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Genome-wide Analysis of Multidrug Resistant *Shigella* spp. Isolated from Clinical Settings in Lebanon.

By

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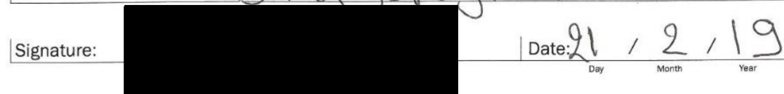

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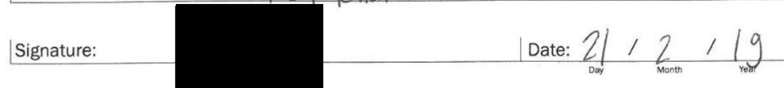

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I dedicate this work to my beloved family.

Genome-wide Analysis of Multidrug Resistant *Shigella* spp. Isolated from Clinical Settings in Lebanon.

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Yara J. Salem

ABSTRACT

Shigella spp. are Gram-negative rod-shaped bacteria belonging to the family *Enterobacteriaceae* and are known to be a major cause of bacillary dysentery worldwide. In this study, whole-genome sequencing was used for the molecular characterization of ESBL producing *Shigella* spp. isolates collected from hospitals in Lebanon. PCRs were performed to detect β-lactam resistance gene reservoirs and to identify genes involved in virulence and host adaptation. PCR-based replicon typing (PBRT) was performed to identify patterns of plasmid distribution. For comparative analysis, multi-locus sequence typing (MLST), whole-genome based SNP analysis, Pan-genome analysis and pulsed-field gel electrophoresis (PFGE) were performed to determine the phylogenic relatedness of the isolates and to trace evolutionary lineages. Our study revealed that, eight of the isolates were ESBL producers; resistance to β-lactam was mainly manifested by the presence of $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-3}}$, and $bla_{\text{TEM-1B}}$ genes. Inc I1α and IncFII were the prevalent incompatibility groups, with IncI1α being detected in all the isolates, and IncFII in eight of the isolates. β-lactam resistance determinants were found mainly on these two plasmids. class 2 integron was the most common integron detected (nine of the isolates) whereas class 1 was only seen in two of the isolates. The ten sequenced isolates were distributed on three multi-locus sequence types (MLST) (ST152, ST145 and ST245) with ST152 being the

predominant. Our study focuses on important virulence determinants and effector proteins used by the bacterium to destroy the host. Individual PCRs were performed to detect the presence of *virA* (motility), *ial* (invasion), *ipaH* (immune system modulation), *set* and *sen* (enterotoxins). The study provided an overview of the major plasmid families circulating in multidrug resistant *Shigella* in Lebanon. The results obtained revealed the role of integrons and gene cassettes in the dissemination of antimicrobial resistant determinants in *Shigella* and gave an insight of the phylogenic relationships between the undertaken isolates and other worldwide-recovered representatives. This study and through the use of genome-based approaches helped in better understanding the diversity of *Shigella,* its pathogenesis and drug resistance at the molecular level, and the risk factors associated with the mobilization of plasmids between *Shigella* spp. and other enteric pathogens

Key Words: *Shigella* spp, ESBL, SNP, MLST, PFGE, Integron.

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LIST OF ABBREVIATIONS

AUBMC: American University of Beirut Medical Center

bla: Beta lactamase

AST: Antibiotic susceptibility testing

AUBMC: American University of Beirut Medical Center

CDC: Centers for Disease Control and Prevention

CTX-M: Cefotaxime resistance

ESBL: Extended-spectrum ß-lactamases.

HGT: Horizontal gene transfer

HUS: Hemolytic uremic syndrome

Ial: Invasion protein

INF: Interferon

IL-8: Interleukin 8

Inc: Incompatibility

Ipa: invasion plasmid antigens

IS: Insertion sequences

LPS: Lipopolysaccharide

M cells: Microfold cells

MDR: Multi-drug resistant

MLST: Multi-locus sequence typing

NCBI: National Center of Biotechnology Information

NK: Natural killers

OmpT: Outermembrane protease

PAI: Pathogenic Island

PBRT: PCR-based replicon typing

PCR: Polymerase Chain reaction

PFGE: Pulsed-field gel electrophoresis

PHAST: Phage Search Tool

PMN: Polymorphonuclear neutrophil leukocyte

PT: Pulsotype

RAST: Rapid Annotation using Subsystem Technology

ShET1: *Shigella* enterotoxin 1

ShET2: *Shigella* enterotoxin 2

SHI-1: *Shigella* Island 1

SHV: Sulfhydryl variable

SNP: Single nucleotide polymorphism

Spa: Surface presentation of Ipa

ST: Sequence type

Stx: Shiga toxin

TEM: Temoneria ß-lactamase

TSA: Tryptic soy agar

T6SS: Type six secretion system

TTSS: Type three secretion system

VF: Virulence factors

WGS: whole genome sequencing

Chapter One

Introduction

1.1. The discovery of *Shigella*

"The discovery of the dysentery bacillus stirred my young heart with hopes of eradicating the disease and saving the suffering of about one hundred thousand cases that occurred yearly in my country at that time. Thirty-eight years have passed since this stirring event. Many thousands still suffer from this disease every year, and the light of hope that once burned so brightly has faded as a dream of a summer night. This sacred fire must not burn out" - Kiyoshi Shiga

During the 19th century in Japan, the *sekiri* dysentery epidemic affected more than 90 000 patients with high mortality rates (over 20%) (Shiga, 1936). In 1897, Dr. Kiyoshi Shiga first isolated a bacillus from the stool of 36 dysentery patients and by employing simple methods of analysis he discovered that the bacteria isolated is Gram-negative, fermenter of dextrose, negative in indole reaction, does not form acid from mannitol, and produces toxic factors. In 1930, the genus of bacillus was named *Shigella* after Kiyoshi Shiga (Trofa et al., 1999).

1.2. Overview of *Shigella* spp.

Shigella is a rod shaped, non-motile, non-spore forming, facultative anaerobic pathogen that belongs to the family of *Enterobacteriaceae.* There are four serogroups of disease-causing *Shigella*: *S. dysenteriae* (Serogroup A), *S. flexneri* (Serogroup B), *S. boydii* (Serogroup C) and *S. sonnei* (Serogroup D). The latter are further divided into serotypes according to their O-antigen structure (Simmons & Romanowska, 1987).

It is thought that *Shigella* spp. evolved from a non-pathogenic *Escherichia coli* ancestor. In fact, this theory suggests that an ancestral *E. coli* strain has acquired a virulence plasmid, now called the *Shigella* virulence plasmid. The genomic plasticity of *E. coli* and *Shigella* spp. and their acquisition of different virulence plasmid has allowed the development of two distantly related bacteria each having highly specific physiological traits and pathogenic properties (Ud-Din & Wahid, 2014; The et al., 2016).

1.3. Epidemiology

Worldwide, there are approximately 1.7 billion cases of diarrheal illnesses yearly and 5-15% of all diarrheal episodes are the outcomes of *Shigella* infections. According to the Centers for Disease Control and Prevention (CDC), 80-165 million cases of shigellosis occur worldwide yearly, resulting in 600,000 deaths annually, with 60% of deaths occurring in children under 5 years old living in low-income countries (Ferreccio et al., 1991; Liang et al., 2007). *S. sonnei* is more common in industrialized countries with improved water supply and sanitation and is one of the major causes of travel-associated diarrheal disease. On the other hand, *S. flexneri* is predominant in developing countries with poor hygienic conditions and limited access to clean water supplies. *S. sonnei* and *S. boydii* cause mild infections whereas *S. dysenteriae* is associated with major complications and high mortality and is known to cause outbreaks and epidemics worldwide (Madiyarov et al., 2010).

1.4. *Shigella* mode of transmission and invasion

Shigellosis is an intestinal infection that causes bacillary dysentery characterized by bloody stools, cramps, dehydration, fever and vomiting. *Shigella* transmission can occur by the fecal-oral route, person-to-person contact or by the ingestion of contaminated food or water (DuPont, Levine, Hornick & Formal, 1989). Ten to hundred bacterial cells are enough to cause an infection; this low infectious dose is due to that fact that *Shigella* is able to tolerate acid and thus it could survive the low pH of the stomach (Gorden & Small 1993). After being ingested the organism crosses the intestinal epithelial barrier via the microfold cells (M cells) to reach the colon and rectum (Ashida et al., 2007; Wassef, Keren & J.L, 1989). At this stage it invades the macrophages, multiplies and induces the death of phagosomes. Apoptosis is accompanied by the release of cytokines (Interleukin β and interleukin 18), which trigger a massive inflammatory response and lead to the activation of natural killer (NK) cells and the release of gamma interferon (IFN-γ) (Sansonetti et al., 2000; Le-Barillec et al., 2005). Once released from the dead macrophages, *Shigella* induces macropinocytosis and cross the basolateral surface of the enterocytes while also secreting factors that promote actin polymerization needed for it to spread freely from cell to cell (Sansonetti et al., 2000). *Shigella* escapes the entry to the vacuole by secreting factors that promote the formation of holes in it, leading to its destruction (Ogawa, 2005). It additionally releases lipopolysaccharides (LPS) and peptidoglycan into the host cell leading to further activation of inflammatory response and resulting in necrotic cell death (Ashida et al., 2007). Moreover, the bacterium secretes factors that mediate the recruitment of polymorphonuclear neutrophil leukocytes (PMN), which in its turn disrupts the integrity of the epithelial lining causing bloody and

watery diarrhea due to impaired adsorption of water, nutrients and solutes (Perdomo, Gounon & Sansonetti, 1994).

1.5. Virulence factors

1.5.1. *Shigella* virulence plasmid

Shigella carries a 220 kb virulence plasmid pINV which has two isoforms pINV A and pINV B of same incompatibility group (Lan, Lumb, Ryan & Reeves, 2001). The virulence plasmid consists of a conserved *mxi-spa* region (encoding components of the Type 3 secretion system) and genes encoding effector proteins such as invasion plasmid antigens (*ipa*s) (The et al., 2016). *Shigella* virulence plasmid is fundamental for virulence; it is needed for invasion, initiation of infection and modulation of the host immune response. *Shigella* spp. acquired an additional 3-kb virulence plasmid pHS- 2 that encodes one gene (*cld* or $wzz_{pHS\text{-}2}$) needed for the determination of O antigen chain length, which is also important as it renders the bacterium resistant to serum killing (Réhel & Szatmari, 1996).

1.5.2. Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) forms the outer membrane of *Shigella* spp. and other Gram-negative bacteria, it is made of a lipid A region, core oligosaccharide and an O-antigen polysaccharide (Passwell, Harlev et al., 2001). The O-antigen polysaccharide chain length and composition differs among *Shigella* species. The interaction between the host and bacterium LPS elicits an immune response, making the LPS an important virulence factor. When the LPS lipid A enters the host cell it leads to endotoxic reactions: fever, hypotension, leukocytosis and coagulation. *Shigella* LPS also confers serum resistance and is required for intercellular spread. In *S. flexneri*, the LPS biosynthesis locus *rfb- rfc- rol* and the LPS core synthesis locus *rfa* are located on the chromosome (Hong & Payne, 2003). On the other hand, O-

antigen synthesis genes are located on the large *Shigella* virulence plasmids or the 3kb pHS-2 plasmid. Previous studies have shown that glucosylation modification of *Shigella* LPS (mediated by *gtrA* and *gtrB*) reduces the O-antigen length, which makes the T3SS more accessible for contact with host epithelial cells (West, 2005). By revealing the T3SS, the LPS contributes in its activation and is indirectly involved in *Shigella* adhesion and invasion. In fact, *wzzB* and $wzz_{\text{pHS-2}}$ are the genes that regulate O-antigen chain length distribution. Moreover, S-OAg (a particular O antigen polymer length) and glucosylation of the O antigen are both required for *Shigella* to resist the acidic pH of the stomach. Notably, in acidic environments the LPS structure gets modified by the addition of phosphoethanolamine to the 1' phosphate of lipid A, leading to acid resistance (Mattock & Blocker 2017; Martinić, Hoare, Contreras, & Álvarez, 2011).

1.5.3. The type three secretion system (T3SS)

Shigella spp. utilize secretion systems to secrete proteins across the inner and outer membrane. To date, seven secretions systems were discovered, out of which six are detected in Gram-negative bacteria and one is usually found in Gram-positive bacteria. T3SS, T4SS, T5SS and T6SS are contact-dependent secretion systems as opposed to T1SS and T2SS which are contact independent.

During the process of host invasion, *Shigella* spp. delivers several different virulence effectors through the type three secretion system. The first group of effectors secreted by the T3SS is plasmid encoded IpaA, IpaB, IpaC and IpaD proteins needed for cell invasion and for the secretion of other effector proteins. A second group of genes encode "membrane expression of *ipa*" (Mxi protein) and "surface presentation of *ipa*" (Spa proteins) important for the functioning and translocation of T3SS effector proteins. Group 3 consists of transcription activators *virB* and *mxiE*, located on the

virulence plasmid whereas group 4, consists of genes encoding chaperones (*ipgA, ipgC, ipgE* and *spa15*) that stabilize T3SS substrates (Schroeder, Hilbi, 2008; Blocker et al., 1999).

