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Characterizations, genomic analysis, and antibioflim efficacy study of novel broadspectrum virulent bacteriophages Sfin-3, Sfin-4, and Sfin-5 targeting MDR clinical isolates of Shigella spp

Chiranjib Guin
Acharya Prafulla Chandra College
Bidisha Das
Acharya Prafulla Chandra College
Srijana Rai
Acharya Prafulla Chandra College
S. K. Tousif Ahamed
University of Pennsylvania
Subhajit Dutta
Bose Institute
V. Balaji
Christian Medical College
Madhumathi Irulappan
Christian Medical College
Anirban Nandi
The University of Burdwan
Somasri Dam
The University of Burdwan
Utpal Basu
University of Kalyani
Nabanita Giri
nabanita@apccollege.ac.in

Acharya Prafulla Chandra College

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Abstract

The increasing prevalence of multidrug-resistant (MDR) *Shigella* species poses a serious global threat to public health and economy. Biofilm formation further complicates treatment due to enhanced resistance to antibiotics and host immune defenses, leading to persistent infections and increased morbidity. In this study, three novel lytic bacteriophages *Sfin-3*, *Sfin-4* and *Sfin-5* were isolated from the river Ganga and evaluated for their therapeutic potential against *Shigella flexneri (S. flexneri)*, *Shigella dysenteriae (S. dysenteriae)*, and *Shigella sonnei (S. sonnei)*. These phages exhibited rapid adsorptions in 5–10 minutes, latent periods of 5–20 minutes, and burst sizes ranging from approximately 95 to 340 plaque-forming units (PFU) per infected cell. They remained stable across a wide pH spectrum and toleated thermal exposure of 60°C for one hour. Morphological analysis identified *Sfin-3*, *Sfin-4*, and *Sfin-5* as members of the family *Siphoviridae*, characterized by isometric heads and long, non-contractile tails. Whole genome sequencing revealed that *Sfin-3*, *Sfin-4* and *Sfin-5* harbor genomes of around 50 kb, with GC contents of around 45%. Comparative genomic and phylogenetic evaluations suggested that phages are genetically distinct from other reported phages and represent novel T1-like phage isolates. Importantly, *Sfin-3*, *Sfin-4*, and *Sfin-5* displayed strong antibiofilm activity against developing and mature Shigella biofilm. Moreover, synergistic interactions with sub-MIC levels of azithromycin or ceftriaxone significantly enhanced biofilm eradication. Compared to earlier Sfin phages, these isolates exhibit broader host range and superior antibiofilm efficacy, highlighting their potential as alternative or adjunct therapeutic agents against MDR *Shigella* infections.

1. Introduction

Shigellosis, an acute gastrointestinal infection, is primarily caused by four *Shigella* species; *S. flexneri*, *S. dysenteriae*, *S. sonnei*, and *S. boydi*, all of which are gram-negative, rod-shaped, non-lactose fermenting facultative anaerobes that do not produce spores. The disease is highly contagious with fecal-oral route. The main mode of transmission especially through infected food and water. Clinical manifestations typically include bloody diarrhea, fever and cramps in the abdomen [1]. Globally, *Shigella* infections affect an estimated 164 million people annually and contribute to significant mortality, most notably in children younger than five [2]. The rise in emergence of MDR *Shigella* strains has presented a considerable concern to current treatment options. Resistance mechanisms in *Shigella* include active antibiotic efflux, decreased membrane permeability, drug-inactivating enzyme production, and target site mutations [3]. Furthermore, *Shigella spp.* via mobile genetic elements such as plasmids, transposons, insertion sequences, and genomic islands can assimilate and disperse resistance genes [4, 5]. As a result, many antibiotics that were previously effective, such as ampicillin, chloramphenicol, tetracycline, and nalidixic acid, have become largely ineffective. Although drugs such as ciprofloxacin, azithromycin, and ceftriaxone retain some efficacy, resistance to these are also increasing [6, 7]. Despite a reduction in mortality from nearly five million to 1.5 million deaths in the last two decades, the threat of MDR *Shigella* persists [8]. In this context, bacteriophage therapy has emerged as a potential option to traditional antibiotics. Lytic bacteriophages are viruses that specifically infect and lyse bacterial cells. Their unique properties, including environmental ubiquity, narrow host specificity, self-replication, and safety profile, make them suitable candidates for therapeutic applications [9]. Additionally, bacteriophages have demonstrated significant potential to disrupt biofilms, a survival strategy commonly adopted by *Shigella spp.* to evade host defenses and antibiotic treatments [10–12]. Biofilm formation is closely associated with the virulence and persistence of *Shigella* infections. Many studies have demonstrated the successful isolation and characterization of lytic phages targeting *Shigella spp*. For example, phages such as vB_SsoS-ISF002, vB_SflS-ISF001, and pSf-1 have shown lytic activity against *S. flexneri* and *S. sonnei* [13]. Likewise, Sfk20 has been shown to infect *S. flexneri* serotypes 1b, 2a, 3a, *S. sonnei* and *S. dysenteriae* 1, but ineffective against serotypes 4, 6, and *S. boydii* [14]. Recently identified lytic phages *Sfin-1*, *Sfin-2*, and *Sfin-6* have also demonstrated activity against MDR strains of *S. flexneri*, *S. dysenteriae*, and *S. sonnei*, and also *Escherichia coli* C [15, 16]. One promising strategy is by using phage cocktails which are association of more than one phages with compatible host ranges and synergistic bactericidal effects. This formulation has the potential to broaden the antibacterial spectrum and reduce the risk of developing bacterial resistance [17]. In view of these challenges, the present study focuses on isolating and characterizing three novel lytic bacteriophages - *Sfin-3*, *Sfin-4*, and *Sfin-5* - that target MDR *Shigella* species, with emphasis on their morphological, biological, and genomic featuresand antibiofilm activity.Despite progress in Shigella phage research, critical gaps remain regarding phage receptor specificity, genome-end organization and packaging mechanisms, and the ability of phages to disrupt EPS-rich biofilms or synergize with clinically relevant antibiotics. This study addresses the gaps by characterizing the host range, receptor usage, headful-packaging strategy, and antibiofilm/synergy profiles of the newly isolated *Sfin-3*, *Sfin-4*, and *Sfin-5* phages against clinically defined MDR Shigella strains.

2. Materials and methods

2.1 Bacterial strains and multidrug resistance test

A total of 42 MDR clinical isolates were examined, including strains of *S. flexneri, S. dysenteriae*, *S. sonnei*, *S. boydii*, and *Salmonella enterica* serovar Typhi, along with various strains of *Escherichia coli* such as AG100, K12, XL1 Blue and *E. coli* C. Thestrains were collected from the patient's stool samples at the National Institute for Research in Bacterial Infections (NIRBI) in Kolkata and the Christian Medical College (CMC) in Vellore, India. These isolates had been characterized in previous studies (Table 1) [18, 19, 20]. For experimental procedure, bacterial cultures were grown in nutrient broth (NB), in presence of specific antibiotic concentrations, at 37ºC. Bacterial growth was evaluated by recording the optical density at 600 nm (OD_{600}).

Table 1
Host specificity test of *Sfin-3, Sfin-4* and *Sfin*-5 phages against several clinically isolated MDRstrains.

Sample No.	Sample ID	Bacterial Sample	Antibiotic Resistance Profile	Lysis by Sfin-3	Lysis by Sfin-4	Lysis by Sfin-5
1	BCH5722	*S. flexneri 2a (1A)*	ACTQNaCipNorOfx	+	+	+
2	BCH4025	*S. flexneri 2a (2A)*	ACQ	+	+	+
3	BCH3651	*S. flexneri 2a (3A)*	ACTQ	+	+	+
4	BCH3557	*S. flexneri 2a (4A)*	CTQNa	+	+	+
5	BCH7151	*S. flexneri 2a (5A)*	ACTQNaCipNorOfx	+	+	+
6	BCH5762	*S. dysenteriae 1(1A)*	ACTQNaCipNor	+	+	+
7	BCH5848	*S. dysenteriae 1(2A)*	ACTQNaCipN	+	+	+
8	BCH5859	*S. dysenteriae 1(3A)*	ACTQNaCipNorOfxAzm	+	+	+
9	BCH5912	*S. dysenteriae 1(4A)*	ACTQNaCipNorOfx	+	+	+
10	BCH5946	*S. dysenteriae 1(5A)*	ACTQNaCipNorOfxCef	+	+	+
11	BCH7084	*S. sonnei (1)*	TQNa	+	+	+
12	BCH7264	*S. sonnei (2)*	TQNa	+	+	+
13	CMCFC2181	*S. flexneri*	AQCipCef	+	+	+
14	CMCFC2358	*S. dysenteriae*	AQCipCef	+	+	+
15	CMCFC87	*S. sonnei*	AQNaCipCef	+	+	+
16	CMCFC1799	*S. sonnei*	AQNaCipCef	+	+	+
17	CMCFC941	*S. dysenteriae*	Cip	+	+	+
18	CMCFC1010	*S. flexneri*	ACefTOfx	+	+	+
19	CMCFC1054	*S. flexneri*	ACipTOfx	+	+	+
20	CMCFC1274	*S. flexneri*	CipTOfx	+	+	+
21	CMCFC745	*S. flexneri*	T	+	+	+
22	CMCFC2113	*S. flexneri 2*	ACip	+	+	+
23	CMCFC868	*S. flexneri*	ATC	+	+	+
24	CMCFC593	*S. flexneri*	TCOfx	+	+	+
25	CMCFC2900	*S. flexneri 2*	CefCip	+	+	+
26	CMCFC526	*S. flexneri*	ACipTCOfx	+	+	+
27	CMCFC2206	*S. sonnei*	ACefAzmOfx	+	+	+
28	CMCFC756	*S. flexneri*	ACip	+	+	+
29	FC970	*S.typhimurium*	ACipCtr	-	-	-
30	FC1391	*S.typhimurium*	ACipCtr	-	-	-
31	FC974	*S.typhimurium*	ACipCtrCef	-	-	-
32	FC988	*S.typhimurium*	ACipCtrCef	-	-	-
33	FC1533	*S.typhimurium*	CipCtr	-	-	-
34	FC1281	*S.typhimurium*	NaCip	-	-	-
35	FC954	*S.typhimurium*	ACipCtr	-	-	-
36	FC1560	*S.typhimurium*	NaCtr	-	-	-
37	FC1482	*S.typhimurium*	NaC	-	-	-
38	FC1428	*S.typhimurium*	ASxtCipCtr	-	-	-
39		*Escherichia coli K12*		-	-	-

Sample No.	Sample ID	Bacterial Sample	Antibiotic Resistance Profile	Lysis by Sfin-3	Lysis by Sfin-4	Lysis by Sfin-5
40		*Escherichia coli C*		-	-	-
41		*Escherichia coli AG100*		-	-	-
42		*XL1 Blue*		-	-	-

2.2 Isolation, amplification, and purification of bacteriophages

Two sites- near Barrackpore in the North 24 Parganas district and Chandannagar in the Hooghly district, both approximately 25 km away from Kolkata, West Bengal, India were selected for collection of environmental water samples from the Ganga River. Initially the samples were filtered through Whatman No.1 filter paper to eliminate suspended particles. Subsequently, an exponential-phase culture of *S. flexneri 2a* was introduced into the water samples, supplemented with 10% peptone (w/v), and incubated at 37ºC with agitation for 24 hours. To eliminate the debris of bacterial cells, 1% chloroform (w/v) was added and thoroughly mixed. Following centrifugation and filtration through 0.22-µm membrane filter (Millipore, USA), the supernatant was collected and filtered. To detect the presence of bacteriophages, 10 µl of the filtrate was spotted on an agar plate seeded with *Shigellaspp.* A lysis zone around the spot indicated lytic activity against *S. flexneri* 2a and other *Shigella* serotypes. In plaque assays, a mixture of 200 µl of *Shigella* culture (OD_{600} = 0.3) and 100 µl of phage filtrate in combination with 3.5 ml of soft agar (0.9%) poured onto NB agar plates and incubated at 37ºC for 24 hours. Distinct plaques appeared, were transferred to fresh *Shigella* plates. Individual plaques were isolated into 500 µl of Sodium Magnesium (SM) buffer and further plaque screening was performed for three successive rounds to purify the bacteriophage. To generate high-titer phage stocks, confluent lysis plates were prepared and scraped; the soft agar was dissolved in cold SM buffer and kept on ice for 24 hours. To obtain the supernatant, the mixture produced was centrifuged at 5,000×g and then, ultracentrifuged at 68,000×g for duration of 2 hours at 4ºC to pellet the phage particles. For further purification, cesium chloride (CsCl) density gradient centrifugation (densities: 1.3, 1.5, and 1.7 g/ml) was carried out at 100,000×g for three hours at 4ºC.The distinct phage band obtained between 1.5 and 1.7 g/mL was carefully collected and dialyzed against Tris-HCl magnesium sulfate (TM) buffer (50 mM Tris-Cl, pH 8.0, containing 10 mM $MgSO_4$). The purified phage preparation was stored at 4°C.

