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Characterization of Antibiotic Resistance and Virulence Genes in *Shigella* Species Isolated from Patient Samples from Southern Palestine

By:

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In Partial Fulfillment of the Requirements for the Degree

Master of Science

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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

Characterization of Antibiotic Resistance and Virulence Genes in *Shigella* Species Isolated from Patient Samples from Southern Palestine

By

Muna Kamal Ismael Salah

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in biotechnology

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Abstract:

Characterization of Antibiotic Resistance and Virulence Genes in *Shigella* Species Isolated from Patient Samples from Southern Palestine

Shigella is a Gram-negative rod-shaped bacteria from the *Enterobacteriaceae* family, classified into four species; *S. dysenteriae, S. flexneri, S. sonnei,* and *S. boydii*. *Shigella* species cause shigellosis, which is transmitted through fecal-oral route causing destruction of colonic epithelium resulting in bloody mucous diarrhea. Infected individuals usually recover without medical intervention. However, severe *Shigella* infections require antibiotic treatment to reduce the severity and duration of illness and to reduce the spread of the disease. For children, ceftriaxone is recommended as first line parenteral therapy, and azithromycin is recommended as oral treatment. While in adults, flouroquinolones are the first choice, in addition to azithromycin and trimethoprim-sulfamethoxazole.

In this study, 502 isolates of *Shigella* species collected from patients seen at Caritas Baby Hospital (CBH) between 2004-2014 were included. The Clinical and Laboratory Standard Institute (CLSI) guidelines were used to determine the antibiograms of the isolates. The presence of macrolide resistance genes {*erm(A), erm(B), erm(C), ere(A), ere(B), mph(A), mph(B), mph(D), mef(A), msr(A)*}, and ESBL producing genes were determined in the resistant isolates by Polymerase Chain Reaction (PCR). Additionally, the six virulence genes (*ipaH, ial, sen, set1A, set1B, stx*) were screened in all isolates by PCR. Moreover, subtyping of the bacterial isolates using pulsed-field gel electrophoresis (PFGE) was performed according to the Centers for Disease Control and Prevention (CDC) standard procedure for 15 isolates

of *S. flexneri* which were resistant to azithromycin to investigate their genetic diversity.

Of the 502 isolates, 40.4% were resistant to ampicillin, 92.2% resistant to trimethoprim-sulfamethoxazole, 78.3% resistant to tetracycline, 3.2% resistant for cephalosporins, 14.7% resistant to naldixic acid, however, 100% were sensitive to ciprofloxacin. For azithromycin, 62 (12.4%) of the isolates showed reduced susceptibility (Breakpoint≤16 mm). Screening for macrolide resistance genes in these isolates showed that only isolates with breakpoint ≤12 mm (49 isolates) were positive for the *mph(A)* gene, and negative for the other genes.

Ten isolates showed reduced susceptibility to cefotaxime, thus indicating the isolates could be ESBL producers. All of them were positive for the *blaTEM* gene; 5 positive for the *blaCTX* gene, 6 were positive for the *blaCTX-M*$_{2\text{-}5}$ and all were negative for all other ESBL genes evaluated.

Virulence genes results showed the presence of invasion plasmid antigen H (*ipaH*) in 479 (95.8 %) isolates, the invasion-associated locus (*ial*) in 374 isolates (75.4%), *set1B* which is responsible for enterotoxins in 39 isolates (7.8%), and exotoxin Shiga toxin (*Stx*) in 8 (1.6%) of the samples. While the other enterotoxin genes (*set1A* and *sen*) were not found in any of the isolates.

The high *Shigella* species resistant patterns to oral antibiotics and the emergence of ESBL producing *Shigella* species, mandates the Palestinian Ministry of Health to control the misuse of antibiotics. In addition, the results of this study can be used to better interpret azithromycin disk diffusion results as no clear guidelines by the CLSI are currently available.

Key words: *Shigella*, *Enterobacteriaceae*, ESBL, Azithromycin, Antibiotics resistance

ملخص:

توصيف مقاومة المضادات الحيوية و الجينات الممرضة في بكتيريا الشيجلا المعزولة من مرضى من جنوب فلسطين

الشيجلا هي يكتيريا سلبية الغرام عصوية من عائلة الانتيروبكتيرياسي (*Enterobacteriaceae*)، تصنف إلى أربعة أنواع: شيجلا الزحار، الشيجلا الفلكسنرية, الشيجلا السونية، والشيجلا البويدية. و تسبب هذه الأنواع داء الشيغلات، وهو مرض ينتقل عن طريق الطعام والشراب الملوث ببراز المريض و يتسبب في تدمير بطانة القولون مؤديا إلى إسهال مخاطي دموي. عادة ما يتماثل المصابون بالمرض للشفاء دون تدخل طبي، لكن التهابات الشيجلا الشديدة تتطلب علاجا بالمضادات الحيوية للحد من شدة ومدة المرض والحد من انتشار المرض. بالنسبة للأطفال، يوصى سيفترياكسون كخط علاج أول بالحقن، وينصح أزيثروميسين كعلاج عن طريق الفم. بينما في البالغين، flouroquinolones هي الخيار الأول، بالإضافة إلى أزيثروميسين وميثوبريم-سلفاميثوكسازول.

في هذه الدراسة تم استخدام 502 عزلة من أنواع الشيجلا التي تم الحصول عليها من عينات البراز للمرضى الواردين الى مستشفى كاريتاس للأطفال في الفترة الواقعة بين عام 2004 و عام 2014. تم استخدام تعليمات ال CLSI لتحديد antibiograms من العزلات, كما تم دراسة وجود جينات المقاومة للmacrolides *erm(A), erm(B), erm(C), ere(A), ere(B), mph(A), mph(B), mph(D), mef(A), msr(A)* في العزلات المقاومة للأزيثروميسين, و الجينات المنتجة لل ESBL في العزلات المقاومة للcefotaxime باستخدام تفاعل الPCR. بالإضافة إلى ذلك، تم دراسة ستة جينات ممرضة { *ipaH* ، *set1A*، *set1B*، *sen*، *ial*، *STX*} في كل العزلات. عدا عن ذلك، فقد تم أيضا إجراء التصنيف الفرعي للعزلات البكتيرية باستخدام طريقة ال PFGE وفقا للإجراءات القياسية ل CDC ل 15 عزلة من الشيجلا الفلكسنرية التي كانت مقاومة للازيثروميسين.

النتائج أظهرت انه من ال502 عزلة، 40.4% كانت مقاومة الأمبيسلين، 92.2% مقاومة ميثوبريم-سلفاميثوكسازول، 78.3% مقاومة التتراسيكلين، 3.2% للالسيفالوسبورين، 14.7% لنالديكسيك اسيد, لكن 100% من العزلات كانت حساسة للسيبروفلوكساسين. أما الأزيثروميسين، فقد أظهرت 62 (12.4%) من العزلات حساسية منخفضة للعلاج (Breakpoint ≤16). فحص الجينات المقاومة للمكروليد في هذه العزلات

أظهر أن فقط العزلات التي كانت فيها الBreakpoint ≥ 12 ملم (49 عزلات) كانت ايجابية لجين *mphA* ال ، وسلبية للجينات أخرى.

أظهرت عشر عزلات حساسية منخفضة للسيفوتاكسيم، مما يدل على ان العزلات يمكن أن تكون منتجة لل ESBL. جميع هذه العزلات كانت إيجابية لجين bla_{TEM}, 5 إيجابية لجين *blaCTX*، 6 إيجابية ل $bla_{CTX-M2-5}$, بينما كان جميعها سلبيا لجميع جينات ال ESBL الأخرى التي تم تقييمها.

نتائج الجينات الممرضة اظهرت وجود جين (*ipaH*) في 479 (95.8%) عزلة, جين ال(*ial*) في 374 (75.4%) من العزلات, جين *set1B* في 39 عزلة (7.8%)، و جين (*STX*) في 8 (1.6%) من العينات. في حين أن الجينات الأخرى *set1A* و *sen* لم تكن إيجابية في أي من العزلات.

الانماط المرتفعة لمقاومة الشيجلا لأنواع المضادات الحيوية المعطاة عن طريق الفم, و ظهور أنواع الشيجلا المنتجة لل ESBL, يتطلب من وزارة الصحة الفلسطينية السيطرة على سوء استخدام المضادات الحيوية. إضافة الى ذلك, فإن نتائج هذه الدراسة يمكن ان تستخدم في وضع تعليمات أفضل لمعاينة نتائج فحص حساسية الشيجلا للازيثروميسين, حيث أنه حتى الان لا توجد تعليمات واضحة من ال CLSI لذلك.

DECLARATION

I declare that the Master Thesis entitled "Characterization of Antibiotic Resistance and Virulence Genes in *Shigella* Species Isolated from Patient Samples from Southern Palestine" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgement is made in the text.

Name and signature: Muna Kamal Ismael Salah

Date: _____

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In presenting this thesis in partial fulfillment of the requirements for the joint master degree in biotechnology at Palestine Polytechnic University and Bethlehem University, I agree that the library shall make it available to borrowers under rules of the library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of the source is made.

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Signature: Muna Kamal Ismael Salah _____

Date:_____

DEDICATION

To all those who have their own dreams and insist to achieve them, the price of success is just dedication, hard work, and an unremitting devotion to the things you want to see happen

ACKNOWLEDGEMENT

After an intensive period of about one year of hard work, now I'm writing the last part of my thesis, remembering all the bittersweet days of this journey. I would like to thank the greatest people who were always there for helping and supporting me during this time.

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PFGE part of this study, thanks for training and teaching me even the detailed steps of the technique, and thanks for his hard work in helping me to get these great results.

In the same context, I would like to acknowledge and thank the staff at the PulseNet US CDC, for their excellent technical assistance.

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Finally, I take this opportunity to express the profound gratitude from my deep heart to my beloved family. Thanks for my dear parents, all the support they have provided over the years was the greatest gift anyone has ever given me, thanks for my father for allowing me to realize my own potential and to achieve my ambitions. Thanks for my mother, my partner in every gain and pain in my life. Thanks for my brother Ahmad and my sisters, Haya, Hind, Basema and Basma. Always I'm overwhelmed with their love and kindness, they were always there for me whenever I needed a helping hand. Thanks for my grandmother for her prayers, thanks for my aunties for infinite support and encouragement. And never forget to mention my niece and nephews, whom I wish to read their thesis someday.

LIST OF ABBREVIATIONS

AMC: Augmentin

AMP: Ampicillin

AZM: Azithromycin

C: Chloramphenicol

CAZ: Ceftazidime

CBH: Caritas Baby Hospital

CDC: Central of Disease Control

CFM: Cefixime

CHEF: Contour-clamped homogeneous electric field

CIP: Ciprofloxacin

CLA: Clavulanic acid

CLSI: Clinical and Laboratory Standard Institute

CN: Gentamycin

CTX: Cefotaxime

DDS: Double Disk Synergy

ECVs: Epidemic cutoff values

EHEC: Enterohemorrhagic *Escherichia coli*

ESBL: Extended Spectrum β-lactamase

ETP: Ertapenem

FAE: Follicle-associated epithelium

HUS: Haemolytic uremic syndrome

ial: Invasion associated locus gene

ipaH: Invasion plasmid antigen gene

IP: Imipenem

MIC: Minimum Inhibitory Concentration

MP: Meropenem

NA: Naldixic acid

NARM: National Antimicrobial Resistance Monitoring System for Enteric Bacteria

PCR: Polymerase Chain Reaction

PFGE: Pulsed Field Gel Electrophoresis

STX: Shiga toxin

SXT: Trimethoprim-sulfamethoxazole (Co-trimaxazole)

T3SS: Type Three Secretion System

TE: Tetracycline

TIFF: Tagged Image File Format

UPGMA: Unweighted Pair-Group Method Arithmetic Averages

WHO: World Health Organization

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CHAPTER 1

INTRODUCTION

Shigella species are aerobic, non-motile, glucose-fermenting, gram-negative rods from the *Enterobacteriaceae* family. *Shigella* species are highly contagious, causing diarrhea after ingestion of about 10 to 100 organisms (Bennish 1991). *Shigella* species are classified into four different species; *S. dysenteriae, S. flexneri, S. sonnei, and S. boydii*.

Shigella causes shigellosis, a disease characterized by the destruction of the colonic epithelium in humans and non-human primates resulting in mucoid and bloody diarrhea (DuPont, Hornick et al. 1969; Echeverria, Sethabutr et al. 1991) and can be transmitted by the fecal-oral route, or via contaminated food or water. Shigellosis is considered as one of the main sources of complication in children with diarrhea in developing countries (Kotloff, Winickoff et al. 1999).

Annual number of epidemic cases of shigellosis throughout the world is estimated to be 80 million cases and 700,000 deaths each year, of which 99% occur in developing nations, while 60-70% of the infections occur in children under five years of age (WHO 2005).

In Palestine, shigellosis is an endemic disease, most commonly caused by *S. sonnei*, *S. flexneri*, and rarely by *S. boydii*. Shigellosis is not a well-documented disease in Palestine, the actual numbers that are reported by the hospital based laboratories to the Palestinian Ministry of Health do not represent the real incidence of the disease.

Infected people with shigellosis usually recover without antibiotic treatment, but some individuals might need antibiotic intervention to reduce the duration, severity of the illness, and to decrease the spread of the infection (Jain, Gupta et al. 2005; RH PC, John et al. 2010). In children with severe shigellosis symptoms such as bloody diarrhea and fever, physicians usually start antimicrobial therapy. The therapy could either be parenteral or oral, depending on the age of the patient. In the absence of susceptibility data, the antibiotics of choice for adults are fluoroquinolones. Oral quinolone achieve high concentrations in serum and stool and have activity against *Shigella*. Ciprofloxacin and parenteral ceftriaxone have been shown to be highly effective for shigellosis, yet, ciprofloxacin is avoided in children (Jain, Gupta et al. 2005). Moreover, first and second generation cephalosporins, and oral aminoglycosides were found to be clinically ineffective, despite their in vitro activity (Jain, Gupta et al. 2005). On the other hand, nalidixic acid may also be effective for shigellosis, in addition to azithromycin which is widely used in children and is recommended as an alternative therapy for the treatment of shigellosis in adults with multidrug-resistant isolates (Cohen, Bingen et al. 2004).

Thus, antibiotic susceptibility testing is crucial for the clinical management of patients (Cohen, Bingen et al. 2004). *Shigella* species antibiotic resistance has been reported to be on the rise all over the world.

1.1 *Enterobacteriaceae*

Enterobacteriaceae is a large family of Gram-negative short rods, non-spore forming, facultative anaerobes bacteria that includes large number of genera which are biochemically and genetically related to each other, such as *Salmonella, Escherichia coli, Yersinia pestis, Klebsiella, Shigella, Proteus, Enterobacter,*

Serratia, and Citrobacter. By definition, all *Enterobacteriaceae* members are dextrose fermenters, oxidase negative, and reduce nitrates to nitrites. Most of these species have multiple flagella, while only few of them are non-motile (Traub, Raymond et al. 1970).

Many of these bacteria are normally present in the intestinal tract as part of the normal flora in humans and other animals, while others are found in water or soil. However, some strains have the ability to colonize, produce endotoxins, and invade tissues, in addition to that some members can possess plasmids that may mediate resistance to antibiotics.

Enterobacteriaceae species can be grown on various types of media including selective and differential media that were originally developed for the selective isolation of enteric pathogens. Selective media are prepared by incorporation of dye and bile salts that inhibit gram positive organisms and may suppress the growth of nonpathogenic species of *Enterobacteriaceae.* In addition, there are other types of media that are differential on the basis of whether the organism is lactose fermenter or nonfermenter or if it produces H_2S or hydrogen gas that can be used to culture *Shigella* species such as XLD and SS agar. (Farmer, Davis et al. 1985).

1.2 Shigella Species

Shigella is a Gram-negative rod shaped bacteria from *Enterobacteriacea*e family, with 0.3 to 1 μm diameter, and 1 to 6 μm in length, appearing singly, in pairs or in chains, nonmotile, nonsporeforming, facultative anaerobic, causes shigellosis disease (Figure 1-1) (Hale and Keusch 1996). *Shigella* was discovered over 100 years ago and named after Kiyoshi Shiga, the Japanese microbiologist who first discovered it in 1897, and then it was adopted as a genus in the 1950s (Yabuuchi 2002). By definition,

Shigella is catalase positive (except *S. dysenteriae* type 1), lactose nonfermenter, oxidase negative, urease negative, ferments glucose and other carbohydrates without producing gas, but does not utilize Simmons citrate, nor produce H_2S (Figure 1-2).

The genus *Shigella* is divided into four species: *Shigella dysenteriae, Shigella flexneri, Shigella boydii,* and *Shigella sonnei*. Also nominated serogroups A, B, C and D, respectively, and each species can be classified into other serotypes as shown in (Ansaruzzaman, Kibriya et al. 1995) (Table 1.1)

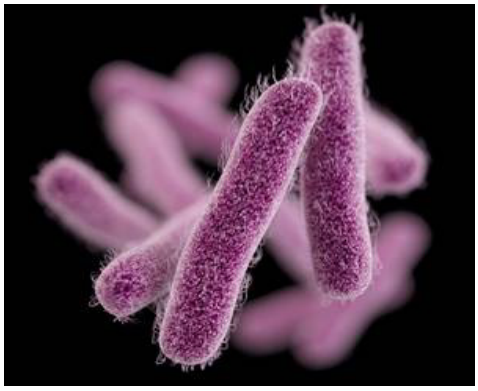


Figure 1-1: Colored scanning electron micrograph of *Shigella* by the Centers for Disease Control and Prevention.

Most cases of shigellosis are caused by *S. sonnei*, while *S. flexneri* comes in second. The other species of *Shigella* are rare, although they are important causes of disease in the developing world, especially deadly epidemics cases that are caused by type 1 *Shigella dysenteriae,* which differs from other *Shigella* species in four important characteristics production of cytotoxin (Shiga toxin); causing more prolonged severe illness that is more frequently fatal than illness caused by other species; resistance to antimicrobials occurs more frequently than in other *Shigella*; and finally it causes large epidemics with high attack and fatality rates (WHO 2005).

Humans and primates are the only reservoirs for *Shigella,* it can be transmitted through direct or indirect fecal-oral contact with patients or carriers, or via fecal contaminated water and foods (DuPont, Hornick et al. 1969).

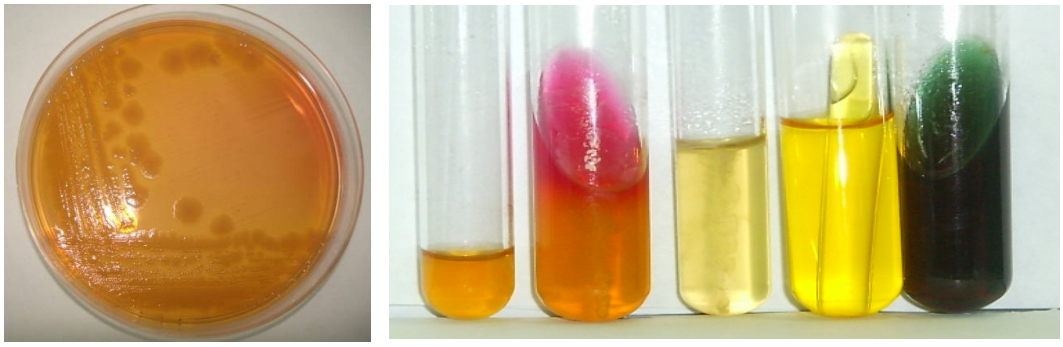


Figure 1-2: A) *Shigella* colonies on MacConkey agar plate. B) Biochemical tests for *Shigella*: Urease negative, K/A without gas or H_2S, nonmotile, mannitol positive, citrate negative

Shigella species are highly contagious; as few as 10 to 100 microorganisms can cause the disease leading to acute bloody diarrhea (Bennish 1991). However, in most cases, symptoms appear as acute non-bloody diarrhea that cannot be distinguished clinically from diarrhea caused by other enteric pathogens.

Table (1-1): Species and serogroups of *Shigella*

Species	Serogroup	Serotypes
Shigella dysenteriae	A	1-15
Shigella flexneri	B	1-6 (with 15 subtypes)
Shigella boydii	C	1-18
Shigella sonnei	D	1

Shigella Pathogenesis

Shigella species have an invasion plasmid of 220 kb, it cause infection by invasion and destruction of the colonic epithelium (Echeverria, Sethabutr et al. 1991). It uses a Type-III secretion system, which has a needle-like shape that allows the insertion of the effectors proteins in the gastrointestinal cells (Schroeder and Hilbi 2008).

