa cells were seeded on culture plates and incubated overnight. Cells were treated with cell lysate *S. flexneri* 1c, BL21, BL21 containing puc plasmid, VTEC-3, and Transformed cell 2. The morphological changes of the HeLa cells with respected cell lysate were confirmed by staining actin filament with FITC conjugated Phalloidin.

Table 3.9 Characterization of Transformed Cell

Strain	Plasmid	Test for invasiveness		Presence of toxin and virulence gene						Presence of TTSS genes							Cytotoxicity
		Sereny test	Congo red binding	ipaH	ial	set	sen	stx1	stx2	ipaBCD	ipaC	ipaD	mxiA1	mxiA2	mxiC	virA	
K-314	3.4, 2.8,2.1,1.8,1.6,1.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
BL21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pUC18	1.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Transformed Cell 1	3.0, 1.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Transformed Cell 2	1.8, 2.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Transformed Cell 3	3.0, 1.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Transformed Cell 4	3.0, 1.8, 2.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Transformed Cell 5	3.0, 1.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

3.15 PFGE analysis of 140 MDa lacking *S. flexneri* 1c

PFGE analysis of *XbaI* digested chromosomal DNA of *S. flexneri* 1c strains yielded 13 to 18 reproducible DNA fragments ranging in size from approximately 20 to 1,050 kb. Three major PFGE types designated A, B, and C were obtained among the serotype 1c strains, of which71% of the strains were of type A, 19% were of type B, and10% were of type C. PFGE analysis of 17 *S. flexneri* 1c strains lacking 140MDa plasmid revealed a closely related banding pattern, belongs to a single PFGE type A with 90% similarity indices (Figure 3.30). This PFGE type is also present in the *S. flexneri* 1c strain containing 140 MDa plasmid. This finding suggested that the cytotoxicity attributed by these particular strains may be due to extra chromosomal DNA.

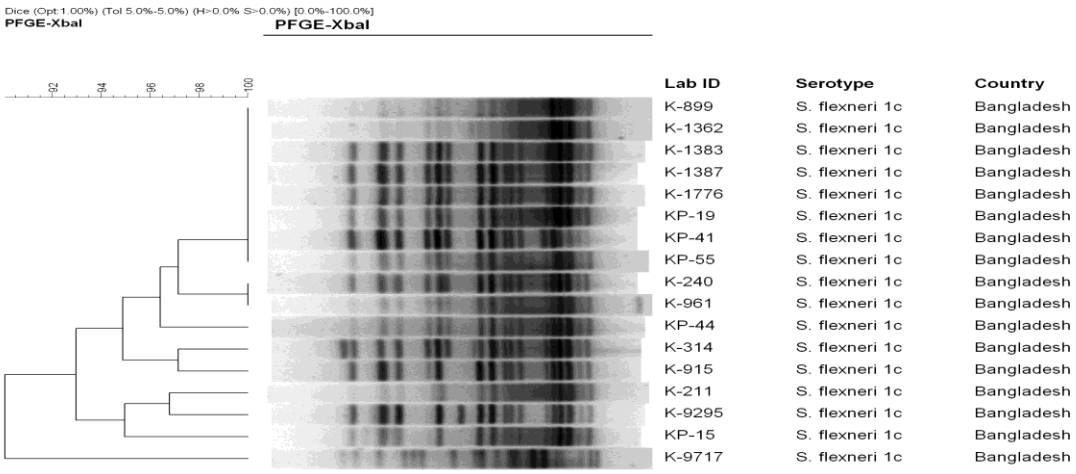


Figure 3.30 PFGE analysis of the XbaI digested chromosomal DNA of *S. flexneri* 1c strains.

4.0 Discussion

Shigellosis remains a major cause of morbidity and mortality among children in developing countries as well as in industrialized countries (Shears, 1996; Red Book, Report of the Committee on Infectious Disease 1997) The higher mortality in developing countries is likely because of certain serotypes of *Shigella* which are common and are frequently associated with severe infections (Khan *et al*., 1999; Nathoo *et al*., 1998; Chopra *et al*., 1997). Clinical manifestation and the associated risk factors have been reported both from developed and developing countries based on the predominant serotypes of *Shigella* isolated from respected regions (DuPont *et al*., 1995; Bennish, 1991). Inspite of the clinical data that are available with *Shigella* infection, there are strong reasons that further such studies are needed to expand the knowledge for better understanding the molecular mechanism of toxin induced mammalian cell deaths.

Most series of case reports in association with *Shigella* infection have suggested that both intestinal and extra intestinal complications are most common in patients infected with *S. dysenteriae* type 1, which is more frequently reported from developing countries (Bennish, 1991; Khan *et al*., 1999). However, previous retrospective studies showed that in Bangladesh fatality rates among hospitalized patients were highest in *S. sonnei* and lowest in *S. dysenteriae* type 1-infected patients (Bennish *et al*., 1990). The most frequent and alarming local and systemic complications of the intestinal obstruction, the hemolytic-uremic syndrome, the leukaemoid reaction, sepsis, hypoglycaemia, convulsion, unconsciousness with *Shigella* infection have been documented independently focusing into one specific complication (Butler *et al*., 1989; Bhattacharya *et al*., 1988; Butler *et al*., 1987; Struelens *et al*., 1985). To our knowledge no one has prospectively examined the clinical manifestation of shigellosis presenting simultaneously both local and systemic complications in areas where all four species of *Shigella* are common.