2.3 Host range determination

To assess the host range of the isolated bacteriophages, various strains of *Shigella*, *Salmonella*, and *Escherichia coli* were tested (Table 1). Firstly, the bacterial cultures were propagated overnight in nutrient broth at 37ºC. After incubation, 200 µl of each culture was combined with 3.5 ml of molten soft agar (0.9% w/v) and evenly layered on solidified NB agar plates (1.8% w/v). The phage suspension of 10 µl containing approximately 1.0 × 10^{10} PFU/ml, was then spotted on the bacterial lawn surface and then incubated overnight at 37ºC. The clear lysis zone indicated the susceptibility of tested bacterial strain into the phage. All experiments were conducted in triplicate. On the basis of the clarity of the lysis zone, results were categorized as either positive (clear zone, +) or negative (no visible reaction, –).

2.4 Lytic activity of Phage against different bacterial strains

Based on CLSI guidelines, 2021 [21], the *Shigella* strains employed in the present study were classified as MDR. To evaluate the bacteriolytic activity of the isolated phages, modified protocol by was used. The cultures of *S. flexneri* 2a and *S. dysenteriae* 1 were grown inpresence of different antibiotics, including ampicillin (32 µg/ml), tetracycline (16 µg/ml), chloramphenicol (32 µg/ml), nalidixic acid (32 µg/ml), cotrimoxazole (25 µg/ml), norfloxacin (16 µg/ml), ciprofloxacin (4 µg/ml), and ofloxacin (8 µg/ml). Similarly, *S. sonnei* was grown in presence of tetracycline (16 µg/ml), nalidixic acid (32 µg/ml) and cotrimoxazole (25 µg/ml). With an OD_{600} of 0.3, 20 ml of each culture was extracted by centrifugation and then again suspended in 1 ml of freshly prepared NB broth. Phages were introduced with multiplicities of infection (MOI) of 0.1, 0.01, and 0.001. In case of *S. flexneri* 2a and *S. dysenteriae* 1, the mixtures were incubated for 5 minutes at 37ºC to allow phage adsorption, while for *S. sonnei*, a 10-minute adsorption period was used. Subsequently each suspension treated with phages was passed on to 20 ml of fresh NB media. Over a 5-hour time course, samples were obtained at designated duration, and bacterial counts were evaluated using the spread plate method. As negative controls, cultures were prepared with antibiotics and phage dilution buffer alone, without phage treatment.

2.5 Thermal and pH stability

To determine the stability of phages at different temperature, the1ml phage suspension (approximately 10^{10}-10^{11} PFU/ml) was allowed for incubation at different temperatures: 4°C, 37°C, 50°C, 60°C, 70°C, and 80ºC. At each temperature, 100 µl aliquots were collected after 5, 15, 40, and 60 minutes. These samples were then evaluated for phage viability using the double layered plaque assay method against *Shigella spp.* For evaluating pH tolerance, phage suspensions (ranging from 10^{10} to 10^{12} PFU/ml) were mixed with 1 ml of TM buffer adjusted to various pH levels, from 2–12. The desired pH values were achieved by adding HCl or NaOH for acidic or alkaline condition. The mixtures were incubated at 37ºC for 1 hour. Subsequently, 100 µl from each pH-treated sample was tested for phage activity against *Shigella spp*. using the double layered plaque assay as described by Wei et al. [22].

2.6 One-step growth curve

To perform one-step growth curve experiment, the modified protocol was used based on the method used by Ahamed et al. [16]. Briefly, *Shigella* strains (*S. flexneri* 2a, *S. dysenteriae* 1, and *S. sonnei* 1) were cultured in NB broth supplemented with the appropriate antibiotics at 37ºC. Once the culture reached on OD_{600} of 0.3, 20 ml of each culture was centrifuged at 5,000 ×g for 10 minutes at 4ºC. The bacterial pellet obtained was then resuspended in 1 ml of freshly prepared NB medium. The phage particles were then added at a multiplicity of infection (MOI) of 0.1 and allowed to

adsorb to bacterial cells for duration of 5 minutes in *S. flexneri* 2a and *S. dysenteriae* 1, and 7 minutes for*S. sonnei* 1 at 37ºC. Following adsorption, the dilution of the mixture was done upto 10^4-fold to a final volume of 10 ml with NB broth and incubated at 37ºC. During the 100-minute incubation, 100 µl aliquots were collected at various time intervals. Each aliquot was mixed with 200 µl of the respective *Shigella* culture, and phage titers were determined using the double layered agar plaque assay. The experiments were performed with three replicates for each *Shigella* strain. To calculate the burst size, the average number of phage particles released after the lysis phase was divided by the average number of initially adsorbed phage particles.

2.7 Transmission electron microscopy

Highly purified phage samples, obtained from cesium chloride density gradient centrifugation (CsCl) were used for analysis of transmission electron microscopy (TEM). The imaging was performed at the Electron Microscopy Laboratory, University of Burdwan, West Bengal. To a carbon coated grid, around 1 × 10^{12} PFU/ml of phage suspension was applied using a Gilson pipette and then stained negatively with 2% (w/v) uranyl acetate. The prepared samples were observed under a JEOL JEM-1400Plus transmission electron microscope operating at an accelerating voltage of 200 kV.

2.8 Genome sequencing and analysis

For DNA extraction procedure 450 µl of phage lysate was taken in a sterile 2 ml centrifuge tube (Tarsons, India). To degrade any host nucleic acids, 1 µl of DNase I (2,000 units/ml, NEB, USA) and 5 µl of RNase A (10 mg/ml, Thermo Scientific, USA) were added and then incubated at 37ºC for 1 hour. To inactivate DNase I, 5 mM EDTA was transferred to individual tube, and then incubated at 78 to 80ºC for 20 minutes. Subsequently, 250 µg of Proteinase K (SRL, Mumbai, India) was added and the samples were incubated at 55ºC for 2 hours to digest phage capsid proteins and release genomic material. Following this enzymatic treatment, genomic DNA from the phage was extracted using a phage DNA isolation kit (Norgen, Canada) as per the protocol provided by manufacturer, with slight modifications as described by Ahamed et al. [16]. The sequencing of whole genome was carried out using the ION Xpress kit (S5-00205), version 5.0.4. Sequence quality was assessed using PRINSEQ and low-quality reads were filtered and trimmed. SPAdes version 3.8.0 were used to assemble high-quality reads into a single contig [23]. The Rapid Annotation Subsystem Technology (RAST) server were used for Genome annotation [24]. The final annotated genome sequences were uploaded to GenBank under accession numbers NC_049831 (*Sfin-3*), MN337573 (*Sfin-4*), and MN342247 (*Sfin-5*). The proteinfunction encoded by numerous coding sequences (CDS) were inferred using the BLASTp tool together with conserved domain analysis accessible at NCBI (http://www.ncbi.nlm.nih.gov/) (Table 2).

Table 2
Characteristics of the protein coding sequences of phage *Sfin-3, Sfin-4* and *Sfin-5*according to the homology to protein database.

Predicted Functional CDSs	Best blastP match and identity (%)	*Sfin-3*				*Sfin-4*				*Sfin-5*			
		CDS	Start	Stop	Length (bp)	CDS	Start	Stop	Length (bp)	CDS	Start	Stop	Length (bp)
Terminase small subunit	Shigella phage *Sfin-1* (100.00%)	18	5534	6058	524	40	13491	14015	524	15	4541	5065	524
Terminase large subunit	Shigella phage *Sfin-1* (100.00%)	19	6098	7666	1568	41	14055	15623	1568	16	5105	6673	1568
Minor capsid protein	Shigella phage *Sfin-5* (100.00%)					43	16953	17714	761	18	8003	8764	761
Tail fibers protein	Shigella phage pSf-2 (100.00%)					53	23186	23854	668	28	14236	14904	668
Minor tail protein	Shigella phage phi2457T Shigella phage *Sfin-1* (100.00%)	35 36 37	19560 19993 20772	19913 20775 21506	353 782 734	58	27952	28734	782	33	19003	19785	782
Tail assembly protein	Shigella phage *Sfin-1* (100.00%)					59 60	28731 29462	29465 30061	731 599	34 35	19782 20513	20516 21112	734 599
Tail fiber protein	Shigella phage *Sfin-1* (100.00%)	46	31472	29688	1784	61	30139	33588	3499	36	21190	24639	3449
lipoprotein	Shigella phage *Sfin-1* (100.00%)					63	34806	34576	230	38	25857	25627	230
						80	47546	47947	401	55	38597	38998	401
Exonuclease	Shigella phage *Sfin-1* (100.00%)					64	35333	36397	1064	39	26384	27448	1064
Recombinase	Shigella phage *Sfin-3* (100.00%)					65	36439	37119	680	40	27490	28170	680
Tail fiber protein	Shigella phage *Sfin-1* (100.00%)					67	39392	37608	1784	42	30443	28659	1784
DNA primase/ helicase	Shigella phage *Sfin-2* (100.00%)	47 62	32508 42425	31573 40857	935 1568	68	40428	39493	935	43	31479	30544	935

Predicted Functional CDSs	Best blastP match and identity (%)	*Sfin-3*				*Sfin-4*				*Sfin-5*			
		CDS	Start	Stop	Length (bp)	CDS	Start	Stop	Length (bp)	CDS	Start	Stop	Length (bp)
DNA adenine methyltransferase	Escherichia phage ADB-2 (100.00%)					72	43535	44248	713	47	34586	35299	713
Holin	Escherichia phage ADB-2 (100.00%)	57	38875	39138	263	78	46795	47058	263	53	37846	38109	263
Lysin	Shigella phage *Sfin-1* (100.00%)									54	38109	38597	488

2.9 Genome end determination of the of isolated phages

To investigate the packaging mechanisms and determine the genome ends of the phages, a phylogenetic analysis was performed focusing on theamino acid sequence of large subunit of the terminase enzyme. This approach, commonly used to infer genome packaging strategies. It was based on sequence comparisons with the other phages of known packaging mechanisms [25]. The large terminase subunit sequences of *Sfin-3*, *Sfin-4*, and *Sfin-5*, along with the sequences from related phages were used for the construction of a phylogenetic tree. The NCBI database was used to obtain the protein sequences. The selected bacteriophages in this analysis are double-stranded DNA (dsDNA) with well-characterized packaging strategies like headful packaging, 5'and 3'cos ends and direct terminal repeats; each associated with different terminase functions. Multiple sequence alignment was performed using ClustalW in MEGA X with default settings. A neighbor-joining phylogenetic tree was generated and the stability of the tree was evaluated bootstrap value of 1,000 replicates [26]. To experimentally assess the nature of the genome ends, around 1 µg of purified phage DNA was digested with the restriction enzymes BglII and MluI separately, using the protocol provided by the manufacturer (NEB, USA). The digested DNA samples were then subjected to heat treatment at 80ºC for 15 minutes, accompanied by rapid cooling on ice or gradual cooling at room temperature. The resulting fragments were separated on a 0.8% (w/v) agarose gel using TAE buffer. The gels stained with ethidium bromide were then visualized under UV light. As a molecular size marker a GeneRuler 1 kb Plus DNA ladder (Thermo Fisher Scientific, USA) was used to assess the presence of circularly permuted or terminally redundant genome structures, as described by Amarillas et al. [25].

2.10 Characterization of the phage receptor

To investigate the nature of receptor of the *Sfin-3*, *Sfin-4*, and *Sfin-5*that involve in phage-host interactions, experiments were carried out with modifications based on the protocol of Ahamed et al. [16]. For identification ofinvolvement of outer membrane protein of bacteria on phage adsorption, proteinase K (0.2 mg/ml; SRL, Mumbai, India) treated *Shigella flexneri* 2a cultures (OD_{600} = 0.3) were incubated at 55ºC for 2 hours. Following treatment, 2 ml of the bacterial suspension was centrifuged at 5,000 × g for 5 minutes and washed with NB medium. These cells were then used in an adsorption assay at a multiplicity of infection (MOI) of 0.0001. As control,no proteinase K was used but incubated with the same other conditions as described before to ensure that any observed effects were due to the enzyme treatment rather than heat exposure. To assess whether LPS of bacteriainvolves in phage host interaction, 1.5 ml of *S. flexneri* 2a culture was centrifuged at 5,000 × g for 5 minutes. The pellet was suspended in 1.5 ml of 50 mM sodium acetate buffer (pH 5.2), in presence and absence of 200 mM sodium periodate ($NaIO_4$), followed by incubation in the dark for 2 hours. After incubation, cells were then centrifuged and resuspended in NB. The control and treated cells were then subjected to phage adsorption assay.

