International Journal of Food Microbiology 305 (2019) 108252

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Prevalence and molecular characterization of multidrug-resistant *Shigella* species of food origins and their inactivation by specific lytic bacteriophages

Khashayar Shahin[a,b], Majid Bouzari[a,*], Ran Wang[b,*], Mahsa Yazdi[a]

[a] Department of Biology, Faculty of Sciences, University of Isfahan, Hezar Jereeb Street, 81746-73441, Isfahan, Iran
[b] State Key Laboratory Cultivation Base of MOST, Institute of Food Safety and Nutrition, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, PR China

ARTICLE INFO

Keywords:
Shigellosis
Foodborne pathogen
Shigella spp.
Antimicrobial resistance genes
Phage therapy
Bio-control

ABSTRACT

Shigella spp. can be isolated from various food sources and is responsible for many outbreaks and sporadic cases of foodborne diseases worldwide. Although *Shigella* species are known as one of the major foodborne pathogens, a few studies have characterized the prevalence and molecular basis of antibiotic resistance of *Shigella* spp. isolated from food origins. This study investigated the prevalence of *Shigella* spp. in a wide range of food samples (1400 samples), and the phenotypic and genotypic basis of antimicrobial resistance of the isolates. In addition, the potential of two *Shigella* specific phages (vB_SflS-ISF001 and vB_SsoS-ISF002) to control the growth of the isolates in food was tested. *Shigella sonnei* and *Shigella flexneri* were detected in 11 (0.8%) and 8 (0.6%) samples, respectively. The highest prevalence of *Shigella* spp. was observed in vegetables. Multidrug resistance phenotypes were noticeably frequent and observed in 17 isolates (89.5%) out of 19 isolates. Moreover, 13 (68.4%), 9 (47.4%) and 17 (89.5%) isolates were positive for β-lactamase-encoding, plasmid-mediated quinolone resistance and tetracycline resistance genes, respectively. Treatment with the phages reduced bacterial counts up to 3 and 4 log when used individually or in cocktail form, respectively. The findings of this study indicate the prevalence of *Shigella* spp. in food sources and also provide useful information for a better understanding of the molecular aspects of antimicrobial resistance in *Shigella* spp.. The results also suggest that the combination of vB_SflS-ISF001 and vB_SsoS-ISF002 phages can effectively reduce contamination of two important species of *Shigella* in food.

1. Introduction

Shigellosis or bacillary dysentery is a bacterial enteric infection caused by *Shigella* bacteria, and is considered as a serious health problem worldwide, especially in developing countries, and also is responsible for epidemic morbidity and mortality (Alizadeh-Hesar et al., 2015; Ram et al., 2008). According to 2014 World Health Organization (WHO) report, *Shigella* spp. are responsible for an estimated 165 million cases of bacillary dysentery and 1 million deaths annually (WHO, 2014). In Iran, *Shigella* spp. are one of the most serious causes of acute diarrhea, particularly in children and young adults (Ranjbar et al., 2016).

Shigella genus consists of four known species, *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei* which also have been classified as subgroups A to D, respectively (Alizadeh-Hesar et al., 2015). Although *S. dysenteriae* causes the most serious form of shigellosis, *S. sonnei* and *S. flexneri* have been identified as the dominant species of *Shigella* in both developed and developing countries (WHO, 2005).

Shigella is generally accepted as a waterborne pathogen; it is also typically transmitted by contaminated food (Warren et al., 2006). A wide variety of foods that are involved in shigellosis include fresh fruits like watermelon (Warren et al., 2006), fresh and raw vegetables such as lettuce, iceberg lettuce, parsley and basil (Taban and Halkman, 2011), raw oyster, and fresh milk and domestic cheese (Warren et al., 2006). In addition, *Shigella* has been detected recently in chicken meat and beef (Ahmed and Shimamoto, 2014).

Although shigellosis is generally treated with fluid and nutrition support, antibiotics are used to reduce the illness duration and risk of organism's excretion (Ahmed and Shimamoto, 2015b; Shiferaw et al., 2012). *Shigella* spp. are becoming resistant to many antimicrobial agents including the first line, inexpensive oral antibiotics (ampicillin, trimethoprim-sulfamethoxazole, and tetracycline) as well as broad spectrum antibiotics such as quinolone, fluoroquinolone and extended-spectrum cephalosporins (Zaidi and Estrada-García, 2014). The emergence of multidrug-resistant (MDR) phenotype (resistant to at least three classes of antibiotics) have been reported among clinical and food

* Corresponding authors.
E-mail addresses: bouzari@sci.ui.ac.ir (M. Bouzari), ranwang@jaas.ac.cn (R. Wang).

https://doi.org/10.1016/j.ijfoodmicro.2019.108252
Received 5 November 2018; Received in revised form 7 May 2019; Accepted 11 June 2019
Available online 13 June 2019
0168-1605/

isolates of *Shigella* in the last decade (Ahmed and Shimamoto, 2015b; Alizadeh-Hesar et al., 2015; Ranjbar et al., 2016; Tajbakhsh et al., 2012). The presence of antibiotic resistant strains makes the treatment of infectious diseases difficult (Shahin et al., 2018). Thus, an urgent need to develop a new strategy to control, inhibit and eliminate bacterial pathogens is essential.

Phages (bacteriophages) are host specific viruses which infect only prokaryotes and have been introduced as safe antibacterial agents to control bacterial pathogens in various conditions without any undesirable effect on human or animal bodies, natural normal flora and plants (Gutiérrez et al., 2010; Jun et al., 2016; Jun et al., 2013). Thus the lytic potential of phages (phage therapy) against bacterial pathogens e. g. *Shigella*, *Salmonella*, *Listeria*, *Campylobacter*, *Escherichia coli*, *Staphylococcus*, *Pseudomonas* have been a progressively interesting subject of investigation (Gutiérrez et al., 2010; Kazi and Annapure, 2015), which up until now lead to introduction of several phage-based products to trade markets e.g. ShigaShield, ListShield, EcoShield, SalmoFresh, etc. (http://www.intralytix.com).

Hence, this aimed to investigate the prevalence of *Shigella* spp. in large-scale of foods, characterize their antibiotic resistance pattern, the molecular basis of resistance and evaluate the lytic effects of two *Shigella* specific phages to eliminate the *Shigella* isolates in food.

2. Materials and methods

2.1. Sample collection

Between March to November 2015, a total number of 1400 food samples were randomly collected from different local supermarkets, groceries, green groceries, vendors, butcheries, and slaughterhouses located in four provinces of Iran including Isfahan, Fars, Hormozgan and Kohkiloyeh va Boyer Ahmad. The samples included 100 ready-to-eat food, 150 fresh meats, 150 frozen meats, 100 raw cow milk, 100 domestic cheeses, and 650 different vegetables. All samples were aseptically collected in sterile bottles or test tubes, labeled and transferred to the laboratory.

2.2. Isolation and biochemical identification of Shigella spp.

The method described previously was followed for isolation of *Shigella* spp. with some modifications (Ahmed and Shimamoto, 2014). Briefly, for each sample 10 g or milliliters was placed in bottles containing 100 ml *Shigella* broth supplemented with novobiocin (2 mg/l) (Sigma Aldrich, Germany). Following homogenization (5 min at 260 rpm) and incubation (anaerobically for 20 h at 42 °C), a loopful of the enriched samples were streaked onto MacConkey and Xylose Lysine Desoxycholate agar (XLD) (Conda, Spain) plates, and then incubated for 20 h at 37 °C, aerobically. Convex colorless to slightly pink colonies on MacConkey agar and red or colorless colonies on XLD agar were selected and subjected to a primary identification process using a recommended list of biochemical tests, including triple sugar iron (TSI), urea, motility, citrate utilization, oxidase, indole production, methyl red (MR), Voges-Proskauer (VP) (Merck, Germany) (Buchanan and Gibbons, 1974; Mikoleit, 2010; Perilla et al., 2003). *S. sonnei* (ATCC 9290), *S. flexneri* (ATCC 12022), *Shigella dysenteriae* (PTCC 1188), *Shigella boydii* (ATCC 9207) and *Escherichia coli* (ATCC 25922) were used as controls.

2.3. Molecular identification and serogroup determination of Shigella spp.

The isolates that biochemically identified as *Shigella* were subjected to amplification of *ipaH* and *invC* genes using PCR to confirm the identification. *Shigella* species were identified by using additional molecular technique and serogrouping methods. For molecular identification of *S. sonnei*, *S. flexneri* and *S. dysenteriae*, the presence of *wbgZ*, *rfc* and *rfpB* genes were checked, respectively (Ahmed and Shimamoto, 2014). The primer names, their sequences, expected amplicon sizes and amplification conditions are summarized in Supplementary Table S1. Serogrouping was done using standard slide agglutination method. A fresh culture of *Shigella* isolates was tested for agglutination on glass slides using polyclonal antisera (Baharafshan Institute of Research & Development, Iran) (Alizadeh-Hesar et al., 2015).

2.4. Antimicrobial susceptibility test

Kirby–Bauer disk diffusion assay was used to determine antimicrobial susceptibilities pattern of each isolate according to the standards and interpretive criteria described by the Clinical and Laboratory Standards Institute (CLSI, 2015). The tested antimicrobial disks (Oxoid, UK) were included cephalothin (30 μg), cefuroxime (30 μg), cefixime (5 μg), cefotaxime (30 μg), cefepime (30 μg), levofloxacin (5 μg), nalidixic acid (30 μg), norfloxacin (10 μg), ciprofloxacin (5 μg), amoxicillin (25 μg), tetracycline (30 μg), trimethoprim – sulfamethoxazole (1.25/23.75 μg), tobramycin (10 μg), amikacin (30 μg), streptomycin (30 μg) and chloramphenicol (30 μg). In addition, detection of extended-spectrum beta-lactamases (ESBL) producing strains and MIC value of the imipenem were determined by double disc synergy method using ceftazidime (30 μg) and ceftazidime (30 μg) + clavulanic acid (10 μg) (Rosco, Denmark) and *E*-test strips (Liofilchem, Italy), respectively (CLSI, 2015). *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Klebsiella pneumoniae* (ATCC 700603) were used for quality control.

