A New Phage Cocktail Against Multidrug, ESBL-Producer Isolates of *Shigella sonnei* and *Shigella flexneri* with Highly Efficient Bacteriolytic Activity

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The globally increasing incidence of antibiotic resistance in pathogenic microorganisms such as *Shigella*, a cause of human acute gastrointestinal infections, calls for developing effective alternatives. In this study, the antibiotic resistance pattern, extended-spectrum β -lactamase (ESBL)-production, and molecular characteristics of 70 multidrug-resistant isolates belong to the two most frequent species of *Shigella* genus, that is, *Shigella sonnei* (44 isolates) and *Shigella flexneri* (26 isolates) were investigated. These isolates were used to evaluate both specificity and activity of *Shigella*-specific bacteriophages, vB_SflS-ISF001, vB_SsoS-ISF002, and a cocktail of both. Twelve out of the 21 tested resistance genes were detected in the isolates. About 59% of *S. sonnei* and 46% of *S. flexneri* isolates were identified as ESBL producers. The bacteriophages showed a high efficiency of plating (EOP ≥ 0.5) in about 75% of the isolates. Moreover, the growth of >85% of the isolates was inhibited by the phage cocktail of vB_SflS-ISF001 and vB_SsoS-ISF002. The phage cocktail was effective against a wide range of ESBL-positive and -negative isolates of *S. sonnei* and *S. flexneri*. Therefore, this phage cocktail has the potential to inhibit or significantly decrease the spread of drug-resistant *Shigella* in humans, food chains, and water/wastewater sanitation systems.

Keywords: shigellosis, *Shigella* spp., multidrug resistance, bacteriophage, phage therapy

Introduction

SHIGELLA IS A GENUS of Gram-negative bacteria belonging to the Enterobacteriaceae family and includes four species, which can cause an acute form of gastrointestinal infection named bloody diarrhea or shigellosis.¹ The global estimation is that at least 80 million bloody diarrhea cases occur annually with about 700,000 mortalities.² Investigations show that about 70% of all the cases and 60% of the mortalities happen in children under the age of 5 years.² Due to two serious risk factors, the easy fecal/oral transmission of *Shigella* (direct contact with the infected individuals or consuming contaminated foods and water) and the low infectious dose (<200 bacteria), several outbreaks have been reported all over the world.^{2,3}

The rapid spreading of antibiotic-resistant phenotypes among microorganisms is a global critical health issue as a consequence of abuse, inappropriate, and extensive use of antimicrobial agents against bacteria.⁴ In 2013, the Centers for Disease Control and Prevention announced that human being stands in the postantibiotic era,⁵ however, just a few

years later (2017), World Health Organization (WHO) released a list of pathogens with an urgent need for new antibiotics or alternative control/treatment approaches. This list categorized the pathogens into three main groups. The extended-spectrum β -lactamase-producing (ESBL-producer) Enterobacteriaceae, responsible for at least 26,000 health care-associated infections and 1,700 death each year, were placed in the critical conditions group.^{4,6} In the case of *Shigella* species, their resistance rate to the available antimicrobial agents dramatically increased over the past 20 years (1999–2019). The National Antimicrobial Resistance Monitoring System (NARMS) of CDC (November 25, 2019) reported that in the United States, the year 1999, only 1%, 0.3%, 1.6%, 56.4%, and 57% of *Shigella* isolates were the resistance phenotypes to ceftriaxone, ciprofloxacin, nalidixic acid, streptomycin, and tetracycline, respectively, while in recent years these rates increased to 10.8% (ceftriaxone), 23.1% (ciprofloxacin), 98.3% (nalidixic acid), 57% (streptomycin), and 87% (tetracycline).⁵

Moreover, antibiotic-resistant infections are economic stress and a critical health issue that increase hospitalization

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periods and decrease the chance of successful treatment. Thus, the discovery and development of alternative treatment approaches to reduce antibiotics consumption, and the emergence rate of resistant isolates is imperative to shorten the treatment duration and to enhance the success rate of treatment. A promising alternative for such a vital task is lytic bacteriophages (phage therapy).^{7,8} Bacteriophages are bacterial viruses with a natural tendency toward specific bacteria despite their resistance or susceptibility toward antibiotics. The specific nature of phage/bacteria interaction makes bacteriophages safe and suitable for use without any concerning about the side effects on other bacteria, in particular, the normal microbiota of a host organism.⁷⁻⁹

The current study aimed to disclose the antibiotic resistance pattern, resistance genes profile, and the integron

genes among multidrug resistance (MDR) isolates of *Shigella sonnei* and *Shigella flexneri*. The second objective was to evaluate the lytic activity of vB_SsoS-ISF002, vB_SflS-ISF001, and a cocktail of both phages against ESBL-producing isolates of *Shigella*.

Materials and Methods

Samples collection

From January 2015 to September 2016, a number of MDR isolates of *S. sonnei* and *S. flexneri* were obtained from several medical centers and diagnostic laboratories in different provinces of Iran, including Isfahan, Fars, Hormozgan, and Kohkiluyeh va BoyerAhmad. To confirm the identity of the isolates, standard biochemical tests, including triple sugar