Infection of *Shigella* results various types of complication especially in young children. Complications in Shigellosis include leukemoid reaction, and haemolytic uraemic syndrome, seizure, encephalopathy, abdominal cramps, rectal pain and septicemia, which significantly increase morbidity and mortality. All these manifestations are difficult to

explain solely on the basis of cytotoxic principles. Neurological (seizure, convulsion) and renal (HUS) complications have been reported to be associated with infection of certain *Shigella Spp*. and *E coli*. However, neurotoxic principle(s) in *Shigella* has not yet been discovered. It is hypothesized that neurotoxin(s) produced by *Shigella* is associated with the development of neurological complications such as seizure, convulsion etc in Shigellosis.

A systematic study to identify and characterize neurotoxic factor(s) present in the *Shigella* spp. was attempted. In order to study the involvement of neurotoxic factor(s) in developing clinical complication; several *Shigella* strains isolated from various types of *Shigella* infection including seizure, convulsion, and HUS patients were screened for the presence of known toxin genes by PCR. Profile is required to understand the role of toxin in developing neurological manifestations in shigellosis. The molecular mechanism of *Shigella* toxin-induced signaling pathway(s) to cause neuronal cell death was studied. To study neurotoxic activity a rat neuron culture model was initially used. Understanding the molecular mechanism of toxin-induced activation of DNA damage signaling pathways is useful in developing inhibitors to block signal(s) that lead to damage of nervous system and other organ failure in shigellosis.

A total of 385 *Shigella* strains isolated from diarrhoeal patients from icddr, b was included in this study. Of these, 238 were *S. flexneri*, 80, *S. dysenteriae*; 42, *S. boydii* and 25, *S. sonnei*. All these strains were analyzed for the large invasive plasmid (140MDa) and subjected to PCR for the presence of known toxin gene in *Shigella* such as *set, sen* and *stx*. Several virulence and genes associated with TTSS were also screened. Cytotoxic (using HeLa cells) and neurotoxic (using cerebellar granule neurons) activity present in *Shigella* cell lysate and culture supernatant were determined. Culture supernatants were analyzed for the ability to cause fluid accumulation in Rabbit ileum. DNA fragmentation and chromatin condensation assay were used as markers of apoptosis. Activation and release of different apoptotic protein such as phosphorylation of H2AX protein and release of Cytochrome C were assayed by western blotting. And the cellular biological status of cells at different phases of cell cycle was analyzed by FACS.

The randomly selected *Shigella* strains were analyzed for the presence of 140MDa plasmid and toxin genes such as *set*, *sen*, stx_1 and stx_2 commonly present in *Shigella* by

PCR. Although there is a little published data available on the association of plasmid DNA of *S. flexneri* and their serotypes, Previous published reports have revealed that heterogeneous plasmid population in *Shigella*, most are smaller than 6MDa (Jamieson *et* al., 1979; Tacket *et* al., 1984). A significant association was observed between the presence of these small plasmids and ecology and/or pathogenecity of the isolates. The presence of additional plasmids patterns related to the particular serotypes suggests that plasmid profile may be useful tool in distinguishing between serotypes of *Shigella*. It may also possible to document the appearance of any new strains in a community by these patterns (Haider *et al*., 1989). Plasmid analysis of 385 *Shigella* strains showed that almost all serotype of *Shigella* contained the 140MDa plasmid except *S. sonnei* and certain strains of *S. flexneri* 1c which did not harbor the virulence plasmid but shared common plasmid pattern (Table 3.1). PCR analysis showed that the strains positive for the 140 MDa plasmid also contained the *sen* gene (located in the 140 MDa plasmid). All *S. dysenteriae* 1 contained stx_1 gene whereas the *set* gene was only present in *flexneri* 2a (Figure 3.2) and some strains of serotype 2b. The 140 MDa plasmid negative *S. flexneri* 1c strains were also negative for the common toxin gene (Table 3.1). Representative strains from these particular *S. flexneri* 1c group were tested for invasiveness (sereny test), indicated that they were noninvasive by sereny and congo red binding ability (Figure 3.4). All strains containing 140 MDa plasmid, showed their invasive trait by sereny and congo red binding. PFGE analysis showed that *S. flexneri* 1c strains yielded three PFGE type irrespective of the presence of large invasive plasmid while the 17 strains belongs to a single PFGE type A (Figure 3.30).

It is known that *Shigella* isolates containing 140 MDa virulence plasmid. It was established that the loss of the virulent plasmid results in avirulent strains (Sansonetti, 2001). But sometimes the genes implicated in virulent functions are localized not only in the virulent plasmid but also in the chromosome. That indicates loss of 140 MDa plasmid from any isolates is not completely avirulent. Over 30 virulent genes are present on the large plasmid of *Shigella* isolates as the determinants for production of invasions, *ipa*B, *ipa*C and *ipa*D; transport functioning ensuring the secretion of the IPA proteins (Menard R., *et al* 1994). TTSS associated genes of *Shigella* spp. were investigated in this study by Polymerase Chain Reaction (PCR). Seven different virulence genes (*mxi*C, *mxi*A1, *mxi*A2, *ipa*BCD, *ipa*C, *ipa*D, and *vir*A) responsible for TTSS in *Shigella* Spp were screened. The major TTSS gene is *mxi* (membrane expression invasion) like *mxi*C, *mxi*A

