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Characterization of Lytic Bacteriophage Specific to *Staphylococcus aureus*: A Step Towards Phage Therapy

[1]Rabia Rahmat, [2]Zarak Khan, [3]Amjid Ali, [4]Amna Ahmed Ali, [5]Naila Menhaj, [6]Razia Misbah, [7]Amna Waqar, [8]Dur.e.kashaf, , [9*]Zia Ullah, [10*]Muhammad Kamil Khan

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Rabia Rahmat
Department of Botany,The Women University Multan, rabiarehmat779@gmail.com
Zarak Khan
Department College of Veterinary Science, University of Agriculture Peshawar.
drzarak@aup.edu.pk
Amjid Ali
Department of MLT, Khyber medical university Peshawar amjidali.labtech@gmail.com
Amna Ahmed Ali
Department of Microbiology, Abbottabad University of Science and Technology Abbottabad
amnashabir94@gmail.com
Naila Menhaj
Department, Centre of biotechnology and microbiology University of Peshawar
Nailamenhaj12@gmail.com
Razia Misbah
Department of Microbiology, Hazara University Mansehra Pakistan.
Misbahmalik00@gmail.com
Amna Waqar
Department of Microbiology, Abbottabad University of Science and Technology Abbottabad
amnawaqarahmed24@gmail.com
Dur.e.kashaf
Department of Microbiology, Abbottabad University of Science and technology Abbottabad
kashafdure3@gmail.com
Zia Ullah
Department of Microbiology, Abbottabad University of Science and Technology Abbottabad. Corresponding Author Email: Ziahamdaed13@gmail.com
Muhammad Kamil Khan
Department of Microbiology, Hazara University Mansehra Pakistan.
Corresponding Auther Email: biologykame9@gmail.com

ABSTRACT

The rising incidence of antibiotic-resistant *Staphylococcus aureus* infections, including methicillin-resistant strains (MRSA), has intensified the demand for alternative antimicrobial strategies. This study focuses on the isolation and characterization of lytic bacteriophages specific to *S. aureus* to assess their potential application in phage therapy. Phages were isolated from sewage and hospital wastewater, purified through plaque assays, and subjected to morphological and molecular characterization. The phages demonstrated notable resilience across a broad pH spectrum (4.0–10.0), with optimal infectivity observed between pH 6.0 and 8.0. A decline in lytic activity was noted at extreme acidic and alkaline values, indicating potential for application in physiological environments. Heat resistance assays showed that phages retained significant activity up to 45°C, with gradual reduction in infectivity observed at higher temperatures. No viable activity was detected beyond 60°C, indicating their suitability for application and storage at ambient and body temperatures. Long-term viability testing revealed that the phages maintained over 80% infectivity when stored at 4°C for up to six months. Lyophilized samples preserved at 20°C demonstrated superior stability, suggesting freeze-drying as an effective method for formulation. The phages exhibited a broad host range, lysing a diverse panel of S. aureus clinical isolates, including multi-drug resistant strains. Specificity tests confirmed negligible activity against non-target Gram-positive and Gram-negative species, affirming their safety and selectivity. In vitro bacterial reduction assays revealed rapid bactericidal action within the first 2 hours post-phage application, achieving 99% reduction of viable bacterial counts. These results underscore the therapeutic potential of the phages in controlling S. aureus infections. Collectively, the characterized phages demonstrate strong lytic activity, stability under diverse conditions, and a broad host range, reinforcing their promise as effective agents in phage therapy against S. aureus.