2.11 Synergistic activity of phages in combination with antibiotics

To evaluate the synergistic bacteriolytic effect of phages paired with antibiotics, cell lysis assay was carried out using the procedure outlined in Materials and Methods (Section 2.4). *S. flexneri* 2a culture was treated with phages *Sfin-3*, *Sfin-4* and *Sfin-5* at an MOI of 0.1, antibiotics such as azithromycin or ceftriaxone were used at half of their MIC (Minimum Inhibitory Condition) values (sub-inhibitory concentrations) to assess potential synergy [27].Four experimental sets were prepared: (i) untreated control, (ii) antibiotic-only, (iii) phage-only, and (iv) phage + antibiotic. Bacterial cultures were initiated at 1×10^6 CFU/ml. After the end of phage adsorption period, antibiotic were added to the antibiotic-only group, while both phage and antibiotic were added to the combination group. The control group received neither phage nor antibiotic, and the phage-only group received only the respective phage. Bacterial viability was monitored for up to 5 hours at 30-minute intervals. 200 µL samples were collected from each group, serially diluted and plated using the spread plate method for colony enumeration.

2.12 Degradation and microscopic analyses of biofilms

To evaluate the antibiofilm activity of *Sfin-3*, *Sfin-4*, and *Sfin-5*, *Shigella flexneri* 2a was grown overnight in nutrient broth at 37°C with shaking and diluted 1:100 in fresh medium. A total of 100 µL of the diluted culture was added to 96-well plates and incubated at 37°C for 24, 48, and 72 h without shaking to allow biofilm formation. After incubation, planktonic cells were removed and wells were washed with PBS. Phage suspensions (MOI 0.1) were added and incubated at 37°C for 24 h. Wells were then washed, air-dried, heat-fixed, and stained with 0.1% crystal violet. The bound dye was

solubilized with 33% acetic acid, and absorbance was measured at 595 nm. Media alone served as a negative control, while untreated biofilm served as a positive control. Background-subtracted OD values were categorized as follows: no biofilm (≤ 0.1), weak (0.1–0.6), moderate (0.6–1.7), and mature (1.7–2.1). Mature biofilms were operationally defined as those formed at 72 h with OD_{595} ≥ 1.7, corresponding to plateau-phase biomass.For microscopic analysis, biofilms were developed on glass coverslips and treated with phages at MOI 0.1. Light microscopy was performed after crystal violet staining. Scanning electron microscopy involved fixation with glutaraldehyde, postfixation with osmium tetroxide, dehydration through ethanol, staining, and visualization to assess structural disruption of the biofilm [28].

3. Result and discussion

Although *Sfin-1*, *Sfin-2*, and *Sfin-6* have been reported as MDR-active Shigella phages, their host range remains comparatively limited. In contrast, *Sfin-3*, *Sfin-4*, and *Sfin-5* exhibit broad polyvalent lytic activity across multiple serotypes of *S. flexneri*, *S. dysenteriae* and *S. sonnei* while retaining strict genus specificity without any effect against non-Shigella species such as *Salmonella Typhi* and *E. coli* strains. This expanded host-range profile addresses a major limitation of earlier *Sfin* series phages. Moreover, identification of phage receptor demonstrated that *Sfin-3* and *Sfin-4* rely on LPS for adsorption, whereas *Sfin-5* requires outer-membrane proteins. Functionally, no data related to antibiofilm or synergy exist for earlier *Sfin*-series phages. In contrast, *Sfin-3, Sfin-4, Sfin-5* exhibited robust degradation of early and mature Shigella biofilms at defined MOIs and showed strong synergistic bacteriolytic activity when combined with azithromycin or ceftriaxone in 5 hour time-kill assays. This combined evidence underscores the novelty and therapeutic advantage of *Sfin-3*, *Sfin-4, Sfin-5* relative to previously described Shigella phages.

3.1 Isolation, amplification, and purification of bacteriophages

Water samples were collected from the river Ganga near Kolkata to isolate *Shigella*-specific bacteriophages, using protocols previously described in the Materials and Methods section. Three distinct phages; designated *Sfin-3*, *Sfin-4*, and *Sfin-5* were successfully isolated from these samples. The phages demonstrated the ability to infect variety of clinically isolated MDR *Shigella* strains. Upon overnight incubation at 37°C, they produced clear and well defined plaques measuring between 1.2 and 2.0 mm in diameter on bacterial lawns (Figs. 1A, D, G). High-titer phage lysates were generated through confluent lysis on agar plates, followed by purification using ultracentrifugation and cesium chloride (CsCl) density gradient centrifugation. The purified phages were then dialyzed into a low-salt buffer at pH 7. Molecular analysis showed the absence of multiple genes encoding phage-specific proteins such as the tail tape measure protein and the large terminase subunit, indicating that each preparation contained a single, distinct phage type.

3.2 Host range determination

The host range of the phages *Sfin-3*, *Sfin-4*, and *Sfin-5* was evaluated using spot-test assays, where purified phage suspensions were tested on lawns of various clinically isolated *Shigella* species, including *S. flexneri*, *S. dysenteriae*, *S. boydii*, and *S. sonnei*. In addition, other enteric pathogens such as *Salmonella typhi* and different *Escherichia coli* (XL1 Blue, AG100, K12, and *E. coli* C) were included in the analysis. The *Shigella* strains tested were MDR, showing resistance to commonly used antibiotics such as amoxicillin, tetracycline, chloramphenicol, norfloxacin, ciprofloxacin, nalidixic acid,ofloxacin, cotrimoxazole, and azithromycin [16] (Table 1). The results of the spot assays demonstrated that all three phages *Sfin-3*, *Sfin-4*, and *Sfin-5* produced distinct zones of lysis in several serotypes of *S. flexneri*, *S. dysenteriae*, and *S. sonnei*, but did not show lytic activity against non-*Shigella* species tested. This selective lytic activity suggests that the phages are polyvalent within the *Shigella* genus, capable of targeting multiple strains. While phages typically display high specificity towards a single bacterial species, some polyvalent phages have previously been reported [29, 19]. The observed broad host range among *Shigella* strains indicates potential applications of these phages in therapeutic settings, particularly in treating shigellosis. This also points to the importance of identifying coding sequences (CDSs) responsible for host specificity and tail structure components. Given that *Shigella* transmission to humans occurs primarily via the fecal-oral route, the successful isolation of *Sfin-3*, *Sfin-4*, and *Sfin-5* from the river Ganga further implies fecal contamination at the sampling site.

3.3 Lytic activity of the phage against different bacterial strains

In vitro bacterial challenge assays were conducted using phages *Sfin-3*, *Sfin-4*, and *Sfin-5* individually, to assess their lytic potential against *Shigella* strains in the presence of multiple antibiotics, including chloramphenicol, ampicillin, tetracycline, ciprofloxacin, cotrimoxazole, norfloxacin, and ofloxacin. Mid-log phase bacterial cultures (OD_{600} = 0.3) were exposed to phage at multiplicities of infection (MOI) of 0.1, 0.01, and 0.001. For each experiment, bacterial host strains were incubated with the respective antibiotics, and phage-free suspensions were used as controls. Bacterial viability was monitored over time by enumerating colony-forming units (CFU), and killing curves were plotted accordingly. For phage *Sfin-3*, a significant reduction in bacterial viability was observed in all MOIs tested. In case of *S. flexneri* 2a, complete lysis was achieved in 3 hours at MOI 0.1, 3.5 hours at MOI 0.01, and 4 hours at MOI 0.001. Similarly, *S. dysenteriae* 1 showed total lysis by 3.5 hours at MOI 0.1 and by 4 hours at the lower MOIs. For *S. sonnei* 1, full lysis occurred within 3.5 hours at MOI 0.1, while MOI of 0.01 and 0.001 required 4 and 4.5 hours, respectively ($p < 0.005$; Figs. 2A - C). Phage *Sfin-4* also demonstrated moderate to strong lytic activity. Complete lysis of *S. flexneri* 2a occurred within 3 hours at MOI 0.1, and at 3.5 and 4 hours for MOIs of 0.01 and 0.001, respectively. A similar trend was observed for *S. dysenteriae* 1, with total lysis occurring within 3.5 hours at MOI 0.1, and by 4 hours at the lower MOIs. For *S. sonnei* 1, complete lysis was observed at 3.5 hours (MOI 0.1) and at 4 hours for both lower MOIs ($p < 0.005$; Figs. 2D - F).*Sfin-5* also displayed strong lytic efficiency. With *S. flexneri* 2a, complete lysis was seen at 3.5 hours across all MOIs. For *S. dysenteriae* 1, lysis was faster occurring within 3 hours at MOI 0.1 and 3.5 hours at MOIs 0.01 and 0.001. In the case of *S. sonnei* 1, complete cell

lysis occurred within 3.5 hours at MOI 0.1 and at 4 hours for both lower MOIs ($p < 0.005$; Figs. 2G - I). A two-way ANOVA confirmed statistically significant differences between the MOI treatments and the control groups. These in vitro challenge results suggest that all three phages possess lytic activity against multidrug-resistant *Shigella* strains and may serve as potential biocontrol agents. Nevertheless, further in vivo studies are needed to evaluate its therapeutic efficacy. It is important to note that prolonged exposure to phages can lead to the development of bacteriophage-insensitive mutants (BIMs). To mitigate this risk, a phage cocktail that combines multiple phages with different infection strategies could be more effective [25, 30]. The data also reveal that the extent of bacterial cell death is closely linked to the MOI. Higher concentrations of phage appear to directly destabilize the bacterial outer membrane, leading to what is termed "lysis from without," a phenomenon distinct from lysis caused by intracellular phage replication and subsequent release [31].

3.4 Thermal and pH stability

The thermal and pH stability of phages *Sfin-3*, *Sfin-4*, and *Sfin-5* was evaluated to determine stability windows relevant to gastrointestinal (GI) transit as well as for food and environmental applications. These phages maintained stable activity after incubation at 37°C for 15 minutes. However, their activity declined progressively with increasing temperatures; at 50°C for 15 minutes, only 4–6% of activity remained; at 60°C and 70°C, activity further dropped to 0.2–2%; and at 80°C, phage activity was nearly undetectable, with only 0.0001% remaining. These findings indicate a temperature-dependent decline in stability. Thermal stability was quantified by measuring phage titers under various temperature conditions (Figs. 3A, B, C). Usually Shigella infections typically occur in the acidic environment of the human intestine [32], evaluating the pH tolerance of these phages is crucial for potential therapeutic applications. The three phages showed optimal stability at pH 7.0 after 1 hour of incubation at 37°C. At pH 4.0 and 12.0, recovery rate varied: *Sfin-3* showed 15% and 14% activity, *Sfin-4* retained 3% and 5%, *Sfin-5* maintained 9% and 4%, respectively ($p < 0.005$; Figs. 3D, E, F). These results show that phages *Sfin-3*, *Sfin-4*, and *Sfin-5* remain stable at temperatures encountered during host colonization, room-temperature storage, and typical environmental conditions. Since the bacteria Shigella passes through the human gastrointestinal tract, phage stability was tested across a wide pH range. All three phages were most stable at neutral pH (7.0) after 1 hour at 37°C, similar to conditions in the intestinal lumen where Shigella grows. They also retained some activity under acidic and alkaline conditions, indicating resilience to short-term exposure to unfavorable environments like stomach acid or alkaline niches. Overall, these findings show that *Sfin-3*, *Sfin-4*, and *Sfin-5* are functional at physiological temperatures and near-neutral pH, with partial tolerance to extreme pH, supporting their potential for oral delivery.

