A statistical correlation between virulence genes and clinical features among patients with shigellosis in Mirzapur, Bangladesh

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Submitted by
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To Whom It May Concern

This is earnestly declared that, the research work embodying the results reported in this thesis, entitled "**A statistical correlation between virulence genes and clinical features among patients with shigellosis in Mirzapur, Bangladesh**", submitted by **Dr. Visnu Pritom Chowdhury**, has been carried out by the undersigned under joint supervision of Professor Naiyyum Choudhury, Biotechnology Program, MNS department, BRAC University and Dr. Kaisar Ali Talukder, Head, Enteric and Food Microbiology Laboratory, Center for Food and Waterborne Diseases, icddr,b. It is further declared that the research work presented here is original and suitable for submission for the fulfillment of the degree of Master of Science in Biotechnology in any other academic institution.

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Working in the realm of molecular biology, exploring relentlessly to find something new, always seemed fascinating to me. To be honest, it always felt more like an adventure than mere “work”. And, looking back at the beginning I can still remember the thrill and daunt I had to go through, while boarding onto this very first step into this vast arena of new and unknown.

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Abstract

Even though, the annual number of *Shigella* episodes or fatalities are not as terrifying as was reported in 1999, these numbers are still unacceptable. *Shigella* spp. is still contributing to about 800,000 deaths globally each year in concert with other diarrheagenic pathogens, affecting predominantly young children during the first 5 years of their lives, mostly in sub-Saharan and south Asian region. Therefore, it is still safe to consider *Shigella* as one of the major public health threats, specially in densely populated developing countries like, Bangladesh. This study was aimed at correlating the status of virulence genes among the *Shigella* strains and the corresponding clinical features observed in patients suffering from shigellosis. To achieve this goal, 61 different serotypes of *S. flexneri* strain, isolated from patients enrolled in Mirzapur study, during a period over 2009 to 2013, were randomly selected, and analyzed to detect the presence of 140 MD large virulence plasmid, and virulence (*ipaH, ial, ipaH7.8*), toxin (*set, sen*), and type 3 secretion system related genes (*virB, ipaBCD, ipaB, ipaC, ipaD, ipgC, ipgB1, ipgA, icsB, ipgD, ipgE, ipgF, mxiH, mxiI, mxiK, mxiE, mxiC, spa15, spa47, spa32, spa24, spa*). Simultaneously, two primers for the *mxiE* and *spa15* genes, were designed and annealing temperatures were optimized. And the predictions were confirmed by PCR gel electrophoresis. Of these 61 strains, 140MD plasmid was detected in 79% (n=48) cases. The most prevalent gene was found to be *ipaBCD* (90%), followed by *ial* (89%), *ipgC, ipgE* (85%); *virB, ipgA* (82%); *sen, mxiH, mxiI, spa15, spa47* (80%); *ipgD* (79%), *ipgF* (77%), *spa32* (75%), *ipgB1* (72%), *mxiK, spa24* (70%); *mxiE* (66%), *set* (58%), *icsB* (44%) and *mxiC* (36%). This data was then compared against the clinical features for each corresponding strain, obtained from icddr,b Mirzapur study database, to detect any statistically significant association in between these two sets of data. Interestingly, 20 statistically significant correlation had been observed ($p < 0.05$) while 5 of them were of high statistical significance ($p < 0.01$). Briefly, the two *Shigella* enterotoxin genes (*set, sen*) were found to be significantly associated with multiple important clinical features frequently observed in shigellosis, such as, presence of blood in stool with a bloody mucoid consistency, rectal strain, dehydration, cough, fever and an overall increased state of disease severity, along with four other virulence genes (*ial, ipaBCD, ipgD* and *spa24*). The finding of this study elucidates the increased level of severity of moderate to severe diarrhea in patients infected with *Shigella* strains containing the enterotoxin genes, and also does it's part in building a gene library of this species.

Contents

List of Tables

List of Figures

List of Abbreviations

µg – Microgram

µl – Microliter

ABC – ATP-binding cassette

AGN - Acute glomerulonephritis

AIDS – Acquired Immunodeficiency Syndrome

ATCC – American type culture collection

ATP – Adenosine Triphosphate

bp – Base-pair

CCF – Congestive Cardiac Failure

CFU – Colony Forming Unit

CM – cytoplasmic membrane

cryo-EM - Cryo-electron microscopy

CTD – Carboxy-terminal domain

df – degrees of freedom

DNA – Deoxyribonucleic acid

DSE – donor strand exchange

EC – epithelial cell

EDTA – Ethylene diamine tetraacetic acid

eg. – for example (Latin: exempli gratia)

EIEC – Enteroinvasive *E. coli*

ER – Endoplasmic reticulum

et al. – And others

g – Gram

HeLa cell - Henrietta Lacks cell (Human cervical epithelial cell)

HUS – Hemolytic-uremic syndrome

ial – invasion associated locus

icddr,b – International Center for Diarrhoeal Disease Research, Bangladesh

ics – intracellular spread

Ig – Immunoglobulin

IL – Interleukin

IM – Intramuscular

IMC – inner membrane component

ipa – invasion plasmid antigen

ipg – invasion plasmid gene

IQR – inter-quartile range

IS – Insertion sequence

kb – Kilobase-pair

KD – Kilo Dalton

l - Liter

LPS – Lipopolysaccharide

M cell – microfold cell

mcg – Microgram

MD – Mega Dalton

mEq – milliequivalent

MFP – membrane fusion protein

mM – millimolar

mmol – millimole

MSD – moderate to severe diarrhea

mxi – membrane expression of ipa

NC – Needle-complex

NG – nasogastric

NK cell – Natural killer cell

NMR – Nuclear magnetic resonance spectroscopy

NS - Nephrotic syndrome

NTD – amino-terminal domain

OM – outer membrane

OMP – outer membrane protein

ORF – Open reading frame

ORS – oral rehydration saline

osp – outer S*higella* proteins

P – Calculated probability

PAI – pathogenicity island

PCR – Plymerase chain reaction

PG – peptidoglycan

pH – Negative logarithm of hydrogen ion concentration

pI – Isoelectric point

PMN – Polymorphonuclear neutrophil

RBC – Red Blood Cell

RIP – ribosome-inactivating proteins

RNA – Ribonucleic acid

RND – Resistance-nodulation-division

rpm – Rotation per minute

RT – Room temperature

SDS – Sodium dodecyl sulfate

set – Shigella enterotoxin 1 (ShET-1)

sen – Shigella enterotoxin 2 (ShET-2)

SmD – Streptomycin-dependent

spa – surface presentation of ipa antigen

spp. – Species

SRL – *Shigella* resistance locus

STEC – Shiga toxin-producing Escherichia coli

Stx – Shiga toxin

T_a – Annealing Temperature

Taq – *Thermophilus aquaticus*

TBE – Tris-borate EDTA

TSB - Trypticase soy broth

T3SS – Type III secretion system

UTI – Urinary tract infection

vir – virulence

WHO – World Health Organization

Dedicated to *My Family*

Chapter 1 : Introduction

1.1 Literature Review

1.1.1 Shigellosis

Shigellosis, also known as Bacillary dysentery, is characterized by loose motion (passage of watery stool more than twice a day) mixed with blood and mucous, accompanied with fever and tenesmus. In many cases, complications may arise, such as hemolytic uremic syndrome, leading to fatality. Shigellosis is caused by one of the four serogroups of *Shigella* species, transmitted by feco-oral route. It is endemic throughout the world, specially in the developing countries, particularly among the age group of under 5 children (Bardhan et al., 2010), with a strong correlation with suboptimal hygiene with a very low infectious dose (as low as 10 micro-organisms) (DuPont, HL, Levine, M M, Hornick, RB and Formal, 1989).

1.1.2 The Organism and it's Classification

1.1.2.1 Shigella

Shigella is a genus of gamma proteobacteria within the family *Enterobacteriaceae*. These micro-organisms are Gram-negative, non-motile, non-spore forming, with an optimum growth temperature of 37°C. They are rod-shaped bacteria and appear singly, in pairs or in chains. These bacteria are facultative anaerobes, oxidase negative. They ferment glucose and other carbohydrates without producing gas.

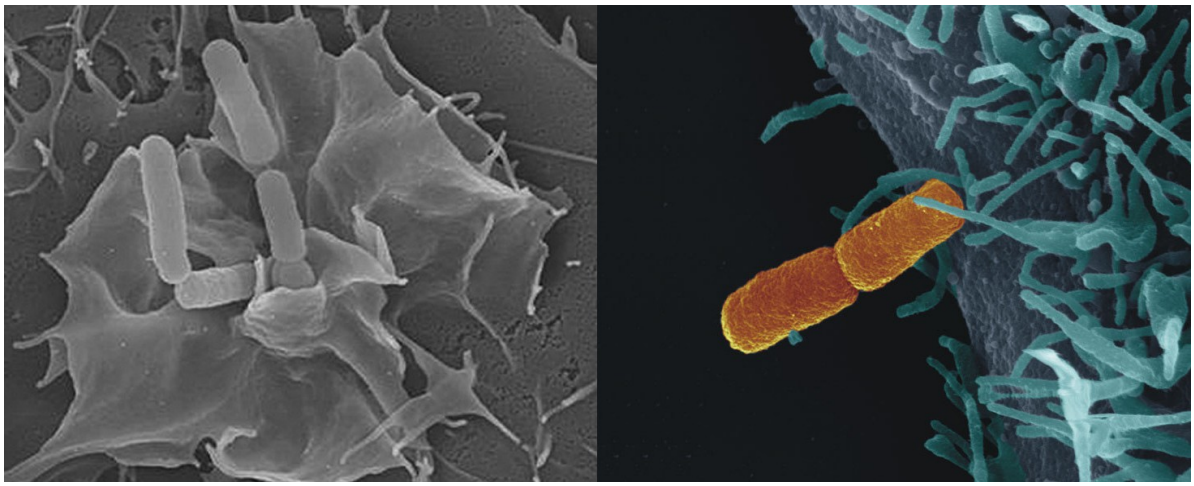

Figure 1.1: Entry by macropinocytosis and contact establishment with host cell by *Shigella*.

Entry point of *Shigella* by macropinocytosis in an epithelial cell (Sansonetti, 2013) (left side); *Shigella flexneri* (orange), establishes contact with a human host cell (blue) (Brinkmann et al., 2010) (right side).

All *Shigella spp.* are lysine decarboxylase negative. Also, they are Voges-Proskaur negative, methyle red positive, do not utilize Simmons citrate, do not produce H_2S. They are arginine dihydrolase and urease negative. *Shigella* are very closely related to *Escherichia coli*. Except *S. dysenteriae* Type 1, rest of the genus are catalase negative.

1.1.2.2 Genome of Shigella

Shigella spp. grows only in human intestine. *Shigella flexneri* is the most prevalent species and 2a is the most abundant serogroup of this species. The circular chromosome (Sf301) is about 4,607,203 bp in length, with 4,434 ORFs (open reading frames), coding percentage of 80.4 and 50.89% G+C content. It consists 314 IS elements (insertion sequence) with 97 tRNA genes.

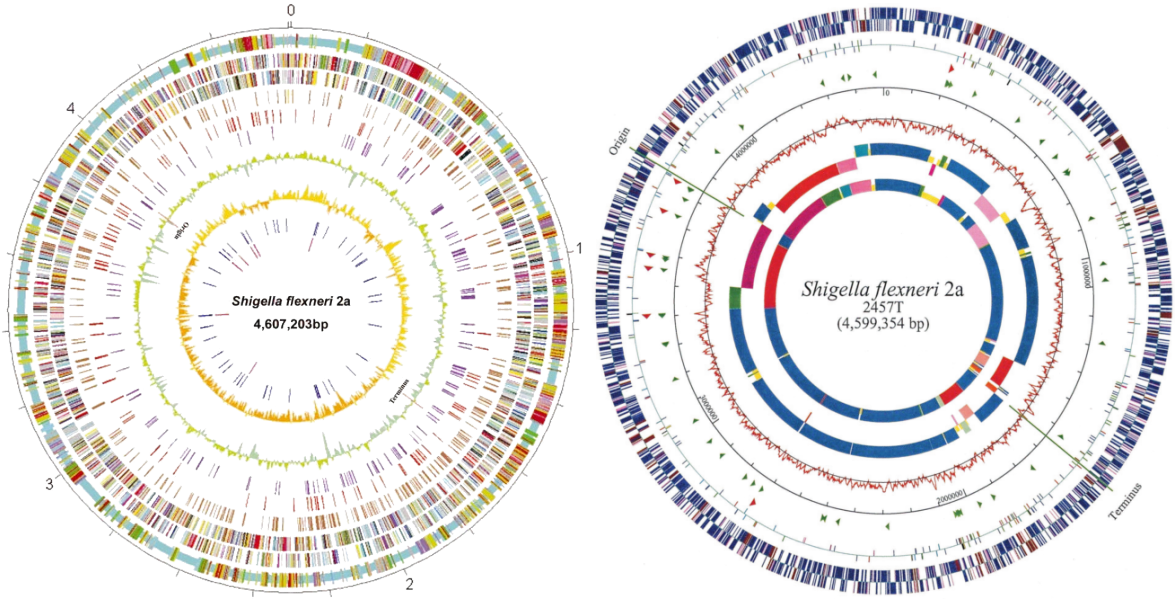

Figure 1.2: Circular chromosomal map of *S. flexneri* 2a compared with E. coli K12.

Circular map of Sf301 chromosome compared with those of *E. coli* K12 MG1655 and 0157 EDL933 (Jin et al., 2002) (left); Circular representation of the *S. flexneri* 2a 2457T genome in comparison with the *E. coli* K-12 genome (Wei et al., 2003) (right).

The plasmid of Sf301 (pCP301) is 221,618 bp long, with 267 ORFs and 76.24% coding sequence. It contains 45.77% G+C content with 88 IS elements. Plasmids from other strains shows similar picture, although with a few deviations, such as, *S. flexneri* 5a plasmid pWR501, has a total length of 221,851 bp, with 293 ORFs, 82.09% coding region and a G+C content of 46.36% and 92 IS elements.

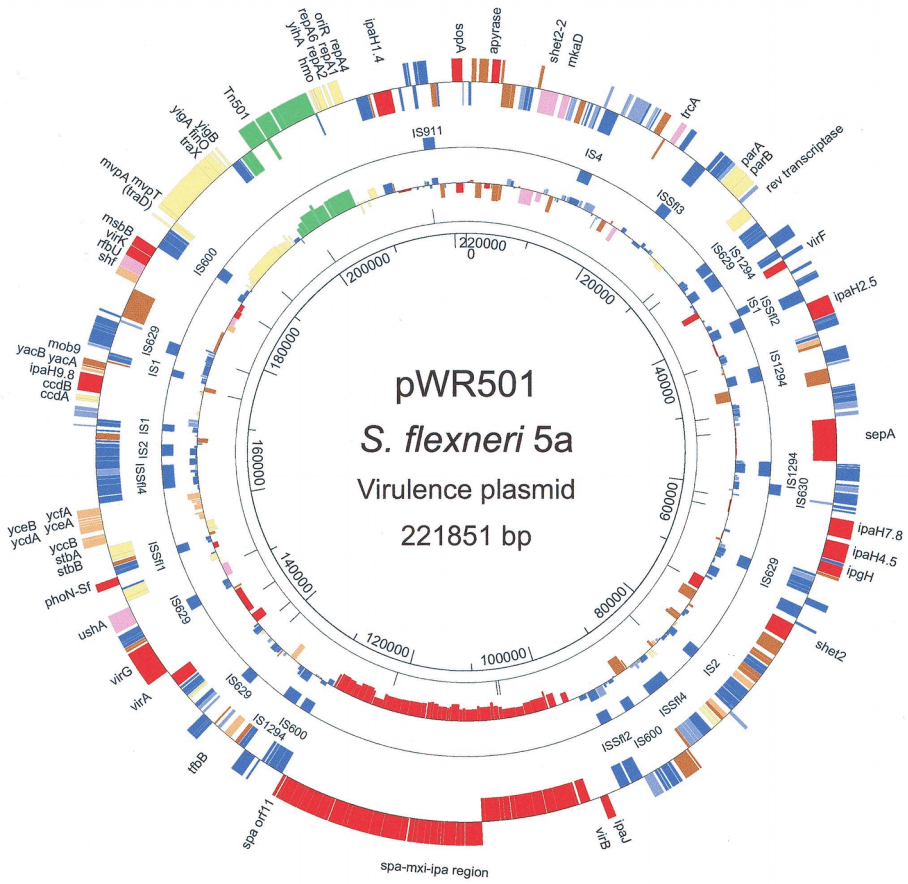

Figure 1.3: Circular map of the virulence plasmid of *S. flexneri* 5a

(Venkatesan et al., 2001).

1.1.2.3 Serological Classification

There are four species in the genus *Shigella* that are responsible for Shigellosis. These species are classified according to serogroups, and thus these two terms are often used interchangeably. *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei* are the four species that can infect and cause bacillary dysentery in humans, which correspond to serogroups A, B, C and D respectively. Further classification can be made depending of the structural differences of the O-antigen found in lipopolysaccharides (LPS) observed on the outer membrane of the call wall (Allison and Verma, 2000). *S. dysenteriae* (group A) has 16 serotypes (Talukder et al., 2007), *S. flexneri* (group B) contains 19 serotypes (Jakhetia et

al., 2014), *S. boydii* has 20 serotypes (Grimont et al., 2007) and *S. sonnei* has a single serotype (Levine et al., 2007).

Table 1.1: Serotypes of *Shigella* spp.

(Grimont et al., 2007; Jakhetia et al., 2014; Levine et al., 2007; Talukder et al., 2007).

Group	Species	Serotypes	Severity
Group A Mannitol non-fermenter	*Shigella dysenteriae*	16 serotypes	Most Severe
Group B Mannitol fermenter, Serologically non-distinct	*Shigella flexneri*	19 serotypes	Mild to Severe
Group C Mannitol fermenter, Serologically distinct	*Shigella boydii*	20 serotypes	Mild to Severe
Group D Late lactose fermenter Mannitol fermenter Serologically distinct	*Shigella sonnei*	1 serotype Phase I & II	Mild

1.1.3 Evolutionary Relationship

Several comparative genomic studies indicate that *Shigella* spp. belong to species *Escherichia coli* rather than being a separate genus (Yang et al., 2007). The divergence of the sequence of *Shigella* and *E. coli* is only 1.5% (Lan and Reeves, 2002), which is only marginal compared to 15% in between *Shigella* and *Salmonella enterica*, which is also closely related to *E. coli*. *Shigella* evolved from *E. coli* by acquiring large virulence plasmid, chromosomal pathogenicity islands and *Shigella* resistance locus (SRL), and

further, by inactivating or deleting genetic loci which are non-functional in intracellular environment (Fukushima et al., 2002; Pupo et al., 1997).

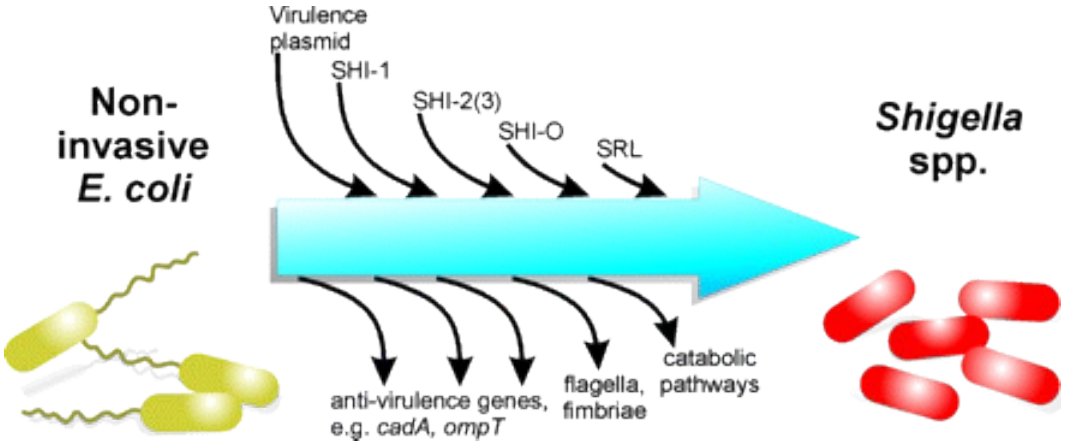

Figure 1.4: Evolution of *Shigella* spp.

Genetic events contributing to the evolution of *Shigella* spp. from non-pathogenic *E. coli* ancestors (Schroeder and Hilbi, 2008).

It also has been observed that, *Shigella* and Enteroinvasive *E. coli* (EIEC) originated through convergent evolution from multiple *E. coli* strains (Rolland et al., 1998; Yang et al., 2007). The *Shigella* spp. began to diverge from *E. coli* about 35,000 to 270,000 years back. Although, *S. sonnei* is more of a recent origin, which began to separate about 10,000 years ago. It might be safe to state that, as EIEC still retains the characteristics of commensal *E. coli*, which may be an earlier stage of evolution of present day *Shigella* (Pupo et al., 2000).

1.1.4 Epidemiology

1.1.4.1 Global Distribution of Shigellosis

Shigella is endemic worldwide, but it affects different groups of population to a varied degree. In developing countries, suddenly displaced population resulting from war, famine, ethnic persecution suffers the most. Insufficient supply of clean water, poor sanitation, over crowding, concomitant malnutrition play a key role for it (Kotloff et al., 1999). Even though

S. dysenteriae Type 1 was predominant in 1918 after its discovery, it has been gradually became replaced by *S. flexneri*.

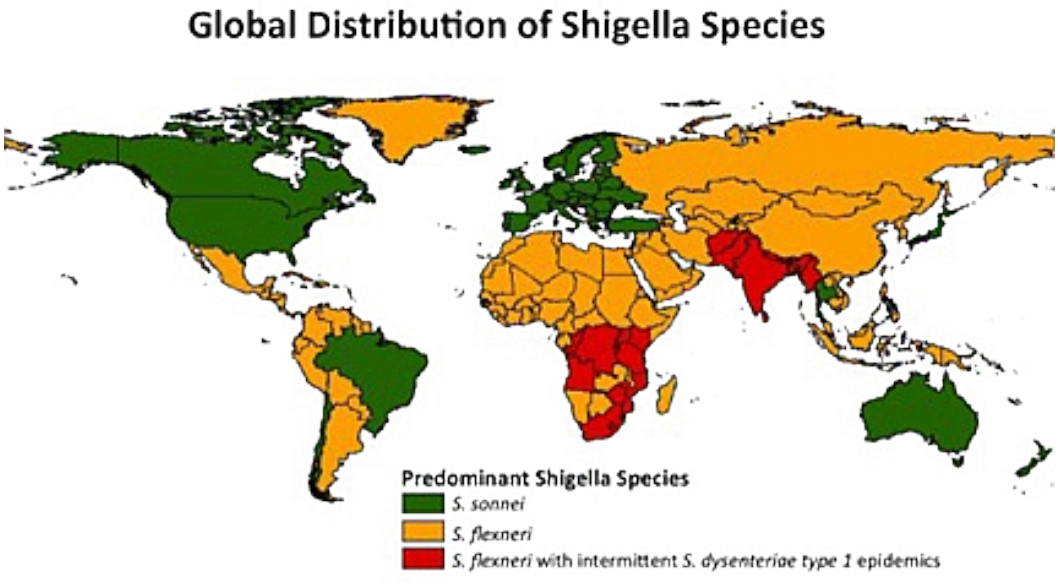

Figure 1.5: Geographic distribution of *Shigella* by species and serotype

(Yale University, 2014).

S. sonnei has become the dominant species of this genus in the industrialized countries. And in developing countries, specially in the Asian countries, it is prevailed by *S. flexneri* except in Thailand, where *S. sonnei* is the most frequent species (von Seidlein et al., 2006). *Shigella boydii* primarily found in Indian subcontinent, as a second most frequent species after *S. flexneri*. Among these countries about 4% of isolates were found as *S. dysenteriae*, but none of these were *S. dysenteriae* Type 1 (von Seidlein et al., 2006).

1.1.4.2 Distribution of Shigellosis in Bangladesh

In a study conducted by Talukder KA and Azmi (2012), it was observed that, of 15,990 isolates obtained from patients attending at icddr,b Dhaka, Bangladesh, of a duration of about 17 years (1997 – 2013), *Shigella flexneri* was the most predominant species (61%),

followed by *Shigella boydii* 17%, *Shigella sonnei* 10% and *Shigella dysenteriae* 8%.

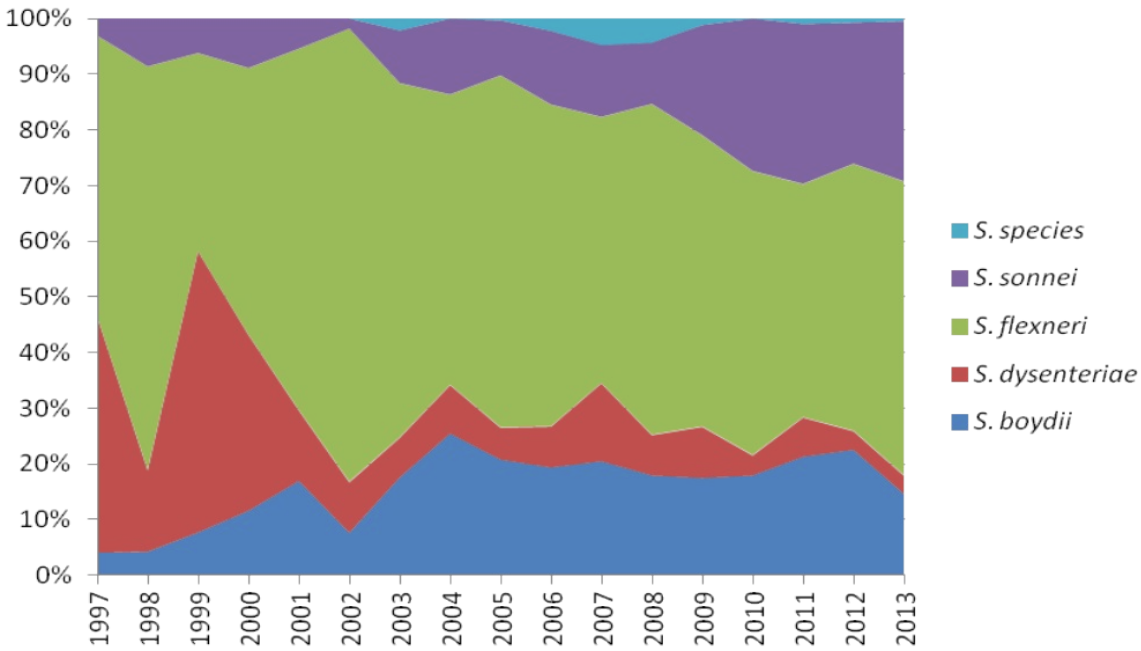

Figure 1.6: Prevalence of *Shigella* spp. in Bangladesh;

Isolated from diarrheal patients in Dhaka, Bangladesh since 1997 to 2013 (n=15,990) (Azmi, 2015).

In last 35 years *S. flexneri* was always found predominant in Bangladesh, and within *S. flexneri*, serotype 2a was found the most frequent (Talukder and Azmi, 2012). Interestingly, the prevalence of *S. sonnei* has been changed to 27% in 2010 from 8% in 2000, and the prevalence of *S. dysenteriae* has been reduced to 3% in 2013. Nevertheless, since 2004 *S. dysenteriae* Type 1 was not found in Bangladesh at all (Azmi, 2015), and that could be attributed to the improvement of the status of sanitation in this region.

1.1.5 Clinical Features

Shigella usually have a window period of 12 hours to 48 hours. Shigellosis is frequently characterized by diarrhea with tenesmus, high fever, generalized weakness, nausea and

anorexia. Diarrheal content is usually consists of high volume of watery stool, followed by frequent scanty, bloody and mucoid stool, associated with urgency and painful defecation (Ashkenazi, 2004) which could be the resulting effect of severe tissue destruction caused by *Shigella*. The *Shigella* Enterotoxin 1 (ShET1) and *Shigella* Enterotoxin 2 (ShET2) is accounted for the watery phase of the disease, which is produced by several strains of *Shigella* spp. *(Fasano et al., 1997; Nataro et al., 1995)*. In contrast, Shiga Toxin can cause wide spread cytotoxic effect and vascular lesions specially in colon, kidney and central nervous system, which could be accounted for the frequent life threatening complications found in *S. dysenteriae* Type 1 infection (Cherla et al., 2003; O'Loughlin and Robins-Browne, 2001). Although *S. flexneri* does not usually cause such severity as *S. dysenteriae*, the predominance of this species in the developing countries could be attributed to the skillful exploitation and evasion of the harmful responses of the immune system (Phalipon et al., 1995). Although, the disease is usually self limiting in adults and older children resolving within a week or so, condition may deteriorate drastically in under 5 children or young infants (Bardhan et al., 2010).

1.1.6 Associated Complications

1.1.6.1 Shigellaemia

Even though, shigellosis remains limited within colonic mucosa and goes to self remission, but bacteremia and septicemia from *Shigella*, also known as shigellaemia, has been reported from time to time. In 1985, Struelens and his co-workers found bacteremia in about 4% of the patients with shigellosis (Struelens et al., 1985). *Shigella* bacteremia was found more frequently in developing countries and particularly in malnourished young infants, and a significant rate of mortality among *Shigella dysenteriae* Type 1 infected cases. The presence of *Shigella* spp. in blood stream has also been reported in immunocompromised patients, such as patients with AIDS (Hitateguy et al., 2013; Keddy et al., 2012) and also in immunocompetent patients associated with other co-morbidities, such as falciparum malaria infection (Grondin et al., 2012) and lung cancer (Liu et al., 2009) Recently, an unusual case has been reported, where the patient was blood culture positive for *Shigella* spp., who had a

previous history of unprotected sexual intercourse with another man (Huynh et al., 2015). Due to the low infectious dose and yet with no effective vaccine against *Shigella*, it is really hard to prevent; which is why, its wise to include shigellosis in the differential diagnosis of sepsis associated with diarrhea in young infants, specially in the *Shigella* endemic areas (Yen et al., 2003).

1.1.6.2 Surgical Complications

In 1980, two patients were reported developing septicemia after renal transplantation (Severn and Michael, 1980). In 1990, another case of sepsis by *Shigella* sonnei was documented by a group of scientists in a patient following splenectomy and portacaval shunt, suggesting that the relatively rare occurrence of *Shigella*emia may be credited to the double protection from liver and the reticuloendothelial cells in spleen (Christensen et al., 1990).

During an epidemic of S. dysenteriae Type 1 in Africa in 1998, another group of scientists recorded several cases of surgical complications and deaths, recommending surgery in the acute phase of shigellosis should be avoided unless there is clear indication (Grant et al., 1998). Miron and his coworkers reviewed and found 57 such cases of surgical complications in last 40 years (Miron et al., 2000).

In 2014, an group of Indian scientists published another case of shigellaemia, in a renal transplant case and proposed for screening for shigellosis in pretransplant period in *Shigella* endemic zones (Appannanavar et al., 2014).

1.1.6.3 Infection of Urogenital System

Although, urinary tract infection (UTI) by *Shigella* spp. is a relatively rare in comparison to *E. coli*, which is about 75% (Farajnia et al., 2009), it has been reported from time to time (Anatoliotaki et al., 2003). Recent publications as well as publications dates back to about half a century, indicates *Shigella* as a causative agent for UTI, and its potential for causing both symptomatic (Baka et al., 2013; Papasian et al., 1995) and asymptomatic (Ekwall et al., 1984; Jao and Jakson, 1963) urinary tract infections.

1.1.6.4 Neonatal Shigellosis

Neonates are relatively less vulnerable in comparison with the older children to shigellosis. The incidence of neonatal shigellosis is about 0.6% of all events of shigellosis up to the age of 10 years. Of all exposed infants 1.6% became ill. The incidence in neonates less than 6 months, is 60 times less than the older children (Viner et al., 2001). Recent studies suggested that, lowest fraction of moderate to severe diarrhea due to *Shigella* was found in age group of 0-11 month. A likely explanation of it, could be the transfer of maternal protective factor to the babies through the placenta during pregnancy or during the breast feeding (Lindsay et al., 2015). In a multi-center study it was found that, the moderate to severe diarrheal (MSD) episodes were found to be peaked as the children started weaning, especially in 24-35 months, when they were introduced to solid foods other than exclusive breast feeding (Lindsay et al., 2015). Similar results were reported in a previous study, where ipaH detection was lowest in age group less than 6 months, and the bacterial load peaked in the second year. The age group varied in different areas worldwide though, such as 12 – 23 months in Gambia and Mali, and 24 – 59 months in Kenya and Bangladesh (Kotloff et al., 2013; von Seidlein et al., 2006).

Neonates with shigellosis may present with mild diarrhea or severe colitis. Compared to the older infants, neonates are more susceptible to have septicemia, meningitis, dehydration, colonic perforation, toxic megacolon and death (Viner et al., 2001). The mortality rate is about one fifth of all deaths in neonates younger than 3 months alone, which could be reaching 30 – 40% in developing countries and less than 1% in developed regions (Bennish et al., 1990).

1.1.6.5 Neurological Manifestations

Central nervous system complication and hemolytic uremic syndrome are among the most common extraintestinal manifestations of shigellosis. The symptoms involving CNS may accompany or precede the intestinal symptoms, and may lead to misdiagnosis (Somech et al., 2001). Seizures and acute transient encephalopathy are manifested by headache,

delirium, lethargy, hallucination, confusion and depressive sensorium, and these are the most commonly reported presentations in pediatric population with shigellosis (Hiranrattana et al., 2005; Ozuah, 1998). Both seizures and transient encephalopathy are common and benign in shigellosis, estimated in 12 – 45% cases (Ashkenazi et al., 1983; Avital et al., 1982), and rarely leave permanent sequelae (Zvulunov et al., 1990).

But, in a rather large study in Bangladesh, Khan and his co-workers observed that 29% cases of death took place in neurologically affected children, while among the unaffected cases the number was only 6%. It was found that, unconscious children with documented seizures were at high risk of fatal outcome. An estimated 76% death was observed of total documented seizures, with no relationship with convulsion and specific *Shigella* spp. (Khan et al., 1999).

Even in developed countries, in a minority of cases, encephalitis may be severe and unresponsive to antibiotics, and even be fatal (Plötz et al., 1999). Unlike developing countries, in developed countries, hypoglycemia and electrolyte imbalance do not play significant pathogenic role, instead early development of brain edema was found to be more predominant (Goren et al., 1992; Khan et al., 1999; Perles et al., 1995).

