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Isolation, characterization, and genomic analysis of three novel *Herelleviridae* family lytic bacteriophages against uropathogenic isolates of *Staphylococcus saprophyticus*

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Abstract

Background *Staphylococcus saprophyticus* (*S. saprophyticus*) is the second most prevalent etiological agent of urinary tract infections (UTIs) in young women. However, there is a paucity of data regarding its bacteriophage (phage). Therefore, this study was conducted to isolate and identify new lytic phages from municipal wastewater with the objective of increasing knowledge about phages and their genomes.

Methods A total of 11 clinical isolates of *S. saprophyticus* and 30 wastewater samples were used to isolate three lytic phages (vB_SsapH-Golestan-100, vB_SsapH-Golestan101-M, and vB_SsapH-Golestan-105-M). The morphology, behavioral characteristics, and complete DNA genomes of these phages were analyzed.

Results The microscopic images of the phages revealed that the sizes of their heads and tail lengths fell within the ranges of 90–111 nm and 234–266 nm, respectively. All phages exhibited high adsorption rates (99.5% in 15 min) and burst sizes (150–210 PFU per infected cell), with a potential for a narrow host range. Genomic analysis of *Staphylococcus* phages indicated a size of 136,433 base pairs (bp) with a guanine-cytosine (GC) content of 33.7% and 192 open reading frames (ORFs) for vB_SsapH-Golestan-100, 144,081 bp with a GC content of 29.6% and 205 ORFs for vB_SsapH-Golestan101-M, and 142,199 bp with a GC content of 30.6% and 203 ORFs for vB_SsapH-Golestan-105-M. A bioinformatics analysis indicated that all three phages belong to the *Twortvirinae* subfamily of *Herelleviridae*. Among the three phages, vB_SsapH-Golestan-100 exhibited the least similarity to previously known phages, with less than 21% similarity with its closest counterparts in genomic databases.

Conclusions This study identified new phages that have the ability to destroy a broad range of *S. saprophyticus* isolates and may potentially be classified as a new genus and species within the *Herelleviridae* family in future studies.

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Keywords *Staphylococcus saprophyticus*, Lytic bacteriophage, *Herelleviridae*, Whole genome sequencing, Urinary tract infection

Background

Coagulase-negative staphylococci (CoNS) are a component of the human and animal skin and mucous membrane microbiota. However, in certain circumstances, they can act as opportunistic pathogens, causing endocarditis, wound infections, and genitourinary tract infections [1]. *Staphylococcus saprophyticus* (*S. saprophyticus*) is one such microorganism that accounts for 15–20% of community-acquired urinary tract infections (UTIs), especially in sexually active young women. Women between the ages of 16 and 25 are particularly susceptible to this infection, and approximately 40% of their UTIs can be attributed to *S. saprophyticus*. In the majority of cases, *S. saprophyticus* is the second most common cause of uncomplicated UTIs, following Uropathogenic *Escherichia coli* (UPEC). The following factors are commonly associated with an increased risk of UTIs: a medical history of recurrent UTIs, female gender, recent sexual intercourse, pregnancy, neurogenic dysfunction of the urinary bladder, the use of an indwelling urinary catheter, and benign prostatic hyperplasia [2]. Despite the efficacy of treatment in many cases, up to 60% of all patients with UTI may experience a recurrence of infection within a year [3, 4]. Furthermore, it has been isolated from several infections including acute pyelonephritis, epididymitis, prostatitis, urethritis, and infection stones. This uropathogenic coccus is gram-positive, facultative anaerobic, non-motile, non-hemolytic, coagulase-negative, catalase-, and urease-positive [5, 6].

The excessive use and misuse of antibiotics has led to a significant increase in the prevalence of antibiotic resistance. This has made the treatment of infections like UTIs, especially those caused by *S. saprophyticus* or other multidrug-resistant (MDR) strains, more challenging for clinicians. While antibiotic treatment remains an option in *S. saprophyticus* infections, there are currently few alternatives currently available for combating infections from this bacterium. It is essential to consistently assess and revise information on alternative treatment options to ensure readiness for their implementation in the event of necessity in the future [4]. Consequently, the potential merits of complementary treatment methods, such as the use of bacteriophages (phages) in conjunction with antibiotics, should be considered as a strategy for the effective management of UTIs [7–12].

Phages are one of the most abundant organisms on the planet and play an important role in controlling bacteria in nature. Scientists have succeeded in using phages in medicine, industries, and agriculture fields artificially to eliminate or manage bacteria [13–16]. In recent years, with the advancement of science, the US Food and Drug Administration (FDA) has approved several products based on phages (in the form of cocktails) in the food production chain or medicine. For example, a phase 1/2 clinical trial evaluated the safety, tolerability, and efficacy of intranasal doses of a phage cocktail (AB-SA01) for the treatment of chronic *S. aureus* infections in patients with recalcitrant chronic rhinosinusitis. The trial results demonstrated that there were no serious adverse events or deaths among the patients, with acceptable tolerability and favorable outcomes in terms of efficacy [17].

The fundamental understanding of phages continues to expand, yet several significant gaps persist within our current knowledge. Further detailed biological and genomic characterization of CoNS-phages, including the understanding of the genetic characteristics of phages, their host specificity and interaction with their bacteria, their metabolism, and their prescription methods, is necessary to increase this knowledge and fully utilize their potential [16, 18, 19]. More favorable outcomes can be attained by expanding the knowledge base regarding phages and enriching the genomic data banks. One of the most effective methods for increasing this knowledge is whole genome sequencing, which can be achieved through the use of techniques such as next-generation sequencing (NGS). NGS can be employed to analyze the genome of a phage, thereby enabling scientists to ascertain with greater confidence that the genome does not contain bacterial virulence genes, and to identify potential characteristics of the virus, such as its taxonomy [20].

The existing literature on lytic phages against *S. saprophyticus* and their genomic characteristics is currently limited. However, Sofy *et* al. conducted a study to identify several phages against CoNS for the purpose of controlling bacteria in food samples in Egypt. In this study, one phage called "CoNShP-3" exhibited a polyvalent behavior and demonstrated the capacity to kill *S. saprophyticus*, *Staphylococcus epidermidis*, *S. aureus*, methicillin-resistant *S. aureus* (MRSA), and vancomycin-resistant *S. aureus* (VRSA) [21]. In a very comprehensive study on the phages of staphylococci, 94 novel staphylococcal phages isolated from wastewater were tested on a diverse panel of 123 staphylococci from 32 species. The study employed network analysis, which revealed the high prevalence of staphylococcal phages in wastewater and their potential role in genetic material exchange. Furthermore, the study demonstrated that staphylococcal phages can have a broad host range, which is a dominant trait. Although this study was very comprehensive, it still provided limited genomic information about

S. saprophyticus phages [22]. A previous report from our team identified VB_SsapS-104, one of the first lytic phages to be isolated and characterized as specific to *S. saprophyticus*. The phage was isolated from a hospital wastewater sample. It had a head size of 50 nm, a tail size of 80 nm, and a neck size of 22 nm, with a morphology shape of siphoviruses. It belonged to the former order *Caudovirales* (currently the class *Caudoviricetes*). Notably, the phage could inhibit the growth of seven out of eight evaluated clinical *S. saprophyticus* isolates [11]. The three previous studies did not comprehensively and fully analyze the genomes of the identified phages. Therefore, this study was conducted to isolate, identify, and genetically analyze three new lytic phages against *S. saprophyticus* to increase the boundaries of knowledge in this field. Furthermore, their genomes were completely checked by NGS technique to cover some of the gaps in this field.

Methods

Bacterial strain information

The ethical committee of Golestan University of Medical Sciences, Gorgan, Iran approved this study (code: IR.GOUMS.REC.1397.339). The bacteria utilized in this study were previously isolated from clinical samples of patients referred to medical centers in Gorgan, Iran, between 2018 and 2020. A total of 11 isolates from 35 available clinical isolates of *S. saprophyticus* were utilized for phage identification. Additionally, all 35 *S. saprophyticus* isolates and eight standard strains were employed in a host range test. The standard strains were *Staphylococcus aureus* ATCC 33,591, *Staphylococcus epidermidis* ATCC 1435, *Escherichia coli* ATCC 2522, *Klebsiella pneumoniae* ATCC 11,296, *Klebsiella pneumoniae* ATCC 13,883, *Enterococcus faecalis* ATCC 29,211, *Proteus mirabilis* ATCC 25,933, and *Pseudomonas aeruginosa* ATCC 27,853.

