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ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGE SPECIFIC FOR *STAPHYLOCOCCUS AUREUS*

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Abstract

There has been a concerning rise in drug-resistant strains of *Staphylococcus aureus*. Consequently, a recurring focus has been on alternative antimicrobial treatments, such as bacteriophages. The present study focused on isolating and characterising a lytic phage that specifically targets *S. aureus* bacteria found in food. This study involved the isolation and titration of a phage using the double-layer agar technique. Additionally, the stability of the phage was assessed under various physical and chemical conditions, including temperature, UV light, alcohol, and isopropanol exposure. The analysis revealed the presence of a lytic phage, which contradicts the presence of an antibiotic-resistant *S. aureus*. The stock titer of the phage is around 3×1010 PFU (plaque-forming units) per milliliter. Based on the characterization properties, it was seen that the highest virus titer was achieved at a temperature of 37°C and a pH of 7.2. However, the activity of the bacteriophage decreased rapidly as the temperature increased progressively to 45°C, 50°C, and 55°C, and it entirely lost its activity at 60°C. The bacteriophage was rendered fully inactive at pH values of 3 and 4 and at pH values of 10 and 11. Additionally, it experienced a partial activity loss at pH values of 5, 6, 8, and 9. It experiences a 10% isopropanol impact but is more significantly impacted by 70% alcohol.

Keywords: *Staphylococcus aureus* phage ; isolation; characterization.

Introduction

Globally, antibiotic resistance is a rapidly intensifying concern that significantly burdens public health and the economy [1, 2]. According to the World Health Organization, if no effective alternatives to current antibiotics are discovered by 2050, antimicrobial resistance (AMR) could result in a global cost of USD 100 trillion and up to 10 million deaths annually. These numbers would exceed the number of deaths caused by cancer and heart diseases [3, 4]. Phage therapy is a relatively unexplored alternative to antibiotic treatment. It involves using bacteriophages to infect and eliminate harmful bacteria resistant to many drugs. Phage therapy is fundamentally distinct from antibiotics because of the inherent ability of phages to self-amplify and generate phage progeny that bind to and cause the lysis of target cells during infection. Bacteriophages exhibit target specificity and generate lower levels of endotoxin upon cell lysis. Furthermore, they possess an enhanced ability to penetrate biofilms. Therefore, the likelihood of phage application leading to unwanted side effects such as disturbance of the gut flora is reduced. In contrast, broad-spectrum antibiotics can lead to the demise of the natural flora [5, 6]. Globally, the Enterococcus faecium, *S. aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and Enterobacter species (ESKAPE) pathogens lead to healthcare-associated infections. *S.aureus* is one of them [7, 8]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is the causative agent of a wide range of disorders, including infections of the skin and soft tissues, as well as severe and potentially life-threatening conditions such as pneumonia, bacteremia, and sepsis. Foodborne sickness arises from the ingestion of tainted food or drinks. Various pathogenic microorganisms can contaminate food, resulting in the existence of numerous foodborne illnesses. The majority of foodborne illnesses are caused by bacterial, viral, and parasitic infections [9, 10]. Phages can be employed for the treatment of MRSA in a localized manner, specifically targeting local infections. Bacteriophages generate endolysins, also known as lysins, which have proven to be highly successful in managing antibiotic-resistant pathogenic bacteria [11, 12]. Multiple phages belonging to the Caudovirales order exhibit strict lytic activity against *S. aureus* and are incapable of transferring bacterial DNA. The majority of these viruses possess an intricate virion structure consisting of a head and an elongated contractile tail. They have been categorized under the Herelleviridae family [13]. Nevertheless, the abundance of phages that infect *S. aureus* is significant. Accurate identification and purification of phages, while ensuring the absence of bacterial cell poisons and other substances, are crucial for developing an effective therapy against antibiotic-resistant bacterial infections [14].