Upon contact with the epithelial cells the bacterium secretes many of the above-mentioned effectors through the T3SS, causing actin polymerization and depolymerization. After crossing the epithelial barrier, it secretes effectors that mediate escape from autophagy and monitor host cell inflammatory response, making the T3SS one of the most important virulence factors needed for invasion, macrophage killing and immune evasion (Ménard, Sansonetti & Parsot, 1994; Killackey, Sorbara & Girardin, 2016).

1.5.4. Type six secretion system (T6SS)

The type 6 secretion system (T6SS) is an essential virulence weapon employed by several bacteria to target host cells but also to compete against bacteria present in the environment. The T6SS machinery is structurally homologous to T4 bacteriophage tail and has core components similar to those found in the bacteriophage (Anderson, Saffarian et al., 2017). The system is widely distributed among Gram-negative bacteria where it plays a pivotal role in bacterial niche adaptation. The proteinaceous T6SS machinery translocates a wide variety of effector proteins all at once, into the eukaryotic host cells (anti-eukaryotic activity) or into competitor bacterial cells (antibacterial activity) (Sana, Lugo & Monack, 2017). T6SS was first described in 2010 in *Pseudomonas aeruginosa* and later in other Gram-negative bacteria (*Vibrio cholera*, *Burkholderia thailandensis* and *Serratia marcescens*). The latter is encoded within a large gene cluster containing three conserved genes that encode for proteins needed to form the basic structure of the machinery. Additionally, T6SS gene cluster encodes system specific proteins (effectors) and accessory proteins. Toxic

antibacterial effectors are found within or outside the T6SS gene cluster, near immunity proteins that protect the secreting cells from their own toxins. Notably, the majority of effectors secreted by T6SS are antibacterial toxins used for competition against rival bacteria found in poly-microbial environments. The first antibacterial effectors to be identified were the peptidoglycan amidase (Tae) and peptidoglycan glycoside hydrolase (Tge) enzymes that target and destroy bacterial cell wall. In addition, several T6SS effectors are lipase/esterase enzymes (Tle). The above-mentioned effectors are grouped into families according to their sequence and phylogenic classification (Durand, Cambillau, Cascales & Journet, 2014). On the other hand, the anti-eukaryotic effectors identified play a role in actin crosslinking and microtubule-dependent invasion. Interestingly, some effectors encoded by H2 and H3 loci, such as phospholipase D (PldA and PldB) could destroy both eukaryotic and prokaryotic cells by targeting their phospholipid bilayer. In fact, H1, H2 and H3 loci mediate non-phagocytic cell invasion (In eukaryotic and prokaryotic cells). It is noteworthy to mention that T6SS also modulates bacterial interaction in the mammalian gut and influences the overall host health. The gut commensal *Bacteroides fragilis* for example, requires T6SS to persist in the gut, making the T6SS a key player in dictating microbial composition in the host gut (Cianfanelli, Monlezun & Coulthurst, 2016). Recently, in an attempt to explain why *S. sonnei* is increasing globally at the expense of *S. flexneri* it was shown that *S. sonnei* but not *S. flexneri* encodes a functional T6SS. The discovery of T6SS uncovered a new mechanism employed by *S. sonnei* for bacterial competition. Bio-informatic analysis of *S. sonnei* sequences revealed the presence of a conserved region consisting of 22 open reading frames that aligned with *E. coli* T6SS, a similar region was detected in *S. flexneri* but was missing genes and was inactivated by stop codons or insertion sequences

(Anderson, Saffarian et al., 2017). This discovery shows that at one point, *S. flexneri* encoded a T6SS that was then lost or inactivated. When *S. sonnei* strains are grown in direct contact with other *Shigella* subgroups and *E. coli* they are able to outcompete the latter by utilizing their T6SS, while *S. flexneri* is unable to do so. Moreover, *Shigella* T6SS is more active near the surface of the intestinal epithelium as opposed to the lumen and is needed in early stages of infection. The finding that *S. sonnei* encodes a T6SS could explain the fact that *S. sonnei* has a competitive advantage over *S. flexneri* and also explains the increasing predominance of *S. sonnei* worldwide (Anderson, Saffarian et al., 2017).

Additionally, a reason why *S. sonnei* is less predominant in low-income countries is that water supplies in such countries are contaminated with *Plesiomonas shigelloides* which express a lipopolysaccharide O antigen similar to that found in *S. sonnei*, so the population has developed immunity against this antigen and thus indirectly became less susceptible to *S. sonnei* infections (Sack et al., 1994; Liu et al., 2008).

1.5.5. Toxins

Shiga toxin (Stx) was first discovered in *S. dysenteriae* and is considered to be one of the most potent bacterial toxins. Stx has two homologues Shiga toxin 1 and Shiga toxin 2, both associated with bloody diarrhea. These homologues are further classified into subtypes depending on their structure and mechanism of action: there are three stx1 and seven stx2 subtypes. Shiga toxins are known to inhibit protein synthesis within host cells, but also induce apoptosis and alter gene and protein expression in host epithelial cells, immune and endothelial cells. By binding to polymorphonuclear leukocytes stx could also reach the kidneys and cause renal endothelium damage and hemolytic uremic syndrome (HUS) in later stages (Mora et al., 2012; Melton- Celsa, 2014).

Additionally, *Shigella* spp. secretes four different enterotoxins (ShET1, ShET2, Pic, SepA) that cause the accumulation of electrolytes in the small intestine and disrupt the host osmotic balance, which results in watery diarrhea. *Shigella* enterotoxin 1 (ShET1) and Pic enterotoxin are found on the chromosome, whereas *Shigella* enterotoxin 2 (ShET2) and SepA are found on the pINV virulence plasmid. ShET1 is only secreted by *S. flexneri*, while ShET2 is secreted by all *Shigella* serogroups and is known to induce the release of IL-8. On the other hand, Pic enterotoxin cleaves mucin (glycoprotein found in the mucous layer of epithelial cells) and causes its release, thus allowing the bacteria to breach the protective mucous layer of the host intestinal epithelial cells (Cherla, Lee & Tesh, 2003; Vargas et al., 1999).

1.5.6. Iron acquisition systems

Most of the iron present in host cells is tightly bound to heme or transferrin and not readily accessible for bacterial usage (Payne, Wyckoff et al., 2006). The concentration of available iron in the host is 10^{-24} M, while *Shigella* require an iron concentration of 10^{-7} M to be able to survive in the host (Raymond, Dertz et al., 2003). The need of iron comes from the fact that it is an essential co-factor used by several enzymes, but an excess of the element is toxic for the bacterium. *Shigella* spp. has developed several strictly regulated mechanisms to counteract the low iron availability in host cells (Imlay, Chin & Linn, 1988). The iron-uptake systems used by *Shigella* spp. are classified into three groups: systems for the use of ferric irons, systems for the use of ferrous iron and finally systems for the use of iron bound to heme.

Shigella spp. has developed several mechanisms to synthesize, secrete and uptake iron-chelating agents called siderephores. The type of siderophores synthetized vary by species but the four *Shigella* species uptake siderephores in the same way (Hider & Kong, 2010). In fact, the siderophore-Fe^{3+} complex binds to receptors on the outer-

membrane then binds to a periplasmic binding protein (PBP) and at the final stage, ABC permease facilitates its transport across the inner-membrane. The transport of siderophore-Fe^{3+} complex across the outer and inner-membranes requires energy. Energy is produced from a proton gradient and is transduced by TonB/ExbB/ExbD complex to outer-membrane receptors, thus providing the needed energy for the siderophore-Fe^{3+} complex to cross the outer-membrane. The complex then needs ATP to cross the inner-membrane. Once inside the bacterial cell, iron is detached from the siderophore and could be used by the bacterium. Several *Shigella* siderophores have been identified, the first being the enterobactin which is known to have the highest affinity for Fe^{3+} (Miethke & Marahiel, 2007; Wei & Murphy, 2016). The *ent* genes are needed for the synthesis and secretion of enterobactin and the *fep* genes (*fepA*, *fepB*, *fepCDG*) encode receptors and proteins needed for the uptake of the enterobactin. *S. sonnei* and *S. dysenteria* possess a functional *ent/fep* locus whereas *S. flexneri* and *S. boydii* have an inactive *ent/fep* locus interrupted by a stop codon or an insertion sequence (Ozenberger, Schrodt & McIntosh, 1987). Nonetheless, Host cells are able to produce lipocalin 2 protein that binds to enterobactin, making iron less readily available for the bacterium to use. To overcome this barrier, *S. dysenteriae* produce salmochelin, a siderophore that does not bind lipocalin 2. In fact, *S. dysenteriae* have the *iro* locus that encode enzymes needed for the production, secretion and internalization of salmochelin (Flo et al., 2004; Wyckoff et al., 2009). Aerobactin is another siderophore used by *S. boydii*, *S. sonnei* and *S. flexneri* and is encoded by *iucABCD* gene found on the same locus with *iutA* which encodes an aerobactin outer-membrane receptor (Lawlor & Payne, 1984; Carbonetti & Williams, 1984).

Shigella spp. also have receptors that recognize siderophores produced by other microorganisms (xenosiderophores), such as the ferrichrome which is produced by

Fungi and has a structure similar to that of aerobactin (Miethke & Marahiel, 2007). Moreover, *S. sonnei* use ferric-dicitrate (iron bound to citrate) as an iron source; this process is mediated by *fecA*, *fecB*, *fecCDE* genes encoded within the *fec* locus (Luck et al., 2001).

The ferrous (Fe^{2+}) iron utilization system is another mechanism used by *Shigella* spp. to capture iron under anaerobic or acidic environment (Kammler, Schon & Hantke, 1993; Wei & Murphy, 2016). To date, three ferrous utilization systems have been identified in *Shigella* with the first being the Feo system (*feoA*, *feoB*, *feoC*) (Marlovits et al., 2002; Kim, Lee & Shin, 2012). The second ferrous-capture system used by *Shigella* is the sit locus that contains *sitA,* encoding the periplasmic binding protein (PBP) and *sitBCD*, encoding the ABC permease protein complex (Fisher et al., 2009; Wei & Murphy, 2016). The sit system is used by *S. flexneri* for iron-uptake but also to from plaques in host cells which renders the bacterium even more pathogenic (Andrew et al., 2003; Runyen-Janecky et al., 2003). On the other hand, *S. sonnei* uses the EfeUOB system that is composed of *efeU,* encoding an inner membrane permeases and *efeO*/*efeB* encoding a periplasmic protein needed for the transport of Fe^{2+}(Payne & Alexandra, 2010; Grobe et al., 2006).

The bacterium not only uses ferric and ferrous iron transport systems to capture free intracellular iron but also uses heme transport systems to transport iron bound-heme into the cytoplasm. Finally, the Shu system, heme-transport system, was first detected in *S. dysenteriae* and then identified in some *S. sonnei* strains. The system includes, *shuA* encoding the outer- membrane heme receptor, *shuT* encoding a PBP and *shuUV* encoding an inner membrane ABC permease (Wyckoff et al.,1998; Burkhard & Wilks, 2008).

1.5.7. Pathogenicity islands (PAIs)

Other factors contributing also to *Shigella's* virulence are the pathogenesis-associated genomic regions, called pathogenicity islands (PAIs). PAIs could be located on the chromosome, plasmid, transposons or phages and are acquired through horizontal gene exchange. These mobile genetic elements are characterized by their nucleotide composition (GC content) and codon usage that differ from the rest of the genome (Al Hasani et al., 2000). *Shigella* has several pathogenicity islands among these is the *Shigella* island 1(SHI-1), which carries several virulent determinants such as: *sigA* (codes for a protease, which causes intestinal fluid accumulation and the release of Shiga toxin), *pic* (encoding for a serine protease which causes mucus permeabilization, serum resistance and hemagglutination) and *set1A* and *set1B* encoding the enterotoxin ShET1 (Behrens, 2002). *Shigella* island 2 (SHI-2) and SHI-3 on the other hand have *iucA, iucB, iucC, iucD* and *iutA* genes needed for the bacteria to sequester iron in an iron deficient environment, whereas SHI-O carries *gtrA*, *gtrB* and *gtrV,* all of which are needed for the modification of O-antigens (serotype modification to escape the host immune response) (Purdy & Payne, 2001; Vokes et al 1999; Huan et al 1997). Other PAIs such as the SRI *Shigella* resistance locus carry tetracycline, chloramphenicol, ampicillin and streptomycin resistance determinants (Shroeder & Hilbi, 2008).