(Parsot and Sansonetti, 1996). These genes play a critical role for TTSS export proteins into the host cells. TTSSs serve to transfer bacterial proteins into host cells. The *mxi* (membrane expression invasion) genes were chosen because *mxi*A and *mxi*C genes are involved in export secretory proteins, effectively a housekeeping function. To identify which serotypes of *Shigella* spp. are more prevalent compared to others this study specially focused on *S. flexneri* strains because *S. flexneri* are more prevalent and more pathogenic than other serogroup of *Shigella* (Figure 3.5). Of *S. flexneri*, all strains of serotype 1b, 2a, 2b, 3a and 4X were positive for all TTSS genes tested. In case of serotype 1c, 35% strains were positive for these genes. The prevalence of *mxi*C, *mxi*A1, *mxi*A2, *ipa*BCD, *ipa*C, *ipa*D, and *vir*A were 38%, 31%, 31in different serotypes of *S. flexneri* except certain strains of *S. flexneri* 1c was high compared to other serogroup. Although *mxi*A1 and *mxi*A2 gene were in same operon, there prevalence varied in different serotypes. The prevalence of *ipa*BCD gene was screened to determine the presence of large plasmid (140 MDa) of *Shigella* Spp. Because it was found only those isolates that have large plasmid. *ipa*C and *ipa*D gene is responsible for the formation of needle tip. Needle tip is required for TTSS to inject effector proteins into the host cells. Data showed that *S. flexneri* was highly prevalent compared to other isolates in needle formation. Prevalence of *vir*A gene is also high in *S. flexneri* compared to *S. boydii* (83%), *S. dysenteriae* (66.66%), and *S. sonnie* (100%). *vir*A gene is expressed in strains having 140 MDa virulent plasmid. Other genes for TTSS were not found in such strains lacking 140 MDa plasmid. These data strongly showed that genes for TTSS are plasmid mediated. Therefore, these *S. flexneri* 1c strains (devoid of all toxin genes) were extensively used for comparative analysis and partially purified toxin fractions from the representative strains of *S. flexneri* 1c (K-314 and K-915) were used as a source of novel cytotoxic factor(s) in this study.

Although the role of most of the plasmids in pathogenesis remains unknown, several studies provided evidence that the 140MDa plasmid bears genes (*ipa* B, *ipa* C, and *ipa* D.) that are associated with the invasive characteristic of *S. flexneri*. This current study did not identify the 140MDa plasmid in the group of clinical isolates of *S. flexneri* 1c (Figure 3.1). This raises various possibilities: i) invasive genes are present in other plasmids; ii) invasive genes are present in the chromosome; or iii) invasive genes are absent in *S. flexneri* 1c. In order to verify (i) and (ii), both plasmid and chromosomal DNA from these *S. flexneri* strains were isolated and analyzed by PCR to identify these

genes. To rule out the possibility of the involvement of the large invasive plasmid, curing of the plasmid from representative *S. flexneri* 1c (K-212) was carried out (Figure 3.26). After the curing of the large plasmid the lysate from the cured strain (lack of 140MDa plasmid) was tested in the HeLa cell line. The cured strain showed strong cytotoxic effect on the cell line which indicates that the toxin gene (novel) may be present in other small plasmid or it may be chromosome mediated. To identify the toxin producing genes are present in small plasmid or in chromosome, the involvement of small plasmids of representative *S. flexneri* 1c (K-314) strains was investigated. For this investigation transformation had been carried out. Bacterial transformation may be referred to as a stable genetic change brought about by taking up naked DNA (DNA without associated cells or proteins), and competence refers to the state of being able to take up exogenous DNA from the environment. Chilling cells in the presence of divalent cations such as Ca2+ (in $CaCI_2$) prepares the cell membrane to become permeable to plasmid DNA. Cells are incubated on ice with the DNA and then briefly heat shocked (e.g. 42°C for 30-120 seconds), which causes the DNA to enter the cell (Che and Dubnau, 2004). Here an *E. coli* (BL21) strain had been used as a competent strain for taking up plasmid (DNA) from the donor (representative *S. flexneri* 1c K-3I4) strain. For the selection of transformed cells ampicillin containing plate was used because *S. flexneri* 1c K-314 strain is ampicillin resistant and the marker plasmid (pUC18) also harbor the ampicillin resistant gene. Transformed cells were randomly picked up and plasmid analysis confirmed the transfer of the desire plasmid. Through the normal transformation process the plasmid from the donor strain *S. flexneri* 1c K-314 were not transferred and only the marker plasmid (pUCI8) was transferred to the *E. coli* BL21 (Table 3.8). In Amp containing plate, bacterial cells that gained pUC18were able to grow revealing that only the pUC18 plasmid was inserted in recipient *E. coli* BL21 strain. Reason for the failure of K-314 mediated transformation may be due to the lower transformation efficiency or lower transmissibility of K-314 donor plasmids. To overcome this problem another method for transferring the desired plasmid to the recipient *E.coli* (BL21), co-transfection process was used. In this process both the plasmids of *S. flexneri* 1c K-314 and the marker plasmid pUC18 were used together. Plasmids of *S. flexneri* 1c K-314 were transferred to *E. coli* Bl2 (Figure 3.27). The transformed cells were randomly picked up and plasmid isolation was performed to confirm the transformation. Plasmid profile revealed that out of 6 plasmids of *S. flexneri* 1c K-314, 2 plasmids (2.8MDa and 2.1 MDa) were transferred to the *E.coli* BL21cells (Figure 3.27; Lane-5 and 6).

Restriction digestion of the transformed plasmid confirmed the single plasmid insertion into the recipient *E. coli* Bl21 (Figure 3.28).

To detect either the transformed cell had cytotoxic effect or not, toxins were prepared from that transformed cells and applied on HeLa cell. It was observed that the transformed cell had severe cytotoxic effect on HeLa cells (Figure 3.29). The epidemiological investigation of the plasmids of 385 *Shigella* showed that all 238 *S. flexneri* strains contained a small plasmids of 2.6, 2.7 and 2.8 MDa size and 72% of *S. flexneri* had cytotoxic effect on HeLa cells. Transfer of this small plasmid (2.8 MDa plasmid) into the avirulent *E. coli* Bl21 and characteristics changes of these strains (transformed cells) also suggested that the cytotoxicity is not due to 140 MDa plasmid (Table 3.9). It indicates primarily that the small plasmid may contain some novel cytotoxin gene which needs to be investigated. Further analysis will be required to identify the involvement of the small plasmids in this cytotoxic activity as well as the chromosomal involvement.