3.5 One-step growth curve assay

The lytic cycle of the *Sfin-3*, *Sfin-4*, and *Sfin-5* phages were analyzed using one-step growth curve experiments. More than 90% of the phage adsorption was completed within 5 to 10 minutes for the three phages. When *S. flexneri* 2a was infected with *Sfin-3*, the latent period was approximately 8 minutes with an average burst size of 102 plaque-forming units (PFU) per cell. When tested against *S. dysenteriae* 1 and *S. sonnei* 1, the latent periods were around 9 and 10 minutes, respectively, producing burst sizes of 148 PFU/cell and 345 PFU/cell (Figs. 4A, D, G). *Sfin-4* showed a latent period of approximately 12 minutes in *S. flexneri* 2a, 10 minutes in *S. dysenteriae* 1, and 14 minutes in *S. sonnei*, with corresponding mean burst sizes of 170, 95, and 340 PFU/cell respectively (Figs. 4B, E, H). Similarly, *Sfin-5* exhibited latent periods of approximately 9 min for *S. flexneri* 2aand *S. sonnei*, and 8minutes for, *S. dysenteriae* 1, with burst sizes of 60, 93, and 200 PFU/cell, respectively (Figs. 4C, F, I).

3.6 Transmission electron microscopy

The morphology of the purified phages *Sfin-3*, *Sfin-4*, and *Sfin-5* was examined using transmission electron microscopy (TEM). Analysis revealed that all three phages possess an isometric head, measuring approximately 59.47 ± 2.08 nm for *Sfin-3*, 57.10 ± 4.03 nm for *Sfin-4*, and 55.04 ± 5.10 nm for *Sfin-5*. Each phage also featured a long, noncontractile tail 121.83 ± 8.29 nm for *Sfin-3*, 162.48 ± 12.30 nm for *Sfin-4*, and 156.20 ± 9.20 nm for *Sfin-5* terminated with a basal tuft (Figs. 1B, C, E, F, H, I). These phages lacked structural components such as a neck, base plate, tail fibers, or spikes. Based on their structural characteristics and following the classification criteria established by the International Committee on Taxonomy of Viruses (ICTV), these phages are identified as members of the *Siphoviridae* family within the *Caudovirales* order [33]. More than 95% of most known bacteriophages are tailed and grouped under *Caudovirales*. Among these, approximately 60% are classified under the *Siphoviridae* family, which is characterized by phages with long and flexible tails [34].

3.7 Genome sequencing and analysis

Genome sequencing plays a crucial role in the deciphering phage biology. The genome of the phage *Sfin-3* spans 50,309 base pairs (GenBank accession: NC_049831) with a GC content of 44.5%. It contains 84 coding sequences (CDSs), of which 19 are transcribed leftward and the remaining are rightward-oriented (Fig. 5A). Functional annotations were identified for 34 CDSs. The *Sfin-4* phage genome is circular and comprises 50,407 base pairs (GenBank accession: MN337573) with a GC content of 45%. Out of 83 CDSs, 19 are oriented leftward and the rest are orientedrightward(Fig. 5B). Functional roles could be assigned to 17 of the CDSs. Similarly, the *Sfin-5* phage also has a circular genome of 50,411 base pairs (GenBank accession: MN342247) and a GC content of 45%. It encodes 83 CDSs, with 19 transcribed leftward and the rest rightward (Fig. 5C). Functional annotation was possible for 18 CDSs. No transfer RNA (tRNA) genes were detected in the genomes of *Sfin-3*, *Sfin-4*, or *Sfin-5*. Whole-genome BLAST comparisons against the NCBI database revealed that all three phages are closely related to previously identified phages, including *Sfin-1* (GenBank: NC_047998), *Sfin-2* (GenBank: MK972831), *Sfin-6* (GenBank: MN393473), and phi2457T (GenBank: MH917278). The genomic similarity of *Sfin-3*, *Sfin-4*, and *Sfin-5* to phi2457T was approximately 95.11%, 99%, and 98.85%, respectively. The mauve allignment *ofSfin-3*,

Sfin-4,*Sfin*-5 resulted into 3 LCB of 41454 bp (green), 1,033 bp (cyan), 1104 bp (red), indicating DNA regions which are homologous among the genomes (Supplementary Fig. 1). Gaps in the graphs indicating non indentical region. Comparative genomic analysis of *Sfin-3*, *Sfin-4*, and *Sfin-5* reveals that these phages are highly similar in terms of genome size, GC content, number of coding sequences (CDSs), and predicted transcription terminator sites. Although the structural and functional protein-coding genes exhibit a high degree of sequence homology, they often differ in genomic arrangement and, in some cases, are transcribed in opposite orientations. The most notable variations among the three genomes are found within the hypothetical protein-coding genes, which remain functionally uncharacterized. It is estimated that 60–80% of the genes in each phage have unknown functions, yet many of these show 78–80% sequence similarity to their counterparts in the phi2457T genome. Significant genetic similarity among these phages, despite being isolated from distinct geographic regions, suggests a shared evolutionary lineage or common ancestral origin. Following genome annotation, the predicted proteins of *Sfin-3*, *Sfin-4*, and *Sfin-5* were grouped into several functional categories, including; DNA metabolism and replication, virion morphogenesis, DNA packaging, phage-host interactions and cell lysis, hypothetical proteins. Among these, the most abundant genes belong to the group of DNA metabolism and replication. Key proteins in this category include 3'-phosphatase, 5'-polynucleotide kinase, phage-associated N-6-DNA adenine methyltransferase, DNA helicase, DNA primase, recombinase, and phage exonuclease. The 3'-phosphatase and 5'-polynucleotide kinase genes identified in the genomes of *Sfin-3*, *Sfin-4*, and *Sfin-5* belong to the Pfam03767 family, which corresponds to the C-terminal domain of the bifunctional T4 polynucleotide kinase/phosphatase (PNKP) enzyme. The phosphatase domain of PNKP is responsible for removing the 3'-phosphate group from DNA, RNA, and deoxynucleoside 3'-monophosphates, facilitating proper DNA end-processing for repair and replication. The enzyme N-6-DNA adenine methyltransferase (Dam), classified under Pfam05869, is also encoded by the three phages. This enzyme specifically methylates the GATC sequence within the phage genome, providing protection against host exonucleases by masking DNA from degradation. All three phages also carry genes that encodeDNA helicases from the Pfam04851 family. These helicases play a vital role in the unwinding of RNA or DNA strands in an ATP-dependent manner, supporting both replication and repair processes. The primase genes found in the Sfin phages are members of the Pfam08273 family. These enzymes contain a zinc finger motif at the N-terminus and an ATP-binding region at the C-terminus that are associated with origin recognition and initiation of DNA replication. A notable feature is the presence of a recombinase gene associated with the ERF superfamily (Pfam04404). This domain contains single-stranded annealing proteins (SSAPs) such as Red-beta, Rad52, ERF, and RecT, which mediate both RecA-dependent and RecA-independent homologous recombination. The recombinase probably facilitates horizontal gene transfer and contributes to genomic rearrangements, promoting evolutionary adaptation through intra-phage gene exchange. Working alongside the recombinase is the phageencoded exonuclease, classified within Pfam12684 and related to the PD-(D/E) XK superfamily. This exonuclease VIII is involved in DNA processing steps that support replication and nucleotide metabolism. Together, these enzymes including 3'-phosphatase, 5'-polynucleotide kinase, recombinase, and exonuclease play integral roles in phage DNA replication, repair, and recombination of the phage DNA allowing efficient genome propagation and adaptability following infection of the host bacterium. Sequence-based annotation of the *Sfin-3*, *Sfin-4*, and *Sfin-5* genomes indicates that several genes are associated with head and tail morphogenesis, key processes in phage assembly. In terms of capsid formation, CDS25 in *Sfin-3*, CDS43 in *Sfin-4*, and CDS18 in *Sfin-5* are predicted to encode capsid proteins that belong to the Phage Mu protein F-like family, which is essential for viral head morphogenesis. For DNA packaging, CDS19 and CDS18 in *Sfin-3*, CDS41 and CDS40 in *Sfin-4*, and CDS16 and CDS15 in *Sfin-5* are predicted to code for large and small subunits of the terminase enzyme complex, respectively. These subunits play a critical role in the ATP-dependent encapsidation of concatemeric DNA into the preformed capsid, as described by Mobberley et al. [35]. The genes responsible for the structure and assemblyof the tail are distributed throughout the genome. In *Sfin-3*, CDS29 to CDS39 and CDS46 are believed to encode various structural components of the tail. For *Sfin-4*, CDS53, CDS58, CDS61, and CDS67 are associated with tail structure, while CDS57 and CDS60 likely function in tail assembly. Similarly, CDS28, CDS33, CDS36 and CDS42 in *Sfin-5* are predicted to encode tail components, while CDS34 and CDS35 are involved in tail assembly processes. Additionally, CDS32, CDS33, and CDS34in*Sfin-3* are predicted to encode the tail tape measure protein, a structural component believed to determine tail length. According to Katsura [36], the length of a phage tail in lambdoid phages may be estimated based on the total number of amino acids in the tape measure protein, using the approximation that each amino acid corresponds to ~ 0.15 nm. Based on this model, the predicted tail length of *Sfin-3* is approximately 173 nm, which is closely aligned with the measured length by electron microscopy of 181.30 ± 8.29 nm. In general, these findings suggest that the capsid, terminase, and tail-related genes of *Sfin-3*, *Sfin-4*, and *Sfin-5* play essential roles in phage assembly and genome packaging, and exhibit a high degree of conservation with known phage structural elements. Genomic analysis of *Sfin-3*, *Sfin-4*, and *Sfin-5* reveals the presence of genes involved in host cell lysis, a critical step in the release of newly formed phage particles. Specifically, CDS58 in *Sfin-3* and CDS54 in *Sfin-5* are predicted to encode endolysins (or lysins); enzymes that degrade the bacterial cell wall. Additionally, CDS57 in *Sfin-3*, CDS78 in *Sfin-4*, and CDS53 in *Sfin-5* encode holin proteins, which are known to form pores in the bacterial inner membrane. Together, these two types of proteins constitute a dual-component lysis system: Holins first disrupt the membrane integrity, creating channels that allow endolysins to access and break down the peptidoglycan layer, leading to cell lysis and phage release. In all three phages, the holin and lysin genesare found adjacent to each other near the terminal regions of their genomes. The lysin genes encode proteins of approximately 162 amino acids, containing conserved domains classified under the Pfam00959 family, which are commonly found in double-stranded DNA (dsDNA) phages. This family includes enzymes capable of cleaving β-1,4-glycosidic bonds within the polysaccharidesbacterial cell wall [37], effectively breaking down the structural integrity of the host. In addition to lysisrelated genes, the BLASTp analysis identified a gene encoding a Cro-like DNA-binding transcriptional regulator in each phage: CDS48 *in Sfin-3*, CDS69 in *Sfin-4*, and CDS44 in *Sfin-5*. These proteins belong to the HTH_XRE superfamily and are likely involved in controlling transcriptional timing and gene expression during the phage replication cycle. Notably, while these regulatory and lytic genes are present, there is no evidence of lysogenyassociated genes in any of the three genomes. This absence, combined with the presence of robust lysis systems, strongly suggests that *Sfin-3*, *Sfin-4*, and *Sfin-5* are strictly lytic phages, lacking the capacity to integrate into the host genome and instead favoring immediate replication and host destruction.

3.8 Genome end determination of isolated phages

Whole genome sequencing followed by the assembly of *Sfin-3*, *Sfin-4*, *Sfin-5*phages revealed that they had a double-stranded DNA genome with a terminal repeat at both of their ends. In tailed bacteriophages, a linear genome is expected within the channel of the portal protein, where only one dsDNA can pass. Therefore, the head contains a linear genome with different types of end. One of the most conserved proteins among phages is the terminase enzyme, particularly its large subunit, which plays a central role in defining the genome ends during packaging. Comparative analysis of theamino acid sequence of the large terminase subunit showed that *Sfin-3*, *Sfin-4*, and *Sfin-5* cluster closely with the terminases of other *Shigella* and *E. coli* phages, including ISF002, Shfl1, psf-2, ISF001, and ADB-2, all of which are members of the T1-like phage family(Fig. 6). Based on their phylogenetic location, it is inferred that the *Sfin-3*, *Sfin-4*, *Sfin-5*phages likely utilize a headful packaging mechanism, characterized by direct terminal repeats (DTRs) and circular permutation. In this system, DNA packaging begins at variable sites along concatemeric DNA, rather than a fixed cleavage point, leading to variation in genome length among individual phage particles. Such phages are known to exhibit complete sets of restriction fragments upon digestion of their circular genomes, as well as submolar-sized terminal fragments similar to those observed in phage P22 [38]. However, in some headful packaging phages assf6 and ES18, these submolar pac-like fragments may be absent due to imprecise initiation cuts, often resulting in a blurred background pattern on electrophoresis gels. To investigate the nature of the genome ends, restriction digestion of *Sfin-3*, *Sfin-4*, and *Sfin-5* was performed using BglII and MluI, followed by heat treatment at 80°C with both slow and rapid cooling. No significant differences were observed between the two treatments, and the appearance of longer fragments in the gels suggested the absence of cohesive (sticky) ends in these phages. Moreover, the gels displayed a diffused background, which supports the presence of various sized terminal fragments. Collectively, these observations indicate that *Sfin-3*, *Sfin-4*, and *Sfin-5* follow a headful DNApackaging strategy similar to T1, lacking cohesive ends and possessing circularly permuted, terminally redundant genomes(Fig. 7).