TABLE 1. ANTIBIOTIC RESISTANCE GENES, INTEGRONS, *wbgZ*, AND *rfc* PRIMERS

Primer	Sequence (5' to 3')	Amplicon size (bp)	Annealing (°C)	Reference
<i>wbgZ</i>	F:TCTGAATATGCCCTCTACGCT R:GACAGAGCCCCGAAGAACCG	430	60	15
<i>rfc</i>	F:TTTATGGCTTCTTTGTCTGGC R:CTGCGTGATCCGACCATG	537	60	15
<i>SHV</i>	F:GATGAACGCTTTCCCATGATG R:CGCTGTTATCGCTCATGGTAA	214	61	17
<i>TEM</i>	F:ATGAGTATTCAACATTTCGG R:CTGACAGTTACCAATGCTTA	867	58	17
<i>CTX-M</i>	F:TTTGCGATGTGCAGTACCAGTAA R:CGATATCGTTGGTGGTGCCATA	590	60	17
<i>OXA</i>	F:GCGTGGTTAAGGATGAACAC R:CATCAAGTTCAACCCAACCG	438	57	18
<i>PER</i>	F:ATGAATGTCTATTATAAAAGC R:AATTTGGGCTTAGGGCAGAA	925	59	20
<i>VEB</i>	F:CGACTTCCATTTCCCGATGC R:GGACTCTGCAACAAATACGC	643	58	16
<i>GES</i>	F:ATGCGCTTCATTACGCAC R:CTATTTGTCCGTGCTCAGG	864	58	18
<i>CMY</i>	F:GACAGCCTCTTTCTCCACA R:TGGAACGAAGGCTACGTA	1,010	61	14
<i>KPC</i>	F:GTCTAGTTCTGCTGTCTTG R:CTTGTCATCCTTGTTAGGCG	798	59	19
<i>NDM</i>	F:GGTTTGGCGATCTGGTTTTC R:GGAATGGCTCATCACGATC	621	59	19
<i>IMP</i>	F:CATGGTTTGGTGGTTCTTGT R:ATAATTTGGCGGACTTTGGC	448	60	19
<i>qnrA</i>	F:ATTTCTCACGCCAGGATTTG R:GATCGGCAAAGGTTAGGTCA	519	60	14
<i>qnrB</i>	F:GATCGTGAAAGCCAGAAAGG R:ACGATGCCTGGTAGTTGTCC	469	60	14
<i>qnrS</i>	F:ACGACATTCTGCAACTGCAA R:TAAATTGGCACCTGTAGGC	417	60	14
<i>aac</i>	F:TTGCGATGCTCTATGAGTGGCTA R:CTCGAATGCCTGGCGTGTTT	482	60	14
<i>tetA</i>	F:GTGAAACCCAACATACCCC R:GAAGGCAAGCAGGATGTAG	888	58	12
<i>tetB</i>	F:CCTTATCATGCCAGTCTTGC R:ACTGCCGTTTTTTCGCC	659	60	12
<i>tetC</i>	F:CTTGAGAGCCTTCAACCCAG R:ATGGTCGTCATCTACCTGCC	418	58	12
<i>tetD</i>	F:TGGGCAGATGGTCAGATAAG R:CAGCACACCCTGTAGTTTTC	787	59	12
<i>int1</i>	5'-CS:GGCATCCAAGCAGCAAG 3'-CS AAGCAGACTTGACCTGA	Variable	57	14
<i>int2</i>	hep74:CGGGATCCCGGACGGCATGCACGATTTGTA hep51:GATGCCATCGCAAGTACGAG	Variable	57	14

iron, urease, motility, citrate utilization, oxidase, indole production, methyl red, and Voges-Proskauer (Merck, Germany) were performed on the isolates.^{10,11} Finally, the identity of each isolate was validated using specific primers for the amplification of *wbgZ* (*S. sonnei*) and *rfc* (*S. flexneri*) genes (Table 1). The standard slide agglutination method was done for serogrouping (Baharafshan Institute of Research & Development, Iran).¹² Standard strains of *S. sonnei* (ATCC 9290), *S. flexneri* (ATCC 12022), *S. dysenteriae* (persian type culture collection [PTCC] 1188), *S. boydii* (PTCC 9207), and *E. coli* (ATCC 25922) were used as the controls.

Antimicrobial Susceptibility and extended-spectrum β -lactamase producer identification

The antibiotic resistance pattern of all isolates was tested against 10 antibiotics from 5 different antibiotic groups (Oxoid, UK), including ampicillin (25 μ g), cefuroxime (30 μ g), ceftazidime (30 μ g), cefotaxime (30 μ g), cefepime (30 μ g), nalidixic acid (30 μ g), ciprofloxacin (5 μ g), tetracycline (30 μ g), streptomycin (30 μ g), and chloramphenicol (30 μ g) by the Kirby/Bauer disk diffusion method following the Clinical and Laboratory Standards Institute (CLSI) guidelines.¹³ After overnight incubation at 37°C, the diameter of the zone of inhibition was measured, and the isolates were classified into three groups of resistant (R), intermediate (I), and susceptible (S) according to CLSI guideline. The isolates that showed resistance phenotype against at least three antibiotic groups were identified as MDR.¹

The minimum inhibitory concentration of imipenem (Liofilchem, Italy) was measured using the *E*-test method following CLSI guidelines.¹³ The ESBL producer isolates were identified with the combined antibiotic disks method using ceftazidime (30 μ g) and ceftazidime (30 μ g)+clavulanic acid (10 μ g) (Rosco, Denmark) according to the CLSI guidelines.¹³ Where compared with the result of the ceftazidime disc (clavulanic acid), a ≥ 5 mm increase in the inhibition zone of the combination disc (with clavulanic acid) was considered as ESBL producer isolate. *E. coli* (ATCC

25922), *Staphylococcus aureus* (ATCC 25923), and *Klebsiella pneumonia* (ATCC 700603) were used as the controls for Kirby/Bauer disk diffusion and combined antibiotic disks method.¹³

Molecular detection of antimicrobial resistance genes and integrons

Presence of the main genes related to antibiotic resistance, including β -lactamase encoding, carbapenem resistance, plasmid-mediated quinolone resistance, tetracycline resistance genes, and integrons was investigated using PCR with specific primers for all of the isolates without considering the antibiotic resistance phenotype^{12,14–20} (Table 1). DNA was extracted using the boiling method.²¹ The PCRs were performed at a final volume of 25 μ L using a green master mix (TSINGKE, China), and the products were visualized by agarose gel (1% w/v) electrophoresis (TSINGKE) stained with ethidium bromide (TSINGKE).