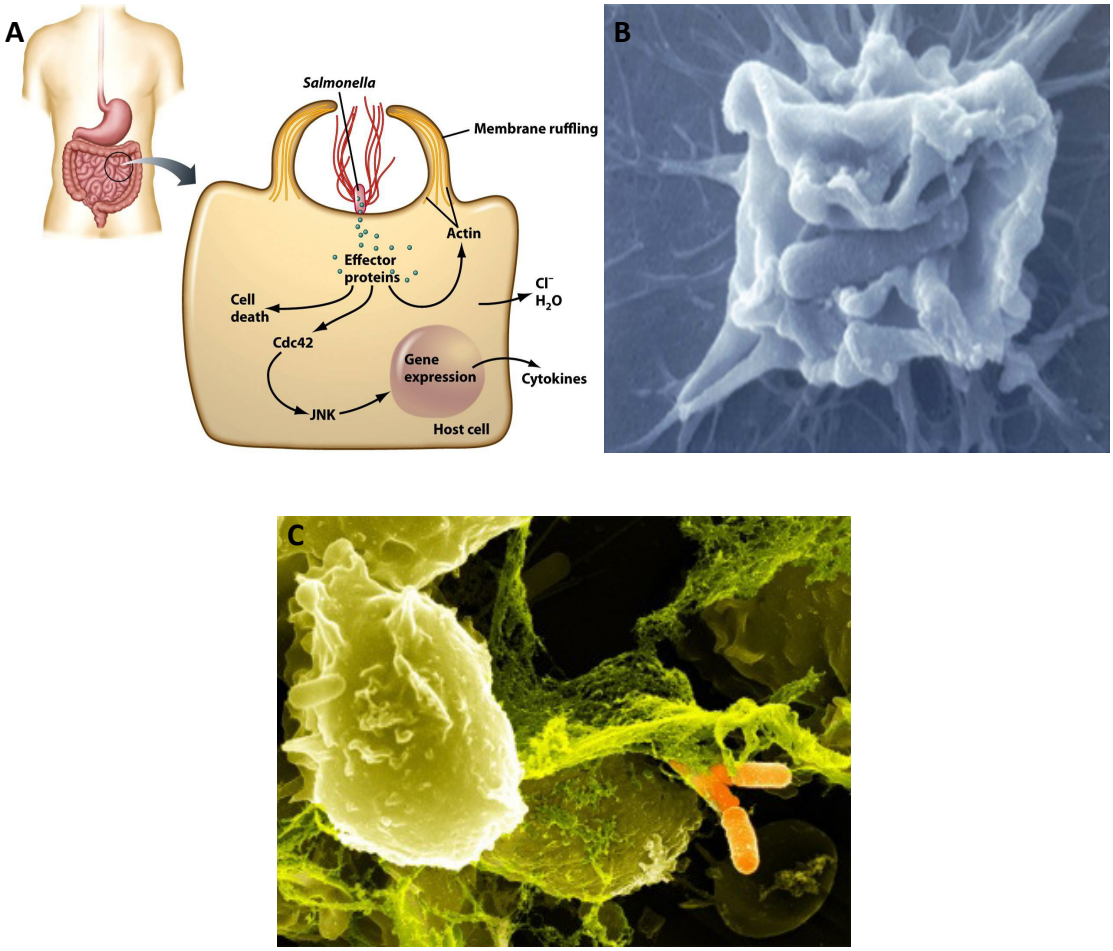


Figure 1-3: A) The insertion process of the effectors proteins in the gastrointestinal cells by Type-III secretion system. B) Scanning electron micrograph (taken by Roger Wepf, Philippe Sansonetti and Ariel Blocker at the EMBL) of *S. flexneri* entering a HeLa cell by interacting with the host cell surface, and injecting its invasions, forming a local actin-rich membrane ruffle at the host cell surface, the ruffle engulfs the bacterium and eventually disassembles, internalizing the bacterium. C) Colored scanning electron micrograph for *Shigella* during the invasion process.

Entrance occurs particularly from the M cells in the follicle-associated epithelium (FAE) that overlies the mucosa-associated lymph nodes by trigger mechanism, which involves the contact between bacteria and cells resulting in cell surface response and bacterial uptake via membrane ruffles (figure 1-3) (Phalipon and Sansonetti 2007; Carayol and Van Nhieu 2013). Invasion results in patchy destruction of the colonic epithelium, and leads to the formation of microulcers and inflammatory exudates,

with continuous appearance of inflammatory cells (polymorphonuclear leucocytes) and blood in the stool (Figure 1-4).

1.3 Shigellosis

Around the world, shigellosis is a considered as major public health problem, especially in the developing countries where sanitation is poor, and is the most important cause of bloody diarrhea. At least, 80 million cases of bloody diarrhea and 700,000 deaths are estimated to be caused by shigellosis each year. 99% of these infections occur in developing countries, about 70% of the cases, and 60% of deaths occurs among children less than five years of age (WHO 2005). Yet, treated cases at hospitals are less than one percent (Bardhan, Faruque et al. 2010)!

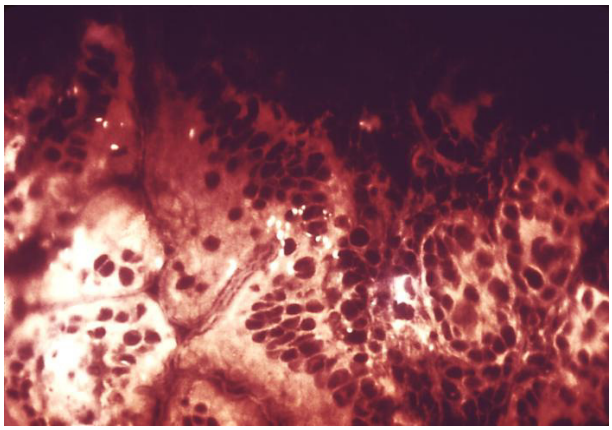


Figure 1-4: The second stage of shigellosis progression; *Shigella* have penetrated the intestinal mucosa causing patchy destruction in the colonic epithelium leads to the formation of microulcers and inflammatory exudates, with continuous appearance of inflammatory cell and blood in the stool. (CDC/Sam Formal/WRAIR)

Shigellosis can be transmitted by fecal-oral contamination, humans are the natural reservoir, while other primates may be infected as well. Additionally, shigellosis can be transmitted by ingestion of contaminated food or water (untreated wading pools, interactive water fountain), or by certain modes of sexual contact (Bennish 1991).

Shigella has a very low infectious dose, for *S. dysenteriae*; only 10 cells can cause the disease, while 100-200 cells are needed for *S. sonnei* or *S. flexneri* infection (Lee, Shapiro et al. 1991). This low dose is thought to be due to the ability of virulent

Shigella to still survive in the low pH of gastric juice, although the real reasons for this are still not completely clear (Schroeder and Hilbi 2008).

The incubation period is about 2-4 days; but it may be prolonged to 12 days according to the load of ingested bacteria (Bennish 1991). The disease is transmissible as long as an infected person excretes the organism in the stool, which may last up to four weeks from the onset of illness. Patients typically present with diarrhea characterized by the frequent passage of small liquid stools, and in most cases stool contains visible blood, and mucus, which associate with abdominal cramps and tenesmus (unproductive, painful straining) (Sansonetti 1992). Other symptoms like fever and anorexia may be observed but are not specific, in addition to dehydration which may occur as a consequence of diarrhea.

Infected individuals usually recover within 7 days of infection without medical intervention, but severe cases require antibiotics to reduce the severity and duration of illness and to reduce the spread of the disease. In rare cases, serious complications may occur, including: metabolic abnormalities, sepsis, convulsions, rectal prolapse, toxic megacolon, intestinal perforation and haemolytic-uraemic syndrome (Bennish 1991).

1.4 Virulence Factors of *Shigella*

Shigella species have a wide variety of virulence factors that mediate its invasion of host cells, adherence to the epithelium of the intestine, and introduction of toxins into the body. Additionally, it has special factors that ease its survival in the stomach acid, and evade the immune responses inside the body.

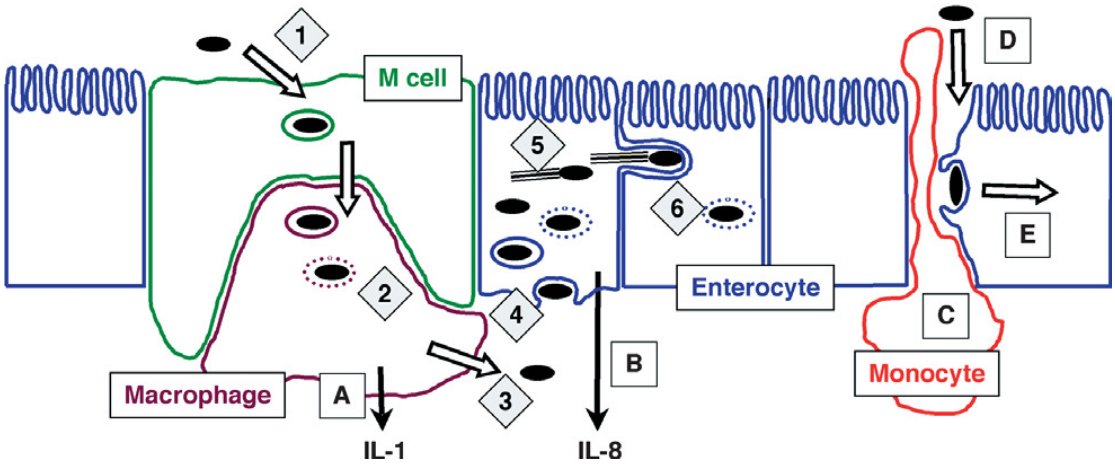


Figure 1-5: Pathogenicity of *Shigella* spp. (1) Bacterial cells pass through the epithelium barrier by entering into M cells and delivered to resident macrophages, in which they induce apoptosis (2), when they reach the basolateral pole of epithelial cells (3), they induce their entry (4), intracellular bacteria moves (5) forming protrusions and dissemination of bacteria within the epithelium (6). Release of cytokines and chemokines, IL-1 by apoptotic macrophages (A) and IL-8 by infected enterocytes (B), promotes recruitment of monocytes that migrate through the epithelial barrier (C), facilitating entry of luminal bacteria into epithelial cells (D) and increasing invasion of the epithelium (E). (From Parsot, C., *Shigella spp*. and enteroinvasive *Escherichia coli* pathogenicity factors(Parsot 2005))

Shigella has a very low infectious dose, this refers to its ability to bear the high acidic conditions inside the stomach, which is found to be dependent on the growth phase (Gorden and Small 1993), stationary phase seems to be necessary for the pathogen in order to survive in the conditions of low pH inside the stomach. When bacterial cells reach the colonic mucosa, it can cross the epithelial cells by invading the M cells that overlay the lymphoid follicles to reach the basolateral pole of epithelial cells where they induce their uptake (Figure 1-5). Bacterial entrance to the epithelial cells involves rearrangements of the cell cytoskeleton in the zone extended between the bacterium and the cell membrane, causing ruffling of the membrane and engulfment of the bacterium within a vacuole (Parsot 2005).

However, the clinical presentation of shigellosis is attributed to the main virulence mechanism of *Shigella*, the type III secretion systems (T3SSs), which are complex structures composed of several subunits made up of approximately 20 bacterial proteins, divided into two groups; the structural proteins, which make up the T3SS

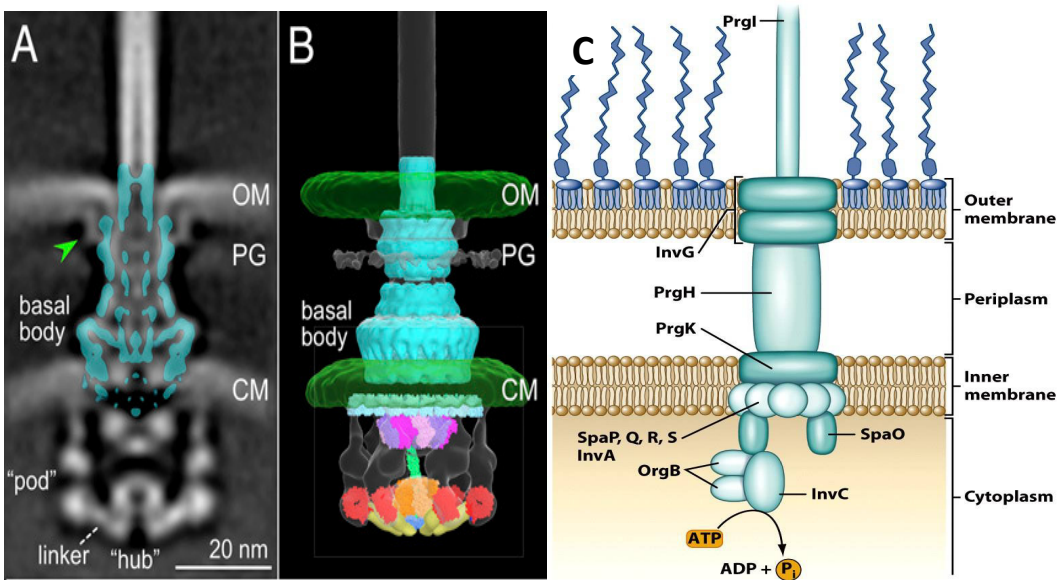


Figure 1-6: The T3SS needle complex

A) and B): A central section and a 3D surface rendering of the intact injectisome shows the outer membrane, cytoplasm, peptidoglycan (PG), basal body, and needle in details (Hu, Morado et al. 2015). C) T33SS mechanism where the effector proteins pass from the *Shigella* cytoplasm through the needle directly into the host cytoplasm. Three membranes separate the two cytoplasms: the double membrane (inner and outer membranes) of *Shigella* and the eukaryotic membrane.

apparatus, and the effectors proteins, which are also called “translocators” as they serve the function of translocating another set of proteins into the host cell cytoplasm (Coburn, Sekirov et al. 2007). The main structural component of T3SS is the needle complex, or as it called "the injectisome", which is composed of three major components: an extracellular needle, a basal body, and a cytoplasmic complex (Figure

1-6). Upon contact with a host cell membrane, the injectisome is activated and starts secreting the effectors into the target cell membrane.

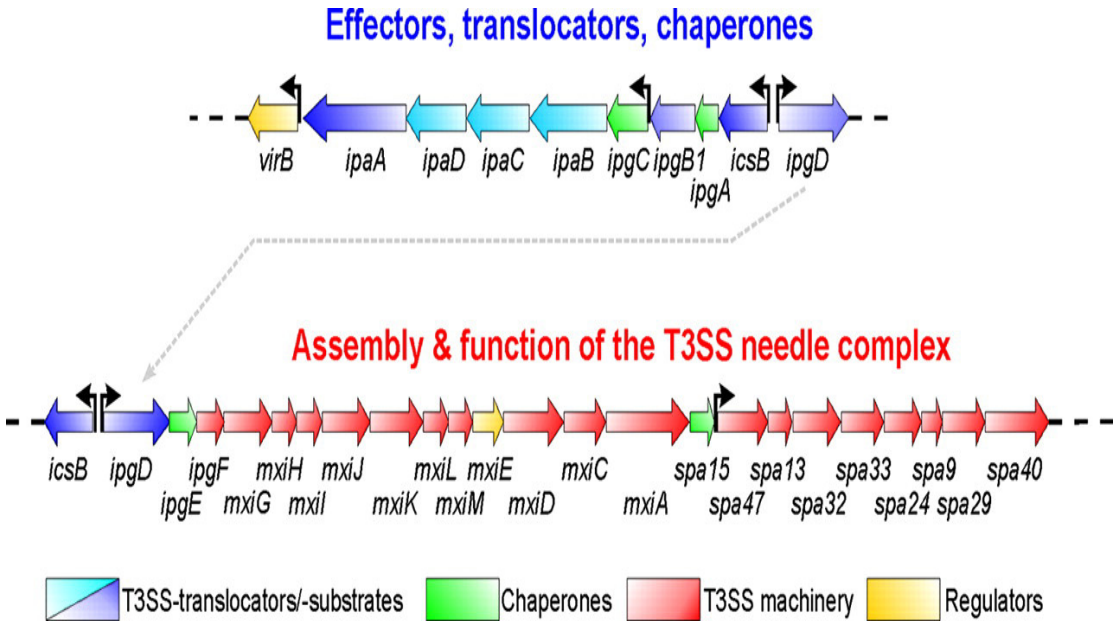


Figure 1-7: Map of the 31-kb "entry region" on *S. flexneri* virulence plasmid pWR100. The genes indicated encode structural components of the Mxi-Spa T3SS, secreted translocator and effector proteins, chaperones, and regulatory proteins. (Schroeder and Hilbi 2008)

At the molecular level, this virulence mechanism is a result of a group of complex action of a large number of bacterial virulence factors, which are encoded by a 220 kb virulence invasion plasmid, with a mosaic of around 100 genes. This plasmid is divided into two main regions; a conserved region of 31 kb at the core of the plasmid, and the PAI-like region, which consists of 34 genes organized into two clusters of opposite directions (Figure 1-7). PAI genes in turn are divided into four groups based on their functions. The first group includes the dominant immunogenic antigens; which are the invasion plasmid antigens *IpaA* to *IpaD*, of which three (*IpaB* to *IpaD*) are the key virulence factors of *Shigella*. The invasion plasmid antigen B (*IpaB*) initiates binding to the host cell, and initiates pathways that kill macrophages upon

infection. While *IpaC*, activates proteins to form the actin-polymerizing complex that allows *Shigella* to move and spread within host cells (Schroeder and Hilbi 2008). The second group of plasmid genes are on the mxi-spa locus, which encodes the components needed for the assembly and function of a T3SS, and collaborates with *IpaB, IpaC*, and *IpaD*, to allow the direct translocation of effector proteins from the bacterial cytoplasm into the host cell. The *mxi-spa* region forms a 45 to 60 nm needle-shape that extends out from the bacterium, nested in a complex composed of seven rings primarily made of *MxiD* genes, which extends to the outer membrane of the pathogen and continues to allow for binding to host cells. When IpaB and IpaC had been translocated to the tip of the needle, IpaB binds to the host cell surface, and thus enduces changes in the cell membrane of the host cell, and works with IpaC to insert the needle into the host cell (Figure 1-8).

In addition to the T3SS genes, there are other genes that contribute to the virulence mechanism of *Shigella*. Invasion associated locus (*ial*) gene, which is found on a 120-140 MDa invasion plasmid, is responsible for invasion-related processes (Nataro, Seriwatana et al. 1995) , while the invasion plasmid antigen H (*ipaH*) gene is present as multiple copies, five on the large plasmid and seven on the chromosome, beside the *virA* gene that was identified in the virulence plasmid of *S. flexneri* 2a, and found to be implicated in invasion and intercellular spreading, thus facilitating the formation of entry structures (Casabonne, González et al. 2016), which indicates that several effectors act to promote the entry of bacteria inside the host cells.

Furthermore, *Shigella* species have a group of enterotoxin genes that account for the early diarrhea. *Shigella* enterotoxin 1 (ShET- 1), which is iron-dependent toxin of 55 kDa, encoded chromosomally by *set1A* and *set1B*, and mainly expressed by *S.*

flexneri 2a, and *Shigella* enterotoxin 2 (ShET-2) encoded by *sen* gene located on invasion plasmid. Shiga toxins 1 and 2 were shown to induce fluid secretion in the intestine and thus thought to produce the watery part of diarrhea. Additionally, ShET-2, the enterotoxin hemolysin, are thought to be involved in eliciting the inflammatory response during *Shigella* invasion (Cruz, Souza et al. 2014). However, the exact mechanism that results in diarrhea is still unknown.