In a relatively recent study in southwestern Iran, spanning from 2006 to 2009, researchers observed similar pathological pattern among children (of age 1 – 4 years) with shigellosis. The study included 154 children suffering from shigellosis, among which neurological manifestation was the second most common clinical features (68.8%) after fever (83.3%), and the third predominant symptom was dysentery (28%). Among the cases of neurological manifestations more than 50% presented with convulsion, and among them 2% developed lethal toxic encephalopathy (Ekiri Syndrome) with fatal outcome (Shamsizadeh et al., 2012).

Ekiri syndrome, was first described in Japanese patients, which is a fulminating form of *Shigella* associated encephalopathy, reported in early 1900, before and just after the World War II. The major clinical features associated with this syndrome are the following, rapidly developing seizures, coma, high fever and few dysenteric symptoms, which was attributed mainly to *S. sonnei* (Dodd et al., 1949; Kobayashi, 1986; Shiga, 1936). But, during the last half of the twentieth century only a few cases of such episodes were reported (Akl and Hamed, 1989; Heldenberg, 1986; Sandyk and Brennan, 1983).

Among them, Goren et al. studied series of fatal *Shigella* encephalopathy consisting of 15 pediatric deaths from 1980 to 1990. The cause of death was found toxic encephalopathy, and cerebral edema was found in CT of brain or autopsy (Bennish and Wojtyniak, 1991; Goren et al., 1992). Headache was the only prominent feature among them who died, and all of them resulted from sudden, severe, acute neurological decompensation.

Unlike the earlier reports where *S. dysenteriae* was found to be predominant, the later cases of fulminating encephalopathy were found to be associated with *S. flexneri* and *S. sonnei* (Akl and Hamed, 1989; Heldenberg, 1986; Khan et al., 1999; Sandyk and Brennan, 1983), which are known to be devoid of genes required for synthesis of Shiga toxin or Shiga-like toxin I, II, essential for neurological manifestation (Bartlett et al., 1986). And for that reason, another neurotoxic protein was implicated (Ashkenazi et al., 1990).

In another study in Bangladesh, in an effort to characterize children with *Shigella* encephalopathy, Chisti et al., (2010), found 29 children with encephalopathy in a time span of about 9 years, from 1997 to 2006, in an age group of 0 – 15 years of age. They observed high fatality among the patients with shigellosis, but lower than the previous studies (Avital et al., 1982; Khan et al., 1999; Waler and Alsaeid, 1990), and found independent association of the following factors, illiterate father, stoppage of breastfeeding, dehydrating diarrhea, short duration of diarrhea (Khan et al., 1999), stunted growth, low use of vitamin A, associated pneumonia and a higher prevalence in male gender (Jha et al., 2006).

In a retrospective study in ICDDR,B between 1999 – 2010 it was found that, *S. flexneri* was the second most prevalent organism following *V. cholerae* isolated from patients with convulsion (Talukder unpublished data).

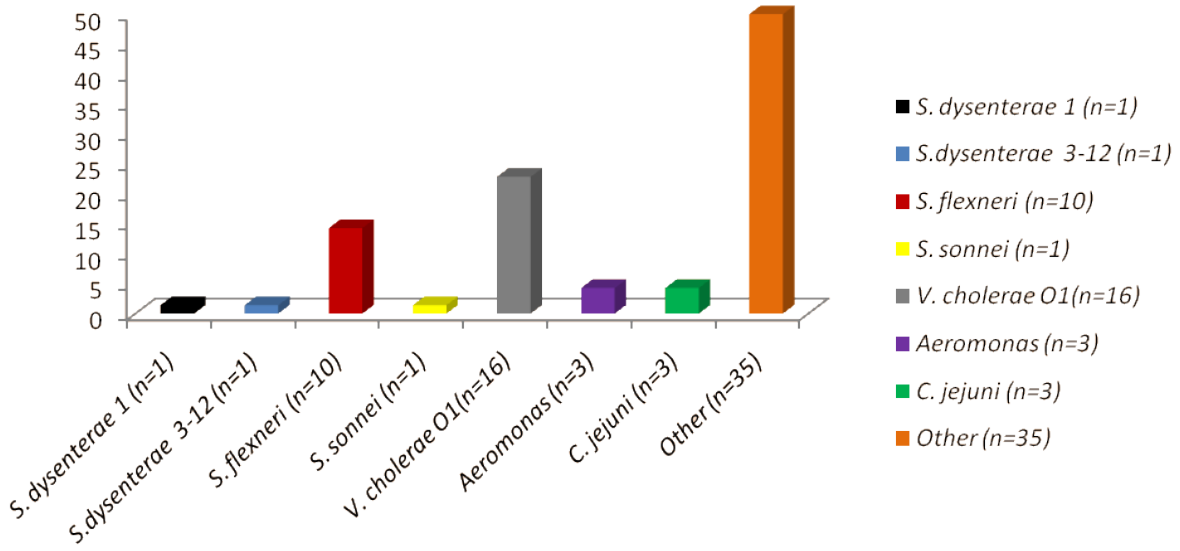

Figure 1.7: Major bacterial pathogens isolated from diarrheal patients with convulsion history (n=70) between 1999 and 2010 (Azmi, 2015).

1.1.7 Diagnosis

Although cases of bacillary dysentery are diagnosed clinically with the following features, such as diarrhea, fresh mucoid bloody stools along with tenesmus, it is inconclusive and sometimes misleading. The symptoms are similar in other enteropathogenic infections such as, *Campylobacter jejuni*, enteroinvasive *E. coli*, *Schistosoma*, *Salmonella* and *Entamoeba histolytica*.

Routine microscopy of fresh stool is used for screening. Due to its low cost its frequently used in the peripheral health centers. It can guide to the bacterial aetiology when numerous PMN cells are detected in the sample.

Confirmatory diagnosis can be made by isolation and serotyping of the organism from stool culture. Culture is also required for antimicrobials sensitivity. PCR (Polymerase Chain Reaction) can be conclusive, but it is regarded impractical due to its high expense.

1.1.8 Management

Prompt and appropriate use of antibiotics usually resolves shigellosis quickly without any further sequelae. However, it can be prevented all together with a practice of a good personal hygiene, even though, an effective *Shigella* vaccine is yet to be available.

In the process of clinical interventions, fluid balance and correction of electrolyte imbalance are primarily focused. During active episodes of shigellosis, frequent feeding especially breastfeeding in young children (3 years) is highly encouraged, as in several studies it was observed that, lactoferrin, a protein abundant in human breast milk, possess antimicrobial, anti-inflammatory and immunomodulatory activity. This protein inhibits bacterial growth by binding with iron, and impair the function of surface expressed virulence factors, by decreasing the ability to adhere/invade host epithelial cells, effecting the loss of type III secretion system (T3SS) of various enteropathogens, including *Shigella* spp. (Ochoa and Cleary, 2009).

1.1.8.1 Prevention

1.1.8.1.1 General Prevention

Shigellosis is a food borne illness, transmitted primarily through feco-oral route. Therefore by ensuring a good personal hygiene, the disease prevalence can be reduced to a great extent, and thus a carefully designed health education always plays a key role. However, during the planning, local terminology, cultural sensitivity, appropriate target population should be considered.

Careful hand-washing before eating and specially after cleaning and/or helping an infected person is vital. Washing hands after defecation, proper stool disposal, and before preparing and handling food is also crucial. If no soap is available ash-earth could be used as a secondary option, and a washed hand should be avoided to be dried with a soiled cloth.

Swallowing water from contaminated sources should be avoided, as water can be contaminated by *Shigella* spp. at all stages of storage and distribution. During traveling, especially the *Shigella* endemic zone, frequent hand-washing should be ensured. Piped water should be chlorinated. Surface water should be avoided as drinking source, but if necessary,

should be used only after chlorination or boiling. It is paramount that no defecation takes place within 10 meters of the water sources (WHO, 2005). Water should be stored in narrow mouthed containers, and should be kept away from small children and animals, which must be covered and cleaned regularly.

Food also, can be contaminated at all stages with *Shigella* spp.. To ensure food safety food handling practice, fly control methods, street food sales, cooking procedures should be monitored closely and carefully. Habit of eating freshly cooked hot food or reheating cold food is a good way to avoid shigellosis.

Breastfeeding should be encouraged during the incidents of *Shigella* attacks, as it has been observed that, fewer and less severe episodes takes place in children, those are breastfed exclusively for 6 months and extended into the 3rd year of the child's age (Chisti et al., 2010; WHO, 2005).

Although shigellosis spread though contaminated food and water, sexual activity with an infected person should also be avoided (CDC, 2016; Huynh et al., 2015).

1.1.8.1.2 Vaccination

Despite 60 years of extensive research no safe and effect vaccine against *Shigella* is available; although, several vaccines are under development especially against *S. flexneri*. Given these backgrounds, studies have demonstrated that measles immunization can substantially reduce the incidence and severity of diarrheal diseases among children (Ashkenazi and Cohen, 2013; WHO, 2005). It has been observed that, natural *Shigella* immunization only ensures limited duration of immunity against this pathogen (Cohen et al., 1991; Ferreccio et al., 1991). Most of the vaccine studies targeted against *Shigella* was focused on the following serotypes, *S. dysenteriae* Type 1, *S. sonnei*, *S. flexneri* 2a, *S. flexneri* 3, *S. flexneri* 6, which constitute about 75% of all cases of shigellosis globally (Levine et al., 2007), and immunization against these serotypes can provide other 11 *S. flexneri* serotypes as cross immunity (Levine et al., 2007; Noriega et al., 1999).

To develop effective *Shigella* vaccines, multiple following approaches were employed for about last half century.

1.1.8.1.2.1 Live Attenuated Vaccines

1.1.8.1.2.1.1 Serial Passage In Vitro

In this process *Shigella* serotypes are passaged on streptomycin containing media until strains become streptomycin-dependent (SmD) and resistant, losing in parallel the capability of mucosal invasion (Sereny, 1957). Protection obtained from this strains persisted several years (Mel and Gangarosa, 1971). Similar live attenuated vaccine using *S. flexneri* 2a strain T32 produced in Romania was found well tolerated and significantly protective (Meitert and Pencu, 1984). But, licensing and further large scale use was prevented due several reasons, especially, need of multiple dosages for primary vaccination, and lack of clear knowledge about exact segment/segments of bacterial genome that was/were changed (Levine and Gangarosa, 1975; Levine et al., 2007).

1.1.8.1.2.1.2 Recombinant DNA Technology

Although the promise was significant in this approach, two major problems arose. Firstly, the narrow window between immunogenicity and safety of the candidates, such as, strain CVD1203 (University of Maryland *Center for Vaccine Development*) was too rectogenic, at a concentration of 108 and 109 CFU (Colony Forming Unit), when it showed good immunogenicity. And on the other hand, CVD1207 was well tolerated at the same concentration, but was poorly immunogenic (Kotloff et al., 2007). Secondly, poor performance in terms of immunogenicity and efficacy in developing countries' children. While live attenuated strain *S. flexneri* SC602 worked well in North American children, poor response was found in Bangladesh (Rahman et al., 2011).

1.1.8.1.2.2 Inactivated Vaccines

1.1.8.1.2.2.1 Whole-cell Vaccines

In early attempts to develop a vaccine, whole-cell *Shigella* preparation were made and administered parenterally, but no significant protection was detected (Levine et al., 2007).

1.1.8.1.2.2.2 LPS-based Vaccines

In this procedure, vaccines were developed demonstrating the association between serum LPS (lipopolysaccharide) and IgG antibody and serospecific protection (Cohen et al., 1991; Passwell et al., 1995). But ultimately it was found that, immune response were limited, and methods to increase immunogenicity were needed.

1.1.8.1.2.2.3 Shigella Conjugate Vaccines

Researchers developed conjugate *Shigella* vaccines by covalently binding serospecific polysaccharides of *Shigella* to a carrier protein, and thus obtaining T-cell dependent antigen, immune memory and better immune response in the process. It was found very safe with minimal adverse effects (Ashkenazi et al., 1999; Passwell et al., 2003). But, it was found that, *S. sonnei* conjugate vaccine had an efficacy of 71% in 3 – 4 year old children but not in younger ones (Passwell et al., 2010). Therefore, further studies especially in human field trials are needed.

1.1.8.1.2.2.4 Other Subunit Shigella Vaccines

S. flexneri 2a and *S. sonnei* LPS were complexed with proteasomes, meningococcal outer membrane protein to boost the immunogenicity of LPS based vaccines, which were administered first orally and then intra-nasally (Fries et al., 2001; Kweon, 2008).

With the idea for developing multivalent subunit *Shigella* vaccine based on type 3 secretion system (T3SS), IpaB, IpaD based *Shigella* vaccines were developed (Heine et al., 2013; Martinez-Becerra and Scobey, 2013; Martinez-Becerra et al., 2012), which showed promising results, especially in pulmonary *Shigella* infections.

In a non-LPS-based approach, outer membrane vesicles released from *Shigella* encapsulated into nanoparticles were found effective in animal models (Camacho et al., 2013).

In another study, genetically derived outer membrane particles, composed of predicted *Shigella* outer membrane and periplasmic protein without LPS also showed promising results (Berlanda Scorza et al., 2012).

1.1.8.2 Clinical Treatment

Usually a patient suffering from shigellosis recovers within 7 – 10 days with proper treatment. Signs of improvement are evident within about 48 hours, if patient is treated early and appropriately. Complications rarely arises as a sequelae of shigellosis, which includes, hemolytic uremic syndrome, toxic megacolon, intestinal perforation, rectal prolapse, encephalopathy, convulsion, and long term complications, such as, persistent diarrhea and prolonged malnutrition, which may result into stunting and wasting (WHO, 2005).

Management of shigellosis consists of proper antimicrobial therapy, correcting the dehydration, and electrolyte imbalance and treatment of complications, if and when arises.

1.1.8.2.1 Antibiotics

With the use of prompt and appropriate antibiotics, improvement is evident within 48 hours and the patient recovers fully within 7 – 10 days. But, during the selection prevalence of local strains and sensitivity should be considered. Ciprofloxacin is the drug of choice (WHO, 2005) irrespective of age. Azithromycin is an alternative choice for adults. Ceftriaxone should be considered for the ciprofloxacin resistant strains.

1.1.8.2.2 Rehydration Feeding and Supportive Care

Rehydration is paramount in case of shigellosis. Oral rehydration is to be ensured with patients with frequent diarrheal episodes, and the state of hydration should be accurately and regularly assessed. Ringer's lactate solution (sodium 130 mEq/L, potassium 4 mEq/L, calcium 2.7 mEq/L, chloride 109 mEq/L, and lactate 28 mEq/L) is preferred for intravenous (IV) infusion, with caution especially in the cases of malnourished children, owing to the risk of hypokalemia and hypoglycemia.

Continued feeding is imperative for all patients with bacillary dysentery to accelerate recovery and to prevent hypoglycemia and malnutrition. Frequent feeding with familiar diets, rich in energy and proteins has to be ensured at least every four hourly. For the breastfed babies, breastfeeding must not be stopped, and should be fed as much and as long as they want. Initially due to the low appetite nasogastric (NG) feeding or IV infusion may

be needed, but condition improves after a day or two. For the young children convalescing from shigellosis, a daily extra meal has to be ensured for at least two weeks for the recovery of the lost weight during the illness.

To control the fever, antipyretic can be used along with tepid sponging. Analgesics may be given for pain management. Zinc supplementation is recommended in children less than 5 years of age for 10 – 14 days.

1.1.8.2.3 Management of Complications

1.1.8.2.3.1 Hypokalemia, Hyponatremia and Hypoglycemia

In young children (<5 years) these metabolic abnormalities could be severe, although could be prevented with continued feeding and by replacing the losses with oral rehydration saline (ORS). In case of severe anorexia, NG feeding and/or IV fluids may be needed. IV normal saline may be needed in hyponatremic patients, and Ringer's lactate solution in hypokalemic cases. However, mild hypokalemia can be easily treated with potassium rich food. If severe hyponatremia (<120mEq/L) is present, then 3% NaCl solution may be administered. Severe hypoglycemia (<2.2 mmol/L) should be treated with intravenous 25% glucose at a dosage of 2 ml/kg.

1.1.8.2.3.2 Convulsion

During bacillary dysentery single brief convulsive episode is common in children. But, anti-convulsive drugs are needed if convulsion is repeated and prolonged. Intramuscular (IM) para-aldehyde, 0.2 ml/kg can be used to treat such cases. Rectal administration of para-aldehyde and diazepam should be avoided, and possible hypoglycemia should be excluded.

1.1.8.2.3.3 Encephalopathy

Encephalopathy may result from metabolic abnormalities, and should be treated accordingly.

1.1.8.2.3.4 Toxic Megacolon

Such complication may develop when mucosal inflammation and ulceration are spread

throughout the colon, resulting in ileus and severe colonic distension. This should be treated with broad spectrum antibiotic, including NG suction. Mortality rate can be as high as 33% in such cases.

1.1.8.2.3.5 Hemolytic-uremic Syndrome

Hemolytic-uremic syndrome (HUS) is a serious complication, associated with *Shigella dysentery* Type 1 and *Escherichia coli* O157:H7, which includes hemolytic anemia, thrombocytopenia and renal failure. Blood transfusion is required in the first two cases. If renal failure is suspected, then fluid intake including ORS and potassium rich foods should be restricted. If renal function does not recover, hemodialysis or peritoneal dialysis may be required.

1.1.8.2.3.6 Intestinal Perforation

Intestinal perforation may be caused by ulceration or vasculitis penetrating colonic wall, resulting in peritonitis or sepsis. In such conditions surgery is required to divert the flow of intestinal contents. Broad spectrum antibiotics and intensive supportive care are mandatory.

1.1.8.2.3.7 Rectal Prolapse

Rectal prolapse usually disappears spontaneously after diarrhea stops, and surgical interventions are rarely required. It can be treated manually by gently pushing the prolapsed rectum back through the anal opening, using surgical glove or soft warm wet cloth putting the affected person in knee-chest position.

1.1.9 Pathogenesis of Shigellosis: Physiology and Cellular Basis

The key factor for *Shigella* to establish shigellosis, is the ability to invade and colonize the intestinal epithelial cells (Sansonetti, 2001). However, most of the studies aimed to determine the pathophysiology of shigellosis were done by *Shigella flexneri*.

The organism gets into the gastrointestinal tract through contaminated food and water. They

pass through stomach and intestine to reach colon. Before reaching colon they multiply during the passage through intestine.

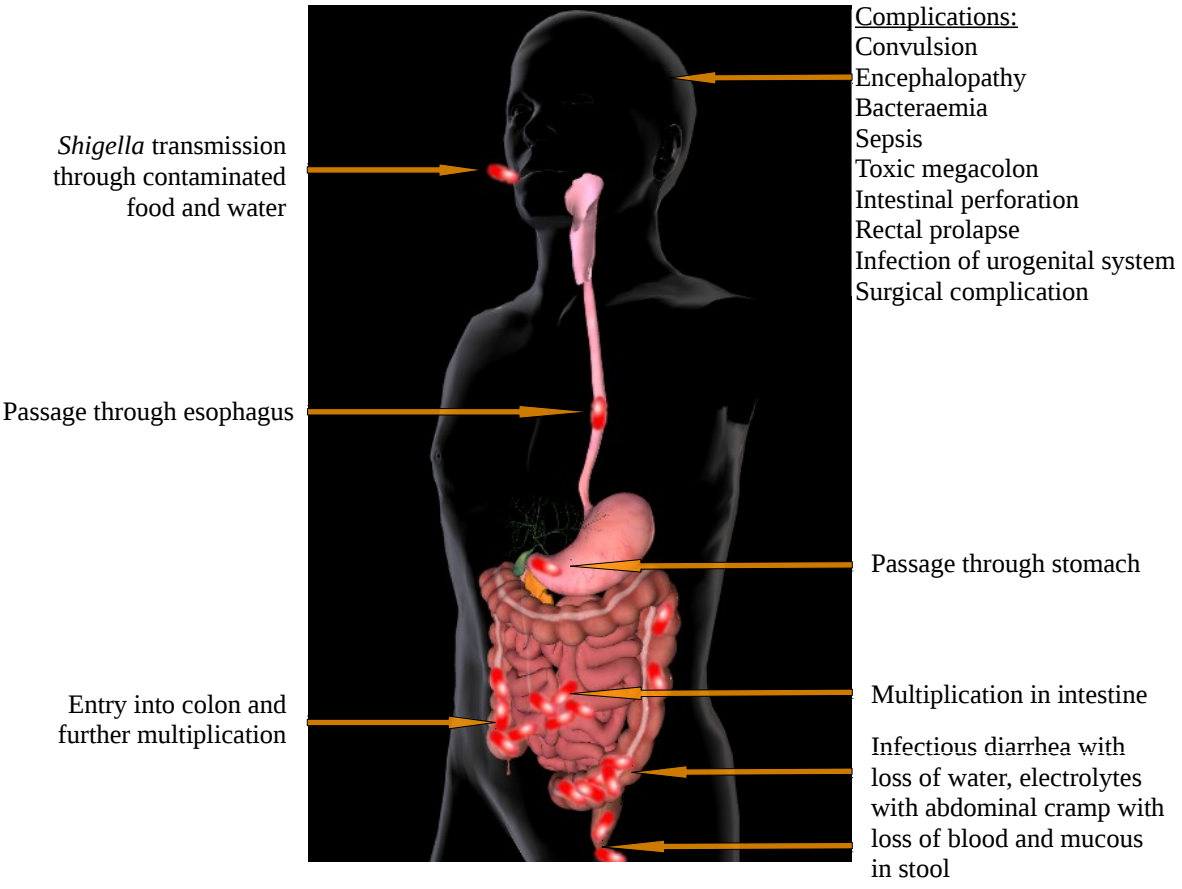

Figure 1.8: Pathophysiology of *Shigella* spp.

The ability to infect the colon is a multi-step process. First, they invade the colonic mucosa via the microfold (M) cells by inducing micropinocytosis. Here, the cell membranes of M cells invaginate around the bacterium, form a vacuole, which are later released into the cytoplasm (Schroeder and Hilbi, 2008; Suzuki and Sasakawa, 2001; Torres, 2004). Then, the bacterium lyses the vacuole, and escape into the cytoplasm where they multiply further. To propel themselves around the cytoplasm, *Shigella* use host cell actin.

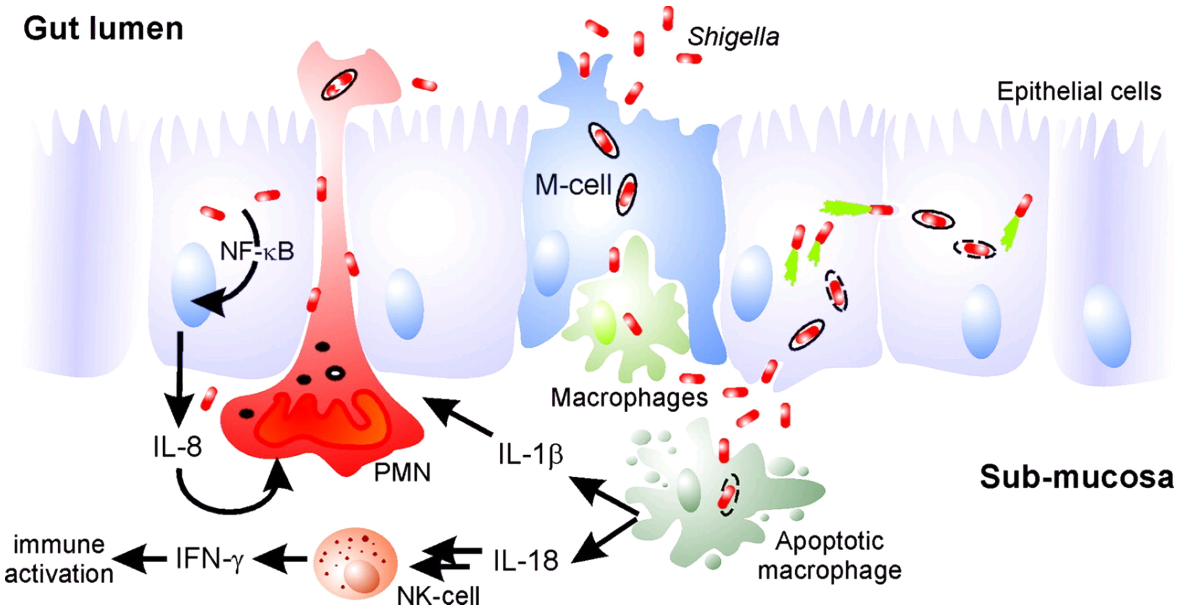

Figure 1.9: Cellular pathogenesis of *Shigella* spp.

(Schroeder and Hilbi, 2008).

These bacteria are taken up by the macrophages, when they are in the intra-epithelial space. Here, they induce the expression of cytokines, including IL-1β, IL-18. While within intracellular space, the bacteria induce apoptosis like cell death of the macrophage cells, causing spilling of it into the extracellular environment. This causes inflammatory reaction, due to the release of proinflammatory cytokines, which attract natural killer (NK) cells and polymorphonuclear (PMN) cells, causing destruction of the cell lining of the gut mucosa, facilitating further and direct invasion by *Shigella*.

Spilled out bacteria also invade adjacent epithelial cells from basolateral sides, and then the next adjacent cells. And ultimately resulting in infectious diarrhea with loss of water and electrolytes with abdominal cramp, fever and loss of blood and mucous as well in stool.

1.1.10 Molecular Determinants of *Shigella* Pathogenesis

1.1.10.1 Virulence Plasmid of Shigella

Cellular pathogenesis and clinical presentation of bacillary dysentery are the output of complex interactions of a large number of *Shigella* virulence factors. The essential molecular machinery required for bacterial invasion and intracellular survival is encoded on the large virulence plasmid of *Shigella* spp. (Sansonetti, 2001; Sasakawa et al., 1986).

Sequencing of virulence plasmid from different *Shigella* strains revealed its length to be approximately 200 kb, with around 100 genes with a comparable number of insertion sequence (IS) (Buchrieser et al., 2000; Venkatesan et al., 2001).

The core of the plasmid is a conserved entry region, 31 kb long, which is both essential and sufficient for epithelial cell (EC) invasion and macrophage killing (Maurelli et al., 1985). The pathogenicity island (PAI) like region, consists of 34 genes, organized into two clusters, transcribed in opposite direction; that is divided into four different groups based on their function.

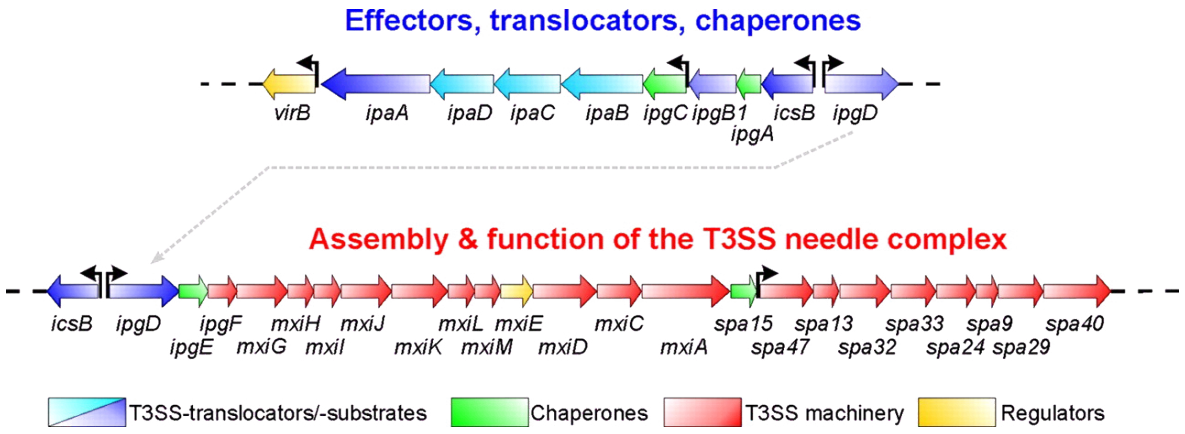

Figure 1.10: Map of the 31-kb "entry region" in *S. flexneri* virulence plasmid pWR100; *ipa*, invasion plasmid antigen; *ipg*, invasion plasmid gene; *mxi*, membrane expression of ipa; *spa*, surface presentation of antigen; *vir*, virulence (Schroeder and Hilbi, 2008).

The first group consists of the genes that encodes the proteins, which act as effectors

secreted by T3SS. These effector proteins manipulates host cell processes in favor of bacteria. These include, invasion plasmid antigen proteins, IpaA, IpaB, IpaC and IpaD (Buysse et al., 1987; Oaks et al., 1986). Among them, IpaB, IpaC and IpaD plays a key role by controlling the secretion and translocation of other effector proteins into the EC, and thus crucial for the host cell invasion and intracellular survival (Blocker et al., 1999; Ménard et al., 1994a, 1996).

The following group occupies more than half of the entry region, and essential for the secretion of Ipa proteins and other effector proteins. They are designated as membrane expression of ipa (*mxi*) and surface presentation of ipa antigen (*spa*) (Hromockyj and Maurelli, 1989; Venkatesan et al., 1992). This mxi-spa locus encodes components required for the assembly and function of the secretion system, that together with IpaB, IpaC and IpaD, permits direct translocation of effector proteins into the host cells (Blocker et al., 1999). About 25 proteins encoded in various locations on virulence plasmid are secreted via this system (Buchrieser et al., 2000).

Group three is comprised of two regulator genes, virB and mxiE. They regulate T3SS associated genes, located within or outside of the entry region, scattered throughout the remainder of the virulence plasmid (Adler et al., 1989; Dorman and Porter, 1998; Kane et al., 2002).

The genes from the last group, ipgA, ipgC, ipgE and spa15, encode the chaperone proteins, which are required to stabilize the T3SS substrates in the bacterial cytoplasm. And, at least two of the corresponding proteins, IpgC and Spa15, has been found functioning as a transcriptional regulator of T3SS effectors located outside of the entry region (Mavris and Page, 2002; Page et al., 1999; Parsot et al., 2003).

Beside the genes within the entry region, several other genes out side of it, are also essential for the organism to be virulent, although most of them take part in post invasion virulence function.

Among them, icsA/virG (*ics* – intracellular spread) encodes proteins, that are directly responsible for the ability of the bacteria to move within the cytoplasm of the infected cells, by actin nucleation at one pole of the organism, and also invade adjacent cells (Bernardini et al., 1989; Makino et al., 1986).

The *virF* gene encodes for transcriptional activator protein, which controls the expression of *icsA* and *virB* genes. Another protein, SepA is a serine protease, coded by the respective gene, found enhancing tissue destruction and inflammation in animal model.

The virulence plasmid contains two copies of *shet2* genes, encoding a putative enterotoxin, and also other genes encoding several secreted proteins, including *virA, ipaH4.5, ipaH7.8, ipaH9.8*. And, additionally, several number of outer S*higella* proteins (*osp*) modulate host immune response in favor of bacteria (Kim et al., 2005; Zurawski et al., 2006).

The genes responsible for virulence on the invasive plasmid remains under strict control of a regulatory network. The primary elements of this regulatory cascade, sensing and reacting towards environmental changes are encoded on the chromosome. These global regulators control the expression of chromosome and the invasive plasmid genes as well (Dorman et al., 2001; Prosseda et al., 2002).

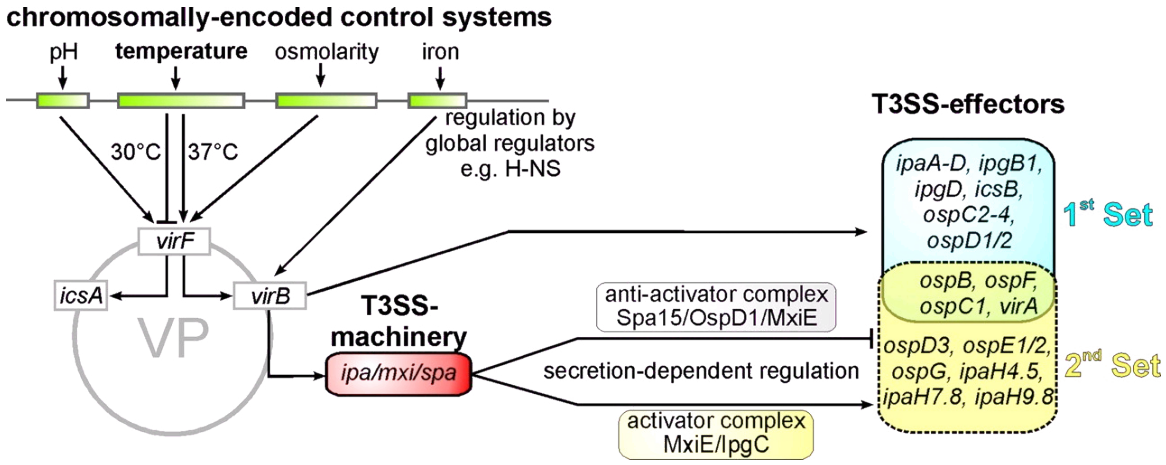

Figure 1.11: Regulatory elements controlling the expression of the T3SS and its substrates on the *S. flexneri* virulence plasmid (Schroeder and Hilbi, 2008).

Activated at 37°C (Tobe et al., 1991), the virulence plasmid-encoded transcriptional activator activates a second virulence plasmid regulator, VirB and the actin nucleator protein IcsA (Adler et al., 1989; Sakai et al., 1988; Tobe and Yoshikawa, 1993). The VirB in turn induces transcription of entry region operons and some additional genes of Osp family (Gall and Mavris, 2005). That enables the bacteria to be equipped with T3SS and the first set of

effector proteins, and also to invade host cells.

The secretion of 'first-set' effector proteins leads to an increased transcription of a subset of already induced protein and an additional 'second-set' T3SS effectors. This second-set effectors are controlled by MxiE, another transcriptional activator, which is blocked by a anti-activator complex consisted of OspD1 and the chaperone, Spa15 (Demers et al., 1998; Gall and Mavris, 2005; Parsot et al., 2003).

After the induction of the type 3 secretion and the first-set effector; OspD1 is secreted along with other substrates, such as IpaB and IpaC, and also in turn the cognate chaperone IpgC (Mavris and Page, 2002), that forms the MxiE-IpgC complex, activating the transcription of the next set of effectors, secreted by the intracellular bacteria.

1.1.10.2 Chromosomal Genes

Beside the core virulence plasmid genes, that dictates the bacterial and host cell interactions, the chromosomal genes also participate in the pathogenic processes of *Shigella*. Studies showed that, after successive conjugative transfer of at least three *S. flexneri* chromosomal regions into virulence plasmid-containing hybrid *E. coli* K12 strain, it conferred full virulence in in vivo infection models (Sansonetti and Hale, 1983).

These chromosomal genes can be classified into two categories.

Firstly, the genes regulating the expression of the virulence genes on plasmid, such as, *virR*, encodes a histone-like molecule which controls the temperature dependent expression of Ipa and Mxi-Spa proteins (Maurelli and Sansonetti, 1988; Sansonetti, 2001).