Bacteriophages

In this study, two specific lytic bacteriophages, vB_SfIS-ISF001 and vB_SsOS-ISF002, against *Shigella* species were used.^{22,23} For phage proliferation, 500 μ L phage suspensions (almost 10^9) were added to the specific early exponential cultures of the host bacteria (*S. sonnei* and *S. flexneri*) and incubated overnight (37°C, 150 rpm). To remove bacterial debris, the phage suspensions were centrifuged at 1,000 g for 10 min, and then the supernatants were filtered using a 0.22 μ m syringe filter (JinTeng, China).²⁴ Phage titers were determined by the double-layer agar method.²⁵

Determination of the lytic activity of the phages against clinical isolates of *Shigella*

The infection and lysis capacity of vB_SfIS-ISF001, vB_SsOS-ISF002, or the cocktail of the two phages against *S. flexneri* and *S. sonnei* isolates were evaluated using the spot test method.²³ First, the lawn cultures of each isolate were

TABLE 2. OCCURRENCE OF ANTIMICROBIAL RESISTANCE AMONG THE *SHIGELLA* ISOLATED

Antimicrobial agents	Resistant n (%)		Intermediate n (%)		Sensitive n (%)	
	<i>S. sonnei</i>	<i>S. flexneri</i>	<i>S. sonnei</i>	<i>S. flexneri</i>	<i>S. sonnei</i>	<i>S. flexneri</i>
β-Lactams						
Ampicillin	42 (95.4)	24 (92.3)	0 (0)	1 (3.8)	2 (4.5)	1 (3.8)
Cefuroxime	26 (59)	10 (38.4)	6 (13.6)	6 (23)	12 (27.2)	10 (38.4)
Ceftazidime	23 (52.2)	10 (38.4)	3 (6.8)	1 (3.8)	18 (40.9)	15 (57.6)
Cefotaxime	27 (61.3)	10 (38.4)	0 (0)	1 (3.8)	17 (38.6)	15 (57.6)
Cefepime	4 (9)	3 (11.5)	4 (9)	1 (3.8)	36 (81.8)	22 (84.6)
Quinolone and fluoroquinolones						
Nalidixic acid	31 (70.4)	16 (61.5)	8 (18.1)	5 (19.2)	5 (11.3)	5 (19.2)
Ciprofloxacin	23 (52.2)	8 (30.7)	4 (9)	4 (15.3)	17 (38.6)	14 (53.8)
Polyketides						
Tetracycline	44 (100)	26 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Aminoglycosides						
Streptomycin	44 (100)	26 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Phenicol						
Chloramphenicol	19 (43.1)	9 (34.6)	5 (11.3)	2 (7.6)	20 (45.4)	15 (57.6)

prepared, and 10 μ L of phage suspension (10^9 pfu/mL) was dropped on the surface of the culture following overnight incubation at 37°C. The formation of a clear zone at the spot area was considered as bacterial susceptibility to the phage. In the case of plaque formation on host culture, the isolate was subjected to the efficiency of plating (EOP) test using the double-layer agar method. Briefly, EOP was calculated as the ratio of the phage titer of each isolate to the phage titer obtained from the reference bacteria.²⁶ The reference bacteria for phage vB_SfIS-ISF001 and phage vB_Ssos-ISF002 were

S. flexneri PTCC 1234 and *S. sonnei* Sh.s-w1, respectively. The test was done in triplicate and results were reported as EOP mean $\pm$ standard deviation.

Preparation of the phage cocktail

vB_SfIS-ISF001 and vB_Ssos-ISF002 phages with a primary concentration of about 10^9 pfu/mL were mixed at 1:1 ratio and the antibacterial activity was evaluated against the isolates using the spot test method as described above.

FIG. 1. PCR amplification of the resistance genes and integrons. The gene names are presented at the top of each well. M, DNA marker (NormalRunTM 250bp-IV DNA ladder, ready-to-use) (Generay Biotech Co., Ltd., China).

Statistical analysis

The bacteriolytic activities of vB_SfIS-ISF001, vB_SsoS-ISF002, and their cocktail against different *S. sonnei* and *S. flexneri* isolates were analyzed by chi-square test and fisher's exact test using GraphPad Prism software, ver 6.0 (GraphPad Software). $p < 0.05$ was considered statistically significant.

Results

Shigella collection

Biochemical, molecular, and serogrouping tests were done for all of the obtained clinical isolates of *Shigella*. The identities of 44 *S. sonnei* and 26 *S. flexneri* isolates were confirmed; thus, only these isolates were used for further experiments.

Antimicrobial Susceptibility

Antimicrobial susceptibility tests showed that all isolates were resistant to tetracycline and streptomycin (Table 2). Without considering these two antibiotics, the lowest (~10%) and highest (~95%) resistance rates were observed for cefepime and ampicillin, respectively. The antimicrobial susceptibility results also showed that at least 50% of the *S. sonnei* isolates were resistant against tetracycline, streptomycin, cefotaxime, ceftazidime, cefuroxime, ampicillin, ciprofloxacin, and nalidixic acid. More than 50% of the *S. flexneri* isolates were resistant against tetracycline, streptomycin, ampicillin, and nalidixic. All the isolates that showed resistance against at least three antibiotic groups were considered as MDR.

Molecular detection of antimicrobial resistance genes and integrons

Of the 21 tested antimicrobial resistance genes, 12 genes (*SHV*, *TEM*, *CTX-M*, *CMY*, *NDM*, *qnrB*, *qnrS*, *aac*, *tetA*, *tetB*, *int1*, and *int2*) were found in at least one of the isolates, while *OXA*, *PER*, *VEB*, *GES*, *KPC*, *IMP*, *qnrA*, *tetC*, and *tetD* genes were not present in any of the isolates (Fig. 1). The frequency of *SHV*, *TEM*, *CTX-M*, *NDM*, *qnrS*, *aac*, *tetA*, and *int2* in *S. sonnei* was higher than *S. flexneri*, whereas the frequency of *CMY*, *qnrB*, and *int1* in *S. flexneri* was higher than *S. sonnei*. However, the observed differences were not statistically significant. Excluding the absent genes, the lowest and highest frequencies were observed for *CMY* and *int1* in *S. sonnei* isolates and *NDM* and *int1* in *S. flexneri* isolates. In addition, the observed frequency of *tetB* was the same in both groups (Table 3).