The mechanism by which *Shigella* produces toxic effects on a variety of human cell types still needs to be understood. A group of scientists believe that the toxin-producing ability of *Shigella* plays a major role in pathogenesis (Vargus *et al.*,1999), although this is still a controversial hypothesis. Concentrated culture supernatant and cell lysate of different serotypes of *Shigella* were assayed in HeLa cells for the cellular cytotoxic potential. The degree of cytotoxicity differs in different species of *Shigella*. Around 72% (172/238) strains of *S. flexneri* possessed the cytotoxic activity whether they contain the *set, sen* or *stx* genes or not (Figure 3.6). Of *S. flexneri*, serotype 1c had severe effect (more than 85% cell death) on HeLa cell followed by serotype 1b and 1a (>85% cell death). Serotype 2a, 2b, X variant and type 4 had almost similar cytotoxic effect (>70% cell death). The culture supernatant from serotype 3a, Y variant, 4X, 6a and 6b showed less cytotoxic effect (40-45% cell death). In case of *S. dysenteriae* (n=80), only serotype 1 and some strains of serotype 4 showed cytotoxic effect (>90%). In contrast, *S. boydii* supernatant caused elongation of the HeLa cells whereas *S. sonnei* caused the rounding of the cell with less frequency. Cytotoxic activity was determined by studying the morphological changes (rounding) under an inverted microscope (Figure3.7) and and later determined by nuclear condensation assay. It is interesting to note here that the 17

S. flexneri 1c strains lacking the 140 MDa plasmid and toxin gene showed strong cytotoxic effect (90%) on HeLa cells (Figure3.8) in a dose dependent manner indicating the presence of cytotoxic factors. Viability of cell was determined by MTT assay (Figure 3.8). Each experiment was repeated more than three times showing similar results and data indicated significant difference between the treatment and the control group ($p \leq 0.0005$). The identification of *S. flexneri* 1c to be deficient in major toxin genes suggested that *S. flexneri* 1c contains toxin genes that are different from those studied here.

To purify and characterize the toxic factor (novel) present in this particular group of *S. flexneri* 1c strains, culture supernatant from the representative strain K-314 (devoid of all known toxin genes) was partially purified with ammonium sulphate. SDS-PAGE analysis of the representative strain of *Shigella* contain different toxin gene such as *set, sen* and *stx*1 showed completely different protein profile compared with the toxin gene negative *S. flexneri* 1c strain (data not shown). HeLa cells were also treated with 40%, 60% and 80% fractions of *S. flexneri* 1c K-314 strain. However, only 60% fraction showed strong cytotoxic activity (Figure 3.9). Again cytotoxic activity of the 60 % toxin fraction in HeLa cells with different doses and time interval were performed and the results showed that percent cell death increased with increasing doses and times. The 60% toxin fraction was further purified and the fractions (F1, F2, F3 and F4) were also studied for cytotoxic activities. The F3 fraction was found to have strong cytotoxic activity and this fraction was considered as the active fraction. This fraction contains a active protein component molecular mass of 100–125kDa which is sensitive to heat and trypsin. Further analysis showed that toxic factors in *S. flexneri* 1c exhibited biochemical properties similar to those of protein. When lysates from *S. flexneri* 1c (K-314 and K-915) were heated and their cytotoxic activity studied in HeLa cells, there was a significantly reduced ($p \leq 0.0002$) cytotoxic activity (Fig. 3.10). *S. flexneri* 1c (K-314 and K-915) lysates treated with DNase, and RNase did not affect cytotoxic activity, while the *S. flexneri* 1c (K-314) lysate treated with protease showed a significant reduction ($p \leq 0.0018$) in its cytotoxic activity (Fig. 3.11). Similarly, protease treatment destroyed the cytotoxic activity of lysates from K-915 while treatment with DNase or RNase had no effect. These results suggest that cytotoxic factors in *S. flexneri* 1c are possibly protein in nature.

Shigella strains are a diverse group in terms of their capacity to cause serious disease in humans, and their ability to adhere to intestinal epithelial cells and to colonize the human gut is undoubtedly one of the key determinants of virulence. At present, the processes involved in establishment and maintenance of gut colonization by *Shigella* are poorly understood. *Shigella* colonization of the gut by adhering to intestinal epithelial cells has been established and survived the harsh conditions of the stomach. It is generally assumed that the colon and perhaps also the distal small intestine are the principal sites of *Shigella* colonization in humans, although this has not been demonstrated directly. *In vitro* adherence of *Shigella* has been examined by using several different epithelial cell lines under a range of experimental conditions, and several adherence phenotypes have been described. However, interpretation of the significance of the studies is complicated by the fact that adherence to non-polarized epithelial cells in tissue culture (even those of human colonic origin) may not be an accurate reflection of molecular interactions that occur between *Shigella* and human colonic epithelium *in vivo*. In this study, total or partially purified culture supernatant from *S. flexneri* bacteria was investigated to find their effect on human cells in culture condition. It was found that these bacterial products induced mammalian cell death, indicating a possible mechanism of some clinical manifestation of shigellosis. In another study with humans, it was suggested that *Shigella* diarrhea results from colonic dysfunction without evidence for an increased small bowel flow rate (Butler *et al*., 1985). Although the intestinal site may be for the secretion responsible for *Shigella* diarrhea, one may postulate that the secretion is a consequence of toxin produced by the *Shigella* spp. If Shiga toxin is responsible for the diarrhea of shigellosis, a key question is how can a protein synthesis inhibiting toxin also elicit fluid secretion in the intestine? Shiga toxin does not appear to act like cholera toxin or *E. coli* heat labile toxin. Although Shiga toxin can elevate the cyclic adenosine monophosphate (cAMP) content of rabbit intestinal mucosa (Donowitz *et al*., 1975), as can cholera toxin and *E. coli* heat-labile toxin, Shiga toxin induced accumulation of cAMP does not occur until long after fluid secretion begins. Shiga toxin also does not appear to act like *E. coli* heat-stable enterotoxin.