And secondly, the genes essential for bacterial survival in both intestinal and intracellular environment. Such as, those encoding the LPS and siderophores. Along with these, it is worth mentioning that, Shiga toxin is encoded by chromosomal locus, although only in *S. dysenteriae* Type 1.

The enterotoxins other than Shiga toxin include, ShET1 and ShET2, which are known to be capable of increasing water accumulation in rabbit ileal loop model, and are encoded by both chromosomal and virulence plasmid genes. It has also been found that, additional enterotoxins other than ShET1 and ShET2, are encoded by *S. flexneri* as well. Among the

four identified factors responsible for enterotoxin activity, ShET1 and Pic, are encoded by chromosome, while ShET2 and SepA are encoded by virulence plasmid (Faherty et al., 2012).

1.1.10.3 Toxins

Toxins one of the major virulence factors in bacteria, and are perhaps the best characterized ones, as they are the easiest to purify by concentrating the supernatant, and also because of distinct phenotypes, often alter and/or kill host cells, usually through enzymatic processes (Schiavo and van der Goot, 2001).

Shigella spp. usually produce two types of toxins, *Shigella* enterotoxin and Shiga toxin. Furthermore, there are two types of enterotoxins elaborated by *Shigella* spp., *Shigella* enterotoxin 1 (ShET1) and *Shigella* enterotoxin 2 (ShET2), which are found to be responsible for the watery phase of diarrhea. However, all of these toxins are exotoxins.

Exotoxins are are usually protein in nature and secreted by bacteria into the extracellular milieu. And, endotoxins are lipopolysaccharides (LPSs), which are primarily carbohydrates, non-protein, non-enzymatic, and not normally secreted. Usually, LPSs are not considered as virulence factors, as they are essential for bacterial survival; even though, they evoke toxic effects in host by inducing immune responses and cytokine production.

1.1.10.3.1 Shigella Enterotoxin 1 (ShET1)

Shigella enterotoxin 1 (ShET1) is a chromosomally encoded, complex, iron dependent protein, that found causing significant fluid accumulation in rabbit ileal loops, indicating it's responsibility for the watery phase of diarrhea. ShET1 also contributes to about 60 – 65% wild type enterotoxic activity in *Shigella flexneri* 2a strains, lacking 140 MD invasive plasmid (Fasano and Noriega, 1995).

ShET1 is composed of two distinct subunits, one 'A' subunit and five identical 'B' subunits, organized in a A_1B_5 configuration. This protein is encoded in *set* gene, which is composed of two contiguous open reading frames (ORFs), of 534 bp (*set1A*) and 186 (*set1B*) bp, operating under same promoter region, separated by only 3 base pairs. The 'A' subunit

possesses a size of about 22 KD (kilodalton), and it is responsible for the secretory activity. And, the 'B' subunits are responsible for the irreversible binding of the toxin to the enterocyte receptor, of size of 7 KD. The holotoxin weighs roughly 55 KD (Fasano and Levine, 1997).

ShET1 is found in 45% of *S. flexneri*, and not found in *S. sonnei* and *S. dysenteriae* species (Vargas and Gascon, 1999). However, it should be noted that, ShET1 is produced universally by *S. flexneri* 2a and rarely (3.3%) in other *Shigella* serotypes, which might explain the epidemiological predominance of *S. flexneri* 2a in developing countries (Noriega and Liao, 1995).

1.1.10.3.2 Shigella Enterotoxin 2 (ShET2)

Shigella enterotoxin 2 (ShET2) is a 62.8 KD, single protein, encoded in *sen* (*Shigella* enterotoxin) gene located in 140 MD invasive plasmid. This T3SS secreted toxin is thought to participate in *Shigella* induced epithelial inflammation (Farfán et al., 2011), by contributing releasing of IL-8 from gut epitheliums. Interleukin-8 (IL-8) is a chemokine that attracts and activates neutrophils in the inflammatory region (Bickel, 1993). ShET2 was first detected in EIEC (enteroinvasive *E. coli*) and was regarded as EIEC enterotoxin. Later, it was also widely detected in *Shigella* spp. and DNA sequencing confirmed 99% homology with EIEC enterotoxin (Fasano and Levine, 1997).

Nataro et al. found *sen* gene in 73% of *S. flexneri* and Vargas et al. found ShET1 along with ShET2 in 36% *S. flexneri*, and overall 83% in all *Shigella* spp. (Nataro et al., 1995; Vargas and Gascon, 1999).

1.1.10.3.3 Shiga Toxin

Shiga toxin (Stx) is produced by *Shigella dysenteriae* Type 1 and certain types of *Escherichia coli*, known as Shiga toxin-producing *E. coli* (STEC) or verocytotoxic *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC), with no available effective treatment against them yet (Bergan et al., 2012). This toxin belong to the family of ribosome-inactivating proteins (RIPs), with two subtypes, Shiga toxin 1 and Shiga toxin 2.

Each molecule of Shiga toxin is composed of two subunits, one enzymatically active 'A' subunit and five identical, non-toxic, receptor-binding 'B' subunits, organized in a pentamer. Stx binds with the target cells through a glycolipid receptor, and endocytosed by clathrin mediated pathway, and transported by a retrograde pathway to endoplasmic reticulum (ER), before the enzymatically active moiety is translocated into cytoplasm (Lauvrak et al., 2004; Nichols and Kenworthy, 2001; Sandvig et al., 2010).

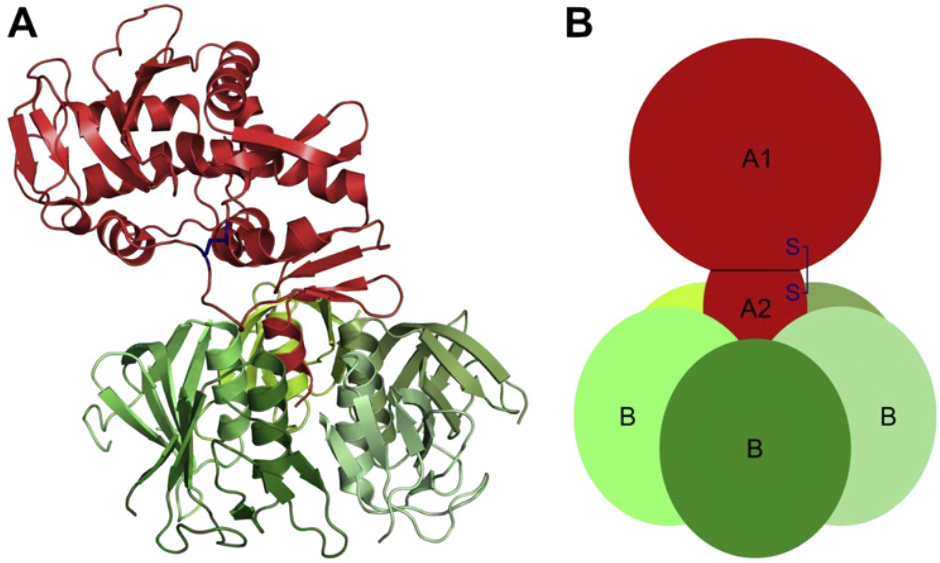

Figure 1.12: Structure of Shiga toxin.

A. The structure of Shiga toxin (holotoxin) as determined by X-ray crystallography (Fraser and Chernaia, 1994). **B.** Schematic representation of the Shiga toxin structure (Bergan et al., 2012).

The 'A' subunit is proteolytically cleaved into catalytically active A_1 and small A_2 polypeptide. The A_1 polypeptide is capable of efficiently inhibiting mammalian protein synthesis. (Endo et al., 1988; Saxena et al., 1989). This inhibition of protein synthesis by RIPs are thought to be responsible for the induction of apoptosis in various cell types, such as in, epithelial, endothelial, leukocytic, lymphoid and neuronal cells (Tesh, 2010). Nevertheless, the *stx* gene is expressed in both *S. dysenteriae* Type 1 and STEC are generally lambdoid bacteriophage-borne, particularly known as Stx-phage (Unkmeir and Schmidt, 2000).

Infection with Shiga toxin-producing bacteria, such as, *S. dysenteriae* Type 1 and STEC, can cause bloody diarrhea with fatal extra-intestinal complications, producing vascular lesions in colon, kidneys and central nervous system. The Shiga toxin-producing bacteria frequently cause hemolytic-uremic syndrome (HUS), characterized by thrombocytopenia, microangiopathic hemolytic anemia and renal failure, capable of causing widespread diarrheal diseases with a substantial death tole (Kaper, 1998; Kitov et al., 2000; Paton et al., 2000).

In between *Shigella* enterotoxin 1 and *Shigella* enterotoxin 2, no nucleotide or protein homology have been observed (Nataro et al., 1995). And further more, gene sequencing showed that, these two enterotoxins (ShET1 and ShET2) are genetically and immunologically unrelated from Shiga toxin that is produced in *S. dysenteriae* Type 1 (Fasano and Levine, 1997).

1.1.10.4 Type III Secretion System

Type 3 secretion systems are found in various Gram-negative bacteria, such as *Salmonella*, *Shigella*, *Yersinia*, *Pseudomonas*, and Enteropathogenic *E. coli* (Galán and Wolf-Watz, 2006). This double membrane spanning secretory cellular machine promotes transferring bacterial effectors to the cytoplasm or plasma membrane of the target eukaryotic cells. Within the host cells, these effectors can modify or subvert specific host functions, and thus facilitate bacterial invasion and colonization (Büttner, 2012; Cornelis, 2006).

Type 3 secretion complex was first isolated and visualized more than 15 years ago in *Salmonella enterica* serovar Typhimurium SPI-1 (Kubori et al., 1998). The main structural component of this system is a protein complex, known as needle complex (NC) of about 3.5 MD (Kubori et al., 1998).

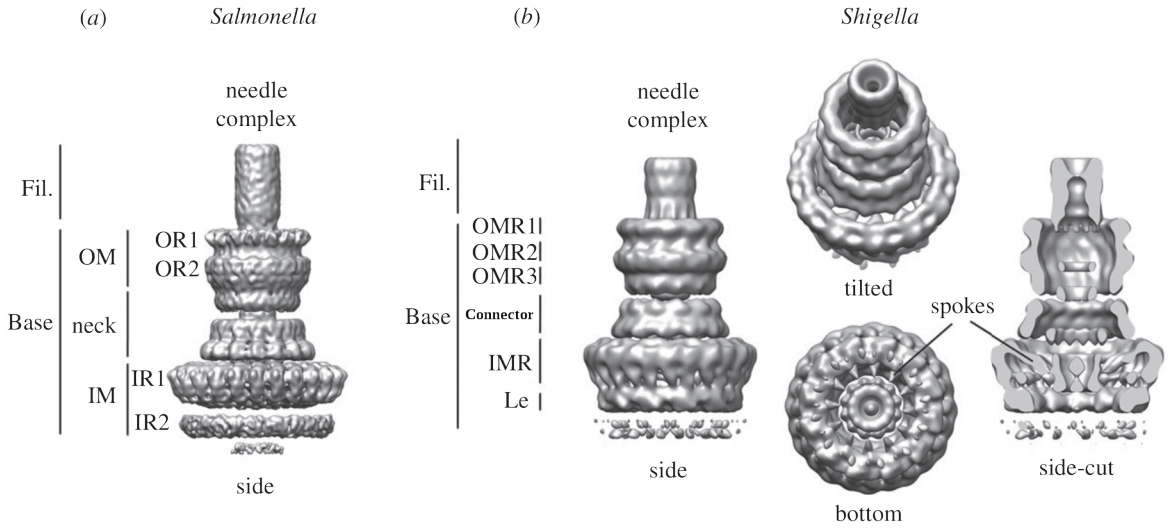

Figure 1.13: Structure of needle-complex of *Salmonella* and *Shigella* spp.

Needle complex structure from (a) *Salmonella* and (b) *Shigella*. (Fil., needle filament; OM, outer membrane; OR, outer ring; OMR, outer membrane ring; IM, inner membrane; IR, inner ring; IMR, inner membrane ring) (Kosarewicz et al., 2012).

1.1.10.4.1 Structure

1.1.10.4.1.1 Base, Needle and Inner Rod

This syringe like structure is made of about 25 protein components, organized in 2 main subunits.

First, a double-membrane spanning base, and secondly, a needle-like filament protruding from bacterial surface to the extracellular space (Marlovits et al., 2004, 2006; Schraidt and Marlovits, 2011). Within base, lower rings are composed of 2 concentric ring proteins, IR1 and IR2, in *Shigella* spp. they are formed by MxiG and MxiJ proteins respectively. While the outer membrane ring proteins are composed of only MxiD protein in *Shigella* spp., which is a member of secretin family of outer membrane protein (Galán et al., 2014).

The needle substructure is assembled from multiple (~100) copies of a single ~80 residue subunit (MxiH), arranged in a helical pattern (Cordes et al., 2003; Loquet et al., 2012). In it's native form the length of the needle ranges from 30 – 70 nm, and it's width ranges from 10 –

13 nm. In *Salmonella* spp., NMR imaging reveals a ~80 Å (1 Å = 10^{-10} m) wide filament with a lumen with a diameter of about ~25 Å (Loquet et al., 2012). The inner rod is made with a single ~90 AA subunit, MxiI (Marlovits et al., 2006). The MxiH and MxiI are thought to have a similar structure, although the actual length is currently unknown.

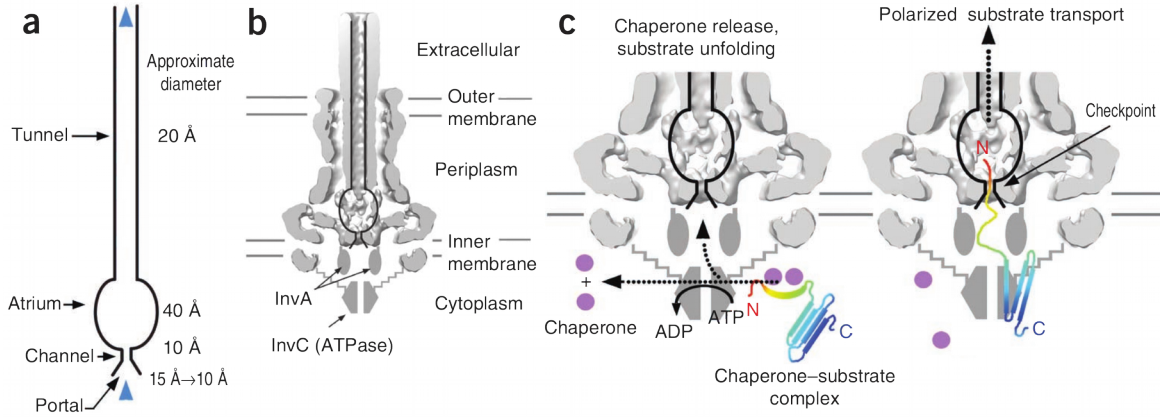

Figure 1.14: Secretion pathway and substrate transport model through needle-complex

a. Secretion path through injectisome with schematic illustration of various spaces of different diameter. The funnel-shaped portal tapers from 15 Å to 10 Å to connect to the channel, the narrowest part for substrate passage (10 Å) through injectisome. The direction of substrate movement is indicated with blue arrowheads; **b.** Sketch of the secretion path through entire needle complexes and cytoplasmic organization of the T3SS-specific ATPase and the export-apparatus protein; **c.** Functional model of substrate transport: release of chaperones and unfolding of substrates is catalyzed by a T3SS-specific ATPase. The free N terminus of a substrate enters the portal of an injectisome, and its passage through the constriction defined by the channel is restricted to completely unfolded proteins (in *Salmonella enterica* serovar Typhimurium SPI-1) (Radics et al., 2014).

1.1.10.4.1.2 Inner Membrane Export Apparatus

All T3SS retain five highly conserved inner membrane proteins, that are essential for the system to be functional. In *Salmonella enterica* SPI-1 these proteins are, InvA, SpaP, SpaQ, SpaR, SpaS and in *Shigella* spp. the corresponding proteins for the secretion system are,

MxiA, Spa24, Spa9, Spa29 and Spa40 (Groisman and Ochman, 1993).

Studies indicate that, at least a subset of these inner membrane protein are located within the NC, and they are thought to serve as a protein channel, to facilitate target proteins through the inner membrane. Even though, they are regarded as a group, they are likely to have specific functions. Such as, Spa40, contains a unique C-terminal domain, that acts as a protease for its own autocatalytic processing (Galán et al., 2014), and thus, it is thought to play role in secretion hierarchy.

1.1.10.4.1.3 Cytosolic Components

A number of cytosolic components are essential for substrate recruitment, unfolding and transport, which associate with the base of the NC following the contact with the host cell (Akeda and Galán, 2005; Eichelberg et al., 1994; Lara-Tejero et al., 2011), which are conserved across all T3SSs (Allaoui et al., 1994; Collazo and Galan, 1996; Morita-Ishihara et al., 2006); although, the organization of these proteins are poorly understood.

One of the highly conserved cytosolic component is an ATPase (Eichelberg et al., 1994; Woestyn et al., 1994), that is thought to be involved in substrate recognition and unfolding (Akeda and Galán, 2005). Again, it's precise subcellular location is still unknown.

In some models, there is a linker protein, MxiN, that recruits the ATPase to the sorting platform (Spa33/MxiK in *Shigella* spp.) (Bo Hu, Dustin R. Morado, William Margolin, John R. Rohde, Olivia Arizmendi, Wendy L. Picking, William D. Picking, 2015). In some alternative models, implication of another cytoplasmic component has been made, Spa13 in *Shigella* spp. (InvI in *Salmonella enterica* SPI-1), that would work in a analogous manner to the stalk that links ATPase to the NC and export apparatus (Ibuki et al., 2011).

1.1.10.4.1.4 Tip Complex

Although variations are found in other T3SS containing Gram-negative spp., in *Shigella* and *Salmonella* spp. the needle filament is capped at its tip, with a single protein (Epler et al., 2012), IpaD and SipD protein respectively. In low resolution cryoEM, IpaD protein exhibits a diameter of 78 Å at its widest point (Epler et al., 2012). It has been also found that, the tip protein undergoes a significant conformational changes upon assembly into the tip complex.

1.1.10.4.1.5 Translocons

The last step of type 3 secretion system is the delivery of effector proteins into the target cells through it's cellular membrane. This step is mediated by the protein translocases, IpaB, IpaC and IpaD in *Shigella* spp., while IpaD plays a dual role by acting as a translocon and also as a signal transducer (Blocker et al., 1999; Roehrich et al., 2013).

After secretion the translocases enter into the target cell membrane, and there they are thought to form a protein channel (Blocker et al., 1999). The mechanism of how the translocases insert into the host cell membrane is poorly understood. In it's absence, the translocases can not get into the target membrane, only can be secreted (Veenendaal et al., 2007).

1.1.10.4.2 Assembly of the Secretion System

The assembly of the needle-complex (NC) takes place in a stepwise fashion. It starts at the inner membrane with the formation of a complex of a subset of the export apparatus component. In *Shigella* spp. it consists of Spa24 and Spa29 proteins (Wagner et al., 2010). It is followed by incorporation of additional export apparatus proteins, that template the assembly of the lower rings, first the inner ring IR1 made of MxiJ, and then the outer ring IR2 made of MxiG protein.

Even though, the assembly of the NC is possible in the absence of the inner membrane export apparatus, the efficiency is greatly reduced. Furthermore, at least 4 of the 5 IM apparatus proteins (Spa24, Spa9, Spa29, Spa40 for *Shigella* spp.) can not be incorporated into previously assembled bases, indicating functional NC assembly is not possible without

the prior deployment of the export apparatus (Wagner et al., 2010).

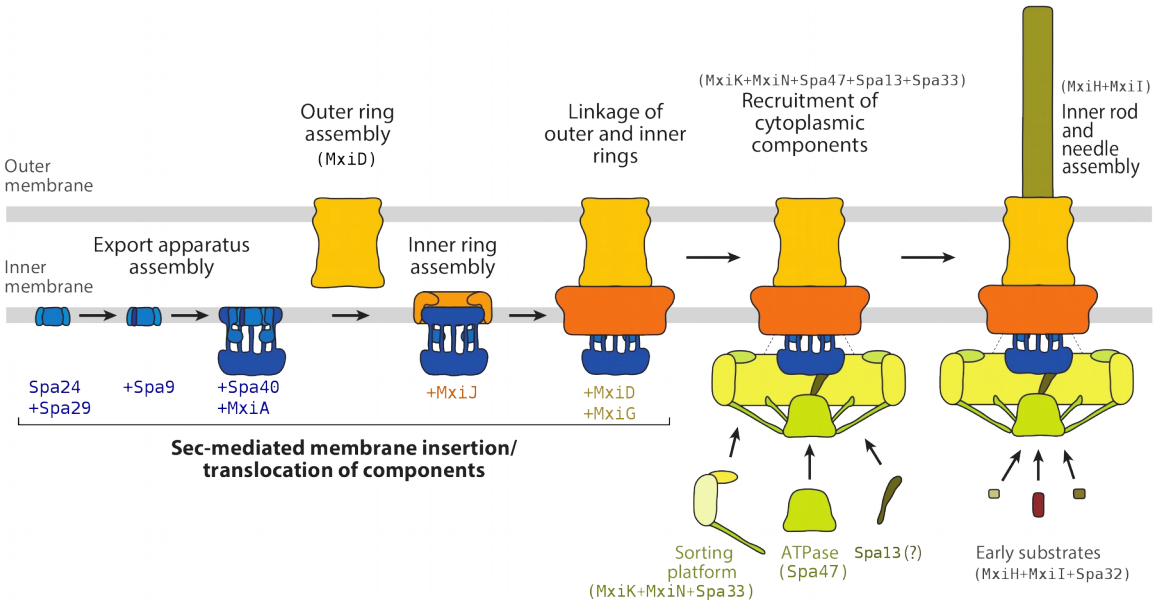

Figure 1.15: Assembly of type 3 secretion (T3S) system.

Model for the assembly of the type 3 secretion needle complex and associated structures, for *Shigella* spp. (De Geyter et al., 2000; Page et al., 1999).

The outer rings and connector are formed with a single protein of the secretin family, MxiD in *Shigella* spp., that frequently requires an accessory lipoprotein of pilotin family (MxiM). Although, the outer and inner rings of the base substructure can assemble independently, their stability is compromised in absence of any one of them (Diepold et al., 2010). Thus it is likely that, although they may be assembled independently, they become linked rapidly to each other to retain stability. It is also likely that, the components of the sorting platform (MxiK and Spa33 for *Shigella* spp.) exist in a preassembled state inside the bacterial cytoplasm.

Whether the ATPase is part of that preassembled complex, is not clear, but it is known that, the recruitment of the ATPase to the NC, requires the components of the sorting platform (Abrusci et al., 2013; Diepold et al., 2010). Although, how the recruitment happens is still unknown. A recent electron microscopic study suggests that, NC itself serve as an anchor for

it, as in it's absence there is no recruitment of cytoplasmic components to the membrane. After completion of cytoplasmic components recruitment, the base structure functions as a limited-specificity type 3 secretion machine, that can only recognize the inner rod and needle promoters, as well as an accessory protein Spa32 (for *Shigella* spp.), required for proper NC assembly. And then, at a certain point, the secretion machine switches substrate specificity, when it would no longer recognize the early substrates (needle and inner rod promoter), and becomes competent for the secretion of needle (the tip protein and translocases) and late substrates (effector proteins). However, how the type 3 machinery changes it's program is not completely understood, although it is observed that this process requires an accessory protein, once again the function of which is not yet clearly understood.

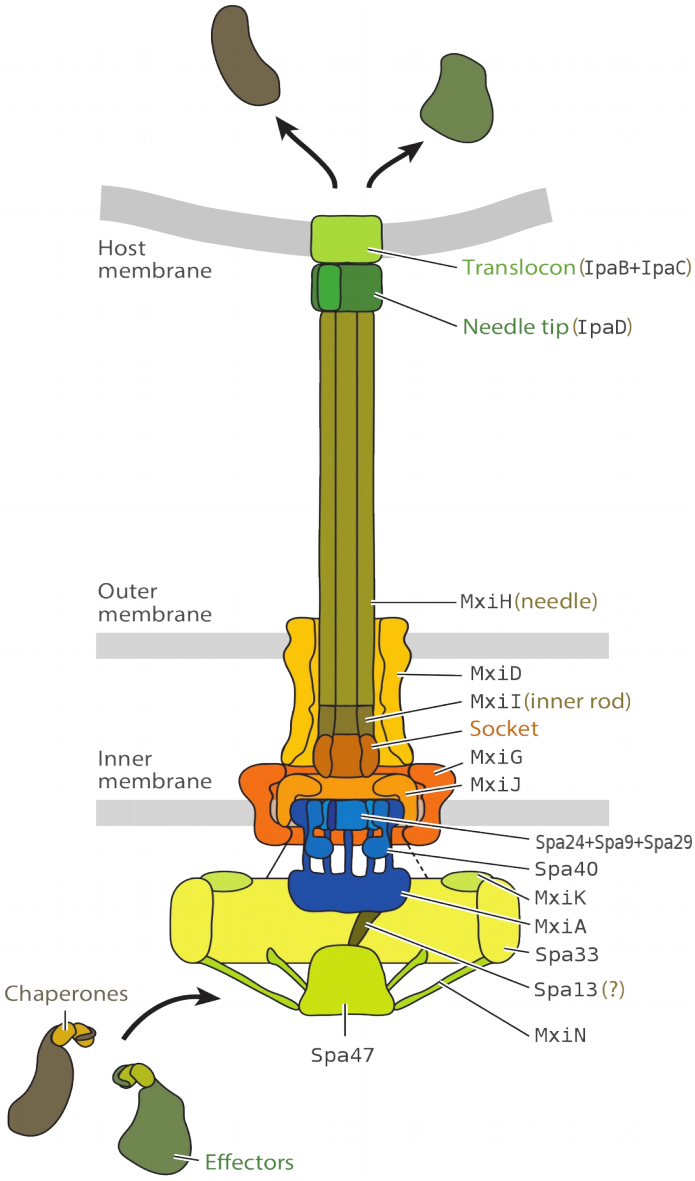

Figure 1.16: Type 3 secretion system schematics.

Schematic diagram of the needle-complex and associated structures of T3SS, for *Shigella* Spp. (Galán et al., 2014; Hueck, 1998).

1.1.10.4.3 Chaperone – the Escort of Substrate

Proteins that are to be secreted through the type 3 secretion pathway are targeted to the machinery by sets of specific secretion signals. One set of secretion signals are the first 20–25 amino acid residues (Michiels and Cornelis, 1991; Sory et al., 1995). And the second signal, at least in some of type 3 secreted proteins, are spanned from residues ~25–100 AA, which serve as a binding site for specific chaperones (Sory et al., 1995; Wattiau et al., 1994). Chaperones associated with type 3 secretion system lack nucleotide-binding or hydrolysis activities. They also show very limited primary amino acid sequence similarities with each other, although sharing structural similarities and similar physical properties, such as, small size and an acidic pI (Stebbins and Galán, 2003).

Based on the tertiary structure and binding specificity, the chaperones are classified into 2 groups, Class I and Class II (Cornelis and Gijsegem, 2000; Parsot et al., 2003; Thomas et al., 2012). Class I chaperones are further classified into two groups. Those bind with a single proteins, known as uni-cargo chaperones, and those bind with several proteins, known as multi-cargo chaperones.

These chaperones transport the substrates in a partially unfolded state aiding the secretion process. In the absence of single cargo chaperones, the cognate effector proteins degrade within the bacterial cytoplasm (Ménard et al., 1994b). But, it is not the same for effector proteins chaperoned by multi-cargo chaperones (Ehrbar et al., 2003). It is thought that, the stability of such effectors may be necessary to facilitate complex assembly, as such proteins are frequently found encoded away from their cognate chaperones. In contrast, single cargo chaperones are encoded in close proximity of their cognate cargo. Furthermore, in some cases they have specific translation regulatory mechanisms in place to co-ordinate their synthesis with that of the cognate effector (Button and Galán, 2011).

Chaperones of class II usually interact with protein translocases, and in some T3S machines, with promoters of the needle, or of the extended appendages (Creasey et al., 2003; Ménard et al., 1994a).

Absence of the chaperones or, removal of the chaperone-binding domain, results into lack of secretion of their corresponding effectors (Schesser et al., 1996). Although, primarily chaperones target their cognate effectors to the secretion pathway and prevent detrimental

interactions and/or stabilize their cognate target proteins, their role in secretion is still unclear, along with the mechanism by which substrates of the secretion system are ultimately recognized and targeted.

The chaperone-effector complexes are most likely recognized and targeted to the secretion machinery by a group of cytoplasmic protein associated with the NC, such as, sorting platform. And it is also likely that, ATPase interacts with the chaperone-effcetor complexes (Gauthier and Finlay, 2003), and capable of dissociating them (Akeda and Galán, 2005). This is necessary before secretion, as the chaperones remain in the cytoplasm after secretion of the cognate effectors. And thus, the chaperones might be involved in establishing the secretion hierarchy by exhibiting different affinity to the relevant sorting platform components.

1.1.10.4.4 ATPase – the System Energizer

The available data indicates that, the proteins move through individual T3S machine quite rapidly, although the precise speed of this process is still unknown (Schlumberger et al., 2005). However, the way the bacteria deliver the proteins directly from its own cytoplasm into the target cell, makes it a energy demanding process.

One of the two possible sources of energy to run this system is the energy derived from ATP hydrolysis by the conserved T3SS associated ATPases. Studies indicate that it's catalytic activity is essential for the secretion (Woestyn et al., 1994). But again, how ATP hydrolysis is coupled with the secretion process is still unknown. Nevertheless, in in vitro studies it has been observed that the ATPase can unfold the effector protein (Akeda and Galán, 2004), and therefore it has been proposed that the energy stored in the unfolded proteins might contribute to the progression of the substrates through the secretion channel.

As suggested by several studies, another possible energy source might be the proton motive force in Gram-negative species such as *Yersinia* (Wilharm et al., 2004). Although, how it coupled with the secretion is also unknown.

1.1.10.4.5 Sensing and Firing – the End of the Beginning

Although the activation process is poorly understood, but evidences indicate that, the activation occurs following the contact with the host cells (Ménard et al., 1994b). Which means, it is most likely that, the effector proteins are delivered directly into the cells instead of the extracellular space. Even though, how cell contact activates the secretion machinery is unknown, it has been indicated that, the tip complex is involved in the sensing process. The signal is thought to be sensed by the tip complex, and transduced to the cytoplasmic side of the secretion machine, which probably mediated by conformational changes in the needle and inner rod structure of the NC (Blocker et al., 2008). This activation of the secretion system ultimately leads to the deployment of the translocases on the target cell membrane, which in turn mediates the passage of effectors through the target plasma membrane.

1.2 Aim of this study

Despite being one of the major public health threats in Bangladesh, no correlation study between clinical features and virulence genes, based on the population of this region has been reported till date. In this context, the present study aims at,

- Exploring the following genomic factors in *S. flexneri* strains:
  - Presence of 140 MD large virulence plasmid,
  - Presence of virulence (ipaH, ial, ipaH7.8), toxin (set, sen) and type 3 secretion related genes (*virB, ipaBCD, ipaB, ipaC, ipaD, ipgC, ipgB1, ipgA, icsB, ipgD, ipgE, ipgF, mxiH, mxiI, mxiK, mxiE, mxiC, spa15, spa47, spa32, spa24, spa*), for each *Shigella flexneri* isolates.
- Analyzing statistical correlation, between the target clinical features and virulence genes for each corresponding strain.

Chapter 2 : Methodology

2.1 Design of the Study

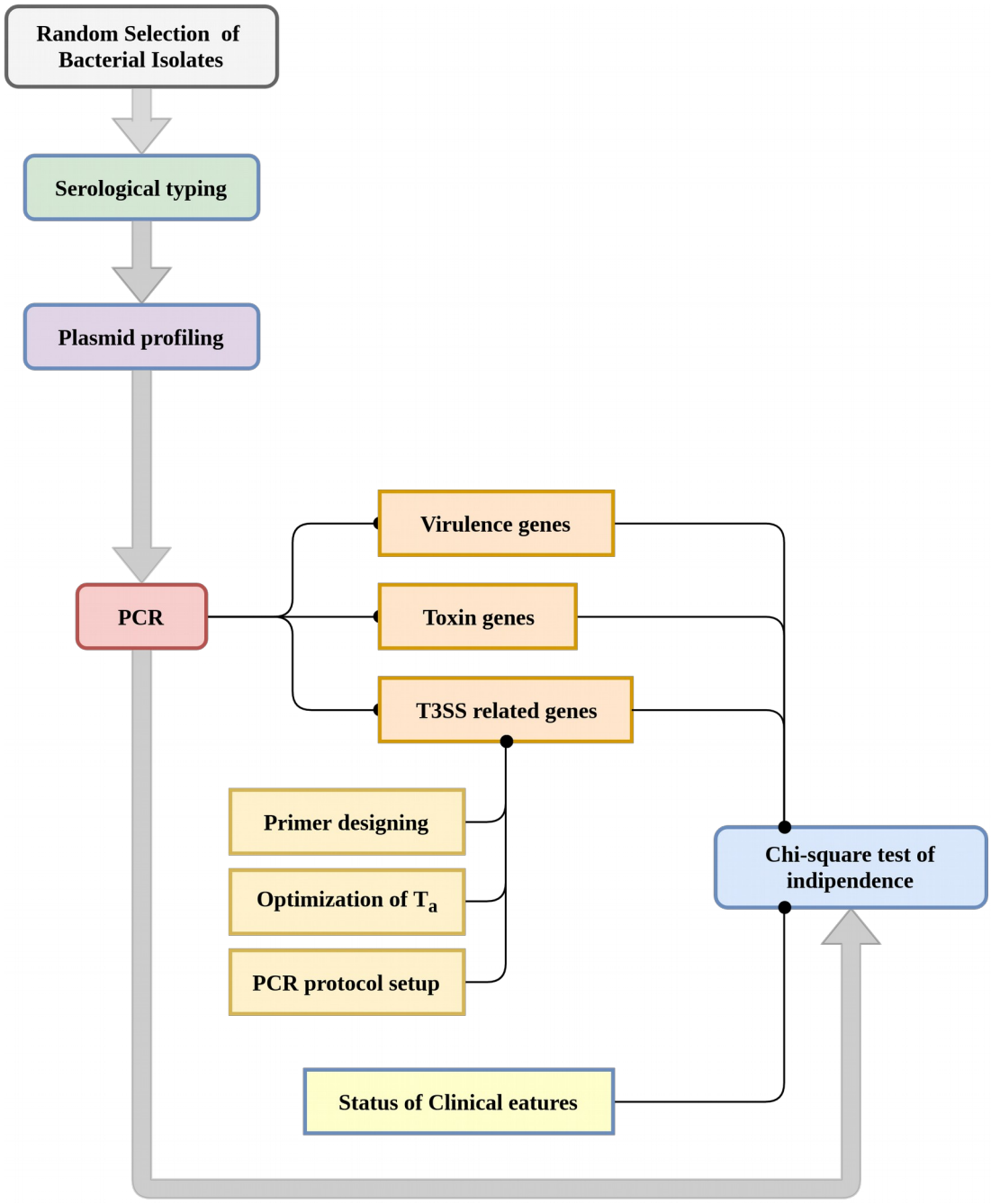

Figure 2.1: Flowchart of this study

2.2 Isolation of Strains

A total 61 clinical isolates of *Shigella flexneri* of different serotypes isolated from patients enrolled in Mirzapur study, during a period over 2009 to 2013, were used in this study. Of these 61 *S. flexneri* strains, 5 were of *S. flexneri* 1b, 9 were *S. flexneri* 1c, 16 of *S. flexneri* 2a, 15 were of *S. flexneri* 2b, 8 of *S. flexneri* 3a, 3 of *S. flexneri* Type-4 and 5 were of *S. flexneri* serotype-6. These strains were isolated and characterized in the Enteric and Food Microbiology Laboratory of icddr,b following standard Microbiological and Biochemical methods (El-Gendy et al., 1999).