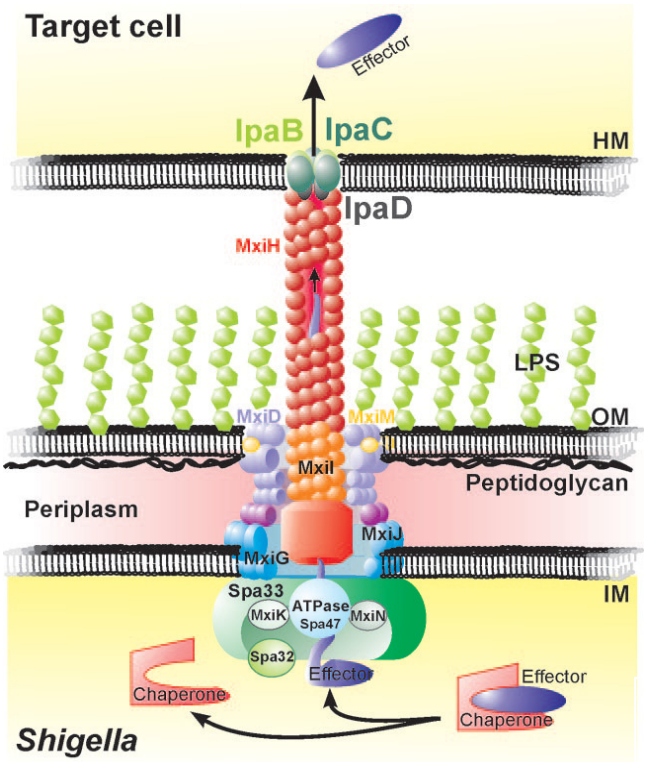


Figure 1-8: Architecture of mxi-spa T3SS (Schroeder and Hilbi 2008)

Finally, Shiga toxins; stxA and stxB , also known as verotoxin, verocytotoxin or Shiga-like toxins, are toxins produced by *Shigella dysenteriae* (serotype 1 only) and EHEC, the two types of toxins are nearly identical but differing by a single amino acid in the catalytic A subunit of the toxin (Johannes and Römer 2010). The active component of the toxin is one A subunit of 32 kDa, bonded non-covalently to five identical B subunits each of 7.7 kDa (Figure 1-9). This toxin forms an important factor in *Shigella* pathogenesis, although it's exact function is still unknown, but it is

thought to be responsible for some of the severe complications such as hemorrhagic colitis and the hemolytic uremic syndrome (HUS) (Obrig 2010).

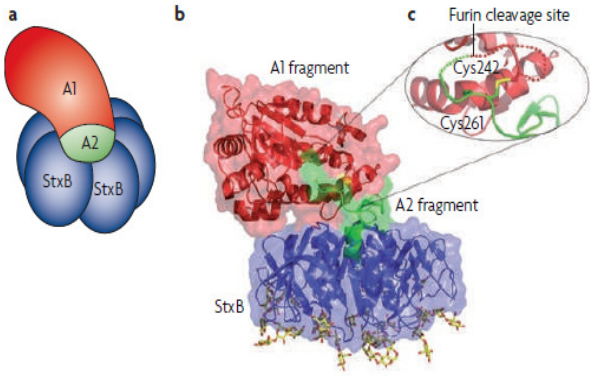


Figure 1-9: Shiga toxin structures (From: Johannes L, et al., 2010. Shiga toxins--from cell biology to biomedical applications)

1.5 Treatment of Shigellosis

Infection with *Shigella* usually resolves without medical intervention within 5 to 7 days, hydration and correction of fluid are usually the mainstay of the treatment, but severe cases require antibiotic treatment to reduce the severity and duration of illness, and to reduce the spread of the disease (Jain, Gupta et al. 2005; RH PC, John et al. 2010). Empiric antimicrobial therapy is recommended for children and adolescents who present with suspected shigellosis and are immunocompromised or have clinical features that suggest bacteremia. Additionally, antimicrobial therapy is also recommended for children and adolescents with culture proven *Shigella* who have bacteremia, require hospitalization, attending day care units, live in institutions, or are involved in food handling. While in adults, it is suggested to give empiric antibiotics for ill patients, elderly patients, or patients with HIV, beside food handlers and health care workers.

The therapy could be either parenteral or oral, depending on the age of the patient (Erdman, Buckner et al. 2008). In the absence of susceptibility data, the antibiotic of

choice for adults is fluoroquinolone, oral quinolones achieve high concentrations in serum and stool and have activity against *Shigella*. In addition to ciprofloxacin, parenteral ceftriaxone, and nalidixic acid. Oral aminoglycosides and first and second generation cephalosporins have been found to be clinically ineffective although they show high in vitro activity. Additionally, azithromycin is widely used in children and is recommended as an alternative therapy for the treatment of shigellosis, it is also effective in the treatment for adults with multidrug-resistant isolates (Cohen, Bingen et al. 2004).

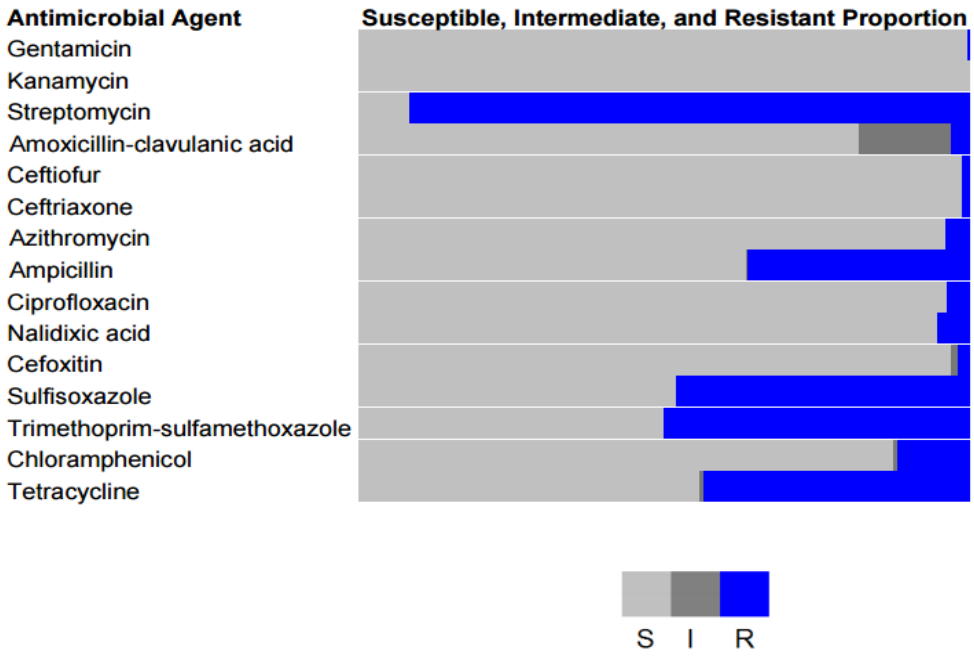


Figure 1-10: Antimicrobial resistance patterns for *Shigella* in United States, 2013 (CDC 2015)

However, *Shigella* species that have antibiotic resistant have been reported to be on the rise. In 2013; the CDC declared antibiotic-resistant *Shigella* as an urgent threat in the United States (CDC 2013). Recently, *Shigella* isolates have been reported to be resistant to common orally given antibiotics such as ampicillin, trimethoprim-sulfamethoxazole, tetracycline and even the macrolide, azithromycin (Khan, Seas et

al. 1997; Heiman, Karlsson et al. 2014). Not only that, but some *Shigella* species have also acquired resistance mechanisms to third generation cephalosporins (extended spectrum β-lactamases) (Cheasty, Skinner et al. 1998), as it was shown in the human isolate final report from National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARM) in 2013 (Figure 1-10).

1.5.1 Azithromycin resistance

Macrolides are a class of antibiotics that mainly affect gram-positive cocci, beside some intracellular pathogens. They are natural lactones with a large ring, consisting of 14 to 20 atoms (Figure 1-11), acting by inhibiting protein synthesis of bacteria by binding to the 50S ribosomal element, leading to inhibition of transpeptidation, translocation, chain elongation, and as a result, inhibit the protein synthesis process (Figure 1-12) (Gaynor and Mankin 2003). Their action is mainly bacteriostatic but at high concentrations it may be bactericidal, or depending on the type of microorganism.

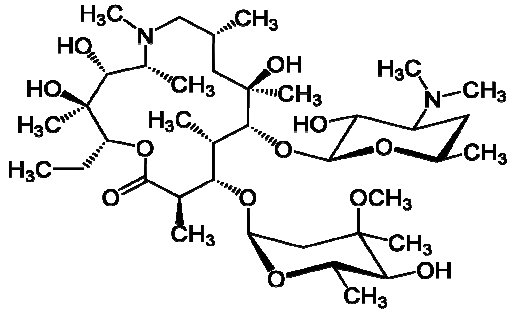


Figure 1-11: Azithromycin chemical structure

Azithromycin is a new subclass of macrolide, called azalidesone (Soares, Figueiredo et al. 2012), it is of the wide spectrum semi-synthetic macrolide antibiotics which inhibits some gram-positive and gram-negative bacteria, it is commonly used in

treating many different types of infections, such as respiratory infections, skin infections, ear infections, and sexually transmitted diseases.

Azithromycin resistance mechanisms

Bacterial species can resist the antimicrobial effect of azithromycin by one of three ways; 1) by modifying the target site through methlation or mutation that prevents the binding of the antibiotic to its ribosomal target, 2) through efflux pump, or finally by 3) drug inactivation (Nguyen, Woerther et al. 2009) (Figure 1-13). However, the impact of these three mechanisms is still not equal in pathogenic organisms, target site modification confers the broad-spectrum of resistance, while efflux and inactivation mechanisms are more limited.

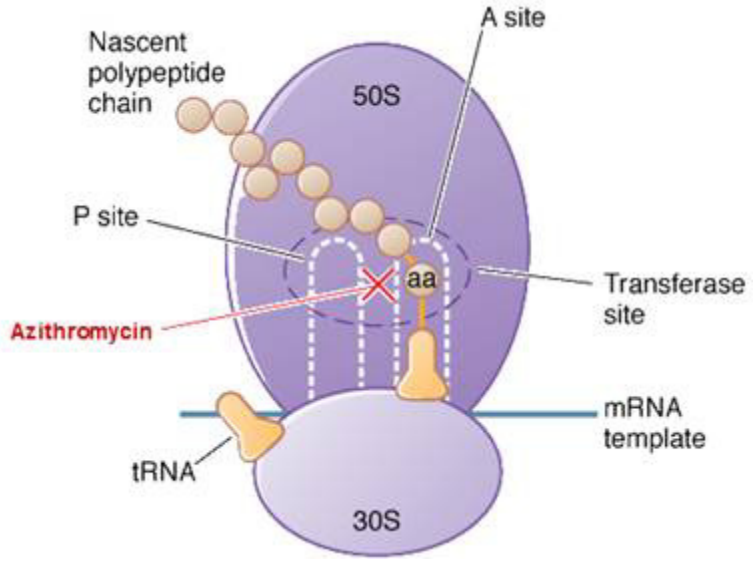


Figure 1-12: Azithromycin mechanism of action: Azithromycin binds to 50S ribosomal subunit of susceptible microorganisms and blocks dissociation of peptidyl t-RNA from ribosomes, causing RNA-dependent protein synthesis to arrest; does not affect nucleic acid synthesis (Brunton LL 2011)

Methylation process of the ribosomal target of the antibiotics is mediated by a variety of *erm* (erythromycin ribosome methylase) genes, which encode *erm* proteins that

cause dimethylation of a single adenine in nascent 23S rRNA, the part of the large (50S) ribosomal subunit. There are about 40 reported *erm* genes, but the major classes that are detected in pathogenic microorganisms includes *erm(A), erm(B), erm(C),* and *erm(F)*. However, *erm(A)* and *erm(C)* are usually found in staphylococcal gene classes, whereas *erm(B)* genes typically appear in *Streptococci* and *Enterococci* (Leclercq 2002). On the other hand, the only efflux proteins that cause resistance to macrolides were characterized in *Staphylococccus* species are ABC transporters encoded by plasmidborne *msr(A)* genes, while *mef(A*) and *mef(E)* genes were found in *Streptococci*.

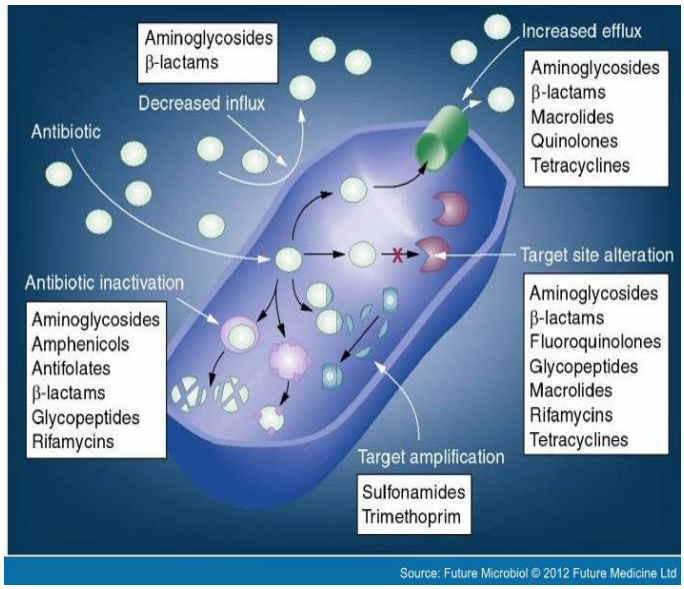


Figure 1-13: Mechanisms of antibiotic resistance in bacteria: Bacteria species use three mechanisms of resistance to macrolides a) limiting the access of macrolides to the cells (efflux pumps); b) altering the ribosome to prevent effective binding of macrolides; c) Inactivating the antibiotic by producing inactivating enzymes. From (Future Microbiol © 2012 Future Medicine Ltd).

Otherwise, *Enterobacteriaceae* species utilize enzymatic drug modification mechanism, which induced by macrolide phosphotransferase gene cluster resulting in high resistance to 14 membered ring macrolides as erythromycin (EM) and oleandomycin (OL) (Noguchi, Takada et al. 2000). This gene cluster consists of *mphA-mrx-mphR* genes; *mphA* gene; which is a phosphotransferase that phosphorylates and thus inactivates erythromycin. Mrx, which is a protein of unknown function but needed for *mphA* expression. And the *MphR* gene that regulates

the expression of *mphA* negatively at the transcriptional level. Yet, this resistance is not considered of a major clinical importance for most *Enterobacteriacea*e, because *Enterobacteriaceae* are normally not targets for macrolides, as they are intrinsically resistant due to drug efflux transporters (Leclercq 2002).

In the past few years, there has been a growing interest in azithromycin as the drug of choice for treatment of most shigellosis cases, as it was found to have in vitro activity against most *Shigella* spp. isolates, beside that it can reach high intracellular concentrations (Boumghar-Bourtchai, Mariani-Kurkdjian et al. 2008). It is recommended by the American Academy of Pediatrics for treatment of shigellosis in children (CDC 2006) , and by the World Health Organization as a second-line treatment in adults (WHO 2005).

However, still there are *Shigella* isolates that are not susceptible to azithromycin (Heiman, Karlsson et al. 2014) (Hassing, Melles et al. 2014), but they are usually not well identified because until 2015, there were no clinical laboratory guidelines for azithromycin susceptibility testing in *Shigella*. Outbreaks caused by azithromycin resistant *Shigella* were found to be related to the emergence of a plasmid-mediated resistance induced by the *mphA* gene (Boumghar-Bourtchai, Mariani-Kurkdjian et al. 2008).

1.5.2 Extended Spectrum B-lactamases

β-Lactams are a group of antibiotics including the penicillins, cephalosporins, carbapenems and monobactams, which act on the cell wall of a bacterial cell by binding to the carboxypeptidases and transpeptidases, the cell wall synthesizing enzymes, and so inhibiting them, resulting in weakening of the cell wall structure, and so cell lysis (Figure 1-13) (Holten 2000). Extended-spectrum beta-lactamases (ESBL)

are group of plasmid-mediated enzymes which cause resistance to most beta-lactam antibiotics (figure 1-14), as they have the ability to hydrolyze third-generation cephalosporins, penicillins, and aztreonam, but not cephamycins or carbapenems, and are inhibited by clavulanic acid (Philippon, Labia et al. 1989).

Enterobacteriaceae producing ESBLs such as SHV and TEM types were known since the 1980s, specially in *Klebsiella spp*., which forms a major cause of hospital-acquired infections. But later in 1990s, other pathogens that commonly cause urinary tract infections and diarrhea have also been found to be ESBL producers, like *Escherichia coli, Salmonella, Shigella* and *Vibrio cholera* (Rawat and Nair 2010).

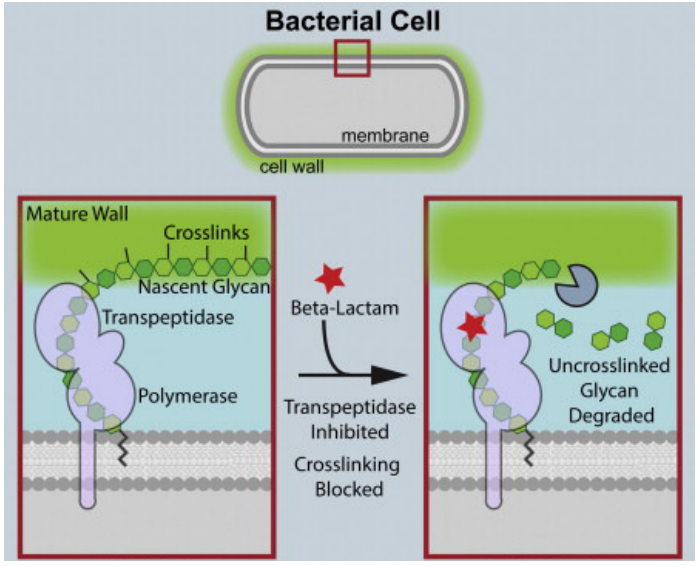


Figure 1-14: β-Lactams mechanism of action: β-Lactam antibiotics inhibit synthesis of the peptidoglycan layer of the bacterial cell wall by blocking the action of transpeptidases (penicillin binding proteins) (Cho, Uehara et al. 2014)

Patients who have the highest risk for developing infection with ESBL producing organisms include seriously ill patients with prolonged hospital stays, especially patients who have invasive medical devices such as urinary catheters, endotracheal tubes, or central venous lines for a prolonged duration.

The first known ESBL enzymes, CTX-M-type β-lactamases, was reported in the late 1980s (Rawat and Nair 2010). In the last years, ESBL-producing bacteria became a major global problem, bacterial isolates usually found to harbor plasmid-mediated enzymes of the TEM, SHV, OXA, PER, and CTX-M types (Samaha-Kfoury, Araj et al. 2003).

In *Shigella*, the first reported case was SHV-11–type appeared in *S. dysenteriae* isolate reported in India in 1999 (Ranjbar, Ghazi et al. 2013). And since that time, CTX-M, SHV, and TEM-type ESBL producing *Shigella* species have been reported worldwide from different regions.

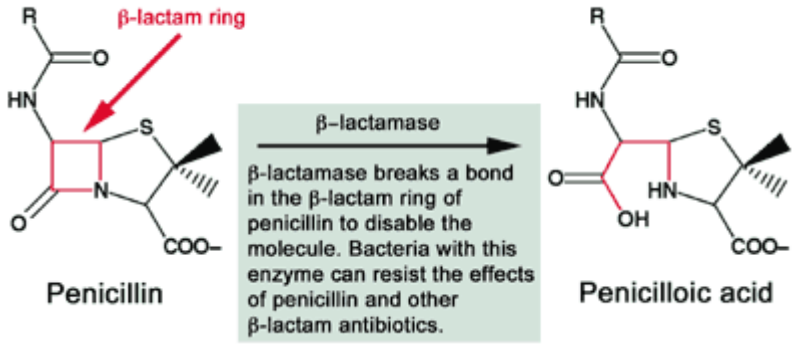


Figure 1-15: β-lactamases mechanism of action to inhibit β-lactam antibiotics (http://www.wiley.com)

1.6 Pulsed Field Gel Electrophoresis

Genotyping techniques for microbial pathogens are particularly important for diagnosis, treatment, and epidemiological surveillance of bacterial infections and bacterial population dynamics (Wolska and Szweda 2012).

Pulsed-field gel electrophoresis (PFGE), the "Gold Standard" method of genotyping, is a highly discriminative molecular typing technique that is used in most epidemiological studies worldwide (Barrett, Gerner-Smidt et al. 2006). It was first

invented in 1984 by Schwartz and Cantor to solve the problem of the large DNA fragments in the conventional gel electrophoresis (Schwartz and Cantor 1984). In this method, bacterial isolates can be classified based on special restriction sites within the bacterial genome, and the variable migration of these restricted DNA fragments by alternating electrical field between spatially distinct pairs of electrodes. The smaller fragments will move faster through the agarose than the larger fragments, resulting in a pattern of DNA bands which forms a fingerprint of each bacterial isolate which can be then used to compare the isolates with each other to investigate if they are belonging to the same strain or they are genetically unrelated.

The process involves embedding a standardized cell suspension of the bacterial isolates in agarose to enhance DNA stability, the resulted plugs are then lysed and digested with rare cutting restriction enzymes that recognize specific sequences in the bacterial genome resulting in 10-20 large restricted DNA fragments (Tenover, Arbeit et al. 1995). The regular changing of the electrical field direction during electrophoresis will facilitate the migration of the large DNA fragments through the agarose gel by allowing the fragments to maneuver through the agarose. Finally, the resulted fingerprints are visualized and documented (Figure 1-15).

