Phylogenetic analysis and antibiotic resistance of *Shigella sonnei* isolates

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Abstract

Shigellosis is one of the most important gastric infections caused by different species of *Shigella* and has been regarded as a serious threat to public health. Lineage/sublineage profile of *S. sonnei* is strongly associated with the antibiotic resistance and population structure of this pathogen. In this study, we determined the phylogeny and antibiotic resistance profiles of *S. sonnei* strains, isolated from 1246 stool and 580 food samples, using multiplex PCR-HRMA genotyping and Kirby-Bauer disk diffusion methods, respectively. A total of 64 *S. sonnei* strains were isolated (13 food and 51 clinical isolates). Multiplex PCR-HMR assay was able to differentiate the lineages II and III, and sublineages IIIb and IIIc strains successfully considering the definite melting curves and temperatures. Lineage I and sublineage IIIa strain were not isolated in this study. We also demonstrated that most of the *S. sonnei* strains isolated from both food and clinical samples clustered within the lineage III and sublineage IIIc. Resistance against trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol and streptomycin antibiotics were the most prevalent phenotypes among the *S. sonnei* lineage III and sublineage IIIc strains.

Keywords: *Shigella sonnei*; PCR-HMRA; Phylogenetic group; Antibiotic resistance

Introduction

Shigella sonnei is a Gram-negative, rod-shaped and facultative intracellular pathogen belonging to *Shigella* and *Enterobacteriaceae* family (Pakbin, *et al.* 2021c, Torraca, *et al.* 2020). *S. sonnei* has been regarded as the main cause of shigellosis in developed countries in North America and Europe. Also, *S. sonnei* has frequently been isolated from shigellosis in those that are economically damaged or developing in the high-income countries (Shad and Shad 2021). However, it is currently expanding in some middle-income countries across the Middle East, Latin America and Asia (Baker and The 2018). This bacterial pathogen causes self-limiting and acute gastrointestinal diseases characterized by fever, watery or bloody diarrhoea and abdominal pain symptoms in adults and children. The infection caused by this pathogen can be strongly life-threatening for infants and children under five years old (Rogawski McQuade, *et al.* 2020). It is estimated that this pathogen causes annually more than 188 million cases and 160 000 deaths, mainly among young children (Hosangadi, *et al.* 2019). *S. sonnei* has been considered a foodborne pathogen and commonly spreads via faecal-oral transmission as it can survives gastric acidity and infects the colonic mucosa (Belotserkovsky and Sansonetti 2018). The main treatment for shigellosis is rehydration therapy and antibiotics such as cephalosporins, fluoroquinolones and azithromycin. However, resistance against different antibiotics is becoming more common among the *S. sonnei* isolated from food and clinical samples. It is widely reported that some specific genotypes of *S. sonnei* are associated with high rates of multi-drug resistance and more frequently contribute to foodborne outbreaks (Pakbin, *et al.* 2020a, Pakbin, *et al.* 2021a, Ranjbar and Farahani 2019).

The genotypic profile of *S. sonnei* isolates is strongly associated with the pathogenicity, resistance against different antibiotics, regional epidemiology and population structure of the pathogen (Pakbin, *et al.* 2021b, Pakbin, *et al.* 2021c, The, *et al.* 2021). Consequently, genotype characterization of *S. sonnei* isolates helps to better investigate the outbreaks in respect of the presence of this pathogen and choose the most efficient strategies to prevent the spread of shigellosis due to the *S. sonnei* isolates. *S. sonnei* is monoclonal, and there is only one serogroup (D) consisting of a single serotype (Ragupathi, *et al.* 2018). Whole-genome sequencing (WGS) has effectively been used for subtyping the *S. sonnei* isolates. According to the WGS studies, *S.*