2.5. Bacterial DNA preparation and PCR screening for ARGs and integrons

The whole DNA from cultured strains was extracted using boiled lysates as previously described (Komijani et al., 2017). The specific primer sequences for detection of β-lactamase encoding, carbapenem resistance, PMQR, tetracycline resistance genes and two classes of integrons (class 1 and 2) are shown in Supplementary Table S1. Amplification reactions were performed in a total volume of 25 μl of green master mix (TSINGKE, China) according to the manufacturer's protocol. Amplification reactions were carried out in an Eppendorf thermal cycler (Eppendorf AG, Germany). The expected amplicons were analyzed by electrophoresis on 1% *w/v* agarose gel stained with ethidium bromide. All expected PCR products were purified from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Japan) before sequencing using an ABI DNA analyzer Model 3730xl (TSINGKE, China). The sequenced fragments were deposited in DDBJ/EMBL/GenBank under the accession numbers LC310935- LC310976.

2.6. Bacteriophage preparation and propagation

Two recently isolated lytic bacteriophages vB_SflS-ISF001 (specific for *S. flexneri* PTCC 1234, *S. flexneri* PTCC 1865 and *S. dysenteriae* PTCC 1188) (Shahin and Bouzari, 2018) and vB_SsoS-ISF002 (specific for different wastewater isolates of *S. sonnei* (Sh.s-w1 to Sh.s-w5), *S. sonnei* ATCC 9290, *S. sonnei* PTCC 1777, *S. flexneri* PTCC 1234 and *S. flexneri* PTCC 1865) (Shahin et al., 2018) were applied against all *Shigella* spp. isolated from food in this study. The vB_SflS-ISF001 and vB_SsoS-ISF002 were propagated individually using *S. flexneri* PTCC 1234 and *S. sonnei* Sh.s-w4 as bacterial host, respectively. The propagation procedure was performed according to Sambrook and Russell protocols (Sambrook and Russell, 2001). The phage suspensions (10^9 PFU/ml) were centrifuged at 25,000 × *g* for 120 min (Beckman Optima L-80 XP ultracentrifuge) to remove media components. The supernatant discarded carefully and the phage pellets were gently resuspended in sterilized sodium chloride-magnesium sulphate (SM) buffer (100 mmol l^{-1} NaCl, 8 mmol l^{-1} MgSO4, 2% gelatin, 50 mmol l^{-1} Tris-HCl, pH 7.5). The double-layer agar method was used to determine the titer of the propagated phages (Kropinski et al., 2009).

Table 1
Frequency of *Shigella*spp. isolated from various food commodities.

Shigella spp.	Ready-to-eat food (n = 250)	Fresh meat (n = 150)	Frozen meat (n = 150)	Cow milk (n = 100)	Domestic cheese (n = 100)	Vegetables (n = 650)	Total (n = 1400)
S. sonnei	2 (0.8%)	1 (0.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	8 (1.2%)	11 (0.8%)
S. flexneri	0 (0.0%)	2 (1.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	6 (0.9%)	8 (0.6%)
S. dysenteriae	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
S. boydii	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Total	2 (0.8%)	3 (2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	14 (2.2%)	19 (1.4%)

2.7. Determination of host range by spot assay

The phages host ranges were determined by the spot test method as described previously (Shahin et al., 2018). This assay was used to find out the ability of the two *Shigella* specific phages to lyse different *Shigella* spp. isolated from foods as well as *S. flexneri* PTCC 1234 and 1865 (Shahin and Bouzari, 2018), *S sonnei* ATCC 9290, PTCC 1777 and Sh-s-w4 (Shahin et al., 2018). Briefly, 10 µl of phages suspension (10^9 PFU/ml) were spotted individually onto the surface of bacterial lawn culture plates and incubated at 37 °C, aerobically, and examined for plaque formation after 18–24 h. *Shigella* strains sensitivity to phages were indicated by cleared zone formation by the spot assay. This assay was performed independently in triplicate for each phage.

2.8. Efficiency of plating (EOP)

The efficiency of plating (EOP = phage titer on tested bacterium / phage titer on the main bacterium host) was used to determine the effectiveness of each phage to cause productive infection in a variety of the bacterial strains. EOP was performed against the *Shigella* isolates with clear zone in the spot test, using a double-layer agar method (Viazis et al., 2011). The experiment was repeated independently three times. Finally, the EOP of each phage/bacterium combination was classified in high (EOP ≥ 0.5), medium (0.1 ≤ EOP < 0.5), low (0.001 < EOP < 0.1) or no (EOP ≤ 0.001) production level according to EOP mean ± SD score (Mirzaei and Nilsson, 2015).

2.9. Preparation of chicken meat for phage/bacterium challenge experiments

The fresh chicken meat was purchased at the local grocery store (Nanjing, Jiangsu province, China). First, 25 g of meat was checked for any contamination with *Shigella* spp. according to WHO recommended protocol (protocol number: 2010GFNLAB001) (Mikoleit, 2010). The meat was finely chopped aseptically before transferring to Petri dishes (5 g) and was kept in the refrigerator at 4 °C until further experiments.

2.10. Phage treatment

The early-exponential culture of the *Shigella* isolates with clear zone in the spot test and EOP > 0.001 were used individually as a bacterial host in the food experiments. The host suspensions were prepared in sterile saline solution (0.85% NaCl) and then were sprayed on the surface of the meat samples to achieve initial levels of about 10^4 CFU/ml. Following 1 h incubation at 4 °C to allow the bacteria to adsorb to the meat, the artificially *Shigella*-contaminated meat samples were treated individually with vB_SflS-ISF001, vB_SsoS-ISF002 or both (phage cocktail) and left again for 1 h to allow attachment of phage particles to the bacteria. The final concentration of added phage (s) was approximately 10^8 PFU/ml in all phage treated meats; thus, the MOI (multiplicity of infection) was constant in all the challenges (MOI = 10^4). Moreover, meat samples with the same concentration of *S flexneri* PTCC 1865 or 1234 (treated by SM buffer) were used as the untreated controls. The Petri dishes were incubated at 4 °C during phage/bacterium challenge experiments.

2.11. Bacterial enumeration

Bacterial cell enumeration was performed as described previously (Shahin and Bouzari, 2018). Briefly, after 2, 24, 48, 72, 96 and 120 h, serial dilution were prepared in PBS buffer and 100 µl of the dilutions were transferred on the surface of XLD agar to count uninjured viable *Shigella* and calculate the bacterial count (CFU/ml). All plates were incubated at 37 °C for 24 h and the plates with no visible colonies were incubated for an additional 24 h at 37 °C. All food experiments were done in triplicate and results were reported as means of the triplicates ± standard deviation.

2.12. Data analysis

The data obtained in food experiments were analyzed statistically by one-way ANOVA method using GraphPad Prism software, ver. 5.0 (Graph Pad Software, USA).

3. Results

3.1. Isolation and identification of Shigella spp.

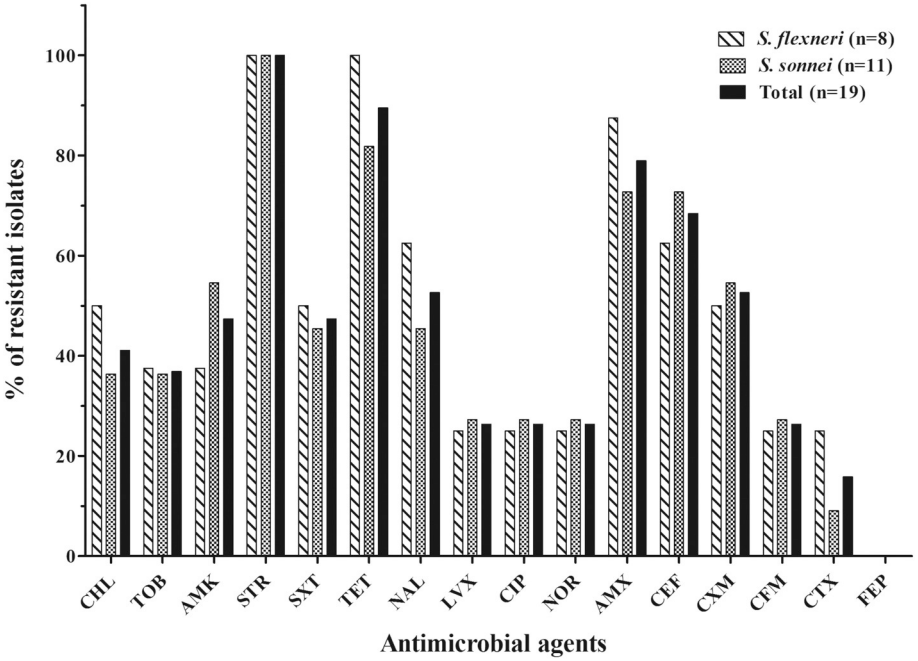
A total of 19 (1.4%) *Shigella* spp. strains were isolated from 1400 tested samples. Among them, 11 (0.8%) and 8 (0.6%) isolates identified as *S. sonnei* and *S. flexneri*, respectively, but no *S. boydii* or *S. dysenteriae* were isolated (Table 1). All the tested frozen meat, raw cow milk and domestic cheese samples in this study were negative for *Shigella* spp. The isolates were primarily identified by biochemical tests and then validated by PCR of *ipaH*, *invC*, *wbgZ* and *rfc* genes as well as serogrouping.