1.5.8. Patho-adaptative mutations and anti-virulence gene inactivation

Shigella spp. has undergone reductive evolution necessary for it to adapt to new milieus. This reductive evolution is not only characterized by the accumulation of pseudogenes but also by the inactivation of anti-virulence genes (Yang, 2005; Feng, Chen & Liu, 2011). The process of gene inactivation is not random and occurs in specific operons and regions of the genome. The loss of flagellum and fimbriae

biosynthesis gene for example is not an accidental event because it helps *Shigella* remain unrecognized by the host immune system (Bravo et al., 2015). Moreover, certain genes are present in *E. coli* but are inactivated or lost in *Shigella* such as the *cadA* anti-virulence gene that encodes a lysine decarboxylase. In *Shigella*, the *cadA* gene produces polyamine cadavrine which interferes with *Shigella* enterotoxin activity and inhibits PMN migration therefore deleting this gene was advantageous for the bacterium (Maurelli et al., 1998; Prosseda et al., 2012). Similarly, *Ompt*, an outer membrane protease was lost in *Shigella* because the protein interfered with T3SS effector protein VirG and consequently its ability to spread from cell to cell. Another similar example, is the disruption of *speG*, spermidine acetyltransferase gene, this resulted in the accumulation of polyamine spermidine intermediate, which allowed *Shigella* to resist macrophage killing (Nakata et al., 1993; Zhao et al., 2010). The loss of the above mentioned anti-virulence genes in *Shigella* spp. gave a selective advantage ensuring niche adaptation and better bacterial fitness.

1.6. Antibiotic resistance

Shigellosis infections were previously treated with sulfonamides, then later tetracycline and chloramphenicol were used. Nowadays, these drugs are no longer effective because *Shigella* has developed resistance against all. Treatment shifted to the use of ampicillin, then nalidixic acid followed by fluoroquinolones, ceftriaxone and then azythromycin with the organism developing resistance against all.

Resistance to tetracycline first appeared in *S. dysenteriae* in 1953 and since then tetracycline resistant *Shigella* increased dramatically. Tetracycline resistance in *Shigella* is mainly mediated by the products of two genes *tetA* and *tetB*, usually present on mobile genetic elements such as plasmids and transposons (Hartman, Essiet, Isenbarger & Lindler, 2003). On the other hand, fluoroquinolone resistance is due to

mutations in chromosomal genes "quinolone resistance determining regions" (QRDRs): *gyrA*, *gyrB* (gyrases), *parC* and *parE* (Type IV topoisomerases) and also plasmid encoded genes: *qnr (qnrA, qnrB, qnrC, qnrD, qnrS, qep), aac(6')lb-cr* (Das, Natarajan & Mandal, 2016).

1.6.1. β-lactam resistance

β-lactam is a class of antibiotic that inhibit enzymes needed for the formation of the bacterial peptidoglycan. β-lactam antibiotics such as penicillin and cephalosporin have long been the most prescribed antibiotics to treat bacterial infections (Shaikh et al., 2015). In 1977, ampicillin resistant *Shigella* was first isolated from an epidemic in Central America, then few years later in African and Asian countries (Barrantes & Achi, 2016). The persistent exposure of bacteria to β-lactam has pushed them to produce enzymes (ESBLs) to counteract the antibiotic's mechanism of action. Extended spectrum β-lactamase (ESBL) is an enzyme capable of hydrolyzing β-lactam antibiotics such as penicillin, cephalosporin and monobactams. The enzyme cleaves the four carbon β-lactam ring of the antibiotic thus rendering it ineffective. β-lactamases are divided into four classes (A, B, C, D) depending on their sequence homology; Classes A, C, D are serine lactamase as opposed to class B that is a metallo-lactamase (Shaikh et al., 2015). The first ESBL-producing bacterium was isolated from a hospital in Germany in 1983 and since then more strains are becoming β-lactam resistant (Rashid & Rahman, 2015). *Shigella* β-lactam resistance is mainly manifested by the presence of bla_{TEM}, bla_{SHV}, bla_{OXA}, and bla_{CTX-M} genes, usually present on transmissible plasmids. The most common ESBL genetic determinant is bla_{CTX-M}., today more than 100 variants of the protein exist. While SHV β-lactamase and TEM β-lactamase are result of a substitution in the original gene, bla_{CTX-M} genes are acquired by horizontal gene transfer of a plasmid or

transposon and are usually found in close proximity to IS*Ecp1* insertion sequence (Shaikh et al, 2015; Poirel, Naas & Nordmann, 2008). A single copy of the IS*Ecp1* insertion sequence is sufficient to mobilize $bla_{\text{CTX-M}}$ and enhance its expression. In 2005, ESBL- producing *S. sonnei* were isolated for the first time in Lebanon, the isolates harbored the $bla_{\text{CTX-M-15}}$ encoded on a IncFII plasmid. Moreover, the gene was found to be flanked by the IS*Ecp1* insertion sequence therefore easily transmitted to other strains by HGT (Matar et al., 2009; Matar et al., 2007).

1.6.2. Carbapenem resistance

Carbapenems have a unique structure made of a carbapenem ring coupled to a β- lactam ring and is a member of the β-lactam class of antibiotics. Carbapenems have a broad spectrum of activity and are structurally related to penicillins. The antibiotic penetrates the bacterial cell wall, then binds and inactivates penicillin-binding proteins (PBPs) which leads to the inhibition of cell wall synthesis and death of the bacterium (Shaikh et al., 2015). The overuse of carbapenem to treat ESBL- producing *Shigella* has led to the emergence of Carbapenem resistant *Shigella* spp. However, carbapenem resistance in *Shigella* spp. still remains an infrequent event (Cadjoe & Donkor, 2017; Papp-Wallace et al., 2011).

Carbapenem resistance may be intrinsic or acquired by the gain of a transferable resistance gene; intrinsic carbapenem-resistance means that the bacterium is naturally resistant to the antibiotic. On the other hand, Carbapenemases are β-lactamase enzymes that hydrolyze imipenem or/and meropenem and are involved in acquired-carbapenem resistance. These enzymes are classified into three groups (Class A, B and D) according to their active site (Nordmann & Poirel, 2002). Class A and D carbapenemases have a serine-based hydrolytic mechanism, as opposed to class B carbapenemases that contain zinc in their active site and are metallo-β-lactamases.

Ambler class A carbapenemase include SME, IMI, NMC, GES and KPC enzymes. *bla*$_{KPC-1}$ is the most prevalent ambler class A carbapenemase and is easily transmissible since it is encoded on a 50kb plasmid. On other hand, class B carbapenemase include IMP, VIM, SPM, GIM and SIM families of enzymes with NDM-1 being the most prevalent one (Queenan & Bush, 2007). The genes encoding these enzymes are often found on class 1 integrons. IMP-3 was first identified in *S. flexneri* isolated from Japan. *S. flexneri bla*$_{IMP-3}$ was located on a class 1-integron found on a transferable plasmid and showed high sequence similarity with *bla*$_{IMP-1,}$ with only two different amino acids (Gly-to-Ser change at position 262) (O'Hara et al., 1998; Iyobe et al., 2000). Class D carbapenemase consists of OXA-type-β-lactamases and are most commonly detected in *A. baumannii* isolates from different parts of the world (Nordmann & Poirel, 2002). Carbapenem resistance is not only due to the production of carbapenemase but is also a result of porin channel alterations or loss, along with an upregulation of β-lactamase genes (*bla*$_{OXA}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$) (Martinez- Martinez et al., 1999; Nordmann & Poirel et al., 2012; Barroud et al., 2012). Another possible mechanism is the upregulation of efflux pumps which leads to the export of carbapenems outside the bacterial cell (Cadjoe & Donkor, 2017).

1.6.3. Colistin resistance

Colistin is the last resort antibiotic used to treat carbapenem resistant strains. The antibiotic associates with the lipopolysaccharide (LPS) outer membrane and causes membrane destabilization which leads to leakage of cellular content followed by lytic cell death (MacNair et al., 2018). The overuse of colistin has led to the appearance of colistin resistant strains. Acquired resistance to polymyxin is associated with LPS modifications that are mediated by chromosomal mutations. However, polymyxin resistance is also mediated by a *mcr-1* gene found on a transferable plasmid

(IncI2, IncF, IncX4, IncP or IncHI2) (Liang et al., 2018). The first *mcr-1* encoding *S. sonnei* strain (EG430) was isolated in 2008 from a hospitalized child in Ho Chi Minh City (Vietnam), but the gene was inactive due to a frameshift mutation caused by the duplication of the open reading frame of *mcr-1*. *mcr-1* encoding *S. sonnei* was also detected in China with the gene being active and conferring polymyxin resistance (Ma, Huang et al., 2018). The study also showed that the *mcr-1* gene was encoded on an IncI2 plasmid and co-existed with other β-lactamase genes such as, $bla_{CTX-M-1}$, $bla_{CTX-M-8}$, $bla_{CTX-M-14}$, bla_{SHV-12}, bla_{NDM-1}, $bla_{CTXM-55}$, bla_{NDM-5} and bla_{TEM-52}. In addition, several insertion sequences were found to be associated with the *Shigella mcr-1* gene such as IS*1*, IS*1294* and IS*Apl1*. Insertion sequences are highly abundant in *Shigella* species and they play an essential role in the horizontal gene transfer of resistance genes (Thanh et al., 2016; Ma, Huang et al., 2018). Later, a plasmid-mediated mcr-1 gene in *S. flexneri* was reported in China, the gene was located on a novel transposon and flanked by inverted repeats of IS*Apl1*(Liang et al., 2018).

1.6.4. Plasmids and Integrons

Mobile genetic elements such as plasmids, transposons but also gene cassettes on integrons are important to understand the mechanism of antimicrobial resistance in *Shigella*. A small spA resistance plasmid found in most *Shigella* strains encodes sulfonamide, streptomycin and tetracycline resistance rendering some strains hard to treat. Furthermore, *S. sonnei* and *S. flexneri* have an extended-spectrum β-lactamase (ESBL) producing plasmids and some strains were shown to have plasmid pKSR1, which carries azithromycin, erythromycin, β-lactams and aminoglycosides resistance genes (Holt et al., 2012; Baker et al., 2015).

Integrons are mobile genetic elements that capture and exchange genes within cassettes. They are composed of a recombination site (*attI*), a promoter (Pc) and an

integrase gene. Integrons are classified into eight groups according to their integrase gene sequence. Class 1 and class 2 integrons are the most widespread integrons in *Shigella* spp. Previous reports have showed that *S. sonnei* and *S. boydii* strains contain class 2 integrons whereas *S. flexneri* and *S. dysenteriae* contain class 1 integrons. Moreover, *Shigella* carry gene cassettes encoding for factors that confer resistance to streptomycin, spectinomycin, trimethoprim and ampicillin (DeLappe et al., 2003; Madiyarov et al., 2010; Ahmed et al., 2006).

Based on the above overview the aims of the study were:

- Describe the genetic and phenotypic characteristics of *Shigella* spp. isolates collected from Lebanon.
- Identify molecular determinants involved in host adaptation and virulence.
- Determine the antimicrobial resistance frequencies for antibiotics commonly used to treat Shigellosis.
- Investigate the antimicrobial resistance profile and the resistance gene reservoirs in the collected isolates.
- Detect the presence of genes conferring resistance to β-lactam antibiotics.
- Identify patterns of plasmid distribution and perform a correlation analysis between plasmid profiles and β-lactam resistance.
- Investigate the prevalence of class 1 and class 2 integrons.
- Study the molecular relatedness among ESBL-producing *Shigella* isolates.
- Perform a comparative analysis between the *Shigella* spp. isolates and find clonality, if present, among isolates.
- Determine the phylogenic relatedness of isolates by using genome sequence data.

Chapter Two

Material and Methods

2.1. *Shigella* spp. Isolates

This study was conducted on ten *Shigella* spp. isolates collected from 2013 to 2016. The clinical isolates were designated as SHS-1- SHS-10 and were recovered from the American University of Beirut Medical Center (AUBMC) and from Sayyidat Al Maounat University hospital. Eight out of ten isolates are *Shigella sonnei*, one is a *S. flexneri* (isolate SHS-2) and one is a *S. boydii* (isolate SHS-4). The bacterial isolates were collected from patients' stool and were cultured on Trypticase Soy Agar (TSA).

2.2. Antimicrobial susceptibility testing

The Kirby Bauer disk diffusion test was performed using Mueller-Hinton agar. The antibiotics used were the following: penicillin (ampicillin), sulfonamide (sulfamethoxazole-trimethoprim), 3rd generation cephalosporins (cefotaxime, ceftazidime, ceftriaxone), quinolones (ciprofloxacin, norfloxacin) and tetracycline. The results obtained were interpreted according to the Clinical & Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017).

2.3. DNA extraction

Bacterial DNA was extracted from fresh colonies using the Nucleospin® Tissue kit (Macherey-Nagel, Germany) according to the manufacturer's instructions and quantified using NanoDrop 1000 spectrophotometer V3.7.

2.4. PCR assays for resistance genes

The ESBL encoding gene *bla*$_{\text{CTX-M}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$ were amplified and sequenced as previously described (Weill et al, 2004; Ashraf et al., 2006). The primers used, annealing temperatures and amplicon sizes are listed in table 2.

Table 1. Resistance genes amplified by PCR, primer sequences and amplicon size. (Ashraf et al., 2006).