sonnei is categorized into five significant lineages and several sublineage phylogenetic groups associated with some phenotypic features, including resistance to different antibiotics, the transmission routes and regional epidemiology (Dallman, *et al.* 2016). For instance, *S. sonnei* isolated from the specimen in Australia, Europe, North America and Asia have been dominated by the Lineage 3 strains for more than 29 years (Holt, *et al.* 2012). These strains demonstrated resistance against tetracycline, trimethoprim-sulfamethoxazole, streptomycin, ampicillin, chloramphenicol, ciprofloxacin, and fluoroquinolones the presence of antibiotic resistance genes and acquisition of point mutations (Hawkey, *et al.* 2021). A single nucleotide polymorphisms (SNPs)-typing scheme based on the high-resolution melting analysis (HRMA) method was developed and described for the first time by Sangal et al. (2013) to identify all lineages and sublineages of *S. sonnei* strains. They detected SNPs in small PCR amplicons (SNPs within *deoA*, *kduD* and *emrA* genes for typing three main lineages I, II and III, and SNPs within *menF* and *fdx* genes for typing sublineages including IIIa, IIIb and IIIc) based on the high-resolution melting patterns. They reported this method is more rapid and cost-effective than WGS for genotyping *S. sonnei* strains (Sangal, *et al.* 2013). Mazi et al. (2015) developed a multiplex PCR-HRMA method to identify and differentiate the lineages I, II, III and sublineages IIIa, IIIb and IIIc of the *S. sonnei* strains using the Qiagen Rotor-Gene 6000 instrument and EvaGreen dye (Mazi, *et al.* 2015). Since the characterization of genotype, lineage, sublineages and antibiotic resistance of *S. sonnei* strains represents an opportunity to understand the ecology, epidemiology and antibiotic resistance of this pathogen to choose the best preventive strategies (Hawkey, *et al.* 2021), this study aimed to determine the lineages, sublineages and antibiotic resistance profile of *S. sonnei* strains isolated from clinical and food samples by using the PCR-HRMA method as a rapid, precise and cost-effective assays.

Materials and methods

Sample collection

Stool samples (N=1246) from children and adults with acute diarrhoea referred to the emergency service and central lab in different hospitals (Qazvin children hospital, Qazvin, Iran and Milad hospital, Tehran, Iran), and the food samples (N=580), including raw milk (n=110), ground meat (n=210) and raw vegetable (n=260) samples from local markets located in various areas of municipalities of Tehran and Qazvin, Iran were collected during Jun 2015 to August 2018. All

samples were immediately transported in cool boxes (4°C ± 0.5) to the central microbiology research laboratory for further analysis. *S. sonnei* PTCC 1777 was purchased from Pasteur Institute (Pasteur In., Tehran, Iran) and used as the positive control and reference strain in this study.

Isolation and identification of *S. sonnei*

S. sonnei was isolated and identified in stool and food samples according to the methods described by Phiri et al. (2021) and Ahmed and Shimamoto (2014), respectively (Ahmed and Shimamoto 2014, Phiri, *et al.* 2021). Suspected colonies (red) on XLD agar were selected and subjected to the biochemical tests, including IMViC, triple sugar iron (TSI), urease production and lysine decarboxylase tests. Slide agglutination method using *Shigella* genus and species polyvalent antisera commercial kits (Difco Co., MI, USA) was used according to the manufacturers' instructions to confirm and determine the genus and species of the presumptive colonies, respectively. All strains were subjected to DNA extraction.

Antibiotic susceptibility testing

Antibiotic resistance of *S. sonnei* isolates was performed using Kirby-Bauer disk diffusion mwthod based on the interpretive criteria and standards previously developed and described by Clinical and Laboratory Standards Institute (Pakbin, *et al.* 2020b). Fourteen commercial antibiotic disks (Oxoid, UK) used in this study included kanamycin (KAN), 30 µg; amoxicillin-clavulanic acid (AMC), 20/10 µg; streptomycin, 10 µg; ampicillin (AMP), 10 µg; amoxicillin (AMX), 25 µg; amikacin (AMK), 30 µg; azithromycin (AZM), 15 µg; gentamicin (GEN), 10 µg; colistin (CST) 10 µg; chloramphenicol (CHL), 30 µg; trimethoprim-sulfamethoxazole (SXT), 1.25/23.75 µg; tetracycline (TET), 30 µg; nalidixic acid (NAL), 30 µg; levofloxacin (LVX), 5 µg and nitrofurantoin (NIT), 300 µg. The results of antibiotic resistance phenotypes were recorded and interpreted according to CLSI guidelines (Pakbin, *et al.* 2020b). Klebsiella pneumoniae ATCC 700603, Staphylococcus aureus ATCC 25923 and Escherichia coli ATCC 25922 were used as the reference strains in this study.

DNA extraction

According to the manufacturers' instructions, the genomic of the bacterial strains was extracted using the Sinaclon Gram-Negative bacterial DNA extraction commercial kit (Sinaclon Co.,

Tehran, Iran). The quantity and quality of the extracted DNA were assessed using the NanoDrop-1000 spectrophotometer (ThermoFisher, MD, USA). Final concentrations of the extracted genomes were adjusted to 50 ng. μL^{-1}. All DNA templates were kept at -20 °C until genotyping analysis.