DOI: Availability

INTRODUCTION

S. aureus is a versatile Gram-positive bacterium frequently colonizing human skin, nasal passages, and mucosal surfaces. Though often benign, it can cause a wide array of infections, ranging from superficial conditions like boils and abscesses to life-threatening diseases such as bacteremia, endocarditis, osteomyelitis, pneumonia, and toxic shock syndrome. The emergence and proliferation of multidrug-resistant strains, especially methicillin-resistant *S. aureus* (MRSA), have turned this once-manageable pathogen into a formidable clinical challenge (Feßler *et al.*, 2018). MRSA infections are not only difficult to treat with conventional antibiotics, but also contribute significantly to morbidity, mortality, and healthcare costs globally. The misuse and overuse of antibiotics in medicine, agriculture, and animal husbandry have accelerated bacterial adaptation, rendering many antibiotic regimens ineffective (Wollesen *et al.*, 2023). The global health crisis caused by antimicrobial resistance (AMR) underscores the urgent need for novel, effective, and sustainable therapeutic alternatives. Bacteriophages (or simply phages) are viruses that specifically infect and destroy bacterial cells. First discovered over a century ago, phages are the most abundant biological entities on Earth, found in virtually every ecosystem including sewage, soil, oceans, and even the human microbiome (Pirolo *et al.*, 2024). They consist of a protein capsid encasing their nucleic acid (DNA or RNA), and they recognize specific receptors on bacterial surfaces to initiate infection. After attachment, the phage injects its genome into the host, hijacking the bacterial machinery for replication (Lopes *et al.*, 2014). Phages follow two major life cycles: the **lytic** cycle, in which the host is rapidly lysed to release new virions, and the **lysogenic** cycle, where the phage integrates its genome into the host's DNA and remains dormant (Mackey *et al.*, 2016). For therapeutic applications, only strictly lytic phages are considered suitable, as they eliminate bacteria without transferring resistance or virulence genes.

Their inherent host specificity ensures targeted action, minimizing collateral damage to beneficial microbiota. The lytic cycle of phages involves a series of steps: adsorption, penetration, biosynthesis, assembly, and lysis (Mackey *et al.*, 2016). The phage binds to a bacterial receptor, injects its genome, and commandeers the host's metabolic pathways to produce viral components. These are assembled into complete virions, culminating in host cell rupture and release of progeny. Phages thrive in environments rich in bacterial populations — such as wastewater, riverbeds, animal intestines, and contaminated hospital surfaces. For isolating *S. aureus*-specific lytic phages, environmental samples are filtered, enriched with target host bacteria, and screened using plaque assays. Each clear plaque signifies a lytic event an indication of a potential

therapeutic agent. These phages are further purified and characterized for stability, host range, and efficacy. The ecological diversity of phages ensures a vast reservoir of antibacterial agents, adaptable to different strains and infection environments (Parasion *et al.*, 2014). Phage therapy, once overshadowed by antibiotics in the 20th century, is experiencing a renaissance amidst rising antibiotic resistance. By exploiting the bactericidal nature of phages, researchers aim to develop targeted, personalized treatments for resistant infections. Phages offer several compelling benefits. Phages targeting *S. aureus* have demonstrated significant activity in in vitro and animal models, including reductions in bacterial load, biofilm clearance, and infection resolution. Their application, however, requires rigorous characterization assessing pH stability**,** thermal resistance**,** long-term storage viability**,** host spectrum**,** and reduction efficacy (Ebrahimizadeh and Rajabibazl, 2014)**.** Combined with advances in genomics and formulation science, phage therapy holds promise as either a standalone or adjunct treatment. As clinical trials and regulatory frameworks evolve, phages may soon become a mainstream weapon against the antibiotic-resistant superbugs of our time (Ebrahimizadeh and Rajabibazl, 2014).

MATERIALS AND METHODS

STUDY AREA

The study was conducted at Abbottabad University of science and technology Microbiology Research Laboratory. The Khyber Teaching Hospital in Peshawar supplied the bacterial samples, while THQ Poran Shangla, DHQ Shangla, and Butt Pull Sewage Drain in Mansehra provided the sewage samples.

BACTERIAL CULTURE

S. aureus clinical samples were gathered from Khyber Teaching Hospital in Peshawar. Fresh nutrient agar plate was streaked with bacterial samples, and they were then incubated for the overnight at 37°C. The bacterium was identified using microscopy and Gram staining. Before every experiment, new bacterial samples were subcultured, and cultures were utilized for eight hours. Regular subculturing helped to preserve the culture's viability and purity.