Material and Methods

1. *Staphylococcus aureus* isolation and characterization

S. aureus was isolated from Milk in General samples using nutrient agar (Oxoid, UK) and Mannitol salt agar (HiMedia, India). The round and golden colonies were recognized on Mannitol Salt Agar (MSA), as shifted the colour of media from light red to yellow (Figure 3). This demonstrated the type of bacteria's resistance to high salt concentrations and the ability to ferment mannitol in media, which was indicated by a shift in media pH to below 6.9. This appeared as a change in the phenol red indicator in the media to yellow [15]. Because of bacteria's ability to

create golden pigment, the golden colour of colonies possesses antioxidant characteristics and is recognized as a useful virulence factor for bacteria [16]. The *S. aureus* isolate's identity was verified by employing particular primers to amplify and sequence the *S. aureus* (*16S rRNA*) gene. The resulting sequences were compared to those in the NCBI database using blast analysis, which showed a 100% match to *S. aureus* species. In addition, the *S. aureus* isolate underwent testing to determine its susceptibility to various antibiotics. The findings indicated that the *S. aureus* isolates exhibited complete resistance (100%) to cefoxitin, penicillin, and ceftazidime. Furthermore, it was discovered that none of the antibiotics examined in this study could eradicate the *S. aureus* isolate. Consequently, the isolate tested in this study was categorized as Methicillin-resistant *S. aureus*.

2. Demonstration of *S. aureus* phage

2.1 Preparation of phage suspension

A suspension of *S.aureus* phage was generated from food samples and then filtered through a cellulose filter with a pore size of 0.8 µm to remove debris particles. The filtered suspension was then centrifuged at 6000rpm for 10 minutes. Finally, the supernatant portion was carefully passed through a filter with a hole size of 0.45 µm in a sterile manner to eliminate bacterial cells and cellular waste [17, 18].

2.2 Phage enrichment and isolation

The enrichment method [19] was used to isolate phages. Below are the main isolation steps:

1. Initially, food samples were centrifuged at 6000 × g for 10 min to remove particles and sterilized through a sterile 0.45 syringe filter.

2. Next, 10 mL of sterile 2x LB broth was transferred to a 50 mL tube, and 10 mL of filtered food samples were added to the medium.

3. Next, the mixture (Luria broth and food samples) was inoculated with 0.1 ml of overnight broth culture of the desired host bacteria (*Staph. aureus*) and incubated at 37°C with shaking at 100 rpm.

4. After overnight incubation, the bacterial culture was centrifuged at 6,000 × g at room temperature for 10 minutes. The supernatant was then collected and filtered using a sterile 0.22 µm syringe filter. Finally, the virus stock was stored in a sterile 50 ml tube at 4°C.

2.3 Testing the suspension of expected phage

The bacteriophage lysis experiment was performed using the spotting technique. A 20 µl sample of bacteria from an overnight culture was added to 2 ml of LB broth media and cultured for 1-2 hours. Next, a 250 µl portion of the culture was combined with 3 ml of LB agar and then put onto a plate. Subsequently, a solitary droplet of the anticipated phage stock solution dispersion was introduced onto the bacterial layer. Subsequently, the plates were allowed to equilibrate at

ambient temperature for approximately 10–15 minutes. Following this, the plates were inverted and subjected to overnight incubation at a temperature of 37°C. Subsequently, the plates were scrutinized for distinct areas of lysis [20, 21]. An equivalent amount of sterile SM buffer was applied onto the plate as a negative control.

2.4 Purification of phage

Mass multiplication removed the materials from the clear zone's centre using a sterile inoculation loop and transferred them to a sterile SM buffer. The SM buffer consisted of NaCl (5.8 g), $MgSO_4.7H_2O$ (2 g), 1 M Tris_Cl pH 7.5 (50 ml), 2% gelatin (5 ml), and the volume was made up to 1,000 ml with water (H_2O). The combination underwent centrifugation at 5000 revolutions per minute for 10 minutes at 25 °C. Subsequently, it was passed through a Millipore Membrane Filter with a pore size of 0.22 micrometres. The liquid that passed through the filter was gathered in bacteria-free containers. The spotting assay was conducted once more, as previously described. This cycle stage was iterated at least three times to guarantee the phage's purity. The phage lysate was kept at a temperature of 4ºC [22, 23].

2.5 Large scale amplification of phage

Phages were concentrated on a small scale by spreading them over the top-agar layer that contained the specific host bacterium via plaque plucking. In summary, the clear zone was excised using a sterile loop and then washed with SM buffer before being stored at a temperature of 4°C. Subsequently, these solutions produced concentrated phage solutions on a bigger scale using broth media with certain alterations, the materials from the centre of the plaques were removed by gently scraping them with a sterile inoculation loop. These materials were transferred to a sterile nutrient broth (100ml) containing the specified organism (Pseudomonas). The mixture was incubated for approximately 24 hours at a temperature of 37ºC. The mixture was centrifugation at a speed of 5000 revolutions per minute for 25 minutes at a temperature of 4 °C. Subsequently, it was passed through a Millipore Membrane Filter with a pore size of 0.22 micrometres. The filtrate passed through the filter was gathered in sterile containers and kept at a temperature of 4°C until needed for later purposes [24, 25].