Phenotypic detection of ESBL and minimum inhibitory concentration of imipenem

The combined antibiotic disks revealed that 59% of *S. sonnei* (26/44 isolates) and 46.1% of *S. flexneri* isolates (12/26 isolates) were ESBL producing. Although the number of ESBL-producing isolates in the *S. sonnei* group was higher, the observed difference was not statistically significant ($p = 0.3292$). PCR results showed that except four isolates of *S. sonnei* (9, 24, 29, and 30) and nine isolates of *S. flexneri* (4, 8, 12, 13, 16, 20, 24, 25, and 26), all the other isolates

had at least one of the ESBL genes. The lowest and highest MIC of imipenem were 0.094 and 2 µg/mL for both *S. sonnei* and *S. flexneri* isolates (Tables 4 and 5).

Bacteriophage activity against the Shigella isolates

Spot assay showed that 36.3% and 72.7% of *S. sonnei* isolates were lysed by vB_SfIS-ISF001 and vB_SsoS-ISF002, respectively (Table 6). The observed difference between the sensitivity of *S. sonnei* to the phages was statistically significant. Moreover, vB_SfIS-ISF001 and vB_SsoS-ISF002 were capable of producing a clear plaque on 65.3% and 53.8% of the *S. flexneri* isolates, respectively, of which the difference was not statistically significant (Table 7).

As shown in Table 6, 11 out of 26 ESBL-producing *S. sonnei* isolates (42.3%) were lysed by vB_SfIS-ISF001, and 20 isolates (76.9%) were lysed by vB_SsoS-ISF002 ($p = 0.0227$). However, each of the vB_SfIS-ISF001 and vB_SsoS-ISF002 bacteriophages lysed 50% of ESBL-producing *S. flexneri* isolates (6 out of 12 isolates).

EOP results showed that a wide range of phage production varied from 0.03 ± 0.15 to 0.02 ± 0.95 in different bacterial hosts (Tables 6 and 7). In other words, among 16 *S. sonnei* isolates susceptible to phage vB_SfIS-ISF001, the EOP of 12 isolates was above 0.5 (high production), and for the other 4 isolates, it was under 0.5 (low production). Of the 33 *S. sonnei* isolates susceptible to phage vB_SsoS-ISF002, the EOP of 24 isolates was above 0.5 and for the other 9, it was under 0.5 (Table 6). Moreover, among the 17 isolates of *S. flexneri* lysed by phage vB_SfIS-ISF001, 11

TABLE 3. OCCURRENCE OF DIFFERENT ANTIMICROBIAL RESISTANCE GENES AND INTEGRONS AMONG THE SHIGELLA ISOLATES

Antibiotic resistance genes	<i>S. sonnei</i> n (%)	<i>S. flexneri</i> n (%)
Extended-spectrum β -lactamases		
<i>SHV</i>	7 (15.9)	3 (11.5)
<i>TEM</i>	25 (56.8)	14 (53.8)
<i>CTX-M</i>	21 (47.7)	7 (26.9)
<i>OXA</i>	0 (0)	0 (0)
<i>PER</i>	0 (0)	0 (0)
<i>VEB</i>	0 (0)	0 (0)
<i>GES</i>	0 (0)	0 (0)
<i>CMY</i>	2 (4.5)	2 (7.6)
<i>KPC</i>	0 (0)	0 (0)
<i>NDM</i>	3 (6.8)	1 (3.8)
<i>IMP</i>	0 (0)	0 (0)
Quinolone resistance		
<i>qnrA</i>	0 (0)	0 (0)
<i>qnrB</i>	10 (22.7)	7 (26.9)
<i>qnrS</i>	14 (31.8)	4 (15.3)
<i>Aac</i>	17 (38.6)	9 (34.6)
Tetracycline resistance		
<i>tetA</i>	19 (43.1)	9 (34.6)
<i>tetB</i>	22 (50)	13 (50)
<i>tetC</i>	0 (0)	0 (0)
<i>tetD</i>	0 (0)	0 (0)
Integron		
<i>int1</i>	28 (63.6)	17 (65.3)
<i>int2</i>	17 (38.6)	10 (38.4)