ISOLATION OF BACTERIOPHAGES FROM SEWAGE

Sewage samples were taken from hospital waste, DHQ hospital in District Shangla and THQ hospital in Poran Shangla. To isolate bacteriophages, the samples were taken to Abbottabad university microbiology lab. A previously described methodology was used to extract bacteriophages from a sewage sample (Liu *et al.*, 2021).

The water samples were agitated for two minutes before being centrifuged for ten minutes at

10,000 rpm to get rid of pathogens and sediment. In a conical flask, 40 mL of the clear sewage supernatant was added to 10 mL of sterile 5 X nutritional broth. A 200 L overnight culture was used to inoculate the flask. Overnight incubation of the infected flask was conducted at 37°C while being shaken at 120 rpm. Following incubation, the flask's contents were centrifuged for five minutes at 10,000 rpm. The clear supernatant was collected in a fresh, clean falcon tube and stored at 4°C for later use after passing through a 0.22 syringe filter. By using a spot test, the presence of bacteriophages in the filtrate was identified (Liu *et al.*, 2021).

DETECTION OF BACTERIOPHAGES IN THE FILTRATE

Following bacteriophage enrichment, a spot test was used to identify the presence of a *S. aureus* specific phage. In the spot test, a nutrient agar plate was covered with 100 L of an overnight-grown *S. aureus* culture. After applying 5 L of the filtrate, the plates were allowed to dry for nearly 10 minutes. The plates were then incubated for the whole night at 37°C. After that, the plates were examined for a distinct bacteriophage-induced lysis zone. The presence of a particular bacteriophage is indicated by the presence of the clear zone, or plaque (Liu *et al.*, 2021).

PURIFICATION OF BACTERIOPHAGES USING A DOUBLE LAYER AGAR ASSAY

Double layer agar overlay method was used to quantify and purify bacteriophage from the lysate (filtrate that caused lysis) (Alvi *et al.*, 2020). Before the experiment began, 100 mL of semisolid nutritional agar was autoclaved and put in a water bath at 48°C in a conical flask. Initially, 900 L of nutritional broths were used to serially dilute the lysate (1:9) up to 1/1012 in microtubes. The chosen dilutions were then supplemented with 100 L of a new *S. aureus* culture. For five minutes, the bacterial culture and phage lysate were incubated to enable the phages to adhere to the bacterial cells. After pouring the chosen dilutions onto nutritional agar plates, 3 mL of semisolid agar was added, and the plates were incubated at 37°C for the whole night. Swirling motion was used to disperse the mixture and semisolid agar on the plate. We looked for countable plaques (30 pfu-200 pfu) on the plates. The chosen plate's plaque forming units (pfu) were counted (Alvi *et al.*, 2021).

Plates with unique plaque were chosen for phage purification. With the use of a sterile micropipette tip, phage was removed from a plaque by gently tapping its surface. For phage propagation, the tip was placed in a test tube with 10 mL of nutritional broth and 1 mL of fresh *S. aureus* strain. It was then cultured for 24 hours at 37°C, after which plaque was visible and purified (Di Martino *et al.*, 2003, Fader *et al.*, 1979). The purifying process was carried out up to ten times. The following formula was utilized to determine the titer of lysate solutions:

Titter (*pfu*/mL) = plaque (*pfu*) dilution number x phage (mL) volume added to plate.

CHARACTERIZATION OF BACTERIOPHAGES

DETERMINATION OF HOST RANGE

An established procedure was used to analyze the host range utilizing bacterial cultures from several strains of *S. aureus* as well as other species (Alvi *et al.*, 2021). On a plate, a bacterial lawn was created and spotted using 5 L of phage lysate. The plates were incubated at 37°C for 24 hours before bacterial lysis was observed (Alvi *et al.*, 2021).

DETERMINATION OF THERMAL STABILITY OF BACTERIOPHAGES

Determining the bacteriophages' temperature stability is crucial since it offers a hint for phage transportation and storage. Aliquots of known titers of phage were placed at 4, 25, 37, 50, and 60°C for an hour in order to assess the thermal stability of the isolated bacteriophages. The two layer agar overlay technique was used to determine the bacteriophage titer following incubation (Alvi *et al.*, 2021).