2.6 Titration by plaque assay

As previously described, using the double-layer agar plate method, the phage titer was quantified by measuring the number of plaque-forming units (PFU) per millilitre. Phage stock was diluted ten-fold, ranging from 10-1 to 10-10. In summary, 20 µl of each dilution was combined with 250 µl of bacterial culture (in the logarithmic growth phase) and incubated for 10 minutes. The mixture was then gently combined with 3 ml of molten soft agar, pre-warmed to 45 ºC in a water bath. The mixture was promptly poured into a Petri dish containing 1.5% nutrient agar. The plates were allowed to equilibrate at ambient temperature for 5 to 10 minutes. Subsequently, the samples were inverted and subjected to overnight incubation at a temperature of 37°C. After incubating the plates for 24 hours, the number of PFU and the phage titer were determined by

counting them on each plate using the following formula: The number of plaque-forming units per millilitre (PFU/ml) can be calculated by multiplying the number of plaques by the dilution factor and the volume of diluted phage given to the plate (ml)[26, 27] .

2.7 Host range

The infectivity of *S. aureus* bacteriophage to multiple bacterial species and genera was assessed. The bacterial strains *Pseudomonas aeruginosa, Escherichia coli, Salmonella,* and *S. aureus* were acquired from food samples. The host range of *S. aureus* phage was determined using a spotting technique. In summary, a 1 ml sample of host strains in the logarithmic growth phase was dispersed on sterile nutrient agar plates. Then, a 0.2 ml portion of phage lysate (containing 3×105 plaque-forming units per millilitre) was injected at the centre of each plate. Then, the plates were subjected to incubation at a temperature of 37°C and evaluated after 24 hours. A host vulnerable area in the bacterial lawn was documented as a clear zone [20].

2.8 Stability to some physical condition

To investigate the purified phage's thermal stability, it was incubated at different temperatures (20, 37, 45, 50, 55, 60, and 70 °C) for 1 hour. A sample of phage was collected at 10-minute intervals throughout 1 hour. The phage concentration was determined using the double-layer plate method. Furthermore, the phage was subjected to ultraviolet light treatment with a power of 30 W and a wavelength of 35 cm. This treatment was carried out in a tube and a petri dish for 5, 15, 25, 35, 45, 55, and 65 minutes. Subsequently, the phage was promptly assessed using the double-layer agar plate method [28, 29]. The results were expressed as the proportions of phages present compared to the initial particles in each treatment .

2.9 Stability to some chemical agent

To assess the chemical stability and pH's impact on phage activity, 10 µl of phage suspension with a concentration of more than 109 PFU/mL was combined with 990 µl of SM buffer. A range of pH values, from 3 to 11, were employed, with pH 7.2 as the control. The specimens were subjected to incubation at a temperature of 37°C for 18 hours. Subsequently, the phage titers were ascertained using the double-layer agar method. The phage underwent treatment with chloroform at varying concentrations (10%, 30%, 60%, and 100% v/v) for 72 hours at a temperature of 4 °C. Additionally, the phage was treated with isopropanol at different concentrations (10%, 30%, 60%, and 100% v/v) for 72 hours. Furthermore, the phage was subjected to alcohol (10%, 30%, 70%, and 100%) for 72 hours. Subsequently, it was promptly quantified using the double-layer agar plate method [29, 30].

Results and discussion

1. Phage isolation

The *S. aureus* phage was enriched and propagated after creating a mixed bacteriophage suspension from food samples (paragraph 2.2.1). Only one specific kind of phage derived from the food samples exhibited significant lytic activity against *S. aureus* isolates. Paragraph 2.2.3 mentions the spot test as the main method (30) for detecting the *S. aureus* phage in food samples (specifically, enrichment samples). The study's findings revealed distinct areas in marked positions, indicating the presence and separation of *S. aureus* phage, as shown in Figure (1). Next, the clear zone is made sterile by suspending it in an SM buffer and passing it through a 0.22µm filter. The spotting approach was repeated three times to guarantee the presence of a clean zone for bacteriophage activity.

Figure 1: Clear Zone

The titration of AP1 was determined by performing a double-layer agar assay on a large-scale filtered suspension of the material following dilution. The analysis revealed that the stock titer was around 3×10^{-10} PFU/ml, as shown in Table (1). Based on this finding, it was determined that the dilution (10^{-8}) yielded the highest countable number of plaques per plate. Therefore, the (10^{-8}) dilution would be utilized for all further investigations.