Recovery and molecular identification of *S. saprophyticus* isolates

Initially, the stored isolates (in a Brain Heart Infusion (BHI) Broth medium containing 15% glycerol at -70 °C; Ibresco, Iran) were sub-cultured on blood agar (Ibresco, Iran), and a single pure colony was selected and incubated for 18 h at 37 °C on blood agar. All isolates belonged to women with UTIs. Subsequently, the DNA of each S. *saprophyticus* isolate intended for phage identification was extracted using a commercial gram-positive bacterial kit (SinaPure, EX6021, SinaClon, Iran). The strains were confirmed by amplification of a fragment of the *16 S* rRNA gene with Polymerase Chain Reaction (PCR), as previously described by Ghebremedhin *et* al. [23]. The *16 S* rRNA gene amplicons from all 11 isolates, utilized in the phage isolation step, were sent to Macrogen Corporation in South Korea for purification and sequencing using the Sanger sequencing method. Subsequently, the isolates were identified and confirmed using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov). The relevant information about them can be found in the GenBank database, with the following accession numbers: MW453014– MW453024. Other *S. saprophyticus* isolates, used in the host range test, were previously identified by sequencing their *16 S* rRNA gene amplicons [11].

Isolation and purification of the lytic phages

Wastewater samples were collected from both municipal and hospital sources with the aim of isolating lytic phages that could effectively infect and eliminate at least one of the 11 *S. saprophyticus* isolates. The samples were collected from the same city where the bacteria used in the study were initially isolated from one of its major hospitals. At each sampling stage, approximately 400 ml of wastewater was collected. Each collected wastewater sample was transferred to the microbiology laboratory, where it was centrifuged at 4,000 rpm for 15 min to remove debris. The supernatant was then purified using a 0.22-µm syringe filter (Gilson, Dunstable, UK). Subsequently, 10 ml of each filtered wastewater sample was added to a falcon tube containing 10 ml of 2X BHI broth medium and one of the 11 selected bacterial suspensions (OD_{600} = 1, the exponential phase), and incubated for 24 h at 37 °C in a shaker incubator (50 rpm). Following incubation, the suspension was centrifuged at 10,000 rpm for 10 min, after which the supernatant was filtered through a 0.22-µm syringe filter. To search for the presence of any possible phage in any enriched supernatant, 100 µl of the filtered supernatant and 100 µl of the *S. saprophyticus* isolate (used in the last enrichment step; OD_{600} = 1, the exponential phase) were gently mixed in a Falcon tube containing 7 ml of 0.7% molten soft BHI agar (45 °C) and dispensed onto the surface of a BHI agar (1.5% agar, base agar) petri dish to create a double-layered agar plate. Following a 24-hour incubation period, a single clear plaque (if present) was selected, and reproduced. This process was repeated three times using double-layer agar method, in accordance with previous studies [11, 24–27].

Following the co-cultivation of the phage with the bacterial host in BHI, the culture was centrifuged at 10,000 g for 10 min at 4 °C, and the resulting supernatant was then filtered using a 0.22-µm syringe filters to obtain a purified phage stock. To eliminate any bacterial nucleic acids, each intact phage stock was treated with DNase I (1 µg/µl) and RNase A (1 µg/µl) enzymes (Thermo Fisher Scientific, USA) for 10 min and one hour at 37 °C, respectively. The treated stock was then combined with 1 M NaCl (Merck, Germany) and 10% polyethylene glycol (PEG) 8000 (Merck, Germany), and then

stored at 4 °C overnight. Following the centrifugation of the phages at 50,000×g for 30 min at 4 °C, the supernatant was decanted and the pellet was resuspended in SM buffer (including 5 ml 2% gelatin; 50 ml 1 M Tris-Cl pH 7.5; (Neutron, Iran); 2 g $MgSO_4$_$7H_2O$ (Merck, Germany); and 5.8 g NaCl, adjusted to 1,000 ml by ddH_2O). Subsequently, the phages underwent purification via glycerol gradient ultracentrifugation. In brief, 3 ml of 40% (w/v) glycerol, 4 ml of 5% (w/v) glycerol (both mixed with SM buffer), and 2 ml of each phage sample were successively added into an ultracentrifuge tube. The sealed tube was then centrifuged at 80,000 × g for 20 h at 4 °C using a Beckman L-series ultracentrifuge (SW28 rotor). Following centrifugation, the supernatant was removed, and the pellet containing purified bacteriophages was resuspended in 1 ml of SM buffer before storage at 4 °C for future use [11, 26–28].

Host range determination of phages

The potential host range of each isolated phage was determined using a standard spot test and plaque assay on the earlier mentioned 35 isolates of *S. saprophyticus* and eight standard strains (related to urinary tract infections), available at the University Microbial Bank, as previously described. Briefly, 100 µl of each 24-hour bacterial culture was mixed with 7 ml of BHI culture medium with 0.7% agar (45 °C). The mixture was then added to a BHI plate with 1.5% agar. After the culture medium had cooled and solidified, 10 µl of each purified phage suspension was placed on the part of the plate and incubated for 24 h. The presence of a clear lytic zone (plaque) indicated that the bacterial isolates were susceptible to the selected phage, whereas the absence of a plaque indicated resistance to that particular phage. Furthermore, the presence of phage plaques was confirmed using a plaque assay in each positive sample in spot tests, whereby the lysate titration of each phage was performed on the double-layer agar plate [11, 29, 30].

Determination of phage adsorption time

To measure the phage adsorption rate, samples were taken at intervals of 0, 5, 10, 15, and 20 min after infection of their host (1 ml in the exponential phase, 10^8 CFU/ml) with 100 µl of each selected phage (Multiplicity of Infection (MOI) of 0.01) in 25 ml of BHI broth, incubated at 37 °C. The samples were immediately centrifuged (5 min at 12,000 × g and 4 °C) to remove the adsorbed phages. Subsequently, the free phage particles in the supernatant were assessed using the double-layer agar method. The initial phage concentration at the onset of the experiment (0 min) served as the baseline of the virus titer. The phage adsorption rate was calculated as 100 × [(baseline concentration - remaining concentration) / baseline concentration] [11, 31].

One-step growth curve

According to our earlier investigation, the latent time and phage burst size were assessed using a one-step growth test with some modifications [11, 26, 31]. After centrifugation, 10^9 CFU/ml of the selected host of *S. saprophyticus* in the exponential stage was added to 2 ml of a new BHI broth. An amount of 0.01 MOI of the related phage was introduced to the BHI broth, and after 15 to 20 min of incubation at 37 °C (to perform the attachment of the phages to bacterial cells), the broth was centrifuged at 12,000 ×g for one minute. The pellet was re-dissolved in 20 ml of fresh BHI broth before incubation at 37 °C. Subsequently, 100 µl of the broth was collected for up to 120 min at 10 min intervals to titrate the number of phages using the double-layer agar method.

Bacteriophage electron microscopy

The morphological characteristics of the isolated phages were investigated using a Transmission Electron Microscope (TEM) in Partow Rayan Rastak's laboratory in Tehran, Iran. Briefly, 10 µl of the purified phage suspension was placed on a carbon-coated copper grid for 1 min, stained with 2% uranyl acetate (pH = 4.5) using a negative-grade staining method for 1 min, and washed with distilled water. The excess liquid was removed using filter paper, and then incubated for an hour in the room. Finally, the virus particles were examined using a Netherlands Philips Em208s TEM with a voltage of 100 kV [24, 32].

Proteome analysis of the isolated *S. saprophyticus* phages

The purified phage particles were analyzed by Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) to ascertain their protein pattern. A 10% acrylamide gel was employed to separate the proteins, which were then stained with Coomassie brilliant blue R250, and de-stained with a 10% methanol and 10% acetic acid solution (all reagents were purchased from Merck, Germany). Subsequently, the proteins were visualized after 24 h of staining and de-staining [11, 24].