DETERMINATION OF PH STABILITY OF BACTERIOPHAGES

HCl and NaOH were used to change the medium's pH. A pH paper was used to measure the pH. To find the pH stability of the isolated bacteriophages, aliquots of known titers of phage were placed at pH 2–10 for an hour. The two layer agar overlay technique was used to determine the bacteriophage titer following incubation (Alvi *et al.*, 2021).

LONG TERM STORAGE STABILITY

For six months, bacteriophages were maintained in LB broth at 4, 25, and 37°C. Prior to storage, bacteriophages phage had respective titers of 7×10^{9}pfu/mL and 8×10^{10}pfu/mL. The two layer agar method was used to measure each bacteriophage's titer both before and after storage (Alvi *et al.*, 2021).

RESULTS

BACTERIAL GROWTH ON NUTRIENT AGAR MEDIA

S. *aureus* is a cocci in shaped, facultatively anaerobic, gram-negative bacteria that is not mobile**.** Bacterial isolate SA1 was then characterized by morphology, by using nutrient agar. They often appear yellowish colonies on nutrient agar media (Figure 1).

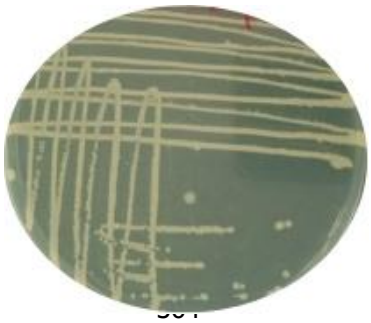

FIGURE 1: GROWTH OF *S. AUREUS* (SA1) ON NUTRIENT AGAR

SPOT TEST FOR THE DETECTION OF *S. AUREUS* SPECIFIC BACTERIOPHAGES

Bacteriophages against *S. aureus* (SA1) was detected in one of five sewage samples. SAP1 was the name of the bacteriophage that was acquired from DHQ Shangla.

FIGURE 2: DETECTION OF BACTERIOPHAGES THROUGH SPOT TEST SAP1

ISOLATED BACTERIOPHAGES PRODUCED CLEAR TRANSPARENT PLAQUES

On a double layer agar plate, the isolated phage produced a circular, clear plaque that was antagonistic to SA1. The plaque is composed of two circular layers, with an outer circle around the inner, completely transparent center. The existence of this hazy layer surrounding plaque is a sign that bacteriophages are producing the depolymerase enzyme. SAP1 had a diameter of 2 mm. The halo surrounding the plaque indicates that the bacterial host cell was decapsulated by soluble enzymes such depolymerase, which was produced by phages SAP1.

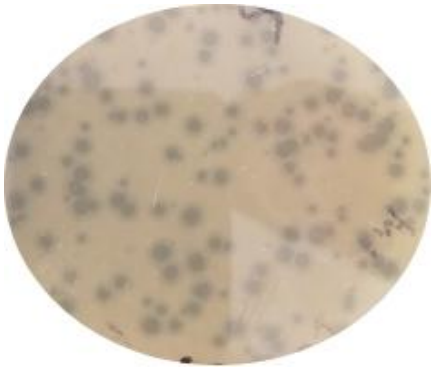

FIGURE 3: ISOLATED BACTERIOPHAGES HAVE TRANSPARENT PLAQUE SAP1

ISOLATED PHAGES HAVE HIGH TITER

The isolated phages' lytic behavior and promise as a therapeutic agent are demonstrated by the translucent, clear plaques. SAP1 titer was reported to be 4×10^{9} pfu/mL following a 24-hour phage propagation period in liquid culture.