A single colony of confirmed *Shigella* strain was grown in Tryptic Soy Broth (TSB) with 0.3% yeast extract and stored at -70°C after addition of 15% glycerol for further use. *S. flexneri* 2a, YSH6000 strain (Sasakawa et al., 1986), and ATCC (American type culture collection) strain (lacking 140 MD invasive plasmid) were used as positive and negative control respectively, in Plasmid analysis and PCR for toxin, virulence, translocator, effector, regulator, chaperone and machinery genes.

E. coli strains PDK-9, V-517, Sa and R1 were used as plasmid molecular weight standard. All these strains used as standard were collected from the Enteric and Food Microbiology Laboratory icddr,b.

All the clinical history of these 61 samples were obtained from icddr,b Mirzapur study database.

2.3 Serological Typing

All the strains were serologically confirmed using commercially available antisera kit (Denka Saiken, Co. Ltd. Japan) as well as, monoclonal antibody (Reagenesia AB, Stockholm, Sweden) reagents specific for all *S. flexneri* type and group-factor antigens.

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 (El-Gendy et al., 1999).

2.4 Plasmid Analysis

2.4.1 Isolation of Plasmid DNA by Alkaline Lysis Method

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

Reagents

1. Solution I : 40 mM tris-NaOAc, 2mM EDTA, pH 7.4
2. Solution II (lysing solution) : 3% SDS, 50 mM Tris, pH 12.6
3. 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. Then it 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 a micropipette, and 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.

2.4.2 Separation of Plasmid DNA by Agarose Gel Electrophoresis

Reagents

1. Agarose (Sigma Chemical Co., St. Louis, Mo)
2. TBE (Tris-borate EDTA) buffer (GIBCO-BRL)
3. Tracking dye (10X concentration)
4. Ethidium bromide (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 (50mA) for 3 hours. Briefly, 30 µl of plasmid DNA solution was mixed with 3 µl of loading dye (Appendix), and was loaded into the individual well of the gel electrophoresis. After the electrophoresis of the gel (5 mm thick) was then stained with 0.5 µg of ethidium bromide for 25 minutes, and then destained with distilled water at room temperature. DNA bands were visualized and photographed using Gel Doc™ XRT with Image Lab™ 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 MD), V-517 (35.8, 4.8, 3.7, 3.4, 3.1, 2.0, 1.8 and 1.4 MD), Sa (23 MD) and R1 (62 MD) were used as molecular weight standard.

2.5 PCR Assay to Detect Virulence and Enterotoxin Genes

Materials

1. 10X PCR buffer (GIBCO-BRL)
2. 50 mM $MgCl_2$ (GIBCO-BRL)
3. 2.5 mM dNTPs (GIBCO-BRL)
4. Taq DNA polymerase (5 U/µl, GIBCO-BRL)
5. Primers
6. Mineral oil (GIBCO-BRL)
7. Filtered deionized water
8. Agarose
9. Ethidium bromide (10 mg/ml)
10. TBE (Tris-borate EDTA) buffer (GIBCO-BRL)
11. 100 bp DNA size standard (Bio-Rad)

Procedure

The method, which was previously described by Vargas, *et al.*, 1999 (Vargas and Gascon, 1999), was followed to detect the virulence, enterotoxin genes. Representative strains were grown on MacConkey agar for overnight. A single colony of each isolate was suspended in 25 µl of reaction mixture containing 2.5 µl of 10X PCR buffer, 1.5 µl of 50 mM of $MgCl_2$, 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 programs:

Table 2.1: PCR programs used for virulence and enterotoxin gene detection.

Gene	Cycle	T_d (°C)	T_a(°C)	T_e (°C)	Final Ext^n (°C)
ipaH	29	95°C, 50 sec	60°C, 90 sec	72°C, 120 sec	72°C, 600 sec
ial	29	95°C, 50 sec	55°C, 30 sec	72°C, 80 sec	72°C, 360 sec
set1A	30	95°C, 50 sec	55°C, 90 sec	72°C, 120 sec	72°C, 420 sec
set1B	30	95°C, 50 sec	55°C, 90 sec	72°C, 120 sec	72°C, 420 sec
sen	34	94°C, 30 sec	55°C, 60 sec	72°C, 60 sec	72°C, 300 sec

Amplification products were subjected to horizontal gel electrophoresis in 1% agarose gel in TBE buffer, at room temperature at 100 volt (50 mA) for 1 hour. Briefly, 10 µl of amplified DNA for each sample was mixed with 1 µl of tracking dye, and loaded into individual well of the gel (5 mm thick). DNA bands were detected by staining the gel with ethidium bromide (0.5 µg) for 30 minutes at room temperature, and photographs were taken using Gel Doc™ XRT with Image Lab™ software. DNA size standard was of 100 bp (Bio-Rad), which was used as a marker to measure the molecular size of the amplified products.

Table 2.2: Primers used for virulence and enterotoxin gene detection.

Gene encoding virulence factor	Oligonucleotide sequence (5' to 3')	Size of amplified product (bp)	Reference
ipaH	TGGAAAAACTCAGTGCCTCT CCAGTCCGTAAATTCATTCT	423	(Talukder et al., 2003)
ial	CTGGATGGTATGGTGAGG GGAGGCCAACAATTATTTCC	320	(Mitra et al., 2012)
set1A	TCACGCTACCATCAAAGA TATCCCCCTTTGGTGGTA	309	(Fasano and Noriega, 1995)
set1B	GTGAACCTGCTGCCGATATC ATTTGTGGATAAAAATGACG	147	(Fasano and Noriega, 1995)
sen	ATGTGCCTGCTATTATTTAT CATAATAATAAGCGGTCAGC	799	(Nataro et al., 1995)

2.6 Primer Designing

2.6.1 Sequence Retrieval from Database

Materials

1. World wide web access
2. NCBI gene database
3. NCBI nucleotide database
4. Primer3plus (web based bioinformatics tool)

Procedure

Desired genes were searched for within the NCBI Gene database. From there, FASTA format of the nucleotide sequence of the target genes were obtained.

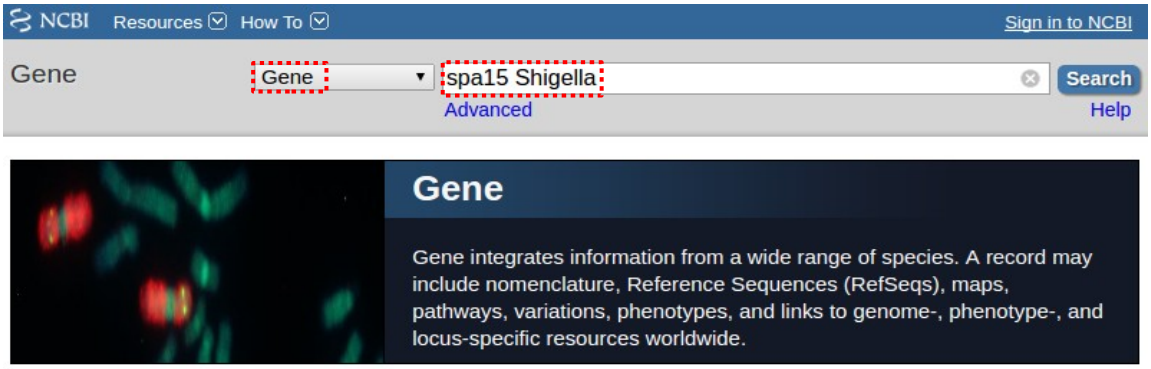

Figure 2.2: Target gene search (spa15) within NCBI gene database

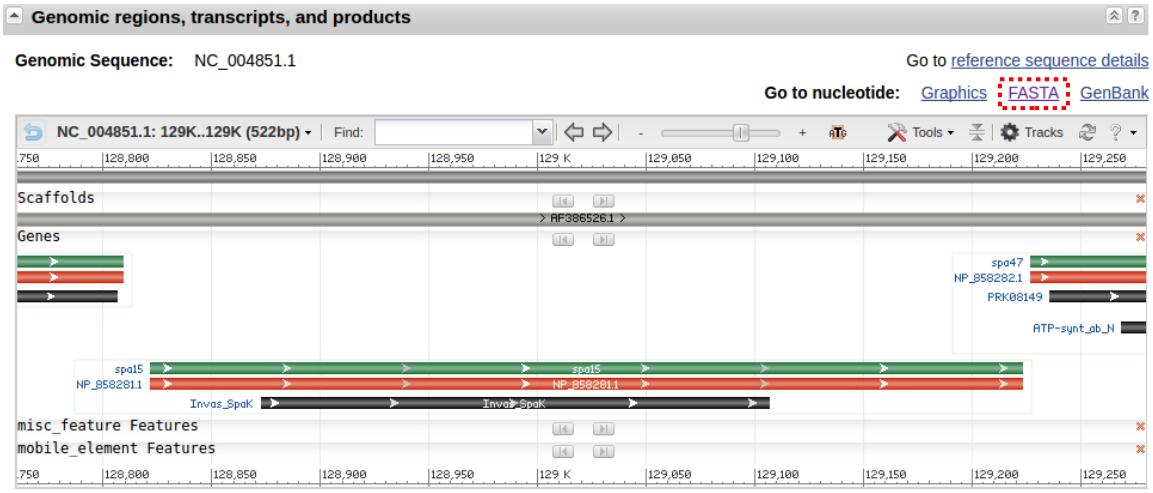

Figure 2.3: FASTA format retrieval of spa15 from NCBI gene database.

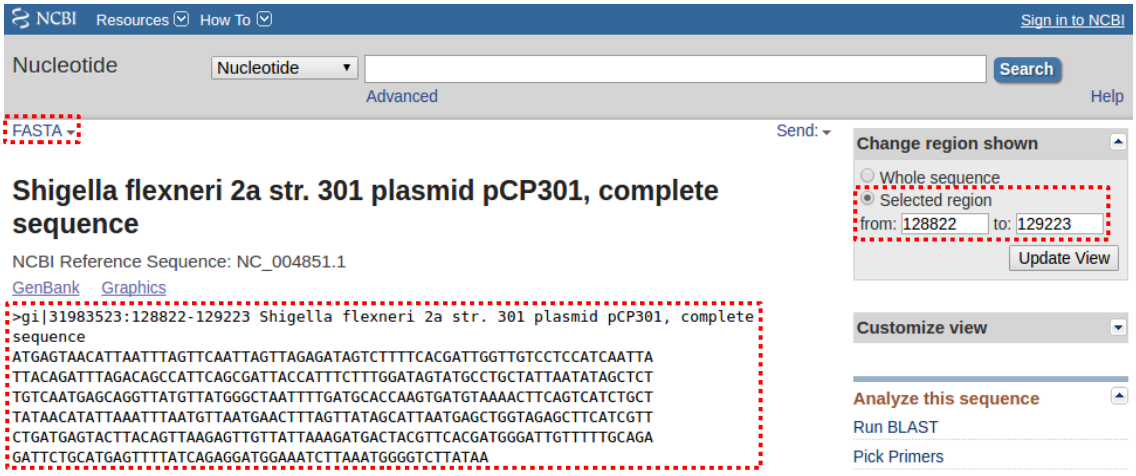

Figure 2.4: DNA sequence of spa15 gene from *S. flexneri* 2a str. 301 plasmid pCP301.

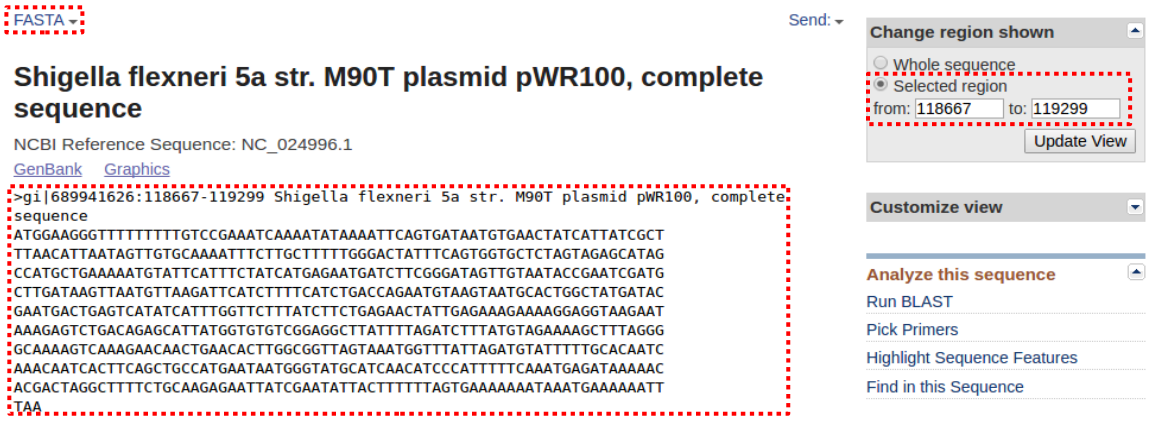

Figure 2.5: DNA sequence of mxiE gene from *S. flexneri* 5a str. M90T plasmid pWR100.

The retrieved sequence was then fed into the web based primer designing tool, primer3plus; for bioinformatic analysis.

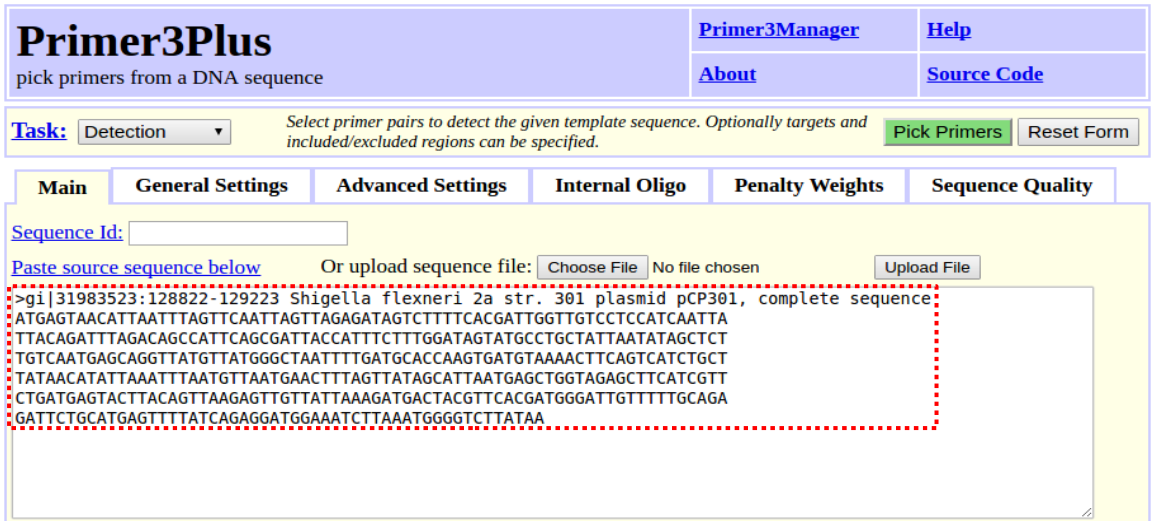

Figure 2.6: Input of spa15 DNA sequence into Primer3plus

(http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) input box for bioinformatic analysis.

2.6.2 Bioinformatic Analysis of Primer Parameters

Materials:

1. World wide web access
2. FASTA formatted DNA sequences of mxiE and spa15 genes of *Shigella* spp.
3. Primer3plus (web based bioinformatics tool)

Procedure:

I. **Primer Length and Specificity:** Primer length was selected to be in between 18 – 30 nucleotides, as the specificity, and the temperature and time of annealing are partially dependent on primer length (Wu et al., 2009). It was chosen as such, so that they had a unique sequence within the template DNA that was to be amplified, as primer designed with a highly repetitive sequence results in a smear when amplifying genomic DNA. During the selection of parameters, primers with long runs of a single base was avoided. It was ensured that, 4 or more G's and C's in a row were not present.

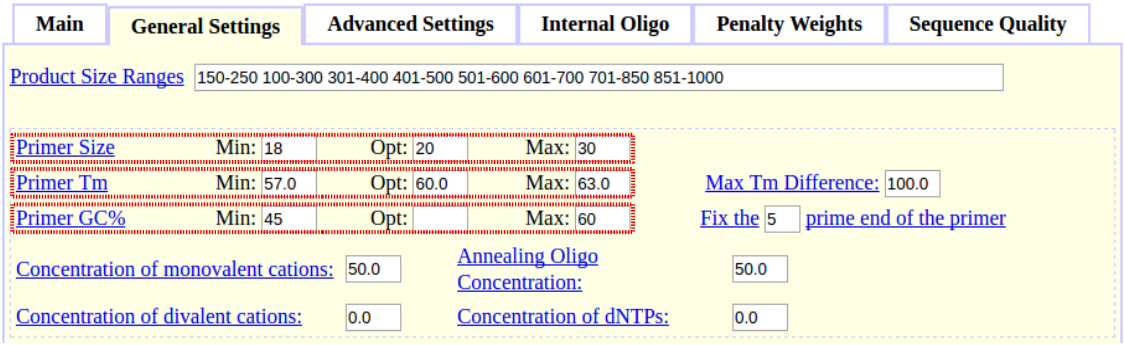

Figure 2.7: General settings parameters optimization for spa15 gene PCR primer design

II. **Melting Temperature (T_m):** The optimal melting temperature was set in the range of 57 – 63°C, as higher melting temperature produce better results than the lower ones. Temperature more than 65°C for T_m was also avoided to circumvent the potential for

secondary annealing. Sequencing reaction with annealing and extension were chosen at 60°C. The formula of Wallace et al., 1979, $T_m = 2(A+T) + 4(G+C)$, was considered for a decent working approximation, valid for 18 – 30 nucleotide oligonucleotides.

III. **GC Contents:** GC content was chosen carefully in between 45 – 60% (Dieffenbach et al., 1993), as less than 50% GC content extends the primer sequence beyond 18 bases to keep the T_m above the recommended limit of 50°C. It was considered cautiously, as GC content, T_m and T_a are strictly dependent on one another (Rychlik et al., 1990).

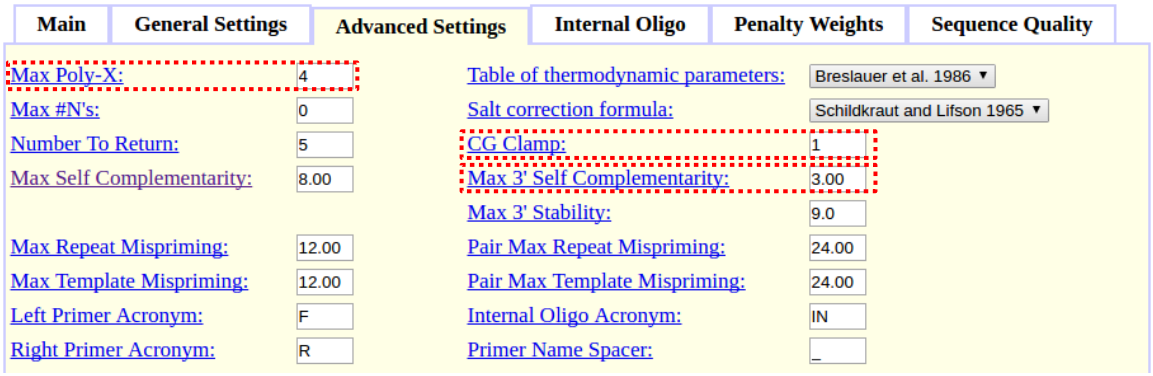

Figure 2.8: Advanced settings parameters optimization for spa15 gene PCR primer design

IV. **GC Clamp (3'-End Sequence):** GC clamp was incorporated into the primer to reduce spurious secondary bands (Sheffield et al., 1989). Theoretically, the GC rich sticky 3'-end anneals at multiple sites on the template DNA and reduces mispriming (Kwok et al., 1990).

V. **Complementary Primer Sequence, Dimer Formation and False Priming:** Any intra-primer homology beyond 3 base-pairs was omitted, so that hairpins were not formed causing the primer to fold back on itself resulting in unproductive priming events, and decreasing the overall obtained signal (Breslauer et al., 1986). Besides, inter-primer homology was also avoided, so that interfere with hybridization and primer dimer formation could not occur.

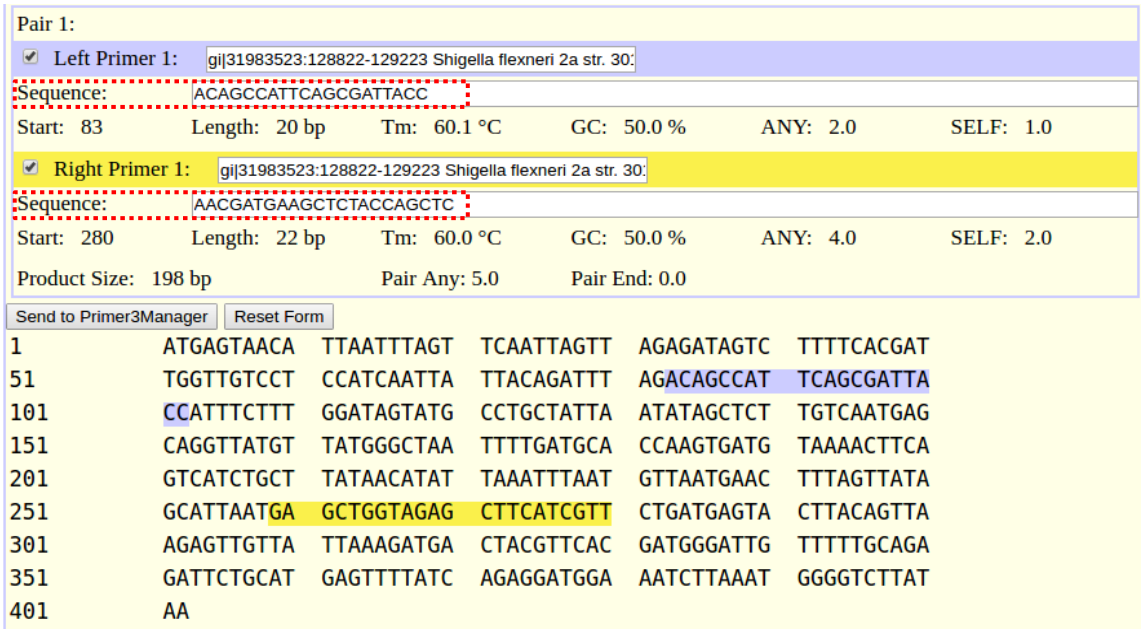

Figure 2.9: Primer sequence output for spa15 gene in Primer3plus software.

Table 2.3: Gene sequence and primer sequences for spa15 and mxiE genes.

Gene	***spa15***
FASTA	>gi|31983523:128822-129223 Shigella flexneri 2a str. 301 plasmid pCP301, complete sequence ATGAGTAACATTAATTTAGTTCAATTAGTTAGAGATAGTCTTTTCACGATTGGTTGTCCTCCATCAATTA TTACAGATTTAGACAGCCATTCAGCGATTACCATTTCTTTGGATAGTATGCCTGCTATTAATATAGCTCT TGTCAATGAGCAGGTTATGTTATGGGCTAATTTTGATGCACCAAGTGATGTAAAACTTCAGTCATCTGCT TATAACATATTAAATTTAATGTTAATGAACTTTAGTTATAGCATTAATGAGCTGGTAGAGCTTCATCGTT CTGATGAGTACTTACAGTTAAGAGTTGTTATTAAAGATGACTACGTTCACGATGGGATTGTTTTTGCAGA GATTCTGCATGAGTTTTATCAGAGGATGGAAATCTTAAATGGGGTCTTATAA
Primer 1	ACAGCCATTCAGCGATTACC
Primer 2	AACGATGAAGCTCTACCAGCTC
Gene	***mxiE***
FASTA	>gi|689941626:118667-119299 Shigella flexneri virulence plasmid pWR100: from 1 to 213494 ATGGAAGGGTTTTTTTTTGTCCGAAATCAAAATATAAAATTCAGTGATAATGTGAACTATCATTATCGCT TTAACATTAATAGTTGTGCAAAATTTCTTGCTTTTTGGGACTATTTCAGTGGTGCTCTAGTAGAGCATAG CCATGCTGAAAAATGTATTCATTTCTATCATGAGAATGATCTTCGGGATAGTTGTAATACCGAATCGATG CTTGATAAGTTAATGTTAAGATTCATCTTTTCATCTGACCAGAATGTAAGTAATGCACTGGCTATGATAC GAATGACTGAGTCATATCATTTGGTTCTTTATCTTCTGAGAACTATTGAGAAAGAAAAGGAGGTAAGAAT AAAGAGTCTGACAGAGCATTATGGTGTGTCGGAGGCTTATTTTAGATCTTTATGTAGAAAAGCTTTAGGG GCAAAAGTCAAAGAACAACTGAACACTTGGCGGTTAGTAAATGGTTTATTAGATGTATTTTTGCACAATC AAACAATCACTTCAGCTGCCATGAATAATGGGTATGCATCAACATCCCATTTTTCAAATGAGATAAAAAC ACGACTAGGCTTTTCTGCAAGAGAATTATCGAATATTACTTTTTTAGTGAAAAAAATAAATGAAAAAATT TAA
Primer 1	GCATAGCCATGCTGAAAAATG
Primer 2	TCCGACACACCATAATGCTC

2.7 Annealing Temperature Optimization with Gradient PCR and PCR Protocol Setup

Materials

1. 10X PCR buffer (GIBCO-BRL)
2. 50 mM $MgCl_2$ (GIBCO-BRL)
3. 2.5 mM dNTP (GIBCO-BRL)
4. Taq DNA polymerase (5 U/µL GIBCO-BRL)
5. Primers
6. Mineral oil (GIBCO-BRL)
7. Filtered deionized water
8. Agarose
9. Ethidium bromide (10 mg/ml)
10. TBE (Tris-borate EDTA) buffer (GIBCO-BRL)
11. 100 bp DNA size standard (Bio-Rad)
12. DNA thermal cycler (Model 480; Perkin-Elmer Cetus, Emeryville, Calif.)

Procedure

The method previously described by (Vargas et al., 1999) was followed, to detect the genes related to type III secretion system (substrate, effector, regulator, chaperone and machinery genes), similarly as before, to detect virulence, enterotoxin genes. Representative strains were grown on MacConkey agar for overnight. A single colony of each isolate was suspended in 25 µl of reaction mixture containing 2.5 µl of 10X PCR buffer, 1.5 µl of 50 mM of $MgCl_2$, 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 programs.

Table 2.4: Programs used for mxiE and spa15 genes' gradient PCR.

Gene	Cycle	T_d (°C)	T_a(°C) Gradient		T_e (°C)	Final Ext^n (°C)
			Lowest T_a(°C)	Highest T_a(°C)		
mxiE	32	94°C, 60 sec	48°C, 60 sec	64°C, 60 sec	72°C, 90 sec	72°C, 600 sec
spa15	32	94°C, 60 sec	48°C, 60 sec	64°C, 60 sec	72°C, 90 sec	72°C, 600 sec

With a predicted product size of 249 bp and 198 bp from the bioinformatic analysis of mxiE and spa15 primers respectively, denaturation temperature (T_d) was set at 94°C for 60 seconds, and extension temperature (T_e) was set at 72°C for 90 seconds, with a temperature for final extension at 72°C for 10 minutes. And, at the annealing stage in between T_d and T_e, a gradient step was incorporated to find out the optimal annealing temperature (T_a).

Annealing temperature was optimized to get a clean product with the following steps. The predicted annealing temperature was calculated based on the melting temperature (T_m) of the primers and template. PCR protocol was created with a gradient in the annealing step. The gradient range was created bracketing the T_a by 5-12°C. A high annealing temperature from the results of the gradient PCR run was chosen, to reduce the chance of non-specific primer binding. The higher annealing temperature was chosen with care, so that yield decrease or inconsistent behavior of primers at high temperature, do not happen.

2.8 PCR Assay to Detect Type III Secretion System Genes

Materials

1. 10X PCR buffer (GIBCO-BRL)
2. 50 mM $MgCl_2$ (GIBCO-BRL)
3. 2.5 mM dNTP (GIBCO-BRL)
4. Taq DNA polymerase (5 U/µL GIBCO-BRL)
5. Primers
6. Mineral oil (GIBCO-BRL)
7. Filtered deionized water
8. Agarose
9. Ethidium bromide (10 mg/ml)
10. TBE (Tris-borate EDTA) buffer (GIBCO-BRL)
11. 100 bp DNA size standard (Bio-Rad)
12. DNA thermal cycler (Model 480; Perkin-Elmer Cetus, Emeryville, Calif.)

Procedure

The method previously described by Vargas et al., 1999, was followed, to detect the genes related to type III secretion system (substrate, effector, regulator, chaperone and machinery genes), similarly as with the detection of virulence, enterotoxin genes. Representative strains were grown on MacConkey agar for overnight. A single colony of each isolate was suspended in 25 µl of reaction mixture containing 2.5 µl of 10X PCR buffer, 1.5 µl of 50 mM of $MgCl_2$, 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 programs:

Table 2.5: PCR programs used for Type III Secretion System related gene detection.

Gene	Cycle	T_d (°C)	T_a(°C)	T_e (°C)	Final Ext^n (°C)
virB	32	94°C, 120 sec	50°C, 120 sec	72°C, 180 sec	72°C, 300 sec
ipaBCD	34	94°C, 120 sec	57°C, 120 sec	72°C, 180 sec	72°C, 600 sec
ipaB	32	94°C, 120 sec	50°C, 120 sec	72°C, 180 sec	72°C, 300 sec
ipaC	32	94°C, 120 sec	58°C, 120 sec	72°C, 180 sec	72°C, 300 sec
ipaD	32	94°C, 120 sec	54°C, 120 sec	72°C, 180 sec	72°C, 300 sec
ipgC	32	94°C, 120 sec	50°C, 120 sec	72°C, 180 sec	72°C, 300 sec
ipgB1	32	94°C, 30 sec	50°C, 30 sec	72°C, 90 sec	72°C, 600 sec
ipgA	32	94°C, 60 sec	50°C, 60 sec	72°C, 60 sec	72°C, 600 sec
icsB	32	94°C, 120 sec	49°C, 120 sec	72°C, 180 sec	72°C, 300 sec
ipgD	32	94°C, 120 sec	48°C, 120 sec	72°C, 180 sec	72°C, 300 sec
ipgE	32	94°C, 120 sec	50°C, 120 sec	72°C, 180 sec	72°C, 300 sec
ipgF	32	94°C, 30 sec	60°C, 60 sec	72°C, 90 sec	72°C, 600 sec
mxiH	32	94°C, 120 sec	49°C, 120 sec	72°C, 180 sec	72°C, 300 sec
mxiI	32	94°C, 120 sec	49°C, 120 sec	72°C, 180 sec	72°C, 300 sec
mxiK	31	94°C, 30 sec	47°C, 60 sec	72°C, 90 sec	72°C, 600 sec
mxiE	32	94°C, 60 sec	60°C, 60 sec	72°C, 90 sec	72°C, 600 sec
mxiC	29	94°C, 60 sec	63°C, 60 sec	72°C, 60 sec	72°C, 600 sec
spa15	32	94°C, 60 sec	60°C, 60 sec	72°C, 90 sec	72°C, 600 sec
spa	32	94°C, 60 sec	52°C, 60 sec	72°C, 60 sec	72°C, 300 sec
spa47	31	94°C, 30 sec	64°C, 60 sec	72°C, 120 sec	72°C, 600 sec
spa32	29	94°C, 60 sec	54.5°C, 60 sec	72°C, 60 sec	72°C, 300 sec
spa24	34	95°C, 60 sec	52°C, 60 sec	72°C, 60 sec	72°C, 600 sec
ipaH7.8	34	95°C, 60 sec	50°C, 60 sec	72°C, 60 sec	72°C, 600 sec

Amplified products were then subjected to horizontal gel electrophoresis, in 1% agarose gel in TBE (Tris-borate EDTA) buffer, at room temperature at 100 volt (50 mA) for 1 hour. Briefly, 10 µl of amplified DNA for each sample was mixed with 1 µl of tracking dye, and loaded into individual well of the gel (5 mm thick). DNA bands were detected by staining the gel with ethidium bromide (0.5 µg) for 30 minutes at room temperature, and photographs

were taken using Gel Doc™ XRT with Image Lab™ software. DNA size standard was of 100 bp (Bio-Rad), which was used as a marker to measure the molecular size of the amplified products.

Table 2.6: Primers used for Type III Secretion System related gene detection.