Genes	Primers	Sequences (5'-3')	Ta (°C)	Amplicon size (bp)
TEM	*TEM-F*	ATAAAATTCTTGAAGACGAAA	50	1080
	TEM-R	GACAGTTACCAATGCTTAATC		
SHV	*SHV-F*	TTATCTCCCTGTTAGCCACC	50	795
	SHV-R	GATTTGCTGATTTCGCTCGG		
OXA	*OXA-F*	TCAACTTTCAAGATCGCA	57	591
	OXA-R	TCAACTTTCAAGATCGCA		
CTX-M	*CTX-M-F*	ATGGTGACAAAGAGAGTGCAA	50	550
	CTX-M-R	TTAGACCCCTTCGGCGAT		

2.5. PCR amplification for virulence determinants *ial*, *ipaH*, *set*, *sen* and *virA*

ial, *ipaH*, *set*, *sen* and *virA* virulence genes were amplified as previously described (Cassabone et al, 2016; Lluque et al, 2015). The primers used are listed in table 2 along with the annealing temperatures and expected amplicon size.

Table 2. PCR amplification of virulence genes, primer sequences, amplicon size and annealing temperatures.

Virulence factor	Primers	Sequences (5'-3')	Ta (°C)	Amplicon size (bp)
ial	*ial*-R	CTGGATGGTATGGTGAGG	56	320
	ial-F	GGAGGCCAACAATTATTTCC		
ipaH	*ipaH*-R	GCCGGTCAGCCACCCTCTGAGAGTAC	60	661
	ipaH-F	GTTCCTTGACCGCCTTTCCGTACCGTC		
virA	*virA*-R	TGATGAGCTAACTTCGTAAGCCCTCC	65	215
	virA-F	CTGCATTCTGGCAATCTCTTCACATC		
set	*set*-R	TATCCCCCTTTGGTGGTA	50	309
	set-F	TCACGCTACCATCAAAGA		
sen	*sen*-R	CATAATAATAAGCGGTCAGC	55	799
	sen-F	ATGTGCCTGCTATTATTTAT		

2.6. Detection of class 1 and 2 integrons

PCR was performed to detect the presence of class 1 and 2 integrons as previously described (Madiyarov et al, 2010; Ahmed et al, 2006). Primers *int1L*-F (ACATGTGATGGCGACGCACGA) and *int1*-R (ATTTCTGTCCTGGCTGGCGA) were used for class 1 integron and resulted in a 569 bp amplicon. Primers *int2*-F (GTAGCAAACGAGTGACGAAATG) and *int2*-R (CACGGATATGCGACAAAAAGGT) were used for class 2 integron detection and resulted in a 789 bp amplicon.

2.7. Plasmid based replicon typing

DIATHEVA PBRT kit (Diatheva, Fano, Italy) was used for PBRT typing. Eight multiplex PCRs were performed for the amplification of 25 Inc Groups found in *Enterobacteriaceae*: A/C, B/O, FIA, FIB, FIB-M, FIC, FII, FIIK, FIIS, HI1, HI2, HIB-M, I1, I2, K, L/M, N, P, R, T, U, W, X1, X2, and Y. Positive controls were used for each multiplex PCR performed. The amplicons were visualized on a 2.5 % agarose gel and ethidium bromide was used for gel staining.

2.8. Pulsed-field gel electrophoresis (PFGE)

PFGE performed according to the procedure described by Pulse Net from the website of the Centers of Disease Control and Prevention. *XbaI* restriction enzyme (Thermo Fisher Scientific, MA, USA) was used and *Salmonella enterica* subsp. *enterica* serovar *Braenderup* (ATCC® BAA664TM) was used as a reference. Bio-Rad laboratories CHEF DR-III system (Bio-Rad Laboratories, Bio-Rad Laboratories Inc., Hercules, CA, USA) was used for electrophoresis with a total run time of 18h and switch time of 5-35s. The gel was stained with ethidium bromide. BioNumerics software version 7.6.1 was used to generate a dendogram (Applied Maths, St-Martens-Latem, Belgium). Fingerprints with three or more different bands were assigned a

different pulsotype.

2.9. Whole Genome Sequencing and assembly

Illumina Nextera XT DNA library preparation kit (Illumina) was used for library preparation. 1µg of genomic DNA (gDNA) was used. Each isolate gDNA was subject to end-pairing, A-tailing, ligation of adaptors and sample-specific barcodes. Qubit® 2.0 fluorometer was used for library quantification (Invitrogen, Carlsbad, CA, USA). Sequencing was performed on an Illumina MiSeq using paired-end 500 cycle protocol to obtain a 250 bp read length. The genome was assembled *de novo* using Spades Genome Assembler Version 3.9.0 (Bankevich et al. 2012). Quality control checks of the raw sequences was done using FastQC version 1.0.0 (Andrews et al., 2010).

2.10. Genome annotation

RAST server (http://rast.nmpdr.org) was used for genome annotation (Aziz et al., 2012). Goseqit (https://www.goseqit.com) and ResFinder 3.0 (www.genomicepidemiology.org) were used to detect resistance genes (Zankari et al., 2012). Virulence factors were determined using RAST, and VirFinder available on the Center for Genomic Epidemiology website. PlasmidFinder 1.3 and Plasmid Constellation Network (PLACNETw) were used to identify the plasmids. Sequence types (ST) were generated using the MLST 1.8 server available on goseqit database (CGE- www.genomicepidemiology.org.) according to the sequence seven housekeeping genes: *adk*, *fumc*, *gyrb*, *icd*, *mdh*, *pura* and *reca* (Larsen et al., 2012). Insertion sequences (ISs) were identified using IS-finder (https://www-is.biotoul.fr/) (Siguier et al., 2006). R-Studio (plot3D v1.1.1 package by Karline Soetaert) was used to generate a 3D plot representing the isolates' genome size, GC content and number of coding sequences. Comparative genome analysis and alignments to reference sequences were performed using BLAST, RAST and BioNumerics version 7.6.1

2.11. wgSNP-based phylogenetic analysis

The genome of all 10 isolates were aligned against five reference genomes: 2015AM-1099 (accession # CP021144), 2015C-3566 (accession # CP022457), Ss046 (accession # CP000038), 53G (accession # HE616528), 75/02 (accession # CP019689). BioNumerics software version 7.6.1 (Applied Maths, Sint-Martens-Latem, Belgium) was used for wgSNP-based phylogenetic analysis. Insertions and deletions were not included and the neighbor-joining (NJ) tree generated was based on categorical differences in SNP between the isolates. Another SNP tree was generated using the kSNP 3.0, a tool that finds SNPs on the basis of k-mer analysis. A k-mer length of 19 nucleotides is needed to generate the tree. In addition, at least 80% of the genomes should have a nucleotide at a given SNP position for this particular SNP to be included in the tree building. The tool uses the pan-genome for SNP detection and generates a SNP-based phylogenic tree (Gardner, Slezak et al., 2015). The reference genomes mentioned above were used for kSNP phylogenic analysis. The resulting tree was visualized on Phandango V 1.1.0 (Hadfield et al, 2017). Interactive Tree of Life v3 tool (iTOL) (http://itol.embl.de), was used for the display, manipulation and annotation of the phylogenic tree generated by kSNP 3.0.

2.12. Pan-genome analysis

Prokka version 1.13 (Seemann, 2014) was used to generate an annotated GFF3 file of the genomes (Stein, 2013). The input file was piped into Roary version 3.12.0. All-against-all comparison is done with BLASTPn. FastTree 2, a tool for inferring phylogenies for core genome alignments was used to generate a maximum-likelihood tree (Price et al., 2010). The pan-genome fingerprints of the isolate and the core genome SNPs were visualized on Phandango V 1.1.0 (Hadfield et al, 2017). The following *S. sonnei* reference genomes were included in the analysis: 2015AM-1099

(accession # CP021144), 2015C-3566 (accession # CP022457), Ss046 (accession # CP000038), 53G (accession # HE616528), 75/02 (accession # CP019689), 4304 (accession # CP029794), AR_0030 (accession # CP032523), ATCC 29930 (accession # CP026802), NCTC7924 (accession # UGZB01000001), NCTC12984 (accession # UGZF01000005). Two *S. dysenteriae* reference genomes were used: 1617 (accession # CP006736), Sd197 (accession # CP000034) along with two *S. boydii* genome references: ATCC9210 (accession # CP011511), CDC 3083-94 (CP001063). Finally, *S. flexneri* 2457T (accession # AE014073) was also included in the analysis.

Chapter Three

Results

3.1. Isolates information

The isolates draft genomes were deposited at the NCBI databases and were assigned the following accession numbers: PYKL00000000, PYKM00000000, PYKN00000000, PYKO00000000, PYKP00000000, PYKQ00000000, PYKR00000000, PYKS00000000, PYKT00000000, PYKU00000000.

Table 3. Strain types of the isolates undertaken in the study, date and place of collection and NCBI accession numbers.

Isolates		Hospital	Date	Accession #
SHS-1	*Shigella sonnei*	Maounat	2016	PYKL00000000
SHS-2	*Shigella flexneri*	Maounat	2016	PYKM00000000
SHS-3	*Shigella sonnei*	Maounat	2016	PYKN00000000
SHS-4	*Shigella boydii*	Maounat	2016	PYKO00000000
SHS-5	*Shigella sonnei*	AUBMC	2013	PYKP00000000
SHS-6	*Shigella sonnei*	AUBMC	2013	PYKQ00000000
SHS-7	*Shigella sonnei*	AUBMC	2014	PYKR00000000
SHS-8	*Shigella sonnei*	AUBMC	2014	PYKS00000000
SHS-9	*Shigella sonnei*	AUBMC	2014	PYKT00000000
SHS-10	*Shigella sonnei*	AUBMC	2014	PYKU00000000

3.2. Genome Content

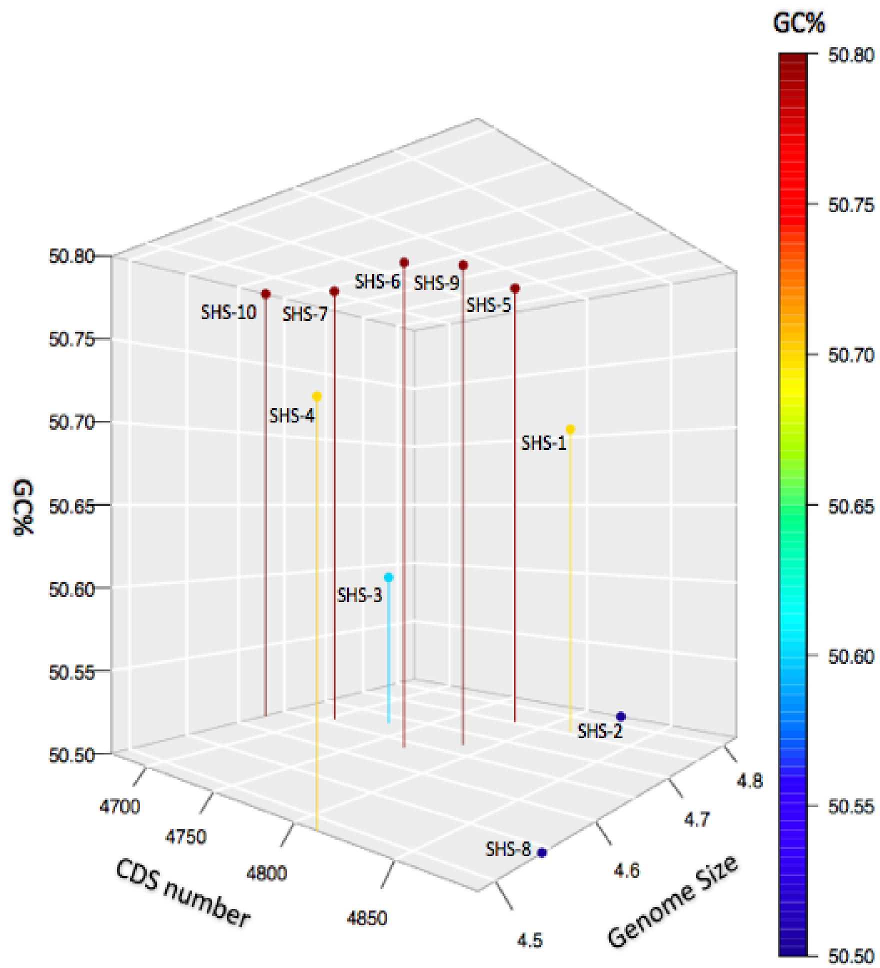

Figure 1. Isolates' Genome content. The 3D plot was generated using R-Studio plot3D (by Karline Soetart). The x axis represents the number of coding sequences of each isolate, y axis is the genome size and the z axis is the GC%.

3.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing revealed that 8/10 of the isolates (SHS-1, SHS-2, SHS-5, SHS-6, SHS-7, SHS-8, SHS-9, SHS-10) were resistant to ampicillin, and 3rd generation cephalosporins (cefotaxime and ceftriaxone). Most of the isolates (9/10 except SHS-2) were sulfamethoxazol-trimethoprim and tetracycline (8/10 except SHS-4 and SHS-10) resistant, while all were susceptible to ciprofloxacin and norfloxacin.

Table 4. Antimicrobial susceptibility testing results. Isolates are mostly resistant to ampicillin, sulfamethoxazole/trimethoprim, cefotaxime, ceftriaxone and tetracycline.