Genotyping by PCR-HRMA

Lineages and sublineages of *S. sonnei* strains isolated from food and clinical samples in this study were determined and differentiated using a PCR-HRMA method previously described by Mazi et al. (2015)(Mazi, *et al.* 2015). Primer sequences used at the present study for PCR-HRMA genotyping of the isolates are summarized in Table 1. PCR-HRMA was performed using a Rotor-Gene Q 6000 real-time PCR instrument (Corbett, Australia) in this study. Genotyping of the strains was performed in two separate runs. In the first run, each reaction contained 20 µL including 4 µL of 5X EvaGreen hot-start PCR-HRM master mix (Solis BioDyne Co, Estonia), 2 µL of DNA template (10 ng. μL^{-1}), 0.5 µL of each primer of *emrA* (100 µM. μL^{-1}), 1 µL of each primer of *deoA* (100 µM. μL^{-1}) and 2.5 µL of each primer of *kduD* (100 µM. μL^{-1}) and nuclease-free water up to the final reaction volume. In the second set, PCR-HRM was performed in a 20-µL final reaction volume containing 4 µL of 5X EvaGreen hot-start PCR-HRM master mix (Solis BioDyne Co, Estonia), 2 µL of DNA template (10 ng. μL^{-1}), 2 µL of each primer of *fdX* (10 µM. μL^{-1}), 1 µL of each primer of *menF* (10 µM. μL^{-1}) and distilled deionized water up to the final reaction volume. The PCR program for both runs was carried out as follow: initial denaturation at 95 °C for 12 min, followed by 35 cycles containing 20 s at 95 °C, 20 s at 60 °C and 22 s at 72 °C. HRM was conducted between 75 to 95 °C, with a data acquisition every 0.1 °C. Rotor-Gene 6000 software version 2.02 (Corbett, Australia) was used to analyze the HRM data.

Statistical analysis

Chi-square and Fisher's exact tests were used to evaluate the significant differences ($P < 0.05$) between the groups using the SPSS version 21.0.1 (IBM Corp., NY, USA) software.

Results

Lineages and sublineages of *S. sonnei* strains by using PCR-HRMA

A total of 64 *S. sonnei* strains were isolated and confirmed from 1246 stool and 580 food samples. 20.3% (n=13) and 79.6% (n=51) of all strains were isolated from food and clinical

samples, respectively. In order to genotype and define the population structure of *S. sonnei* strains, we used PCR-HRMA method previously developed and optimized by Sangal et al. (2013) and Mazi et al. (2015), respectively (Mazi, *et al.* 2015, Sangal, *et al.* 2013). As it is shown in Figure 1A, the first HRMA-genotyping set run was clearly and successfully able to separate and differentiate lineages II and III regarding the distinct melting curves. Lineage I was not detected and identified among the strains. The second HRMA-genotyping set run completely distinguished the sublineages IIIb and IIIc with significantly distinct melting curves (Fig. 1B). Sublineage IIIa was not detected also among the *S. sonnei* strains. Melting temperatures of all alleles are summarized in Table 2. Significant differences ($P < 0.01$) were observed among the melting temperatures of all alleles. Sequencing of all SNPs verified the reliability of the melting temperatures of the amplicons associated with each lineage and sublineage of *S. sonnei* strains as previously described by Mazi et al. (2015)(Mazi, *et al.* 2015). Consequently, lineages and sublineages of *S. sonnei* strains were identified and differentiated correctly in this study.