Genome extraction, whole genome sequencing, and bioinformatics analyses

Genomic DNA was extracted from each purified phage using a PureLink Viral DNA mini kit (Thermo Scientific Fisher, Germany) according to the manufacturer's protocol. The purity and concentration of each extracted DNA were assessed using NanoDrop TM (DeNovix, DE, USA). The whole genome of each phage was sequenced using Illumina HiSeq. 2005 high-throughput sequencing (Macrogen Company, South Korea), following the Illumina NextEra XT library preparation protocol. The sequencing was carried out in paired-end with an average read length of 150 base pairs (bp). The Paired-ended

FASTQ files were polished for chimeric sequences, barcode errors, and duplication using the Trimmomatic tool [33]. Trimmed data were assembled using Unicycler [34], employing default parameters for optimal genome reconstruction. Contigs were checked for their quality through QUality ASsessment Tool (QUAST) [35]. After assembly, the phage genomes were searched for Open Reading Frames (ORFs) by Prokka [36]. The ORF functions were annotated using the NCBI server's protein Basic local alignment search tool (Blastp: https://blast.ncbi.nlm.nih.gov/Blast.cgi). Physical maps of the annotated phages' genomes were generated using the DNA plotter software [37]. The isoelectric points (pI) and molecular weights (MW) of the proteins were assessed by the protein isoelectric point calculator (http://isoelectric.org/index.html) and Sequence Manipulation Suite (https://www.genecorner.ugent.be/protein_mw.html), respectively [38]. Both tRNA Scan-SE (http://lowelab.ucsc.edu/tRNAscan-SE) and GtRNAdb (http://gtrnadb.ucsc.edu) were used to predict putative tRNAs [39, 40]. In addition, the existence of any possible antimicrobial resistance or virulence genes was evaluated by ResFinder v2.1 (https://cge.cbs.dtu.dk/services/ResFinder/) and VirulenceFinder v2.0 (http://cge.cbs.dtu.dk/services/VirulenceFinder/) [41–43]. Using the PHASTER (PHAge Search Tool–Enhanced Release) servers, the lysogenic sequences, integrase genes, or attR and attL sites were also checked [44]. Progressive Mauve software was used for progressive multiple genome alignment and comparison of the phages' nucleic and amino acid sequences with that of the homolog phages' sequences available in the NCBI database to ascertain the degree of relatedness between the phages' genomes and the homolog phages [45]. Phylogenetic analysis of the phages was performed using the Viptree tool [46]. Furthermore, the inter-genomic similarities of these three phages with related phages were analyzed by the VIRIDIC tool [47]. Finally, the complete genomic sequences of the phages were submitted to the NCBI database under accession numbers LC647030.1, LC557520.1, and LC648442.1.

Statistical analyses

In the experiments, if necessary, three repetitions were conducted and the results were reported as the mean ± standard deviation (SD). The statistical analyses were conducted using either a T-test or a one- or two-way ANOVA repeated measures, with the software employed being either GraphPad Prism v8 or SPSS software 16.0.

Results

Molecular confirmation of the clinical isolates of *S. saprophyticus*

All isolates were obtained from women with UTIs. These clinical isolates were stored in a BHI broth medium containing 15% glycerol at -70 °C until further use. Furthermore, the BLAST results confirmed that all isolates were *S. saprophyticus*, with more than 99% coverage and 99.5% similarity rates with other *S. saprophyticus* isolates.

Isolation of lytic *S. saprophyticus* phages

A total of 30 wastewater samples were collected, comprising 18 municipal and 12 hospital samples. Of these, three candidate phages with clear plaques were selected. They were obtained from the municipal wastewater samples. Plaques formed by the candidate phages were 1–2.5 mm in diameter, distinct, lacking a halo, and with clearly defined margins. Isolates, including *S. saprophyticus* 100, *S. saprophyticus* 101, and *S. saprophyticus* 105, were selected as the hosts for the next propagation of phages vB_SsapH-Golestan-100, vB_SsapH-Golestan101-M, and vB_SsapH-Golestan-105-M, respectively. The phage nomenclature system was based on the method proposed by Kropinski *et* al. method [48].

Host range of the isolated *S. saprophyticus* phages

The host ranges of these three phages were determined by examining 35 *S. saprophyticus* isolates. The results of plaque assay showed that phages vB_SsapS-105-M and vB_SsapS101-M inhibited 17/35 (48.57%) and 16/35 (45.71%) bacterial isolates, respectively. The other phage, vB_SsapS-100, could lyse 12 *S. saprophyticus* isolates (34.28%). All eight standard bacteria were resistant to these phages and the phages can only lyse *S. saprophyticus* strains. The host range results are presented in Table 1.

The morphological characteristics of the isolated phages

Following TEM imaging, the components of phages were measured using the IMAGE J software (Fig. 1). Figure 1A depicts vB_SsapH-Golestan-100, which has a head measuring approximately 85 nm and a contractile tail spanning about 220 nm. Furthermore, phage vB_SsapH-Golestan101-M (Fig. 1B) exhibited a head size of approximately 95 nm and a contractile tail measuring about 235 nm. Another phage, phage vB_SsapH-Golestan-105-M, (Fig. 1C) exhibited a head size of approximately 100 nm and a contractile tail measuring approximately 245 nm.

The adsorption rate, latent time, and burst size of the phages

The adsorption rates were almost similar for all three phages. After 5 min of vB_SsapH-Golestan-100 infection

Table 1 Host spectrum of bacteriophages against clinical isolates of *Staphylococcus saprophyticus*

Isolates	vB_SsapH-Golestan-100	VB_SsapS-Golestan101-M	VB_SsapS-Golestan-105-M
S. Saprophyticus 23	-	+	-
*S. Saprophyticus 46**	+	-	-
S. Saprophyticus 4643	-	-	-
S. Saprophyticus 100*€	+	+	+
S. Saprophyticus 101*€¥	-	+	+
S. Saprophyticus 103	-	+	+
S. Saprophyticus 105*€	+	-	+
S. Saprophyticus 107	-	-	+
S. Saprophyticus 108	+	+	+
S. Saprophyticus 109	-	+	+
S. Saprophyticus 110*	-	-	-
S. Saprophyticus 111*	-	+	+
S. Saprophyticus 112	-	+	+
S. Saprophyticus 113	+	-	-
S. Saprophyticus 114	+	+	+
S. Saprophyticus 115	+	-	-
S. Saprophyticus 116	-	-	-
S. Saprophyticus 117	-	+	+
S. Saprophyticus 118	+	-	+
S. Saprophyticus 119	-	-	+
S. Saprophyticus 120	+	-	-
S. Saprophyticus 121	-	+	+
S. Saprophyticus 122*¥	-	-	-
S. Saprophyticus 123*	-	-	-
S. Saprophyticus 124	-	-	-
S. Saprophyticus 125	+	-	-
S. Saprophyticus 126	+	-	-
S. Saprophyticus 127*¥	-	-	-
S. Saprophyticus 128	-	+	-
S. Saprophyticus 129¥	-	+	+
S. Saprophyticus 130*	-	-	-
S. Saprophyticus 131	+	+	+
S. Saprophyticus 132*	-	-	-
S. Saprophyticus 190	-	+	+
S. saprophyticus 541	-	+	-
Sum	12/35 (34.28%)	16/35 (45.71%)	17/35 (48.57%)

* Use as the first source of phages finding, € use as the main host for studying, ¥ multi-drug resistance strain

with isolate number 100, 35.3% of the phage was free in the medium. The other two phages had lower adsorption rates and approximately 40% of the phages were still free after 5 min. The highest adsorption rate occurred at 15 min after infection for three phages.

Furthermore, based on the results, the latent period of vB_SsapH-Golestan-100 was approximately 30 min, and that for vB_SsapH-Golestan101-M and vB_SsapH-Golestan-105-M phages was approximately 20 min. The burst sizes of the phages were about 150–210 PFU per infected cell. The largest burst size was observed for vB_SsapH-Golestan-105-M. The adsorption time and one-step growth curves are shown in Fig. 2.