3.9 Characterization of the phage receptor

A critical step in the phage infection cycle is the recognition and binding to specific receptors on the surface of the host cell. These receptors can vary significantly depending on the phage-host pair and can include peptides, polysaccharides, or even capsular structures and slime layers [39–44]. *Shigella* species, being Gram-negative bacteria, have a complex outer membrane composed primarily of lipopolysaccharides (LPS) and proteins, making both components potential targets for phage adsorption [45]. Therefore, identifying the specific surface structure used by a phage for attachment is essential to understand host specificity. To determine the receptor used by *Sfin-3, Sfin-4* and *Sfin-5*, an experimental approach based on Ahamed et al. [16] was adopted, in which the outer membrane components of *S. flexneri* 2a were selectively degraded using sodium periodate to target LPS and proteinase K to degrade proteins prior to phage infection [46]. For *Sfin-3* and *Sfin-4*, a significant number of unadsorbed phages were detected when periodate was used to treat the host cells, while proteinase K treatment had no noticeable impact on infection efficiency. This implies that *Sfin-3* and*Sfin-4* primarily use LPS as its receptor, and outer membrane proteins are not essential for its binding (Fig. 8A and B). In contrast, for *Sfin-5*, a significant number of unadsorbed phages were detected when proteinase K was used to treat the host cells, while periodate treatmentdid not have a noticeable impact on infection efficiency. This implies that *Sfin-5* primarily utilizes outer membrane proteins as its receptor, and LPS are not essential for its binding, (Fig. 8C). Observed reductions in adsorption efficiency were correlated with specific receptor binding protein (RBP) encoding genes identified in the genomes of *Sfin-3* (CDS29–39, 46), *Sfin-4* (CDS53, 58, 61, 67), and *Sfin-5* (CDS28, 33, 36, 42).These results highlight the diverse receptor usage strategies among closely related phages and underscore the importance of characterizing host-phage interactions at the molecular level.

3.10 Synergistic lytic activity of phage in combination with antibiotics

The time-kill assay revealed a clear synergistic interaction between the phages *Sfin-3*, *Sfin-4*, and *Sfin-5* and sub-inhibitory concentrations of azithromycin or ceftriaxone. For all three phages, the untreated control exhibited a typical exponential growth of *S. flexneri* 2a, with bacterial counts increasing steadily throughout the 5-hour incubation period. The antibiotic-only groups showed minimal to moderate inhibition, resulting in slight reductions or stabilization of CFU compared to the rapidly increasing control, indicated that half-MIC concentrations alone provide limited bactericidal activity. Phage-only treatments produced a marked decline in bacterial counts for all phages, confirming their strong lytic potential. Particularly, thecombination of phage + antibiotic consistently resulted in the most pronounced reduction, with CFU values decreasing by 3 to 5 log units more than phage alone over the same duration. In each panel, the combination curve dropped more sharply and rapidly, achieving nearly complete bacterial clearance by approximately 150 to 180 minutes. In case of*Sfin-3*, complete lysis was achieved within 3 hours in the phage-only group and within 2.5 hours in the phage + antibiotic group ($p < 0.005$; Fig. 9A). Similarly, for *Sfin-4* and *Sfin-5*, complete lysis occurred within 3.5 hours in the phage-only groups and within 3 hours in the respective combination groups ($p < 0.005$; Figs. 9B–C). A two-way ANOVA confirmed statistically significant differences between treatment groups. These in vitro challenge results suggest that all three phages possess strong lytic capabilities against MDR *Shigella* strains and may serve as potential biocontrol agents. Overall, the results clearly indicate synergistic bacteriolytic activity: the presence of sub-inhibitory antibiotic concentrations significantly enhances the killing efficiency of the phages, accelerating the rate and extent of bacterial decline far beyond what either agent achieves independently.

3.11 Spectroscopic and microscopic evaluation of biofilm disruption

The antibiofilm activity of bacteriophages, alone and in combination with antibiotics, was evaluated against mature MDR *Shigella* biofilms using a crystal violet assay at 24, 48, and 72 h. Untreated controls showed progressive biofilm development with maximum biomass at 72 h, while SM buffer had no effect on biofilm formation. Phage treatment at an MOI of 0.1 for 24 h significantly reduced biofilm biomass across all stages, demonstrating efficacy against both developing and mature biofilms (Fig. 10A–C). The combined phage + antibiotic treatment exhibited the strongest antibiofilm effect, indicating a synergistic interaction. Statistical analysis (one-way ANOVA) confirmed significant reductions in biofilm biomass in treated groups compared to controls. Mechanistically, phages inhibited early biofilm formation by lysing planktonic cells and disrupted established biofilms through EPS degradation. Light microscopy revealed dense, multilayered biofilms in controls, partial disruption with phage treatment, and near-complete eradication with combination therapy (Fig. 11A–E). High-resolution scanning electron microscopy (SEM) further confirmed these observations (Fig. 11F–J), showing abundant EPS and bacterial clusters in controls, but reduced bacterial adhesion and loss of EPS in phage-treated samples. Overall, the results demonstrated that bacteriophages, particularly when combined with antibiotics, are highly effective in disrupting *Shigella* biofilms and represent a promising strategy to manage biofilm-associated infections.

4. Phage safety considerations

In silico genomic analysis confirmed the safety of phages *Sfin-3*, *Sfin-4*, and *Sfin-5* for therapeutic or biocontrol use. Genome annotation using RAST, BLASTp, and conserved domain analysis revealed no genes linked to bacterial virulence, antibiotic resistance, lysogeny, or horizontal gene transfer. Notably, no Shigella virulence factors or lysogeny associated elements such as integrases or repressors were detected. All three phages possess complete holin–endolysin lysis modules, indicating a strictly lytic lifestyle. Although some hypothetical proteins were present, none showed similarity to known virulence-related genes. Overall, these findings demonstrate that *Sfin-3*, *Sfin-4*, and *Sfin-5* are obligately lytic and genomically safe, supporting their potential as effective therapeutic or environmental biocontrol agents against MDR *Shigella* species.

5. Therapeutic implications

The distinct biological and genomic features of *Sfin-3*, *Sfin-4*, and *Sfin-5* underscore their potential relevance across multiple practical and clinical contexts. In regions with high burdens of MDR shigellosis particularly low- and middle income countries (LMICs) where access to effective antibiotics is limited, these phages may serve as alternative or adjunctive therapeutics capable of rapidly reducing pathogen load. Their ability to disrupt mature EPS rich biofilms suggest utility in severe or persistent infections, including dysentery cases with mucosal invasion where biofilm associated persistence contributes to treatment failure. Beyond clinical settings, the strict genus specificity and non-lytic effect on non-*Shigella* enteric bacteria highlight their suitability for food safety applications, such as decontamination of raw products or food processing surfaces contaminated via fecal contamination.

6. Rational integration into a Shigella phage cocktail

The biological features of *Sfin-3*, *Sfin-4*, and *Sfin-5* support their use together as an effective Shigella phage cocktail. S*fin-3* and *Sfin-4* utilize LPS based adsorption, while *Sfin-5* employs outer membrane proteins, providing a multi-receptor approach that lowers the risk of resistance through single receptor changes. Their partially overlapping host ranges increase coverage across different clinical serotypes of *S. flexneri*, *S. dysenteriae*, and *S. sonnei*, ensuring that loss of susceptibility to one phage does not compromise the activity of the entire cocktail. Including all three phages would therefore improve robustness, reduce the emergence of phage-insensitive mutants, and maintain strong lytic activity across diverse strains and settings.

7. Limitations and Future Work

Despite their promising biological properties, the present study has several limitations. First, the findings are based entirely on in vitro assays, and no in vivo infection models were used to evaluate pharmacokinetics, therapeutic clearance, mucosal stability, or immunological interactions. Second, the isolates screened represent a single center or region specific clinical collection, and broader global serotype diversity was not evaluated. Third, the study did not incorporate pharmacokinetic/pharmacodynamic (PK/PD) modeling or long-term surveillance of resistance emergence, both of which are essential for advancing these phages toward clinical translation. Future work should include animal models of shigellosis, expanded global isolate panels, evaluation of optimized dosing strategies, cocktail based resistance suppression studies, and detailed PK/PD characterization to support eventual therapeutic deployment.

8. Conclusion

The isolated bacteriophages target multidrug-resistant (MDR) *Shigella spp.*Biological, morphological, and genomic analyses confirmed that these phages belong to the Siphoviridae family. They are lytic in nature and do not contain genes related to lysogeny. The phages remain stable over a wide range of pH and temperature conditions. They show rapid adsorption, a short latent period, and a high burst size. These features indicate strong lytic activity and good adaptability for therapeutic use. The *Sfin* phages also exhibit good thermal and pH stability. This stability supports their potential use in oral or encapsulated phage delivery systems. Genomic analysis revealed conserved structural and lytic gene modules. Spectroscopic and microscopic studies showed that the phages alone, as well as in combination with antibiotics, effectively disrupted both developing and mature Shigella biofilms. They reduced bacterial attachment and degraded the extracellular polymeric matrix. These results demonstrate that the phages can

prevent biofilm formation and also break down established biofilms. This ability is important for controlling persistent Shigella infections. Overall, *Sfin-3*, *Sfin-4*, and *Sfin-5* emerge as promising candidates for phage therapy and biocontrol against MDR Shigella. Further in vivo studies and phage cocktail development are required to evaluate their clinical potential and expand their application in treating resistant infections.

Declarations

Competing of interests

The authors declare that there are no competing of interests.The authors have no relevant financial or non-financial interests to disclose.

Supplementary Figure S1

Comparative genome analysis of *Sfin-3*, *Sfin-4, Sfin*-5. Mauve alignment resulted into 3 LCBs of 41454 bp (green), 1,033 bp (cyan), 1104 bp (red), indicating DNA regions which are homologous among the genomes. Gaps in the graphs indicating non indentical region.

Author Contribution

CG, BD, TA, and NG conceived and designed the entire study, CG, BD performed the practical experiments, accumulated data, and wrote the main manuscript text. CG, SR, and NG analyzed the results and revised the manuscript. MI and VB supplied the clinical samples. CG, AN, SD, and NG analyzed the phage structure. SD helped in genome analysis. All authors wrote, read and approved the final manuscript.

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Data Availability

The datasets generated during the current study are publicly available. The whole-genome sequences of bacteriophages Sfin-3, Sfin-4, and Sfin-5 have been deposited in the NCBI GenBank database under accession numbers NC_049831, MN337573 and MN342247, respectively. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Figures