Lineage and sublineage prevalence of *S. sonnei* strains

S. sonnei strains are categorized into three main whole-genome sequence analysis types called 'lineages', which were identified and differentiated by using the PCR-HRMA assay in this study. Phylogenetic analysis of *S. sonnei* strains isolated from food and stool samples were performed to determine its population structure. The prevalence of different *S. sonnei* lineages and sublineages in this study are shown in Figures 2A and 2B, respectively. 100% (13 out of 13 isolates) and 96% (49 out of 51 isolates) of *S. sonnei* strains isolated from food and stool samples, respectively clustered within lineage III. Only two strains, which were isolated from stool samples, were divided into lineage II. The strains in lineage III were further divided into the sublineages IIIb and IIIc. 18.3% (9 out of 49) and 81.6% (40 out of 49) *S. sonnei* lineage III strains isolated from stool samples clustered within sublineages IIIb and IIIc, respectively. Most of the *S. sonnei* lineage III strains isolated from food samples (92.3%, 12 out of 13 isolates) were categorized into the sublineage IIIc and only one food strain (7.6%, 1 out of 13 isolates) was identified the sublineage IIIb. This study did not detect *S. sonnei* strains belonging to lineage I and sublineage IIIa. The majority of *S. sonnei* strains isolated from food and stool samples were significantly ($P < 0.05$) belonging to the lineage III. Notably, most of the *S. sonnei* lineage III strains isolated from both food and stool samples significantly ($P < 0.05$) belonged to the sublineage IIIc.

Antibiotic susceptibility profile of *S. sonnei* lineages/sublineages

All 64 *S. sonnei* isolates were evaluated for antibiotic susceptibility against 9 classes and 14 antibiotics. The results of antibiotic susceptibility profiles of different *S. sonnei* lineages and sublineages are presented in Table 3. Trimethoprim-sulfamethoxazole, amoxicillin, tetracycline, ampicillin, amoxicillin-clavulanic acid, streptomycin and chloramphenicol resistance were dominant phenotypes among the *S. sonnei* strains. However, the lowest antibiotic resistance was observed against kanamycin and nitrofurantoin. *S. sonnei* lineage III and sublineage IIIc strains were mostly resistant to trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol and streptomycin antibiotics.

Discussion

Genotypic characteristics of *S. sonnei* strains are strongly associated with population structure, pathogenicity and antibiotic resistance properties of this pathogen (Hawkey, *et al.* 2021). Different types of *S. sonnei* have been identified by whole-genome sequencing analysis of this pathogen, categorized into several lineages and sublineages (Baker, *et al.* 2017). Alternative methods such as PCR-HRMA have been designed and developed to differentiate these phylogenetic groups among the *S. sonnei* strains (Mazi, *et al.* 2015). This study used PCR-HRMA assay to identify lineages and sublineages of 64 *S. sonnei* strains isolated from food (n=13) and stool samples (n=51). HRMA method differentiated lineages II and III from each other, considering significantly different melting curves and temperatures. Also, this method determined the sublineages IIIb and IIIc among the strains. The reliability of the melting temperatures of the amplicons was verified by sequencing. Sangal et al. (2013), for the first time, designed and used PCR-HRMA assay for identification of *S. sonnei* phylogenetic groups (lineages/sublineages). They showed that *S. sonnei* phylogeny can be accurately determined with limited SNPs by using this method and different *S. sonnei* lineages/sublineages can be identified successfully as previously defined by the variations of whole-genome (Sangal, *et al.* 2013). Mazi et al. (2015) developed and optimized the multiplex-PCR-HRMA method to differentiate *S. sonnei* lineages and sublineages using Rotor-Gene 6000 HRM real-time PCR instrument (Qiagen) EvaGreen intercalating dye as we used the same dye and instrument in this study. They found that this method is a robust and straightforward genotyping assay for rapid and cost-effective identification of *S. sonnei* lineages and sublineages and might be regarded as an

alternative method of whole-genome sequencing (Mazi, *et al.* 2015). The differences between the melting temperatures reported in this and previous studies might be due to the variations in the experimental conditions, such as differences in PCR-HRM master mix and $MgCl_2$ concentration (Reed, *et al.* 2007, Wittwer 2009).

The results of this study provide novel information about the prevalence of different lineages and sublineages of *S. sonnei* strains isolated from food and stool samples. We found that the prevalence of *S. sonnei* lineage III and sublineage IIIc was significantly higher than that of other lineages and sublineages among the strains isolated from food and clinical samples. Sangal et al. (2013) also reported that 58 out of 68 *S. sonnei* isolates (85.2%) belonged to lineage III. They found 41.1% (28 out of 68) and 38.2% (26 out of 68) of the strains belonged to sublineages IIIb and IIIc which were mostly isolated from Brazil and North Africa, respectively (Sangal, *et al.* 2013). Baker et al. (2018) investigated the genomic epidemiology of *S. sonnei* isolated from clinical samples in the UK by using the WGS technique, and they also found that most of the *S. sonnei* strains (96.7%; 181 out of 187) belonged to the lineage III (Baker, *et al.* 2018). Hawkey et al. (2021) studied the global population structure of *S. sonnei* strains and they revealed that most of the *S. sonnei* strains isolated in Asia, Africa and Latin America are clustered within lineage III, and the sublineages IIIa and IIIb were mostly identified in Central Asia and Latin America (Hawkey, *et al.* 2021). This is the first study that investigated the *S. sonnei* lineages/sublineages in food samples, and we found the same results compared to those from the clinical samples. Lineage III was the most common lineage followed by lineage II that researchers have reported among the *S. sonnei* strains worldwide (Baker, *et al.* 2017, Hawkey, *et al.* 2021, The, *et al.* 2021); also, we reported at the present study in Iran among the isolates from food and stool samples.