Preliminary results indicated that toxic factors in *S. flexneri* 1c are different from STX, ShET-1 and ShET-2. Identification and characterization of toxic factors in *S. flexneri* 1c may provide critical information in understanding the molecular basis of *Shigella*-induced cytotoxic effect on different types of human cells. *Shigella* infected patients

initially suffer from watery diarrhea, followed by bloody diarrhea and hemorrhagic colitis (O'Brien *et al*., 1983; Riley, 1987; Riley *et al.*,1983). Consistent with clinical manifestations, these strains induce fluid accumulation in experimental rabbit ileal loops. In order to study the fluid accumulation by *S. flexneri* 1c (K-314) partially purified culture supernatant fractions were tested for its ability to induce fluid accumulation in rabbit ileal loop. Results showed that Different toxin fraction of *S. flexneri* 1c did not induce fluid accumulation in the rabbit ileal loop (Figure 3.12), however, lysates from *S. flexneri* 2a, *S. dysenteriae* 1 did induce fluid accumulation (data suggesting a possible difference between *S. flexneri* 1c toxins (novel cytotoxic factor) and other toxin genes (*set, sen, stx* 1 and 2). It was also found that mucus diarrhea was produced within the first 48 hours and continued for 2 days in the RITARD model test (Table 3.3), when rabbits were inoculated with *S. flexneri* 1c (K-314). Although it is impossible to ignore a possible contribution of additional toxin(s), novel cytoxic factor present in *S. flexneri* 1c is considered to be the major, if not the only contributor in exhibiting cytotoxic activity.

An alternative mechanism by which Shiga toxin could cause net fluid secretion is to block (or inhibit) fluid absorption. Indeed, Keenan *et al*., (1986) reported that purified Shiga toxin and purified *E. coli* Shiga like toxin selectively destroyed the mature absorptive epithelial cells of the rabbit ileum. Shiga toxin binds to mature absorptive epithelial cells through glycolipid receptors, inactivates protein synthesis in the host cells, and ultimately causes the death of those cells. However, diarrhea would result from inhibition of absorption rather than from active secretion. The histopathological hallmark of disease caused by Stxs is the destruction of blood vessels serving the colon, kidney (especially renal glomeruli), and central nervous system. However, the histological sections obtained from the ligated ileal segments treated with *S. flexnei* 1c showed moderate inflammatory infiltrate in lamina propria and in submucosa of the wall of the intestine. It also shows sheared off of tips villi in some places with Grade-3 inflammation (Figure 3.13, A1&A2) while S. dysenteriae caused severe tissue necrosis with Grade-4 inflammation (Figure 3.13, B1&B2). The recently described Shiga like toxin elaborating strains of *E coli* O157:H7 have been identified as the etiologic agents of hemorrhagic colitis, but the organisms are not believed to penetrate the epithelial cells. It is likely that these strains possess attachment factors which allow them to colonize the colonic epithelium, bring them in close association with the epithelial cell, and thus allow them to deliver toxin directly to the cell.

Previously, it was accepted that enteropathogens caused either watery or bloody diarrhea depending on the virulence factors they express. However, the number of microorganisms associated with a rather broad clinical spectrum, from watery to bloody diarrhea, is increasing (Black *et al*., 1988). It is now known that invasive pathogens may also produce enterotoxins, which are able to cause hyper secretion in the absence of an inflammatory response, and this could be the reason for the watery phases often seen in shigellosis (Keusch and Benish, 1989). Therefore, the *S. flexneri* 1c toxin probably has a damaging effect on the intestinal epithelium, and the fluid accumulation could then be a secondary effect. However, toxins may also participate in the pathogenesis, although the extent of their role is not yet clear. Shiga toxin causes damage to villus cells destroying absorptive cells and causing net fluid secretion (Keenan *et al*., 1986).

Neurological complications, particularly seizures, are common in shigellosis, often preceding onset of diarrhea, and are estimated to occur in 12% to 45% of children (Avital *et al*., 1982; Ashkenazi *et al*., 1983). Other complications, mainly transient encephalopathy and hallucinations, are less common (Avital *et al*., 1982; Ashkenazi *et al*., 1989). Peripheral neuritis is a rare complication reported mainly in adult patients (Barret Connor *et al*., 1970). Both seizures and transient encephalopathy are considered benign and rarely recur or leave permanent sequelae (Avital *et al*., 1982; Ashkenazi *et al*., 1983; Zvulunov *et al*., 1990). Fatal shigellosis characterized by a rapid onset of neurological abnormalities with only mild colitis was first described in Japanese patients during the first half of this century and was considered to be a distinct clinical entity named the Ekiri syndrome (Shiga K, 1936; Dodd *et al*., 1949). It occurred mainly due to *S. sonnei* infection and was attributed to hypocalcemia, coinfection, or a toxin, but the pathogenesis was never established. Only five cases of fatal toxic encephalopathy due to shigellosis have been described in the past few years. One report of three cases aged 9 to 11 years, all due to *S. flexneri*, showed a typical encephalopathic clinical and pathological pattern (Sandyk *et al*., 1983). Another report is of a 3-year old who had encephalopathy and a "Reye-like" metabolic derangement also due to *S. flexneri* (Heldenberg *et al*., 1986). The latest report is of a preschool girl who also had a fulminating encephalopathy associated with *S. flexneri* infection (Akl and Haned, 1989). The neurological symptoms in human shigellosis have often been attributed to a cell-free neurotoxin, the "Shiga toxin." It has been shown to cause focal brain hemorrhage and limb paralysis in rabbits but its pathogenic role in human shigellosis has never been