Isolates	Ampicillin	Sulfamethoxazol+ Trimethoprim	Cefotaxime	Ceftazidime	Ceftriaxone	Ciprofloxacin	Norfloxacin	Tetracycline
SHS-1	■	■	■		■			■
SHS-2	■		■		■			■
SHS-3		■						■
SHS-4		■						
SHS-5	■	■	■		■			■
SHS-6	■	■	■		■			■
SHS-7	■	■	■		■			■
SHS-8	■	■	■		■			■
SHS-9	■	■	■		■			■
SHS-10	■	■	■		■			

Penicillin
Sulfamide
3rd Generation Cephalosporins
Quinolones
Tetracycline

3.4. Resistance genes

Antibiotic resistance genes were detected through PCR assays and *in silico*. A total of 13 genes conferring resistance to aminoglycosides, β-lactams, sulfonamides, trimethoprim, tetracycline and phenicol were detected. (Table 2).

Aminoglycoside resistance was manifested by four resistance determinants: *strA*, *strB*, *aadA1*, *aac(3)-IId,* with all isolates except SHS-2 being positive for *strA* and *strB*. SHS-2 along with SHS-4 and SHS-5 additionally carried the *aadA1*gene, while SHS-7, SHS-8, SHS-9 and SHS-10 had the *aac(3)-IId* gene.

β-lactam resistance was linked to the presence of four resistance determinants which were detected *in silico* and confirmed through PCR assays. $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B}}$ were the most common β-lactamase encoding genes and detected in half of the studied isolates (SHS-5, SHS-7, SHS-8, SHS-9 and SHS-10). It is important to note that isolates harboring the $bla_{\text{CTX-M-15}}$ ESBL variant were also positive for $bla_{\text{TEM-1B.}}$ Only one isolate (SHS-2) had $bla_{\text{OXA-1}}$ ESBL variant and two (SHS-1 and SHS-6) had the $bla_{\text{CTX-M-3.}}$

Sulfonamide and trimethoprim resistance can be conferred by the presence of only one resistant determinant, *sul2* and *dfrA1*, respectively. All isolates, except SHS-2 harbored the *sul2* gene, and most (8/10 except SHS-2, and SHS-10) had *dfrA1* trimethoprim resistance determinant.

Tetracycline resistance also showed little variability with only two resistance genes being detected: *tetA* and *tetB*. *tetA* was the most common and was identified in 7/10 of the isolates, whereas *tetB* was only detected in SHS-2.

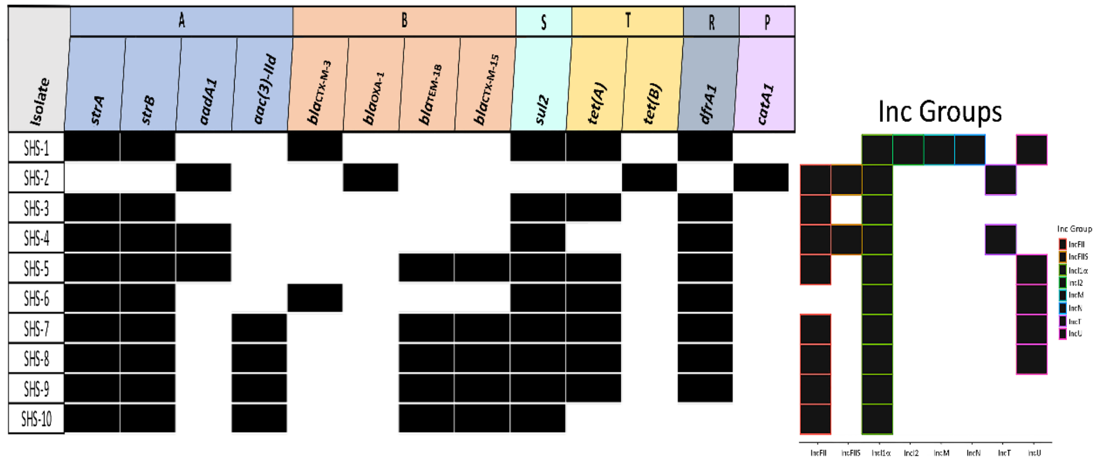

Table 5. Antibiotic resistance genes were detected by PCR and *in silico*. Classes of antibiotic resistance genes were designated as: A for aminoglycoside resistance determinants; B for β-lactam resistance determinants; S for sulfonamide resistance determinants; T for tetracycline resistance determinants; R, trimethoprim resistance determinants and P for phenicol resistance determinants. Incompatibility groups were identified in silico using plasmid finder and by PCR-based replicon typing.

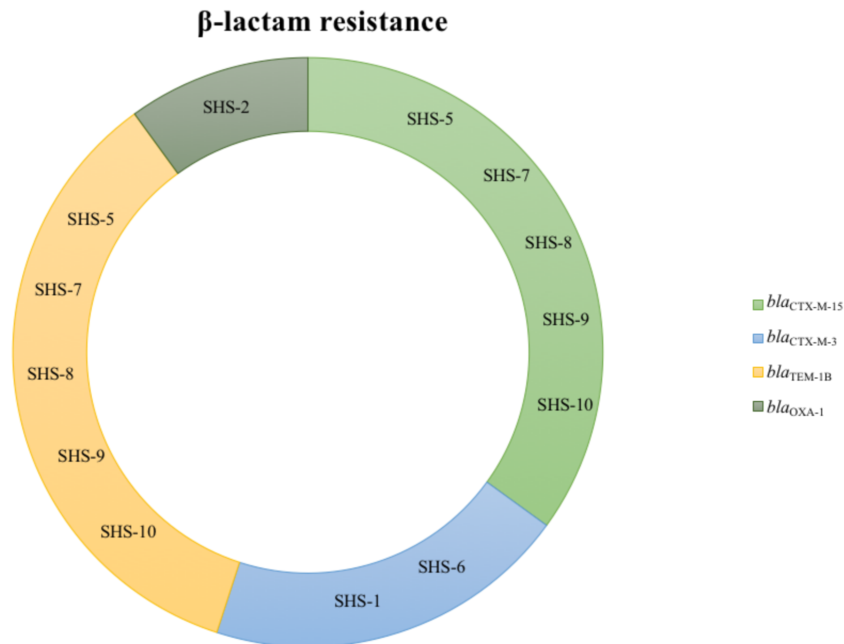

Figure 2. Doughnut chart showing the β-lactam resistance profile. β-lactam resistance was mainly mediated by *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM-1B}}$.

3.5. Plasmid typing

PCR-base replicon typing (PBRT) was performed to determine the Inc groups. PlasmidFinder was also used to confirm the results obtained through the PCR assays (Table 2). A total of eight Inc groups were identified with all isolates having at least one. The highest number of Inc groups was detected in SHS-1, with it being the only isolate positive for Inc M and IncN. IncI1α and IncFII were the two most commonly identified; IncI1α was detected in all the isolates whereas IncFII was detected in 8/10 of the isolates. Alignments using BLAST, RAST and BioNumerics revealed that SHS-2 bla_{OXA-1} is encoded on a class 1 integron that is integrated on IncFII type plasmid, while SHS-1 $bla_{CTX-M-3}$ was detected on an IncI1α plasmid. $bla_{CTX-M-15}$ and bla_{TEM-1B} in isolates SHS-5, SHS-7, SHS-8, SHS-9 and SHS-10 were on an IncFII plasmid. The genetic environments of $bla_{CTX-M-3}$, $bla_{CTX-M-15}$ and bla_{TEM-1B} were elucidated by aligning the reads to the following reference plasmids: pEK204 (accession # EU935740) and reference plasmids pSH4469 (accession # KJ406378) and pKHSB1 (accession # HF572032) respectively (Figure 3 and 4).

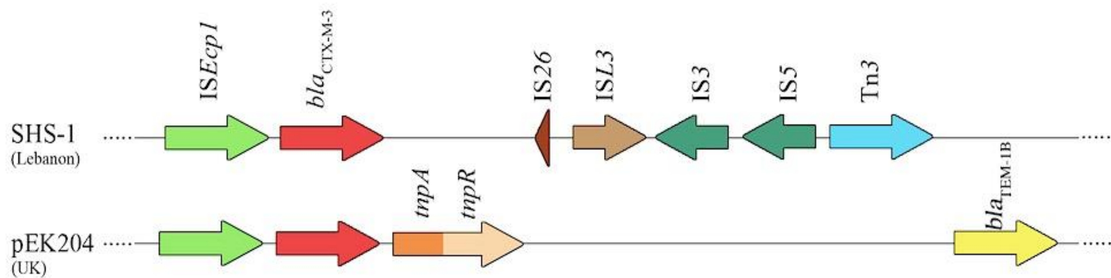

Figure 3. Genetic environment of $bla_{CTX-M-3}$ encoded on IncI1α plasmid in SHS-1. Alignment of SHS-1 with plasmid pEK204 indicate that IS*Ecp*1 is in close proximity to $bla_{CTX-M-3}$ in both plasmids. In pEK204, $bla_{CTX-M-3}$ co-existed with bla_{TEM-1B}.

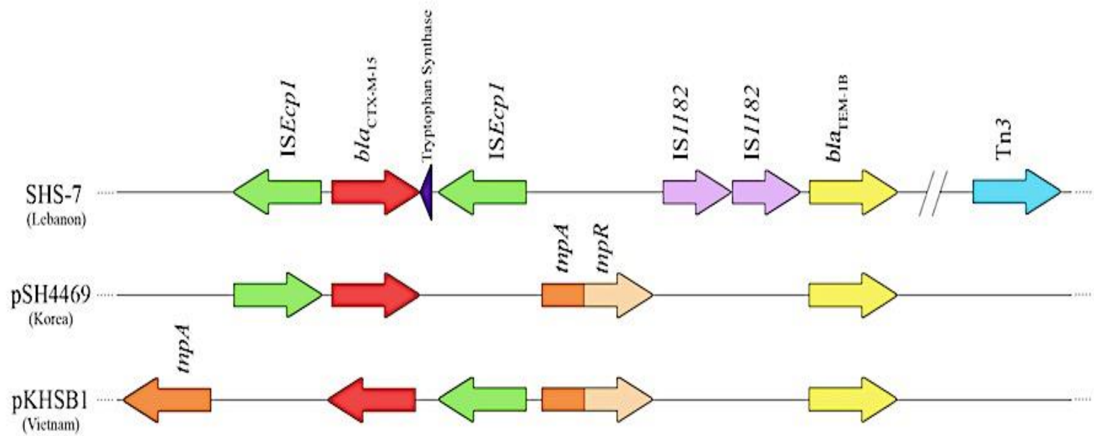

Figure 4. Genetic environment of *bla*CTX-M-15 and *bla*TEM-1-B in SHS-7. Alignment of SHS-7 with plasmids pSH4469 and pKHSB1 indicate that *bla*CTX-M-15 and *bla*TEM-1-B are close to each other with *bla*CTX-M-15 being flanked by IS*Ecp1* insertion sequence in all three plasmids. SHS-7 ⁄⁄ : IS*21*, *parA*, Error prone DNA polymerase, IS*5*.

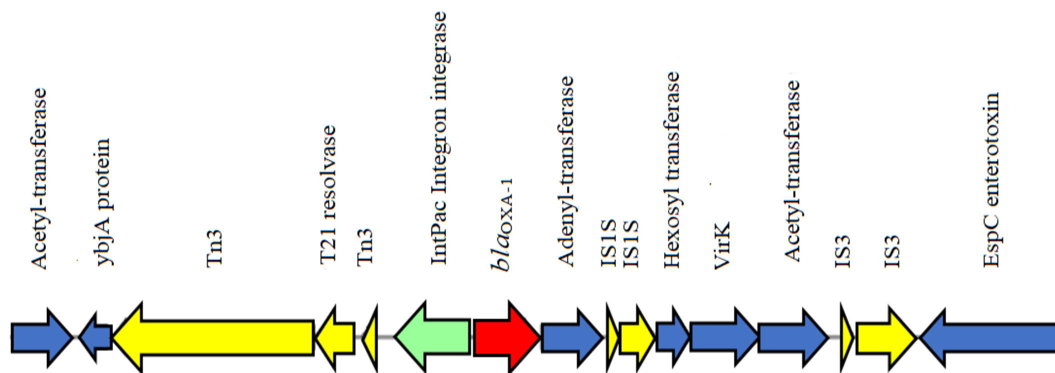

Figure 5. Genetic environment of *bla*OXA-1 encoded on class 1 integron integrated on an IncFII plasmid.