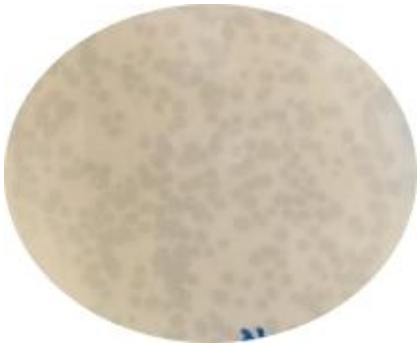

FIGURE 4: TITER OF PURIFIED BACTERIOPHAGE SAP1

ISOLATED BACTERIOPHAGE WAS FOUND TO HAVE NARROW SPECTRUM

It was discovered that the isolated *S. aureus* phage was very strain-specific. While no infectivity was seen for the other examined genera, SAP1bacteriophage was able to infect and create a lytic zone against isolates of *S. aureus*. According to the findings of the host range specificity test, the SAP1phage was able to infect four out of eight *S. aureus* but not bacteria from other genera (*E. coli*, *S. typhi*, *Enterobacter*, *P. aeruginosa*.

TABLE 1: HOST RANGE SPECTRUM OF KPP1 AND KPP2 BACTERIOPHAGES AGAINST VARIOUS BACTERIAL CULTURES

S.No	Bacterial culture	Spot test SAP1 Phage	Source
1	*S. aureus*1	+	Clinical source
2	*S. aureus*2	-	Clinical source
3	*S. aureus*3	+	Clinical source
4	*S. aureus*4	+	Clinical source
5	*S. aureus*5	-	Clinical source
6	*S. aureus*6	+	Clinical source
7	*S. aureus*7	-	Clinical source
8	*S. aureus*8	-	Clinical source
9	*E. coli* isolate	-	Clinical source
10	*Salmonella* isolate	-	Clinical source
11	*Enterobacter* isolate	-	Clinical source
12	*Pseudomonas* isolate	-	Clinical source

THE ISOLATED BACTERIOPHAGE WAS FOUND THERMALLY STABLE

The stability of bacteriophages is significantly impacted by temperature. It impacts adhesion, penetration, and proliferation in all aspects of phage replication. It was discovered that the SAP1 phage remained stable at 50ºC without experiencing any titer changes. For SAP1, there was no drop in phage titer at 4 or 37ºC, however there was a 2-fold drop at 25 and 50ºC while at 60ºC, the SAP1 titer decreased 6 times (Figure 4). Since SAP1 phage was stable at 4°C, 25°C and 37°C, respectively, and since the average human body temperature is 37°C, these phages may be readily used if they were to be utilized as a therapeutic agent. Moreover, these phages may be transported without the need for specific heating conditions.

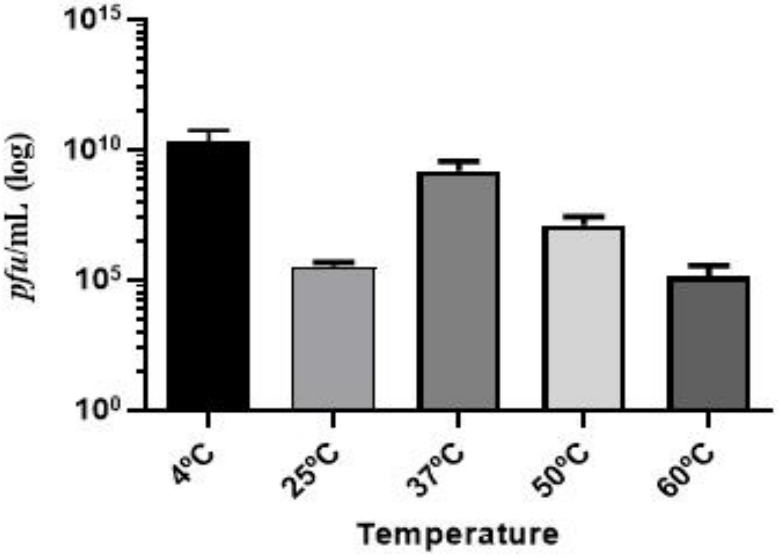

FIGURE 5: THERMAL STABILITY OF SAP1 BACTERIOPHAGE AT DIFFERENT TEMPERATURES

PH STABILITY OF BACTERIOPHAGES

The environment's acidity and alkalinity have a crucial role in phage stability. After an hour, the SAP1 phage showed resistance to the pH range of 3.0 to 9.0. It was discovered that the ideal pH range for phage SAP1 was 3–9. Additionally, at pH 9, 8, 6, 5, 4 and 3, a drop of 2, 3, 2, 2, 3 and 4 log were noted, respectively (Figure 6). Very few phages can withstand such a wide pH range.