Moreover, interpretation for PFGE results became easier after the establishment of standard methods of analysis suggested by Tenover et al. (1995) (Table 1-2), bacterial isolates yielding the same PFGE pattern with the same numbers of bands and the corresponding bands are the same apparent size are designated as indistinguishable. Closely related isolates are the isolates that differ by a single genetic event, usually caused by point mutation or an insertion or deletion of DNA, which appears as a difference of one to three bands. Isolates differing by two independent genetic

changes resulting in difference of four to six bands, are possibly related. Bacterial isolates containing six or more band differences, representative of three or more genetic changes, are considered unrelated (Tenover, Arbeit et al. 1995).

Table (1-2): Criteria for interpreting PFGE patterns by (Tenover, Arbeit et al. 1995)

Category	Criteria	Fragment differences	Epidemiologic interpretation
Indistinguishable	The restriction patterns have the same numbers of bands and the corresponding bands are the same apparent size.	0	Isolate is part of the outbreak
Closely related	The restriction pattern differs from the outbreak pattern by a single genetic event, most probably resulted from a point mutation or an insertion or deletion of DNA.	2-3	Isolate is probably part of the outbreak
Possibly related	The restriction pattern differs from the outbreak pattern by changes consistent with two independent genetic events.	4–6	Isolate is possibly part of the outbreak
Unrelated	The restriction pattern differs from the outbreak pattern by changes consistent with three or more independent genetic events.	≥7	Isolate is not part of the outbreak

PFGE is highly preferable genotyping technique because it can be easily applied to different species resulting in consistent patterns within and between laboratories, in addition to the yields of high amount of pattern diversity. On the other hand, it is labor and time intensive method that needs relatively long working time. Moreover, it cannot identify the differences resulting from mutations, one mutation can yield

differences in several fragments and so different fingerprints (Wolska and Szweda 2012).

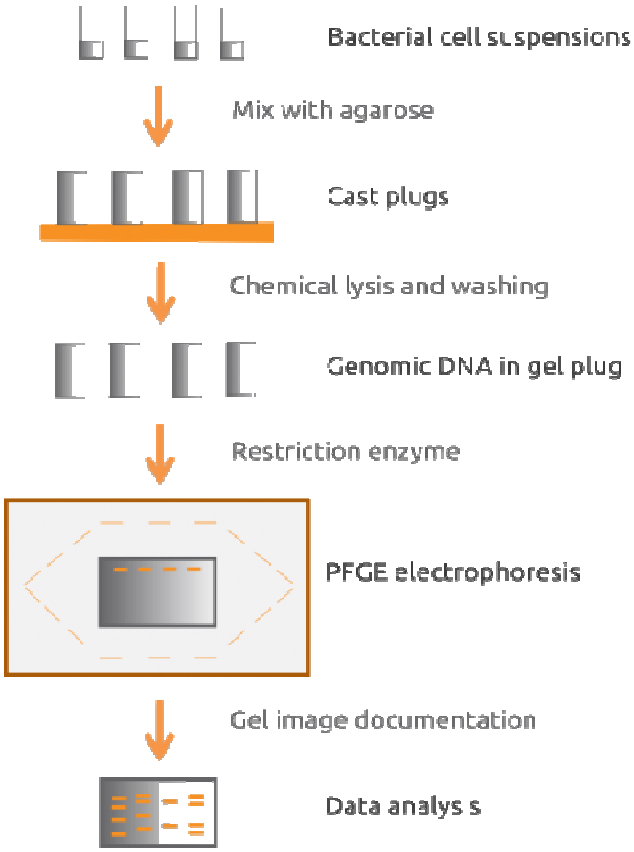


Figure 1-16: PFGE steps: Cell suspension is embedded in agar, then the bacterial cells are lysed to generate large fragments of the bacterial chromosome by macro-restriction analysis. DNA fragments are then separated using an electrical field of alternating polarity, after which the fingerprints are visualized and documented. (Applied Maths © 2016 NV)

Results of PFGE can be analyzed using BioNumerics software which offers a special integrated platform for the analysis of PFGE fingerprint, through the industry leading database engines that allow storing all the epidemiological info and gel images in one database. Then it is easy to define new fingerprint types, choose the optimal settings for normalization, resolution, background subtraction, smoothing, and band finding,

with easy access to re-edit the processing at any stage without losing any editing in another step.

However, the resulted patterns should be compared to a molecular weight standard to establish reference positions that are used for normalization and adjustments in the PFGE gel images by matching the standard in the gels to the electronic reference standard. The images of the standard reference and the marked positions to be used for normalization are present in the PulseNet customization database setup files provided to all PulseNet participating labs. *Salmonella* serotype Braenderup (H9812) restricted with *XbaI* is the "universal" standard strain, because of its even distribution of bands over the entire range of band sizes normally seen in the foodborne pathogens tracked by PulseNet. (Hunter, Vauterin et al. 2005).

CHAPTER 2: METHODOLOGY

2.1 Study population

Clinical strains of different *Shigella* species were isolated from stool samples of 502 patients presenting with diarrhea and seen at Caritas Baby Hospital in the period between 2004 and 2014. Patients age ranged from less than one month up to 14 years, and were mainly from Bethlehem and Hebron districts.

2.2 *Shigella* species identification

Stool specimens were inoculated on 5% sheep blood agar, MacConkey agar, selenite broth, and XLD agar plates (Oxoid Ltd, Hampshire, UK). Suspected *Shigella* colonies were identified according to the Gram stain appearance, oxidase test, and biochemical analysis (Triple Sugar Iron (TSI), Sulfide Indole Motility (SIM), Simmon's Citrate, Mannitol, and Urea), (Oxoid Ltd, Hampshire, UK). Identification of *Shigella* species was performed by serotyping the isolate using specific *Shigella* antisera (Denka Sekien Co, Tokyo, Japan). Samples with unclear biochemical or morphological results were confirmed using API 20E test (BioMérieux, France) according to the manufacturer guidelines.

2.3 Antibiotic sensitivity testing

2.3.1 Disc diffusion test

Sensitivity testing was performed for all isolates using Kirby–Bauer disk diffusion method on Muller-Hinton agar plates (Becton, Dickinson and company, Sparks, MD, USA) according to the Clinical and Laboratory Standard Institute guidelines (CLSI 2016) for the following antibiotics: ampicillin (AMP) (10µg), cefotaxime (CTX) (30

µg), cefixime (5 µg), chloramphenicol (C) (30 µg), gentamycin (CN) (10 µg), trimethoprim-sulfamethoxazole (SXT) (1.25/ 23.75 µg), tetracycline (TE) (30 µg), nalidixic acid (NA) (30 µg), ciprofloxacin (CIP) (5 µg), and azithromycin (AZM) (15 µg). (Oxoid Ltd, Hampshire, UK). Isolates which were resistant or intermediate to cefotaxime were confirmed by phenotypic ESBL confirmation using double disk synergy (DDS) method for the antibiotics (cefotaxime, Augmentin, ceftazidime, ceftazidime/clavulanic acid, cefotaxime /clavulanic acid)

2.3.2 Minimum Inhibitory Concentration (MIC) test

Azithromycin disk diffusion results were confirmed using MIC E-test for all isolates (N=82) with reduced susceptibility (breakpoint ≤18mm). In addition to another 166 susceptible isolates (breakpoint ≥18mm) which were chosen randomly. Moreover, cefotaxime intermediate or resistant isolates were tested for their sensitivity to the carbapenems (imipenem, meropenem, and ertapenem) using the E test method on Muller-Hinton agar plates.

For all the sensitivity testing, bacterial suspension were prepared to a density of 0.5 McFarland units, which is equivalent to 1.5×10^8 CFUs/ml, from fresh 18-24 hours old bacterial colonies in saline solution, and spread on Muller Hinton plates, then incubated for 18-24 hours at 35 ºC.

2.4 Total nucleic acid extraction

2.4.1 Spin column extraction

Total nucleic acid was extracted from fresh well-isolated colonies of 102 *Shigella* samples using the High Pure Nucleic Acid extraction kit (Roche, Switzerland) according to the manufacturer's guidelines. DNA was extracted from 200 µl of 2

MacFarland bacterial suspension (6×10^8 CFUs/ml) prepared in saline solution. Extracted DNA was eluted in 50 µl elution buffer, and stored at -20ºC.

2.4.2 Bacterial cell disruption using boiling method

DNA was released from the cells of 398 isolates using boiling method. 2 MacFarland bacterial suspension was prepared from freshly streaked well-isolated *Shigella* colonies. The bacterial cells were subjected to boiling at 100 ºC for 15 min in a PTC-100 Peltier Thermal Cycler (Bio-Red). The cell lysates were stored at -20°C pending analysis.

2.5 Polymerase Chain Reaction (PCR)

2.5.1 Detection of macrolide resistance genes

82 azithromycin non-susceptible isolates were screened for macrolide resistance by amplifying the *erm* genes [*erm(A), erm(B), erm(C)*], esterases genes [*ere(A), ere(B)*], phosphotransferases [*mph(A), mph(B), mph(D)*], and acquisition of efflux pumps [*mef(A), msr(A)*] by polymerase chain reaction (PCR). PCR reactions were performed in three multiplex reactions (Table 2-1). For this analysis, extracted bacterial DNA was used.

Table (2-1): PCR multiplex reactions for macrolide resistance genes

Multiplex Reaction 1	V/ 1 sample (µl)
Thermo Fisher Scientific Master Mix	12.5
H_2O	6
erm B R	1
erm B F	1
erm C R	1
erm C F	1

DNA	2.5
Total	25
Multiplex Reaction 2	**V/ 1 sample (µl)**
Thermo Fisher Scientific Master Mix	12.5
H_2O	4
mef (A) F	1
mef (A) R	1
erm A R	1
erm A F	1
ere (B) R	1
ere (B) F	1
DNA	2.5
Total	25
Multiplex Reaction 3	**V/ 1 sample (µl)**
Thermo Fisher Scientific Master Mix	12.5
H_2O	2
mph (A) F	1
mph (A) R	1
mph (B) R	1
mph (B) F	1
ere (A) R	1
ere (A) F	1
msr (A) (R)	1
msr (A) (F)	1
DNA	2.5
Total	25

2.5.2 Detection of ESBL producing genes

Bacterial strains showing non-susceptible phenotype to third generation cephalosporins were tested for the presence of ESBL producing genes. Four multiplex PCR reactions were performed as shown in (Table 2-2) for the genes *(TEM, SHV, CTX-M-2 and 5, CTX-M, OXA, OXA-2 and -20, OXA-5 and -10, PER-1 and 2, ACT-1, MIR-1,*

CMY and LAT, FOX-1 to -5, DHA-1 and -2, ACC-1 and -2). All genes were tested on extracted bacterial DNA.

Table (2-2): PCR multiplex reactions for ESBL genes

Multiplex Reaction 1	**V/ 1 sample (µl)**	**Multiplex Reaction 2**	**V/ 1 sample (µl)**
Thermo Fisher Scientific Master Mix	12.5	Thermo Fisher Scientific Master Mix	12.5
H_2O	2	H_2O	2
TEM R	1	*SHV R*	1
TEM F	1	*SHV F*	1
CTX M 2/5 R	1	*CTX M R*	1
CTX M 2/5 F	1	*CTX M F*	1
OXA 1 R	1	*ACT 1 R*	1
OXA 1 F	1	*ACT 1 F*	1
MIR 1 R	1	*CMY & LAT R*	1
MIR 1 F	1	*CMY & LAT F*	1
DNA	2.5	DNA	2.5
Total	25	Total	25
Multiplex Reaction 3	**V/ 1 sample (µl)**	**Multiplex Reaction 4**	**V/ 1 sample (µl)**
Thermo Fisher Scientific Master Mix	12.5	Thermo Fisher Scientific Master Mix	12.5
H_2O	4	H_2O	4
OXA 2/20 R	1	Primers	
OXA 2/20 F	1	*OXA 5/10 R*	1
PER 1/2 R	1	*OXA 5/10 F*	1
PER 1/2 F	1	*FOX 1/5 R*	1
DHA1/2 R	1	*FOX 1/5 F*	1
DHA 1/2 F	1	*ACC R*	1
DNA	2.5	*ACC F*	1
Total	25	DNA	2.5
		Total	25

2.5.3 Screening of virulence genes

The main six virulence genes of *Shigella* (*ipaH, ial, sen, set1A, set1B, stx*) were screened in bacterial lysate using 2 multiplex PCR reactions as shown in (Table 2-3).

Table (2-3): PCR multiplex reactions for virulence genes

Multiplex Reaction One	V/ 1 sample (µl)	Multiplex Reaction Two	V/ 1 sample (µl)
Thermo Fisher Scientific Master Mix	12.5	Thermo Fisher Scientific Master Mix	12.5
H_2O	1.5	H_2O	1.5
ial R	1	*iph (A) F*	1
ial F	1	*iph (A) R*	1
sen R	1	*set 1 A R*	1
sen F	1	*set 1 A F*	1
stx R	1	*set 1 B R*	1
stx F	1	*set 1 B F*	1
DNA	5	DNA	5
Total	25	Total	25

For all PCR assays, the reactions were performed in 25 µl volume reactions containing 2X PCR Master Mix with 1.5 mM $MgCl_2$ (Thermo Fisher Scientific), with primer concentrations of 20 pmol/µl at standard PCR conditions in a PTC-100 Peltier Thermal Cycler (Bio-Red). Assays were validated by negative and positive controls. In addition, during the validation of the PCR assays, the amplified PCR products were sequenced using the BigDye® Terminator v3.1 Cycle sequencing kit (Life technology, USA) on the ABI Prism sequencer 3130x/ Genetic Analyzer (Applied Biosystems). Target genes and their corresponding primers for PCR amplification are noted in Table (2-4).

Table 2-4: Details for the primers, annealing temperature, and amplicon size for the various PCR amplifications performed in this study

Group	Target gene	Sequence	Amplicon size	Annealing Temp
Macrolide Resistance genes	*mph(A)*	F: GTGAGGAGGAGCTTCGCGAG R: TGCCGCAGGACTCGGAGGTC	403	60
	mph(B)	F: GATATTAAACAAGTAATCAGAATAG R: GCTCTTACTGCATCCATACG	494	58
	erm(A)	F: TCTAAAAAGCATGTAAAAGAAA R: CGATACTTTTTGTAGTCCTTC	533	52
	erm(B)	F: GAAAAAGTACTCAACCAAATA R: AATTTAAGTACCGTTACT	639	45
	erm(C)	F: TCAAAACATAATATAGATAAA R: GCTAATATTGTTTAAATCGTCAAT	642	45
	ere(A)	F: GCCGGTGCTCATGAACTTGAG R: CGACTCTATTCGATCAGAGGC	420	60
	ere(B)	F: TTGGAGATACCCAGATTGTAG R: GAGCCATAGCTTCAACGC	537	55
	mef(A)	F: AGTATCATTAATCACTAGTGC R: TTCTTCTGGTACTAAAAGTGG	345	54
	msr(A)	F: GCACTTATTGGGGGTAATGG R: GTCTATAAGTGCTCTATCGTG	384	58

ESBL Producing genes	*Tem*	F:GTGCGCGGAACCCCTATT R:TTACCAATGCTTAATTAATCAGTGAGGC	968	58
	SHV	F:CTTTACTCGCCTTTATCGGC R:TTACCGACCGGCATCTTTCC	982	58
	CTX-M-2 and 5	F: ATGTGCAGYACCAGTAAGG R:TAAGTGACCAGAATMAGCGG	564	58
	CTX-M	F: ATGTGCAGYACCAGTAAAG R: GGTCACCAGAAGGAGC	562	58
	OXA	F: CAGATTCAACTTTCAAGATCG R: GTGTTTAGAATGGTGATCG	612	58
	OXA-2 and -20	F: GCRTCSACATTCAAGATWCC R: TCWTCCATYCTGTTTGGCG	524	58
	OXA-5 and -10	F: AGCATCAACATTYAARATYCC R:ATGATGCCYTCACTTKCC	597	58
	PER-1 and 2	F: GGCCTGACGATCTGGAACC R:TAACTGCATAACCTACTCC	855	58
	ACT-1	F:ACAGGCAAGCAGTGGCAGG R:GGATTCACTTCTCTCGCAGGC	619	58
	MIR-1de	F: TCGGTAAAGCCGATGTTGCG R: CTTCCACTGCGGCTGCC	301	58
	CMY and LAT	F: ATTCCGGGTATGGCCGT R:GGGTTTACCTCAACGGC	835	58

	FOX-*1 to -5*	F:GACGGCATTATCCAGCCG R:GTAACCGGATTGGCCTGGAAGC	856	58
	DHA-*1 and -2*	F:ATCTGCAACACTGATTTCCG R:GCACTCAAAATAGCCTGTGC	1115	58
	ACC-*1 and -2*	F: AACAGCCTCAGCAGCCGG R: GCCGCAATCATCCCTAGC	342	58
Virulence genes	*ipaH*	F: TGGAAAAACTCAGTGCCTCT R: CCAGTCCGTAAATTCATTCT	423	55
	Ial	F: CTGGATGGTATGGTGAGG R: GGAGGCCAACAATTATTTCC	320	55
	Sen	F: ATGTGCCTGCTATTATTTAT R: CATAATAATAAGCGGTCAGC	799	55
	set1A	F: TCACGCTACCATCAAAGA R: TATCCCCCTTTGGTGGTA	309	55
	set1B	F: GTGAACCTGCTGCCGATATC R: ATTAGTGGATAAAAATGACG	147	55
	Stx	F: ACCCTGTAACGAAGTTTGCG R: ATCTCATGCGACTACTTGAC	140	55

2.6 Pulsed Field Gel Electrophoresis (PFGE)

Sub-typing bacterial strains using Pulsed Field Gel Electrophoresis method was performed for 15 *S. flexneri* isolates, which were chosen from the samples that were found to be resistant to azithromycin. The isolates were taken from the years 2006,

2007 and 2014 and are from different geographic areas. PFGE was performed according to the standardized and international validated PulseNet PFGE protocol employed by PulseNet laboratories of the US CDC for *E. coli O157:H7, Salmonella*, and *Shigella*. Genomic DNA of the 15 isolates was digested with *NotI* as a primary restriction enzyme, and *XbaI* as secondary enzyme (New England Biolabs), and separated in 1% SeaKem Gold agarose gel, with contour-clamped homogeneous electric field (CHEF-DRIII system, Bio-Rad, Hercules, USA) using electrophoresis conditions of initial switch time 5 seconds and final switch time 35 seconds, the voltage used was 6 V/cm, and the included angle for the CHEF Mapper system was 120° and the ramp factor was linear, over a total running time of 18.5 hours. *Xba1*-digested *Salmonella* serotype Braenderup H9812 was used as the molecular weight standard.

The Gel was stained for 25 min with GelRed™ Nucleic Acid Stain (Biotium Inc., Hayward, CA). Then the gel was visualized with Bio-Rad Gel Documentation System (Bio-Rad, Molecular Imager Gel Doc™, XR Imaging System). Digital images were stored electronically as TIFF files, and the resulting TIFF images were analyzed using the BioNumerics software version 5.10 software (Applied Maths, Kortrijk, Belgium).

The PFGE band patterns were compared based on the Dice-coefficient, a dendogram with a setting of 1.5% position tolerance for band comparison was constructed by UPGMA (Unweighted Pair-Group Method Arithmetic Averages) algorithm.

Band similarity determined according to the criteria suggested by Tenover et al, 1995 (Table 1-2).

CHAPTER 3: RESULTS

3.1 *Shigella* species identification

502 isolates were identified as *Shigella* species by biochemical tests. *Shigella* species isolates were citrate negative, urease negative, non-motile, mannitol positive, and K/A in TSI test (Figure 3-1).

Figure 3-1 Biochemical test results for *Shigella*; Urease negative, nonmotile, Mannitol positive without gas, K/A, citrate negative.

3.2 Epidemiological analysis for the sample population

Of the 502 isolates, 373 (74%) were *S. Sonnei*, 119 (24%) were *S. flexneri*, and 10 (2%) isolates were *S. boydii* (Figure 3-2).

The yearly distribution of the 502 *Shigella* isolates between 2004 and 2014 shows that the most cases were reported in the period between the end of 2005 to the beginning of 2007 (Figure 3-3), while the geographic distribution shows that most of the isolates were for patients from Bethlehem and Hebron districts (Figure 3-4). Finally, the patients age distribution between less than one year and 14 years old is presented in (Figure 3-5).