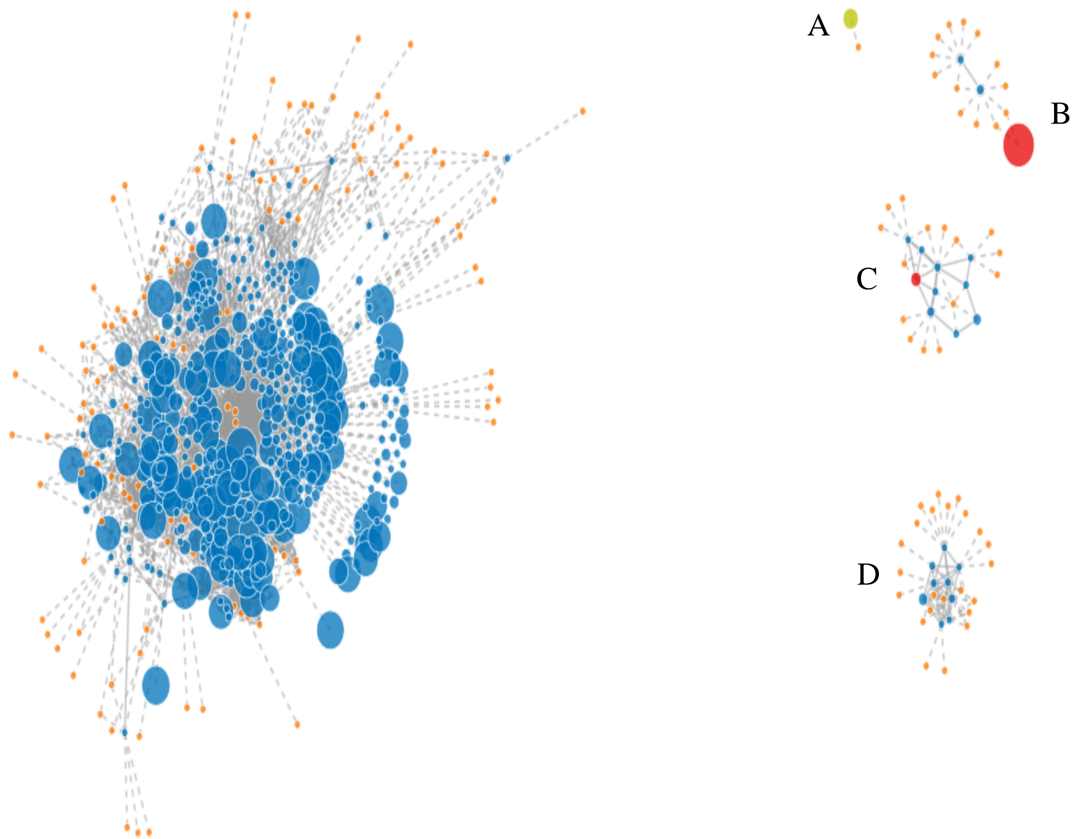

Figure 6. Schematic representation of the SHS-5 genome generated by PLACNETw. The network obtained contains two types of nodes: references (orange) and SHS-5 assembled contigs (blue, green or red). The red circles correspond to contigs containing relaxase proteins whereas the green circle corresponds to contigs having relaxase and replication initiator site. Three plasmids were detected (B, C and D). The nodes are connected by solid lines or dotted lines. Solid lines correspond to scaffold links connecting contigs whereas dotted lines correspond to BLAST results connecting nodes to reference sequences. A: fragment of IncI1 plasmid, B: IncI1 plasmid, C: IncFII plasmid and D: pINV B (*Shigella* virulence plasmid isoform B).

3.6. Class 1 and 2 integron detection

Class 2 integron was more prevalent than class 1 integron. Class 2 was detected in all the isolates except SHS-2, whereas class 1 was less abundant and was only seen in SHS-2 and SHS-5 isolates. *in silico* analysis was also performed to confirm the results obtained though PCR assays. Alignments using Blast and Bionumerics revealed that isolates SHS-5 and SHS-4 were positive for class 2 integrons carrying an aminoglycoside resistant gene *aadA1*. Class 1 integron in SHS-2 was integrated in an IncFII plasmid and carried the β-lactam resistant gene bla_{OXA-1}.

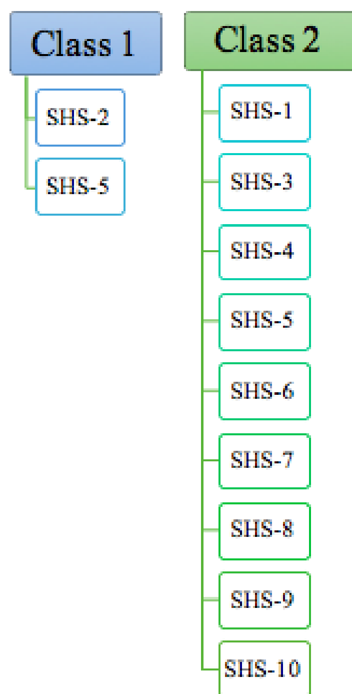

Figure 7. Class 1 and class 2 integrons detected by individual PCRs and confirmed *in silico*.

3.7. Virulence factors

Virulence genes were detected *in silico* with *ial*, *ipaH*, *virA*, *set* and *sen* being detected through individual PCR assays, as they are the most commonly detected virulence determinants in *Shigella* spp. All the isolates were positive for the acid resistance determinant *gad* and *ipaH* genes. *sigA* cell altering toxin was the most common enterotoxin seen in 9/10 (except *S. flexneri* SHS-2). On the other hand, *Shigella* enterotoxin 2 (*sen* gene) was detected in 5/10 of the isolates (SHS-2, SHS-3, SHS-5, SHS-8 and SHS-9), whereas *Shigella* enterotoxin 1 (*set* gen) was absent from all. Invasion *ipaD* gene, another important virulence determinant, was detected in 4/10 of the isolates (SHS-2, SHS-3, SHS-4 and SHS-5), *ial* (invasion) and *sepA* (invasion-related) were only detected in *S. boydii* SHS-4 and *S. flexner*i SHS-2, respectively, and *iha* (invasion and adhesion) only in isolate SHS-4. Finally, *ipfa* and *virA* genes (Fimbriae and mobility determinants) were detected in 8/10 (except SHS-2 and SHS-4) and 5/10 (except SHS-1, SHS-2, SHS-6, SHS-7, SHS-10) of the isolates.

Table 6. Virulent determinants detected *in silico* and by PCR.

Isolate	*gad*	*ipfa*	*virA*	*ipaD*	*ial*	*sepA*	*ipaH*	*iha*	*sen*	*set*	*sigA*
SHS-1	■	■					■				■
SHS-2	■			■		■	■		■		
SHS-3	■	■	■	■			■		■		■
SHS-4	■		■	■	■		■	■			■
SHS-5	■	■	■	■			■		■		■
SHS-6	■	■					■				■
SHS-7	■	■					■				■
SHS-8	■	■	■				■		■		■
SHS-9	■	■	■				■		■		■
SHS-10	■	■					■				■

Acid Resistance
Fimbriae
Motility
Invasion
Host Immune System Modulation
Adhesion
Enterotoxins

3.6. Pan genome analysis

Roary was used to build pan genomes and identify core and accessory genes of *Shigella* spp. isolates. The isolates core and accessory genomes identified were compared to thirteen *Shigella* spp. reference genomes. Using Roary we were able to determine the conserved genes among the different chosen reference *Shigella* isolates along with the ones undertaken in this study. *S. flexneri* reference 2457T and SHS-2 both belong to ST245 while on the other hand *S. boydii* reference ATCC9210 and SHS-4 belong to ST145. The remaining *S. sonnei* isolates aligned with *S. sonnei* reference sequences 53G, NCTC12984, AR_0030, NCTC7924, Ss046, 2015AM-1099, 2015C-3566, 4303 and 75/02 all of which belong to ST152 except for AR_0030 that belongs to ST5479. In addition, the two *S. dysenteriae* reference sequences Sd197 and 1617 clustered together, separately from the remaining genomes and both belong to ST146. Notably, a core genome of 40.98kb was shared between all genomes (*S. sonnei*, *S. boydii*, *S. flexneri* and *S. dysenteriae*), being mainly composed of metabolism-related proteins (glucose-6-phosphatase, glycerol- 3- phosphate regulator etc.), ribosomal proteins, signal transduction proteins, transcription activators/repressors, vitamin binding proteins and stress-response related proteins. *S. sonnei* had a larger core genome (approximately 78kb) compared to *S. flexneri*, *S. dysenteriae* and *S. boydii* genomes, which had a smaller core genome but a wider variety of accessory genes. Species-specific regions were mostly composed of effector proteins and virulence genes for attachment, colonization and propagation. Accessory genes on the other hand, were mostly insertion sequences, multidrug resistance proteins, outer and inner membrane proteins and porins and secretion system proteins.

Pan-genome analysis gave a better understanding of the diversity within different *Shigella* spp. genomes.

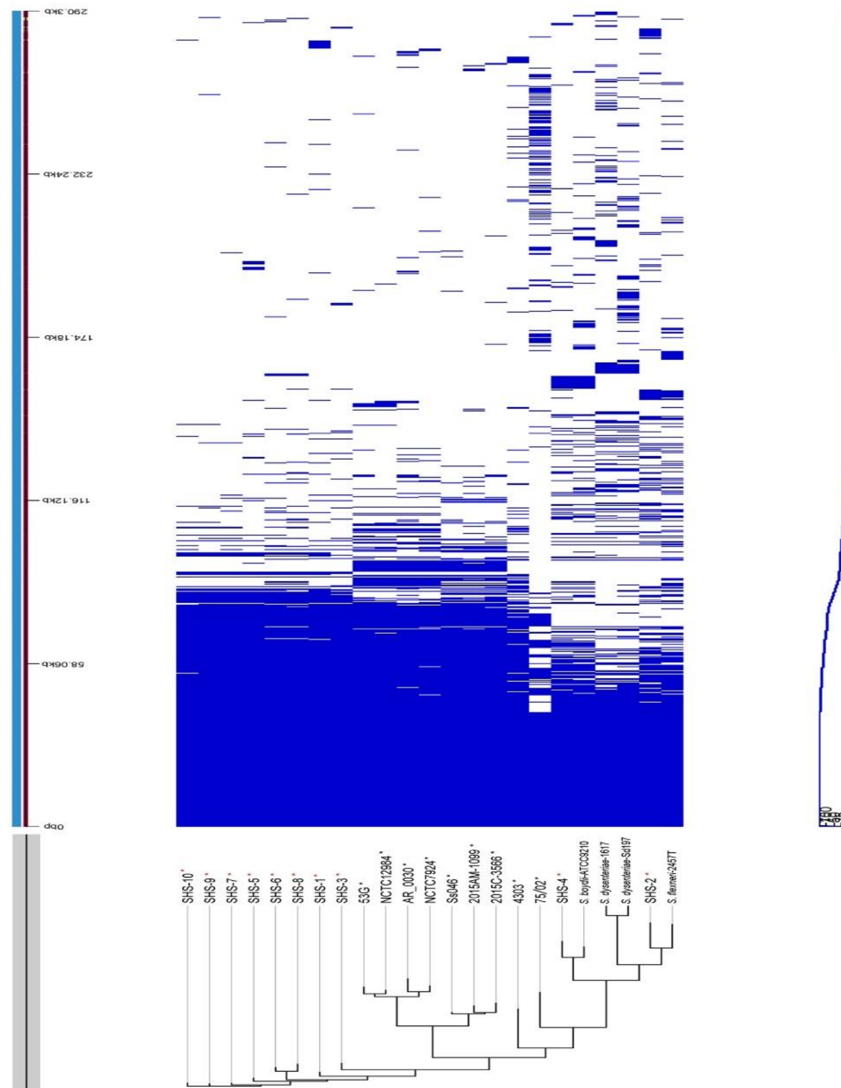

Figure 8. Pan genome analysis using Roary (Rapid large- scale prokaryote pan genome analysis). The core genome and accessory genes of the ten isolates were identified and compared to thirteen *Shigella* spp. reference genomes. * was used to designate the ten isolates of this study. * was used to designate nine *S. sonnei* reference genomes obtained from NCBI. Two *S. dysenteriae*, one *S. boydii* and one *S. flexneri* references were also used for comparison.

3.8. Multi-locus sequence typing

The MLST results showed that the majority of the isolates (8/10) were typed as ST152. However, two other MLST allelic profiles were also detected and were designated as belonging to ST245 and ST145. It is important to note that *S. boydii* SHS-4 was typed as ST145, *S. flexneri* SHS-2 as ST245, whereas all *S. sonnei* isolates as to ST152 (Table 7).

Table 7. MLST profiles. Sequencing results of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*). The isolates were distributed on three multi-locus sequence types (ST152, ST145 and ST245) with ST152 being the predominant.

Isolates	*adk*	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	ST
SHS-1	11	63	7	1	14	7	7	152
SHS-2	6	61	6	11	13	3	50	245
SHS-3	11	63	7	1	14	7	7	152
SHS-4	1	10	1	1	1	1	1	145
SHS-5	11	63	7	1	14	7	7	152
SHS-6	11	63	7	1	14	7	7	152
SHS-7	11	63	7	1	14	7	7	152
SHS-8	11	63	7	1	14	7	7	152
SHS-9	11	63	7	1	14	7	7	152
SHS-10	11	63	7	1	14	7	7	152

3.9. Pulsed-field gel electrophoresis

A total of eight different pulsotypes (PT) were identified. Isolates having ST152 were grouped together showing 80% similarity pattern or more. Isolates SHS-2 and SHS-4 had different ST and pulsotypes from all the remaining and clustered separately (Figure 4).