3.2. Antimicrobial susceptibility analysis of Shigella spp.

All the 19 *Shigella* spp. isolates were examined for their antimicrobial susceptibility against 16 different classes of antibiotics. As it is demonstrated in Fig. 1, streptomycin (19 isolates, 100%), tetracycline (17 isolates; 98.5%), amoxicillin (15 isolates; 78.9%), cephalothin (13 isolates; 68.4%), cefuroxime (10 isolates; 52.6%) and nalidixic acid (10 isolates; 52.6%) resistance were the predominant phenotypes among *Shigella* spp. isolates. While the lowest resistance were observed for cefotaxime (3 isolates; 3.8%), cefixime (5 isolates; 26.3%), norfloxacin (5 isolates; 26.3%), ciprofloxacin (5 isolates; 26.3%), levofloxacin (5 isolates; 26.3%), tobramycin (7 isolates; 36.8%), amikacin (9 isolates; 47.4%) and sulfamethoxazole-trimethoprim (9 isolates, 47.4%), all isolates were susceptible to cefepime. Seventeen out of 19 isolates (89.5%) were resistant to at least 3 different classes of antimicrobial agents and considered as MDR.

3.3. Identification of β-lactamase and carbapenemase genes

PCR results demonstrated that 13 out of 19 isolates (68.4%) carried at least one of the β-lactamase-encoding genes (Fig. 2). PCR identified among 8 *S. flexneri* isolates, 4 (50.0%), 1 (12.5%), 1 (12.5%) and 1 (12.5%) were positive for bla_{TEM}, bla_{CTX-M}, bla_{TEM}/bla_{CTX-M} and bla_{TEM}/bla_{SHV}, respectively and among 11 *S. sonnei* isolates, 4 (36.4%) and 2



AMK, amikacin; **AMX**, amoxicillin; **CHL**, chloramphenicol; **CEF**, cephalothin; **CXM**, cefuroxime; **CFM**, cefixime; **CTX**, cefotaxime; **FEP**, cefepime; **LVX**, levofloxacin; **NAL**, nalidixic acid; **NOR**, norfloxacin; **CIP**, ciprofloxacin; **TET**, tetracycline; **SXT**, trimethoprim-sulfamethoxazole; **TOB**, tobramycin; **STR**, streptomycin.

Fig. 1. Frequency of antimicrobial resistance among *Shigella* spp. isolated from various food commodities.

(18.2%) were positive for bla_{TEM} and bla_{TEM}/bla_{CTX-M}, respectively. Moreover, DNA sequencing revealed that the β-lactamase-encoding genes were belonged to bla_{TEM-1} (12 isolates), bla_{SHV-2} (1 isolate), $bla_{CTX-M-15}$ (2 isolates) and $bla_{CTX-M-14}$ (2 isolates) (Table 2). However, bla_{PER}, bla_{OXA}, bla_{VEB}, bla_{GES} and carbapenemase encoding genes (bla_{KPC}, bla_{NDM} and bla_{IMP}) were not detected in any isolates.

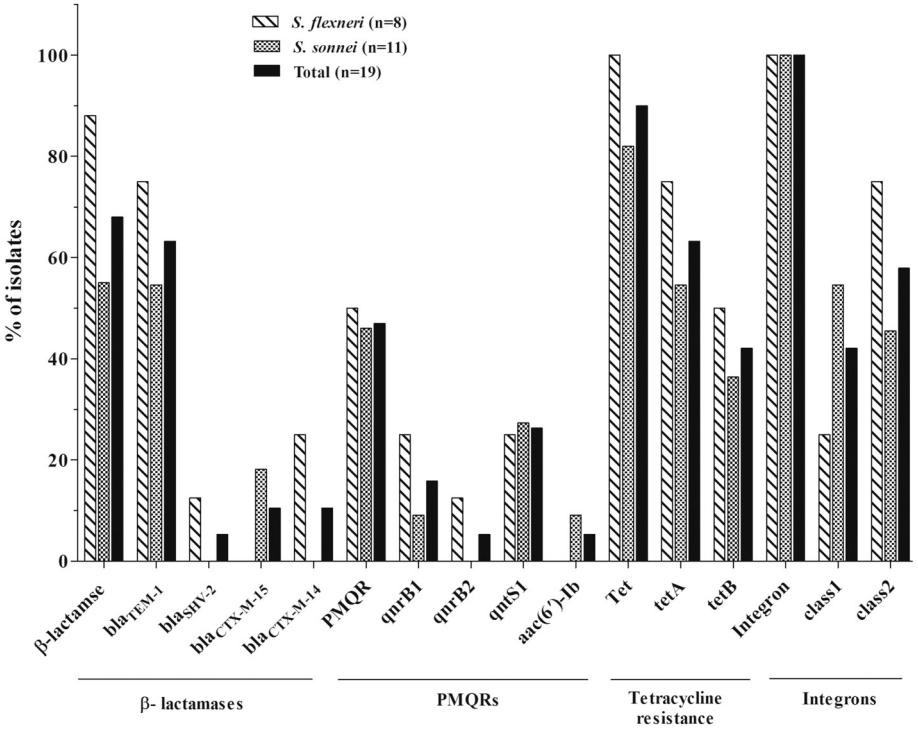


Fig. 2. Frequency of different resistance genes and integrons among *Shigella* spp. isolated from various food commodities.

Table 2
Resistance phenotypes and resistance genes in *Shigella* spp. isolated from various food commodities.

Sample	Source[a]	Resistance phenotype[b]	MIC IMI[c] (μg/ml)	ESBL[d]	Resistance genes	Integrons (variable region content)
S. flexneri						
Sh.f-8	V	STR, CHL, TOB, SXT, TET, NAL, AMX, CFM, CTX	0.125	+	$bla_{CTX-M-14}$, *qnrB-1, tetA*	Class 2 (*dfrA1-sat2-aadA1*)
Sh.f-13	V	STR, CHL, AMK, TET, NAL, AMX, CEF, CXM	0.125	−	bla_{TEM-1}, bla_{SHV-2}, *tetA,*	Class 2 (*dfrA1-sat2-aadA1*)
Sh.f-15	FM	STR, TET, NAL, LVX, NOR, CIP, AMX, CEF	0.094	−	bla_{TEM-1}, *qnrB-2, qnrS-1, tetA*	Class 2 (*dfrA1-sat2-aadA1*)
Sh.f-17	V	STR, TOB, AMK, TET	0.125	−	*tetA, tetB*	Class 1 (*dfrA17*)
Sh.f-20	FM	STR, TET, NAL, AMX, CEF, CXM, CFM, CTX	0.75	+	bla_{TEM-1}, $bla_{CTX-M-14}$, *qnrS-1, tetB*	Class 2 (*dfrA1-sat2-aadA1*)
Sh.f-21	V	STR, CHL, AMK, SXT, TET, AMX, CEF, CXM	0.094	−	bla_{TEM-1}, *tetB*	Class 1 (*dfrA17, aadA5*)
Sh.f-22	V	STR, TOB, SXT, TET, AMX, CEF	0.19	−	bla_{TEM-1}, *tetA*	Class 2 (*dfrA1-sat2-aadA1*)
Sh.f-26	V	STR, CHL, SXT, TET, NAL, LVX, NOR, CIP, AMX, CXM	0.094	−	bla_{TEM-1}, *qnrB-1, tetA, tetB*	Class 2 (*dfrA1-sat2-aadA1*)
S. sonnei						
Sh.s-3	V	STR, TOB, AMK, TET, NAL	0.094	−	*qnrS-1, tetA*	Class 2 (*dfrA1-sat2-aadA1*)
Sh.s-18	RF	STR, CHL, AMK, AMX, CEF, CXM	0.125	−	bla_{TEM-1}	Class 1 (*dfrA17, aadA5*)
Sh.s-26	FM	STR	0.094	−		Class 1 (*dfrA17, aadA5*)
Sh.s-31	RF	STR, TOB, SXT, TET, NAL, LVX, NOR, CIP, AMX, CEF, CXM, S, CFM, CTX	0.19	+	bla_{TEM-1}, $bla_{CTX-M-15}$, *qnrS-1, tetA*	Class 1 (*dfrA17, aadA5*)
Sh.s-37	V	STR, AMK, SXT, TET, AMX, CEF	0.094	−	bla_{TEM-1}, *tetA*	Class 2 (*dfrA1-sat2-aadA1*)
Sh.s-40	V	STR, TOB, SXT, TET, NAL, CEF, CXM, CFM	0.125	+	*qnrB-1, tetA, tetB*	Class 1 (*dfrA17, aadA5*)
Sh.s-77	V	STR, CHL, AMK, TET, AMX, CEF, CXM	0.125	−	bla_{TEM-1}, *tetB*	Class 2 (*dfrA1-sat2-aadA1*)
Sh.s-79	V	STR, AMK, SXT, TET, NAL, LVX, NOR, CIP, AMX, CEF, CXM	0.094	−	bla_{TEM-1}, *aac(6′)-Ib, tetA*	Class 1 (*dfrA17, aadA5*)
Sh.s-80	V	STR, TOB, AMK, SXT, TET, NAL, LVX, NOR, CIP, AMX, CEF	0.19	−	*qnrS-1, tetB*	Class 1 (*dfrA17, aadA5*)
Sh.s-87	V	STR, CHL, TET, AMX, CEF, CXM, CFM	0.125	+	bla_{TEM-1}, $bla_{CTX-M-15}$, *tetA*	Class 2 (*dfrA1-sat2-aadA1*)
Sh.s-93	V	STR, CHL, TET, AMX	0.094	−	*tetB*	Class 2 (*dfrA1-sat2-aadA1*)

[a] V, vegetables; FM, fresh meat; RF, ready-to-eat food.
[b] AMK, amikacin; AMX, amoxicillin; CHL, chloramphenicol; CEF, cephalothin; CXM, cefuroxime; CFM, cefixime; CTX, cefotaxime; FEP, cefepime; LVX, levofloxacin; NAL, nalidixic acid; NOR, norfloxacin; CIP, ciprofloxacin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; TOB, tobramycin; STR, streptomycin.
[c] MIC, minimum inhibitory concentration; IMI, imipenem.
[d] +, ESBL positive; −, ESBL negative.