Gene encoding virulence factor	Oligonucleotide sequence (5' to 3')	Size of amplified product (bp)	Reference
virB	GCGAAAGTCACTCGTC CCATCATGCCGCATCC	255	(Berlutti et al., 1998)
ipaBCD	GCTATAGCAGTGACATGG ACGAGTTCGAAGCACTC	500	(Faruque et al., 2002)
ipaB	GTAAGCACCACAAACACTGG TGAAGTTGAGTGTGCATTAG	208	(Marteyn et al., 2010)
ipaC	CCCCCGGGGGAAATTCAAAACACAAAACCAACC CCAAGCTTCGCACGAATATTACCCGCAATCTGACT	400	(Faruque et al., 2002)
ipaD	ATGAATATAACAACTCTGACT ATGGACAAAAAGTTTATCTGT	450	(Faruque et al., 2002)
ipgC	CACCATGTCTTTAAATATCACCG ATACTCCTTGATATCCTGAATTG	475	(Lane and Gantt, 2004)
ipgB1	CGGGATCCCATATAGGGGGTATCATG GCGTCGACTTAATTTGTATTGCTTTG	650	(Ohya et al., 2005)
ipgA	CGCGGATCCATGTGTCGCAAACTATATG CCGGAATTCTTAGTTCACTTCTGAAGTG	435	(Ogawa et al., 2003)
icsB	CGCGGATCCATGAGCCTCAAAATTAGCAA CCGGAATTCCTATATATTAGAATGAG	1568	(Ogawa et al., 2003)
ipgD	TCGGCGTCAGAAGAGAAGTC TATTAGCACATCATCATCAA	560	(Lan et al., 2001)
ipgE	CCACCGGTCGACTTAATACCCCTTCATTCTTCG CCACGCGGATCCATGGAAGATTTAGCAGATGTT	390	(Niebuhr et al., 2000)
ipgF	GCTTGAATTCGATTGTTGGGATAAGGCTGG GAGCAAGCTTTTATTATATCCTTCGATTATTCTGCTTGCTC	441	(Zahrl et al., 2005)
mxiH	AGCGGATCCAGTGTTACAGTACCG CGCGTCGACTGGATTATCTGAAGT	283	(Magdalena et al., 2002)
mxiI	CATGCCATGGTTTACATTTATCCAGTC CCCAAGCTTAGACTTTAATAAAGTTTC	318	(Magdalena et al., 2002)
mxiK	GGCGTAATCAGAAGTGAG TATACGCAGTGATTCAGC	575	(Yang et al., 2003)

mxiE	GCATAGCCATGCTGAAAAATG TCCGACACACCATAATGCTC	249	This study
mxiC	GTAGGTGATGTATGCTTG GATCACTTTCATCTCCTG	1067	(Lan et al., 2001)
spa15	ACAGCCATTCAGCGATTACC AACGATGAAGCTCTACCAGCTC	198	This study
spa47	CTCGGGGCCAATCTTGGAAAC CGGCCGACTTGCGTATGAAGA	237	(Jouihri et al., 2003)
spa32	GCTCGCATGCCTTTTGGAGGATGAT GGCCGGATCCAAGAACCATTTACT	958	(Magdalena et al., 2002)
spa24	TTCTGATTCTGGCTTGATG GTGTCTGCTCTTGGAGTTG	547	(Yang et al., 2003)
spa	ATCATTTCTTTACTCCCGGGA CGCTATTACCATTTTTAGTTGC	350	(Beloin and Dorman, 2003)
ipaH7.8	GCAGAGAAACTTCAGCTC TATTGAGCGAGGATTTACG	533	(Yang et al., 2003)

2.9 Statistical analysis

Cross tabulations were done, and then Pearson Chi-square tests of independence were conducted to study, whether the presence of a particular clinical feature does not depend upon the presence of a specific gene. The results were considered to be statistically significant if P value was found <0.05 at 5% level of significance, and to be highly statistically significant if P value was found <0.01 at 1% level of significance. Median value and inter quartile ranges (IQR) for certain variables were also obtained as per requirement. All statistical calculations were carried out with IBM® SPSS® Statistics Version 22.0 software. Graphs and figures were prepared using Image Lab Version 5.2.1, IBM® SPSS® Statistics Version 22.0, ImageJ 1.48k, GNU Image Manipulation Program Version 2.8.10 softwares.

Chapter 3 : Results

3.1 Plasmid Profile Analysis

Of 61 randomly selected *S. flexneri* strains, the virulence plasmid of 140 MD, carrying virulence genes were present in 49 strains, except in following 13 strains (79% 140 positive, n=48), K-3336 *S. flexneri* 1b, K-3253 *S. flexneri* 1c, K-3229 *S. flexneri* 1c, K-3127 *S. flexneri* 1b, K-2982 *S. flexneri* 1b, K-2680 *S. flexneri* 1c, K-2401 *S. flexneri* 1c, K-2067 *S. flexneri* 1c, K-1940 *S. flexneri* 1b, K-1721 *S. flexneri* 1b, K-1080 *S. flexneri* 2a, K-1044 *S. flexneri* 2b, K-842 *S. flexneri* 1c.

Among 16 *S. flexneri* 2a, 15 contained 140 virulence plasmid (94%). In *S. flexneri* 2b, 14 out of 15 strains was 140 positive (93%). In contrast, none of *S. flexneri* 1b was found positive for 140 MD virulence plasmid (100% 140 negative, 5 out of 5), and in *S. flexneri* 1c about 67% was found 140 negative (6 out of 9 lacked 140 MD plasmid). Amid rest of the strains, 8 *S. flexneri* 3a, 3 *S. flexneri* Type-4 and 5 *S. flexneri* 6a, all were found positive for 140 MD virulence plasmid.

Small plasmids of sizes around 2.7 MD and 2.1 MD were found in 94% strains, and therefore regarded as core plasmids. These strains showed a heterogeneous plasmid population over all. And further, at the serotype level, the pattern based on molecular weight was also found quite diverse.

The plasmid pattern of the representative *S. flexneri* strains were shown in Figure 3.1.

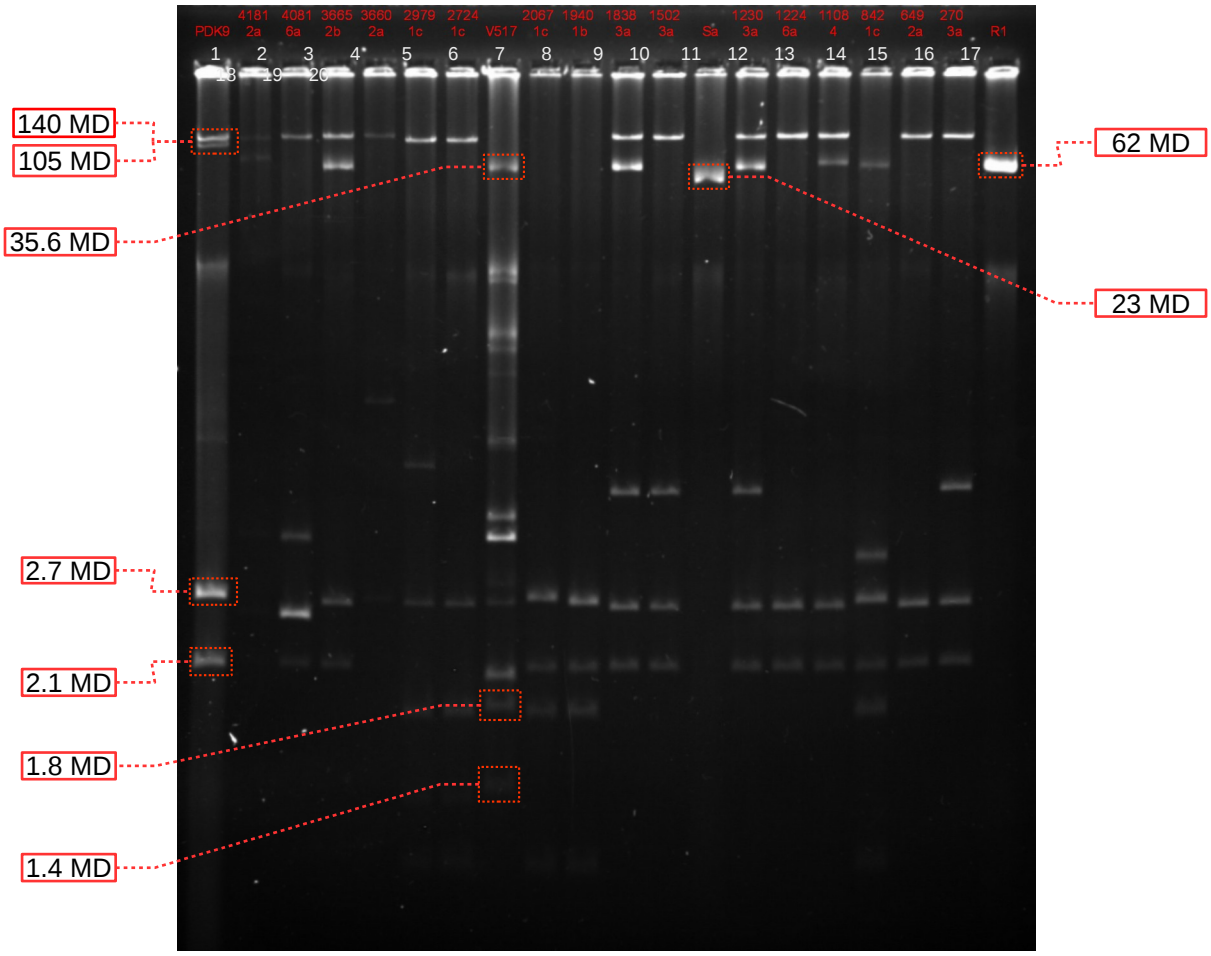

Figure 3.1: Plasmid DNA analysis of *S. flexneri* strains.

Plasmid DNA was isolated and then characterized by agarose gel electrophoresis for each strain. Lane 1, *E. coli* PDK-9; Lane 2, *S. flexneri* 2a K-4181; Lane 3, *S. flexneri* 6a K-4081; Lane 4, *S. flexneri* 2b K-3665; Lane 5, *S. flexneri* 2a K-3660; Lane 6, *S. flexneri* 1c K-2979; Lane 7, *S. flexneri* 1c K-2724; Lane 8, *E. coli* V-517; Lane 9, *S. flexneri* 1c K-2067; Lane 10, *S. flexneri* 1b K-1940; Lane 11, *S. flexneri* 3a K-1838; Lane 12, *S. flexneri* 3a K-1502; Lane 13, *E. coli* Sa; Lane 14, *S. flexneri* 3a K-1230; Lane 15, *S. flexneri* 6a K-1224; Lane 16, *S. flexneri* Type 4 K-1108; Lane 17, *S. flexneri* 1c K-842; Lane 18, *S. flexneri* 2a K-649; Lane 19, *S. flexneri* 3a K-270; Lane 20, *E. coli* R_1. The reference position of the reference strains at 140 MD, 105 MD, 62 MD, 35.6 MD, 23 MD, 2.7 MD, 2.1 MD, 1.8 MD and 1.4 MD plasmid DNA shown by indicators.

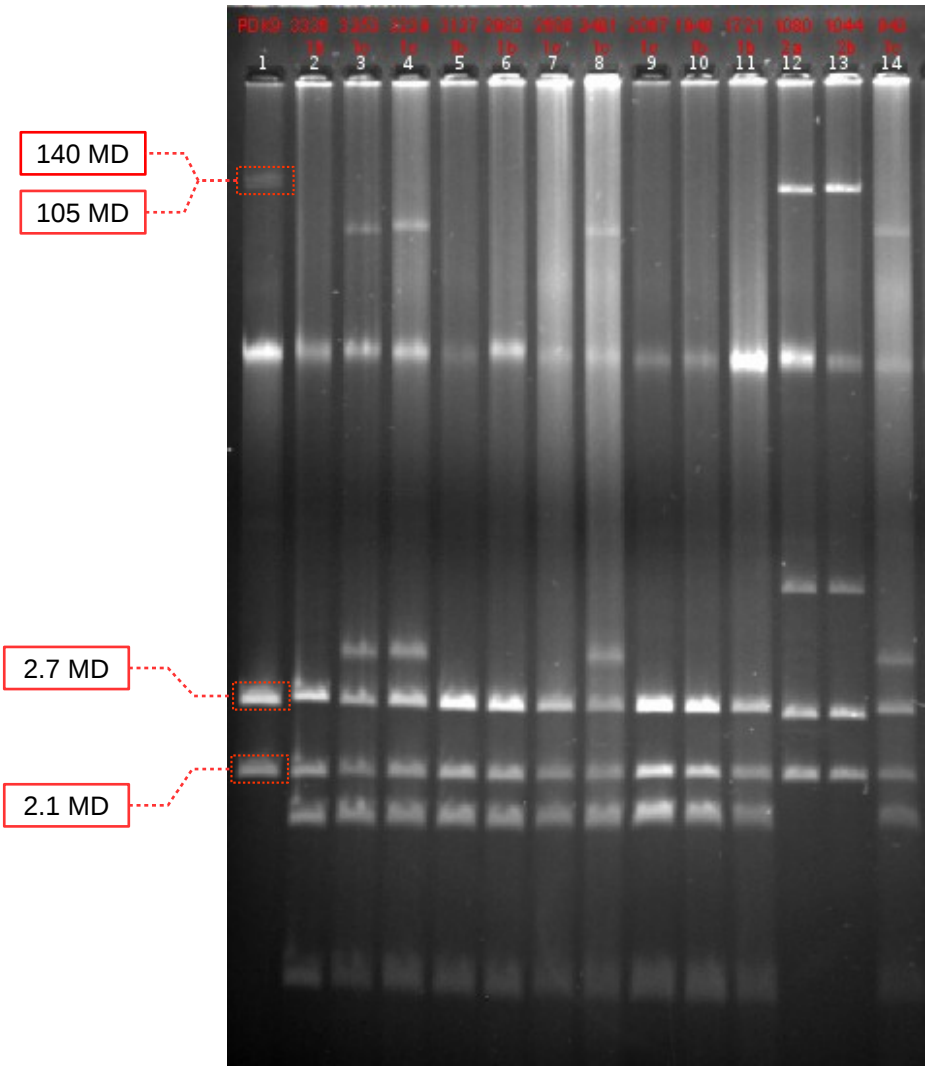

Figure 3.2: Plasmid profile of 140 MD negative representative strains

of *S. flexneri*. Lane 1 *E. coli* PDK-9, Lane 2 K-3336 *S. flexneri* 1b , Lane 3 K-3253 *S. flexneri* 1c, Lane 4 K-3229 *S. flexneri* 1c, Lane 5 K-3127 *S. flexneri* 1b, Lane 6 K-2982 *S. flexneri* 1b, Lane 7 K-2680 *S. flexneri* 1c, Lane 8 K-2401 *S. flexneri* 1c, Lane 9 K-2067 *S. flexneri* 1c, Lane 10 K-1940 *S. flexneri* 1b, Lane 11 K-1721 *S. flexneri* 1b, Lane 12 K-1080 *S. flexneri* 2a, Lane 13 K-1044 *S. flexneri* 2b, Lane 14 K-842 *S. flexneri* 1c.

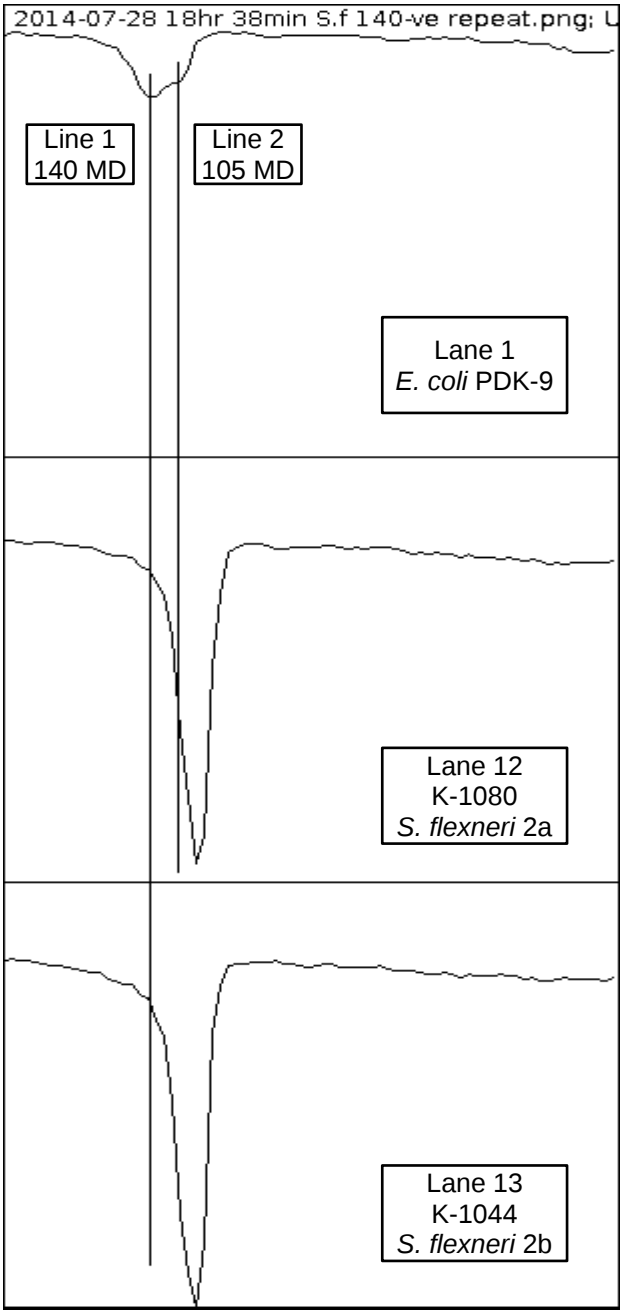

Figure 3.3: Plasmid profile band analysis

of two representative 140 MD negative strains with a reference strain; *E. coli* PDK-9 (reference strain), K-1080 *S. flexneri* 2a and K-1044 *S. flexneri* 2b.

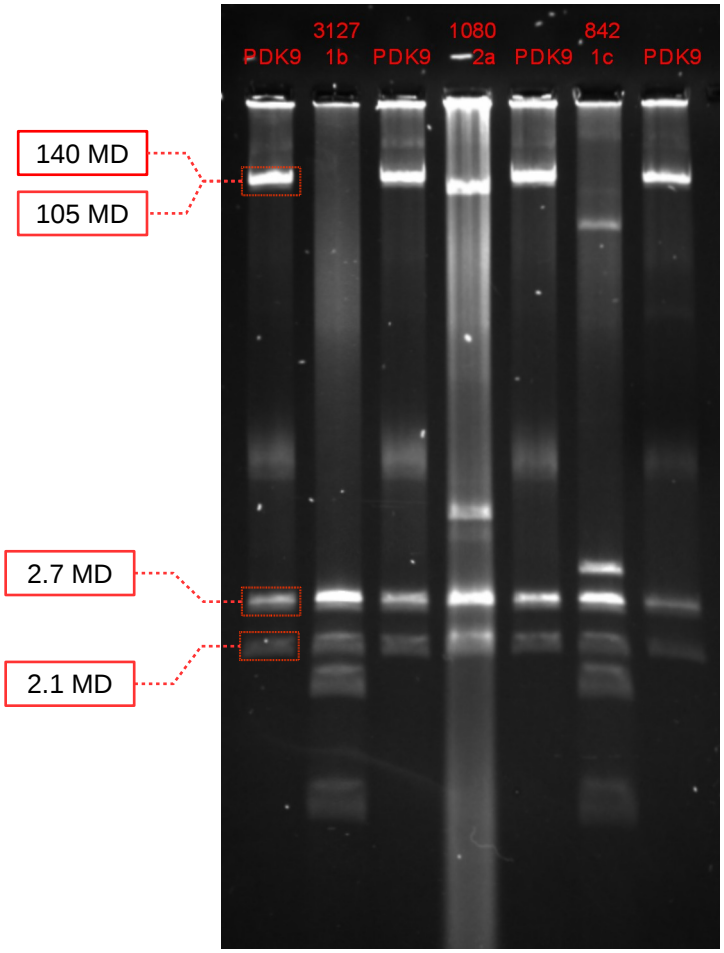

Figure 3.4: Plasmid profiling and confirmation of 140 MD plasmid absence in representative *S. flexneri* strains. Lane 1, *E. coli* PDK9 (reference strain), Lane 2, K-3217 *S. flexneri* 1b, Lane 3, *E. coli* PDK9, Lane 4, K-1080 *S. flexneri* 2a, Lane 5, *E. coli* PDK9, Lane 6, K-847 *S. flexneri* 1c, Lane 7, *E. coli* PDK9

3.2 Detection of *Shigella* Enterotoxin (*set, sen*) and virulence (*ipaH, ial*) genes

The 61 randomly selected strains of *S. flexneri* were analyzed for the presence of virulence (*ipaH, ial*) and toxin (*set, sen*) genes by polymerase chain reaction (PCR). Here, all of them were found positive for *ipaH* gene.

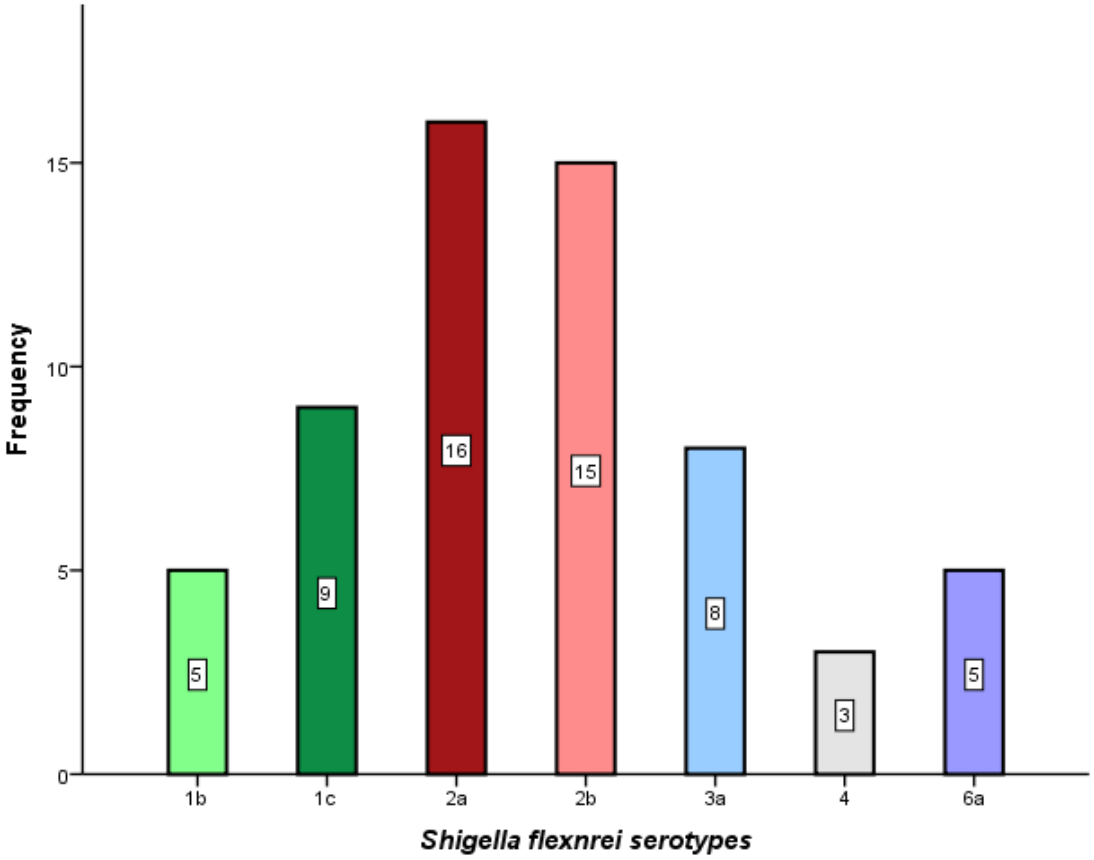

Figure 3.5: Frequency of different serotypes of *S. flexneri* strains taken in this study.

Among the two plasmid born genes *ial* and *sen*, *ial* was found in 54 *S. flexneri* strains except for 7, while all of the 7 strains lacked 140 MD large plasmid. Although, 6 *S. flexneri* strains were found positive for *ial* gene, even though they were negative for 140 MD plasmid. And, the *sen* gene was found positive in 49 strains, while devoid of in 12 strains. All of the *sen*

negative strains were 140 MD plasmid negative.

In case of *set* gene, 34 strains were detected positive, leaving negative for 27 *S. flexneri* strains, among which 22 isolates were detected negative for both *set1A* and *set1B* genes, while the rest of the 5 were negative for either one of the gene components. And further, all of the *S. flexneri* 1b and 1c strains were found negative for *set* gene. Figure 3.6 below shows the prevalence for 140 plasmid, virulence (*ipaH, ial*) and toxin (*set, sen*) genes in *S. flexneri*.

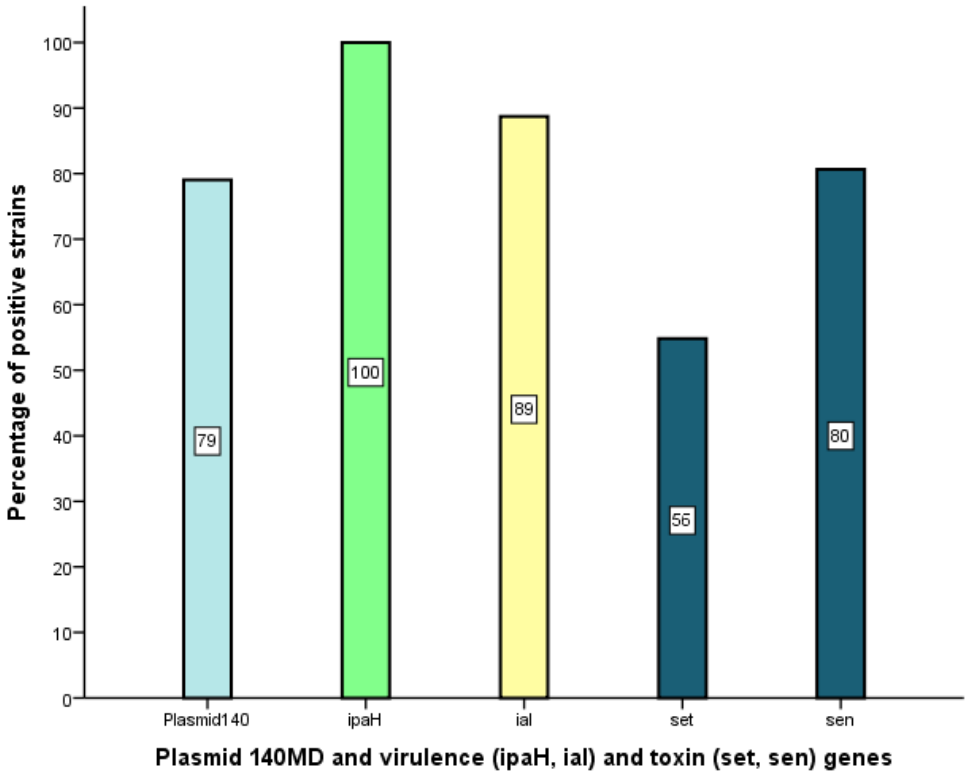

Figure 3.6: The prevalence of 140 MD plasmid, virulence and toxin genes in *S. flexneri.*

Positive results found in, Plasmid 140 (n=48), *ipaH* (n=61), *ial* (n=54), *set* (n=34), *sen* (n=49).

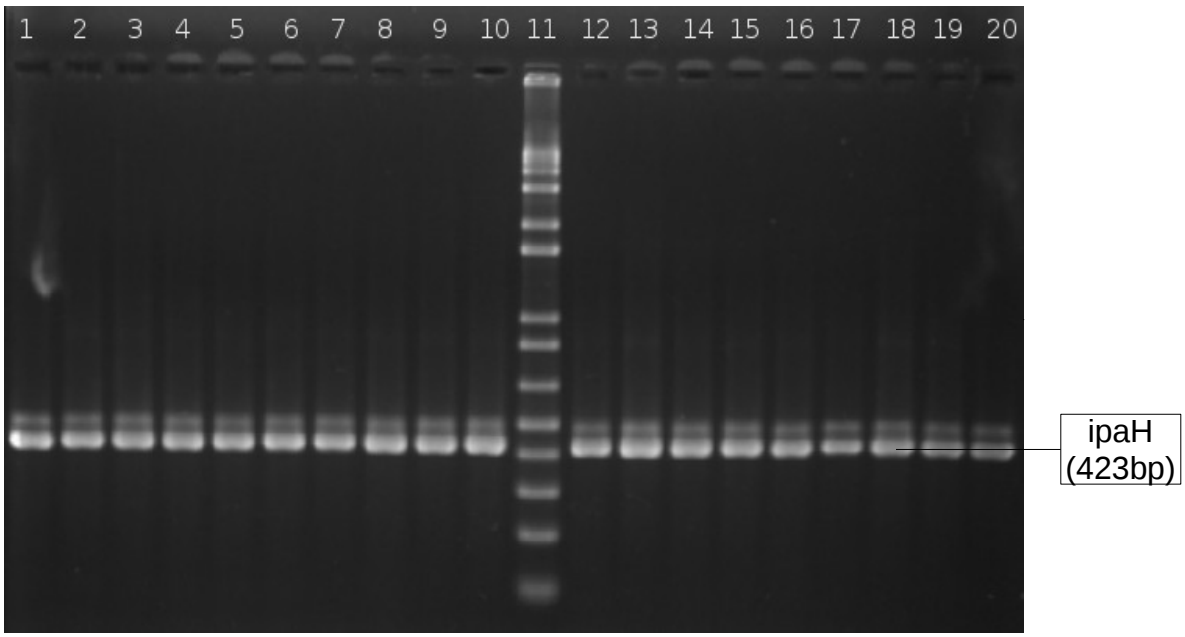

Figure 3.7: Gel electrophoresis of ***ipaH*** PCR product

of representative *S. flexneri* strains. Expected position of the PCR product of *ipaH*, shown with a black line. Lane 1, K-4193, Lane 2, K-4181, Lane 3, K-4179, Lane 4, K-4081, Lane 5, K-3687, Lane 6, K-3665, Lane 7, K-3662, Lane 8, K-3661, Lane 9, K-3660, Lane 10, K-3650, Lane 11, 1 kb plus DNA ladder, Lane 12, K-3644, Lane 13, K-3612, Lane 14, K-3597, Lane 15, K-3579, Lane 16, K-3496, Lane 17, K-3336, Lane 18, K-3253, Lane 19, K-3229, Lane 20, K-3127.

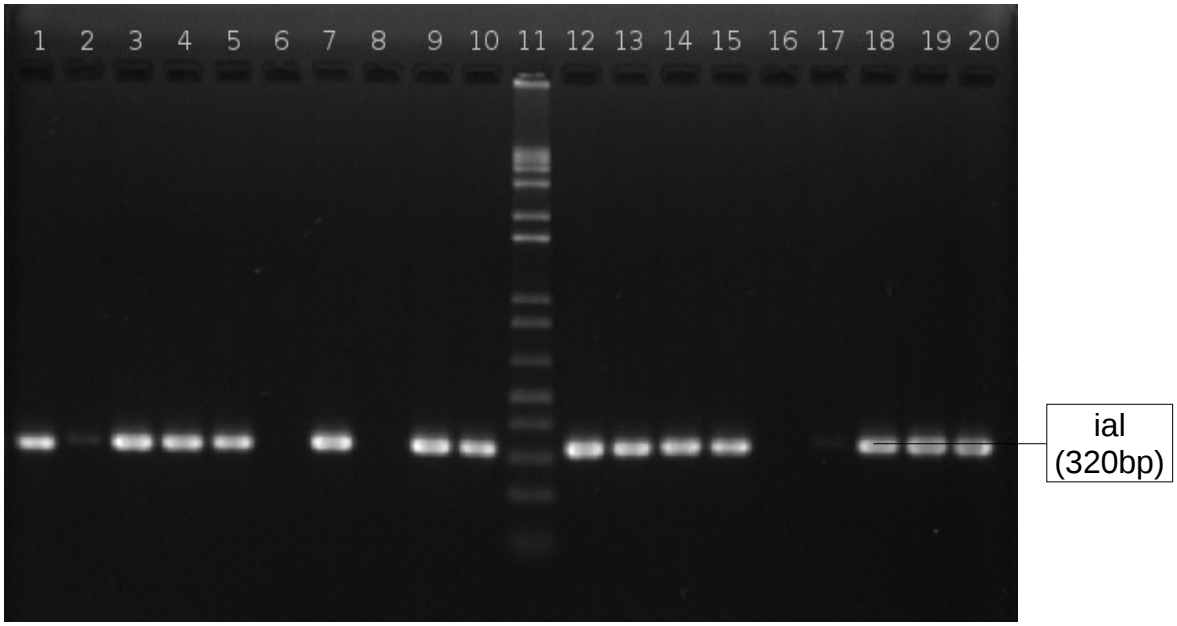

Figure 3.8: Gel electrophoresis of ***ial*** PCR product

of representative *S. flexneri* strains. Expected position of the PCR product of *ial*, shown with a black line. Lane 1, K-3644, Lane 2, K-3612, Lane 3, K-3597, Lane 4, K-3579, Lane 5, K-3496, Lane 6, K-3336, Lane 7, K-3229, Lane 8, K-2982, Lane 9, K-2980, Lane 10, K-2979, Lane 11, 1 kb plus DNA ladder, Lane 12, K-2960, Lane 13, K-2889, Lane 14, K-2724, Lane 15, K-2552, Lane 16, K-1721, Lane 17, K-1692, Lane 18, K-1502, Lane 19, K-1230, Lane 20, K-1224.

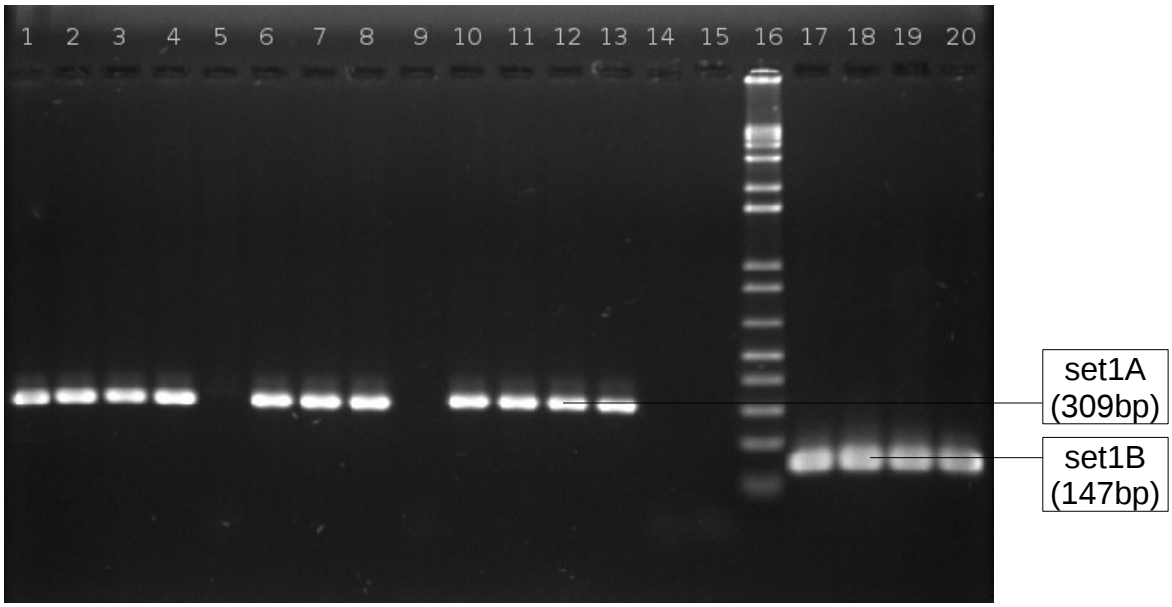

Figure 3.9: Gel electrophoresis of ***set1A*** and ***set1B*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of these genes are shown with two lines. Lane 1, K-1063, Lane 2, K-1057, Lane 3, K-1053, Lane 4, K-1044, Lane 5, K-842, Lane 6, K-662, Lane 7, K-658, Lane 8, K-649, Lane 9, K-632, Lane 10, K-629, Lane 11, K-583, Lane 12, K-570, Lane 13, Positive control, YSH6000 *S. flexneri* 2a, Lane 14, Negative control, *E. coli* ATCC-25922, Lane 15, Reagent blank, Lane 16, 1 kb plus DNA ladder, Lane 17, K-3687, Lane 18, K-3665, Lane 19, K-3662, Lane 20, K-3661.