proven (Avital *et al*., 1982; Levine, 1982). A study from Bangladesh noted a 29 percent mortality rate in affected children compared with 6 percent in those without neurologic symptoms (Khan *et al*., 1999). In a retrospective study in icddr,b Bangladesh between 1990 and 2010, 70 diarrhoeal patients were admitted in the Dhaka treatment center operated by icddr,b with the history of neurological complication. Among those patients, *S. flexneri* was the major pathogen isolated, which indicate that this complains may be due to the neurotoxic effect of *S. flexneri* toxin. In this study, it was observed that after administration of the *S. flexneri* 1c intraperitoneally in mice, the signs of toxin effect appeared after 36 hours including hind limb paralysis, rapid breathing and finally mice died (Figure 3.14). These results are consistent with Richardson *et al*., (1992), whose studies produced disruption of the nerve supply to the limbs and caused paralysis. More recently, Obata *et al*., (2008) reported that the neuron is a primary target of *Shigella* toxin, affecting neuronal function and leading to paralysis. The clinical illness was dominated by neurologic involvement and frequently culminated in death and limb paralysis.

Toxins are potent molecules used by various bacteria to interact with a host organism. Some of them specifically act on neuronal cells (clostridial neurotoxins) leading to characteristics neurological affections. But many other toxins are multifunctional and recognize a wider range of cell types including neuronal cells. Various enterotoxins interact with the enteric nervous system, for example by stimulating afferent neurons or inducing neurotransmitter release from enterochromaffin cells which result either in vomiting, in amplification of the diarrhea, or in intestinal inflammation process. Other toxins can pass the blood brain barrier and directly act on specific neurons.Multiple types of toxin and their corresponding protein products have been discovered in *Shigella* spp and EIEC (O'Brien *et al.*, 1992; Sansonetti, 1998); a clear understanding of the role of toxin in Shigellosis remains to be studied. Shiga toxin has also been shown to induce nuclear DNA damage in human endothelial cells and releases single-stranded DNA (Brigotti *et al.*, 2002). This toxin is mostly present in *S. dysenteriae* 1 and Shiga-toxin producing *E. coli*. In addition, two enterotoxins have been identified in *S. flexneri*: *Shigella* enterotoxin 1 (ShET-1) encoded by the *set*1 chromosome gene and *Shigella* enterotoxin 2 (ShET-2) encoded by the *sen* gene (140 MDa plasmid) (Vargas *et al*., 1999). Interestingly, a group of clinical isolates of *S. flexneri* strains have been identified that do not contain any of *stx, set,* or *sen* genes. However, these strains exhibit very

strong cytotoxic activity against HeLa cells. With the help of rat brain neuron culture model the neurotoxicity of these strains were studied. The granular neurons were isolated from the rat pups and plated on to the PLL coated plate. After administration of the lysate from *S. flexneri* 1c and overnignt incubation the average total neurite length per neuron and its branches was determined. The average length per neuron was determined from a sample of at least 100 neurons from random fields. The total neurite length was calculated as the sum of the lengths of individual neurites. Reduction of the neurite length indicates the presence of neurotoxic factor. Using rat brain (cerebellar) neurons culture model it was found that these strains bear strong neurotoxic activity (Figure 3.15). The neurotoxic principle was studied by biochemical fractionation. Recently, Shiga toxin and Shiga-like toxins I and II were proven not to be essential for the development of neurological manifestations in five patients who presented with seizures or encephalopathy. Another neurotoxic protein was implicated in the pathogenesis of the neurological disease (Ashkenazi *et al*.,1990). The data generated from this indicate that the neurotoxic activity is not due to *stx, set*, or *sen* indicating that some *Shigella* strains may have additional cytotoxic factor(s). These results are consistent with Ashkenazi *et al.*, 1990, whose studies indicated the presence of other neurotoxic factor in *Shigella*.

In order to study more detail on the molecular basis of toxin induced mammalian cell death, it was attempted to investigate how mammalian cells are killed by *S. flexneri* 1c. In this study it was found that toxin induced morphological changes of cells and activation of signaling pathways of apoptosis indicating that *Shigella* most likely kills mammalian cells by apoptosis. Apoptosis or programmed cell death is a genetically regulated process, which is characterized by plasma membrane blebbing, cell shrinkage, nuclear condensation, chromosomal DNA fragmentation and formation of apoptotic bodies (Wyllie *et al*., 1997). Toxin induced apoptosis is an important process in the pathophysiological response of humans to the bacterial toxin. Stx kill epithelial cell (e. g., Vero cell) *in vitro* and a concept that Stxs may directly target epithelial and vascular endothelial cells for damage has emerged. Apoptosis has been reported in several different cell types as a result of Stx1 and Stx2 action associated with enterohemorrhagic *E. coli* disease, the phases of hemorrhagic diarrhea (Michino *et al*., 1999; Riley *et al*., 1983), hemolytic uremic syndrome (Karmali *et al*., 1985) and neurological damage (Hamano *et al*., 1993; Tesh *et al*., 1991; Yasuhara *et al*., 2000). The relative importance of Stx induced apoptosis in human disease is becoming apparent. Indeed, the Stxs cause

apoptosis in human renal proximal tubular epithelial cells (Kodama *et al*., 1999), renal tubular cells (Kaneko *et al*., 2001; Karpman *et al*., 1998), human renal cortical epithelial cells (Karpman *et al*., 1998; Kiyokawa *et al*., 1998), lung epithelial cells (Uchida *et al*., 1999), human endothelial cells (Kita *et al*., 2000), astrocytoma cells (Arab *et al*., 1998), and vero cells (Inward *et al*., 1995). But the mechanisms of apoptosis have not been fully elucidated. To understand the molecular mechanism of *S. flexneri* induced mammalian cell death, it was studied whether the cytotoxicity induced by *S. flexneri* was due to activation of apoptosis.