3.4. Identification of PMQR genes

PCR-screening and sequencing demonstrated that 9 out of 19 isolates (47.39%) carried PMQR genes (Fig. 2). Out of 8 *S. flexneri* isolates, 2 (25.0%), 1 (12.5%), and 1 (12.5%) were positive for *qnrB* (*qnrB-1*), *qnrS* (*qnrS-1*) and *qnrB/qnrS* (*qnrB-2/qnrS-1*), respectively. Moreover, out of 11 *S. sonnei* isolates, 1 (9.1%) and 3 (27.3%) and 1 (9.1%) were positive for *qnrB* (*qnrB-1*), *qnrS* (*qnrS-1*) and *aac(6′)-Ib*, respectively, while *qnrA* gene was not detected in any of the isolates (Table 2).

3.5. Identification of tet genes

PCR results and sequencing demonstrated that 17 out of 19 isolates (89.5%) carried at least 1 of the 4 tetracycline resistance genes (*tetA*, *tetB*, *tetC*, *tetD*) (Fig. 2). Out of 8 *S. flexneri* isolates, 4 (50%), 2 (25%), and 2 (25%) were positive for *tetA*, *tetB and tetA/tetB*, respectively. Moreover, out of 11 *S. sonnei* isolates, 5 (45.5%), 3 (27.3%) and 1 (9.1%) were positive for *tetA*, *teB and tetA/tetB*, respectively, while *tetC*, *tetD* genes were not detected in any of the isolates (Table 2).

3.6. Identification of class 1 and 2 integrons

Class 1 integron was detected in 8 (42.1%) and class 2 integron in 11 (57.9%) isolates (Fig. 2). DNA sequencing of the PCR products showed that the inserted gene cassette fragment of class 1 integron consist of 2 arrangements; *dfrA17* (resistance to trimethoprim, aminoglycoside adenyltransferase) and *dfrA17/aadA5* (resistance to streptomycin and streptothricin) (Table 2). In addition, DNA sequencing of the detected class 2 integron was a *dfrA1/sat2/aadA1* fragment, *a* classical type of this integron, which confer resistance to trimethoprim, streptothricin and streptomycin/spectinomycin, respectively (Table 2).

3.7. Phage host range and efficiency of plating analysis (EOP)

Spot assay revealed that most of the *Shigella* isolates (15 out of 19 isolates, 78.9%) were lysed by vB_SflS-ISF001, vB_SsoS-ISF002 or a cocktail of both phages (Table 3). vB_SflS-ISF001 phage formed clear plaques on 11 out of the 19 isolates (8 *S. flexneri* and 3 *S. sonnei*) and vB_SsoS-ISF002 resulted in completely cleared plaques on all the *Shigella* spp. isolates (Table 3). The susceptible isolates from the spot test were subjected to EOP analysis. Despite the observed sensitivity in the spot test a wide range of EOP (0.28 ± 0.03–0.90 ± 0.02) was obtained for the host bacteria. vB_SflS-ISF001 and vB_SsoS-ISF002 infections resulted in productive infection in all tested isolates, from low production level (Sh.s-f79/vB_SflS-ISF001 combination) to high production level (Sh.f-f21/vB_SflS-ISF001 combination) (Table 4). Moreover, the high (EOP ≥ 0.5) and medium (0.1 ≤ EOP < 0.5) production levels were observed in about 75 and 25% of the tested isolates, respectively (Table 4).

3.8. Efficacy of the phages on Shigella experimentally contaminated meat

To determine the bacteriolytic effects of vB_SflS-ISF001, vB_SsoS-ISF002 or their mixture, the meat samples were first contaminated with the host bacteria (*Shigella* food isolates) and after a short incubation time were treated with the phage samples. The result demonstrated that phage treatment of the *Shigella* contaminated meat samples significantly decreased the count of the host bacteria which had EOP ≥ 0.001 (Tables 5–7). In control samples (without phage/phages treatment), no remarkable change was observed and the count of the host bacteria remained close to the initial concentration during the experiment. In contrast, the host CFU on samples treated by phage/phages were decreased after 2 h of the treatment, and the highest decrease rate was recorded after 48 h. After this point, the bacterial cell count was continued to decline in a decreasing rate up to 120 h. The host cell count after treatment with only vB_SflS-ISF001 or vB_SsoS-ISF002 samples were reduced from 4.2 log CFU/g to 1.4 and 1.1 log CFU/g, respectively ($P < 0.01$) (Tables 5 and 6). In the phage cocktail sample (consist of the both phages) the bacterial count was dropped from 4.2 to 0.3 log CFU/g ($P < 0.001$) (Table 7), indicating a higher

Table 3
The host range and efficiency of plating (EOP) of two *Shigella* specific phages against *Shigella* spp. isolated from food.

Bacterial species	Strain	Infectivity (spot test)		Efficacy of plating (EOP) (mean ± SD)	
		vB_SflS-ISF001	vB_SsoS-ISF002	vB_SflS-ISF001	vB_SsoS-ISF002
S. flexneri	PTCC 1234	+[a]	+[b]	1.00	0.59 ± 0.03
S. flexneri	PTCC 1865	+[a]	+[b]	0.84 ± 0.04	0.87 ± 0.04
S. flexneri	Sh.f-8	+	+	0.56 ± 0.04	0.40 ± 0.05
S. flexneri	Sh.f-13	+	+	0.54 ± 0.07	0.36 ± 0.04
S. flexneri	Sh.f-15	+	+	0.84 ± 0.03	0.74 ± 0.07
S. flexneri	Sh.f-17	+	+	0.73 ± 0.06	0.50 ± 0.06
S. flexneri	Sh.f-20	+	+	0.42 ± 0.08	0.66 ± 0.04
S. flexneri	Sh.f-21	+	+	0.90 ± 0.02	0.74 ± 0.05
S. flexneri	Sh.f-22	+	+	0.64 ± 0.04	0.86 ± 0.05
S. flexneri	Sh.f-26	+	+	0.62 ± 0.04	0.89 ± 0.04
S. sonnei	Sh.s-w4	−	+[b]	0	1.00
S. sonnei	ATCC 9290	−[a]	+[b]	0	0.90 ± 0.06
S. sonnei	PTCC 1777	−[a]	+[b]	0	0.78 ± 0.05
S. sonnei	Sh.s-3	−	+	0	0.48 ± 0.07
S. sonnei	Sh.s-18	+	+	0.61 ± 0.08	0.54 ± 0.08
S. sonnei	Sh.s-26	−	+	0	0.76 ± 0.03
S. sonnei	Sh.s-31	−	+	0	0.69 ± 0.02
S. sonnei	Sh.s-37	−	+	0	0.79 ± 0.02
S. sonnei	Sh.s-40	−	+	0	0.78 ± 0.07
S. sonnei	Sh.s-77	−	+	0	0.57 ± 0.04
S. sonnei	Sh.s-79	+	+	0.28 ± 0.03	0.34 ± 0.08
S. sonnei	Sh.s-80	−	+	0	0.72 ± 0.09
S. sonnei	Sh.s-87	−	+	0	0.87 ± 0.05
S. sonnei	Sh.s-93	+	+	0.72 ± 0.07	0.49 ± 0.06

+, clear plaque; −, no plaque.
[a] The spot test results are extracted from our previous study (Shahin and Bouzari, 2018).
[b] The spot test results are extracted from our previous study (Shahin et al., 2018).

Table 4
Categorization of the results of spot test assays and efficiency of plating (EOP) on the isolated *Shigella* spp.

	vB_SflS-ISF001	vB_SsoS-ISF002
S. flexneri (n = 8)		
Production of clear plaque in spot assay	8 (100%)	8 (100%)
EOP ≥ 0.5 (High production)	7 (87.5%)	6 (75%)
0.1 ≤ EOP < 0.5 (Medium production)	1 (12.5%)	2 (25%)
0.001 < EOP < 0.1 (Low production)	0 (0.0%)	0 (0.0%)
EOP ≤ 0.001 (No production)	0 (0.0%)	0 (0.0%)
S. sonnei (n = 11)		
Production of clear plaque in spot assay	3 (27.3%)	11 (100%)
EOP ≥ 0.5 (High production)	2 (18.2%)	8 (72.7%)
0.1 ≤ EOP < 0.5 (Medium production)	1 (9.1%)	3 (27.3%)
0.001 < EOP < 0.1 (Low production)	0 (0.0%)	0 (0.0%)
EOP ≤ 0.001 (No production)	8 (72.7%)	0 (0.0%)
Total (n = 19)		
Production of clear plaque in spot assay	11 (57.9)	19 (100%)
EOP ≥ 0.5 (High production)	9 (47.4%)	14 (73.7%)
0.1 ≤ EOP < 0.5 (Medium production)	2 (10.5%)	5 (2%)
0.001 < EOP < 0.1 (Low production)	0 (0.0%)	0 (0.0%)
EOP ≤ 0.001 (No production)	8 (42.1%)	0 (0.0%)

efficiency of treatment with phage cocktail compared to treatments with only the other two phage samples which were consist of just an individual phage.