TABLE 4. RESISTANCE PHENOTYPES AND RESISTANCE GENES IN CLINICAL ISOLATES OF *S. SONNEI*

<i>S. sonnei</i> isolates	Resistance phenotypes	ESBL	MIC of IMI ($\mu\text{g/mL}$)	Resistance genes/integron
C1	TET, STR, CHL, AMP, NAL, CIP, CTX, FEP	+	0.19	CTX-M, qnrB, aac, tetB, int1
C2	TET, STR, AMP, NAL, CIP, CXM, CAZ, CTX, FEP	+	0.125	TEM, CTX-M, qnrS, tetA, int1
C3	TET, STR, CHL, AMP, NAL, CIP, CXM, CAZ, CTX	+	0.125	CTX-M, qnrS, tetB, int1
C4	TET, STR, AMP, NAL, CXM, CTX	+	0.094	TEM, SHV, qnrB, tetB, int2
C5	TET, STR, CHL, AMP	–	0.5	TEM, int1
C6	TET, STR, AMP	–	0.125	TEM, int1
C7	TET, STR, CHL, AMP, NAL, CIP, CXM, CAZ, CTX	+	0.125	CMY, qnrB, tetB, int2
C8	TET, STR, AMP, NAL, CIP, CXM, CAZ, CTX, FEP	+	2	CTX-M, NDM, qnrB, tetB, int2
C9	TET, STR, AMP, NAL, CIP	–	0.125	qnrS, tetA, int1
C10	TET, STR, CHL, AMP, NAL	–	0.125	TEM, CTX-M, aac, tetB, int2
C11	TET, STR, AMP, NAL, CXM, CAZ, CTX	+	1	TEM, NDM, aac, tetA, int1
C12	TET, STR, CHL, AMP, NAL, CIP, CXM, CAZ, CTX	+	0.75	CTX-M, qnrS, aac, tetB, int1
C13	TET, STR, AMP, NAL, CXM, CAZ, CTX, FEP	+	0.125	TEM, SHV, qnrS, tetA, int2
C14	TET, STR, CHL, AMP, NAL, CAZ, CTX	+	0.125	CTX-M, aac, tetB, int1
C15	TET, STR, AMP, CXM, CAZ, CTX	+	0.5	TEM, CTX-M, tetA, int1
C16	TET, STR, CHL, AMP, NAL, CIP, CXM, CTX	+	0.125	CTX-M, qnrS, tetB, int2
C17	TET, STR, AMP, CIP	–	0.125	TEM, aac, tetB, int2
C18	TET, STR, AMP, NAL, CIP, CXM, CAZ, CTX	+	0.125	SHV, CTX-M, qnrB, int1
C19	TET, STR, AMP, CXM, CAZ, CTX	+	0.125	SHV, CTX-M, aac, tetB, int1
C20	TET, STR, AMP, NAL	–	0.125	TEM, aac, tetA, int2
C21	TET, STR, AMP, CXM, CAZ, CTX	+	0.5	TEM, CTX-M, aac, tetB, int1
C22	TET, STR, CHL, NAL, CIP, CXM, CAZ, CTX	+	0.125	CTX-M, qnrS, tetA, int2
C23	TET, STR, CHL, AMP, NAL, CIP, CXM, CAZ, CTX	+	0.125	TEM, CTX-M, qnrS, tetA, int2
C24	TET, STR, NAL	–	0.125	aac, int1
C25	TET, STR, AMP	–	0.125	TEM, tetA, tetB, int1
C26	TET, STR, AMP, CXM	–	0.125	SHV, int2
C27	TET, STR, AMP, NAL, CIP, CXM, CAZ, CTX	–	0.125	TEM, SHV, CTX-M, qnrB, qnrS, tetB, int1
C28	TET, STR, CHL, NAL, CIP, CXM, CAZ	–	0.125	TEM, qnrS, tetA, int1
C29	TET, STR, NAL	–	0.125	tetA, int2
C30	TET, STR, CHL, AMP, NAL, CXM, CTX	+	0.19	aac, tetB, int1
C31	TET, STR, AMP, CXM, CAZ, CTX	+	0.125	SHV, CTX-M, aac, int1
C32	TET, STR, CHL, AMP	–	0.125	TEM, aac, tetB, int1
C33	TET, STR, CHL, AMP, NAL, CIP	–	0.094	TEM, qnrB, tetA, int2
C34	TET, STR, AMP, NAL, CIP	–	0.75	TEM, qnrS, tetA, int2
C35	TET, STR, AMP	–	0.125	TEM, tetB, int2
C36	TET, STR, AMP, NAL, CIP, CTX	+	0.19	TEM, qnrS, tetA, tetB, int2
C37	TET, STR, AMP, CHL, NAL, CIP, CXM, CAZ, CTX	+	0.125	TEM, CTX-M, qnrS, tetA, int1
C38	TET, STR, AMP, NAL, CIP	–	0.125	TEM, aac, tetA, tetB, int1
C39	TET, STR, CHL, AMP, NAL, CIP, CXM, CAZ, CTX	+	0.094	TEM, CMY, qnrS, tetB, int1
C40	TET, STR, CHL, AMP, NAL, CIP, CXM, CAZ, CTX	+	0.75	TEM, CTX-M, qnrB, aac, tetB, int2
C41	TET, STR, CHL, AMP, NAL, CIP, CXM, CAZ, CTX	+	0.125	CTX-M, qnrB, tetA, int1
C42	TET, STR, AMP	–	0.125	TEM, aac, tetA, int1
C43	TET, STR, CHL, AMP, NAL, CIP, CXM, CAZ, CTX	+	1.5	CTX-M, NDM, qnrB, tetB, int1
C44	TET, STR, AMP, NAL, CXM, CAZ, CTX	+	0.125	CTX-M, aac, tetA, int1

AMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; CXM, cefuroxime; ESBL, extended-spectrum β -lactamase; –, ESBL negative; +, ESBL positive; FEP, cefepime; IMI, imipenem; MIC, minimum inhibitory concentration; NAL, nalidixic acid; STR, streptomycin; TET, tetracycline.

and 6 isolates had an EOP of above and under 0.5, respectively; whereas, for the 14 *S. flexneri* isolates susceptible to phage vB_SsoS-ISF002, the EOP of 10 and 4 isolates were above and under 0.5, respectively (Table 7).

Phage cocktail

Among all of the 44 *S. sonnei* and 26 *S. flexneri* isolates, only 5 isolates were not found sensitive to the phage cocktail. In other words, 88.6% of the *S. sonnei* isolates and

80.7% of *S. flexneri* isolates were lysed by the cocktail. In ESBL-producing isolates, 88.4% of the *S. sonnei* (23 out of 26), and 75% of the *S. flexneri* isolates (9 out of 12) were lysed by the cocktail.