This study found that resistance against trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol and streptomycin antibiotics were the dominant phenotypes among the *S. sonnei* strains grouped into the lineage III and sublineage IIIc. As previously described, the strains clustered within lineage III are predominantly resistant to tetracycline, trimethoprim-sulfamethoxazole and streptomycin because of a chromosomal Tn7-like transposon and the small plasmid spA. 74.1 and 61.2% of *S. sonnei* lineage III isolates in this study were resistant to ampicillin and chloramphenicol antibiotics, respectively. Resistance to ampicillin and chloramphenicol antibiotics mainly was observed among the lineage III strains. Several studies

also previously reported resistance against fluoroquinolones and ciprofloxacin among *S. sonnei* lineage III strains due to the acquisition of point mutations within the *gyrA* and *parC* genes (Hawkey, *et al.* 2021, Phiri, *et al.* 2021, Shad and Shad 2021, The, *et al.* 2021). It is extremely important to monitor different *S. sonnei* lineages/sublineages and the antibiotic resistance profile of these strains as the new emerging threat to public health and global concern of food safety (Hawkey, *et al.* 2021, Pakbin, *et al.* 2021a, Pakbin, *et al.* 2021c).

Conclusions

This study determined the phylogeny and antibiotic resistance profiles of *S. sonnei* strains isolated from food and stool samples. HMR assay correctly differentiated the lineages II and III, and sublineages IIIb and IIIc strains regarding the distinct melting curves and temperatures. We did not detect any lineage I and sublineage IIIa strain in this study. *S. sonnei* strains isolated from both food and clinical samples in this study clustered dominantly within the lineage III and sublineage IIIc. We also found that *S. sonnei* lineage III and sublineage IIIc strains mainly were resistant to trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol and streptomycin antibiotics.

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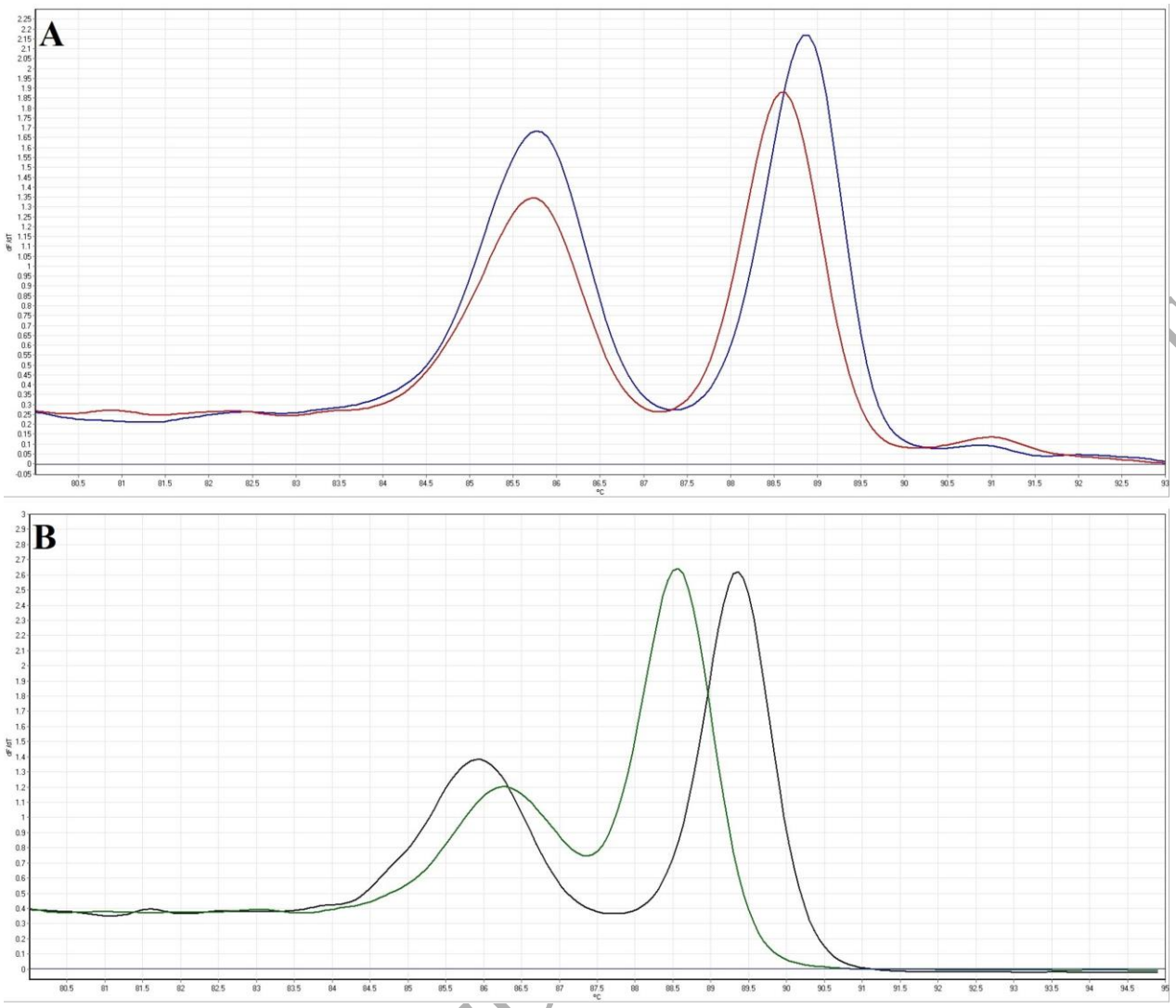