4. Discussion

Shigella spp. are one of the major foodborne bacterial pathogens worldwide (Ahmed and Shimamoto, 2015b). For instance, potato salad, carrots, fresh vegetables e.g. lettuce, iceberg lettuce and parsley were introduced as the source of shigellosis outbreaks in different parts of the world (Davis et al., 1988; Frost et al., 1995; Hedberg et al., 1992; Hyams et al., 1991; Kapperud et al., 1995; Kuo et al., 2009; Lew et al., 1991; Taban and Halkman, 2011). In the current study, we estimated the frequency of *Shigella* spp. in meat, dairy products and vegetables collected in Iran by using conventional culturing methods, molecular assays and serogrouping and determined their antimicrobial resistance profile and prevalence of resistance genes among the isolates.

From 1400 food collected samples, 19 *Shigella* isolates were recovered. This indicated that 1.4% of the samples were contaminated with *Shigella* spp. which is less than the recent report from Egypt (27 out of 1600, 1.7%) (Ahmed and Shimamoto, 2014), while a study in Turkey reported the absence of *Shigella* spp. in a range of foodstuffs (Cetinkaya et al., 2008). The differences in the isolation rate of *Shigella* would be related to the sample size, source and season of the sampling, geographical locations and the level of public hygiene.

Our results showed that the frequency of *S. sonnei* (11 isolates, 0.8%) was higher than *S. flexneri* (8 isolated 0.6%). Although *S. flexneri* has been reported to be the main cause of shigellosis in developing countries, recent reports revealed that *S. sonnei* has become the predominant species of *Shigella* in Iran (Alizadeh-Hesar et al., 2015; Eftekhari et al., 2013; Farshad et al., 2006; Ranjbar et al., 2016; Ranjbar et al., 2008; Tajbakhsh et al., 2012). This discloses a direct correlation between the prevalence of *Shigella* spp. isolated from human patients and different food sources in Iran. Moreover, improvement in personal and public hygiene level and industrialization may justify the increasing frequency of *S. sonnei* in both clinical and food samples in Iran (Alizadeh-Hesar et al., 2015).

S. sonnei was isolated from the 0.8% of the ready-to-eat food, 0.7% of the fresh meat and 1.2% of the vegetable samples, while *S. flexneri* was isolated from 1.3% of the fresh meat and 0.9% of the vegetable samples. In Egypt, Ahmed and Shimamoto (2014) reported *S. sonnei* and *S. flexneri* in 0.2% and 0.7% of fresh meat samples, respectively while only *S. flexneri* was reported in frozen meat (0.1%) and cow milk (0.1%). They also reported the presence of *S. sonnei* (0.4%) and *S. flexneri* (0.5%) in cheese and *S. flexneri* (0.13%) in raw cow milk

Table 5
Treatment effects of vB_SflS-ISF001 on the CFU of *Shigella* isolates.

Isolates	Survival numbers of *Shigella* spp. isolates at different times (log CFU/g ± SD)					
	2 h	24 h	48 h	72 h	96 h	120 h
S. flexneri PTCC 1234	4.2 ± 0.24	4.2 ± 0.12	4.1 ± 0.15	4.0 ± 0.21	3.9 ± 0.13	4.0 ± 0.15
S. flexneri Sh.f-8	3.1 ± 0.02	2.8 ± 0.30	2.0 ± 0.14	2.1 ± 0.03	2.1 ± 0.15	2.3 ± 0.17
S. flexneri Sh.f-13	2.9 ± 0.12	2.7 ± 0.40	1.9 ± 0.10	2.0 ± 0.13	1.9 ± 0.18	1.9 ± 0.50
S. flexneri Sh.f-15	3.0 ± 0.18	1.9 ± 0.10	1.5 ± 0.13	1.4 ± 0.19	1.3 ± 0.02	1.3 ± 0.08
S. flexneri Sh.f-17	3.1 ± 0.10	2.3 ± 0.20	2.0 ± 0.11	1.8 ± 0.40	2.0 ± 0.20	1.9 ± 0.07
S. flexneri Sh.f-20	2.9 ± 0.27	2.8 ± 0.07	2.4 ± 0.16	2.2 ± 0.04	2.2 ± 0.08	2.2 ± 0.13
S. flexneri Sh.f-21	3.0 ± 0.30	1.7 ± 0.25	1.4 ± 0.06	1.2 ± 0.14	1.2 ± 0.04	1.1 ± 0.20
S. flexneri Sh.f-22	3.1 ± 0.08	2.6 ± 0.40	2.0 ± 0.01	1.7 ± 0.12	1.6 ± 0.00	1.7 ± 0.14
S. flexneri Sh.f-26	3.0 ± 0.21	2.0 ± 0.14	1.8 ± 0.20	1.8 ± 0.08	1.9 ± 0.12	1.9 ± 0.16
S. sonnei Sh.s-3	-[a]	-	-	-	-	-
S. sonnei Sh.s-18	3.2 ± 0.21	2.4 ± 0.04	1.6 ± 0.02	1.5 ± 0.12	1.9 ± 0.23	2.0 ± 0.04
S. sonnei Sh.s-26	-	-	-	-	-	-
S. sonnei Sh.s-31	-	-	-	-	-	-
S. sonnei Sh.s-37	-	-	-	-	-	-
S. sonnei Sh.s-40	-	-	-	-	-	-
S. sonnei Sh.s-77	-	-	-	-	-	-
S. sonnei Sh.s-79	3.1 ± 0.40	2.7 ± 0.40	2.3 ± 0.15	2.1 ± 0.02	2.1 ± 0.23	2.2 ± 0.40
S. sonnei Sh.s-80	-	-	-	-	-	-
S. sonnei Sh.s-87	-	-	-	-	-	-
S. sonnei Sh.s-93	3.3 ± 0.02	2.5 ± 0.21	1.8 ± 0.16	1.8 ± 0.20	1.6 ± 0.09	1.9 ± 0.17

The initial count of the host bacteria (time: 0 h) were approximately 4.2 log CFU/g.
[a] -, strain with no clear plaque indicating an EOP of <0.001.

(Ahmed and Shimamoto, 2014). In our study, no *Shigella* isolates were recovered from dairy products (raw cow milk and domestic cheese), which is similar to the study in Turkey (Cetinkaya et al., 2008). Due to the fact that only human is considered as the primary source of *Shigella* spp., it is believed that *Shigella* may have been transferred to meat, ready-to-eat foods and dairy products by contaminated equipment or workers' hands during collecting, processing and packing (Ahmed and Shimamoto, 2014; Ghosh et al., 2007). Moreover, in this study, the frequency of *Shigella* spp. in raw vegetables (2.2%) was higher than other food samples. This indicates a high potential risk of different raw vegetables as *Shigella* spp. carriers which probably is due to utilization of untreated wastewater or sewage, abnormal organic manure and contaminated ground and surface water because of proximity to residential areas (Taban and Halkman, 2011).

One of the most critical aspects of food safety is the appearance of antimicrobial resistant bacterial strains. These strains are capable of horizontal transferring of resistance genes from environmental and animal food resources to human normal flora and pathogens through the food chains (Ahmed and Shimamoto, 2015b). Recent study of Ahmed and Shimamoto (2015b) in Egypt showed a high resistance to tetracycline (95.8%), nalidixic acid (95.8%), sulfamethoxazole/trimethoprim and ampicillin (87.5%), chloramphenicol (58.5%) and ciprofloxacin, ceftriaxone and cefotaxime (37.5%) among food isolates of *Shigella* spp. (Ahmed and Shimamoto, 2015b). The unsupervised use of antibiotics by the community and animal health professionals may partially elucidate the differences between resistance patterns observed in different studies. In a regional point of view, our results are in contrast with previous studies in Iran that reported complete resistance to ampicillin, chloramphenicol, streptomycin, tetracycline and trimethoprim and a 50% resistance to cefotaxime among *Shigella* clinical isolates

Table 6
Effects of vB_SsoS-ISF002 treatment on the CFU of *Shigella* isolates.

Isolates	Survival numbers of *Shigella* spp. isolates at different times (log CFU/g ± SD)					
	2 h	24 h	48 h	72 h	96 h	120 h
S. flexneri PTCC 1865	4.1 ± 0.13	4.2 ± 0.04	4.1 ± 0.02	3.9 ± 0.19	3.9 ± 0.10	4.0 ± 0.11
S. flexneri Sh.f-8	3.0 ± 0.04	2.8 ± 0.22	2.2 ± 0.06	2.2 ± 0.16	2. 2 ± 0.10	2.1 ± 0.09
S. flexneri Sh.f-13	3.1 ± 0.11	2.7 ± 0.21	2.3 ± 0.14	2.3 ± 0.20	2.2 ± 0.00	2.4 ± 0.10
S. flexneri Sh.f-15	3.1 ± 0.03	2.0 ± 0.05	1.6 ± 0.07	1.5 ± 0.10	1.5 ± 0.20	1.4 ± 0.0.6
S. flexneri Sh.f-17	3.0 ± 0.15	2.5 ± 0.03	2.0 ± 0.05	1.9 ± 0.05	2.0 ± 0.20	2.0 ± 0.12
S. flexneri Sh.f-20	3.0 ± 0.40	2.4 ± 0.17	1.7 ± 0.13	1.7 ± 0.1	1.7 ± 0.20	1.6 ± 0.22
S. flexneri Sh.f-21	3.1 ± 0.11	1.9 ± 0.05	1.6 ± 0.02	1.5 ± 0.15	1.55 ± 0.2	1.4 ± 0.07
S. flexneri Sh.f-22	2.8 ± 0.03	2.4 ± 0.05	2.0 ± 0.12	1.8 ± 0.2	1.9 ± 0.10	1.8 ± 0.04
S. flexneri Sh.f-26	2.9 ± 0.10	1.5 ± 0.14	1.5 ± 0.30	1.3 ± 0.21	1.4 ± 0.30	1.0 ± 0.13
S. sonnei Sh.s-3	3.2 ± 0.13	2.8 ± 0.11	2.3 ± 0.15	2 ± 0.02	2.1 ± 0.20	2.1 ± 0.22
S. sonnei Sh.s-18	3.0 ± 0.2	2.4 ± 0.18	1.9 ± 0.03	1.6 ± 0.09	1.6 ± 0.10	1.7 ± 0.05
S. sonnei Sh.s-26	3.1 ± 0.05	2.1 ± 0.03	1.7 ± 0.05	1.7 ± 0.18	1.6 ± 0.10	1.5 ± 0.11
S. sonnei Sh.s-31	3.1 ± 0.03	2.2 ± 0.1	2.0 ± 0.13	1.9 ± 0.02	2.0 ± 0.20	1.9 ± 0.06
S. sonnei Sh.s-37	3.2 ± 0.01	1.6 ± 0.1	1.3 ± 0.14	1.4 ± 0.21	1.3 ± 0.20	1.2 ± 0.06
S. sonnei Sh.s-40	3.0 ± 0.05	1.3 ± 0.01	1.3 ± 0.20	1.2 ± 0.03	1.3 ± 0.10	1.2 ± 0.03
S. sonnei Sh.s-77	3.0 ± 0.21	2.7 ± 0.07	2.0 ± 0.11	2.0 ± 0.20	2.1 ± 0.20	2.1 ± 0.2
S. sonnei Sh.s-79	3.1 ± 0.26	2.4 ± 0.1	2.0 ± 0.20	2 ± 0.06	2.1 ± 0.10	2.1 ± 0.23
S. sonnei Sh.s-80	3.3 ± 0.1	2.5 ± 0.02	1.9 ± 0.10	1.9 ± 0.13	1.8 ± 0.20	1.7 ± 0.10
S. sonnei Sh.s-87	2.8 ± 0.14	1.3 ± 0.12	1.1 ± 0.10	1.2 ± 0.13	1.3 ± 0.2	1.3 ± 0.33
S. sonnei Sh.s-93	2.9 ± 0.26	2.8 ± 0.04	2.0 ± 0.21	2 ± 0.12	2.0 ± 0.2	2.1 ± 0.07