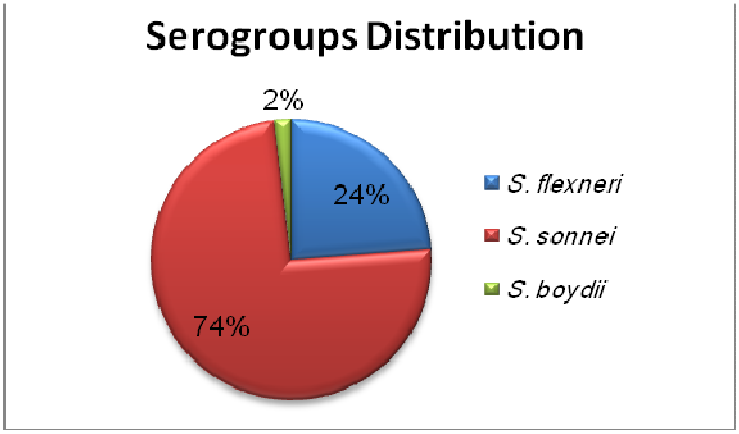


Figure 3-2: Histogram showing serogroup distribution for the tested isolates

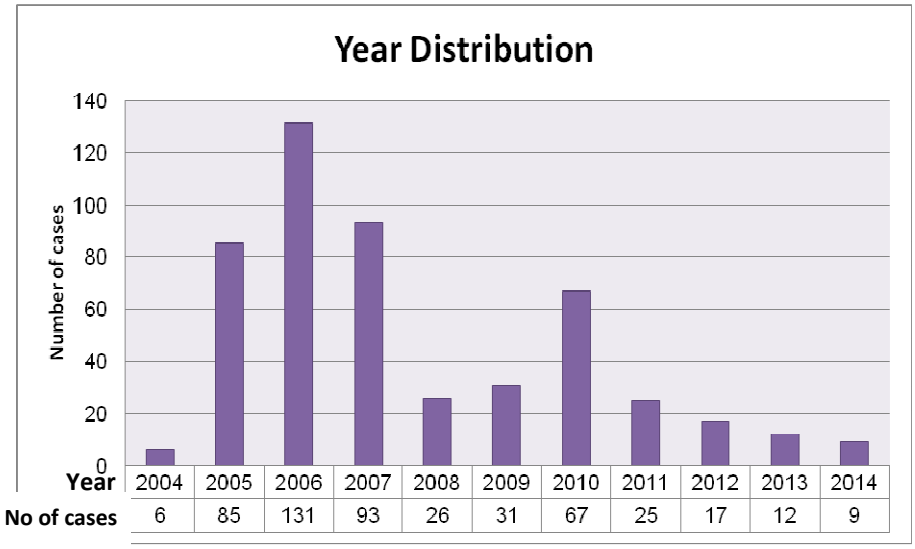


Figure 3-3: Histogram showing the number of samples collected in each year

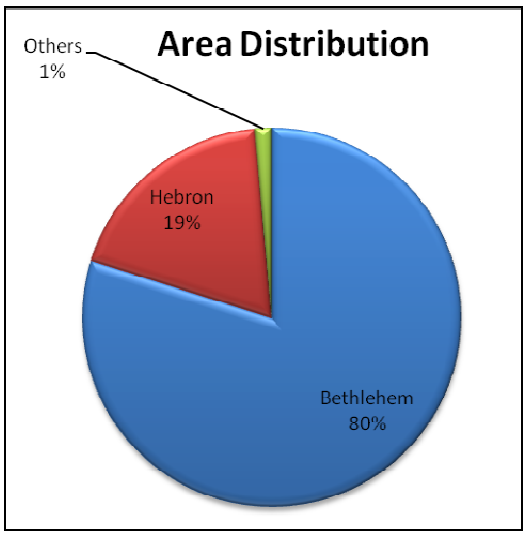


Figure 3-4: Sample distribution among the included districts

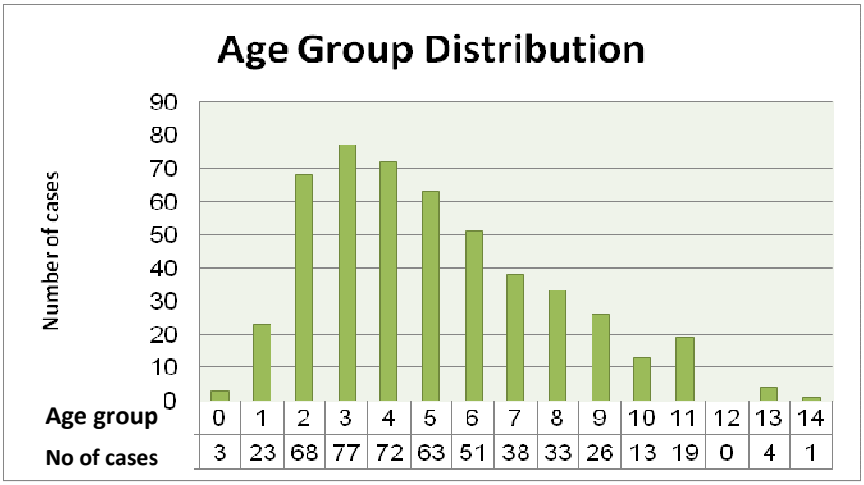


Figure 3-5: Distribution of patients age

3.3 Virulence factors

Screening for six main virulence genes *(ipaH, ial, sen, set1A, set1B, STX*) in the 502 isolates showed high prevalence of the genes among the *Shigella* species tested. The invasion plasmid antigen (*ipaH*) gene was found in 95.4% of the isolates, where the invasion associated locus gene (*ial*) was found in 75.1% of them. For enterotoxin genes, (*set1B)* was positive in 7.8% of isolates, but (*set1A)* and *(sen)* genes were negative in all of the isolates tested. Not only that, but, 8 isolates of *S. sonnei* were positive to Shiga toxin gene *(STX*) (Figure 3.6). The distributions of these genes according to the serotypes are shown in figures (3.7).

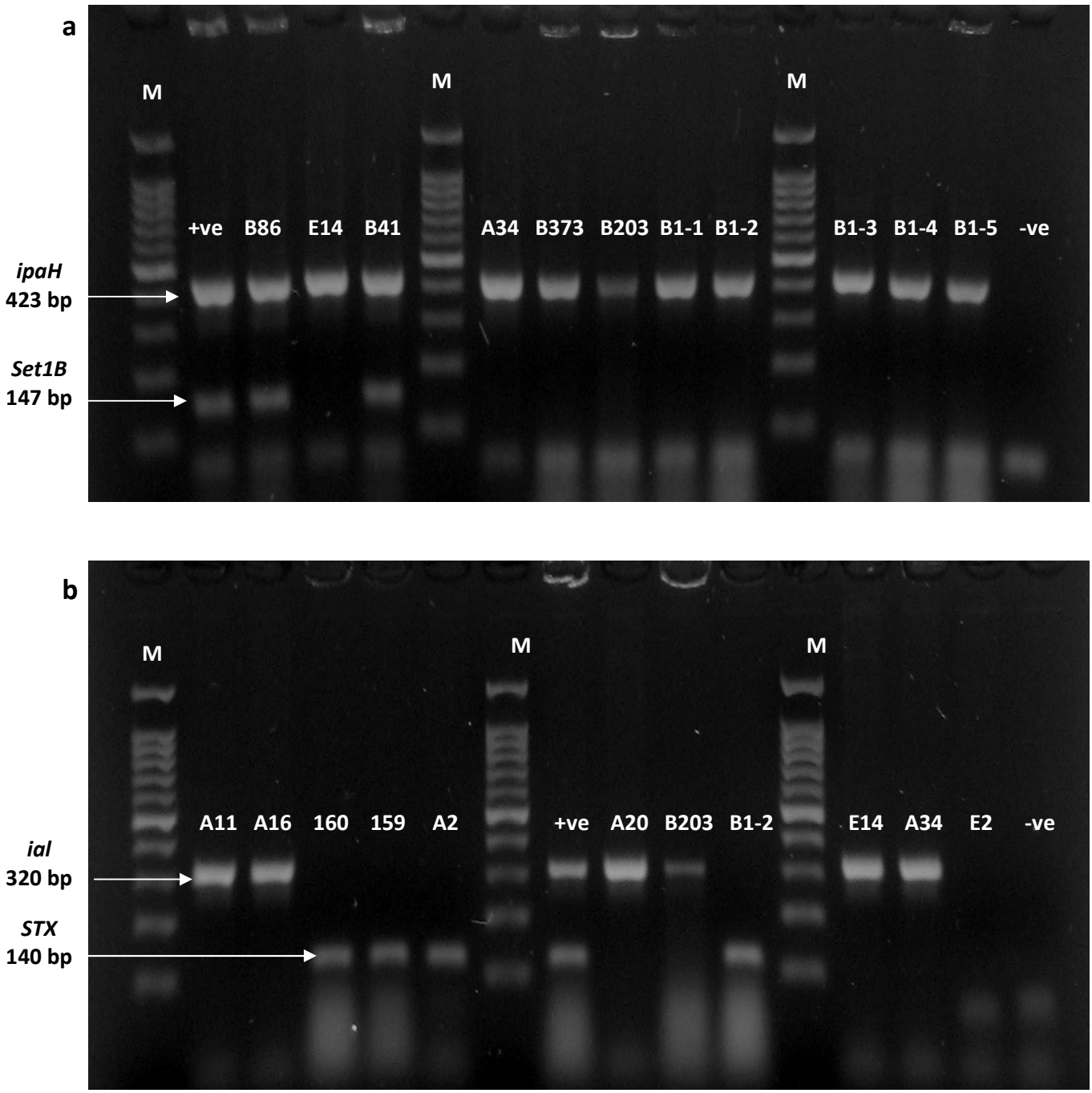


Figure 3-6: 1.5% Gel electrophoresis results for the virulence genes, **A)** showing the results for multiplex PCR 1 for *ipaH* and *ial* genes, where *set1A* was negative in all samples, M = 100 bp DNA ladder. **B)** showing the results for multiplex PCR 2 for *ial* and *stx* genes while *sen* gene was negative in all samples, M = 100 bp DNA ladder

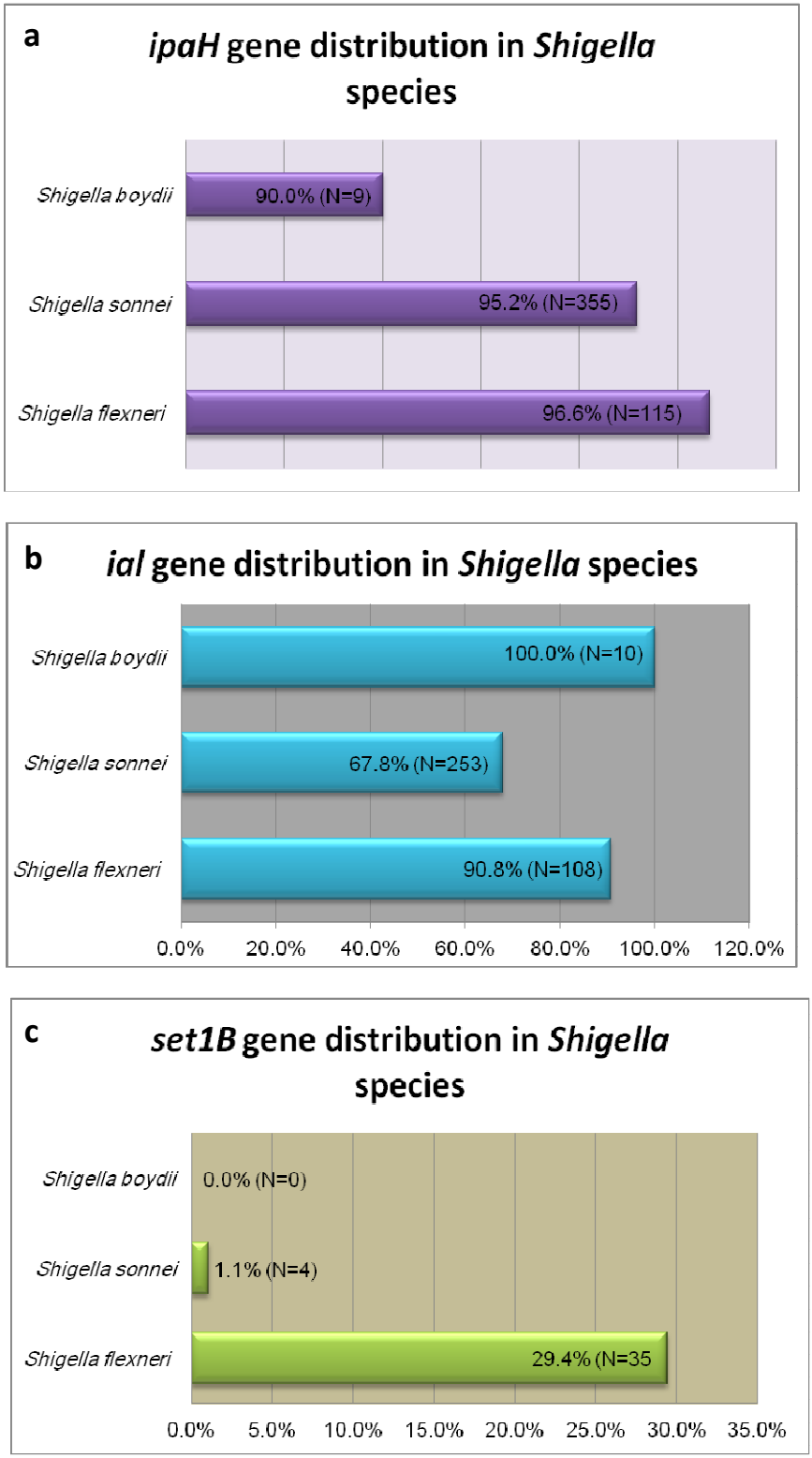


Figure 3-7: Prevelance of virulence genes among *Shigella* serotypes, **A)** *ipaH* gene was present in 96.6% of *S. flexneri*, 95.2% of *S. sonnei*, and 90% of *S. boydii* isolates. **B)** *ial* genes was present in 90.8% in *S. flexneri* isolates, 67.8% of *S. sonnei* and 100% of *S. boydii*. **C)** *set1B* was present in 29.4% of *S. flexneri*, 1.1% of *S. sonnei*, but in non of *S. boydii* isolates.

3.4 *STX* positive samples

8 isolates of *S. sonnei* were positive to *Shiga Toxin (STX)* gene, which is a gene restricted in *Shigella dysenteriae serotype 1* and *enterohaemorrhagic Escherichia coli (EHEC)* strains only. Referring to CBH archive, the clinical presentation for the 8 patients showed that these isolates were highly virulent with severe symptoms (Table 3-1). Four of the eight patients were from the same family (patient's #'s: 64733, 82312, 90569, 75589), which indicates that this strain was highly infectious and spread rapidly, while three other patients were admitted to the hospital for four and three days (patient's #'s: 68292, 65248, 73829), which suggests the high severity of the illness caused by these isolates.

Table (3-1): Clinical presentation for patients infected with *stx* positive *S. Sonnei* isolates

Patient #	Year	Admission	Region	Age /yr	Symptoms	Fever
68292	2004	4 Days	Dhesheh		No information available	
65248	2005	3 Days	Nahhalin	4.7	Convulsion	
73829	2010	4 Days	Dar Salah	9.5	Fever and bloody diarrhea , abdominal pain	39
64733	2010	1 Day	Halhoul	10.2	Vomiting and bloody diarrhea of one night duration, ill presentation	Fever
82312	2010	1 Day	Halhoul	6.1	Vomiting and diarrhea of one night Duration, ill presentation	38.5
68814	2010	3 Days	Aroub	10.5	No information available	
90569	2010	1 Day	Halhoul	4.5	Vomiting and bloody diarrhea	
75589	2010	1 Day	Halhoul	7.4	Vomiting and bloody stool	38.5

3.5 Antibiogram results

3.5.1 Disk Diffusion Test

Disk diffusion test was performed on all isolates for various classes of antibiotics (figure 3-8). Overall, the results showed that 12.4% of the isolates were resistant to azithromycin, 0.6% were resistant to gentamicin, 14.7% for naldixic acid, 1.8% for cefixime, 91.4% resistant to trimethoprim-sulfamethoxazole, 12% for chloramphenicol, 78.3% resistant to tetracycline, 3.2% for cefotaxime, 40.4% ampicillin, but 100% of them were sensitive to ciprofloxacin (Figure 3-9).

Among the different serogroups, there was high variance in antibiotics resistance patterns, *S. sonnei* isolates showed the lowest resistance rates for azithromycin, trimethoprim-sulfamethoxazole, and ampicllin, 87% resistant to tetracycline, and only 24% of the isolates were resistant to ampicillin, while most of the isolates were susceptible to all other antibiotics. On the other hand, all *S. boydii* isolates were resistant to ampicillin, where for *S. flexneri* isolates, most isolates were resistant to amplicillin (86%), trimethoprim-sulfamethoxazole (79%), and tetracycline (50%) (Figure 3-10).

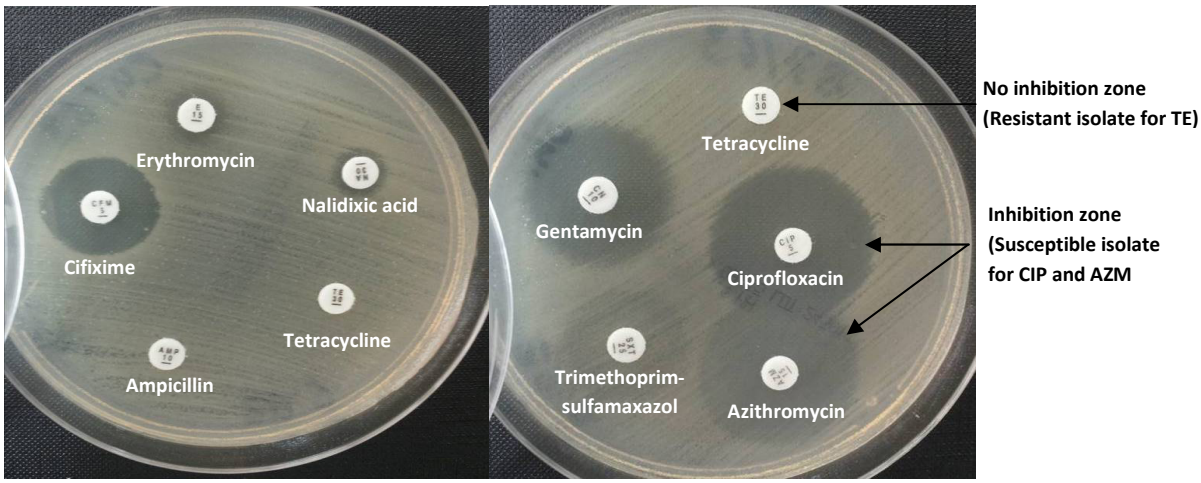


Figure 3-8: Disk diffusion test for different *Shigella* isolates on Muller Hinton agar