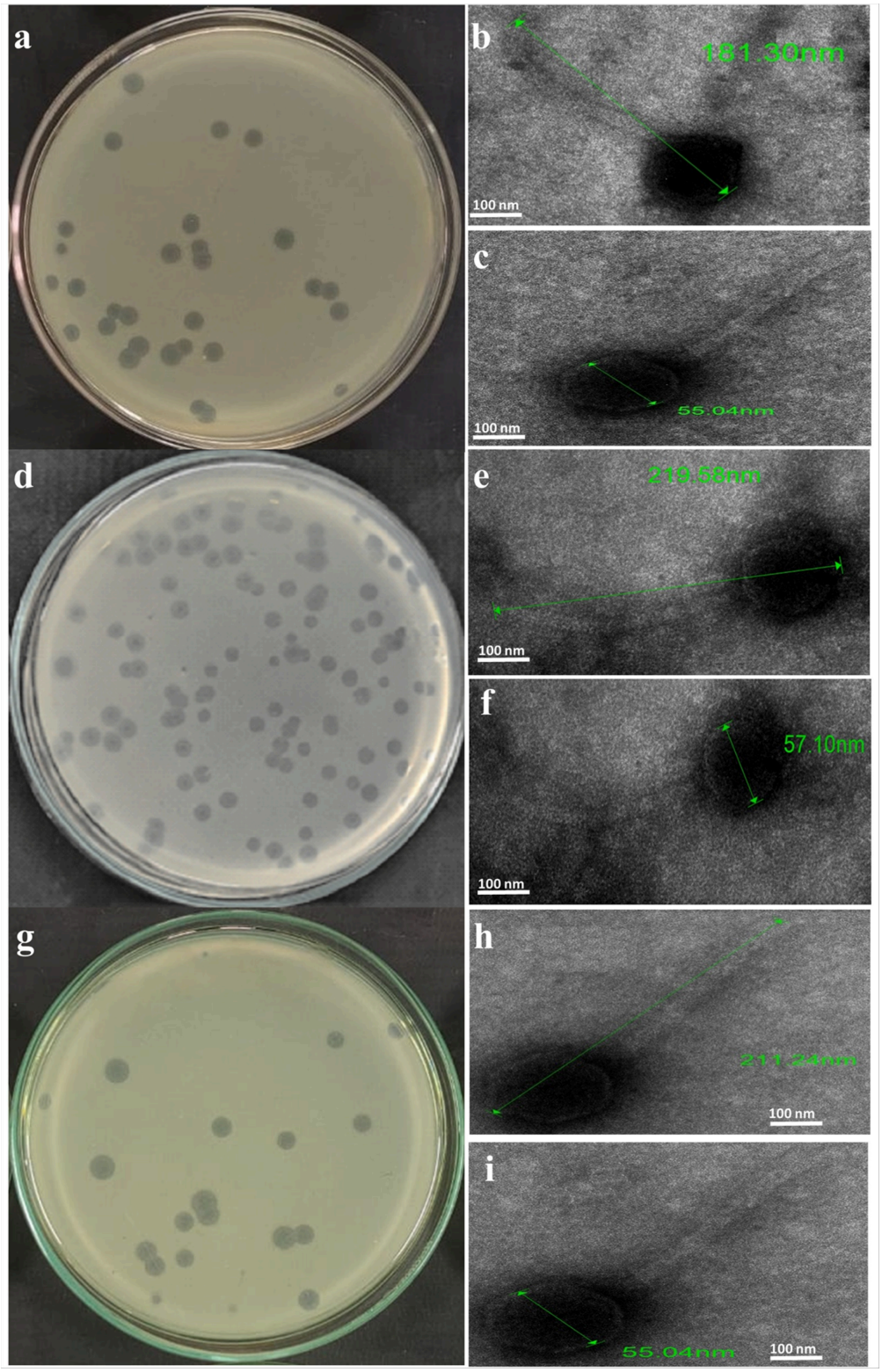


Figure 1

Plaques of *Shigella spp.* specific phages *Sfin-3* (a), *Sfin-4* (d) and *Sfin*-5 (g) in the lawn of *Shigella spp.* The electronmicrograph view of the phages *Sfin-3* (b, c), *Sfin-4* (e, f) and *Sfin-5* (h, i) in 100 and 200 nm scales.

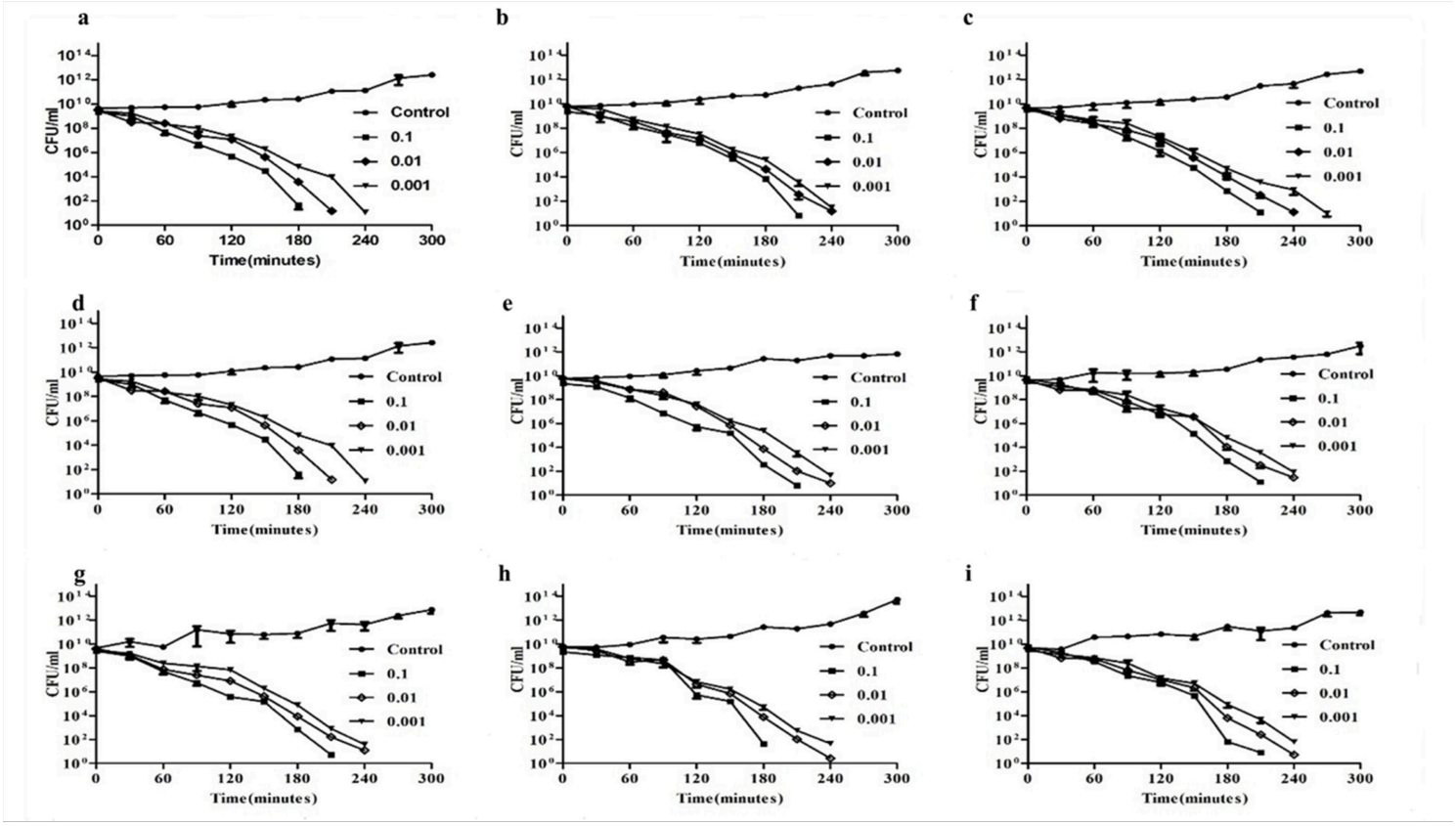


Figure 2

Bacterial challenge test of phage *Sfin-3, Sfin-4* and *Sfin*-5 on *S. flexneri* 2a (a, d, g), *S. dysenteriae* 1 (b, e, h) and *S. sonnei* 1 (c, f, i). The two-way ANOVA indicated significant difference between control and phage-infected sets ($p < 0.0001$, $n = 3$).

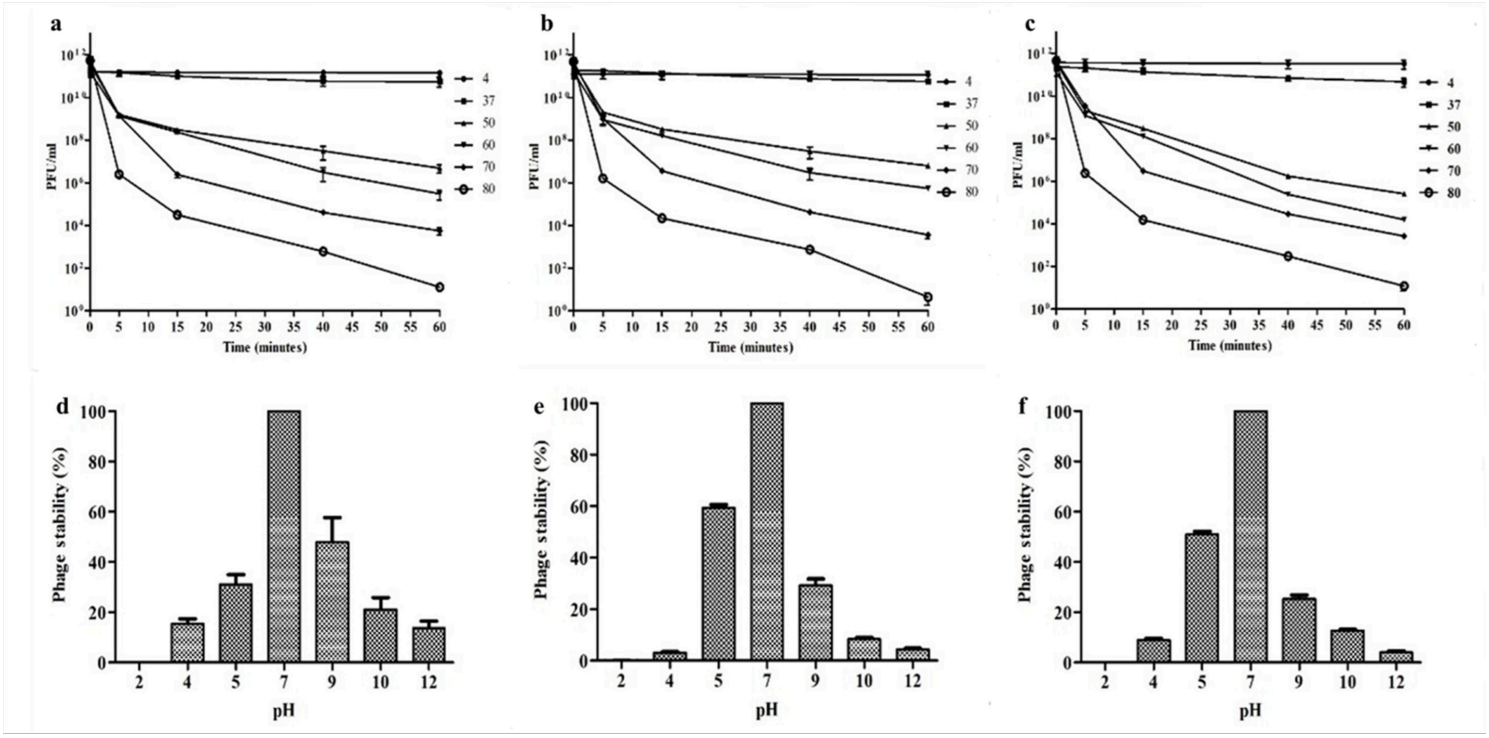


Figure 3

Stability of phage *Sfin-3, Sfin*-4 and *Sfin*-5 in wide temperature and pH ranges. Thermal stability of *Sfin-3, Sfin-4* and *Sfin*-5 phages at various temperatures (a, b, c). pH stability of phage *Sfin-3, Sfin*-4 and *Sfin*-5 (d, e, f). The result was plotted as mean ± SD ($n = 3$).