The initial count of the host bacteria (time: 0 h) were approximately 4.2 log CFU/g.

Table 7
The effects of treatment with vB_SflS-ISF001/vB_SsoS-ISF002 phage cocktail on the CFU of *Shigella* isolates.

Isolates	Survival numbers of *Shigella* spp. isolates at different times (log CFU/g ±SD)					
	2 h	24 h	48 h	72 h	96 h	120 h
***S. flexneri* Sh.f-8**	3.2 ± 0.10	2.2 ± 0.05	1.7 ± 0.11	1.5 ± 0.15	1.5 ± 0.10	2.1 ± 0.09
***S. flexneri* Sh.f-13**	3.1 ± 0.15	2.7 ± 0.09	1.9 ± 0.05	1.7 ± 0.07	1.8 ± 0.02	1.8 ± 0.14
***S. flexneri* Sh.f-15**	3.0 ± 0.05	1.5 ± 0.08	0.9 ± 0.11	0.7 ± 0.10	0.8 ± 0.14	0.7 ± 0.20
***S. flexneri* Sh.f-17**	3.2 ± 0.11	1.9 ± 0.15	1.5 ± 0.12	1.5 ± 0.20	1.3 ± 0.02	1.3 ± 0.16
***S. flexneri* Sh.f-20**	3.0 ± 0.21	2.2 ± 0.10	1.4 ± 0.06	1.4 ± 0.19	1.4 ± 0.25	1.3 ± 0.03
***S. flexneri* Sh.f-21**	3.0 ± 0.12	1.1 ± 0.14	0.8 ± 0.11	0.6 ± 0.20	0.5 ± 0.12	0.5 ± 0.10
***S. flexneri* Sh.f-22**	3.2 ± 0.06	1.0 ± 0.10	0.4 ± 0.15	0.4 ± 0.16	0.3 ± 0.03	0.3 ± 0.21
***S. flexneri* Sh.f-26**	3.0 ± 0.10	1.0 ± 0.05	0.3 ± 0.10	0.3 ± 0.14	0.3 ± 0.12	0.3 ± 0.10
***S. sonnei* Sh.s-3**	-[a]	-	-	-	-	-
***S. sonnei* Sh.s-18**	3.0 ± 0.05	2.0 ± 0.06	1.6 ± 0.12	1.3 ± 0.10	1.2 ± 0.01	1.2 ± 0.11
***S. sonnei* Sh.s-26**	-	-	-	-	-	-
***S. sonnei* Sh.s-31**	-	-	-	-	-	-
***S. sonnei* Sh.s-37**	-	-	-	-	-	-
***S. sonnei* Sh.s-40**	-	-	-	-	-	-
***S. sonnei* Sh.s-77**	-	-	-	-	-	-
***S. sonnei* Sh.s-79**	3.0 ± 0.15	1.9 ± 0.03	1.5 ± 0.10	1.5 ± 0.01	1.5 ± 0.04	1.4 ± 0.13
***S. sonnei* Sh.s-80**	-	-	-	-	-	-
***S. sonnei* Sh.s-87**	-	-	-	-	-	-
***S. sonnei* Sh.s-93**	3.1 ± 0.20	1.9 ± 0.21	1.5 ± 0.03	1.5 ± 0.10	1.3 ± 0.11	1.3 ± 0.05

The initial count of the host bacteria (time: 0 h) were approximately 4.2 log CFU/g.
[a] -, strain with no clear plaque indicating an EOP of <0.001 for at least one of the tested phages.

(Tajbakhsh et al., 2012). Moreover, Jafari et al. (2009) study in Iran reported the highest resistance to tetracycline, sulfamethoxazole/trimethoprim, ampicillin and chloramphenicol, while there was no resistance to cefotaxime or ceftazidime (Jafari et al., 2009). Various resistance levels to nalidixic acid and ciprofloxacin have been reported among *Shigella* ssp. in Asia–Africa regions (high frequency) and Europe–America regions (low frequency) (Gu et al., 2012). Furthermore, comparing the obtained results in this study in the case of quinolone and fluroquinolone resistance rate with earlier clinical studies from Iran suggests that there is a noticeable increase in resistance to these classes of antibiotics (Rahbar et al., 2007; Ranjbar et al., 2016; Tajbakhsh et al., 2012). Hence, it is essential to come up prevention strategy to avoid probable casualties as well as providing alternative control approaches such as phage therapy.

Our finding demonstrated that 89.5% of *Shigella* isolated from ready-to-eat food, fresh and frozen meat and vegetable samples exhibited MDR phenotype to at least three different classes of antibiotics. Almost the same incidence of MDR (88.9%) strains among food isolated *Shigella* spp. was reported in Egypt (Ahmed and Shimamoto, 2015b). It is noticeable that recent studies on clinical isolates of *Shigella* spp. reported MDR prevalence from 45% (Eftekhari et al., 2013) to 98.5% (Alizadeh-Hesar et al., 2015) and 100% (Tajbakhsh et al., 2012).

β- lactamases are known to be associated with resistance development to β-lactam antibiotics particularly in *Enterobacteriaceae* (Bradford, 2001). In this study, bla_{TEM} (63.2%), bla_{SHV} (5.3%) and bla_{CTX-M} (21.1%) were detected among *Shigella* isolates. This differs from the frequencies (16.6%, 5.5%, 22.2% and 7.18% for bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{OXA} genes, respectively) reported for food isolated *Shigella* spp. from Egypt (Ahmed and Shimamoto, 2015b). The observed differences may be correlated to the geographical locations of sampling. Moreover, in this study among the five isolates of *Shigella* spp. resistant to third generation cephalosporins (TGCs), one harbored bla_{CTX-M} gene (1 *S. flexneri*), three harbored bla_{TEM} and bla_{CTX-M} genes (2 *S. sonnei* and 1 *S. flexneri*) and one (*S. sonnei*) harbored no genes. This observation is in general agreement with earlier reports in which bla_{CTX-M} gene had been identified as the most common type for cefotaxime (TGCs) resistance among clinical *Shigella* isolates in Iran (Tajbakhsh et al., 2012) and some other regions (United States, Japan, China) (Ahmed and Shimamoto, 2015b).

According to the PMQR genes PCR results, *qnrB*, *qnrS* and *aac(6′)-Ib* genes were detected among 21.1%, 26.3% and 5.3% of *Shigella* spp., respectively. In Egypt, *qnrB* (7.4%), *qnrS* (25.9%) and *aac(6′)-Ib* (11.1%) had been identified among food isolates of *Shigella* spp. (Ahmed and Shimamoto, 2015b). This result indicated that the PMQR genes frequencies are strongly dependent on the geographical location of sampling and people drug consumption pattern. Among PMQR genes, only *qnrS* was observed among *Shigella* spp. in earlier reports from Iran (Ranjbar et al., 2016; Tajbakhsh et al., 2012). Moreover, recent studies in Iran reported other PMQR determinants in other members of *Enterobacteriaceae* such as *E. coli* (Pakzad et al., 2011; Sedighi et al., 2015). The natural ability of mobile resistance genetic elements to spread among *Enterobacteriaceae* members might be the cause of introducing new resistance genes such as PMQR from other bacteria to *Shigella* spp. (Ranjbar et al., 2016).

All tetracycline resistant strains in this study were positive for at least one of the tetracycline resistance genes (A–D). The *tetA* and *tetB* genes were detected in 63.2% and 42.1% of the isolates, demonstrating *tetA* as the main tetracycline resistance-related gene in food isolates of *S. sonnei* and *S. flexneri*. Although, the result is entirely consistent with an earlier study on resistance of clinical isolates of *Shigella* in Iran (Alizadeh-Hesar et al., 2015), it is in contrast with the reports from Brazil, USA, Canada, Thailand and Bangladesh in which *tetB* was the major tetracycline resistance-related gene determinant (Hartman et al., 2003). Moreover, From 19 isolates in this study, 3 (15.8%) were positive for both *tetA* and *tetB*. The prevalence of *tetA/tetB* among 5.7% of clinical isolates of *Shigella* in Iran was previously reported (Alizadeh-Hesar et al., 2015). The *tetC* and *tetD* were not detected in the current study, which is consistent with the previous investigations in Iran and other regions all over the world (Alizadeh-Hesar et al., 2015; Hartman et al., 2003).