Discussion

As one of the most important pathogens associated with diarrhea, *Shigella* is a global health concern in particular in children under the age of 5 years.²⁷ Shigellosis is generally

TABLE 5. RESISTANCE PHENOTYPES AND RESISTANCE GENES IN CLINICAL ISOLATES OF *S. FLEXNERI*

<i>S. flexneri</i> isolates	Phenotypic resistance profile	ESBL	MIC of IMI (µg/mL)	Resistance genes/integron
C1	TET, STR, TET, STR, CHL, NAL, CXM, CTX	+	0.5	<i>TEM, CMY, qnrB, tetA, int1</i>
C2	TET, STR, AMP	–	0.125	<i>TEM, aac, tetB, int1</i>
C3	TET, STR, AMP, CAZ	+	0.125	<i>SHV, CTX-M, aac, tetB, int2</i>
C4	TET, STR, AMP	–	0.125	<i>qnrS, int1</i>
C5	TET, STR, AMP, CXM, CTX	+	0.125	<i>TEM, CTX-M, tetA, int1</i>
C6	TET, STR, NAL	–	0.094	<i>TEM, qnrS, tetA, int2</i>
C7	TET, STR, AMP, NAL, CIP, CXM, CAZ, CTX	+	0.19	<i>CMY, qnrB, tetB, int1</i>
C8	TET, STR, CHL, AMP	–	0.125	<i>int1</i>
C9	TET, STR, AMP, CAZ	+	0.125	<i>TEM, CTX-M, tetA, int1</i>
C10	TET, STR, AMP	–	0.094	<i>TEM, tetA, int2</i>
C11	TET, STR, AMP, NAL, CIP, CXM, CAZ, CTX, FEP	+	2	<i>TEM, CTX-M, NDM, qnrB, aac, tetA, tetB, int1</i>
C12	TET, STR, CHL, AMP, NAL, CIP	–	0.125	<i>aac, tetB, int1</i>
C13	TET, STR, AMP	–	0.125	<i>tetB, int2</i>
C14	TET, STR, AMP, NAL, CXM, CAZ, CTX	+	0.125	<i>TEM, SHV, qnrS, tetA, int1</i>
C15	TET, STR, AMP, NAL, CIP, CXM, CAZ, CTX, FEP	+	0.5	<i>TEM, qnrB, int1</i>
C16	TET, STR, AMP, NAL	–	0.125	<i>aac, tetB, int2</i>
C17	TET, STR, AMP, NAL, CIP, CXM, CAZ, CTX, FEP	+	0.38	<i>TEM, CTX-M, aac, tetA, int1</i>
C18	TET, STR, AMP, NAL, CXM, CAZ, CTX	–	0.125	<i>TEM, aac, tetB, int2</i>
C19	TET, STR, CHL, AMP, NAL, CIP	–	0.125	<i>TEM, qnrB, tetB, int2</i>
C20	TET, STR, CHL, AMP	–	0.094	<i>tetB, int1</i>
C21	TET, STR, CHL, AMP, NAL, CXM, CTX	+	0.19	<i>CTX-M, qnrS, tetB, int1</i>
C22	TET, STR, CHL, AMP, NAL, CIP, CXM, CAZ, CTX	+	0.19	<i>TEM, SHV, aac, tetB, int2</i>
C23	TET, STR, AMP, NAL, CAZ	+	0.125	<i>TEM, CTX-M, qnrB, tetA, int2</i>
C24	TET, STR, AMP	–	0.094	<i>tetB, int1</i>
C25	TET, STR, CHL, AMP, NAL	–	0.094	<i>aac, int2</i>
C26	TET, STR, CHL, AMP, NAL, CIP	–	0.094	<i>qnrB, int1</i>

introduced as the disease for poor, crowded populations who lack adequate public or personal hygiene, clean water, and proper wastewater sanitation systems. This pathogen has become resistant to several classes of antibiotics, such as cephalosporins and quinolones, which further burden the clinical procedures to identify effective antibiotics against this microorganism,⁶ of which the ESBL-producing strains are of critical importance.⁶ Therefore, there is an urgent need in finding and developing new strategies to control or eliminate the MDR and/or ESBL-producing isolates of *Shigella*. Using lytic bacteriophages against bacterial pathogens (phage therapy) is one of the promising alternatives in which its effectiveness is independent of the strain being MDR or not, or being an ESBL positive or an ESBL negative.²⁸

The objectives of our study were to determine the presence of any of the 21 antimicrobial resistance genes and integrons among the clinical MDR isolates of *Shigella*, identification of the ESBL-producing isolates, and finally, studying the efficiency of vB_SflS-ISF001, vB_SsoS-ISF002, and their cocktail on the ESBL-positive and -negative isolates.

In a study by Nuesch *et al.* during 2004–2014 on 344 *Shigella* spp isolates, 78.5% of the isolates were identified as MDR. In detail, 83.8% of *S. sonnei* and 78.5% of *S. flexneri* isolates were MDR. Moreover, the resistant levels to ampicillin, cefotaxime, nalidixic acid, streptomycin, tetracycline, ciprofloxacin, and chloramphenicol were 17.3%, 4.5%, 27.4%, 91%, 81%, 15%, and 3.4% in the *S. sonnei* isolates and 68.2%, 0%, 14%, 75.7%, 77.6%, 8.4%, and 52.3% in the *S. flexneri* isolates, respectively.²⁹ The study by Ghosh *et al.* during 2007–2010 reported 90% of the

Shigella isolates as MDR, and the resistance measures to nalidixic acid, tetracycline, ciprofloxacin, chloramphenicol, and ampicillin in these isolates were recorded as 93.4%, 88.7%, 85.8%, 62.7%, and 53.8%, respectively.³⁰

The emergence of antibiotic-resistant pathogens, such as *Shigella* species, has become a global concern.² Comparing the antimicrobial susceptibility obtained in this study with the previous reports disclose a catastrophical increase in the frequency of antibiotic-resistant *Shigella* species over the past few years. Williams and Berkley reported that resistance to the third generation of the cephalosporins due to increased number of the ESBL-producing bacteria has been increasingly prevalent in Asia, and suggested monitoring plans for such strains.³¹ The relatively high rate of ESBL isolates in this study (59% and 46.1% for *S. sonnei* and *S. flexneri*, respectively) indicates a significant increase in the population of these superbugs and calls for urgent actions in developing new efficient alternatives for rapid detection, monitoring, and control/treatment strategies.