Lysates from *S. flexneri* 1c cytotoxic activity against HeLa cells suggest the presence of cytotoxic factors. Research has demonstrated that pathogenic bacteria activate different death signaling pathways depending on the type of bacteria. For example, *Shigella*, *Salmonella*, *Yersinia*, and *Mycobacterium* induce apoptotic pathways in host cells (Fratazzi *et al*., 1997, Monack *et al*., 1996, Ruckdeschel *et al*., 1997., Zychlinsky *et al*., 1992.), *S. flexneri* activate necrosis in human macrophages (Fernandez-Prada *et al*., 2000.), and *S. flexneri* activates the autophagy signaling pathway (Suzuki *et al*.,2008). Although *S. flexneri* 1c lacks major bacterial toxin genes, results of this current study in *S. flexneri* 1c treated HeLa cells showed an increase in chromatin condensation, a marker for apoptosis (Figure 3.17).

In the present study, to describe a pathway of *S. flexneri* induced apoptosis in one cell type, HeLa cells, served as a model. The apoptotic program is characterized by certain morphological features. *S. flexneri* induced morphological changes (Figure 3.16) including plasma membrane such as loss of membrane asymmetry and attachment, a condensation of the cytoplasm and nucleus (Figure 3.17) and also internucleosomal cleavage of DNA (Figure 3.18). Kerr *et al*., (1971) reported that apoptotic cells round up along with chromatin condensation, leading to the formation of crescent shaped masses aggregating at the membrane. These initial changes are followed by the cytoplasmic and nuclear codensation and ultimately by DNA fragmentation of the cells. DNA fragmentation is one of the critical markers, which is used to identify apoptotic cells (Fraziska *et al*., 1994). Members of the ribosome inactivating proteins (RIPs) that includes Stx and ricin have been shown to deadenylate ribosomal RNA (Barbieri *et al*., 1994, 1997, 1998 and 2000; Nicolas *et al*., 2000) suggesting that inhibition of protein synthesis may play a key role in RIP induced cell death. However, protein synthesis

inhibitory role of Stx in inducing apoptosis in mammalian cells has remained controversial (Sakamoto *et al*., 2003). Stx has also been found to induce expression of additional signaling pathway(s) leading to apoptosis. Recently, RIPs have been shown to induce DNA and RNA fragmentation *in vitro* and *in vivo* (Barbieri *et al*., 1997; Brigotti *et al*., 2002). It has also been shown that small pieces of DNA induce apoptosis in mammalian cells (Schiavone *et al*., 2000; Tidd *et al*., 2000; Milyavsky *et al*., 2001; Nur-E-Kamal *et al*., 2003). In this study it was also found that *S. flexneri* induced the DNA fragmentation another indication of apoptosis (Figure 3.18). These results clearly indicated that apoptosis is the major; if not the sole pathway involved in *S. flexneri* induced mammalian cell death. In this study, markers of necrosis, autophagy was not studied. Therefore, activation of other signaling pathways for mammalian cells could not be ignored. Studying activation of these signaling pathways will be useful to confirm contribution of apoptosis in *S. flexneri* induced mammalian cell death. Similar to other study by Talukder *et al*., 2012 it was observed that some of the proapoptotic marker protein that is phosphorylation of H2AX (Figure 3.20) and cytochrome C was released (Figure 3.19) that leads to to activation of caspase pathway and apoptotic cell death of mammalian cells. These results suggested that toxin from *S. flexneri* might activate additional pathways leading to apoptosis. Although the DNA fragmentation proved that the cellular death is probably mediated by apoptosis, it was unable to depict the degree of DNA fragmentation in this study.

Genomic (DNA) of a cell is under constant surveillance to monitor the damage in it. If any damage is detected, a cell halts it's progression of cell cycle until repaired. Extensive research has been carried out to understand the molecular basis of signal produced during genome damage and cell cycle arrest. In recent years it has been demonstrated that cell cycle checkpoints are layers of control that act to delay CDK activation when defects in the division program occur. As the CDKs functioning at different points in the cell cycle are regulated by different means, the various checkpoints differ in the biochemical mechanisms by which they elicit their effect. However, all checkpoints share a common hierarchy of a sensor, signal transducers and effectors that interact with CDKs. Cells whose DNA is damaged by irradiation with UV light or chemical modification become arrested in G1 and G2 until the damage is repaired. Arrest in G1 prevents copying of damaged bases, which would fix mutation in the genome. Arrest in G2 prevents copying of double stranded breaks to be repaired before mitosis. If

the DNA damage is too extensive to repair, the cell commits suicide via apoptosis.