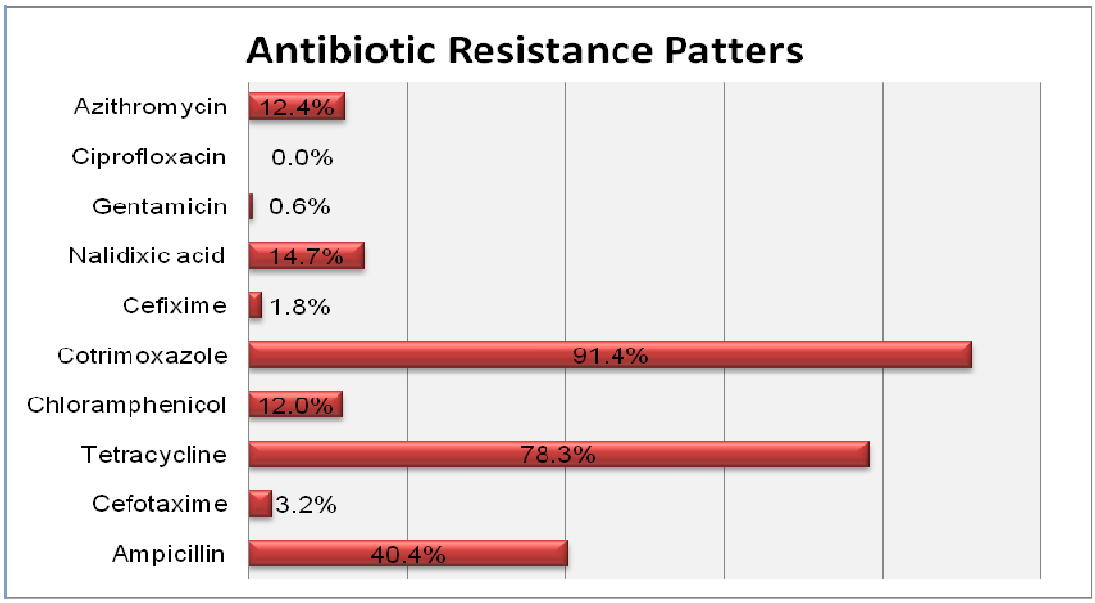
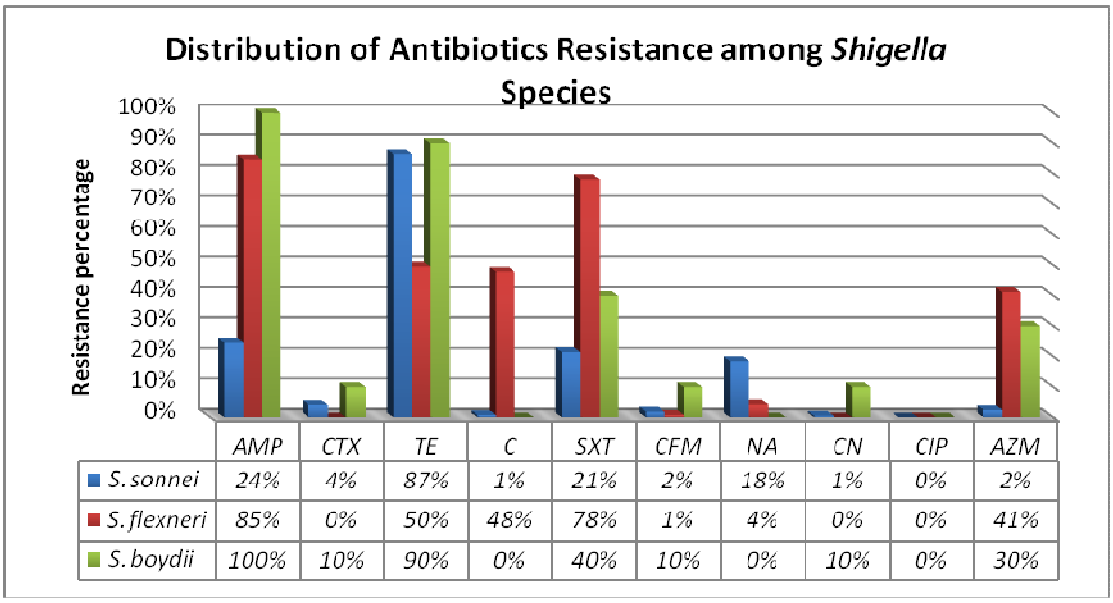


Figure 3-9: The percent resistance of *Shigella* isolates to each antibiotic according to CLSI guidelines



	AMP	CTX	TE	C	SXT	CFM	NA	CN	CIP	AZM
S.sonnei	24%	4%	87%	1%	21%	2%	18%	1%	0%	2%
S.flexneri	85%	0%	50%	48%	78%	1%	4%	0%	0%	41%
S.boydii	100%	10%	90%	0%	40%	10%	0%	10%	0%	30%

Figure 3-10: Distribution of antibiotics resistance among *Shigella* serogroups

3.6 Cefotaxime resistance isolates

3.6.1 ESBL phenotypically confirmation test

For the purpose of confirming ESBL producing isolates, double disk synergy test was performed for the 15 isolates which were intermediate or resistant to cefotaxime. A

clear DDS zone appeared in ten of them which confirms ESBL producing phenotype, while in 5 of them no double disk synergy appeared (Figure 3-11). On the other hand, MIC values for carbapenems E-test showed that all of them were sensitive to imipenem, meropenem, and ertapenem (Figure 3-12)

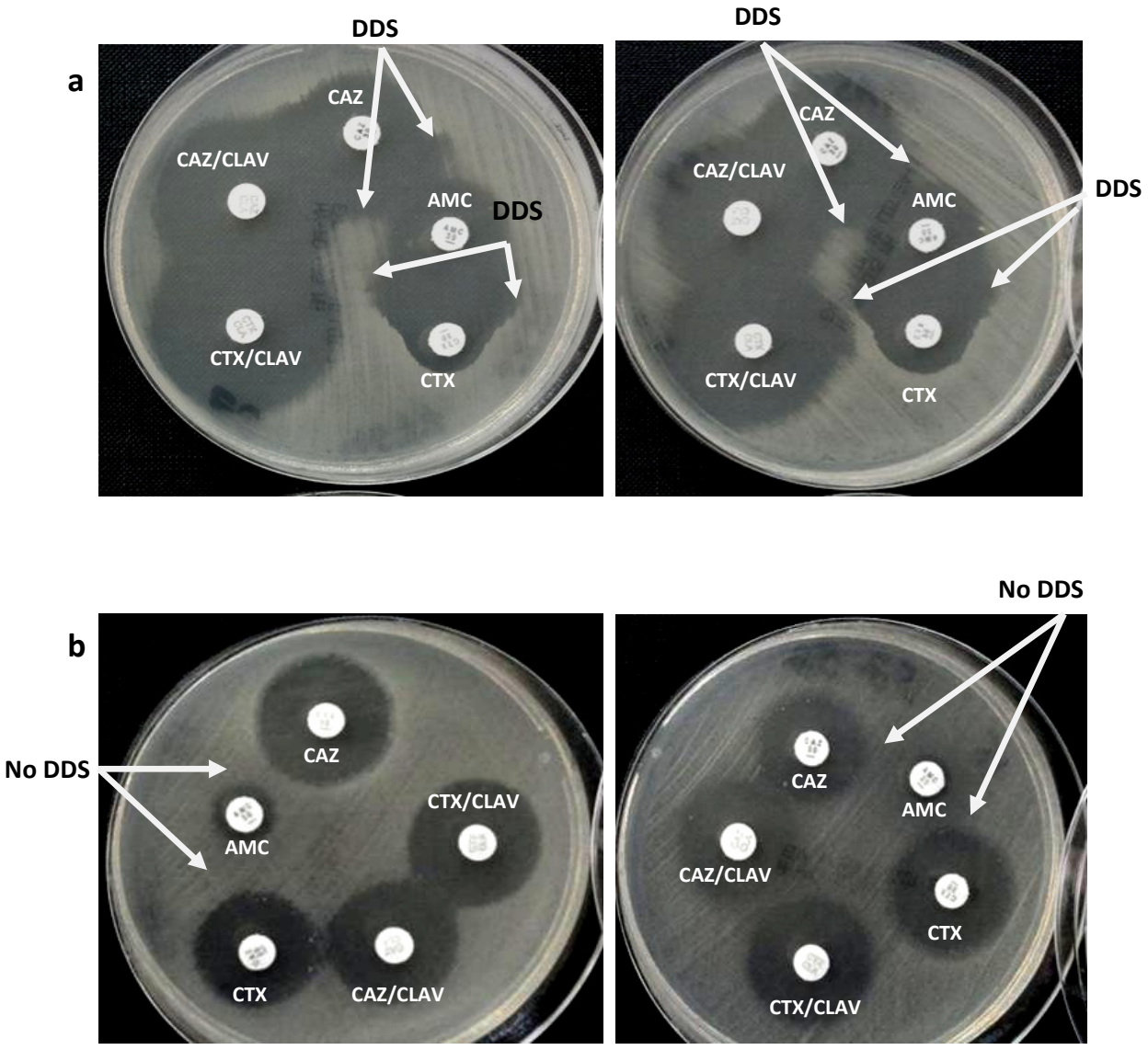


Figure 3-11: Double Disk Synergy test for phenotypically ESBL confirmation, **a)** ESBL producing Samples, **b)** non-ESBL producing samples

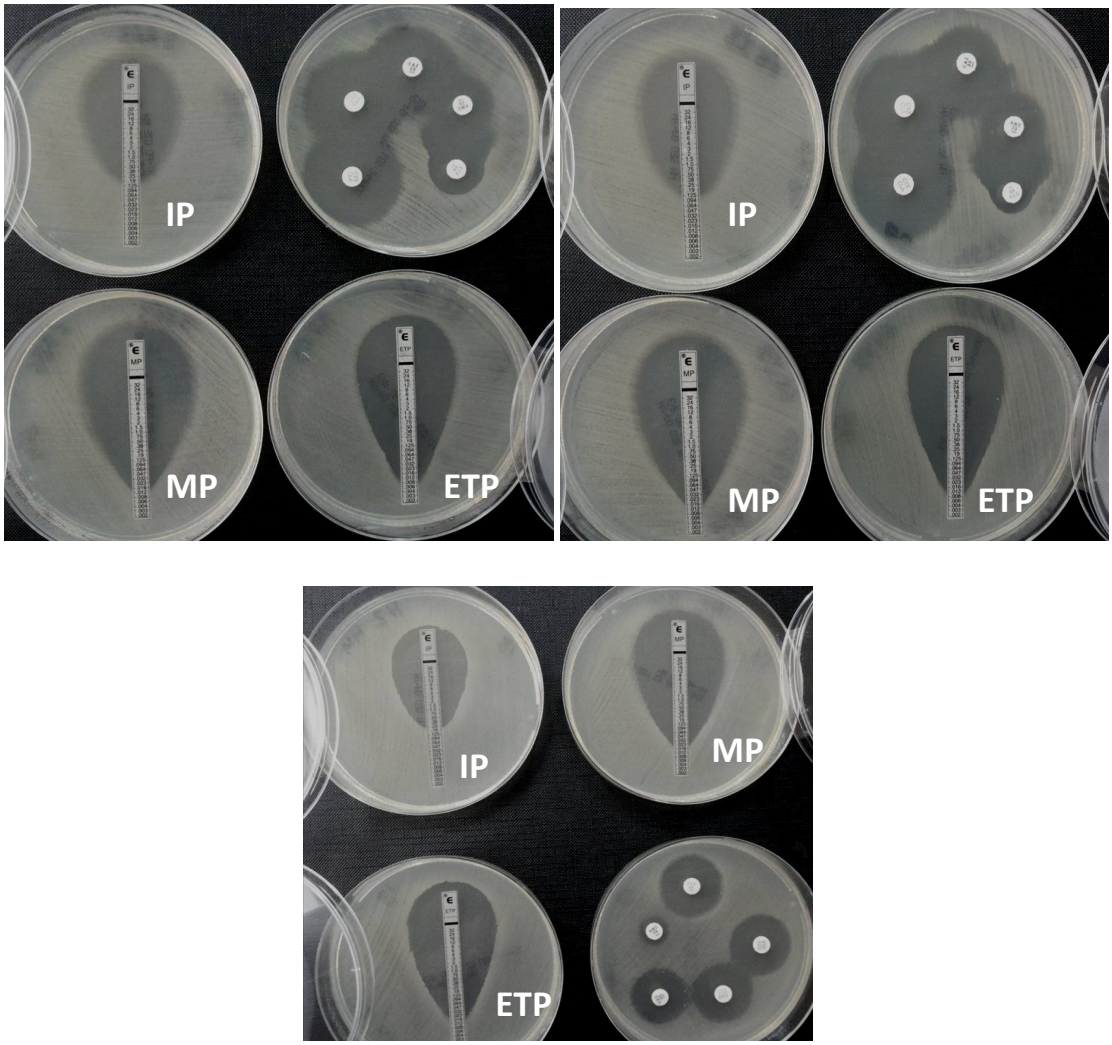


Figure 3-12: Minimum Inhibitory Concentration E-test for Carpapenems in ESBL producing Samples and Cefotaxime resistance samples.

3.6.2 ESBL producing genes

Of the 15 isolates which were non-susceptible to cefotaxime, 10 isolates were ESBL producing according to the phenotypic ESBL confirmatory test. Consequently, ESBL producing genes were screened in these isolates. Results showed that 10 isolates were positive for *TEM* gene but negative for *SHV*, while *CTX* and *CTX-M-*$_{2\ and\ 5}$ genes were positive in 5 and 6 isolates respectively (Figure 3-13).

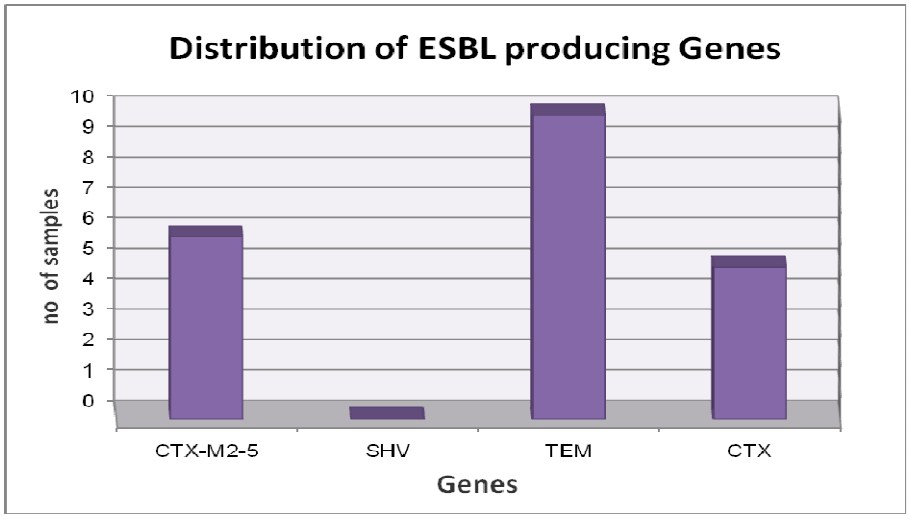


Figure 3-13: Histogram showing the prevalence of ESBL producing genes in the ESBL samples tested

3.7 Azithromycin resistant isolates

3.7.1 Azithromycin MIC E-test

As a consequence for testing azithromycin sensitivity, results for Disk Diffusion test were confirmed using MIC E-test for all reduced susceptibility isolates (N=82), in addition to another 166 susceptible isolates which were chosen randomly. Fifty isolates showed reduced susceptibility (MIC≤16), while 198 isolates were sensitive (Figure 3-14).

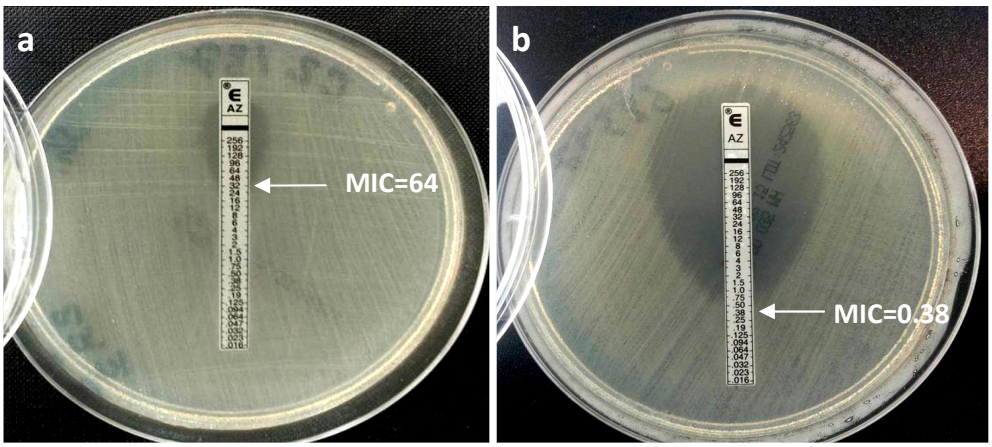


Figure 3-14: Minimum Inhibitory Concentration E test of Azithromycin for 2 isolates; **a)** resistant isolate, **b)** sensitive isolate

3.7.2 Detection of macrolide resistance genes

Isolates which showed reduced susceptibility to azithromycin (breakpoint ≤18 mm) were screened for macrolide resistance genes by polymerase chain reaction (PCR). Of the 82 isolates tested, only isolates with breakpoint ≤12 mm, which was equivalent to MIC ≥24, were positive for *mph(A)* gene (49 isolates), while the other genes [*erm*(A), *erm*(B), *erm*(C), *ere*(A), *ere*(B), m*ph*(B), m*ph*(D), *mef(A), msr(A)*] were negative in all of them (Figures 3-15 and 3-16). Results were validated against a positive control and using sequencing.

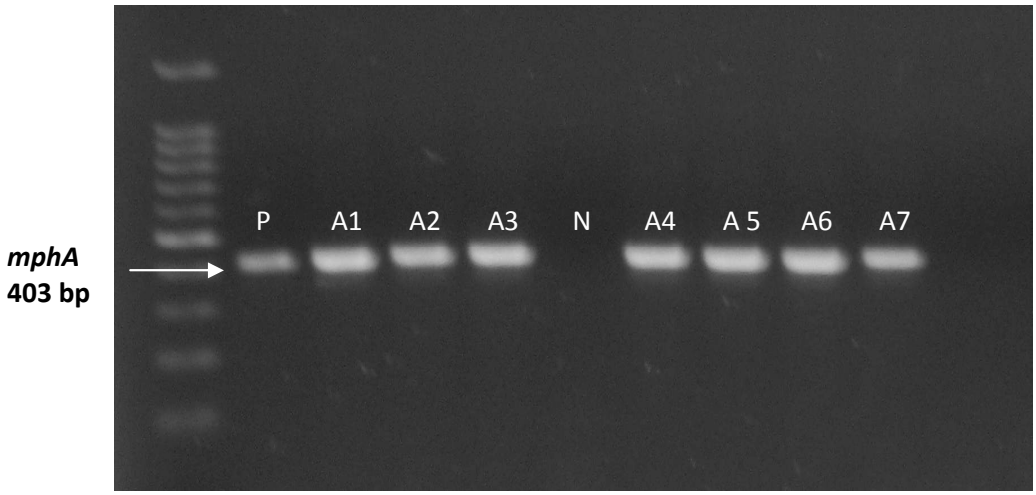
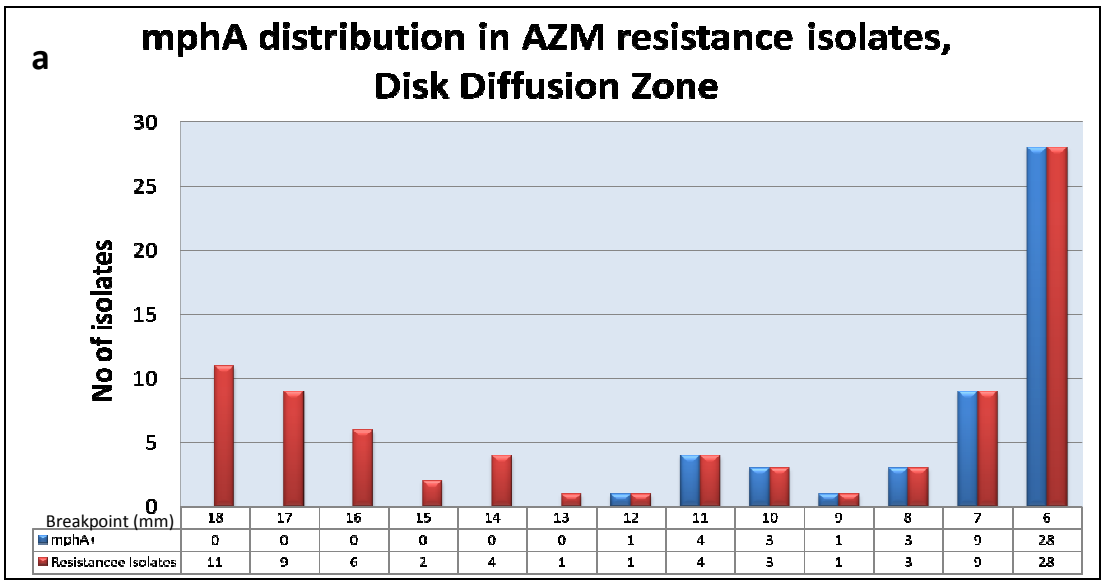


Figure 3-15: 1% Gel electrophoresis results for macrolide resistance gene (*mphA*) in 7 AZM resistant isolates, M= 100bp DNA ladder, P= positive control, N= negative control



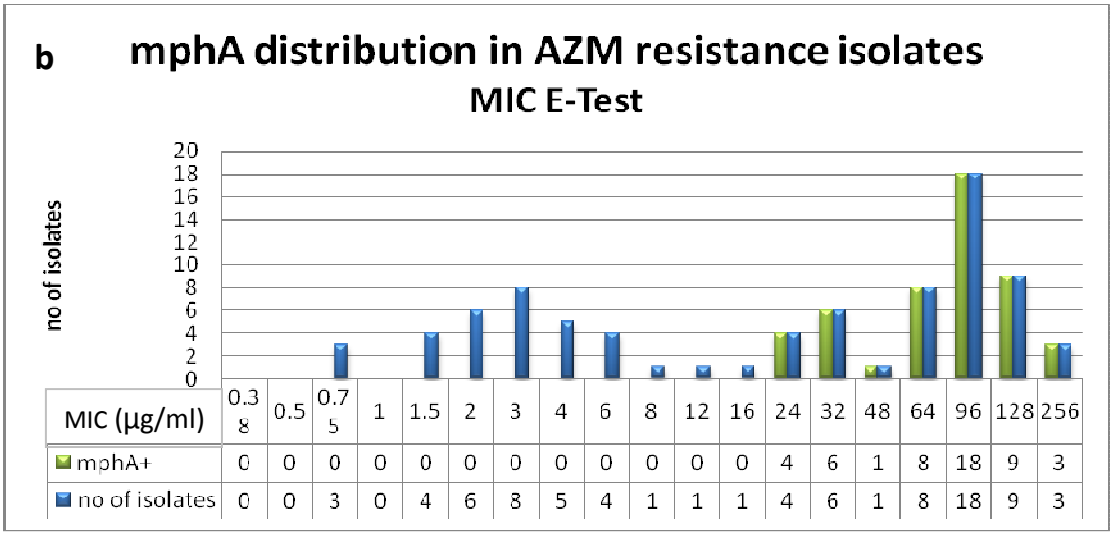


Figure 3-16: Histograms showing the distribution of *mphA* gene in the azithromycin resistant isolates tested a) according to disc diffusion test results, b) according to the MIC E-test results.