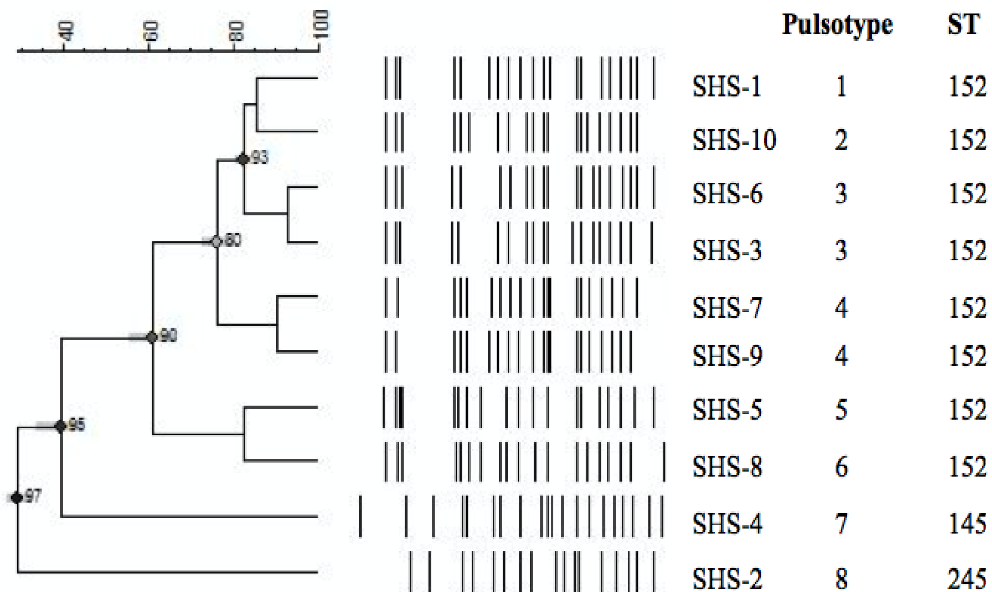

Figure 9. PFGE patterns, Pulsotypes and MLST profiles. The dendogram was generated using BioNumerics software version 7.6.1. The banding patters shows the phylogenic relatedness between the isolates. The restriction enzyme used to generate this banding pattern was XbaI.

3.10. wgSNP-based phylogenic typing

The ten isolates along with five *S. sonnei* reference strains downloaded from NCBI were used to build a wgSNP-based phylogenic tree. Results obtained showed very similar clustering and in accordance with PFGE and MLST typing results. Another SNP tree was generated using the kSNP tool and was edited using iTOL. Comparable clustering was obtained in both trees, with SHS-2 and SHS-4 clustering again separately from all the rest.

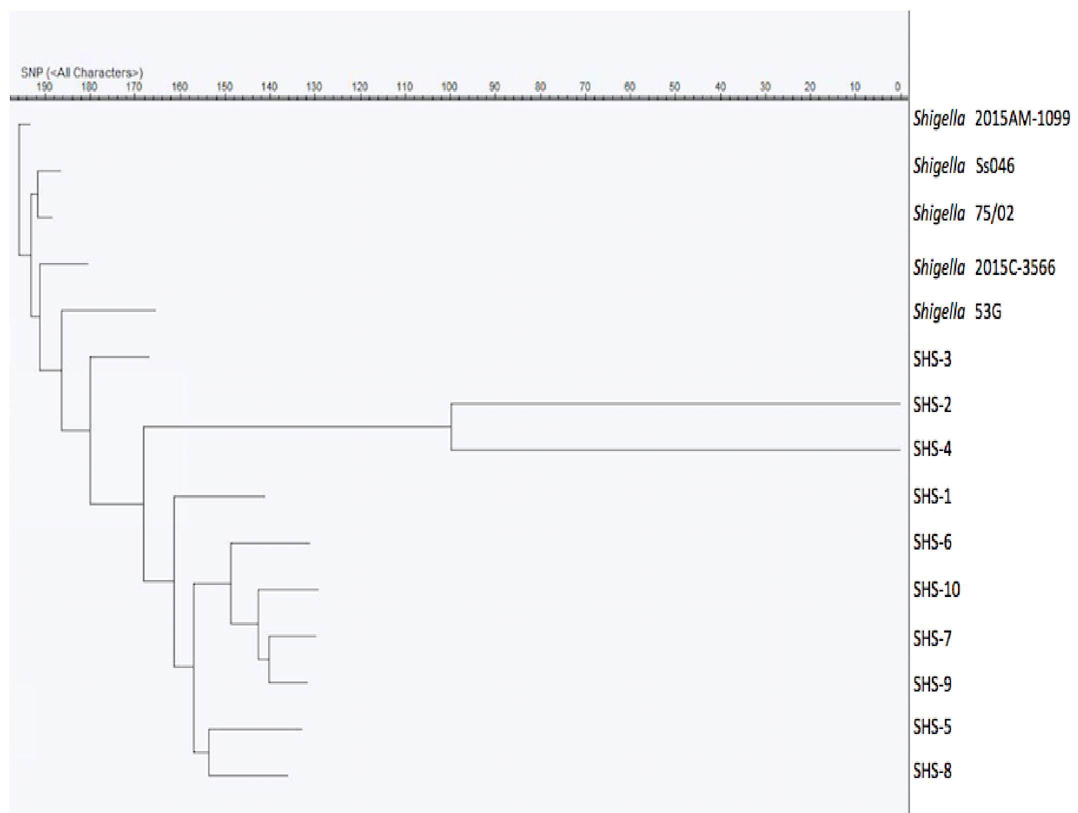

Figure 10. wgSNP-based phylogenic tree using Bionumerics, UPGMA algorithm. To generate a better comparison five reference genomes were used in addition to the ten isolates of this study.

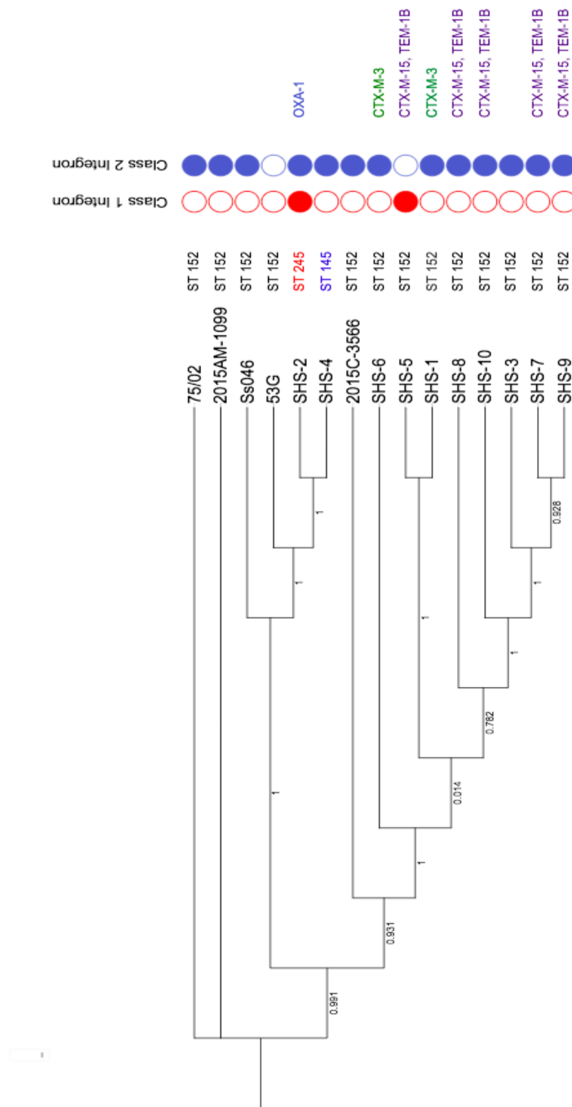

Figure 11. wgSNP-based phylogenic tree with bootstrap values was generated using kSNP 3.0. Five *S. sonnei* reference genomes were used for comparison. MLST, class 1 and 2 and β-lactam resistance profiles were added to the tree using iTOL tool.

Chapter Four

Discussion

This study presents the whole-genome sequencing and genome comparative analysis of ten *Shigella* spp. isolates. Eight of the isolates were ESBL-producers with β-lactam resistance mainly mediated by the presence of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1B}$ both of which were carried by an IncFII plasmid (Table 5). On the other hand, $bla_{CTX\text{-}M\text{-}3}$ was encoded on an IncI plasmid. Class 2 integrons were highly prevalent among the *S. sonnei* isolates as opposed to class 1 integrons, that were detected only in two isolates (SHS-2 and SHS-5) (Figure 7). Moreover, aminoglycoside resistance was integron-mediated and one isolate (SHS-2) had an OXA-1 that was carried by class 1 integron found on an IncFII plasmid. Our study focused on important virulence determinants and effector proteins used by the bacterium to destroy the host, such as *ial*, *ipaH*, *IpaD, sigA*, *gad*, *sen* and *virA* (Table 6). We shed the light on the prevalence of *Shigella* enterotoxin 2 (ShET2) in all *Shigella* serotypes as opposed to ShET1 that was not detected in any of the undertaken isolates. We showed the importance and accuracy of using a combination of typing techniques to study the relatedness among *Shigella* isolates. We revealed through wgSNP-based phylogenic typing, PFGE, MLST and Roary that *S. flexneri* and *S. boydii* clustered separately and had different fingerprints, ST, accessory and core genes than the remaining *S. sonnei* isolates.

Antimicrobial susceptibility testing revealed that eight of the isolates were ampicillin, cefotaxime, ceftriaxone and tetracycline resistant while on the other hand nine isolates were sulfamethoxadol/trimethoprim resistant (Table 4). Notably, these are the most common resistance patterns detected in MDR *S. sonnei* isolated worldwide (Cohen et al., 2014). A study conducted on 335 *S. sonnei* isolated from cases of diarrheal disease

in England and Wales revealed similar resistance patterns with 98.5% of the isolates being resistant to trimethoprim/sulfamethoxazole, 30.2% to ampicillin resistant and 79.2% to tetracycline (Sadouki et al., 2017). Similarly, Arcilla et al. showed that 98.7% of the isolates were resistant to trimethoprim, 92.1% to ampicillin and 86.8% to tetracycline (Arcilla et al., 2017). Isolates collected from Iran however, were mainly resistant to ampicillin (AMP) (95.1%) and trimethoprim/sulfamethoxazole (SXT) (Aminshahidi et al., 2017). Over the decades, an increasing incidence of resistance to tetracycline, trimethoprim/sulfamethoxazole, ampicillin and third generation cephalosporins has been described in *Shigella* (Cohen et al., 2014, Seol et al., 2006). Ampicillin and third generation cephalosporin resistance are mainly due to bla_{TEM} or $bla_{\text{CTX-M}}$ in *S. sonnei* while related to bla_{OXA} in *S. flexneri*, which was in harmony with our findings with SHS-2 (*S. flexneri*) being the only isolate encoding $bla_{\text{OXA-1}}$ while β-lactam resistance in all remaining *S. sonnei* isolates was CTX-M or TEM-mediated (Toro et al., 2005; Puzari, Sharma & Chetia, 2018). CTX-M ESBLs are the most commonly detected enzymes in *Shigella* and have been previously reported in Argentina, Korea, India and in China (Nhu et al., 2010). Similarly, our findings showed that resistance to β-lactam antibiotics was manifested mostly by the presence of $bla_{\text{CTX-M}}$ genes and specifically $bla_{\text{CTX-M-15}}$. The emergence of plasmid encoded CTX-M-15-producing S. *sonnei* in Lebanon was first reported in 2007 and since then more CTX-M producing *Shigella* spp. were detected from clinical settings (Matar et al., 2007), and β-lactamases mediated resistance was reported to be the most important and common resistance mechanism employed by *Shigella* spp (Zhang et al., 2014). ESBL-encoding genes are most commonly found on conjugative plasmids which is why it can easily spread (Brolund & Sandegren, 2015). In Lebanon infections caused by ESBL-producing *Enterobacteriaceae* are increasing (Moubareck et al., 2005). A

study conducted by Daoud et al. on *Enterobacteriaceae* isolated from community and hospital settings in Lebanon revealed that the majority of the isolates were ESBL producers and resistance was mainly manifested by *bla*$_{CTX\text{-}M\text{-}15}$. This was in harmony with the results obtained in this study with 8 of the isolates being ESBL-producers and mostly expressing *bla*$_{CTX\text{-}M\text{-}15}$ (Daoud et al., 2018). Additionally, Tokajian et al. showed that ESBL-producing *E. coli* collected from wastewaters in refugee camps in Lebanon were mainly positive for the *bla*$_{CTX\text{-}M\text{-}15}$ and *bla*$_{TEM\text{-}1B}$. CTX-M-15 was the most prevalent in sewage and wastewater worldwide and similar to our findings, was usually associated with TEM-1 (Dolejska et al., 2011). Moreover, Rashid et al revealed a potential danger of drug resistance due to transfer of ESBL producing plasmid between *E. coli* and *S. sonnei* which may explain why ESBL-producing *E. coli* and *S. sonnei* have very similar β-lactam resistance determinants (Rashid & Rahman, 2015). We found that *bla*$_{CTX\text{-}M\text{-}15}$ was found in close proximity to *bla*$_{TEM\text{-}1B}$ and both genes were carried on IncFII type plasmid (Figure 4). In addition, the insertion sequence IS*Ecp1* was detected also upstream of *bla*$_{CTX\text{-}M\text{-}15}$; a promoter enhancing *bla*$_{CTX\text{-}M\text{-}15}$ expression (Poirel, Naas & Nordmann, 2008). Previous studies revealed that *bla*$_{TEM\text{-}1B}$ was carried on transposon Tn*3* the sequence of which was also disrupted by the IS*Ecp1*-mediated transposition of *bla*$_{CTX\text{-}M\text{-}15}$ (Smet et al., 2010). On the other hand, *bla*$_{CTX\text{-}M\text{-}3}$ detected on an IncI1 type plasmid and, similar to our findings, was flanked by the IS*Ecp1* insertion sequence enhancing its expression (Zhao & Hu, 2012). Caratolli et al. suggested that β-lactam resistant determinants were usually encoded on IncFII or IncI1 type plasmids, which where the two prevalent types in the isolates undertaken in this study.