It remains to be known whether *S. flexneri* could mimic DNA damage signaling and causes cell cycle arrest. In this study, *S. flexneri* 1c treatment affected progression of cell cycle in HeLa cells as detected by FACS analysis at different time intervals (Figure 3.24 & 3.25). The data from the FACS analysis indicated that untreated HeLa cells were progressed through normal cell cycle (Fig. 3.21 & 3.25) while *S. flexneri* treated HeLa cells were arrested at G0/G1 phase. The percentage increased gradually from 17.25 to 51.52 (Figure 3.24 & 3.25) indicated that *S. flexneri* 1c induced G0/G1 cell cycle arrest and was responsible for apoptosis. Mammalian cells respond to DNA damaging agents by activating cell cycle checkpoints. These control mechanisms determine a temporary arrest at a specific stage of the cell cycle to allow the cell to correct possible defects (Hartwell and Weinert, 1989; Hartwell and Kastan, 1994). At least two checkpoints monitor DNA damage: one at the G1/S transition and the other at the G2/M transition. More studies are required to confirm and understand these preliminary results of the molecular mechanism of *S. flexneri* induced cell cycle arrest and apoptosis.

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Appendix-I

Media composition

The composition of the media used in this thesis work is given below.

MacConkey Agar (Difco)

Ingredients	Amounts (g/l)
Peptone	17.0
Protease peptone	3.0
Lactose	10.0
Bile salts no. 3	1.5
Sodium Chloride	5.0
Agar	13.5
Neutral red	0.03
Crystal Violet	0.001
Distilled Water	1l
pH 7.1 ± 0.2, auclaved at 120^{0} C for 15 minutes at 15 lbs pressure.	

Trypticase soy borth (Difco)

Ingredients	Amounts (g/l)
Pancreatic digest of casein	15.0
Papaic digest of soyabean meal	5.0
Sodium Chloride	5.0
Distilled Water	1l
pH 7.3 ± 0.2, autoclaved at 120^{0} C for 15 minutes at 15 lbs pressure.	

Dulbecco's Modified Eagles Medium (DMEM)*

Ingredients	Amounts (g/l)
Glucose	High
L-glutamine	Supplemented
Pyridoxine HCl	Supplemented
Na-pyruvate	110 mg/L
$NaHCO_3$	3.7 g/L
Distilled water	**1L**
pH : 7.2 ± 0.2	

Neuron Culture Media

Ingredients	Amounts (ml)
Neurobasal Media (Gibco)	500ml
Glutamine	1.25 ml
B27	5 ml
Penicillin-Streptomycin Solution	5 ml

Appendix-II

Buffer and solutions

The preparation of buffer and solutions used in this thesis work are given below.

Tris-HCl

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

3 M NaCl

175.3 g of NaCl was dissolved in distilled water to a final volume of 1 Litre. The solution was autoclaved and stored at room temperature.

Phosphate buffered saline (PBS)

PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 2 g of $KH_2\ PO_4$ in 800 ml of distilled water. pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 Litre by distilled water. The solution was sterilized by autoclave and stored at room temperature.

RNase A stock solution (10 mg/ml)

RNase A stock solution was prepared by dissolving 100 mg of RNase A in 10 ml of distilled water and stored in $-20^{\circ}C$ until use.

TE buffer (pH 8.0)

10 mM tris-Cl (pH 8.0), 1 mM EDTA was prepared by diluting concentrated stocks of 1M tris-Cl (pH 8.0) and 0.5 M EDTA. The buffer was stored at $4^{\circ}C$.

TBE buffer (GIBCO-BRL)

The total content of a bag having the formula of 100 mM tris, 90 mM Boric acid, 1.0 mM EDTA was mixed with 0.99 Liter of distilled water to make the 1X concentrated TBE buffer. The buffer was stored at room temperature.

Ethidium bromide solution

Ethidium bromide was dissolved in distilled water at a concentration of 10 mg/ml and stored at 4^0C in the dark.

Gel loading buffer

6% concentrated loading buffer consisted of 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (type 400; Pharmacia), 0.5 mg/ml RNase in water. It was stored at 4^0C in 1 ml aliquot.

0.5 M EDTA

186.1 gm of $Na_2EDTA.2H_2O$ (Disodiun ethylene diamine tetra-acetic acid) and 20 gm of NaOH pellets were added to 800 ml distilled water and dissolved by stirring on a magnetic stirrer. The pH was adjusted to 8.0 with a few drops of 10 M NaOH and final volume was made up to 1 litre with distilled water. The solution was sterilzed by autoclave and stored at room temperature. .

10 M NaOH

40 gm of NaOH pellet was dissolved in distilled water to make the final volume of 100 ml. The solution was stored in air tight bottle at room temperature.

Sodium Pentobarbital solution

Sodium pentobarbital	16.19 gm
Ethyl alcohol	25 ml
Propylene glycol	50 ml
Benzyl alcohol	5 ml
Distilled water	250 ml

Harris Haematoxylin and Eosin stain solution

Haematoxylin crystal	5 gm
Absolute alcohol	50 ml
Ammonium alum	100 gm
Murcuric oxide	2.5 gm
Glacial acidic acid	40 ml
Distilled water	1l

Haematoxylin crystals were dissolved in alcohol at 56°C. Alum was dissolved in distilled water using gentle heat with stirring. The alcoholic haematoxylin solution was added to the hot alum solution with stirring and mercuric oxide was then added slowly. On cooling, 0.2 ml acetic acid was added per 100 ml of solution to enhance precision of nuclear stain.

Acid alcohol

1 ml hydrochloric acid (HCl) was added in 99 ml of 70% ethyl alcohol

Eosin solution

1 gm eosin was dissolved in 100 ml distilled water and added 0.2 ml glacial acetic acid and filtered before use.

10% Fetal bovine

10 ml FBS was added in 90 ml DMEM

4% Paraformaldehyde

4 ml paraformaldehyde was added in 96 ml PBS

0.5% Triton X solution

0.5 ml was added in 99.5 ml PBS