Proteomic analysis of the phages' proteins

Protein profiling of the phage isolates indicates a distinct, prominent band likely corresponding to the major capsid protein (50–52 kDa) in each phage. Furthermore, a band within the 30–35 kDa range, potentially attributable to capsid proteins, was present in all the phages. Additionally, tail proteins exhibit a range of band sizes, with some shared similarities across all phages (Supplementary Fig. 1).

Genomics of the *S. saprophyticus* phages

The genomes of the *Staphylococcus* phages vB_SsapH-Golestan-100, vB_SsapH-Golestan101-M, and vB_SsapH-Golestan-105-M were 136,433 bp in length with a Guanine-Cytosine (GC) content of 33.7%, 144,081 bp

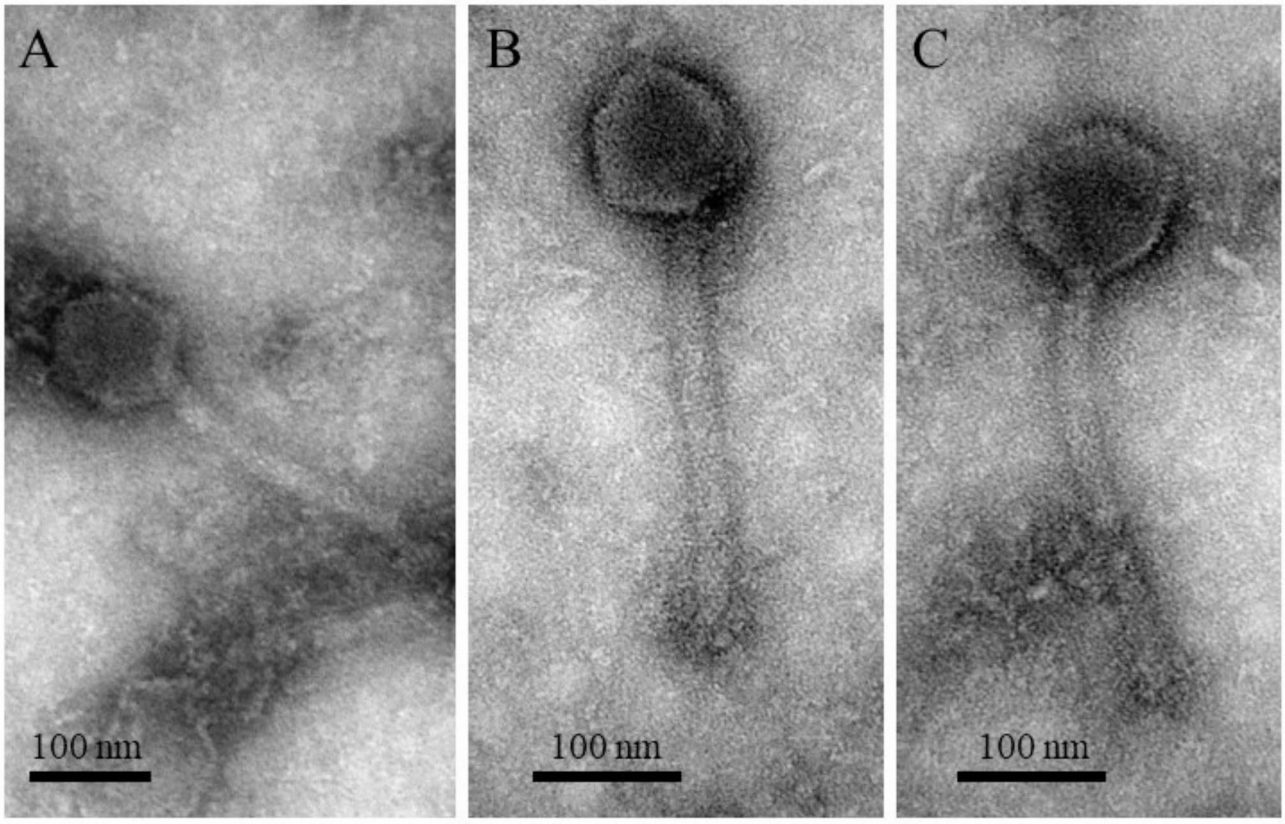

Fig. 1 Electron micrographs of phages that infect *S. saprophyticus*. Newly identified phages were named vB_SsapH-Golestan100 (**A**), vB_SsapH-Golestan101-M (**B**), and vB_SsapH-Golestan105-M (**C**).

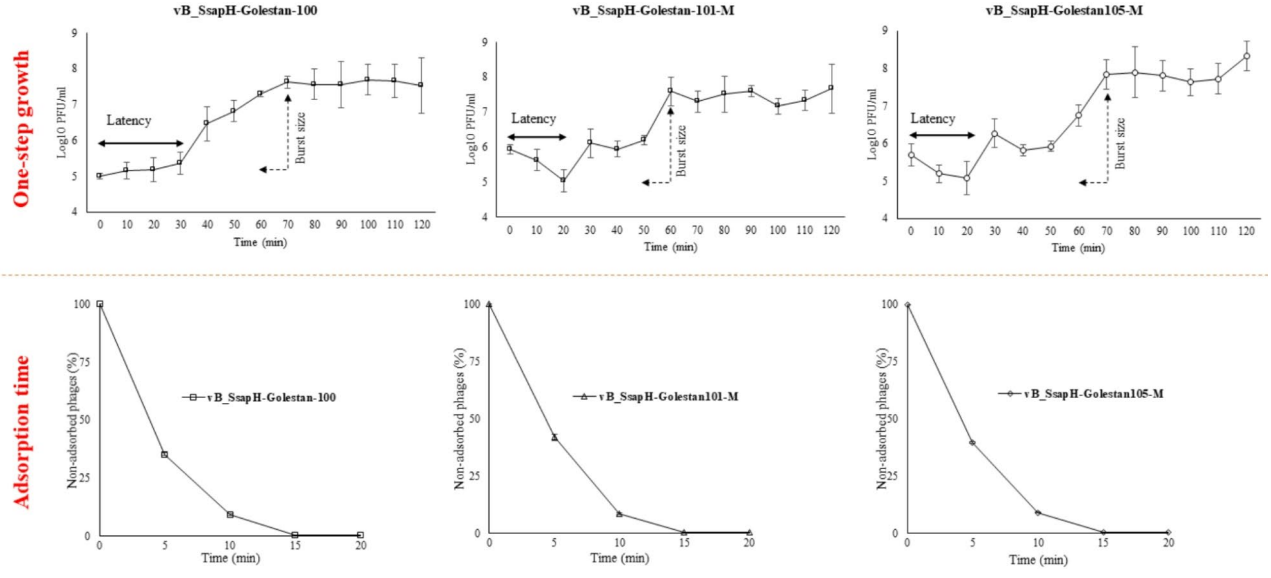

Fig. 2 The one-step growth and adsorption times curves of three identified phages.

in length with a GC content of 29.6%, and 142,199 bp in length with a GC content of 30.6%, respectively. In addition, the vB_SsapH-Golestan-100 phage comprised 192 ORFs, of which 134 were located on the forward strand and 58 on the reverse strand. Additionally, the tail morphogenetic protein, comprising 1337 amino acids, was the largest predicted protein in vB_SsapH-Golestan-100 (Supplementary Table 1). The vB_SsapH-Golestan101-M

phage had 205 ORFs in both the forward (64/205) and reverse (141/205) strands. The average length of the vB_SsapH-Golestan101-M proteins was 200 amino acids. Moreover, the tail length tape-measure protein, with a predicted length of 1450 amino acids, was the largest vB_SsapH-Golestan101-M protein (Supplementary Table 2). The other vB_SsapH-Golestan-105-M phage also contained 203 ORFs. Accordingly, 51 ORFs were on the forward strand, and 152 were on the reverse strand. Tail lysin, containing 1375 amino acids, was the largest predicted protein in vB_SsapH-Golestan-105-M (Supplementary Table 3). Upon analyzing the genomes of the three phages, no sequences related to antibiotic resistance, toxins, lysogeny, or virulence were identified. The genomes of vB_SsapH-Golestan-100, vB_SsapH-Golestan101-M, and vB_SsapH-Golestan105-M phages were submitted to NCBI under accession numbers LC647030.1, LC557520.1, and LC648442.1, respectively.