3.8 PFGE Results

For PFGE analysis, genomic DNA of 15 azithromycin resistant *S. flexneri* isolates was digested with two restriction enzymes, *NotI* as a primary restriction enzyme, and *XbaI* as secondary. *NotI* digestion yielded 16 to 18 DNA fragments, while those digested with *XbaI* yielded 20 to 23 DNA fragments (Figure 3-17). As shown in Figure 3-18, the fifteen isolates were distributed into four PFGE groups, arbitrarily

designated as patterns I, II, III and IV. Two of these patterns (I & II), representing 13 isolates, were more closely related to each other with similarity value of 92.3%. The third pattern (III) represents one isolate appeared different from patterns I & II with 86.4 % similarity. The fourth pattern (IV) which is isolate (A3/80) was uniquely different from the rest with a distinct *NotI/XbaI*-PFGE patterns (Figure 3-18).

Furthermore, PFGE pattern (II) of the four nonsusceptible isolates (antibiogram was different from the rest) digested with *NotI* restriction enzyme, were shown to be indistinguishable. This distinct PFGE patterns showed no obvious differences in their resistance patterns. Even though, the PFGE patterns with the secondary digest restriction enzyme (*XbaI*) of these isolates showed 92.5% similarity. This may indicate that these isolates most probably were from of the same common origin.

According to criteria for interpreting PFGE patterns, and based on the PFGE patterns with *NotI* and *XbaI* digests, around 93.0% of the isolates recovered during 2006 and 2007 with an outbreak or sporadic cases, were either indistinguishable or closely related strains.

Figure 3-17 below shows the TIFF images for the resulting gel, while Figures 3-18 and 3-19 are phylogenetic dendrogram for the isolates typed by PFGE and digested with *NotI* restriction enzyme *NotI* and *XbaI* respectively.

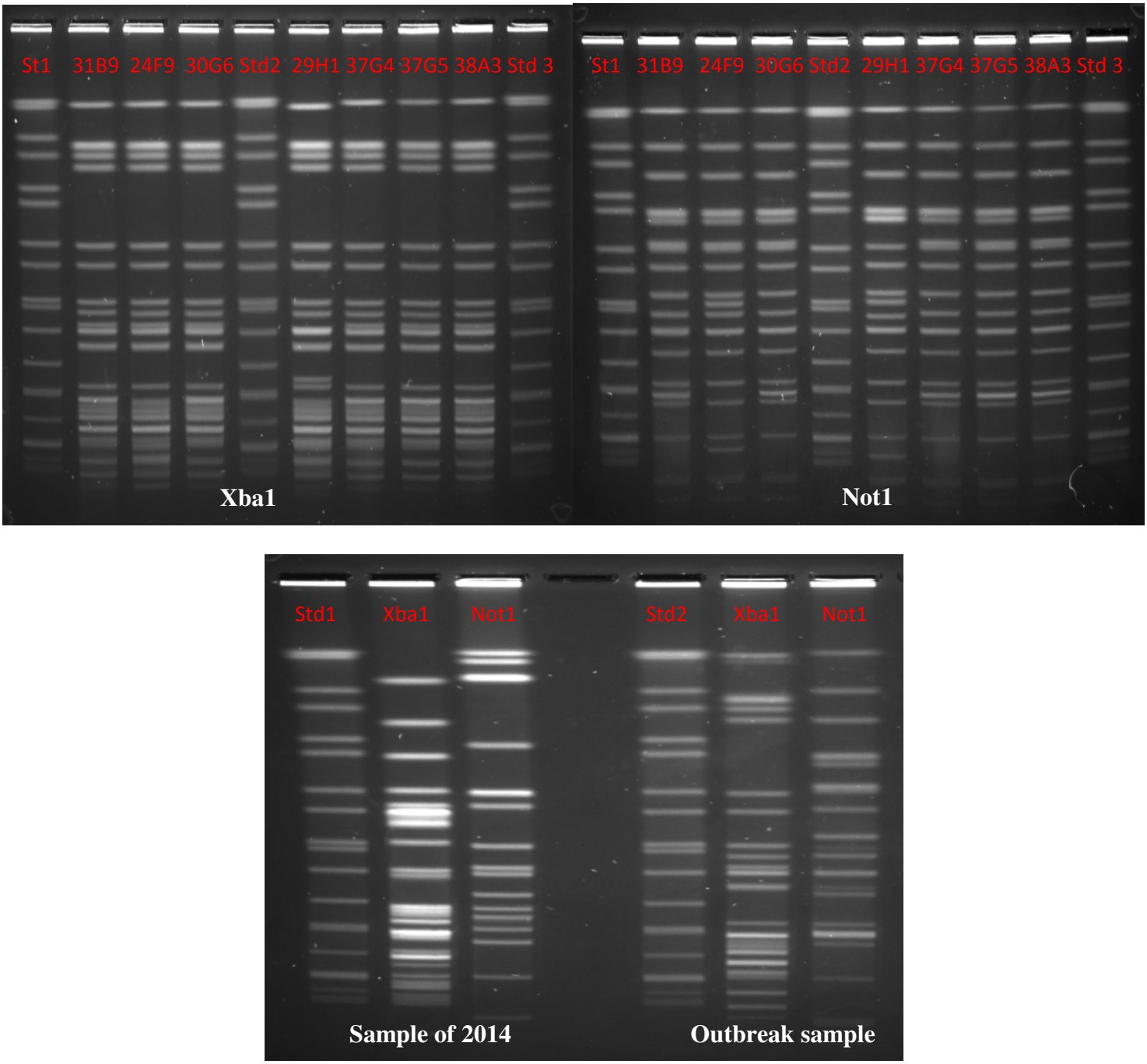


Figure 3-17: TIFF images for the gels results of a group of *S. flexneri* isolates, A) Digestion using *XbaI* enzyme yielded 20 to 23 DNA fragments, B) Digestion using *NotI* yielded 16 to 18 DNA fragments. Lanes 1,5,10 of each gel are for the standard strain *Salmonella enterica* serotype Braenderup H9812. C) The resulted patterns for the isolates recovered during 2006 and 2007 show that they were indistinguishable or closely related, which suggests that they form an outbreak, while the resulted patterns for the isolate recovered from 2014 show that it is unrelated.

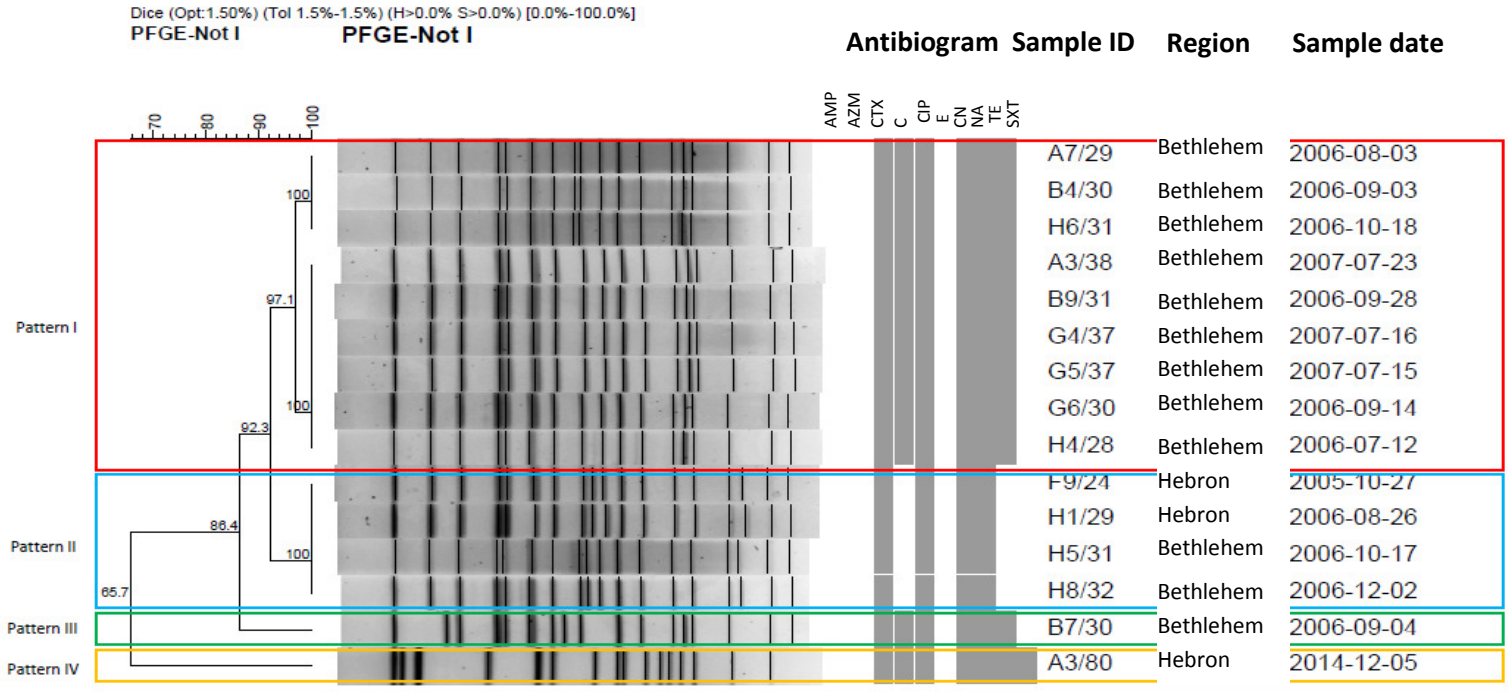


Figure 3-18: Phylogenetic dendrogram of *NotI*-digested chromosomal DNA from of *Shigella flexneri* isolates along with Antibiogram results.

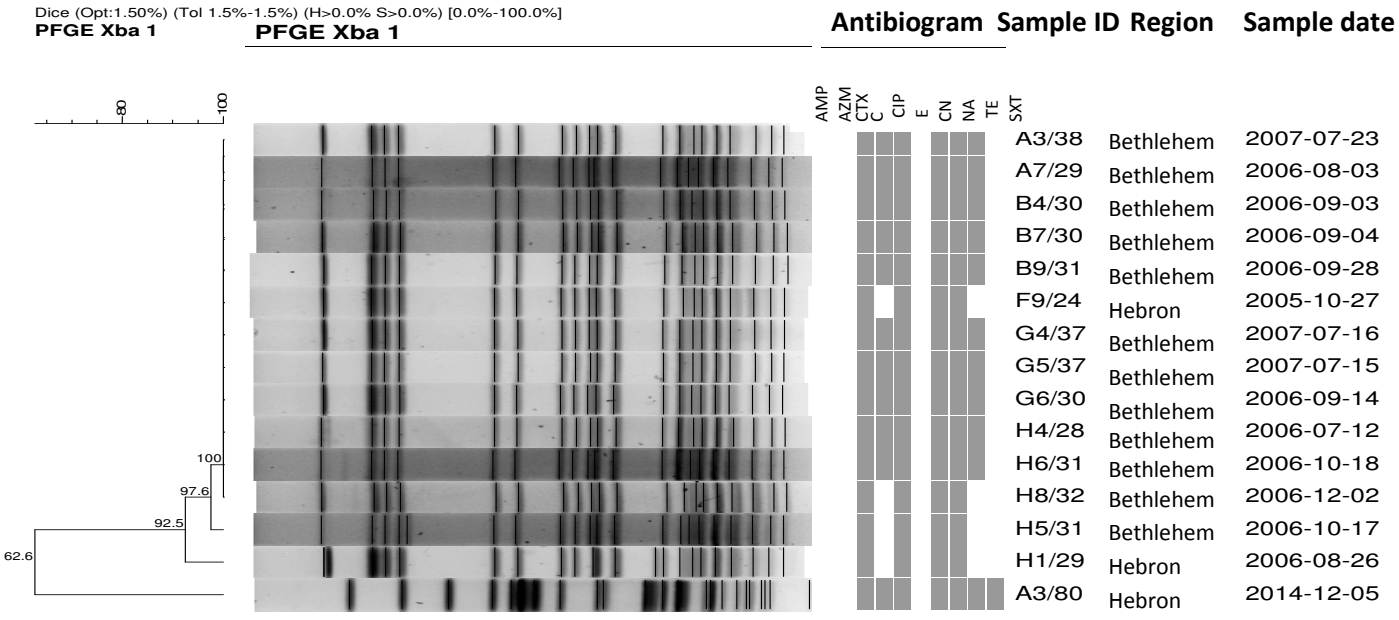


Figure 3-19: Phylogenetic dendrogram of *XbaI*-digested chromosomal DNA from of *S. flexneri* isolates along with Antibiogram results.

CHAPTER 4: DISCUSSION

Shigella species are gram-negative facultative rod bacteria from the *Enterobacteriaceae* family, and are divided into four species, *Shigella dysenteriae (*serogroup *A), Shigella flexneri (*serogroup *B), Shigella boydii (*serogroup *C), and Shigella sonnei (*serogroup *D).*

Shigellosis is a major public health problem, especially in the developing countries where it remains the most common cause of bloody diarrhea. *S. sonnei*, is the most commonly isolated species, especially in industrial countries.

In Palestine, shigellosis is an endemic disease. As it appears from our data, it is commonly caused by *S. sonnei,* and *S. flexneri*, but rarely by *S. boydii*, while there have been no reported cases for *S. dysenteriae* since more than 15 years. Most cases were from children less than five years of age, as well as the reported statistics worldwide, that's because usually young children have less effective immune responses to *Shigella* infections than adults, and so they are less likely to control bacterial infection and proliferation. Nevertheless, few cases were reported in children less than one year because children in this age have maternal antibodies that protect them from such infections.

According to the period included in this study, the highest rate of infection was reported in the period between 2006 and 2007, the same outbreak was reported in Jordan and Israel, where the highest rates of infection were during the period 2006-2008 (Berger 2016; Berger 2016). The reported cases in the following years decreased, which could be in part due to the improved hygiene practices in the Palestinian society, in particular the availability and chlorination of water supplies, which is the most important way to eliminate and prevent the disease.

Despite the fact that *Shigella* species are considered as a major cause of diarrheal disease, still little is known about their genetic diversity and virulence mechanism. The main virulence factor of *Shigella* is its ability to colonize and invade the intestinal cells, the process that is mediated by the large invasion plasmid, which carries an operon that encodes the type III-secretion-system (T3SS). In addition to type III-secretion system, *Shigella* has another virulence factors that enhance it's pathogenicity, the invasion-associated locus *(ial)*, which is carried on a plasmid of 120 to 140 MDa, and the invasion plasmid antigen H (i*paH*) gene, which is present in both the plasmid and chromosomal copies, are mainly involved in the invasion process, beside the enterotoxins *Shet1* and *Shet2* genes. Screening for these virulence genes in our sample population showed vast genetic diversity among the tested species. Although *ipaH* gene has multiple copies, still 23 isolates were negative to this gene. Similar report for Bhattacharya, et al. and Cruz et al showed that the main virulence gene is *ipaH* (Bhattacharya, Bhattacharya et al. 2014) (Cruz, Souza et al. 2014). Worth mentioning that in these studies also not 100% detection of virulence genes was noted. On the other hand, because the *ial* gene is exclusively located on the plasmid, it was detected only in 75.1% of the isolates, while *ShET*-2 genes, mainly *sen* gene, were negative in all the isolates. However, although *Set1* genes were detected in S. *flexneri* species only (mainly in type 2a) and supposed to be not found in other *Shigella spp*. In this study, 4 *S. sonnei* isolates were positive for *set1B* gene, the most likely explanation for this is the occurrence of integrase-mediated excision for the *she PAI* like region where the two genes are located, resulting in the formation of a circular excision product, packaged into phage particles and integrated into the bacterial chromosome (Zhang, Liu et al. 2014).

Nevertheless, the finding that was quite unexpected was the presence of shiga toxin gene (stx) in 8 *S. sonnei* samples. This gene is known to be related for *Shigella dysenteriae serotype 1* and enterohaemorrhagic *Escherichia coli (EHEC)* strains only. Clinical profile for the 8 patients showed that these isolates were highly virulent causing severe symptoms, four of the eight patients were from the same family, which indicates that this strain was highly infectious and spread rapidly, while three other patients were admitted in the hospital for four and three days, which suggests the high severity of the illness caused by these isolates. Beutin et al. and Nyholm et al. had been previously reported similar cases for stx2 –positive *S. sonnei* isolates (Beutin, Strauch et al. 1999; Nyholm, Lienemann et al. 2015). These isolates thought to be emerged from an *S. sonnei* isolate that was subsequently lysogenized by a free stx2 phage, previous studies revealed that the *STX* gene of the *S. sonnei* phage and a published *STX* sequence of *S. dysenteriae* type 1 are highly similar, which suggests that the *S. sonnei* phage might have acquired its *STX* gene from an *S. dysenteriae* type 1 strain (Strauch, Lurz et al. 2001).

Antibiotic resistance patterns for *Shigella* species in Palestine were also investigated in this study. The antibiotics ampicillin, cefotaxime, cefixime, gentamycin, chloramphenicol, trimethoprim-sulfamethoxazole, tetracycline, nalidixic acid, ciprofloxacin, and azithromycin were evaluated for all the isolates. Results showed the high incidence of resistance for ampicillin, tetracycline, and trimethoprim-sulfamethoxazole, which conforms with the worldwide reported cases of the increasing antimicrobial resistance of *Shigella* species. Ashkenazi et al. reported resistance against tetracycline and ampicillin in 87% and 81% of *Shigella* isolates that appeared in Israel (Ashkenazi, Levy et al. 2003). For trimethoprim-sulfamethoxazole,

Belay et al. demonstrated 55% resistance rate in Ethiopia (Belay, Solomon et al. 2000), while in Trinidad the reported rate was only 2.7% (Khan-Mohammed, Adesiyun et al. 2005), the highest recorded rates were 87.8% in Yaoundé, 90% in Cameron (Njunda, Assob et al. 2012), and 100% in Iran and Ghana (Belay, Solomon et al. 2000). While in England and Wales, almost 50% of *S. sonnei* isolates were resistant to ampicillin or trimethoprim, and 15% were resistant to both of these antibiotics (Cheasty, Skinner et al. 1998). Later in 2002, about 13% of *Shigella* cases that appeared in the same region were resistant to nalidixic acid (Cheasty, Day et al. 2004). Moreover, increasing levels of ceftriaxone and azithromycin resistance have been reported in Asia and Europe (Boumghar-Bourtchai, Mariani-Kurkdjian et al. 2008; Jue, Hardee et al. 2010).

Additionally, results show the high variance of resistance patterns among the different serogroups. All *S. boydii* isolates were resistant to ampicillin, while *S. flexneri* isolates were mostly resistant to ampicillin (86%), trimethoprim-sulfamethoxazole (79%), and tetracycline (50%). However, *S. sonnei* isolates showed less resistance rates, 87% were resistant to tetracycline, and only 24% of the isolates were resistant to ampicillin, while most of the isolates were susceptible to all other antibiotics. That's why *S. sonnei* infections are commonly mild and easily to be treated and managed.