We additionally detected an OXA-1-mediated β-lactam resistance but in only one isolate (SHS-2) and further analysis revealed that *bla*$_{OXA\text{-}1}$ was encoded on a class 1

integron found on an IncFII type plasmid. In line with our finding, studies have showed that *bla*$_{OXA-1}$ is usually found in *S. flexneri* isolates on a class 1 integrons (Puzari, Sharma & Chetia, 2018).

Furthermore, we aligned the SHS-1 *bla*$_{CTX-M-3}$ to pEK204 reference plasmid, and showed that IS*Ecp1* was upstream of *bla*$_{CTX-M-3}$ gene (Figure 3). Plasmid pEK204 (IncI) was isolated from a multiresistant ST131 *E. coli* (United Kingdom), carrying two resistance genes *bla*$_{CTX-M-3}$ and *bla*$_{TEM-1B}$ as opposed to only *bla*$_{CTX-M-3}$ in SHS-1 (Kim et al., 2014). pEK204 CTX-M-3 differed from the CTX-M-15 by only a Asp240 → Gly substitution (Woodford et al., 2009; Kim et al., 2014). Moreover, pEK204 TEM-1B was carried by Tn*3* which was disrupted by the IS*Ecp1*- mediated transposition of *bla*$_{CTX-M-3}$ that was initially found on pCOLIb-P9-like plasmid (IncI type plasmid) (Woodford et al., 2009). On the other hand, we aligned SHS-7 *bla*$_{CTX-M-15}$ to pKHSB1 plasmid (IncFII) isolated from *S. sonnei* in Vietnam and pSH4469 reference plasmid (IncI1) isolated from Korea. In all three plasmids, the gene co-existed with *bla*$_{TEM-1B}$ and had a similar IS*Ecp1*-related *bla*$_{CTX-M-15}$ transposable unit. In 2008, an outbreak of *S. sonnei* occurred in a school for disabled children in South Korea, the ESBL-producing isolates belonged to the same clone and carried the pSH4469 CTX-M-15 encoding plasmid. The plasmid showed high sequence homology with pEK204 isolated from the UK, with only two region differences (*bla*$_{CTX-M-3}$ and IS*66*) (Kim et al., 2014). IncI1 and IncFII plasmids are both successful platforms for efficient horizontal gene transfer mediating the spread of β-lactam resistance determinants among different bacteria (Novais et al., 2006). These plasmids were considered to be “epidemic” since they were widely spread between *Enterobacteriaceae* and across countries (Sullivan & Schaus, 2015). Aside from carrying resistant determinants these plasmids carried virulence determinants too;

IncI1 plasmids for example have a cluster encoding type IV pili needed for adhesion and invasion (Carattoli, 2009).

Gene cassettes on integrons also play a role in the dissemination of resistance genes, they are often embedded in plasmids or transposons and are transferred with them (Ke, Gu, Pan & Tong, 2011). In this study, all detected integrons were chromosomal except for the class 1 integron in one of the isolates (SHS-2), which we showed was integrated in an IncFII type plasmid. Chromosomally associated integrons are more stable hence limiting the spread of resistance determinants (Dubois et al., 2007). Class 1 and class 2 integrons were extensively studied because of their association with antibiotic resistance (Chang et al., 2011). In this study, class 2 integrons were widely spread among the *S. sonnei* isolates. Class 1 integrons are usually associated with *S. flexneri* and *S. dysenteriae* while the class 2 are abundant in *S. boydii* and *S. sonnei* (Ke, Gu, Pan & Tong, 2011). Alignments using Blast and BioNumerics revealed that class 2 integron carried the aminoglycoside resistant gene *aadA1* in two of the studied isolates (SHS-4 and SHS-5). Class 1 integron was less abundant and only detected in two isolates (SHS-2, SHS-5). Gene cassettes within class 1 integrons are usually integrated on chromosomes or plasmids and similar to our finding, carry $bla_{\mathrm{OXA\text{-}1}}$ or *aadA* genes conferring resistance to ampicillin and trimethoprim, respectively (DeLappe et al., 2003; Madiyarov et al., 2010; Ahmed et al., 2006). Class 2 integron usually consisted of *dfrA1*, *sat1* and *aadA1* genes cassettes conferring resistance to trimethoprim, streptothricin and streptomycin, respectively and their transposition was mediated by Tn*7*, which was in contrast with our results with class 2 integrons not carrying any of the above mentioned resistance gene cassettes (Mammina, Pontello, Dal Vecchio & Nastasi, 2005). A recent study conducted in Iran on 142 *Shigella* spp. isolates collected from different parts of Iran revealed that 80.8% of the isolates resistant to extended-

spectrum cephalosporin carried class 2 integrons and these were mostly *S. sonnei*, these findings were in accordance with our results with class 2 integrons being predominant among *S. sonnei* isolates (Zamanlou et al., 2018). The study also suggested that 66.7% of the detected class 2 integrons harbored gene cassettes carrying *aadA1* while 14.1% harbored the *dfrA17/sat1* gene cassette which was not the case in this study. Moreover, 27% of the isolates harbored the class 1 integron which carried *dfrA17/aadA1* and *dfrA7* gene cassettes; notably, two of the undertaken isolates harbored a class 1 integron that carried *aadA1* cassette (Zamanlou et al., 2018).

Virulence factors in *Shigella* spp. include several iron chelating systems, O-antigen modifying proteins, enterotoxins, and secretion system proteins. All isolates had the chromosomally encoded *gad* gene (glutamate-dependent resistance gene); one of the mechanisms that *Shigella* spp. uses to resist acid (Waterman & Small, 2003). Retention of the enzyme encoded by *gad* was necessary for the bacteria to pass through the stomach (Grant, Weagant & Feng, 2001). Additionally, *Shigella* spp. has several VF needed for adhesion, among these are *ipfa* and *iha* genes. The *ipfa*, a fimbriae-encoding factor, was detected in most of the studied isolates (8/10) and on an IncFII type plasmid similar to previous findings (Germane et al., 2008). On the other hand, *iha* which encoded an adhesion factor, was only detected in isolate SHS-4 (*S. boydii*). Previous studies have demonstrated that *ipaH* (invasion-plasmid antigen H) and to a lesser extent *ial* (invasion-associated locus) were highly prevalent among all four *Shigella* serogroups (Yaghoubi et al., 2017; Ranjbar, Bolandian & Behzadi, 2017). *ipaH* was highly conserved in various serotypes and many copies of the gene were available; 7 copies of *ipaH* could be found on the chromosome and 5 copies on the large invasion plasmid (pINV) (Vargas et al., 1999; Zhang et al., 2014). The latter may explain why *ipaH* was usually detected in all *Shigella* isolates studied while on the

other hand *ial* (plasmid encoded) was less frequently detected than *ipaH*; plasmid on which *ial* was encoded could be lost (Vargas et al., 1999). *ipaH* was detected in all the undertaken isolates while *ial* was only detected in *S. boydii* SHS-4, which was not in accordance studies which showed that *ial* was prevalent among all *Shigella* serogroups (Ghosh, Nataro, Ramamurthy, Pazhani & Niyogi, 2014). *virA*, an essential virulence factor in *Shigella*, was detected in half of the isolates and was essential for cell entry, actin-based motility and cell to cell spread (Germane et al., 2008). *ipaD* another T3SS effector needed for invasion was found in only four of the isolates. Previously, however, *virA* and *ipaD* were detected by Lluque et al. in all studied *Shigella* isolates. It is important to mention that *virA* and *ipaD* are encoded on the large virulence plasmid pINV that may be lost, which could possibly explain the differences in their detection frequencies (Yaghoubi et al., 2017).

Shigella IgA-like protease homologue (sigA) is stably integrated on the chromosome SHI-1 pathogenecity island and are less likely to be lost explaining their detection in all the studied isolates except in SHS-2 (Lluque et al., 2015). In line with our results, Fan et al. and Lluque et al. reported that *sigA* was prevalent among all *Shigella* isolates and was detected at frequencies of 78.9% and 69%, respectively (Fan et al., 2017; Lluque et al., 2015).

Shigella produces a variety of enterotoxins, such as, *Shigella* enterotoxin 1 (ShET-1) encoded by *set* gene and *Shigella* enterotoxin 2 (ShET-2) encoded by the *sen* gene found on the pINV plasmid (Moosavian, 2018). ShET-1 was located on the chromosome SHI-1 island and we couldn't detect it in any of the studied isolates which was consistent with other studies showing that *set* was not detected in *S. sonnei* or *S. boydii* but might be found in *S. flexneri* (Casabonne, Gonzalez, Aquili & Balague, 2016). On the other hand, *sen* gene was detected in half of the undertaken isolates.

This finding was similar to other studies reporting the prevalence of *sen* in all *Shigella* serogroups (Noriega et al., Yaghoubi et al., Ghosh et al., Telli et al.) as opposed to *set* gene, which was less commonly detected (Noriega et al., 1995; Yaghoubi et al., 2017; Ghosh et al., 2014; Telli et al., 2010).

Multiple techniques could be used to type *Shigella*: antimicrobial susceptibility testing, phage typing, biotyping, pulsed- field gel electrophoresis (PFGE), ribotyping, plasmid typing, and multi-locus sequence typing (MLST) (Ranjbar & Memariani, 2015). PFGE is the gold standard technique among the molecular typing methods and is based on the migration of large DNA restriction fragments in an electrical field of alternating polarity (Herschelb, Ananiev & Schwartz, 2007). In this study eight pulsotypes (PT) were detected, six of which in *S. sonnei* (Figure 9). This finding confirmed that PFGE had a great discriminatory power and was a suitable method for studying the population structure of *S. sonnei*, being also in line with the study conducted by Seribelli et al. On the other hand, multi-locus sequence typing (MLST) relied on the typing of seven housekeeping genes (*arcA, aroE, icd, mdh, mtlD, pgi, rpoS),* it is used to explore the clonal lineages and evolutionary pathways of bacteria and was known to be a powerful tool for epidemiological investigations of *Shigella* infections (Li et al., 2016). All *S. sonnei* isolates typed as ST152 which was also consistent with previous findings (Shokoohizadeh et al., 2017; Chattaway et al., 2017; Nemoy et al., 2016). ST152 was the most frequent and dominant type detected and was considered to be a "founder" type (has the highest number of single-locus-variants as compared to ST145, ST245) (Shahsavan et al., 2016). *S. flexneri* SHS-2 typed as ST245, while on the other hand *S. boydii* SHS-4 was typed as ST145, while in other studies ST245 was seen in both *S. boydii* and *S. flexneri* (Table 7) (Chattaway et al.,

2017). ST245 was detected among *S. flexneri* recovered from Africa, America, Asia and Europe and in *S. boydii* from China (Shokoohizadeh et al., 2017). However, as previously described by Wirth et al. and Cao & Wei, ST145 and ST245 encompassed both *S. boydii* and *S. flexneri* (Wirth et al., 2006; Cao & Wei, 2012).

Our study revealed that PFGE had a greater discriminatory power than MLST, as all *S. sonnei* isolates belonged to the same ST (152). MLST may not always have enough discriminatory power to distinguish between closely related strains (Shokoohizadeh et al., 2017). Therefore, a combination of typing approaches could better reflect the epidemiological relatedness.

This study presents an in depth-analysis of the whole-genome of ten *Shigella* isolates collected from clinical settings in Lebanon. WGS has become an important tool for genome analysis and helped us to have a better understanding of the characteristics, genome profiles, diversity and relatedness among ESBL-producing *Shigella* isolates. Several typing techniques and comparative analytical approaches were used to reveal the clonality, if existent, among our isolates. We also provided an insight of virulence and antibiotic resistance determinants helping the bacterium better survive within the host. A clear virulence and resistance gene profiles might help in clinical diagnosis and treatment. For future studies, it would be of great interest to focus on secretion systems and effector proteins, targeting specifically the T6SS clusters. Recent studies emphasized the need of *S. sonnei* T6SS for the bacterium to survive in the gut and outcompete the rival gut microbiome thus it would be of great importance to assess the functionality of the T6SS (Anderson et al., 2017).

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