Genomic analysis showed that our *Staphylococcus* phage isolates, vB_SsapH-Golestan-100, vB_SsapH-Golestan101-M, and vB_SsapH-Golestan-105-M, belonged to *Herelleviridae*, and subfamily of *Twortvirinae*. The Mauve alignment of the genomes of these three phages, with those of three reference *Staphylococcus* phages (Twort, phiSA_BS1, and vB_SauM_Remus) revealed a predominantly collinear genome organization, characterized by several large conserved blocks (LCBs). These LCBs represent regions of homologous sequence shared among the phages. Notably, the majority of the genomic content is arranged in the same order across five of the six phages. Phages vB_SsapH-Golestan101-M, vB_SsapH-Golestan-105-M, phiSA_BS1, Remus, and Twort share multiple extensive LCBs that span most of their genomes, demonstrating high genomic collinearity. Within these shared blocks, the nucleotide sequences were highly conserved. Accordingly, pairwise comparisons support this close relatedness. For instance, phage vB_SsapH-Golestan101-M shares ~ 96.6% nucleotide identity over ~ 68% of its genome with *Staphylococcus* phage phiSA_BS1, underscoring the close clustering of these *Herelleviridae*, *Twortvirinae* phages. In contrast, vB_SsapH-Golestan-100 is more divergent, with its genome aligning only partially with the others based on the Mauve results. Additionally, it lacks substantial homology across large genomic regions, consistent with the low similarity to any known phage noted previously. The three conserved domains of vB_SsapH-Golestan-100, including Terminase Large Subunits (TLS), DNA polymerase, and portal protein, indicated that this phage could be entirely new, and no genetic correlation was observed with other homologous proteins. This phage had two separate TLS, 83 and 118 amino acids in length. Homology analysis revealed that these TLS proteins had near similarity to homologs in *Staphylococcus* phage Twort (89% coverage, 55.70% identity) and *Staphylococcus* phage pSco-10 (100% coverage, 63.90% identity). This phage had one portal protein that was similar to that of the *Staphylococcus* phage Stab22 (84% coverage, 58.51% identity). Its DNA polymerase with 358 amino acids had a similarity with the DNA polymerase in *Staphylococcus* phage vB_SscM-1 (99% coverage, 49.54% identity). Based on tRNA scanning, the genome had no tRNA and depended entirely on the host (Figs. 3 and 4, Supplementary Table 1).

The vB-SsapH-Golestan101-M phage was also found to belong to the *Baoshanvirus* genus, which is a member of the *Twortvirinae* subfamily. Based on the total genome alignment with the submitted genome in databases (NCBI, DDBJ, and EMBL), vB-SsapH-Golestan101-M was similar to *Staphylococcus* phage phiSA_BS1 (96.61% identity and 68% coverage) and *Staphylococcus* phage phiSA_BS2 (96.61% identity and 67% coverage) but had low similarity to other aligned phages. Phage vB-SsapH-Golestan101-M had three DNA polymerases that were highly similar to the DNA polymerase in *Staphylococcus* phage phiSA_BS1 and *Staphylococcus* phage phiSA_BS2. Additionally, vB-SsapH-Golestan101-M had two separate TLSs at ORFs 157 and 159. These TLSs were similar to the TLSs in *Staphylococcus* phage phiSA_BS1 and *Staphylococcus* phage IME-SA2. VB-SsapH-Golestan101-M was free of tRNA sequences, so it depended on host behavior during protein synthesis (Figs. 3 and 5, Supplementary Table 2).

The phage vB_SsapH-Golestan-105-M exhibited the highest degree of similarity with *Staphylococcus* phage Stab23, with 76% coverage similarity in length and 96.59% identity in the genome. Additionally, homology with other phages was also assessed using three conserved domains: TLS, DNA polymerase, and portal protein. In ORF19, a DNA polymerase consisting of 356 amino acids shows similarity to the DNA polymerase of Staphylococcus phage Stab23, with 100% coverage and 98.88% identity. Furthermore, vB_SsapH-Golestan-105-M contains two TLSs that are similar to those found in *Twortvirinae* phages, exhibiting 100% similarity in length, amino acids, and nucleotides when compared with Stab23 and *Staphylococcus* phage vB_Sau_S24. Additionally, its genome harbors two tRNA genes for L-aspartate and threonine, which are located approximately between 85,000 and 110,000 bp in the genome (Figs. 3 and 6, Supplementary Table 3).

Taxonomic relationships of the *S. saprophyticus* phages

Taxonomic analysis based on the similarity of nucleic acid sequences of the genomes with the NCBI database and comparison of all ORFs in the database showed that vB_SsapH-Golestan-100 is genetically distant from vB-SsapH-Golestan101-M, vB_SsapH-Golestan-105-M,

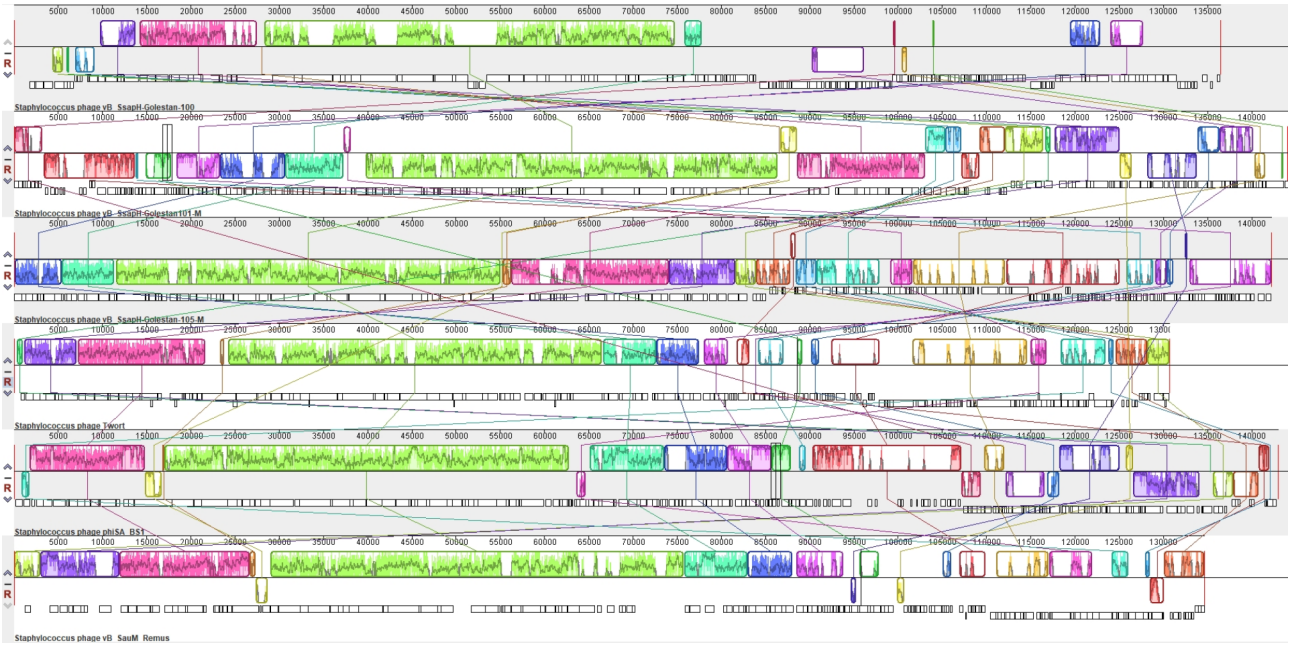

Fig. 3 Mauve alignment of six *Staphylococcus* phage genomes. Each genome is represented by a horizontal bar labeled with the corresponding phage name (genome lengths: 136,433 bp, 144,081 bp, 142,199 bp, ~ 139 kb, ~ 149 kb, and 134,643 bp, listed from top to bottom). Colored blocks along each genome denote large conserved blocks (LCBs). Homologous DNA segments are shared among the genomes. Blocks of the same color are connected between genomes to highlight conserved regions. The position of a block—either above or below the center line of a genome—indicates its orientation. Blocks drawn below the line in a given genome are inverted relative to the reference orientation. For this analysis, all genomes have been oriented to maximize collinearity.