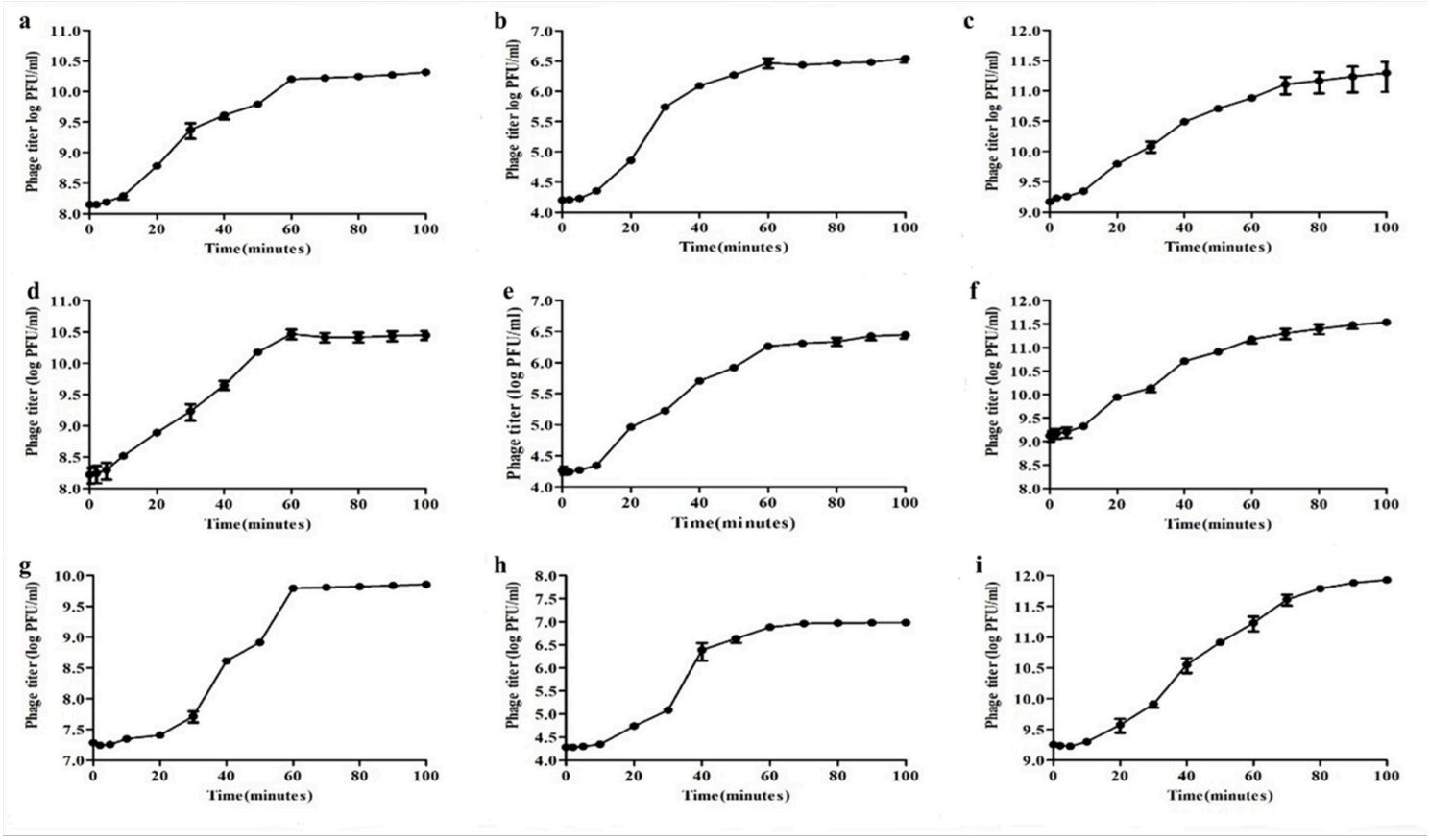


Figure 4

One-step growth curve of phage *Sfin-3, Sfin-4* and *Sfin*-5. *Shigella flexneri* 2a, *Shigella dysenteriae* 1, and *Shigella sonnei* 1. The result was plotted as mean ± SD (*n* = 3). (a, d, g), (b, e, h), and (c, f, i) Present one-step growth curves of *Sfin-3, Sfin*-4 and *Sfin*-5 in *S. flexneri* 2a, *S. dysenteriae* 1, and *S. sonnei* 1, respectively

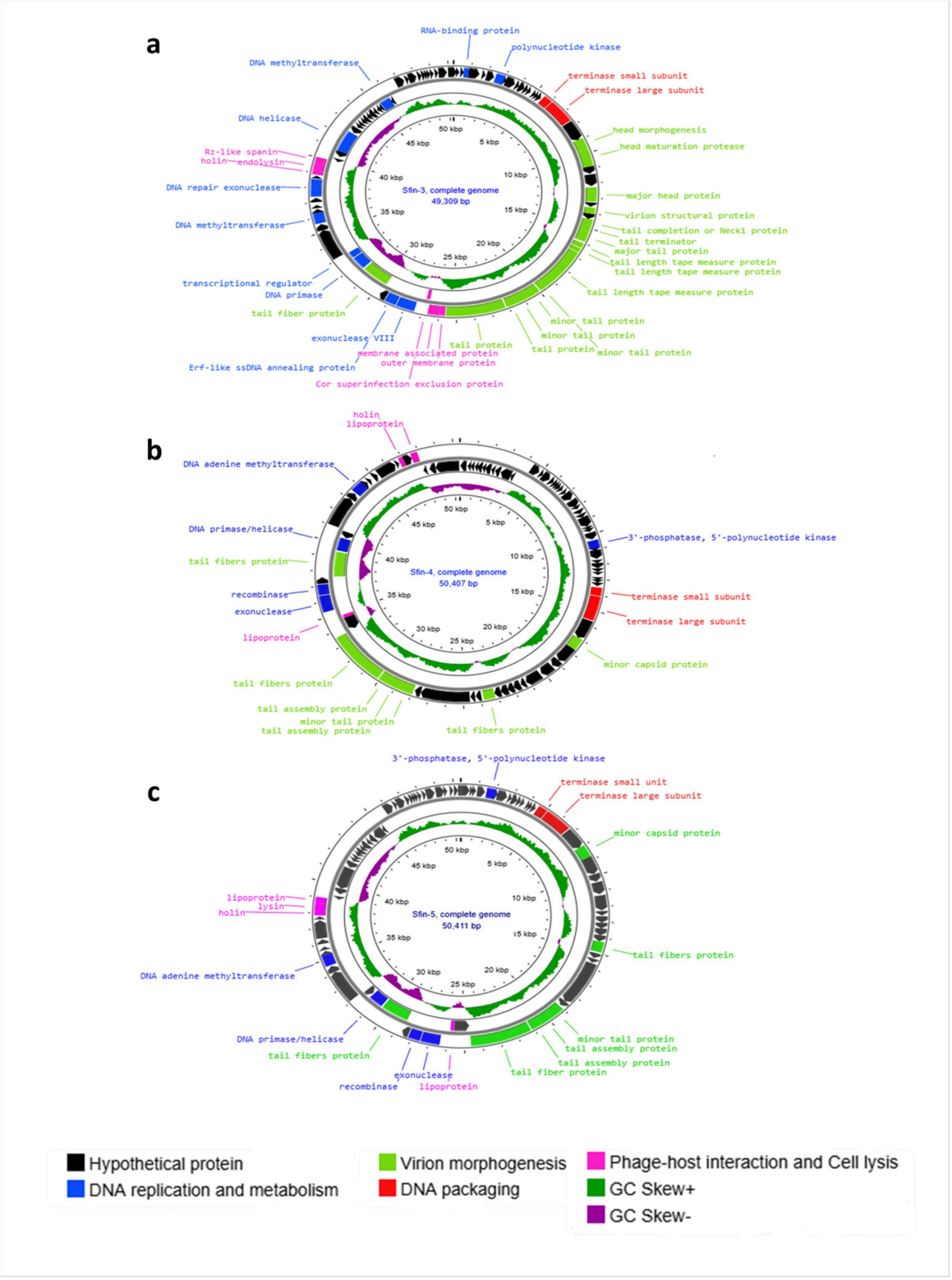


Figure 5

Genome organization of *Sfin-3, Sfin-4* and *Sfin*-5. *Sfin-3* (a), *Sfin-4* (b), and *Sfin*-5 (c), genome maps were schematically presented. The arrows indicate the predicted CDSs and the orientation of the transcription. Predicted molecular functions of CDS were indicated by different colors; Virion morphogenesis (green arrows), DNA metabolism and replication (blue arrows), DNA packaging (red arrows), Phage-host interaction and cell lysis (pink arrows), and hypothetical proteins (blue arrows).

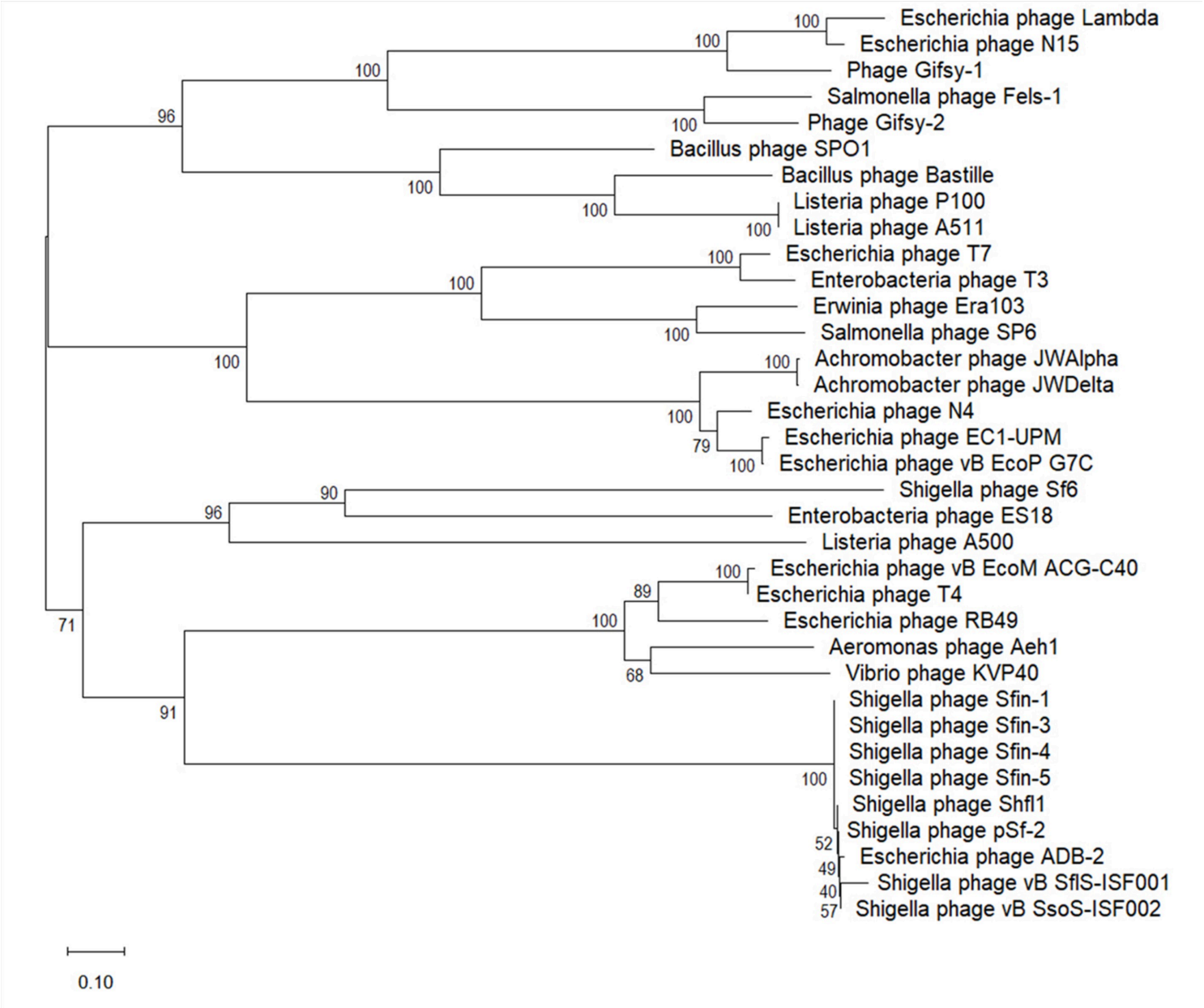


Figure 6

Phylogenetic study of *Sfin-3, Sfin-4* and *Sfin*-5 phages with related phages. The phylogenetic analysis based on the large terminase subunit of known packaging mechanisms phages. The bootstrap analysis was performed with 1,000 repetitions. The terminase large subunits were compared in the MEGA 7.0 version using the neighbor-joining method.