Table 1: AP1 Titration

Plate no.	Dilution	Plaque no	Stock titer per ml (plaque no. × invert dilution)/ 0.1*	Dilution titer (Plaque no.×DF**)/0.1*
1	10^{-1}	Clear	-	-
2	10^{-2}	Sime Clear	-	-
3	10^{-3}	TMTC	-	-
4	10^{-4}	167	1.67×10^{7}	1.67×10^{4}

5	10^{-5}	90	9×10^{7}	9×10^{3}
6	10^{-6}	39	3.9×10^{8}	3.9×10^{3}
7	10^{-7}	28	2.8×10^{9}	2.8×10^{3}
8	10^{-8}	12	1.2×10^{10}	1.2×10^{3}
9	10^{-9}	3	3×10^{10}	3×10^{2}
10	10^{10}	0	-	-

Additional studies have demonstrated that the concentration of phage SPW S. *aureus* in a large-scale preparation exceeded 109 pfu/ml [31]. In contrast, Ahiwale and coworkers (2012)[32], reported a concentration of 6.6×109 PFU/ml for *Pseudophage*. The variations may arise from factors such as the sample, bacterial enumeration, working conditions, and characteristics of each phage**.**

3. Host Range

The cross-infectivity of phage AP1 was assessed to determine its spectrum of activity against other bacterial taxa. The spectrum of activity of phage AP1 was assessed by testing *P. aeruginosa*, *E. coli*, *Salmonella ssp*., and *S. aureus*. AP1 exhibited specific lytic activity against *S. aureus*, while it did not demonstrate lytic activity against other bacterial taxa mentioned above. It was demonstrated that Phage AP1 exhibits a high level of specificity towards the *S. aureus* bacteriophage. These findings are consistent with a prior study [32] but in contrast to the literature [33], which reported that *Salmonella ssp*. phages infected many genera, indicating a wide host range of phages.

5. Stability to some physical condition and chemical agents

Its physical and chemical stability were analyzed to assess the appropriateness of phage AP1 for prospective therapeutic applications in the future. The phage stock was subjected to incubation at various temperatures to evaluate the impact of temperature on the stability of the AP1 phage. The findings indicated that the phage titer remained constant when incubated at 20°C and 37°C. Phage titers exhibited a gradual decline at temperatures of 45°C, 50°C, and 55°C, ultimately resulting in the full loss of phage activity at 60°C (Figure). The bacteriophage's chemical makeup was denatured due to the elevated temperature. Prior research has demonstrated similar findings [23, 32].

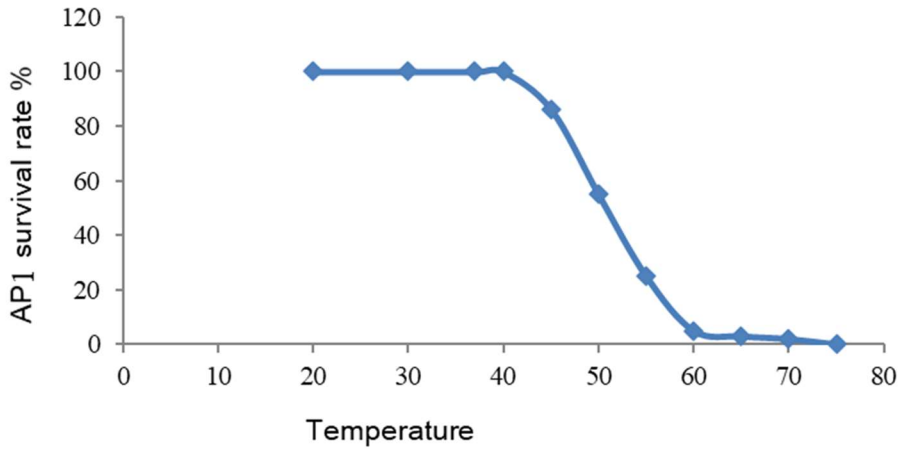

Figure 2: Stability of AP1 to Temperature

The phage AP1 inserted in a petri dish was immediately inactivated by direct UV radiation, while the phage AP1 in a tube barely lasted for 5 minutes. The results from [30, 31] indicated that the *S. aureus* phage SPW could endure UV light exposure for a maximum duration of 40 minutes. In addition, the presence of chloroform at various concentrations (10, 30, 60, and 100%) had minimal impact on the viability of phage AP1. This finding is consistent with previous studies. On the other hand, the viability of phage PAP was slightly affected by 30% and 60% isopropanol, moderately affected by 100% isopropanol, and significantly affected by 10% isopropanol. Figure 3 demonstrates that the viability of phage SPW was minimally impacted by a 5% concentration of isopropanol, in contrast to other conditions.