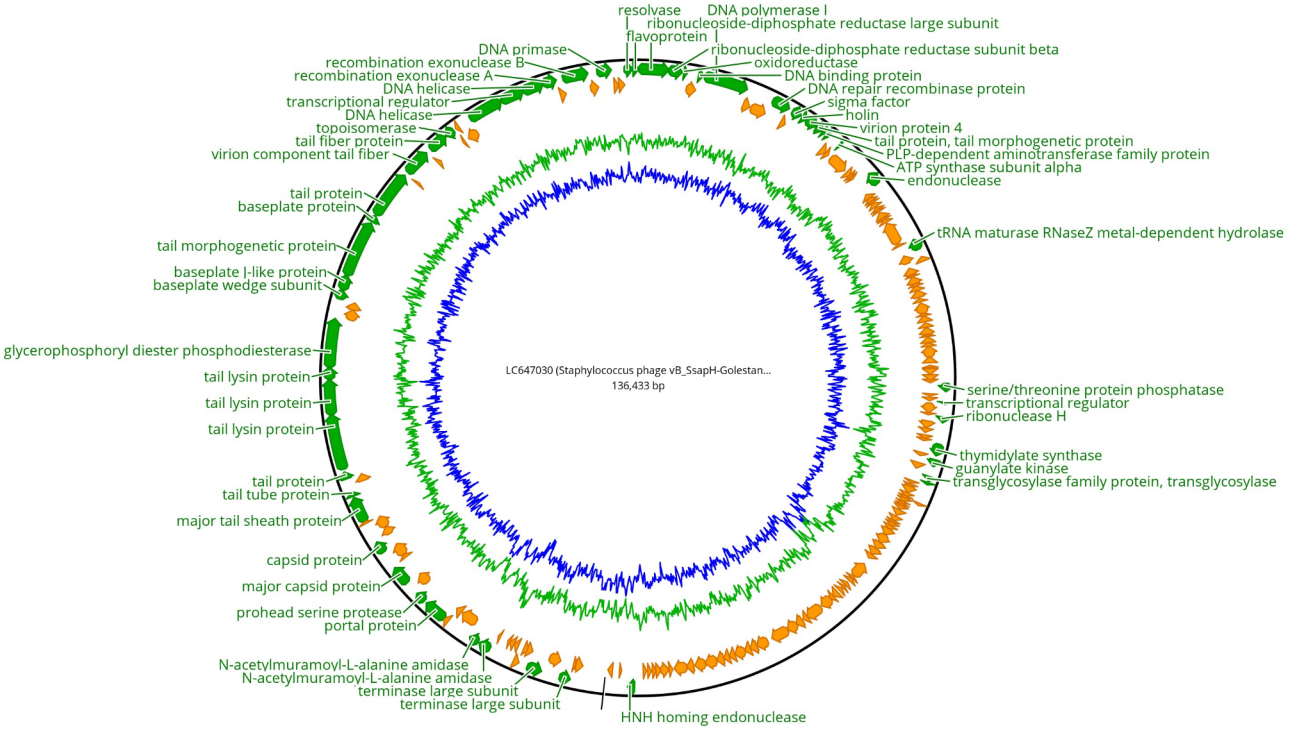

Fig. 4 Genome representation of vB_SsapH-Golestan-100 (LC647030.1) possible novel phages isolated from sewage waters. The inner rings illustrate the GC% and GC skew of the predicted CDS. The arrows represent the putative ORFs on forward or reverse strands. For enhanced visualization purposes, the linear genome maps have been represented in a circular format by the DNA plotter software.

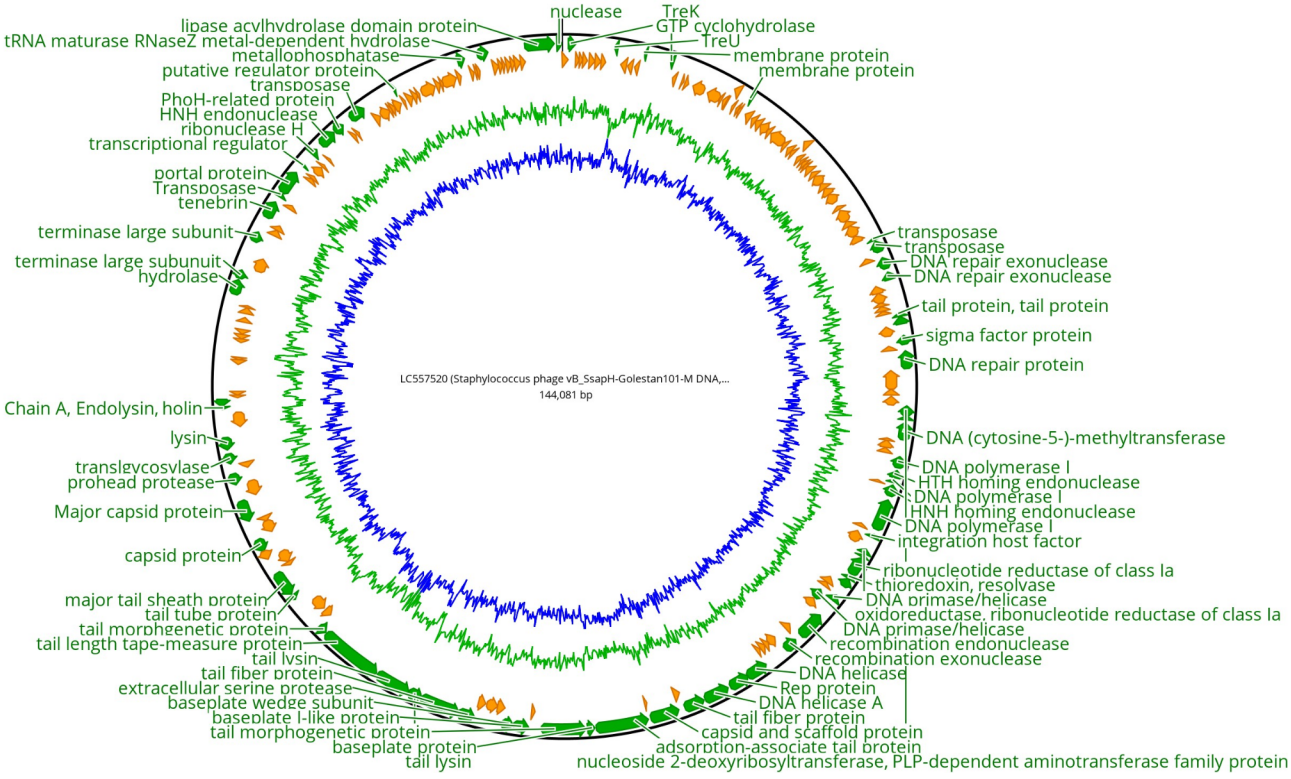

Fig. 5 Genome representation of vB_SsapH-Golestan101-M (LC557520.1) possible novel phages isolated from sewage waters. The inner rings illustrate the GC% and GC skew of the predicted CDS. The arrows represent the putative ORFs on forward or reverse strands. For enhanced visualization purposes, the linear genome maps have been represented in a circular format by the DNA plotter software.