Horizontal transmission of antibiotic resistance genes between close species or even genera of bacteria can convert susceptible isolates to resistant ones, thus, it is an important cause of the progressive increase in the frequency of the resistant isolates. Mandomando *et al.* reported frequency of 71% and 2% for the *tetA* in *S. sonnei* and *S. flexneri*, respectively, whereas it was 0% and 98% for *tetB*, respectively.³² Beladi *et al.* reported the frequencies of *TEM*, *CTX-M*, and *SHV* genes in *S. sonnei* isolates as 60.87%, 21.74%, and 0%, and 58.3%, 16.6%, and 0% in *S. flexneri* isolates, respectively.³³ Hartman *et al.* study on 459 tetracycline-resistant *Shigella* isolates reported the frequency of *tetA* and

TABLE 6. THE HOST RANGE AND EFFICIENCY OF PLATING OF vB_SfLS-ISF001 AND vB_SsoS-ISF002 PHAGES AGAINST THE CLINICAL ISOLATES OF *S. SONNEI*

Isolates	Spot test				EOP				Spot test				EOP	
	MDR	ESBL	001	002	Mix	001	002	Mix	MDR	ESBL	001	002	001	002
W4	—	—	—	1.00	1.00	—	—	—	—	—	—	—	—	—
1234	—	—	1.00	0.60 ± 0.50	—	—	—	—	—	—	—	—	—	—
C1	—	—	0.28 ± 0.05	0.61 ± 0.08	—	—	—	—	—	—	—	—	—	—
C2	—	—	—	0.79 ± 0.07	—	—	—	—	—	—	—	—	—	—
C3	—	—	0.56 ± 0.04	0.68 ± 0.02	—	—	—	—	—	—	—	—	—	—
C4	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C5	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C6	—	—	0.57 ± 0.05	—	—	—	—	—	—	—	—	—	—	—
C7	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C8	—	—	0.55 ± 0.03	—	—	—	—	—	—	—	—	—	—	—
C9	—	—	0.24 ± 0.04	—	—	—	—	—	—	—	—	—	—	—
C10	—	—	0.65 ± 0.03	—	—	—	—	—	—	—	—	—	—	—
C11	—	—	—	0.88 ± 0.07	—	—	—	—	—	—	—	—	—	—
C12	—	—	0.34 ± 0.02	0.53 ± 0.05	—	—	—	—	—	—	—	—	—	—
C13	—	—	0.65 ± 0.01	0.35 ± 0.01	—	—	—	—	—	—	—	—	—	—
C14	—	—	0.74 ± 0.04	0.65 ± 0.03	—	—	—	—	—	—	—	—	—	—
C15	—	—	0.54 ± 0.05	0.78 ± 0.07	—	—	—	—	—	—	—	—	—	—
C16	—	—	0.61 ± 0.07	0.70 ± 0.04	—	—	—	—	—	—	—	—	—	—
C17	—	—	—	0.68 ± 0.05	—	—	—	—	—	—	—	—	—	—
C18	—	—	—	0.58 ± 0.09	—	—	—	—	—	—	—	—	—	—
C19	—	—	—	0.72 ± 0.01	—	—	—	—	—	—	—	—	—	—
C20	—	—	—	0.60 ± 0.05	—	—	—	—	—	—	—	—	—	—
C21	—	—	—	0.42 ± 0.02	—	—	—	—	—	—	—	—	—	—
C22	—	—	—	0.95 ± 0.02	—	—	—	—	—	—	—	—	—	—

The represented numbers were the mean ± SD of three independent repeats; 001, vB_SfLS-ISF001; 002, vB_SsoS-ISF002; W4, *Shigella sonnei* W4; PTCC 1234, *Shigella flexneri* PTCC 1234. The positive results, high production (EOP ≥ 0.5) and low production (EOP ≤ 0.5) were shown as black (■), dark gray (■), and light gray (■), respectively. EOP, efficiency of plating; MDR, multidrug resistance.

TABLE 7. THE HOST RANGE AND EFFICIENCY OF PLATING OF vB_SfLS-ISF001 AND vB_SsoS-ISF002 PHAGES AGAINST THE CLINICAL ISOLATES OF *S. FLEXNERI*

Isolates	MDR	ESBL	Spot test			EOP	
			001	002	Mix	001	002
W4	—	—	—	■	■	—	1.00
PTCC 1234	—	—	■	■	■	1.00	0.60 ± 0.50
C1	■	■	■	■	■	0.53 ± 0.02	0.37 ± 0.05
C2	■	■	■	■	■	0.15 ± 0.03	0.65 ± 0.06
C3	■	■	■	■	■	0.40 ± 0.05	0.67 ± 0.01
C4	■	■	■	■	■	0.72 ± 0.02	0.62 ± 0.03
C5	■	■	■	■	■	—	0.80 ± 0.05
C6	■	■	■	■	■	0.52 ± 0.05	0.44 ± 0.02
C7	■	■	■	■	■	—	0.68 ± 0.06
C8	■	■	■	■	■	0.37 ± 0.05	0.29 ± 0.04
C9	■	■	■	■	■	—	0.75 ± 0.01
C10	■	■	■	■	■	—	—
C11	■	■	■	■	■	—	—
C12	■	■	■	■	■	0.65 ± 0.02	0.53 ± 0.05
C13	■	■	■	■	■	0.59 ± 0.03	0.67 ± 0.01
C14	■	■	■	■	■	—	—
C15	■	■	■	■	■	—	—
C16	■	■	■	■	■	—	—
C17	■	■	■	■	■	0.50 ± 0.07	0.15 ± 0.02
C18	■	■	■	■	■	0.62 ± 0.01	0.73 ± 0.05
C19	■	■	■	■	■	—	0.87 ± 0.02
C20	■	■	■	■	■	0.42 ± 0.01	—
C21	■	■	■	■	■	0.73 ± 0.06	—
C22	■	■	■	■	■	0.81 ± 0.01	—
C23	■	■	■	■	■	0.55 ± 0.05	—
C24	■	■	■	■	■	0.27 ± 0.08	—
C25	■	■	■	■	■	0.45 ± 0.01	—
C26	■	■	■	■	■	0.64 ± 0.03	—