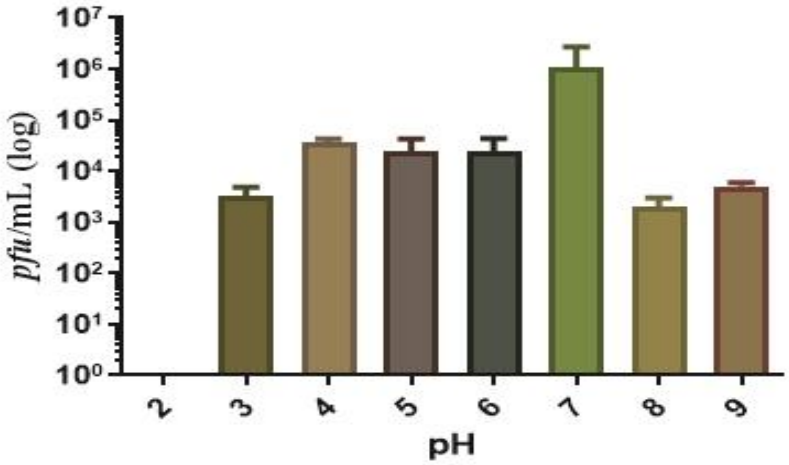

FIGURE 6. EFFECT OF VARIOUS PH ON THE VIABILITY OF SAP1 BACTERIOPHAGE

4°C SAP1 PHAGE WAS FOUND BEST FOR STORAGE

After three months of storage, the phage SAP1 titer was determined to be steady. For phage SAP1, there was no titer drop at 4°C, but at 25°C and 37°C, there was a decrease of 1 and 2 log, respectively (Table 2).

TABLE 2. STORAGE STABILITY OF BACTERIOPHAGE AT DIFFERENT TEMPERATURE

Phage	Titer Before Storage	Titer After Storage		
		4°C	25°C	37°C
SAP1	8×10^{9} *pfu*/mL	5×10^{9}	7×10^{8}	4×10^{7}

SAP1 PHAGE REDUCED *S. AUREUS GROWTH*

The bacterial growth reduction test was used to evaluate the antibacterial activity of the SAP1. Using a spectrophotometer to record the OD600 every two hours for the following twenty-four hours, the capacity of SAP1 phage to inhibit bacterial growth was tracked and contrasted with the growth of the control (Figure 7). SAP1 phage was proposed as a possible phage treatment option based on the suppression of bacterial growth, provided that the required clinical and animal model investigations are conducted.