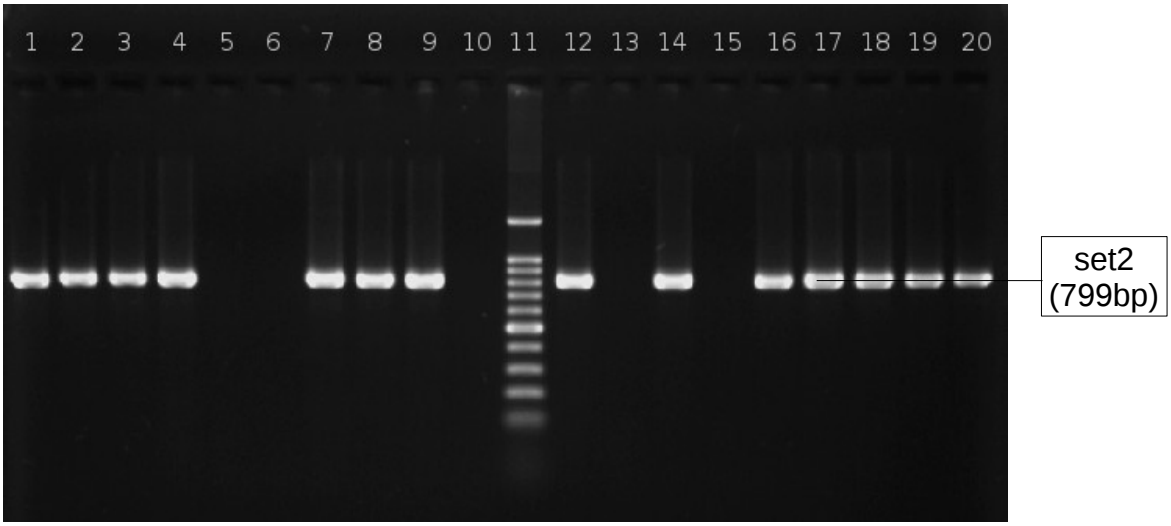

Figure 3.10: Gel electrophoresis of ***sen*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with a black line. Lane 1, K-3612, Lane 2, K-3597, Lane 3, K-3579, Lane 4, K-3496, Lane 5, K-3336, Lane 6, K-3253, Lane 7, K-2980, Lane 8, K-2979, Lane 9, K-2960, Lane 10, K-2680, Lane 11, 1 kb plus DNA ladder, Lane 12, K-2552, Lane 13, K-2401, Lane 14, K-2336, Lane 15, K-2067, Lane 16, K-1692, Lane 17, K-1502, Lane 18, K-1230, Lane 19, K-1224, Lane 20, K-1108.

3.3 Optimization of Annealing Temperature (T_a) with Gradient PCR

A temperature range of 16°C, from 48°C to 64°C was taken to optimize the annealing temperature (T_a) for PCR assay of *mxiE* and *spa15* genes. Here, YSH6000 *S. flexneri* 2a strain was used for each cases. At temperature from 54.2°C to 61.3°C, solid bands with no non-specific bands were obtained in agarose gel electrophoresis for both genes. And, thus the annealing temperature (T_a) of 60°C was chosen for PCR assay for both cases.

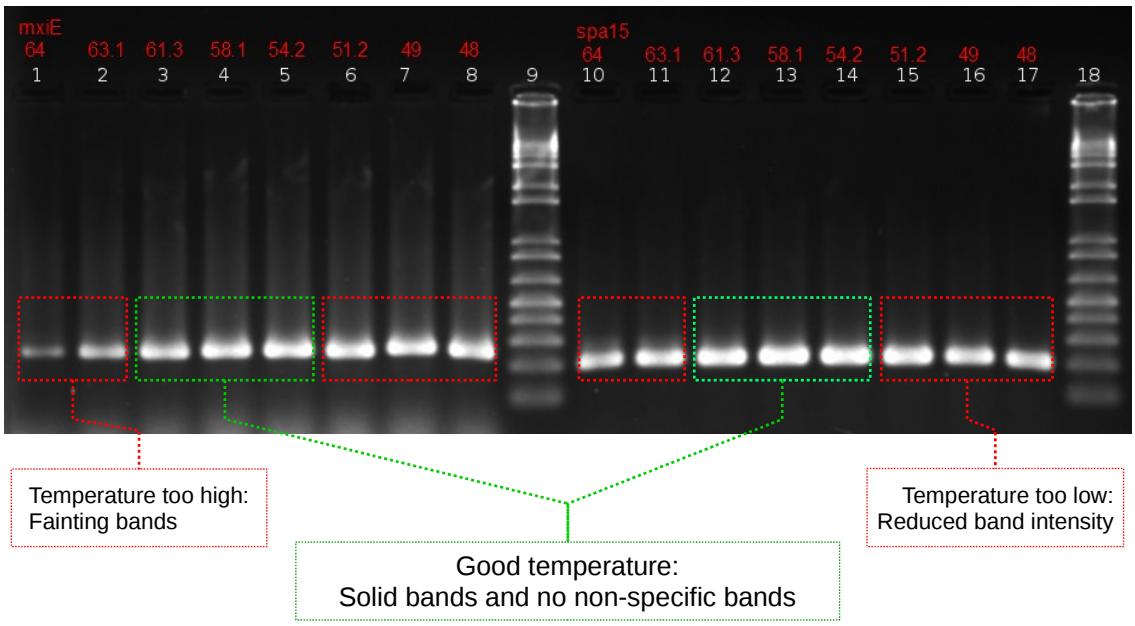

Figure 3.11: Optimization of T_a of *mxiE* and *spa15* genes with gradient PCR.

Each lane contains YSH6000 *S. flexneri* 2a. (T_a, Annealing Temperature)

3.4 Detection of T3SS related virulence genes

Out of total 32 genes responsible for type 3 secretion system (T3SS) in *Shigella* spp., 20 different virulence genes (*virB, ipaB, ipaC, ipaD, ipgC, ipgB1, ipgA, icsB, ipgD, ipgE, ipgF, mxiH, mxiI, mxiK, mxiC, spa15, spa47, spa32* and *spa24*), were analyzed by PCR assay (Figure 3.12).

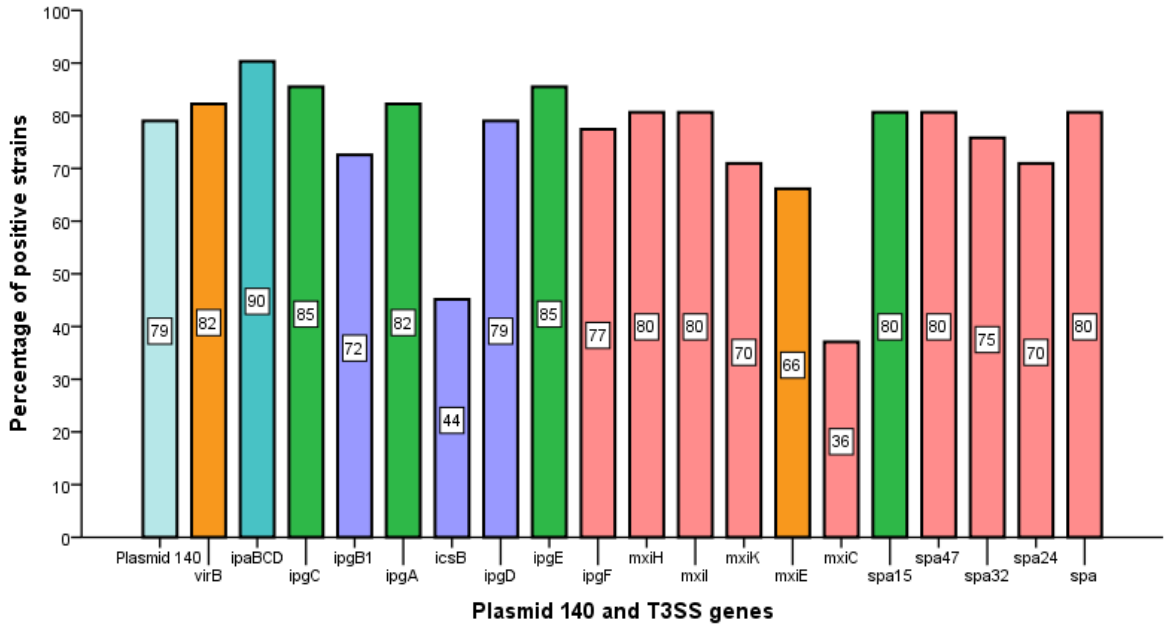

Figure 3.12: Prevalence of T3S system related genes among *S. flexneri* isolates.

Positive results found in, Plasmid 140 (n=48), *virB* (n=50), *ipaBCD* (n=55), *ipgC* (n=52), *ipgB1* (n=44), *ipgA* (n=50), *icsB* (n=27), *ipgD* (n=48), *ipgE* (n=52), *ipgF* (n=47), *mxiH* (n=49), *mxiI* (n=49), *mxiK* (n=43), *mxiE* (n=40), *mxiC* (n=22), *spa15* (n=49), *spa47* (n=49), *spa32* (n=46), *spa24* (n=43), *spa* (n=49).

Among these strains, most prevalent was, *ipaBCD* (90%), followed by *ipgC, ipgE* (85%); *virB, ipgA* (82%); *mxiH, mxiI, spa15, spa47* (80%); *ipgD* (79%), *ipgF* (77%), *spa32* (75%), *ipgB1* (72%), *mxiK, spa24* (70%); *mxiE* (66%), *icsB* (44%) and *mxiC* (36%).
None of the plasmid 140 negative strains were completely devoid of T3SS virulence genes. Only one gene count was positive in the following strains, K-3336 *S. flexneri* 1b (*ipgD*), K-

3253 *S. flexneri* 1c (*ipgD*), K-2680 *S. flexneri* 1c (*ipgD*), K-1940 *S. flexneri* 1b (*ipaBCD*) and K-1721 *S. flexneri* 1b (*ipgD*). And the rest of the 140 negative strains were found positive for multiple T3SS genes, K-3127 *S. flexneri* 1b and K-2982 *S. flexneri* 1b both were positive for 2 genes, *ipaBCD* and *ipgD*; K-3229 *S. flexneri* 1c was found positive for 3 genes, *ipgC, ipgD* and *ipgE*; K-2401 *S. flexneri* 1c was positive for 4 genes, *ipaBCD, ipgC, ipgD* and *ipgE*; K-2067 *S. flexneri* 1c was detected positive for 5 genes, *ipaBCD, ipgC, ipgA, ipgD* and *ipgE*; and K-842 *S. flexneri* 1c gave positive results for 6 genes, *virB, ipaBCD, ipgC, ipgD, ipgE* and *spa24*. Although, K1080 *S. flexneri* 2a was also 140 negative, it was found positive for every T3SS related genes this thesis has worked upon, except for *ipgD*. Status of T3SS genes in 140 negative strains are summarized in the table below.

Table 3.1: Status of tested genes in 140 MD negative *S. flexneri* strains.

ID	K-3336	K-3253	K-3229	K-3127	K-2982	K-2680	K-2401	K-2067	K-1940	K-1721	K-1080	K-1044	K-842
Str	1b	1c	1c	1b	1b	1c	1c	1c	1b	1b	2a	2b	1c
p140	–	–	–	–	–	–	–	–	–	–	–	–	–
ipaH	+	+	+	+	+	+	+	+	+	+	+	+	+
ial	–	–	+	+	–	–	+	+	+	–	+	–	–
set	–	–	–	–	–	–	–	–	–	–	+	+	–
sen	–	–	–	–	–	–	–	–	–	–	+	–	–
virB	–	–	–	–	–	–	–	–	–	–	+	–	+
ipaBCD	–	–	–	+	+	–	+	+	+	–	+	–	+
ipgC	–	–	+	–	–	–	+	+	–	–	+	–	+
ipgB1	–	–	–	–	–	–	–	–	–	–	+	–	–
ipgA	–	–	–	–	–	–	–	+	–	–	+	–	–
icsB	–	–	–	–	–	–	–	–	–	–	+	–	–
ipgD	+	+	+	+	+	+	+	+	–	+	–	+	+
ipgE	–	–	+	–	–	–	+	+	–	–	+	–	+
ipgF	–	–	–	–	–	–	–	–	–	–	+	–	–
mxiH	–	–	–	–	–	–	–	–	–	–	+	–	–
mxiI	–	–	–	–	–	–	–	–	–	–	+	–	–
mxiK	–	–	–	–	–	–	–	–	–	–	+	–	–
mxiE	–	–	–	–	–	–	–	–	–	–	+	–	–
mxiC	–	–	–	–	–	–	–	–	–	–	+	–	–
spa15	–	–	–	–	–	–	–	–	–	–	+	–	–
spa47	–	–	–	–	–	–	–	–	–	–	+	–	–
spa32	–	–	–	–	–	–	–	–	–	–	+	–	–
spa24	–	–	–	–	–	–	–	–	–	–	+	+	+

Table 3.2: Status of clinical features in associated 140 negative *S. flexneri* strains.

Interpretation code for each clinical features are following: 1. Severity (1 – mild, 2 – mod – severe, 9 – NA (control)); 2. Stool description (1 – simple watery, 2 – rice watery, 3 – mucoid, 4 – bloody mucoid); 3. Blood in stool (1 – yes, 2 – no); 4. Abdominal pain (1 – yes, 2 – no, 7 – don't know); 5. Rectal strain (1 – yes, 2 – no, 7 – don't know); 6. Vomiting (1 – yes, 2 – no); 7. Cough (1 – yes, 2 – no, 7 – don't know); 8. Fever (1 – yes, 2 – no, 7 – don't know); 9. Dehydration status (1 – no sign, 2 – some, 3 – severe); 10. Eye status (1 – normal, 2 – sunken); 11. Mouth status (1 – normal, 2 – somewhat dry, 3 – very dry); 12. Skin pinch (1 – normal, 2 – slow return, 3 – very slow return); 13. Convulsion (1 – yes, 2 – no, 7 – don't know); 14. Altered mental status (1 – Normal, 2 – restless, irritable, 3 – lethargic/ unconscious); 15. Pedal edema (1 – yes, 2 – no, 9 – not applicable).

ID	K-3336	K-3253	K-3229	K-3127	K-2982	K-2680	K-2401	K-2067	K-1940	K-1721	K-1080	K-1044	K-842
Severity	2	1	2	2	1	2	2	2	2	2	2	2	2
Stool description	4	1	1	4	1	4	4	4	4	4	4	4	4
Blood in stool	1	2	2	1	2	1	1	1	1	1	1	1	1
Abdominal pain	2	2	7	1	2	1	1	1	1	1	1	1	1
Rectal strain	2	2	2	2	2	1	1	1	1	1	1	1	1
Vomiting	1	1	1	2	2	2	1	2	1	2	1	1	1
Cough	2	1	2	1	1	1	2	1	2	2	2	1	2
Fever	1	2	1	2	1	1	2	1	1	2	2	1	1
Dehydration status	1	1	2	1	1	1	1	1	1	1	1	1	2
Eye status	1	1	2	1	1	1	1	1	1	1	1	1	1
Mouth status	1	1	2	1	1	1	1	1	1	1	1	1	1
Skin pinnch	1	1	1	1	1	1	1	1	1	1	1	1	1
Convulsion	2	2	2	2	2	2	2	2	2	2	2	2	2
Altered mental status	1	1	1	1	1	1	1	1	1	1	1	1	1
Pedal edema	2	2	2	2	2	2	2	2	2	2	2	2	2

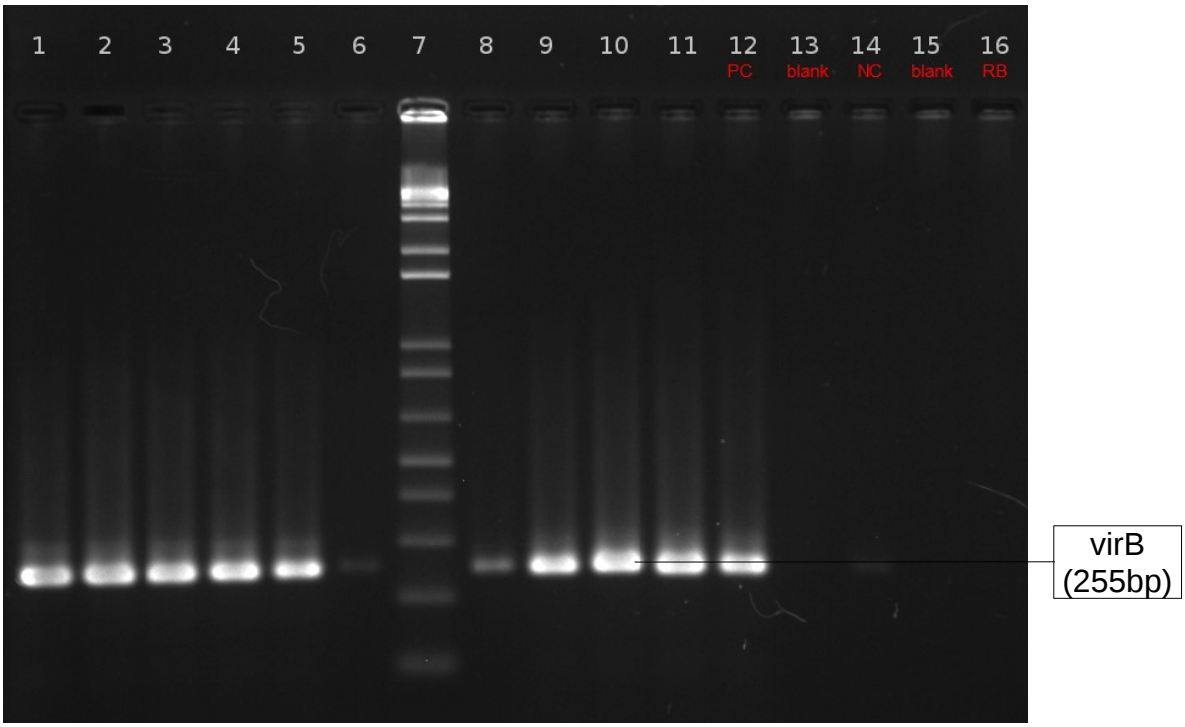

Figure 3.13: Gel electrophoresis of ***virB*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with a blunt arrow. Lane 1, K-4193, Lane 2, K-4181, Lane 3, K-4179, Lane 4, K-4081, Lane 5, K-3687, Lane 6, K-3665, Lane 7, 1 kb plus DNA ladder, Lane 8, K-3662, Lane 9, K-3661, Lane 10, K-3660, Lane 11, K-3650,Lane 12, Positive control, YSH6000 *S. flexneri* 2a, Lane 13, blank, Lane 14, Negative control, *E. coli* ATCC-25922, Lane 15, blank, Lane 16, Reagent blank.

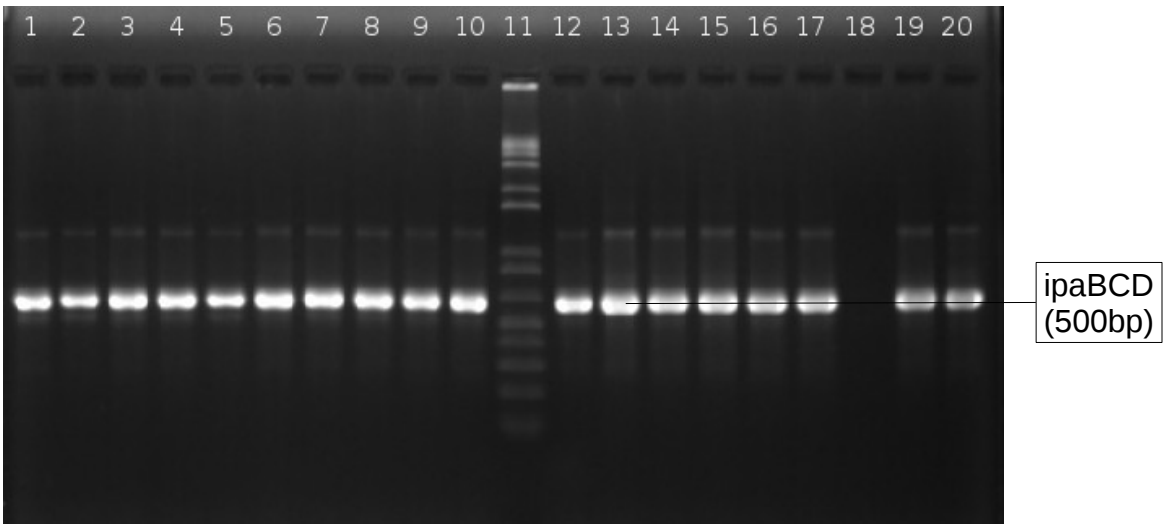

Figure 3.14: Gel electrophoresis of ***ipaBCD*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with a blunt arrow; Lane 1, K-4193, Lane 2, K-4181, Lane 3, K-4179, Lane 4, K-4081, Lane 5, K-3687, Lane 6, K-3665, Lane 7, K-3662, Lane 8, K-3661, Lane 9, K-3660, Lane 10, K-3650, Lane 11, 1 kb plus DNA ladder, Lane 12, K-3644, Lane 13, K-3612, Lane 14, K-3597, Lane 15, K-3579, Lane 16, K-3496, Lane 17, K-3127, Lane 18, K-2680, Lane 19, K-2552, Lane 20 Positive control, YSH6000 *S. flexneri* 2a.

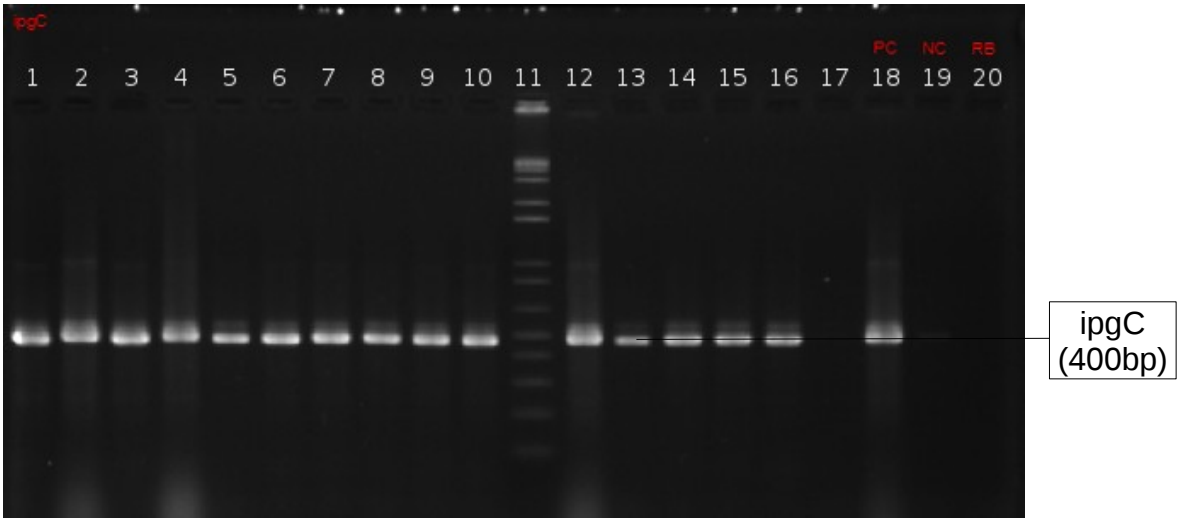

Figure 3.15: Gel electrophoresis of ***ipgC*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with a blunt arrow; Lane 1, K-4193, Lane 2, K-4181, Lane 3, K-4179, Lane 4, K-4081, Lane 5, K-3687, Lane 6, K-3665, Lane 7, K-3662, Lane 8, K-3661, Lane 9, K-3660, Lane 10, K-3650, Lane 11, 1 kb plus DNA ladder, Lane 12, K-3644, Lane 13, K-3612, Lane 14, K-3597, Lane 15, K-3579, Lane 16, K-3496, Lane 17, K-3336, Lane 18, Positive control, YSH6000 *S. flexneri* 2a, Lane 19, blankLane 14, Negative control, *E. coli* ATCC-25922, Lane 20, Reagent blank.

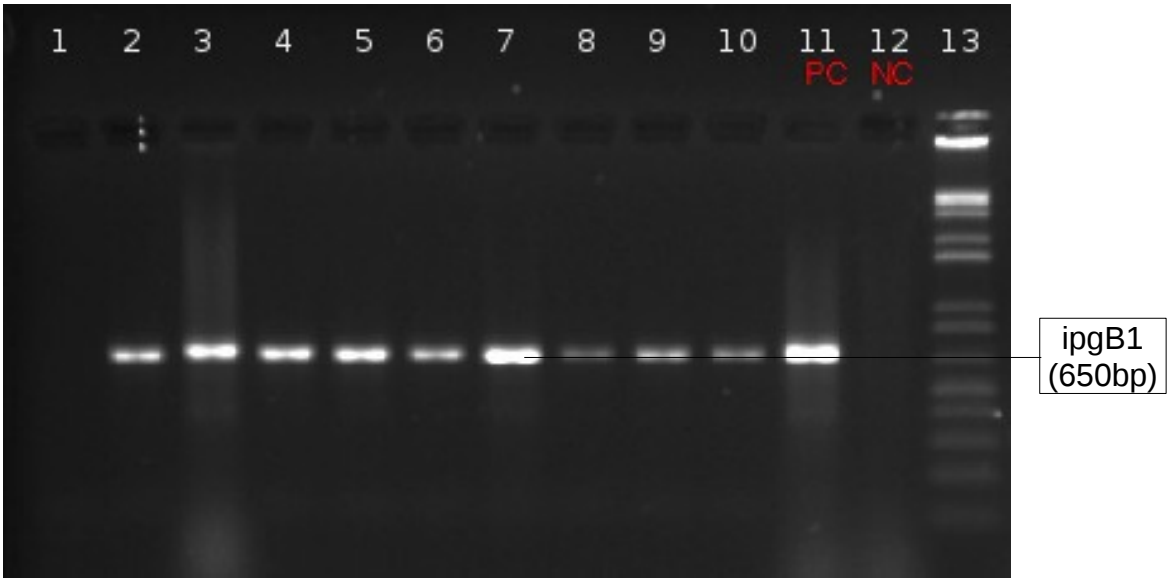

Figure 3.16: Gel electrophoresis of ***ipgB1*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with a blunt arrow; Lane 1, K-1721, Lane 2, K-1692, Lane 3, K-1502, Lane 4, K-1230, Lane 5, K-1224, Lane 6, K-1108, Lane 7, K-1080, Lane 8, K-1063, Lane 9, K-1057, Lane 10, K-1053,Lane 11, Positive control, YSH6000 *S. flexneri* 2a, Lane 12, Negative control, *E. coli* ATCC-25922,Lane 13, 1 kb plus DNA ladder.

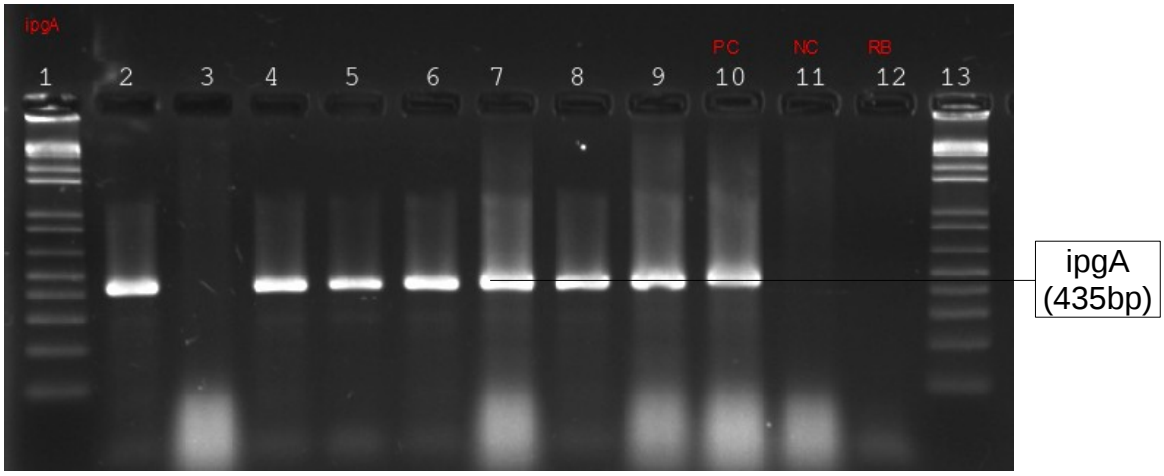

Figure 3.17: Gel electrophoresis of ***ipgA*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with a blunt arrow; Lane 1, 1 kb plus DNA ladder, Lane 2, K-2552, Lane 3, K-2401, Lane 4, K-2336, Lane 5, K-2278, Lane 6, K-2198, Lane 7, K-2111, Lane 8, K-2067, Lane 9, K-1838, Lane 10, Positive control, YSH6000 *S. flexneri* 2a, Lane 11, Negative control, *E. coli* ATCC-25922, Lane 12, Reagent blank, Lane 13, 1 kb plus DNA ladder.

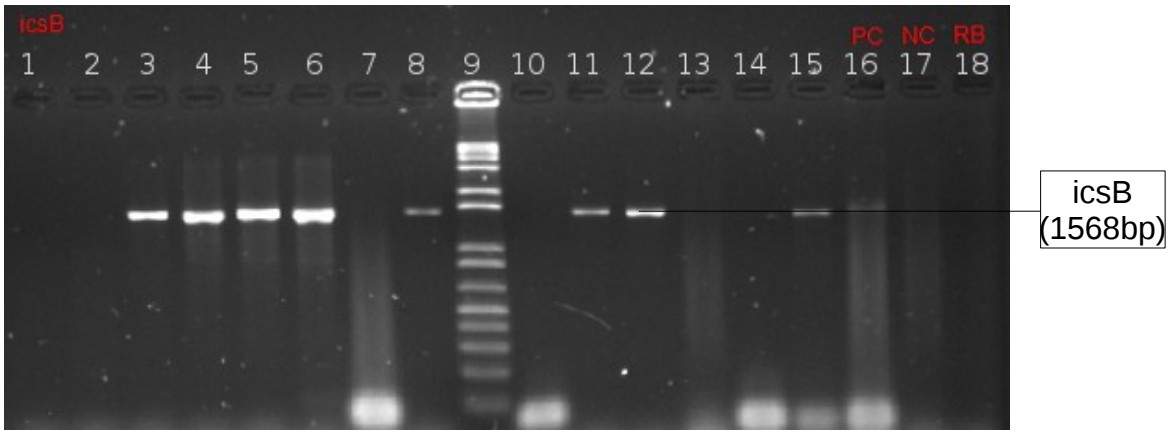

Figure 3.18: Gel electrophoresis of ***icsB*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with a blunt arrow; Lane 1, K-1044, Lane 2, K-842, Lane 3, K-662, Lane 4, K-658, Lane 5, K-649, Lane 6, K-645, Lane 7, K-632, Lane 8, K-629, Lane 9, 1 kb plus DNA ladder, Lane 10, K-583, Lane 11, K-570, Lane 12, K-569, Lane 13, K-425, Lane 14, K-151, Lane 15, K-102, Lane 16, Positive control, YSH6000 *S. flexneri* 2a, Lane 17, Negative control, *E. coli* ATCC-25922, Lane 18, Reagent blank.

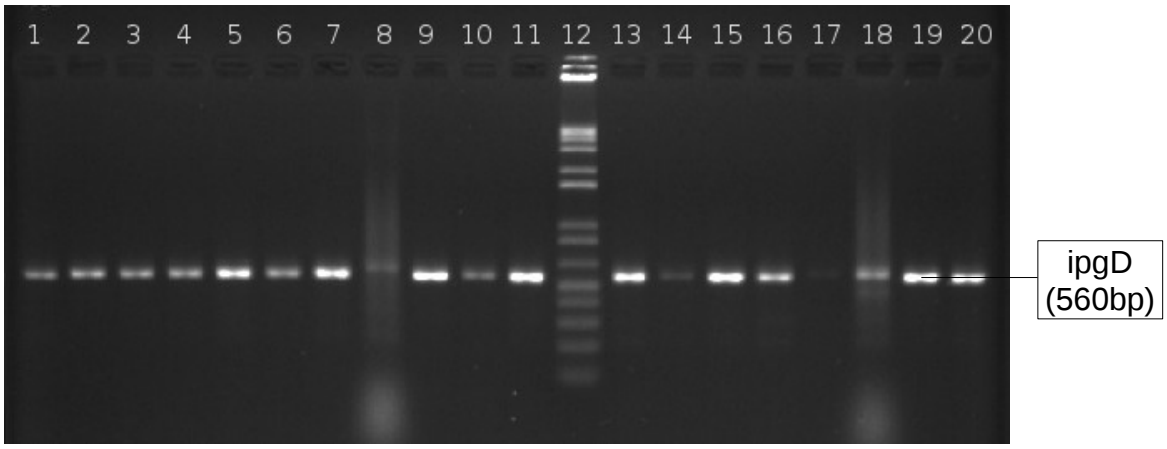

Figure 3.19: Gel electrophoresis of ***ipgD*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with a blunt arrow; Lane 1, K-3597, Lane 2, K-3579, Lane 3, K-3496, Lane 4, K-3336, Lane 5, K-3253, Lane 6, K-3229, Lane 7, K-3127, Lane 8, K-2982, Lane 9, K-2980, Lane 10, K-2979, Lane 11, K-2960, Lane 12, 1 kb plus DNA ladder, Lane 13, K-2889, Lane 14, K-2724, Lane 15, K-2680, Lane 16, K-2552, Lane 17, K-2336, Lane 18, K-2278, Lane 19, K-2198, Lane 20, K-2111.