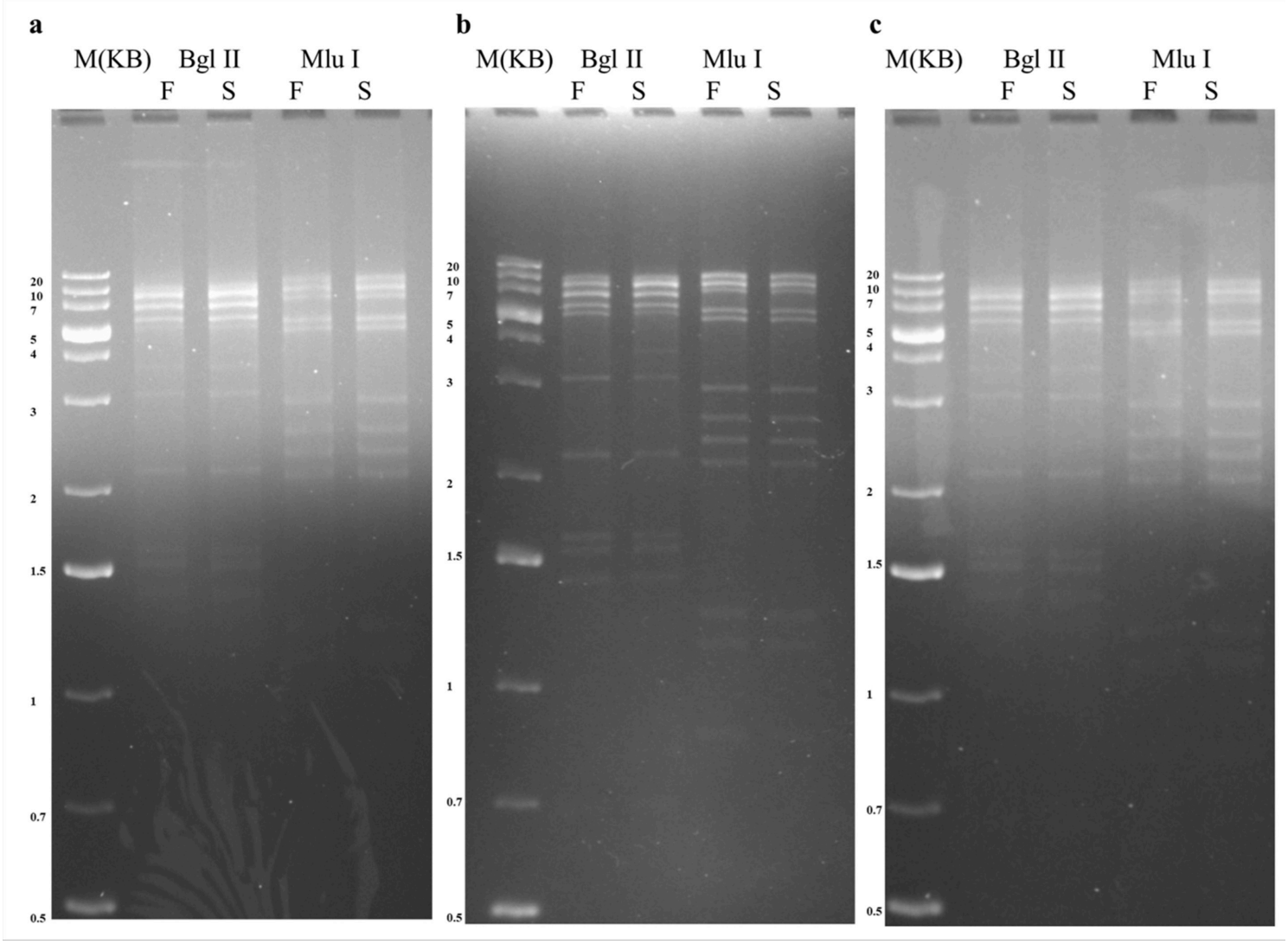


Figure 7

Enzymatic analysis of *Sfin-3, Sfin-4* and *Sfin*-5 genomic DNA. Phage *Sfin-3* (a), *Sfin-4* (b) and *Sfin-5* (c) DNA was completely digested with BglII and MluI and the products were analyzed by 0.8% agarose gel electrophoresis. Lane M indicates the 1 kb Plus DNA Ladder. Lanes F and S indicate that the digests were heated to 80°C for 15 min and then cooled fast on ice or slow at room temperature, respectively

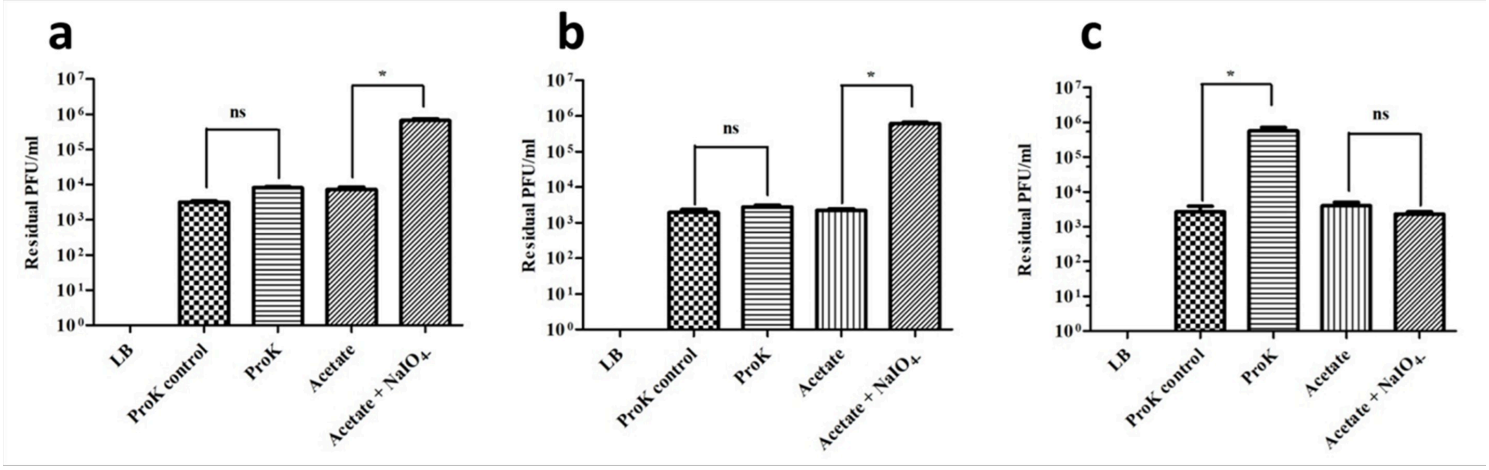


Figure 8

Sfin-3, Sfin-4 and *Sfin*-5 infections on proteinase K and periodate-treated host. The effect of proteinase K and sodium periodate on *Sfin-3* (a). The effect of proteinase K and sodium periodate on *Sfin-4* (b). The effect of proteinase K and sodium periodate on *Sfin-5* (c). The mean ± SD of three

independent experiments is indicated. To determine the significance of the differences between group means, unpaired *t*-tests were performed between the controls and the tests. (ns indicates no significance, asterisks indicates Significance).

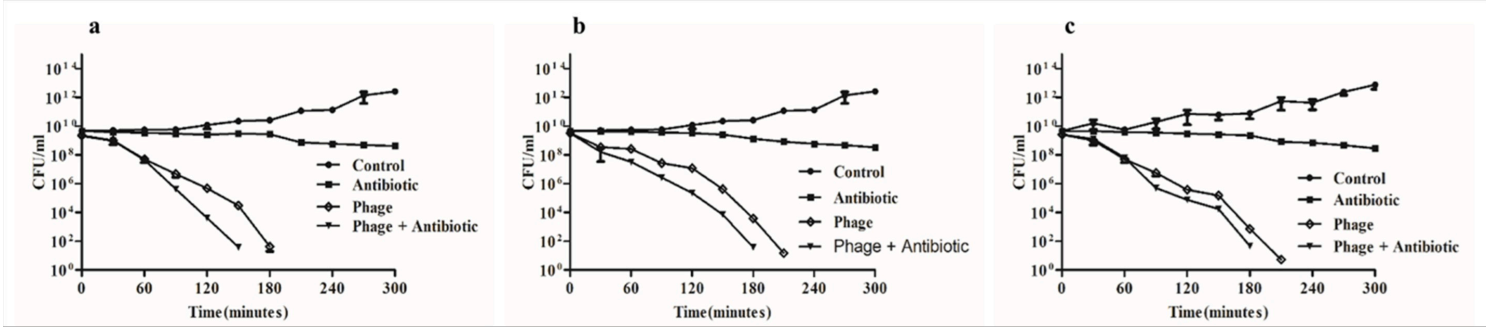


Figure 9

Time killing assay showing *synergistic* bacteriolytic activity of Phage *Sfin-3*(a)*, Sfin-4* (b) and *Sfin*-5 (c)*in combination with antibiotics.* The two-way ANOVA indicated significant difference between control and phage-infected sets ($p < 0.0001$, $n = 3$).

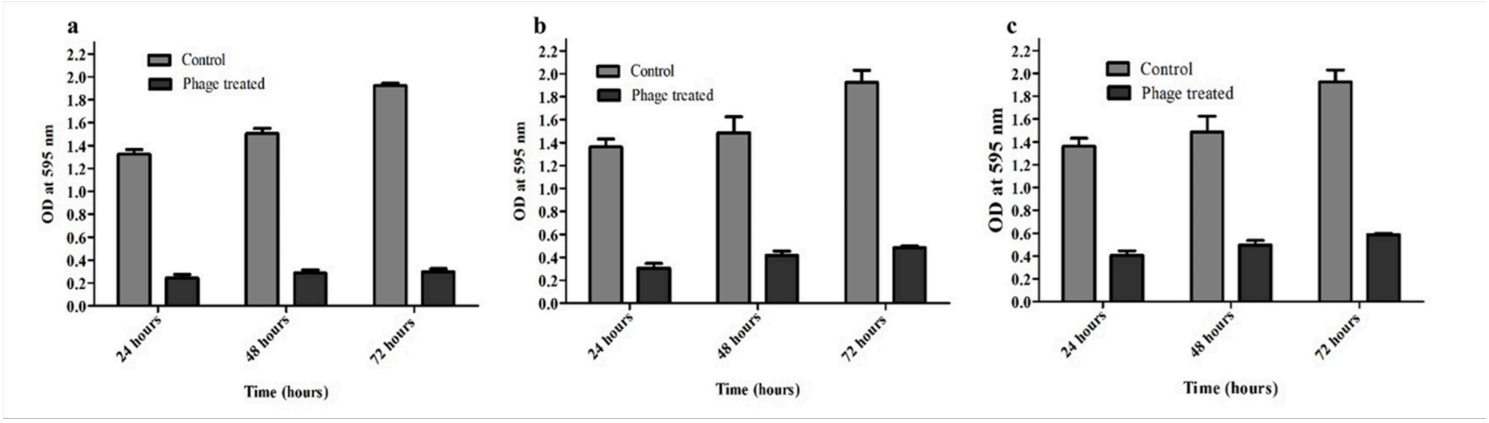


Figure 10

Effect of *Sfin-3* (a), *Sfin-4* (b), and *Sfin-5*(c) on preformed biofilm disruption at 24, 48, and 72 h (OD595). Grey bars indicate control, and black bars indicate phage-treated biofilm. Data are presented as mean ± SD

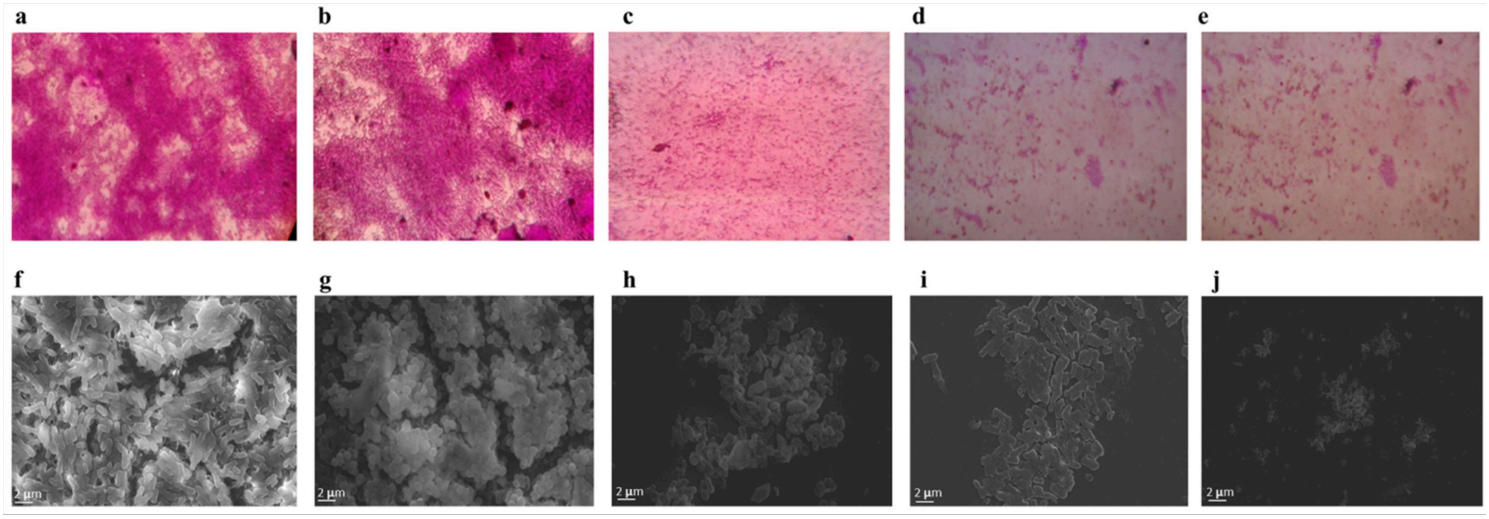


Figure 11

Light microscopic analysis of anti-biofilm activity of phage and antibiotic on *Shigella* biofilm; Control (a), Antibiotic (b), *Sfin-3* (c), *Sfin*-4 (d), and *Sfin*-5 (e).

Supplementary Files

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