The represented numbers are the mean ± SD of three independent repeats; W4, *Shigella sonnei* W4; PTCC 1234, *Shigella flexneri* PTCC 1234. The positive results, high production (EOP ≥ 0.5) and low production (EOP ≤ 0.5) were shown as black (■), dark gray (■) and light gray (■), respectively.

tetB genes as 26% and 75% for the *S. flexneri* isolates, whereas no *tetC* and *tetD* genes were detected. The frequencies of *tetA*, *tetB*, *tetC*, and *tetD* genes in *S. sonnei* isolates were recorded as 16%, 36%, 3%, and 42%, respectively.³⁴ Sow *et al.* reported the frequencies of class 1 and class 2 integrons in *Shigella* spp. as 92.8% and 57.1%, respectively.³⁵ Barrantes and Achí review on *Shigella* spp. found a significant geographical difference in the prevalence of class 1 and class 2 integrons in Brazil, Bolivia, Chile, Costa Rica, and Peru.³⁶ The observed differences among these studies can be due to geographical differences (e.g., different antibiotic consumption pattern in different countries), the number of samples, human errors, and the type of materials in experiments and sampling; nevertheless, the overall global pattern indicates an increasing trend in the spread of antibiotic resistance genes. Enterobacteriaceae family members, as one of the most clinically significant microorganisms, are capable of horizontal gene transmission among the family members and contain many of these resistance genes in their genome.³⁷ Most of these genes have extrachromosomal DNA origins, and exposure to the related antibiotics, activates the expression of these genes; while, in the absence of such environmental pressures, bacteria lose these plasmids due to the fitness cost. Therefore, in theory, the use of alternative methods can lead to a significant decrease in antibiotic con-

sumption, and over time may decrease the antibiotic-resistant incidence, which, as a result, may prevent the epidemics of antibiotic-resistant infectious diseases.^{38–40}

As early as the discovery of the bacteriophage in the 1910s, their potential use in treatment of bacterial infections, also known as phage therapy, was recognized.²⁷ Nowadays, phage therapy is one of the most promising alternative methods for antibiotic therapy. The remarkable properties of phages, including having specific hosts and as a result having no harmful influences on the normal flora or natural microbial communities of an ecosystem, productive potential, and having no side effects on eukaryotic cells, make them a highly efficient and suitable alternative for antibiotic agents.^{7,9} Several phage-based antibacterial products, such as Salmo-fresh, Ecolshield, Listshield, and Phaeurn that are already approved by the FDA and are available in the trade market, demonstrate the confirmation of successful application.

The application of bacteriophages in phage cocktail(s) requires specific criteria. An essential criterion for the application of bacteriophages in phage therapy or biocontrol purposes is being safe to use. The appropriate potential of vB_SfLS-ISF001 to be used as a biocontrolling agent in raw or cooked food had been described previously.²² In addition, no undesirable or harmful genes, example, antimicrobial resistance, bacterial virulence, or lysogeny genes were found

in the whole genome sequences of vB_SfIS-ISF001 (GenBank accession number: MG049919) and vB_SsoS-ISF002 (GenBank accession number: MF093736).^{23,41}

Having a specific phage/bacteria interaction and yet having the capacity to lyse different pathogenic strains/species of the same bacterial genus is another important criterion for the efficient application of bacteriophages. We demonstrated that both vB_SfIS-ISF001 and vB_SsoS-ISF002 were lytic bacteriophages against most of the *S. sonnei* and *S. flexneri* isolates, that is, both phages have a wide host range. Nevertheless, not being able to lyse a few of the isolates of both species does not affect such capacity. Adding one or more specific and safe bacteriophage(s) to the cocktail is a solution to overcome this shortage.

Being active against either antibiotic-resistant or susceptible strains is another critical feature for an effective bacteriophage product. vB_SfIS-ISF001 and vB_SsoS-ISF002 ability to lyse ESBL-producing MDR isolates of both *S. sonnei* and *S. flexneri* isolates is an indication of a high potential of these phages to be used as a phage product, in particular, a phage cocktail. ShigActive™, a phage cocktail consisting of five lytic *Shigella* bacteriophages, could inhibit the growth of about 92% of *S. flexneri* strains.⁴² Bernasconi *et al.* evaluated three commercial phage cocktails (PYO, INTESTI, Septaphage) against *Shigella* spp., and reported 95% susceptibility (19 out of 20 isolates) for the tested *Shigella* isolates to PYO and INTESTI, and 55% susceptibility (11 out of 20 isolates) to Septaphage.⁴³ However, none of these studies focused on the effects of these phage cocktails on MDR and ESBL-producing isolates. Also, we observed a high EOP for many of the bacterial isolates, which indicate desirable phage production in most of the samples and can ensure effective production and a high number of newborn phage release. The results of our study demonstrate that vB_SfIS-ISF001 and vB_SsoS-ISF002 are highly potent means of elimination for *Shigella* contaminations. Thus, the high efficiency of elimination against the two important *Shigella* species hosts by these phages is an indication of their potential for further development and commercial use of the phage cocktail against *Shigella*.

In summary, antibiotic resistance and high prevalence of ESBL-producing *Shigella* and the resistance genes should be considered as a serious public health issue. With the increasing number of antibiotic-resistant bacteria, bacteriophages are a powerful option for the postantibiotic era. Using vB_SfIS-ISF001 and vB_SsoS-ISF002 bacteriophages or a cocktail of both phages have the potential of further development to an antimicrobial agent against *Shigella* superbugs despite the MDR and/or ESBL profile.

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