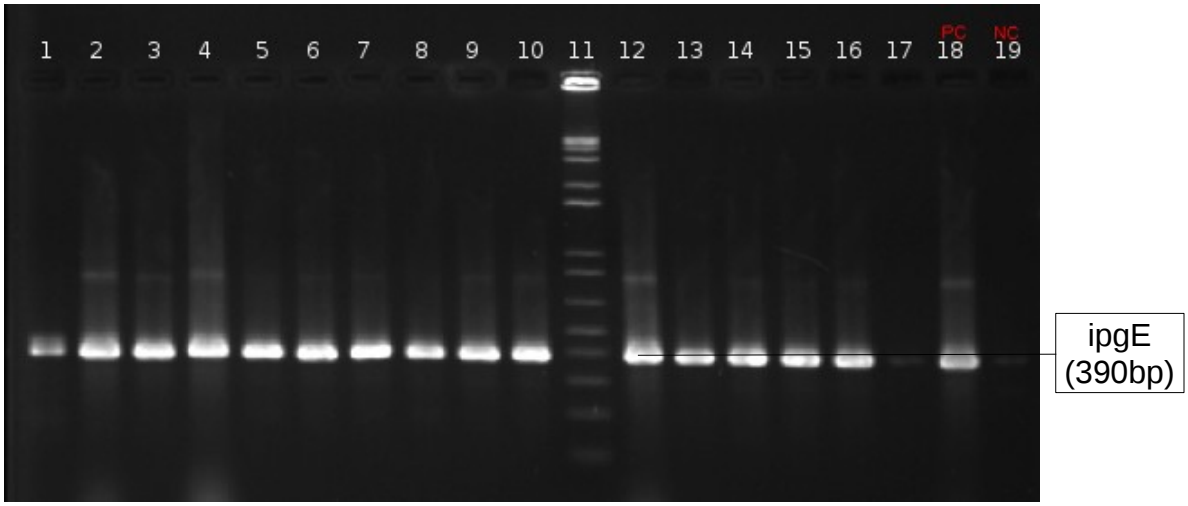

Figure 3.20: Gel electrophoresis of ***ipgE*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with a blunt arrow; Lane 1, K-1224, Lane 2, K-1108, Lane 3, K-1080, Lane 4, K-1063, Lane 5, K-1057, Lane 6, K-1053, Lane 7, K-842, Lane 8, K-662, Lane 9, K-658, Lane 10, K-649, Lane 11, 1 kb plus DNA ladder, Lane 12, K-645, Lane 13, K-632, Lane 14, K-629, Lane 15, K-583, Lane 16, K-570, Lane 17, K-569, Lane 18, Positive control, YSH6000 *S. flexneri* 2a, Lane 19, Negative control, *E. coli* ATCC-25922.

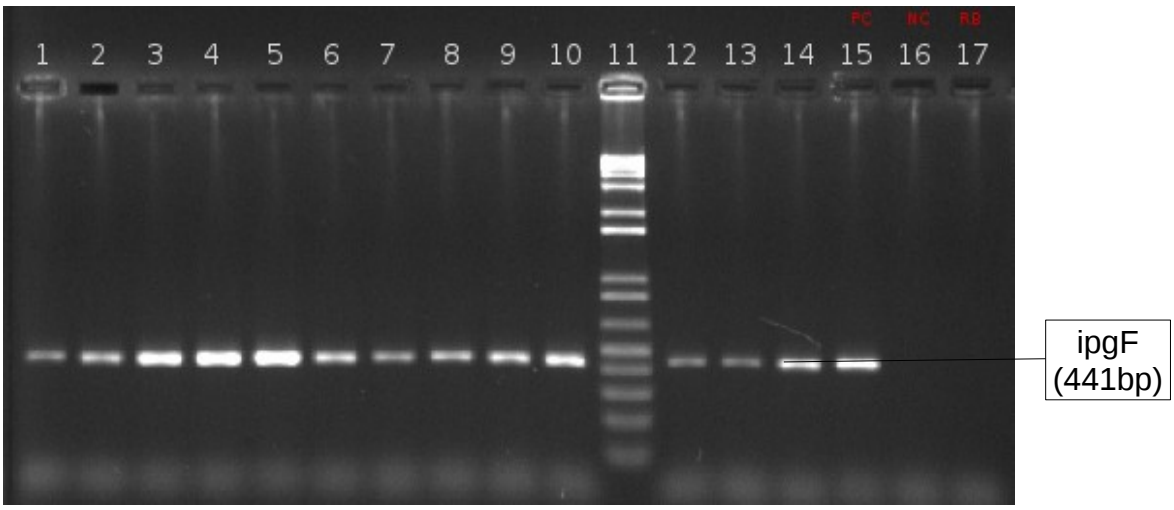

Figure 3.21: Gel electrophoresis of ***ipgF*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with a blunt arrow; Lane 1, K-662, Lane 2, K-658, Lane 3, K-649, Lane 4, K-645, Lane 5, K-632, Lane 6, K-629, Lane 7, K-583, Lane 8, K-570, Lane 9, K-569, Lane 10, K-425, Lane 11, 1 kb plus DNA ladder, Lane 12, K-151, Lane 13, K-102, Lane 14, K-53, Lane 15, Positive control, YSH6000 *S. flexneri* 2a, Lane 16, Negative control, *E. coli* ATCC-25922, Lane 17, Reagent blank.

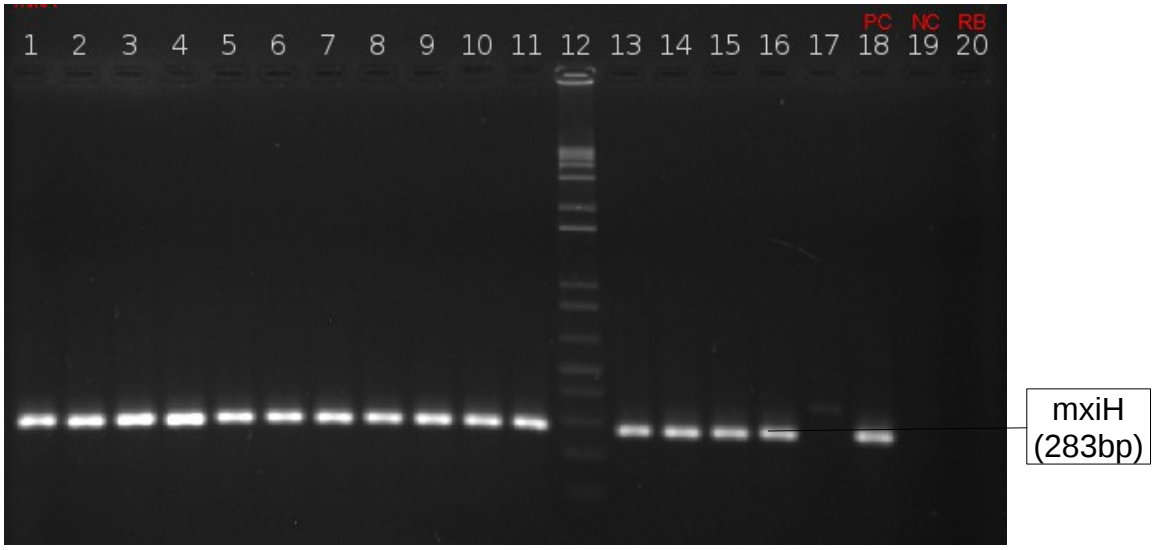

Figure 3.22: Gel electrophoresis of ***mxiH*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with a blunt arrow; Lane 1, K-4193, Lane 2, K-4181, Lane 3, K-4179, Lane 4, K-4081, Lane 5, K-3687, Lane 6, K-3665, Lane 7, K-3662, Lane 8, K-3661, Lane 9, K-3660, Lane 10, K-3650, Lane 11, K-3644, Lane 12, 1 kb plus DNA ladder, Lane 13, K-3612, Lane 14, K-3597, Lane 15, K-3579, Lane 16, K-3496, Lane 17, K-3336, Lane 18, Positive control, YSH6000 *S. flexneri* 2a, Lane 19, Negative control, *E. coli* ATCC-25922, Lane 20, Reagent blank.

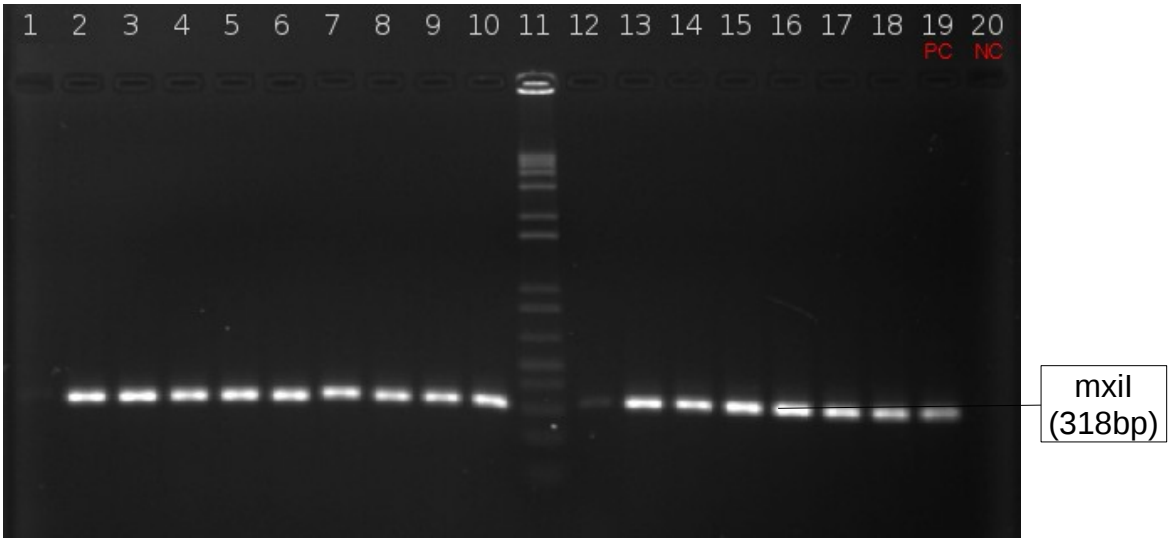

Figure 3.23: Gel electrophoresis of ***mxiI*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with indicator; Lane 1, K-2982, Lane 2, K-2980, Lane 3, K-2979, Lane 4, K-2960, Lane 5, K-2889, Lane 6, K-2724, Lane 7, K-2552, Lane 8, K-2336, Lane 9, K-2278, Lane 10, K-2198, Lane 11, 1 kb plus DNA ladder, Lane 12, K-2111, Lane 13, K-1838, Lane 14, K-1692, Lane 15, K-1502, Lane 16, K-1230, Lane 17, K-1224, Lane 18, K-1108, Lane 19, Positive control, YSH6000 *S. flexneri* 2a, Lane 20, Negative control, *E. coli* ATCC-25922.

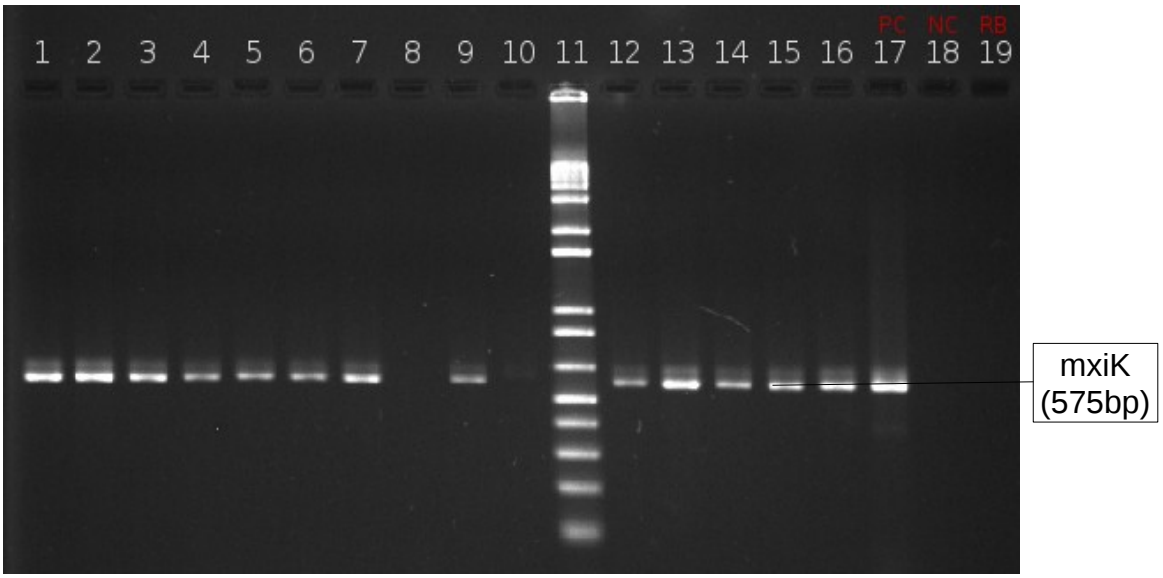

Figure 3.24: Gel electrophoresis of ***mxiK*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with an indicator; Lane 1, K-3579, Lane 2, K-3496, Lane 3, K-2980, Lane 4, K-2979, Lane 5, K-2960, Lane 6, K-2889, Lane 7, K-2724, Lane 8, K-2680, Lane 9, K-2552, Lane 10, K-2401, Lane 11, 1 kb plus DNA ladder, Lane 12, K-2336, Lane 13, K-2278, Lane 14, K-2198, Lane 15, K-2111, Lane 16, K-1838, Lane 17, Positive control, YSH6000 *S. flexneri* 2a, Lane 18, Negative control, *E. coli* ATCC-25922, Lane 19, Reagent blank.

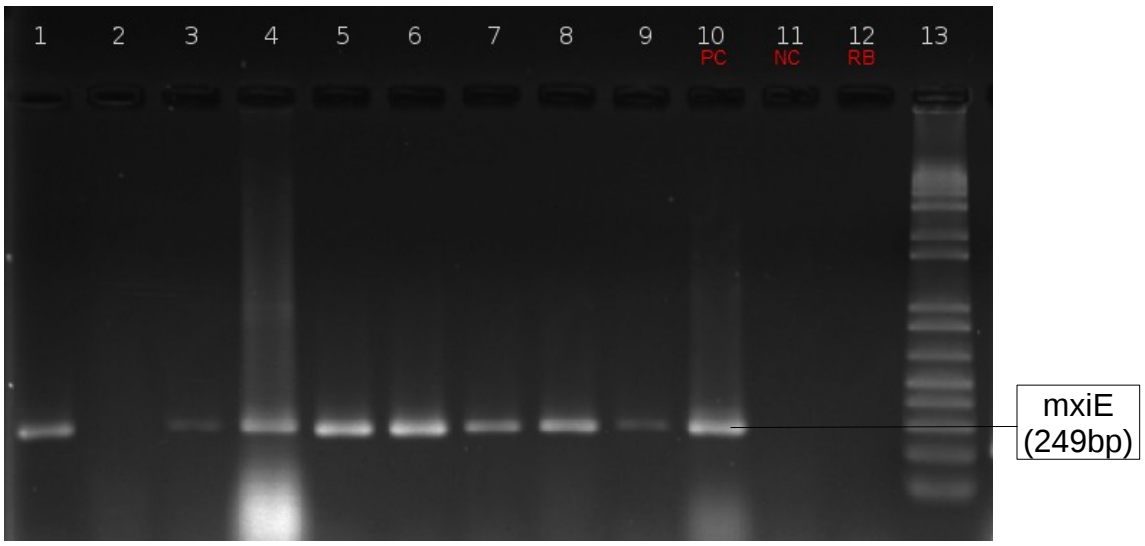

Figure 3.25: Gel electrophoresis of ***mxiE*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with an indicator; Lane 1, K-1838, Lane 2, K-1721, Lane 3, K-1692, Lane 4, K-1502, Lane 5, K-1230, Lane 6, K-1224, Lane 7, K-1108, Lane 8, K-1080, Lane 9, K-1063, Lane 10, Positive control, YSH6000 *S. flexneri* 2a, Lane 11, Negative control, *E. coli* ATCC-25922, Lane 12, Reagent blank, Lane 13, 1 kb plus DNA ladder.

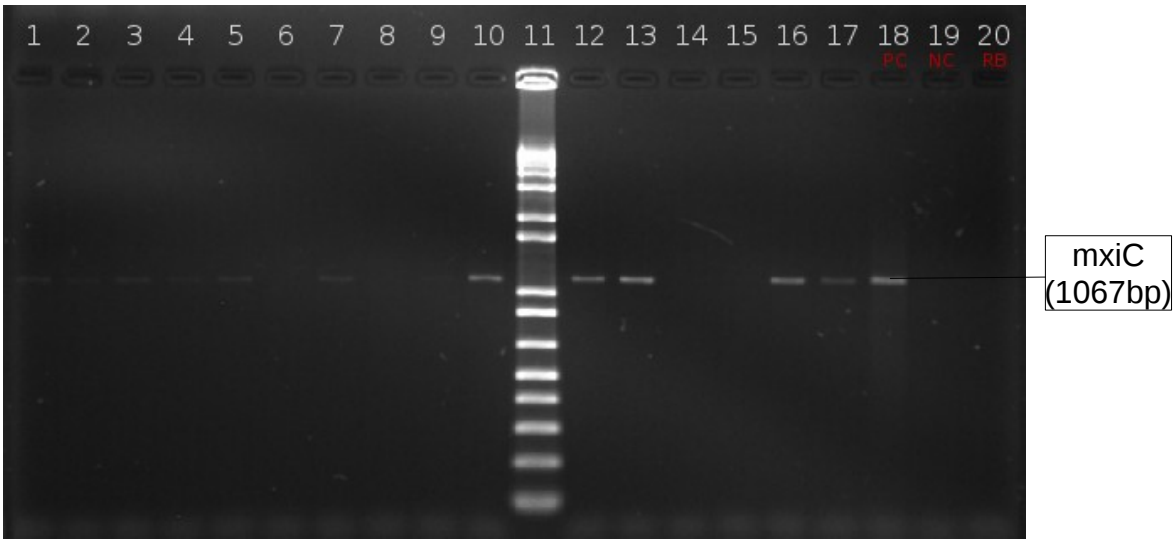

Figure 3.26: Gel electrophoresis of ***mxiC*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with an indicator; Lane 1, K-2980, Lane 2, K-2979, Lane 3, K-2960, Lane 4, K-2889, Lane 5, K-2724, Lane 6, K-2680, Lane 7, K-2552, Lane 8, K-2067, Lane 9, K-1940, Lane 10, K-1838, Lane 11, 1 kb plus DNA ladder, Lane 12, K-1692, Lane 13, K-1230, Lane 14, K-1224, Lane 15, K-1108, Lane 16, K-1080, Lane 17, K-658, Lane 18, Positive control, YSH6000 *S. flexneri* 2a, Lane 19, Negative control, *E. coli* ATCC-25922, Lane 20, Reagent blank.

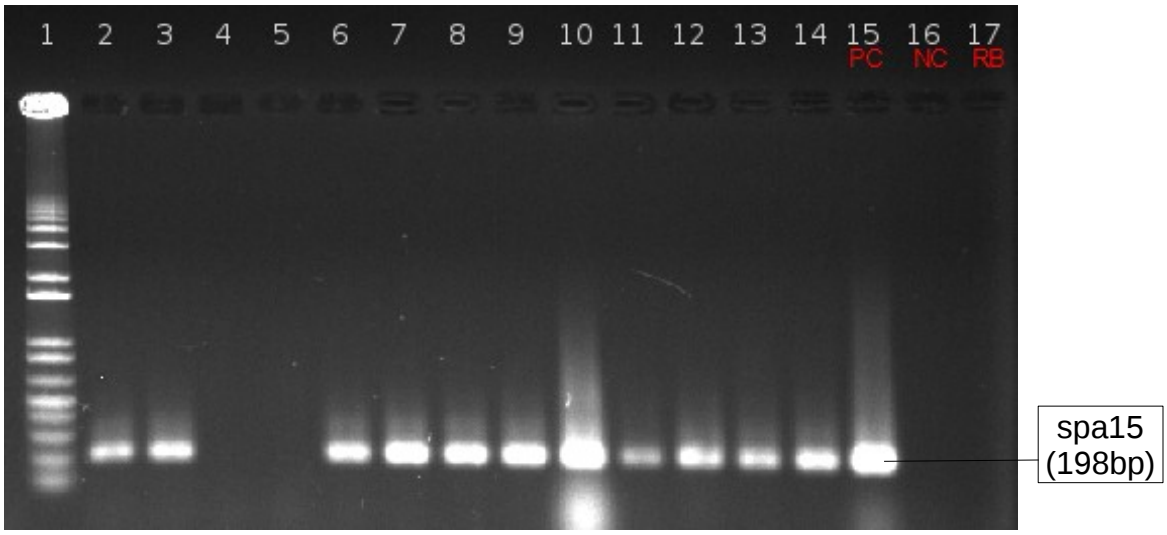

Figure 3.27: Gel electrophoresis of ***spa15*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with an indicator; Lane 1, 1 kb plus DNA ladder, Lane 2, K-2198, Lane 3, K-2111, Lane 4, K-2067, Lane 5, K-1940, Lane 6, K-1692, Lane 7, K-1502, Lane 8, K-1230, Lane 9, K-1224, Lane 10, K-1108, Lane 11, K-1080, Lane 12, K-1063, Lane 13, K-1057, Lane 14, K-1053, Lane 15, Positive control, YSH6000 *S. flexneri* 2a, Lane 16, Negative control, *E. coli* ATCC-25922, Lane 17, Reagent blank.

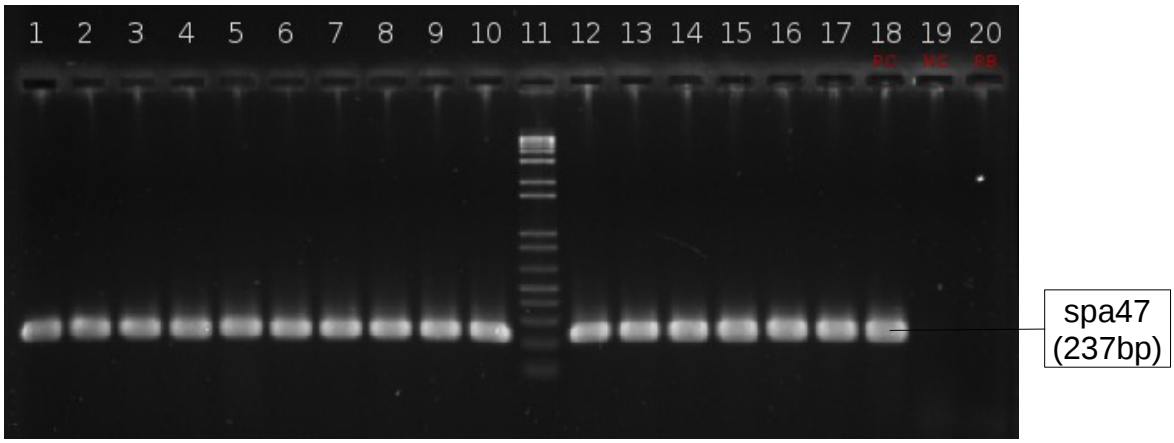

Figure 3.28: Gel electrophoresis of ***spa47*** PCR products

of representative *S. flexneri* strains.Expected position of the PCR product of this gene are shown with an indicator; Lane 1, K-4193, Lane 2, K-4181, Lane 3, K-4179, Lane 4, K-4081, Lane 5, K-3687, Lane 6, K-3665, Lane 7, K-3662, Lane 8, K-3661, Lane 9, K-3660, Lane 10, K-3650, Lane 11, 1 kb plus DNA ladder, Lane 12, K-3644, Lane 13, K-3612, Lane 14, K-3597, Lane 15, K-3579, Lane 16, K-3496, Lane 17, K-2980, Lane 18, Positive control, YSH6000 *S. flexneri* 2a, Lane 19, Negative control, *E. coli* ATCC-25922, Lane 20, Reagent blank.

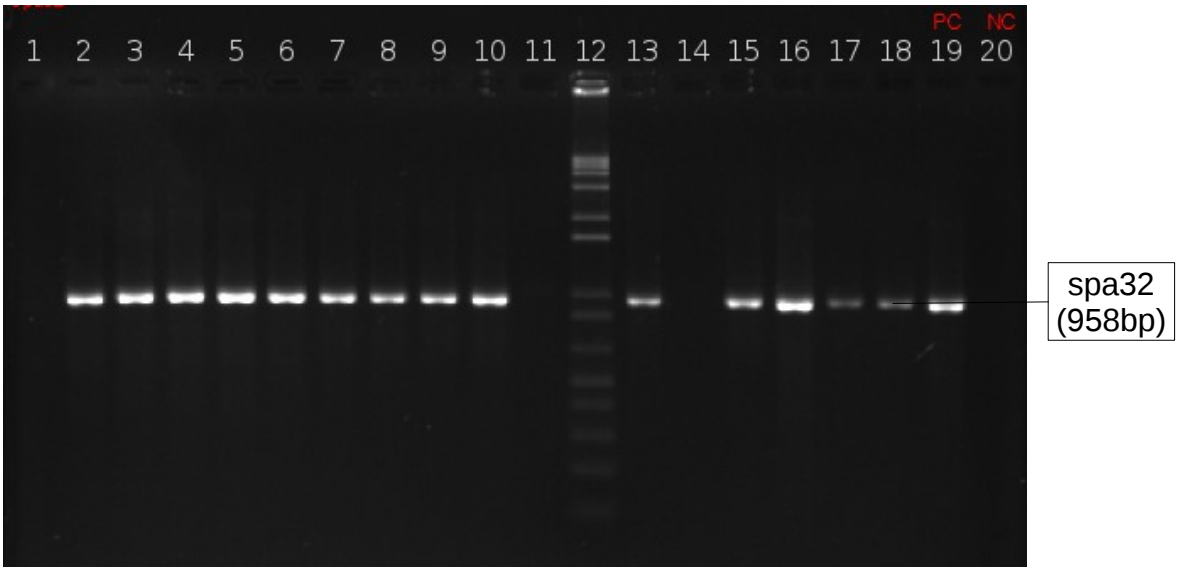

Figure 3.29: Gel electrophoresis of ***spa32*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with an indicator; Lane 1, K-3687, Lane 2, K-3665, Lane 3, K-3662, Lane 4, K-3661, Lane 5, K-3660, Lane 6, K-3650, Lane 7, K-3644, Lane 8, K-3597, Lane 9, K-3579, Lane 10, K-3496, Lane 11, K-2680, Lane 12, 1 kb plus DNA ladder, Lane 13, K-2552, Lane 14, K-2401, Lane 15, K-2336, Lane 16, K-2278, Lane 17, K-2198, Lane 18, K-2111, Lane 19, Positive control, YSH6000 *S. flexneri* 2a, Lane 20, Negative control, *E. coli* ATCC-25922.

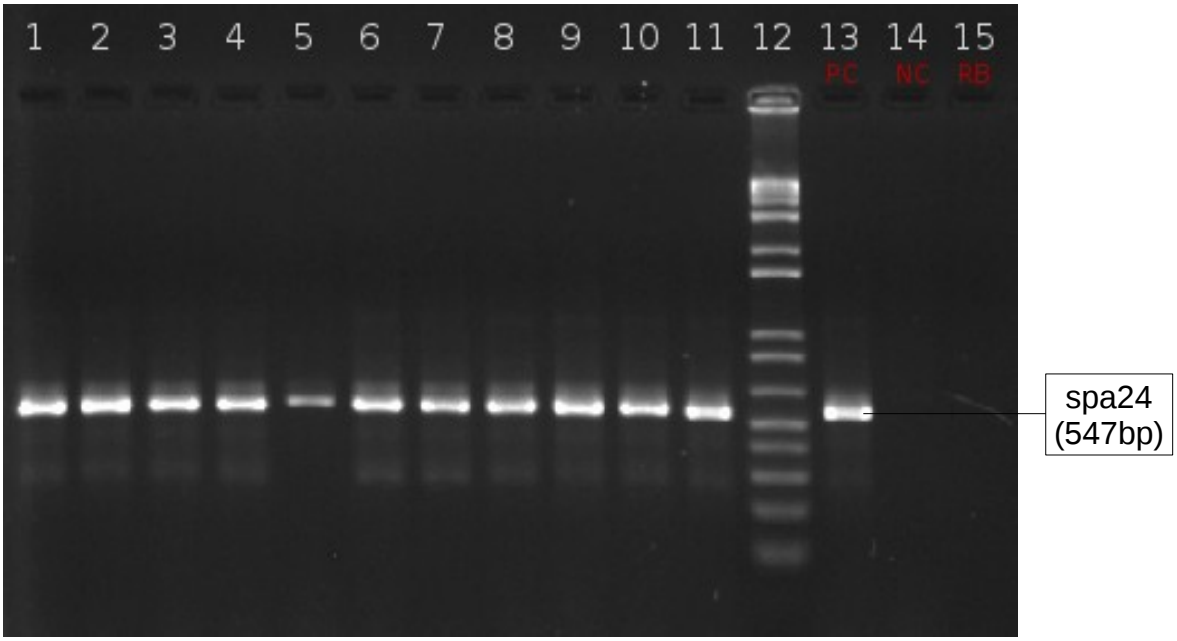

Figure 3.30: Gel electrophoresis of ***spa24*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene are shown with an indicator; Lane 1, K-3687, Lane 2, K-3665, Lane 3, K-3662, Lane 4, K-3661, Lane 5, K-3660, Lane 6, K-3650, Lane 7, K-3644, Lane 8, K-3612, Lane 9, K-3597, Lane 10, K-3579, Lane 11, K-3496, Lane 12, 1 kb plus DNA ladder, Lane 13, Positive control, YSH6000 *S. flexneri* 2a, Lane 14, Negative control, *E. coli* ATCC-25922, Lane 15, Reagent blank.

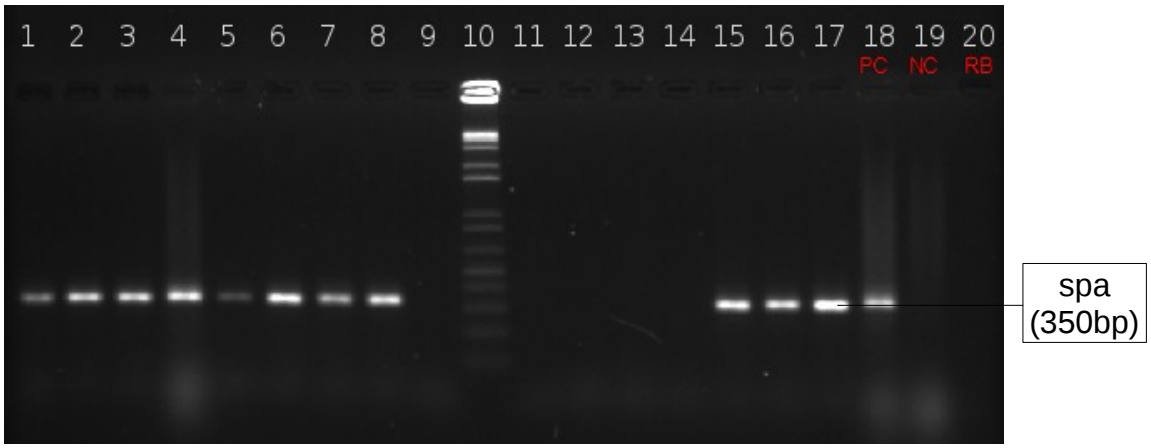

Figure 3.31: Gel electrophoresis of ***spa*** PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this gene cluster are shown with an indicator; Lane 1, K-3661, Lane 2, K-3660, Lane 3, K-3650, Lane 4, K-3644, Lane 5, K-3612, Lane 6, K-3597, Lane 7, K-3579, Lane 8, K-3496, Lane 9, K-3336, Lane 10, 1 kb plus DNA ladder, Lane 11, K-3253, Lane 12, K-3229, Lane 13, K-3127, Lane 14, K-2982, Lane 15, K-2980, Lane 16, K-2979, Lane 17, K-2960, Lane 18, Positive control, YSH6000 *S. flexneri* 2a, Lane 19, Negative control, *E. coli* ATCC-25922, Lane 20, Reagent blank.

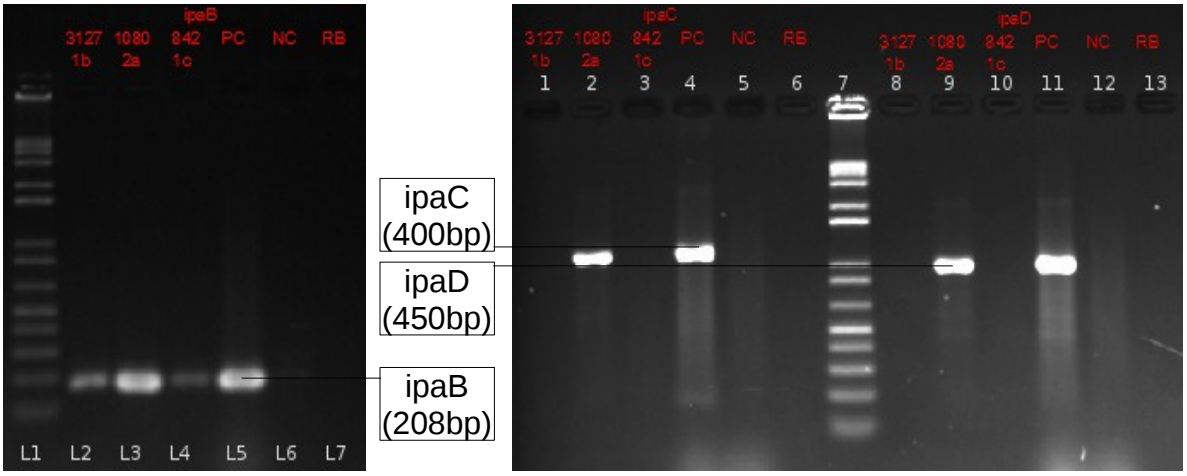

Figure 3.32: Gel electrophoresis of ***ipaB***, ***ipaC***, ***ipaD*** PCR products

of representative strains *of S. flexneri*. Expected position of the PCR product of these genes are shown with indicators; (On left) Lane 1, K-3127, Lane 2, K-1080, Lane 3, K-842, Lane 4, Positive control, YSH6000 *S. flexneri* 2a, Lane 5, Negative control, *E. coli* ATCC-25922, Lane 6, Reagent blank; (On right) Lane 1, K-3127, Lane 2, K-1080, Lane 3, K-842, Lane 4, Positive control, YSH6000 *S. flexneri* 2a, Lane 5, Negative control, *E. coli* ATCC-25922, Lane 6, Reagent blank, Lane 7, 1 kb plus DNA ladder, Lane 8, K-3127, Lane 9, K-1080, Lane 10, K-842, Lane 11, Positive control, YSH6000 *S. flexneri* 2a, Lane 12, Negative control, *E. coli* ATCC-25922, Lane 13, Reagent blank.