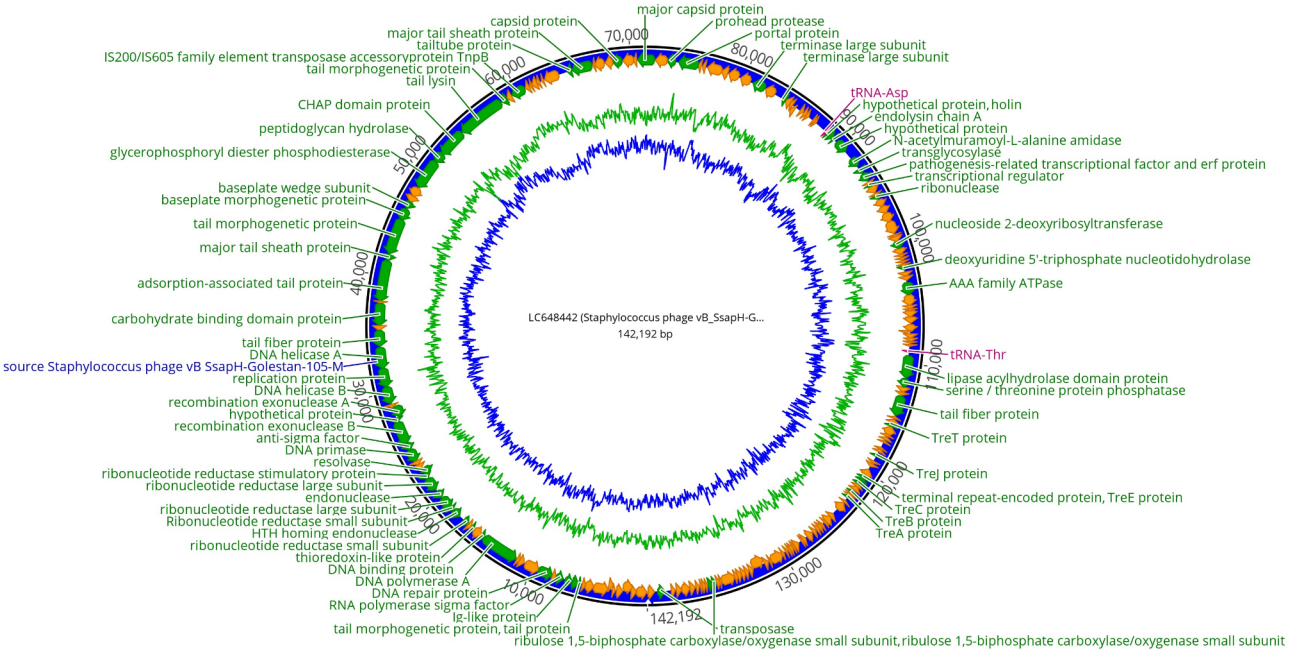

Fig. 6 Genome representation of vB_SsapH-Golestan-105-M (LC648442.1) possible novel phages isolated from sewage waters. The inner rings illustrate the GC% and GC skew of the predicted CDS. The arrows represent the putative ORFs on forward or reverse strands. For enhanced visualization purposes, the linear genome maps have been represented in a circular format by the DNA plotter software.

and other genera in *Herelleviridae* and can be a new genus in this family (the phylogenetic tree shown in the Supplementary Figure S2). The analysis of intergenomic similarities among phages using the VIRIDIC tool revealed that vB_SsapH-Golestan-100 (LC647030.1) exhibits less than 21% similarity with its closest counterparts in genomic databases. Conversely, *Staphylococcus* phages vB_SsapH-Golestan-101-M (LC557520.1) and vB_SsapH-Golestan-105-M (LC648442.1) demonstrate 72.8% and 73.1% similarity, respectively, with their nearest phage relatives (Fig. 7). However, despite these varying degrees of similarity, all three phages belong to the *Herelleviridae* family.

Discussion

In the era of antibiotic-resistant outbreaks, it is logical to consider alternative options for combating bacterial infections [49]. Lytic phages have shown promising therapeutic activities against *S. aureus* and *S. epidermitis* infections, as evidenced by studies [50, 51]. However, there is a paucity of information regarding the characteristics of phages with lytic activities against uropathogenic *S. saprophyticus* [11, 52]. In our previous study, we demonstrated that vB_SsapS-104, a lytic phage, was capable of efficiently lysing uropathogenic *S. saprophyticus* isolates [11]. Given the lack of complete genome information about lytic bacteriophages of *S. saprophyticus*, and in continuation of this research path, we sought to isolate and characterize additional phages with lytic activities against uropathogenic *S. saprophyticus* isolates through the application of additional comparative genomic and phylogenetic analyses.

According to studies, source selection, temperature, the quantity of disinfectant used in sewage, the flow rate of sewage, exposure to sunlight or radiation, and source choice affect the number of phages in the raw material [51]. In the present study, the phages were only isolated from municipal wastewater samples. A variety of disinfectants are routinely used for hospital wastewater treatment, including hydrogen peroxide, chlorine bleach, ethanol, quaternary ammonium compounds, and formaldehyde [53]. Therefore, the failure to isolate phages in the hospital wastewater might be due to the high volume of bleaches or antiseptics in those waters along with the wastewater treatment plant process. However, as introduced in previous research, there is always a chance to isolate the phages from all types of wastewater, including hospital sewage [11]. Anyway, according to the results of present and other studies, there might be a higher chance of isolating phage from municipal sewage or waters with minimal contact with disinfectants. The municipal wastewater samples are rich, partly because of late water exchange. This type of sewage is also stagnant and is generally filled with dirt. These characteristics of water are favorable for bacteria and phages to propagate. In this regard, Elahi *et* al. showed that wastewater from municipal sources is suitable for phage isolation against *Enterococcus* spp [54]. Further study by Gunathilaka *et* al. isolated 29 phages against antibiotic-resistant *E. coli* from urban wastewater as a rich source of lytic phages [55]. Our study, along with the aforementioned studies, supports the potential of recovering lytic phages from urban or municipal water resources.

Candidate phages for use in biotechnology in medical sciences and the food industry should possess certain characteristics [16, 56]. These include the absence of toxins, drug resistance, bacterial pathogenicity, or lysogeny genes. In addition, they should exhibit a large intraspecies host range and a low inter-species host range. Moreover, a short latent period and adsorption time, as well as a considerable burst size in a lytic phage, enhances the potential of the virus for biotechnology applications. Consequently, the phages, under investigation in this research, have the capacity to be employed in biotechnology, both in the phage therapy and medical industry. In combination with each other (in a possible cocktail) or with the simultaneous use of antibiotic drugs, they may be effective against more than two-thirds of the clinical strains of *S. saprophyticus*. Further studies are required to substantiate this claim, but other studies have demonstrated the efficacy of using multiple phages simultaneously or in conjunction with antibiotic drugs to eradicate bacteria [11, 57, 58].

The introduced phages belonged to the *Herelleviridae* family. Members of this viral family mainly infect Gram-positive human gut bacteria, especially members of the Bacillota phylum. Currently, *Herelleviridae* family has five subfamilies, 34 genera, and 92 species [59]. The genome of viruses from this family comprises a linear, terminally redundant, and non-permuted dsDNA with 106–170 kbp size. The results indicated that our phages belonged to the *Twortvirinae* subfamily, which has a genome range of 127–153 kbp with approximately 30% GC content and can generally infect *Staphylococcus* species [59]. The majority of the genomic characteristics of the phages identified in this study were identical to those of this family. Consequently, similar to other phages in the *Herelleviridae* members, the majority of the ORFs were transcribed on the negative strand. The morphology of *Herelleviridae* members is typically comprised of an icosahedral head (approximately 85–100 nm) and a contractible tail (approximately 130–220 nm length) replicable with a small collar [59]. In accordance with the aforementioned observations, the morphology of *Herelleviridae* was demonstrated through the TEM results of our phages. It is noteworthy that the length of the tails exceeded the standard size for this family, measuring approximately 220 to 245 nm in length. This variation