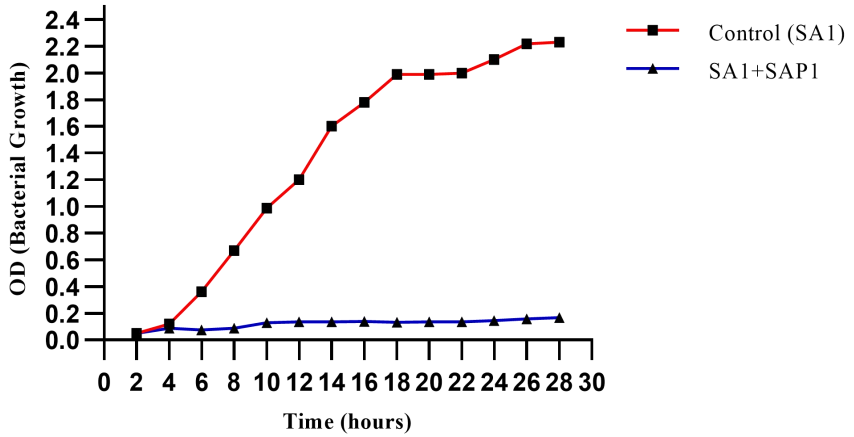

FIGURE 7. BACTERIAL GROWTH REDUCTION POTENTIAL OF SAP1 BACTERIOPHAGE

DISCUSSION

Staphylococcus aureus represents one of the most clinically significant bacterial pathogens affecting both human and animal health worldwide. This Gram-positive, facultative anaerobic coccus has demonstrated remarkable adaptability to various ecological niches, including the gastrointestinal tract of poultry. *S. aureus* infections are a leading cause of morbidity and mortality worldwide, with both community-associated (CA) and healthcare-associated (HA) strains contributing to the disease burden. Key virulence determinants include surface proteins (e.g., protein A, which interferes with antibody-mediated immunity), toxins (e.g., alpha-hemolysin and Panton-Valentine leukocidin, which lyse host cells), and enzymes (e.g., coagulase, which promotes fibrin clot formation) (Wendlandt *et al.*, 2013).

S. aureus show resistance towards wide range of antibiotics. A viable substitute for fighting resistant *S. aureus* strains is the use of bacteriophages, which are naturally occurring predators of bacteria. Without altering the host microbiome, phage treatment uses the specificity of phages to infect and lyse bacterial cells. In the current study *S. aureus* clinical samples were gathered from Khyber Teaching Hospital in Peshawar. Sewage samples were taken from hospital waste of DHQ hospital in District Shangla and THQ hospital in Poran Shangla for bacteriophage isolation. Bacteriophages against *S. aureus* (SA1) was detected in one of five sewage samples. SAP1 was the name of the bacteriophage that was acquired from DHQ Shangla. Similar study was repored by Rehman *et al.*, (2016) which shows 10 sewage samples were taken from hospital

waste of district Malakand out of 10 samples bacteriophages against *S. aureus* was detected in five of ten sewage samples. On a double layer agar plate, the isolated phage produced a circular, clear plaque that was antagonistic to SA1. Similar investigation by Ali *et al.*, (2016) which shows *S. aureus* phage SPA produced a circular, clear plaque.

Host range specificity test was performed using spot test method, the SAP1phage was able to infect four out of eight *S. aureus*. Similar study by Fri *et al.*, (2013) which shows phage SPA only infect strains of *S. aureus*. It was discovered that the SAP1 phage remained stable at 50ºC without experiencing any titer changes. For SAP1, there was no drop in phage titer at 4 or 37ºC, however there was a 2-fold drop at 25 and 50ºC while at 60ºC, the SAP1 titer decreased 6 times. After an hour, the SAP1 phage showed resistance to the pH range of 3.0 to 9.0. It was discovered that the ideal pH range for phage SAP1 was 3–9. Similar study by Ruya *et al.*, (2014) which shows *S. aureus* Phage SAB remains stable up to 50 degrees and at 3-9 pH range.

After three months of storage, the phage SAP1 titer was determined to be steady. For phage SAP1, there was no titer drop at 4°C, but at 25°C and 37°C, there was a decrease of 1 and 2 log, respectively SAP1 phage was proposed as a possible phage treatment option based on the suppression of bacterial growth, provided that the required clinical and animal model investigations are conducted. Similar study by Ruya et al., (2014) which shows *S. aureus* Phage SAB there was no titer drop at 4°C, but at 25°C and 37°C, there was a decrease of 2 and 4 log, respectively

CONCLUSION

A significant health risk now exists from infections and the consequences of diseases brought on by multidrug-resistant (MDR) bacterial pathogens. Common infections become challenging to cure. Although there are several alternatives to traditional therapeutic methods, bacteriophages continue to be a powerful substitute. They are able to be separated from the natural habitat of bacteria. Following the required animal modeling and clinical trials, bacteriophages SAP1 appear to be promising candidates for phage therapy due to them in vitro effectiveness in reducing bacterial growth, causing stability at a wide pH and temperature range, and promoting long-term storage stability without the use of preservatives.

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