Figure 1.

(A) Multiplex-PCR-HRM curves of lineage II (Red curve) and lineage III (Blue curve) *S. sonnei* strains; (B) Multiplex-PCR-HRM curves of sublineage IIIb (Black curve) and sublineage IIIc (Green curve) *S. sonnei* strains.

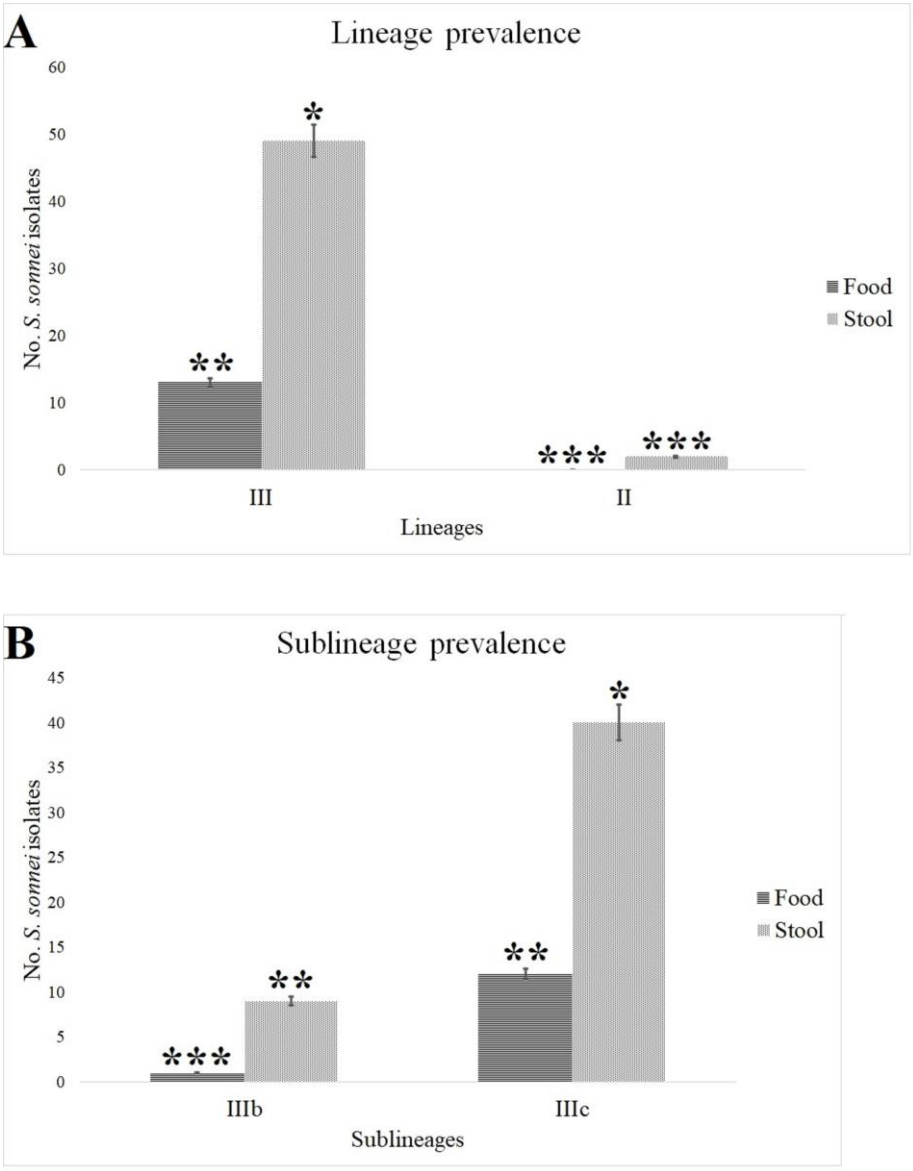

Figure 2.

Prevalence rate of different *S. sonnei* strain lineages (A) and sublineages (B) isolated from food and stool samples.

Table 1

The primers used in this study for PCR-HRM genotyping of *S. sonnei* isolates

Target gene	Primer	Sequence (5′→3′)
kduD	kduDF	CGACGGCGAAACACTTTATC
	kduDR	CGCGTATAAGAAGGCACACG
emrA	emrAF	TGCCACCGAAGTACGTAACG
	emrAR	CATCCACCCACATATTGGTG
deoA	deoAF	GGAGATGCTTATCTCCGGCAA A
	deoAR	AGTCGGTTGGGCCTTTT
menF	menFF	TATTCTCGCGCTGGTTTTTA
	menFR	GCTTTTCTTGGCTCTTCACC
fdx	fdxF	CAAAGCCTGGGACTGGA
	fdxR	CATGGTTGATAGTGTAACGC

Table 2.

T_m profile for each *S. sonnei* lineage detected in this study

	T_m (°C) (±0.02°C)				
Lineage	*kduD*	*deoA*	*emrA*	*fdx*	*menF*
Main lineages					
II	85.8	88.6	91.1		
III	85.8	88.9	90.9		
Sublineages					
IIIb				85.9	89.4