Although azithromycin is recommended as a first line treatment for children, still there are no clear guidelines for interpreting it's sensitivity test results. In 2016, CLSI added a separated section for Epidemiological Cutoff Values (ECVs) for *Shigella flexneri* and *Shigella sonnei*. ECVs are MIC values that separate bacterial populations into those with and without acquired or mutational resistance mechanisms, mostly determined according to the in-vitro data. For azithromycin, ECVs were determined

as ≤16 µg/ml S; ≥32 µg/ml R, but still these ECVs should be reported only after discussion with a patient's clinician. In our study, disk diffusion breakpoints for azithromycin were compared to the MIC's of the E-test, and then it was correlated with the presence of the macrolide resistance genes. Macrolide resistance genes *(erm(A), erm(B), erm(C), ere(A), ere(B), mph(A), mph(B), mph(D), mef(A), msr(A))* were screened in all the isolates which showed reduced susceptibility to azithromycin (inhibition zone ≤18 mm). Results showed that only isolates with breakpoint ≤12 mm, which was equivalent to MIC≤24 µg/ml, were positive for *mph(A)* gene, while the other genes were negative in all of the isolates tested. *Mph(A)* is the gene that encodes macrolide 2-phosphotransferase which inactivates macrolide antimicrobial drugs, it was first reported in an *E. coli* isolate from Japan (Boumghar-Bourtchai, Mariani-Kurkdjian et al. 2008). Drug modification and so inactivation of the antibiotics via esterases and phosphotransferases is the inherited mechanism for macrolide resistance in *Enterobacteriaceae*. However, this resistance mechanism is not considered of major clinical importance because *Enterobacteriaceae* species are not the main target for macrolide antibiotics (Leclercq 2002), but it is important to consider this for azithromycin as it is recommended as an alternative therapy for shigellosis, because it can be highly concentrated within the intracellular compartment of phagocytes and other cells, and so it is found to be effective in vivo for *Shigella*, which is a facultative intracellular pathogen (Jain, Gupta et al. 2005). To our knowledge, this is the first study that seeks to correlate the MIC and the breakpoint of azithromycin with the presence of the resistance genes in the isolates.

Screening for ESBL producing genes in ESBL producing isolates showed that all isolates had *TEM* β-lactam type but none was of *SHV* β-lactamases, while *CTX* and

CTX-M-2 and 5 genes were positive in 5, 6 isolates respectively. Worldwide, *TEM-1, OXA-1* and *OXA-3* are the most frequent reported types of β-lactamase in *Shigella* species (Navia, Capitano et al. 1999). However, there were reported *S. dysenteriae* case with *SHV-11*, the variant of the narrow-spectrum β-lactamase *SHV-1* (Ahamed and Kundu 1999), *S. sonnei* strain with a *CTX-M-2*-type ESBL from Argentina (Radice, Gonzealez et al. 2001), and a recent study from Lebanon reported four ESBL isolates that harbored the *CTX-M-15* gene (Sabra, Araj et al. 2009). Yet, *TEM* β-lactam is still the most common gene because it is plasmid and transposon mediated, so it was rapidly spread worldwide and now it is found in many different species of the *Enterobacteriaceae* family (Rawat and Nair 2010).

PFGE is one of the most reproducible and highly discriminatory typing techniques available for molecular characterization of several enteric bacteria including *Shigella*. It is used as a reference for epidemiological studies combined with other genotyping methods like plasmid profile analysis, which is the most common typing procedure currently used with *Shigella spp*. beside PFGE, that is the current standard subtyping method certified by the PulseNet.

Here we used PFGE method to perform subtyping for 15 *S. flexneri* isolates, which were chosen from the samples that were found to be resistant to azithromycin and recovered from 2006, 2007 and 2014 from different areas. DNA digestion was performed using two restriction enzymes, *NotI* as a primary, and *XbaI* as secondary. *NotI*-digested chromosomal DNA yielded 16 to 18 reproducible DNA fragments, while those digested with *XbaI* yielded 20 to 23 DNA fragments, which indicates that *Not1* is a better differentiating restriction enzyme for *S. flexneri* because it gives more distinguishable patterns with more differences, in addition to that, the resulted

patterns were much easier to analyze and less prone to the band marking errors. The results showed overall similarity of 93.0% for the isolates recovered during 2006 and 2007, isolates were indistinguishable or closely related strains. The most different pattern was for the isolate recovered from a patient in December 2014, this PFGE finding suggests that a new *S. flexneri* clone which was resistant to AZM appeared in 2014 in Palestine.

This study represents a long-term surveillance and molecular characterization concerning antimicrobial resistance for *Shigella* species from southern Palestine. Moreover, the study confirms the worldwide findings about the continued emergence of drug resistant *Shigella* species as a result of the widespread antimicrobial use. The high *Shigella* species resistance patterns to oral antibiotics and the emergence of ESBL producing *Shigella* species, mandates the Palestinian Ministry of Health to control the use of antibiotics. In addition, the results of this study can be used to better interpret azithromycin disk diffusion results as no clear guidelines by the CLSI are currently available.

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APPENDIX 1

Disk diffusion test and MIC E test for *mphA* positive *Shigella* Isolates

Sample ID	Year	Disk Diffusion Zone (mm)	MIC E test (µg/ml)
C2/24	2005	10	32
F9/24	2005	12	32
B6/25	2005	11	32
H6/26	2006	6	256
H4/28	2006	6	96
I9/28	2006	6	96
A7/29	2006	6	96
A8/29	2006	6	96
C1/29	2006	6	96
C4/29	2006	6	96
H1/29	2006	8	32
I5/29	2006	6	64
I6/29	2006	6	64
I7/29	2006	6	96
B4/30	2006	7	64
B7/30	2006	6	64
B8/30	2006	6	96
C8/30	2006	6	96
D4/30	2006	6	128
G3/30	2006	8	96
G6/30	2006	6	128
B3/31	2006	6	96
B9/31	2006	6	96
F7/31	2006	6	96
F6/31	2006	6	128
F8/31	2006	6	128
G7/31	2006	6	64
H5/31	2006	10	24
H6/31	2006	6	64
B7/32	2006	6	24
D4/32	2006	11	32
E2/32	2006	6	64
E6/32	2006	6	128
F2/32	2006	11	24
G2/32	2006	7	256

H6/32	2006	8	256
H7/32	2006	7	128
H8/32	2006	10	48
A4/33	2006	11	24
C4/33	2006	6	64
G7/36	2007	7	96
C2/37	2007	6	128
G5/37	2007	9	32
G4/37	2007	7	128
A3/38	2007	7	96
A5/38	2007	7	96
B9/38	2007	7	96
C2/38	2007	7	96
80/A3	2014	6	128

Appendix 2

Protocol for isolating nucleic acids

1. To a nuclease-free 1.5 ml microcentrifuge tube, add 200 µl bacterial suspension.
2. Add 200 µl working solution, freshly prepared
3. Add 50 µl Proteinase K solution; mix immediately.
4. Incubate for 10 min at +72°C.
5. Add 100 µl Binding Buffer and mix well.
6. To transfer the sample to a High Pure Filter Tube: Insert one High Pure Filter Tube into one Collection Tube, pipette the entire sample into the upper reservoir of the Filter Tube.
7. Insert the entire High Pure Filter Tube assembly into a standard table-top centrifuge, centrifuge 1 min at 8,000 × g.
8. After centrifugation, remove the Filter Tube from the Collection Tube; discard the flowthrough and the Collection Tube.
9. Combine the Filter Tube with a new Collection Tube, after combining the Filter Tube with a new Collection Tube, add 500 µ l Inhibitor Removal Buffer to the upper reservoir of the Filter Tube.
10. Centrifuge 1 min at 8,000 × g.
11. After centrifugation, remove the Filter Tube from the Collection Tube; discard the flowthrough and the Collection Tube.
12. Combine the Filter Tube with a new Collection Tube, add 450 µl Wash Buffer to the upper reservoir of the Filter Tube.
13. Centrifuge 1 min at 8,000 × g and discard the flowthrough.

14. After the first wash and centrifugation, remove the Filter Tube from the Collection Tube; discard the flowthrough and the Collection Tube.

15. Combine the Filter Tube with a new Collection Tube.

16. Add 450 µl Wash Buffer to the upper reservoir of the Filter Tube.

17. Centrifuge 1 min at 8,000 × g and discard the flowthrough.

18. Leave the Filter Tube-Collection Tube assembly in the centrifuge and spin it for 10 s at maximum speed (approx. 13,000 × g) to remove any residual Wash Buffer. The extra centrifugation time ensures removal of residual Wash Buffer.

19. Discard the Collection Tube and insert the Filter Tube into a nucleasefree, sterile 1.5 ml microcentrifuge tube.

20. To elute the viral nucleic acids, add 50 µl Elution Buffer to the upper reservoir of the Filter Tube.

21. Centrifuge the tube assembly for 1 min at 8,000 × g.

22. The microcentrifuge tube contains the eluted, purified viral nucleic acids.

APPENDEX 3

Standard Operating Procedure for PulseNet PFGE of *Escherichia coli* O157:H7, *Escherichia coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri*

Grow the culture:

Streak an isolated colony from test cultures onto Trypticase Soy Agar with 5% defibrinated sheep blood (TSA-SB) plates (or comparable media) for confluent growth. It is recommended that a storage vial of each culture be created. To do this, stab small screw cap tubes of TSA, HIA, or similar medium.with the same inoculating loop used to streak the plate. This will ensure that the same colony can be retested if necessary. Incubate cultures at 37°C for 14-18 hours.

Making plugs

1. Turn on shaker water bath or incubator (54-55°C), stationary water baths (55-60°C) and spectrophotometer (or equivalent instrument such as the Dade Microscan Turbidity meter or bioMérieux Vitek colorimeter).

2. Prepare TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0) as follows:

2.1. 10 ml of 1 M Tris, pH 8.0

2.2. 2 ml of 0.5 M EDTA, pH 8.0

2.3. Dilute to 1000 ml with sterile Ultrapure Clinical Laboratory Reagent Water (CLRW)

3. Prepare 1% SeaKem Gold agarose in TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0) for PFGE plugs as follows:

3.1. Weigh 0.50 g (or 0.25 g) SeaKem Gold (SKG) agarose into 250 ml screw-cap flask.

3.2. Add 50.0 ml (or 25.0 ml) TE Buffer; swirl gently to disperse agarose.

3.3. Loosen or remove cap, cover loosely with clear film, and microwave for 30 seconds; mix gently and repeat for 10 seconds intervals until agarose is completely dissolved.

3.4. Recap flask and return to 55-60°C water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

4. Label small tubes (12mm x 75mm Falcon tubes or equivalent) with culture numbers.

5. Prepare Cell Suspension Buffer (100 mM Tris:100 mM EDTA, pH 8.0) as follows:

5.1. 100 ml of 1 M Tris, pH 8.0

5.2. 200 ml of 0.5 M EDTA, pH 8.0

5.3. Dilute to 1000 ml with sterile Ultrapure water (CLRW)

6. Transfer 2 ml of Cell Suspension Buffer (CSB) to small labeled tubes. Use a sterile polyester-fiber or cotton swab that has been moistened with sterile CSB to remove some of the growth from agar plate; suspend cells in CSB by spinning swab gently so cells will be evenly dispersed and formation of aerosols is minimized.

7. Adjust concentration of cell suspensions to one of values given below by diluting with sterile CSB or by adding additional cells.

7.1. Spectrophotometer: 610 nm wavelength, absorbance (Optical Density) of 1.00 (range of 0.8-1.0)

7.2. Dade Microscan Turbidity Meter:

7.2.1. 0.40-0.45 (measured in Falcon 2054 tubes)

7.2.2. 0.58-0.63 (measured in Falcon 2057 tubes)

7.3. bioMérieux Vitek colorimeter: 17-18% transmittance (measured in Falcon 2054 tubes)

Casting Plugs

1. Label wells of PFGE plug molds with culture number. When reusable plug molds are used, put strip of tape on lower part of reusable plug mold before labeling wells.

2. Transfer 400 µl adjusted cell suspensions to labeled 1.5-ml microcentrifuge tubes.

3. Add 20 µl of Proteinase K (20 mg/ml stock) to each tube and mix gently with pipet tip. (200 µl are needed for 10 cell suspensions.)

4. Add 400 µl melted 1% SeaKem Gold agarose to 400 µl cell suspension; mix by gently pipetting mixture up and down a few times. Over-pipetting can cause DNA shearing. Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60°C).

5. Immediately, dispense part of mixture into appropriate well(s) of reusable plug mold. Do not allow bubbles to form. Two plugs of each sample can be made from these amounts of cell suspension and agarose and are useful if repeat testing is required. Allow plugs to solidify at room temperature for 10-15 minutes. They can also be placed in the refrigerator (4°C) for 5 minutes.

Lysis of Cells in Agarose Plugs:

1. Label 50ml polypropylene screw-cap or 50ml Oak Ridge tubes with culture numbers.

2. Prepare Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) as follows:

2.1. 50 ml of 1 M Tris, pH 8.0

2.2. 100 ml of 0.5 M EDTA, pH 8.0

2.3. 100 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt)

2.4. Dilute to 1000 ml with sterile Ultrapure water (CLRW)

3. Calculate the total volume of Cell Lysis/Proteinase K Buffer needed as follows:

3.1. 5 ml Cell Lysis Buffer is needed per tube e. g., 5 ml x 10 tubes = 50 ml

3.2. 25 µl Proteinase K stock solution (20 mg/ml) is needed per tube of the cell lysis buffer e. g., 25 µl x 10 tubes = 250 µl

4. Prepare the master mix by measuring the correct volume of Cell Lysis Buffer and Proteinase K into appropriate size test tube or flask and mix well.

5. Add 5 ml of Proteinase K/Cell Lysis Buffer to each labeled 50 ml tube.

6. Trim excess agarose from top of plugs with scalpel, razor blade or similar instrument. Open reusable plug mold and transfer plugs from mold with a 6-mm wide spatula to appropriately labeled tube. If disposable plug molds are used, remove white tape from bottom of mold and push out plug(s) into appropriately labeled tube. Be sure plugs are under buffer and not on side of tube.

7. Remove tape from reusable mold. Place both sections of the plug mold, spatulas, and scalpel in 90% ethanol, 1% Lysol/Amphyll or other suitable disinfectant. Soak them for 15 minutes before washing them. Discard disposable plug molds or disinfect them in 90% ethanol for 30-60 minutes if they will be washed and reused.

8. Place tubes in rack and incubate in a 54-55°C shaker water bath or incubator for 1.5-2 hours with constant and vigorous agitation (150-175 rpm). If lysing in water bath, be sure water level is above level of lysis buffer in tubes. 9. Pre-heat enough sterile Ultrapure water (CLRW) to 54-55°C so that plugs can be washed two times with 10-15 ml water (200-300 ml for 10 tubes)

Washing of Agarose Plugs After Cell Lysis

1. Remove tubes from water bath or incubator, and carefully pour off lysis buffer into an appropriate discard container; plugs can be held in tubes with a screened cap or spatula.

2. Add at 10-15 ml sterile Ultrapure water (CLRW) that has been pre-heated to 54-55°C to each tube and shake the tubes in a 54-55°C water bath or incubator for 10-15 minutes.

3. Pour off water from the plugs and repeat wash step with pre-heated water (Step 2) one more time.

4. Pre-heat enough sterile TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0) in a 54-55°C water bath so that plugs can be washed four times with 10-15 ml TE (400-600 ml for 10 tubes) after beginning last water wash.

5. Pour off water, add 10-15 ml pre-heated (54-55°C) sterile TE Buffer, and shake the tubes in 54-55°C water bath or incubator for 10-15 minutes.

6. Pour off TE and repeat wash step with pre-heated TE three more times.

7. Decant last wash and add 5-10 ml sterile TE. Continue with step 1 in "Restriction Digestion" section or store plugs in TE Buffer at 4°C until needed. Plugs can be transferred to smaller tubes for long term storage.

Restriction Digestion of DNA in Agarose Plugs

1. Label 1.5-ml microcentrifuge tubes with culture numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for Salmonella ser. Braenderup H98121 standards.

2. Pre-Restriction Incubation Step: Prepare a master mix by diluting the appropriate 10X restriction buffer (Roche Applied Science or equivalent) 1:10 with sterile Ultrapure water (CLRW) according to the following table:

Reagent	µl/Plug Slice	µl/10Plug Slices	µl/15 Plug Slices
CLRW	180 µl	1800 µl	2700 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
Total Volume	200 µl	2000 µl	3000 µl

3. Add 200 µl diluted restriction buffer (1X) to labeled 1.5-ml microcentrifuge tubes.

4. Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on large glass slide.

5. Cut a 2.0 to 2.5mm wide slice from each test samples and the appropriate number of S. ser. Braenderup H9812 standards with a single edge razor blade (or scalpel, cover slip, etc.) and transfer to tube containing diluted restriction buffer. Be sure plug slice is under buffer. Replace rest of plug into the original tube that contains 5 ml TE buffer and store at 4°C.

5.1. Incubate sample and control plug slices in a 37°C water bath for 5-10 minutes or at room temp for 10-15 minutes.

5.2. After incubation, remove buffer from plug slice using a pipet fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to damage the plug slice with pipet tip and that plug slice is not discarded with pipet tip.

6. Prepare the restriction enzyme master mix according to the following table. May mix in the same tube that was used for the diluted restriction buffer.

7. Add 200 µl restriction enzyme master mix to each tube. Close tube and mix by tapping gently; be sure plug slices are under enzyme mixture.

8. Incubate sample and control plug slices in 37˚C water bath for 1.5-2 hours.

9. If plug slices will be loaded into the wells (Option B, page 9), continue with Steps 1-4 of the next section (Casting an Agarose Gel) approximately 1 hour before restriction digest reaction is finished so the gel can solidify for at least 30 minutes before loading the restricted PFGE plugs.

Casting an Agarose Gel

1. Confirm that water bath is equilibrated to 55-60°C.

2. Make volume of 0.5X Tris-Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis running buffer according to one of the following tables:

Reagent	Volume (ml)	Volume(ml)
5X TBE Stock	200	220
CLRW	1800	1980
Total Volume	2000	2200

Reagent	Volume (ml)	Volume (ml)
10X TBE Stock	100	110
CLRW	1900	2090
Total Volume	2000	2200

3. Make 1% SeaKem Gold (SKG) Agarose in 0.5X TBE as follows:

3.1. Weigh appropriate amount of SKG into 500 ml screw-cap flask. Mix 1.0 g agarose with 100 ml 0.5X TBE for 14cm-wide gel form (10 wells) Mix 1.5 g agarose with 150 ml 0.5X TBE for 21cm-wide gel form (15 wells)

3.2. Add appropriate amount of 0.5X TBE; swirl gently to disperse agarose.

4. Loosen cap and microwave for 60 seconds; mix gently and repeat for 15 second intervals until agarose is completely dissolved.

5. Return flask to 55-60°C water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

6. A small volume (2-5 ml) of melted and cooled (55-60°C) 1% SKG agarose may be wanted to seal wells after plugs are loaded. Prepare as described above. Unused SKG agarose can be kept at room temperature, melted, and reused several times.

7. Remove restricted plug slices from 37°C water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 minutes.

8. Remove plug slices from tubes; put comb on bench top and load plug slices on the bottom of the comb teeth as follows:

8.1. Load S. ser. Braenderup H9812 standards on teeth (lanes) 1, 5, 10 (10 well gel) or on teeth 1, 5, 10, 15 (15 well gel).

8.2. Load samples on remaining teeth and note locations.

9. Remove excess buffer with a kimwipe. Allow plug slices to air dry on the comb for 3-5 minutes or seal them to the comb with 1% SKG agarose (55-60°C).

10. Position comb in gel form and confirm that the plugs slices are correctly aligned on the bottom of the comb teeth, that the lower edge of the plug slice is flush against the black platform.

11. Carefully pour the agarose (cooled to 55-60°C) into the gel form and remove any bubbles or debris.

12. Put black gel frame in electrophoresis chamber. Add 2 -2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer needed depends on whether residual buffer was left in tubing or if unit was flushed with water after the last gel was run).

13. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (setting of about 70) and cooling module (14°C) approximately 30 minutes before gel is to be run.

14. Remove comb after gel solidifies for 30-45 minutes.

15. Fill in wells of gel with melted and cooled (55-60°C) 1% SKG Agarose (optional). Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a kimwipe. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

Electrophoresis Conditions *Shigella flexneri* strains restricted with NotI or XBAI:

Select following conditions on CHEF Mapper o Auto Algorithm o 50 kb:

low MW o 400 kb: high MW o Select default values except where noted by pressing "Enter."

Change the switch times to the following values:

Initial switch time: 5 seconds o Final switch time: 35 seconds

Change run time to 18-19 hours (Default values: Initial switch time = 6.76 s; Final switch time = 35.38 s)

Select following conditions on CHEF DR-III

Initial switch time: 5 s , Final switch time: 35 s , Voltage: 6 V , Included Angle: 120°, Run time: 18-19 hours

Select following conditions on CHEF DR-II.

Initial A time: 5 s o Final A time: 35 s , Start Ratio: 1.0 (if applicable) , Voltage: 200 V , Run time: 19-20 hours