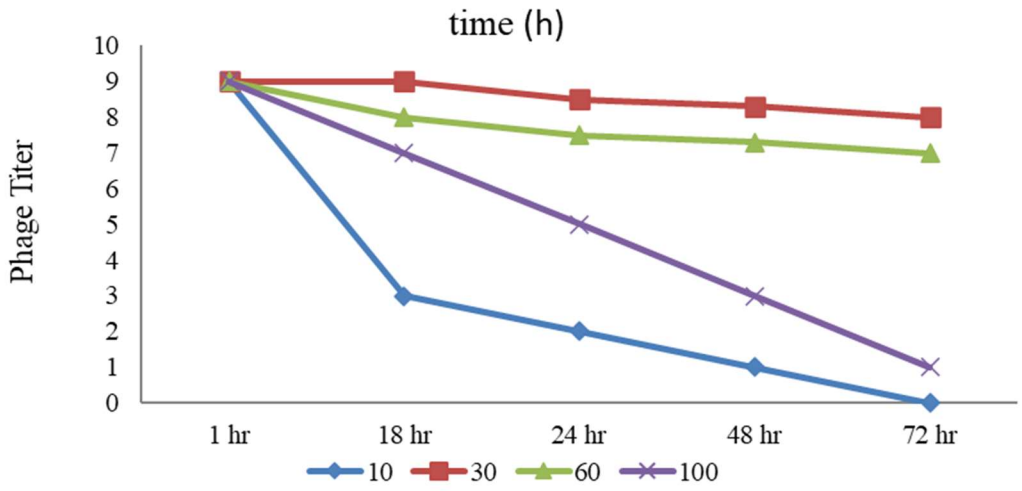

Figure 3: Stability of AP1 to Isopropanol

The viability of phage AP1 remained unchanged when exposed to a 10% alcohol solution for three days. However, the survivability of AP1 was moderately damaged when exposed to 30%

and 100% alcohol solutions. Interestingly, the viability of AP1 was significantly more affected when exposed to a 70% alcohol solution, as depicted in Figure (4). This results from the dilution of alcohol by 10% and the rapid volatilization of 100%.

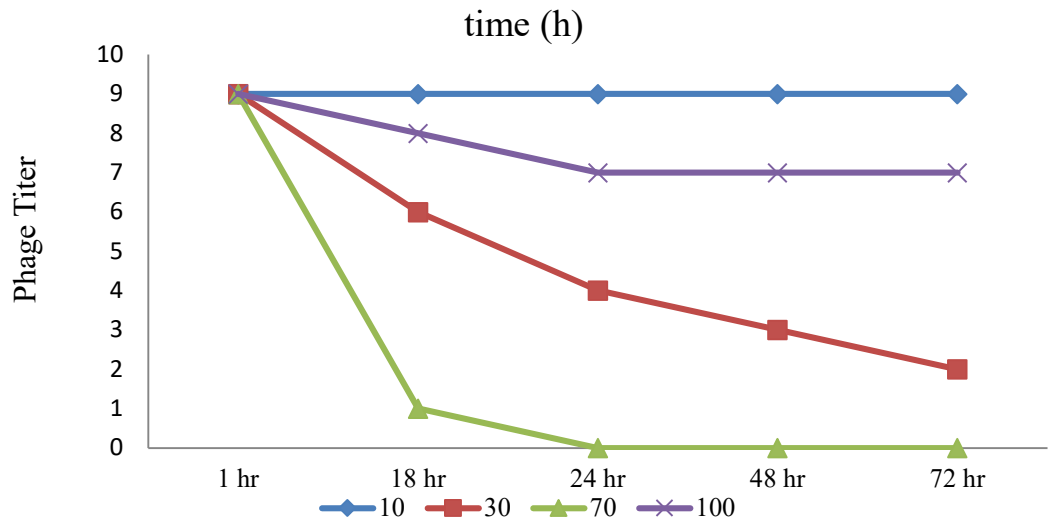

Figure 4: Stability of AP1 to Alcohol

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