Integrons are a type of mobile genetic elements in bacteria that are associated with resistance to different antimicrobial agents especially in *Enterobacteriaceae* family (Ahmed and Shimamoto, 2015a). Interestingly, all the isolates were positive either for class 1 (42%) or class 2 (58%) integrons. Almost the same frequency had been reported earlier among *Shigella* isolated from feces in Iran (Alizadeh-Hesar et al., 2015; Eftekhari et al., 2013), and foods in Egypt (Ahmed and Shimamoto, 2015b). A higher prevalence of class 2 than class 1 of integron had been reported in recent studies in different parts of the world, for examples, Eftekhari et al. (2013) reported that 78.1% and 40.6% of clinical isolates of *Shigella* spp. in Iran harbored class 2 and class 1 of integrons, respectively. Moreover, Alizadeh-Hesar et al. (2015) demonstrated that

class 1 and class 2 integrons were existed in 22.9% and 87.7% of clinical isolates of *Shigella* spp. in Iran, respectively. Similar reports in Japan (Ahmed et al., 2006), China (Pan et al., 2006), and Bangladesh (Ud-Din et al., 2013) declared a higher frequency of class 2 than class 1 integrons in clinical isolates of *Shigella* spp.

In our previous studies, the *Shigella* specific phages vB_SflS-ISF001 and vB_SsoS-ISF002, were characterized biologically and genomically (Shahin and Bouzari, 2018; Shahin et al., 2018). The host range results showed that 100% of the *S. flexneri* and *S. sonnei* strains were sensitive either to vB_SflS-ISF001 and vB_SsoS-ISF002 or both. In addition, EOP method was used against all the phage sensitive bacteria as a tool to determine the killing potential of the individual phages on each of the isolates, in which it allows to study the phage-bacteria interaction in a large collection of bacteria in a time and cost effective manner. The results of EOP assay showed that the 50% infection yield of the isolates with vB_SflS-ISF001 and 75% infection yield with vB_SsoS-ISF002 can be considered as high production category ($\geq$ 0.5). Differences in resistance systems which are genetically encoded by bacterial strains like transcription dependent systems (R/M, CRISPR-cas, Abi and various defense genes encoded by plasmids or prophages) could justify the variation in EOP assay result (Mirzaei and Nilsson, 2015). Basically, immediately after phage attachment to the surface of bacteria and genome injection, a phage begins to produce phage progenies despite the fact that the host genome contains genetic resistance systems. Due to the activation of resistance systems, different levels of phage production were measured in EOP method (Mirzaei and Nilsson, 2015). According to the data obtained from EOP assay indicating high production capacity; vB_SflS-ISF001 and vB_SsoS-ISF002 are theoretically appropriate candidates for biocontrol purposes and phage cocktail development.

In addition, in the current study, the lytic efficiency of the phages in the elimination of bacteria in food matrix supports the potential use of either vB_SflS-ISF001, or vB_SsoS-ISF002 or a cocktail of the two as a biocontrol approach for *Shigella* contamination in food, for various ranges of *S. sonnei* and *S. flexneri*. These phages significantly reduced the CFU of *S. sonnei* and *S. flexneri* isolates in the food matrix, which was in parallel to the EOP results, in other words, the reduction was greater in the isolates with high EOP. Overall, individual phage application reduced the CFU number of *Shigella* ($\geq$ 2 log). In our previous study, we reported that vB_SflS-ISF001 was capable of reducing *S. flexneri* (PTCC 1234) count in the food matrix, and the current study demonstrated its elimination potential on wild strains of *S. flexneri* and *S. sonnei* which were isolated from foodstuffs. Moreover, the individual application of another *Shigella* specific phage, vB_SsoS-ISF002, showed promising potential in reducing *Shigella* in the contaminated food. Phage cocktail, which is using more than one phage at the same time, is the best way to improve antibacterial rates and host ranges of phage-based products (Chan et al., 2013). In the current study, the combination of vB_SflS-ISF001 and vB_SsoS-ISF002 phages was applied to *Shigella* elimination in contaminated food. The results demonstrated that CFU of the bacteria reduced greatly in the treated food by the phage cocktail compare to that of the treated food by individual phages. Thus, it can be concluded that vB_SflS-ISF001 and vB_SsoS-ISF002 have a synergistic effect on each other functions. Different commercial phage-based products are available in the trade market and almost all of them contain phage cocktail e. g. ListShield, EcoShield, etc. (http://www.intralytix.com). In addition, the phage-based product Shigashield, active against *Shigella* spp., was introduced to the market recently (Soffer et al., 2017). The combination of vB_SflS-ISF001 and vB_SsoS-ISF002 has the potential to be considered as a new product of this category.

5. Conclusion

In conclusion, this study is the first to describe the incidence of *Shigella* spp. in food in Iran. It demonstrated that the incidence of *Shigella* spp. is more frequent in raw vegetables. The presence of this pathogen may be due to inappropriate irrigation of farms with contaminated water. This study also provides useful information about antimicrobial resistance pattern in non-clinical *Shigella* spp. isolates in which a high resistance to streptomycin, tetracycline, nalidixic acid and ampicillin was observed. Although the frequency and types of β-lactamase coding genes and tetracycline resistance genes of the current study are relatively consistent to previous reports, it differs in the case of PMQR determinants. It is possible that *Shigella* spp. obtained other types of PMQR from other genera of gram-negative bacteria. To develop a better insight about *Shigella* in food sources, regular studies on a wide range of foods is suggested. Moreover, the combination of vB_SflS-ISF001 and vB_SsoS-ISF002 showed a promising potential to be developed as a product for *Shigella* elimination in the food industry. Moreover, isolation of new virulent phages to enhance lytic rates and host range ensures elimination of the pathogen.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108252.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.Acknowledgment

Authors would like to pay special thankfulness, warmth and appreciation to all persons who made this research successful.

Financial support

This research was supported by an operating grant of the Dean of Research and Graduate Studies at the University of Isfahan (No: A/94/32650) and Jiangsu Agricultural Science and Technology Foundation (No. CX(16)1060).

References

Ahmed, A.M., Shimamoto, T., 2014. Isolation and molecular characterization of *Salmonella enterica*, *Escherichia coli* O157: H7 and *Shigella* spp. from meat and dairy products in Egypt. Int. J. Food Microbiol. 168, 57–62.
Ahmed, A.M., Shimamoto, T., 2015a. Molecular analysis of multidrug resistance in Shiga toxin-producing *Escherichia coli* O157: H7 isolated from meat and dairy products. Int. J. Food Microbiol. 193, 68–73.
Ahmed, A.M., Shimamoto, T., 2015b. Molecular characterization of multidrug-resistant *Shigella* spp. of food origin. Int. J. Food Microbiol. 194, 78–82.
Ahmed, A.M., Furuta, K., Shimomura, K., Kasama, Y., Shimamoto, T., 2006. Genetic characterization of multidrug resistance in *Shigella* spp. from Japan. J. Med. Microbiol. 55, 1685–1691.
Alizadeh-Hesar, M., Bakhshi, B., Najar-Peerayeh, S., 2015. Clonal dissemination of a single *Shigella sonnei* strain among Iranian children during fall 2012 in Tehran, IR Iran. Infect. Genet. Evol. 34, 260–266.
Bradford, P.A., 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. 14, 933–951.
Buchanan, R.E., Gibbons, N.E., 1974. Bergey's Manual of Determinative Bacteriology, 8 ed. Williams and Wilkins, Baltimore.
Cetinkaya, F., Cibik, R., Soyutemiz, G.E., Ozakin, C., Kayali, R., Levent, B., 2008. *Shigella* and *Salmonella* contamination in various foodstuffs in Turkey. Food Control 19, 1059–1063.
Chan, B.K., Abedon, S.T., Loc-Carrillo, C., 2013. Phage cocktails and the future of phage therapy. Future Microbiol 8, 769–783.
CLSI, 2015. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. vol. 31 Lab. Stand. Inst (M02-A12, M07-A10, and M11-A8).
Davis, H., Taylor, J.P., Perdue, J.N., Stelma, G.N., Rowntree, R., Greene, K.D., 1988. A shigellosis outbreak traced to commercially distributed shredded lettuce. Am. J. Epidemiol. 128, 1312–1321.
Eftekhari, N., Bakhshi, B., Pourshafie, M.R., Zarbakhsh, B., Rahbar, M., Hajia, M., Ghazvini, K., 2013. Genetic diversity of *Shigella* spp. and their integron content. Foodborne Pathog. Dis. 10, 237–242.
Farshad, S., Sheikhi, R., Japoni, A., Basiri, E., Alborzi, A., 2006. Characterization of *Shigella* strains in Iran by plasmid profile analysis and PCR amplification of *ipa* genes. J. Clin. Microbiol. 44, 2879–2883.
Frost, J.A., McEvoy, M.B., Bentley, C.A., Andersson, Y., 1995. An outbreak of *Shigella sonnei* infection associated with consumption of iceberg lettuce. Emerg. Infect. Dis. 1,