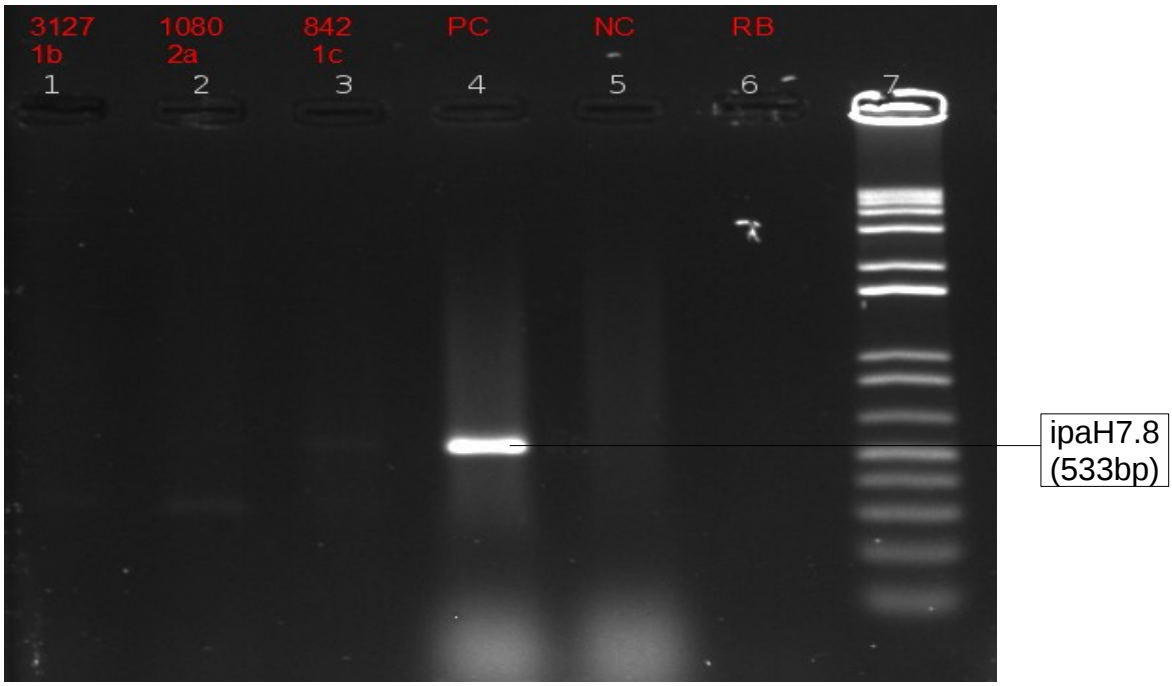

Figure 3.33: Gel electrophoresis of *ipaH7.8* PCR products

of representative *S. flexneri* strains. Expected position of the PCR product of this plasmid specific gene is shown with an indicator; Lane 1, K-3127, Lane 2, K-1080, Lane 3, K-842, Lane 4, Positive control, YSH6000 *S. flexneri* 2a, Lane 5, Negative control, *E. coli* ATCC-25922, Lane 6, Reagent blank, Lane 7, 1 kb plus DNA ladder.

Table 3.3: Summary of virulence plasmid and gene prevalence in *S. flexneri* strains (n=61) of 140 MD plasmid, virulence and toxin genes on the basis of plasmid profiling and PCR assay.

ID	Str	p140	ipaH	ial	set	sen	virB	ipaBCD	ipgC	ipgB1	ipgA	icsB	ipgD	ipgE	ipgF	mxiH	mxiI	mxiK	mxiE	mxiC	spa15	spa47	spa32	spa24	spa
K-4193	2a	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K-4181	2a	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K-4179	2a	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+
K-4081	6a	+	+	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+	–	+	+	+	+	–	+
K-3687	3a	+	+	+	+	+	+	+	+	–	+	–	–	+	+	+	+	–	–	–	+	+	–	+	+
K-3665	2b	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	+	–	–	+	+	+	+	+
K-3662	2a	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
K-3661	2a	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	+	+
K-3660	2a	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+	–	+	+	+	+	+
K-3650	2a	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
K-3644	2a	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	+	+
K-3612	4	+	+	+	+	+	+	+	+	–	+	–	–	+	+	+	+	–	–	–	+	+	–	–	+
K-3597	6a	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	+
K-3579	6a	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	+
K-3496	3a	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
K-3336	1b	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
K-3253	1c	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
K-3229	1c	–	+	+	–	–	–	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–
K-3127	1b	–	+	+	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
K-2982	1b	–	+	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
K-2980	2a	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+
K-2979	1c	+	+	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+
K-2960	2a	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K-2889	2a	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K-2724	1c	+	+	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+
K-2680	1c	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
K-2552	2a	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+
K-2401	1c	–	+	+	–	–	–	+	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–
K-2336	6a	+	+	+	–	+	+	+	+	–	+	–	–	+	+	+	+	+	–	+	+	+	+	–	+
K-2278	2b	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+
K-2198	2b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K-2111	3a	+	+	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+
K-2067	1c	–	+	+	–	–	–	+	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–
K-1940	1b	–	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
K-1838	3a	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K-1721	1b	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
K-1692	3a	+	+	+	–	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+
K-1502	3a	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
K-1230	3a	+	+	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+
K-1224	6a	+	+	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+	–	+	+	+	–	+
K-1108	4	+	+	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+	–	+	+	+	–	+

ID	Str	p140	ipaH	ial	set	sen	virB	ipaBCD	ipgC	ipgB1	ipgA	icsB	ipgD	ipgE	ipgF	mxiH	mxiI	mxiK	mxiE	mxiC	spa15	spa47	spa32	spa24	spa
K-1080	2a	–	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+
K-1063	2a	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–	+	+	+	+	+
K-1057	2a	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	–	+	+	+	+	+
K-1053	2b	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–	+	+	+	+	+
K-1044	2b	–	+	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	–
K-842	1c	–	+	–	–	–	+	+	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	+	–
K-662	2b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
K-658	2b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K-649	2a	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+
K-645	2b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K-632	4	+	+	+	–	+	+	+	+	+	+	–	–	+	+	+	+	+	+	–	+	+	+	–	+
K-629	2b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+
K-583	2b	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	+	+	+	+	+
K-570	2b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+
K-569	2b	+	+	+	+	+	+	+	–	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+
K-425	2b	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	–	+	+	+	+	+
K-270	3a	+	+	+	–	+	+	+	+	–	+	+	+	+	–	+	+	–	+	–	+	+	–	+	+
K-151	2b	+	+	+	+	+	+	+	+	–	+	–	–	+	+	+	+	+	+	–	+	+	+	+	+
K-102	2b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K-53	1c	+	+	+	–	+	+	+	+	+	+	–	–	+	+	+	+	+	+	–	+	+	+	+	+

3.5 Statistical Analysis

The median age of the patients (in months) was found to be 24 months, with an IQR (inter-quartile range) of 14 – 32.5 months. And, for the episodes of loose motion the median value was found 10 with an IQR of 8 – 15 times a day.

Cross-tabulation and Pearson Chi-Square test of independence were done at two levels of significance in between important clinical features and tested genes. The result was considered statistically significant at 5% level of significance, and at 1% level of significance the result was considered highly statistically significant (Table 3.4 – 3.24).

Table 3.4: Cross-tabulation of - *set* * Blood in stool

			Blood in stool		
			Yes	No	Total
set	Negative	Count	17	10	27
		% within set	63.0%	37.0%	100.0%
	Positive	Count	32	2	34
		% within set	94.1%	5.9%	100.0%
Total		Count	49	12	61
		% within set	80.3%	19.7%	100.0%

Table 3.5: Cross-tabulation of - *sen* * Blood in stool

			Blood in stool		
			Yes	No	Total
sen	Negative	Count	17	10	27
		% within sen	63.0%	37.0%	100.0%
	Positive	Count	32	2	34
		% within sen	94.1%	5.9%	100.0%
Total		Count	49	12	61
		% within sen	80.3%	19.7%	100.0%

Table 3.6: Cross-tabulation of - *set* * Stool consistency

			Stool consistency				
			Simple watery	Rice watery	Sticky/ mucoid	Bloody mucoid	Total
set	Negative	Count	8	1	1	17	27
		% within set	29.6%	3.7%	3.7%	63.0%	100.0%
	Positive	Count	2	0	0	32	34
		% within set	5.9%	0.0%	0.0%	94.1%	100.0%
Total		Count	10	1	1	49	61
		% within set	16.4%	1.6%	1.6%	80.3%	100.0%

Table 3.7: Cross-tabulation of - *sen* * Stool consistency

			Stool consistency				
			Simple watery	Rice watery	Sticky/ mucoid	Bloody mucoid	Total
sen	Negative	Count	8	1	1	17	27
		% within sen	29.6%	3.7%	3.7%	63.0%	100.0%
	Positive	Count	2	0	0	32	34
		% within sen	5.9%	0.0%	0.0%	94.1%	100.0%
Total		Count	10	1	1	49	61
		% within sen	16.4%	1.6%	1.6%	80.3%	100.0%

Table 3.8: Cross-tabulation of *set* * Rectal strain

			Rectal strain		
			Yes	No	Total
set	Negative	Count	15	12	27
		% within set	55.6%	44.4%	100.0%
	Positive	Count	30	4	34
		% within set	88.2%	11.8%	100.0%
Total		Count	45	16	61
		% within set	73.8%	26.2%	100.0%

Table 3.9: Cross-tabulation of - *sen* * Rectal strain

			Rectal strain		
			Yes	No	Total
sen	Negative	Count	15	12	27
		% within sen	55.6%	44.4%	100.0%
	Positive	Count	30	4	34
		% within sen	88.2%	11.8%	100.0%
Total		Count	45	16	61
		% within sen	73.8%	26.2%	100.0%

Table 3.10: Cross-tabulation of - *spa24* * Rectal strain

			Rectal strain		
			Yes	No	Total
spa24	Negative	Count	10	8	18
		% within spa24	55.6%	44.4%	100.0%
	Positive	Count	35	8	43
		% within spa24	81.4%	18.6%	100.0%
Total		Count	45	16	61
		% within spa24	73.8%	26.2%	100.0%

Table 3.11: Cross-tabulation of - *ial* * Abdominal pain

			Abdominal pain			
			Yes	No	Don't know	Total
ial	Negative	Count	4	3	0	7
		% within ial	57.1%	42.9%	0.0%	100.0%
	Positive	Count	48	4	2	54
		% within ial	88.9%	7.4%	3.7%	100.0%
Total		Count	52	7	2	61
		% within ial	85.2%	11.5%	3.3%	100.0%

Table 3.12: Cross-tabulation of - *ipaBCD* * Abdominal pain

			Abdominal pain			
			Yes	No	Don't know	Total
ipaBCD	Negative	Count	3	2	1	6
		% within ipaBCD	50.0%	33.3%	16.7%	100.0%
	Positive	Count	49	5	1	55
		% within ipaBCD	89.1%	9.1%	1.8%	100.0%
Total		Count	52	7	2	61
		% within ipaBCD	85.2%	11.5%	3.3%	100.0%

Table 3.13: Cross-tabulation of - *set* * Dryness of mouth

		Mouth		
		Normal	Somewhat dry	Total
set	Negative	22	5	27
	Positive	33	1	34
Total		55	6	61

Table 3.14: Cross-tabulation of - *sen* * Dryness of mouth

		Mouth		
		Normal	Somewhat dry	Total
sen	Negative	22	5	27
	Positive	33	1	34
Total		55	6	61

Table 3.15: Cross-tabulation of - *ipgD* * Pedal edema (<5years)

			Pedal edema (<5years)		
			No	Not Applicable	Total
ipgD	Negative	Count	10	3	13
		% within ipgD	76.9%	23.1%	100.0%
	Positive	Count	47	1	48
		% within ipgD	97.9%	2.1%	100.0%
Total		Count	57	4	61
		% within ipgD	93.4%	6.6%	100.0%

Table 3.16: Cross-tabulation of - *set* * Fever

			Fever		
			Yes	No	Total
set	Negative	Count	17	10	27
		% within set	63.0%	37.0%	100.0%
	Positive	Count	30	4	34
		% within set	88.2%	11.8%	100.0%
Total		Count	47	14	61
		% within set	77.0%	23.0%	100.0%

Table 3.17: Cross-tabulation of - *sen* * Fever

			Fever		
			Yes	No	Total
sen	Negative	Count	17	10	27
		% within sen	63.0%	37.0%	100.0%
	Positive	Count	30	4	34
		% within sen	88.2%	11.8%	100.0%
Total		Count	47	14	61
		% within sen	77.0%	23.0%	100.0%

Table 3.18: Cross-tabulation of - *icsB* * Fever

			Fever		
			Yes	No	Total
icsB	Negative	Count	23	11	34
		% within icsB	67.6%	32.4%	100.0%
	Positive	Count	24	3	27
		% within icsB	88.9%	11.1%	100.0%
Total		Count	47	14	61
		% within icsB	77.0%	23.0%	100.0%

Table 3.19: Cross-tabulation of - *set* * Cough

			Cough		
			Yes	No	Total
set	Negative	Count	15	12	27
		% within set	55.6%	44.4%	100.0%
	Positive	Count	10	24	34
		% within set	29.4%	70.6%	100.0%
Total		Count	25	36	61
		% within set	41.0%	59.0%	100.0%

Table 3.20: Cross-tabulation of - *sen* * Cough

			Cough		
			Yes	No	Total
sen	Negative	Count	15	12	27
		% within sen	55.6%	44.4%	100.0%
	Positive	Count	10	24	34
		% within sen	29.4%	70.6%	100.0%
Total		Count	25	36	61
		% within sen	41.0%	59.0%	100.0%

Table 3.21: Cross-tabulation of - *ipgD* * Cough

			Cough		
			Yes	No	Total
ipgD	Negative	Count	2	11	13
		% within ipgD	15.4%	84.6%	100.0%
	Positive	Count	23	25	48
		% within ipgD	47.9%	52.1%	100.0%
Total		Count	25	36	61
		% within ipgD	41.0%	59.0%	100.0%

Table 3.22: Cross-tabulation of - *set* * Disease severity

			Disease severity		
			Mild	Moderate - Severe	Total
set	Negative	Count	5	22	27
		% within set	18.5%	81.5%	100.0%
	Positive	Count	1	33	34
		% within set	2.9%	97.1%	100.0%
Total		Count	6	55	61
		% within set	9.8%	90.2%	100.0%

Table 3.23: Cross-tabulation of - *sen* * Disease severity

			Disease severity		
			Mild	Moderate - Severe	Total
sen	Negative	Count	5	22	27
		% within sen	18.5%	81.5%	100.0%
	Positive	Count	1	33	34
		% within sen	2.9%	97.1%	100.0%
Total		Count	6	55	61
		% within sen	9.8%	90.2%	100.0%

Table 3.24: Chi-Square test of independence at 1% and 5% level of significance

	Gene	Clinical Features	Pearson Chi-Square Test		
			Value	df	P
1.	***set***	**Blood in stool**	**9.244**[c]	1	**.002**
2.	***sen***	**Blood in stool**	**9.244**[c]	1	**.002**
3.	*set*	Stool consistency (bloody mucoid)	9.514[b]	3	.023
4.	*sen*	Stool consistency (bloody mucoid)	9.514[b]	3	.023
5.	***set***	**Rectal Strain**	**8.306**[f]	1	**.004**
6.	***sen***	**Rectal Strain**	**8.306**[f]	1	**.004**
7.	*spa24*	Rectal Strain	4.378[g]	1	.036
8.	*ial*	Abdominal pain	7.778[d]	2	.020
9.	*ipaBCD*	Abdominal pain	7.378[e]	2	.025
10.	*set*	Dryness of mouth	4.118[a]	1	.042
11.	*sen*	Dryness of mouth	4.118[a]	1	.042
12.	***ipgD***	**Pedal edema**	**7.358**[k]	1	**.007**
13.	*set*	Cough	4.253[h]	1	.039
14.	*sen*	Cough	4.253[h]	1	.039
15.	*ipgD*	Cough	4.476[i]	1	.034
16.	*set*	Fever	5.435[j]	1	.020
17.	*sen*	Fever	5.435[j]	1	.020
18.	*icsB*	Fever	3.840[j]	1	.050
19.	*set*	Disease severity	4.118[a]	1	.042
20.	*sen*	Disease severity	4.118[a]	1	.042

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.66. b. 5 cells (62.5%) have expected count less than 5. The minimum expected count is .44. c. 0 cells (.0%) have expected count less than 5. The minimum expected count is 5.31. d. 3 cells (50.0%) have expected count less than 5. The minimum expected count is .23. e. 3 cells (50.0%) have expected count less than 5. The minimum expected count is .20. f. 0 cells (.0%) have expected count less than 5. The minimum expected count is 7.08. g. 1 cells (25.0%) have expected count less than 5. Minimum expected count is 4.72. h. 0 cells (.0%) have expected count less than 5. Minimum expected count is 11.07. i. 0 cells (.0%) have expected count less than 5. Minimum expected count is 5.33. j. 0 cells (.0%) have expected count less than 5. Minimum expected count is 6.20. k. 2 cells (50.0%) have expected count less than 5. The minimum expected count is .85. df – degrees of freedom; P – calculated probability.

The two *Shigella* enterotoxin genes, *set* (*Shigella* Enterotoxin 1) and *sen* (*Shigella* Enterotoxin 2), both were found highly statistically significant at 1% level of significance, associated with both of the following clinical features, blood in stool (P 0.002 <0.01) and rectal strain (P 0.004 <0.01). Additionally, an effector gene *ipgD* was also found of high statistical significance, associated with pedal edema (<5 years) at 1% level of significance (P 0.007 <0.01) (Table – 3.24).

At 5% level of significance, additional 17 correlations were found statistically significant (P ≤0.05) (Table – 3.24). Both of the two *Shigella* enterotoxin genes, *set* (*Shigella* Enterotoxin 1) and *sen* (*Shigella* Enterotoxin 2) were equally found statistically significant, associated with the following clinical features, stool with bloody mucoid consistency (P 0.023 < 0.05), dryness of mouth (P 0.042 <0.05), fever (P 0.02 <0.05), cough (P 0.039 <0.05), and overall increased disease severity (P 0.042 <0.05).

Along with these enterotoxin genes, 5 separate genes were found to be significantly associated with 4 different clinical features. An inner membrane export-apparatus protein gene of T3SS, *spa24* was found associated with rectal strain of the patient (P 0.036 <0.05), *ial* gene was found linked with abdominal pain (P 0.02 <0.05), *ipaBCD* gene (responsible for inducing apoptosis, actin polymerization, control of protein flux through T3SS of *Shigella)* with abdominal pain (P 0.025 <0.05), *icsB* (which protein is culpable for camouflaging IcsA, responsible for *Shigella* invasiveness) with fever (P 0.02 <0.05) and *ipgD* (encodes an effector protein responsible for host cell membrane blebbing) with cough.

The rest of the calculations produced values with P >0.05, and thus regarded as statistically insignificant in this study (Data not shown).

Chapter 4 : Discussion

Although, the incidence of shigellosis has been decreased significantly throughout the world, with improved sanitation, better health care facilities, and safer water supply, it is still one of the major causes of moderate to severe diarrheal diseases, contributing to 800,000 fatalities worldwide each year, mostly in sub-Saharan Africa and south Asian region, in children less than 5 years of age (Kotloff et al., 1999, 2013).

Given that *S. dysenteriae* is responsible for the most severe form of bacillary dysentery, among the four species constituting the genus *Shigella*, *S. dysenteriae, S. flexneri, S. boydii* and *S. sonnei;* in Bangladesh for the last 35 years, *S. flexneri* was always found as the predominant species, and *S. flexneri* 2a as the most frequent serotype (Talukder et al., 2012). However, since 2004 no *S. dysenteriae* incidence was reported in Bangladesh (Azmi, 2015).

With a low infectious dose of about 10 – 100 pathogens, and yet with no effective vaccine, it is still wise to consider *Shigella*, as one of the major public health threats, specially in densely populated developing countries, and for displaced population suffering from war, or natural disasters (DuPont, HL, Levine, M M, Hornick, RB and Formal, 1989).

In this study, 61 randomly selected strains of *S. flexneri* were analyzed. Plasmid profiling was done for all of them, to detect the presence or absence of 140 MD large virulence plasmid. Then, PCR assay was done for virulence (*ipaH, ial, ipaH7.8*), toxin (*set1A, set1B, sen*) and type 3 secretion system related genes (*virB, ipaBCD, ipaB, ipaC, ipaD, ipgC, ipgB1, ipgA, icsB, ipgD, ipgE, ipgF, mxiH, mxiI, mxiK, mxiE, mxiC, spa15, spa47, spa32, spa24, spa*) for each strain, constituting a total count of 27 separate gene PCR assays.

Simultaneously, primers for two of the genes, *mxiE*, a T3SS regulator gene, and *spa15*, a T3SS chaperone gene, were designed with web based bioinformatics tool, and the annealing temperature (T_a) optimization was done by gradient PCR, and eventually PCR protocols for those genes were set up. The validity of the primers were confirmed, when the predicted product size correlated with the band size of the PCR product in gel electrophoresis analysis.

Among these 61 isolates, the most prevalent gene was found to be *ipaBCD* (90%), followed by *ial* (89%), *ipgC, ipgE* (85%); *virB, ipgA* (82%); *sen, mxiH, mxiI, spa15, spa47* (80%); *ipgD* (79%), *ipgF* (77%), *spa32* (75%), *ipgB1* (72%), *mxiK, spa24* (70%); *mxiE* (66%), *set* (58%), *icsB* (44%) and *mxiC* (36%). And, among the 48 cases of 140MD positive strains, the status of *sen* and T3SS related genes are the following, *sen, virB, ipaBCD, ipgA, mxiH, mxiI,*

spa15, *spa47* (100%), *ipgC*, *ipgE* (98%), *ipgF* (96%), *spa32* (94%), *ipgB1* (90%), *mxiK* (88%), *spa24* (83%), *mxiE* (81%), *ipgD* (77%), *icsB* (54%) and *mxiC* (44%).

The disease burden data obtained from the icddr,b Mirzapur study database, was analyzed for the most frequently encountered clinical features associated with shigellosis. Surprisingly, most recurrent clinical feature was found to be the presence of pedal edema in 93.4% cases, followed by an overall increased disease severity in 90.2%, abdominal pain in 82.5%, presence of blood in stool and bloody mucoid stool consistency in 80.3%, fever in 77%, rectal strain in 73.8%, vomiting in 45.9%, cough in 41%, sunken eye, dry mouth and moderate to severe dehydration respectively in 11.5%, 9.8%, 21.3% cases, convulsion in 3.3% and altered level of mental status in 1.6% cases (Data not shown).

However, although theoretically T3SS related virulence genes resides with in a conserved 32 kb entry region within the 140 MD virulence plasmid (Schroeder and Hilbi, 2008), none of the thirteen 140 negative strains were found to be completely devoid of T3SS related genes, tested in this work (Table 3.1). These strains were confirmed negative for 140 MD plasmid for three separate times of plasmid isolation. Only one gene was returned positive in 5 of these strains, followed by 2 genes counts in three strains, 3, 4 and 5 counts of genes in three separate strains. But, on the top of them all, two strains, K-842 *S. flexneri* 1c and K-1080 *S. flexneri* 2a were detected positive for an intriguing 6 and 19 counts of genes, against the total 20 plasmid specific genes tested.

Moreover, the clinical data for this 13 virulence plasmid negative strains also showed a similar picture (Table 3.2). Two separate clinical symptoms were found positive in 2 correspondent cases, and in rest of the affected patients more than 4 separate target clinical features were found positive. The two strains aforementioned, K-1080 and K-842, were found positive in patients with high severity of the disease (MSD), presence of blood in stool, bloody mucoid stool consistency, abdominal pain, rectal strain, vomiting, fever and moderate to severe dehydration status. It could be a possibility that the virulence genes, could had been integrated into the chromosome. Therefore, further study is required to resolve this matter.

To determine if there was any significant association in between any particular clinical feature and tested genes, Chi-square test of independence with two different cutoff values (P

<0.05 and P <0.01) along with cross-tabulation of important clinical features and the presence of target genes were done. It revealed 20 statistically significant correlations (P <0.05), among which 3 were highly statistically significant (P <0.01).

The two *Shigella* enterotoxin genes, *set* and *sen* were found highly significantly associated with the presence of blood in stool (P 0.002 <0.01) and presence of rectal strain in patient (P 0.004 <0.01). They were also found to be significantly associated (P <0.05) with bloody mucoid stool consistency (P 0.023 <0.05), dryness of mouth (P 0.042 <0.05), fever (P 0.02 <0.05), cough (P 0.039 <0.05) and an overall increased severity of the disease (P 0.042 <0.05).

The chromosomal *set* gene consists of two separate units, *set1A* and *set1B*, encoding ShET1A and ShET1B proteins respectively. They (*set1A*, *set1B*) are individually culpable for, secretory activity and the irreversible binding of toxin to the enterocyte receptors respectively (Fasano et al., 1997). And the Shigella enterotoxin 2 (ShET2) coding *sen* gene, located in 140 MD large plasmid, is responsible for, the epithelial inflammation by contributing to the release of Interleukin-8 (IL-8) from the gut epithelium (Farfán et al., 2011; Fasano and Levine, 1997). The findings in this study are compatible with the previous studies, as Interleukin-8 is a chemokine, and it attracts and activates the neutrophils at the inflammatory region (Bickel, 1993). These chain of events are likely to be responsible for the following clinical features found here, presence of blood in stool and bloody mucoid stool consistency in the affected patients, rectal strain, and ultimately an increased disease severity. It is highly probable that these events resulted from the rectal epithelial injury, produced by the activated neutrophils. Nevertheless, the secretory activity of ShET1 and inflammatory activity of ShET2 correlate with mouth dryness, indicating a state of dehydration among the affected individuals. Although, a larger sample size and extended clinical data would give a better insight into it.

Here, another gene *spa24* was seen to be associated with rectal strain (P 0.036 <0.05), which is known to be an essential component of inner membrane complex of the export apparatus. Although, without the inner membrane export apparatus the assembly of the NC (needle complex of T3SS) is possible, but the efficacy is greatly reduced without it (Wagner et al., 2010), which backs up the significance of the association.

Moreover, the association of observed fever with the two toxin genes (P 0.02 <0.05), may also be an inevitable outcome of the repeated occurrence of the inflammatory event. Along with it, another gene, *icsB* was also seen to be correlated with the fever (P 0.05 ≤0.05). This gene (*icsB*) is essential to evade the host autophagy defense system by binding with the autophagy protein, Atg5 (Allaoui et al., 1992; Kayath et al., 2010).

Though, the two enterotoxin gene, *set*, *sen*, and a T3SS effector gene *ipgD* were found significantly associated with cough (P 0.039 <0.05 and P 0.034 <0.05 respectively), the data was insufficient to further verify the finding. Even though, *ipgD* is known to alter the cellular response causing host cell membrane blebbing, once it is injected into the host cell (Niebuhr et al., 2000), more data is required to clarify whether there was any relevant co-infections, such as previous streptococcal or similar infections; or, co-morbidities, such as, CCF (congestive cardiac failure) consequenced from such infections. In this regard, bioinformatic analysis such as, multiple sequence alignment to detect any conserved epitope among these pathogens, could be a feasibility.

Moreover, *ipgD* was also found of highly statistical significance in association with pedal edema, in children less than 5 years old (P 0.007 <0.01); at which age, mild metabolic abnormalities could bring a fatal consequence very rapidly. The clinical feature found to be associated here, pedal edema could result from any of the following problems, from decreased albumin content in blood to congestive cardiac failure to impaired renal function, such as glomerulonephritis or nephrotic syndrome. However, nephrotic syndrome is characterized by a group of symptoms that includes, high level of protein in urine, low blood protein levels, high level of cholesterol and triglyceride (TG) in blood, and local or generalized swelling; while glomerulonephritis predominantly presents with hematuria.

Two other virulence genes *ial* and *ipaBCD*, were also found significantly associated with abdominal pain in patients (P values, 0.02 <0.05 and 0.025 <0.05, respectively). The *ial* gene has been reported to direct the epithelial cell penetration by the organism (Hale, 1991; Ménard et al., 1996). And, *ipaBCD* gene cluster is known for coding three proteins, IpaB, IpaC and IpaD; all acting as translocon, ensuring the delivery of specific proteins into the target cells, while IpaD carrying out an additional role of a signal transducer (Blocker et al., 1999; Roehrich et al., 2013).

Even though, Shigellosis was found to be associated with convulsion in previous studies (Khan et al., 1999), no significant statistical relationship was detected with convulsion, or with higher cerebral function in this study. A larger sample size with additional detailed clinical data is required to further verify this aspect.

In summary, the two Shigella enterotoxin genes (*set*, *sen*) were found to be significantly associated with multiple important clinical features frequently observed in shigellosis, such as, presence of blood in stool with a bloody mucoid consistency, rectal strain, dehydration, cough, fever and an overall increased state of diarrheal severity, along with four other virulence genes (*ial*, *ipaBCD*, *ipgD* and *spa24*).

In future, further study with a larger sample is required, with detailed and organ specific clinical data, to get a better insight into the association of the clinical features, such as pedal edema, level of dehydration and *Shigella* spp.. More information is needed to rule out the existence of any previous pathogenic involvement, or associated co-morbidities. In silico approaches, could be made to explore the possibilities of any conserved DNA sequences among *Shigella* spp. and those organisms specially responsible for immune system mediated cross reactions in cardiac or renal system. And, lastly the possibilities of the integration of virulence genes into the chromosomal DNA should also be resolved.

4.1 Limitations of this Study

This study has a number of drawbacks as follows:

1. The absence of 140 MD virulence plasmid would have been more conclusive, if several plasmid isolation and/or confirming techniques, such as electroextraction or Sereny test, could have been implemented instead of only one method (alkaline lysis method).
2. The status of previous infections, such as *S. pyogenes*, (known to cause immune system mediated cross reactions), or the existence of relevant co-morbidities, such as CCF, NS or AGN were not available in the clinical data.
3. The available data regarding the patients' systemic examinations and particular clinical features, such as liver, spleen, lungs, status of sore throat, were inadequate. Additional detailed organ specific clinical data related to these features and organs were needed.
4. The lack of statistical association between the tested virulence genes and clinical features, such as convulsion, altered level of mental status, is likely to be attributed to the relatively small sample size.

4.2 Recommendations for Future Work

In future additional work regarding this project may include,

- Confirming the absence of 140 MD large virulence plasmid with alternative methods, for particularly 2 strains, K-1080 *S. flexneri* 2a, *and* K-842 *S. flexneri* 1c.
- Investigating the possibilities of chromosomal integration of plasmid borne virulence genes.
- Determination of any conserved regions or epitope in between *S. flexneri* and organisms known for causing immune system mediated cross-reactions in humans.
- A more comprehensive analysis with a larger sample size with extended clinical information.

Chapter 5 : Reference

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Appendix

1) Laboratory Apparatus

1. Eppendorf tubes and micropipette tips were taken from Eppendorf® and Sigma, and were sterilized by autoclaving at 121°C for 20 minutes.
2. Petridishes used in the experiments were provided by Gibco.
3. Plastic tubes and pipettes were of Falcon®; both were the brands of Becton, Dickinson and Company.
4. Micropipettes were from Thermo Labsystems.
5. Mini scale centrifugations were carried out in a Sorvall® pico microfuge.
6. C1000™ thermal cycler from Bio-Rad Laboratories was used for PCR reaction.
7. Sub-Cell® GT Cell electrophoresis system was used for agarose gel electrophoresis from Bio-Rad Laboratories.
8. Gel Doc™ XR+ Gel Documentation System from Bio-Rad Laboratories was used for PCR gel electrophoresis imaging, documentation and analysis.

2) Chemical Reagents

Preparations of the stock solutions used in this thesis work are the following:

(All the working solutions used in the work were prepared from the stock solutions).

1. **1 M 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 1L with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

2. **3 M NaCl**

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

3. **10 M NaOH**

   40 g of NaOH pellet was dissolved in distilled water to final volume of 100 ml. The solution was stored in an airtight bottle at RT.

4. **0.5 M EDTA**

   186.1 g of $Na_2EDTA.2H_2O$ (disodium ethylene diamine tetra-acetic acid) and 20g of NaOH pellets were added to 800 ml distilled water and dissolved by stirring on a magnetic stirrer. pH was adjusted to 8.0 with few drops of 10 M NaOH and final volume was made up to 1l with distilled water. The solution was sterilized by autoclaving and stored at RT.

5. **10% SDS**

   10 g of SDS (Sodium dodecyl sulfate) (Sigma) was added to 80 ml of distilled water and dissolved by stirring on a magnetic stirrer slowly to avoid foaming. The final volume was adjusted to 100 ml with distilled water and stored at RT.

6. **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_2PO_4 in 800 ml of distilled water. pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1l by distilled water. The solution was sterilized by autoclaving for 20 minutes and stored at RT.

7. **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 4^0 C.

8. **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 l of distilled water to make the 1x concentrated TBE buffer. The buffer was stored at RT.

9. **0.5% Triton X solution**

   0.5 ml was added in 99.5 ml PBS

10. **Ethidium bromide solution**

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

11. **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°C in 1 ml aliquot.

3) Media Composition

The compositions of the media used in this thesis are given below (Unless specified otherwise all media were autoclaved at 120°C for 15 minutes at 15 lbs pressure).

1. **Trypticase Soy agar (TSA), (Difco™)**

Ingredients	**Amount (g/L)**
Pancreatic digest of casein	15.0
Papaic digest of soyabean meal	5.0
Sodium chloride	5.0
Agar	15.0
Distilled Water	1L
Final pH = 7.3 ± 0.2	

2. **Tryptic Soy Broth (TSB), (Difco™) + Yeast Extract (0.3%), (Difco™)**

Ingredients	**Amount (g/L)**
Pancreatic digest of casein	17.0
Papaic digest of soybean	3.0
Dextrose	2.5
Sodium chloride	5.0
Dipotassium phosphate	2.5
Yeast extract	0.3
Final pH = 7.2 ± 0.2	

3. **MacConkey Agar (Difco™)**

Ingredients	**Amount (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
Final pH = 7.1 ± 0.2	

4) Software

1. Libreoffice – Text Document
2. Libreoffice – Spreadsheet
3. Libreoffice – Drawing
4. Image Lab™ – Bio-Rad
5. GIMP – GNU Image Manipulation Program
6. ImageJ 1.48k – Image Processing and Analysis in Java
7. Mendeley – Free reference manager
8. IBM® SPSS® Statistics version 22.0