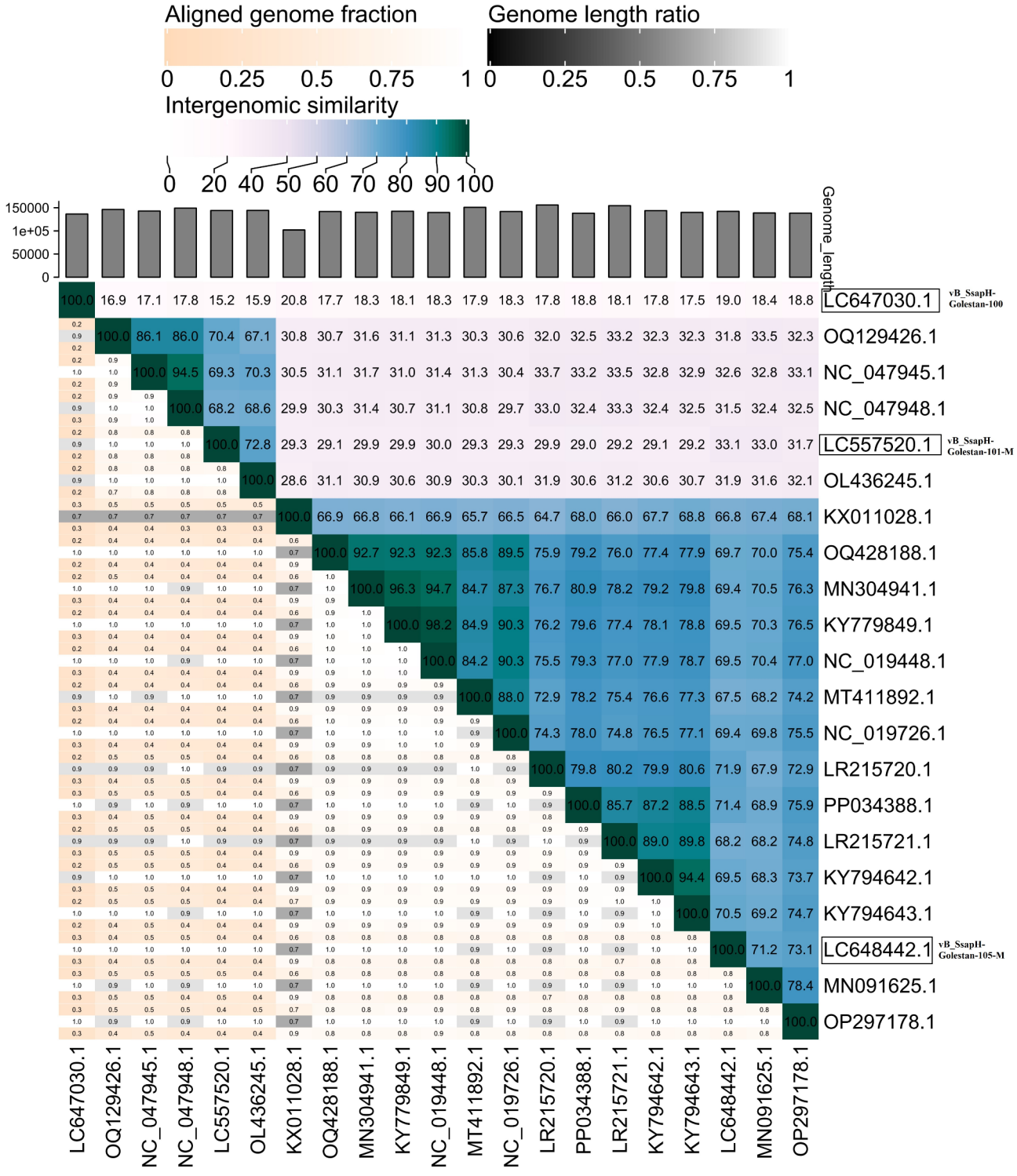

Fig. 7 The analysis reveals that vB_SsapH-Golestan-100 (LC647030.1) displays less than 21% similarity with its closest genomic counterparts. In contrast, *Staphylococcus* phages vB_SsapH-Golestan-101-M (LC557520.1) and vB_SsapH-Golestan-105-M (LC648442.1) exhibit 72.8% and 73.1% similarity, respectively, with their nearest phage relatives.

in tail length may indicate a unique adaptation characteristic of these particular phages. However, further confirmation is required, and therefore, higher-quality microscopic images are necessary.

The genomic profiles of two isolated phages, vB-SsapH-Golestan101-M and vB-SsapH-Golestan-105-M, exhibit 15% coverage and 97.23% identity. This indicates a significant genetic difference between the two phages. As a result of this difference, vB-SsapH-Golestan101-M and vB-SsapH-Golestan-105-M are classified into different taxonomic lineages. Additionally, another phage, vB_SsapH-Golestan-100, with a genome size of 136,433 bp, only shares 1% and 4% genome length coverage with vB-SsapH-Golestan101-M and vB-SsapH-Golestan-105-M, respectively. This pronounced disparity in genomic coverage indicates that vB_SsapH-Golestan-100 is genetically distinct from both vB-SsapH-Golestan101-M and vB-SsapH-Golestan-105-M. Indeed, vB_SsapH-Golestan-100 exhibits a greater degree of genetic divergence than other known Staphylococcus phages. Nevertheless, a more comprehensive phylogenetic analysis, such as network analysis, is necessary to elucidate the precise position and classification of these three phages. Numerous hypothetical protein-encoding genes were found within the genomes. In order to elucidate the functions of these proteins, a potential approach involves utilizing homology modeling and in-silico studies. These methods enable the identification of protein similarity and other functional aspects when compared to closely related protein families. Subsequent steps would include sequence cloning, protein expression, and functional analysis in ex-vivo and in-vivo models. Further investigation into the functions of these hypothetical proteins can be carried out in future studies involving these and similar phages.

It should be noted that this study is subject to a number of limitations. The most significant limitation was the suboptimal quality of the microscopic images, which was a consequence of the limitations of the available facilities. To address this issue, we attempted to accurately diagnose the taxonomy of the virus by conducting a complete genomic analysis. Additionally, the study did not examine the resistance of phages under varying temperature and pH conditions, nor did it investigate their efficacy in combination with antibiotics or against bacterial biofilms. Furthermore, to ascertain the comprehensive range of *Staphylococcus* species susceptible to lysis by these viral agents, a much larger number of bacterial species should be examined. Furthermore, employing advanced bacteriophage purification techniques, such as cesium chloride or sucrose density gradient centrifugation followed by dialysis in SM solution, can significantly improve the quality of the final purified viral samples compared to the method used in this study. By doing so, more accurate and comprehensive protein analyses can be conducted on these purified samples. These aspects could be subjects of future research studies.

Conclusion

The present study isolated and characterized three phages from municipal wastewater that exhibited lytic activity against *S. saprophyticus* isolates. To the best of our knowledge, these isolated phages represent one of the first reported members of the *Herelleviridae* family to exhibit lytic activity against *S. saprophyticus*, whose genome has been fully evaluated. Of the 35 *S. saprophyticus* isolates tested, 26 (74.28%) were found to be susceptible to at least one of the isolated phages. Our NGS analyses revealed that one of the isolated phages, vB_SsapH-Golestan-100, may potentially represent a new genus of the *Herelleviridae* family in future viral taxonomy.

Abbreviations

UTIs	Urinary Tract Infections
UPEC	UroPathogenic *Escherichia coli*
MDR	Multi Drug-Resistant
phages	Bacteriophages
FDA	US Food and Drug Administration
PCR	Polymerase Chain Reaction
MOI	Multiplicity of Infection
TEM	Transmission Electron Microscope
SDS-PAGE	SDS polyacrylamide gel electrophoresis
QUAST	QUality ASsessment Tool
ORFs	Open Reading Frames
pI	Isoelectric points
MW	Molecular weights
CDS	CoDing Sequence
NGS	Next-generation sequencing

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-025-02710-0.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Acknowledgements

Not applicable.

Author contributions

HSA, MY, and EAG designed the experiments; MY, HSA, and MR did the experiments; EAG and AT supervised the work; HAS, MY, AM, AS, and EAG analyzed the data; HAS, AM, and MR wrote the manuscript. HAS, AT, and EAG revised the paper. All authors read and approved the final manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. The complete genomic sequences of the phages were submitted to the NCBI database under accession numbers LC647030.1, LC557520.1, and LC648442.1.

Declarations

Ethics approval and consent to participate
The ethics committee of Golestan University of Medical Sciences approved the present study (Ethics committee reference number: IR.GOUMS.REC.1397.339).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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