IIIc				86.3	88.6

Table 3.

Antibiotic resistance profile of *S. sonnei* lineages/sublineages

Antibiotic group	Antibiotic Agent	n (%)			
		Lineage II (N=2)	Lineage III (N=62)	Sublineage IIIb (N=10)	Sublineage IIIc (N=52)
β-Lactams	amoxicillin	2 (100)	37 (59.6)	4 (40.0)	33 (63.4)
	ampicillin	2 (100)	38 (61.2)	5 (50.0)	33 (63.4)
	amoxicillin-clavulanic acid	1 (50)	35 (56.4)	7 (70.0)	28 (53.8)
Aminoglycosides	streptomycin	1 (50)	45 (72.5)	5 (50.0)	40 (76.9)
	kanamycin	0 (0)	31 (50.0)	2 (20.0)	29 (55.7)
	amikacin	0 (0)	0 (0)	0 (0)	0 (0)
Quinolones and fluoroquinolones	nalidixic acid	0 (0)	30 (48.3)	3 (30.0)	27 (51.9)
	levofloxacin	0 (0)	33 (53.2)	6 (60.0)	30 (57.6)
Macrolides	azithromycin	0 (0)	32 (51.6)	7 (70.0)	25 (48.0)
Tetracyclines	tetracycline	1 (50)	49 (79.0)	9 (90.0)	40 (76.9)
Lipopeptides	colistin	0 (0)	12 (19.3)	2 (20.0)	10 (19.2)
Phenicols	chloramphenicol	0 (0)	46 (74.1)	8 (80.0)	38 (73.0)
Nitroheterocyclics	nitrofurantoin	0 (0)	10 (16.1)	3 (30.0)	7 (13.4)
Folate pathway antagonists	trimethoprim-sulfamethoxazole	1 (50)	54 (87.0)	10 (100)	44 (84.6)