26–29.
Ghosh, M., Wahi, S., Kumar, M., Ganguli, A., 2007. Prevalence of enterotoxigenic *Staphylococcus aureus* and *Shigella* spp. in some raw street vended Indian foods. Int. J. Environ. Health Res. 17, 151–156.
Gu, B., Cao, Y., Pan, S., Zhuang, L., Yu, R., Peng, Z., Qian, H., Wei, Y., Zhao, L., Liu, G., 2012. Comparison of the prevalence and changing resistance to nalidixic acid and ciprofloxacin of *Shigella* between Europe–America and Asia–Africa from 1998 to 2009. Int. J. Antimicrob. Agents 40, 9–17.
Gutiérrez, D., Martínez, B., Rodríguez, A., García, P., 2010. Isolation and characterization of bacteriophages infecting *Staphylococcus epidermidis*. Curr. Microbiol. 61, 601–608.
Hartman, A.B., Essiet, I.I., Isenbarger, D.W., Lindler, L.E., 2003. Epidemiology of tetracycline resistance determinants in *Shigella* spp. and enteroinvasive *Escherichia coli*: characterization and dissemination of *tet (A)-1*. J. Clin. Microbiol. 41, 1023–1032.
Hedberg, C.W., Levine, W.C., White, K.E., Carlson, R.H., Winsor, D.K., Cameron, D.N., MacDonald, K.L., Osterholm, M.T., 1992. An international foodborne outbreak of shigellosis associated with a commercial airline. JAMA 268, 3208–3212.
Hyams, K.C., Bourgeois, A.L., Merrell, B.R., Rozmajzl, P., Escamilla, J., Thornton, S.A., Wasserman, G.M., Burke, A., Echeverria, P., Green, K.Y., 1991. Diarrheal disease during operation desert shield. N. Engl. J. Med. 325, 1423–1428.
Jafari, F., Hamidian, M., Rezadehbashi, M., Doyle, M., Salmanzadeh-ahrabi, S., Derakhshan, F., Reza Zali, M., 2009. Prevalence and antimicrobial resistance of diarrheagenic *Escherichia coli* and *Shigella* species associated with acute diarrhea in Tehran, Iran. Can. J. Infect. Dis. Med. Microbiol. 20, e56–e62.
Jun, J.W., Kim, J.H., Shin, S.P., Han, J.E., Chai, J.Y., Park, S.C., 2013. Characterization and complete genome sequence of the *Shigella* bacteriophage pSf-1. Res. Microbiol. 164, 979–986.
Jun, J.W., Kim, H.J., Yun, S.K., Chai, J.Y., Lee, B.C., Park, S.C., 2016. Isolation and comparative genomic analysis of T1-like *Shigella* bacteriophage pSf-2. Curr. Microbiol. 72, 235–241.
Kapperud, G., Rørvik, L.M., Hasseltvedt, V., Høiby, E.A., Iversen, B.G., Staveland, K., Johnsen, G., Leitao, J., Herikstad, H., Andersson, Y., 1995. Outbreak of *Shigella sonnei* infection traced to imported iceberg lettuce. J. Clin. Microbiol. 33, 609–614.
Kazi, M., Annapure, U.S., 2015. Bacteriophage biocontrol of foodborne pathogens. J. Food Sci. Technol. 53, 1–8.
Komijani, M., Bouzari, M., Rahimi, F., 2017. Detection and characterization of a novel lytic bacteriophage (vB-KpneM-Isf48) against *Klebsiella pneumoniae* isolates from infected wounds carrying antibiotic-resistance genes (TEM, SHV, and CTX-M). Iran Red Crescent Med J 19, e34475.
Kropinski, A.M., Mazzocco, A., Waddell, T.E., Lingohr, E., Johnson, R.P., 2009. Enumeration of bacteriophages by double agar overlay plaque assay. Methods Mol. Biol. 501, 69–76.
Kuo, H., Kasper, S., Jelovcan, S., Höger, G., Lederer, I., König, C., Pridnig, G., Luckner-Hornischer, A., Allerberger, F., Schmid, D., 2009. A food-borne outbreak of *Shigella sonnei* gastroenteritis, Austria, 2008. Wien. Klin. Wochenschr. 121, 157–163.
Lew, J.F., Swerdlow, D.L., Dance, M.E., Griffin, P.M., Bopp, C.A., Gillenwater, M.J., Mercatante, T., Glass, R.I., 1991. An outbreak of shigellosis aboard a cruise ship caused by a multiple-antibiotic-resistant strain of *Shigella flexneri*. Am. J. Epidemiol. 134, 413–420.
Mikoleit, M., 2010. Laboratory Protocol: Biochemical Identification of *Salmonella* and *Shigella* Using an Abbreviated Panel of Tests. WHO Global Foodborne Infections Network, Geneva, Switzerland.
Mirzaei, M.K., Nilsson, A.S., 2015. Isolation of phages for phage therapy: a comparison of spot tests and efficiency of plating analyses for determination of host range and efficacy. PLoS One 10, e0118557.
Pakzad, I., Ghafourian, S., Taherikalani, M., Abtahi, H., Rahbar, M., Mansory Jamshidi, N., 2011. Qnr prevalence in extended spectrum beta-lactamases (ESBLs) and none-ESBLs producing *Escherichia coli* isolated from urinary tract infections in central of Iran. Iran. J. Basic. Med. Sci. 14, 458–464.
Pan, J.C., Ye, R., Meng, D.M., Zhang, W., Wang, H.Q., Liu, K.Z., 2006. Molecular characteristics of class 1 and class 2 integrons and their relationships to antibiotic resistance in clinical isolates of *Shigella sonnei* and *Shigella flexneri*. J. Antimicrob. Chemother. 58, 288–296.
Perilla, M., Ajello, G., Bopp, C., Elliott, J., Facklam, R., 2003. Manual for the laboratory identification and antimicrobial susceptibility testing of bacterial pathogens of public health importance in the developing world. WHO/CDS/CSR/RMD/2003.6. , 121–141.
Rahbar, M., Deldari, M., Hajia, M., 2007. Changing prevalence and antibiotic susceptibility patterns of different *Shigella* species in Tehran, Iran. Internet J Microbiol 3, 1–3.
Ram, P.K., Crump, J.A., Gupta, S.K., Miller, M.A., Mintz, E.D., 2008. Part II. Analysis of data gaps pertaining to *Shigella* infections in low and medium human development index countries, 1984–2005. Epidemiol. Infect. 136, 577–603.
Ranjbar, R., Dallal, M.M.S., Talebi, M., Pourshafie, M.R., 2008. Increased isolation and characterization of *Shigella sonnei* obtained from hospitalized children in Tehran. Iran. J. Health Popul. Nutr. 4, 426–430.
Ranjbar, R., Behnood, V., Memariani, H., Najafi, A., Moghbeli, M., Mammina, C., 2016. Molecular characterisation of quinolone-resistant *Shigella* strains isolated in Tehran. Iran. J. Glob. Antimicrob. Re. 5, 26–30.
Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual, 2 ed. Cold Spring Harbor Laboratory, NY.
Sedighi, I., Arabestani, M.R., Rahimbakhsh, A., Karimitabar, Z., Alikhani, M.Y., 2015. Dissemination of extended-spectrum β-lactamases and quinolone resistance genes among clinical isolates of Uropathogenic *Escherichia coli* in children. Jundishapur. J. Microbiol. 8.
Shahin, K., Bouzari, M., 2018. Bacteriophage application for biocontrolling *Shigella flexneri* in contaminated foods. J. Food Sci. Technol. 55, 550–559.
Shahin, K., Bouzari, M., Wang, R., 2018. Isolation, characterization and genomic analysis of a novel lytic bacteriophage vB_SsoS-ISF002 infecting *Shigella sonnei* and *Shigella flexneri*. J. Med. Microbiol. 67, 376–386.
Shiferaw, B., Solghan, S., Palmer, A., Joyce, K., Barzilay, E.J., Krueger, A., Cieslak, P., 2012. Antimicrobial susceptibility patterns of *Shigella* isolates in foodborne diseases active surveillance network (foodnet) sites, 2000–2010. Clin. Infect. Dis. 54, S458–S463.
Soffer, N., Woolston, J., Li, M., Das, C., Sulakvelidze, A., 2017. Bacteriophage preparation lytic for *Shigella* significantly reduces *Shigella sonnei* contamination in various foods. Plo S one 12, e0175256.
Taban, B.M., Halkman, A.K., 2011. Do leafy green vegetables and their ready-to-eat [RTE] salads carry a risk of foodborne pathogens? Anaerobe 17, 286–287.
Tajbakhsh, M., Migura, L.G., Rahbar, M., Svendsen, C.A., Mohammadzadeh, M., Zali, M.R., Aarestrup, F.M., Hendriksen, R.S., 2012. Antimicrobial-resistant *Shigella* infections from Iran: an overlooked problem? J. Antimicrob. Chemother. 67, 1128–1133.
Ud-Din, A.I., Wahid, S.U., Latif, H.A., Shahnaij, M., Akter, M., Azmi, I.J., Hasan, T.N., Ahmed, D., Hossain, M.A., Faruque, A.S., 2013. Changing trends in the prevalence of *Shigella* species: emergence of multi-drug resistant *Shigella* sonnei biotype g in Bangladesh. PLoS One 8, e82601.
Viazis, S., Akhtar, M., Feirtag, J., Brabban, A., Diez-Gonzalez, F., 2011. Isolation and characterization of lytic bacteriophages against enterohaemorrhagic *Escherichia coli*. J. Appl. Microbiol. 110, 1323–1331.
Warren, B.R., Parish, M.E., Schneider, K.R., 2006. *Shigella* as a foodborne pathogen and current methods for detection in food. Crit. Rev. Food Sci. Nutr. 46, 551–567.
WHO, 2005. Shigellosis: disease burden. epidemiology and case management. Wkly. Epidemiol. Rec 80, 94–99.
WHO, 2014. Antimicrobial resistance: global report on surveillance 2014. Switzerland, Geneva.
Zaidi, M.B., Estrada-García, T., 2014. *Shigella*: a highly virulent and elusive pathogen. Curr. Trop. Med. Rep. 1, 81–87.