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Article

Isolation and Characterization of *Staphylococcus* Phage Rih21 and Evaluation of its Antibacterial Activity against Methicillin-resistant *Staphylococcus aureus* Clinical Isolates

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Abstract: From the hospital waste-water, a novel bacteriophage was isolated and characterized. According to characterization properties, this bacteriophage belongs to the Siphoviridae family, the maximum bacteriophage titer was recorded at 37°C and a pH of 7.2, had a 44,789 bp linear doublestrand DNA genome, and within the genome sequence, there are 61 genes, all of which are encoded into proteins. Although this bacteriophage does not have any virulence factors or antimicrobial resistance genes and had specific lytic activity against some antimicrobial resistance S. aureus clinical isolates.

Keywords: phage Rih21; MRSA; novel bacteriophage; S. aureus; bacteriophage; phage genome analysis

1. Introduction

Staphylococcus aureus (*S. aureus*) is a major cause of a wide range of diseases from simple skin infections to lethal pneumonia and sepsis. It generates a remarkable diversity of virulent factors. These include toxins and immune evasion factors, as well as a multitude of protein and non-protein components that allow them to colonize and disseminate to various parts of the body during infection [1]. Bacteraemia caused by *S. aureus* has been found to kill more people than Human immunodeficiency virus (HIV), tuberculosis, and viral hepatitis combined [2]. *S. aureus* is one of the most important organisms in terms of resistance to antibiotics. Since the early 1940s, when penicillin resistant in *S. aureus* was first documented, it has gradually acquired resistance to nearly all currently used antibiotics [3]. The virulence factors as well as antimicrobial resistance in *S. aureus* lead to more effective colonization and dissemination in a population. As a result, it is not difficult to imagine a synergic effect of these traits on infection. As a consequence, many antimicrobial effects on the regulation of virulence factors of *S. aureus* have indeed been revealed in the past few years [4].

The bacteriophage is expected to be ten times greater than the number of bacterial cells in total [5]. Because phages are a natural element of the human microbiome, they are generally well-tolerated whenever employed in therapeutic applications [6, 7, 8]. Phages are the most interesting antibiotic alternatives that can be employed in medicine and other fields [9]. The emergence of pan-drug-resistant and multidrug-resistant bacteria pose a serious danger to global infectious disease control, as a result, new therapeutic strategies are urgently required. Phages, according to the National Institutes of Health in the United States, are promising methods for fighting microbial resistance [10]. Phage host specificity (range) vary, with certain phages infecting many species while others only thriving on a defined isolate. Their specificity, however, is far greater than that of antibiotics. The major factors that define specificity are receptors on the cell surface of phage hosts and antiviral defence mechanisms (genetic and physical) [11]. To cure severe infections, cocktails of phages containing phages that cover the entire spectrum of possible strains are

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recommended. An alternate method is to employ lytic enzymes from phages (endolysins), which have broad host specificity at species and genus levels [12].

In this study, we performed genome sequencing and biological characterization of *S. aureus* phage Rih21 isolated from the hospital waste-water.

2. Methods

2.1. Isolation of S. aureus

In this study, 129 clinical specimens were collected from blood, nasal, ear, wound, oral, pus, and urine infections using sterile swabs. The specimens were taken from three different hospitals (Baghdad Teaching Hospital, Al Shaheed Ghazi Hospital, and AL-Kadhimiya Hospital) in Baghdad during the period from October, 2020, to January, 2021. The specimens were transferred directly to the laboratory by transport media (CNWTC/China). And all samples were subjected on Mannitol salt agar (MSA) and blood agar media at 37°C for 24 h. after that examining to different biochemical tests (catalase, oxidase and coagulase test). The identity of *S. aureus* isolates was further confirmed by using specific primers (nuc-F: 5′-GCGATTGATGGTGATACGGTT-3′; nuc-R: 5′AGCCAAGCCTTGACGAACTAAAGC-3′) for amplification and sequencing of the *S. aureus* thermonuclease (nuc) gene [13]. Also, the disc diffusion method was used to examining the sensitivity of *S. aureus* isolates to cefoxitin (30 μg).

2.2. Phage Isolation

The sewage samples were collected in 50 ml falcon tubes from three different places (Medicine City hospitals, AL-Rusafa municipality T1 station, and AL- Kadhimiya Hospital), transferred to the laboratory and stored at 4°C. The enrichment method [14] was used for the isolation of phage from wastewater. As follow: At the beginning, the wastewater samples were centrifuged at 6000 × g for 10 min to remove particulates and sterilized through a 0.45 sterile syringe filter. Then, 10 ml of sterile 2xLB Broth was transferred into a 50-ml Falcon tube, and 10 ml of filtered wastewater was added to the media. After that, the mixture (Luria broth and wastewater) was inoculated with 0.1 ml of an overnight broth culture of the desired host bacterium (*S. aureus*) and incubated at 37°C with shaking at 50 rpm. After overnight incubation, the bacterial culture was centrifuged at 6000×g at room temperature for 10 min. Then the supernatant was collected and filtrated using a 0.22μm sterile syringe filter. Finally, the virus stock was stored in a 50-ml sterile Falcon tube at 4°C.

2.3. Phage Purification (Plaque Assay for Purification)

Aliquot of 20 μl of overnight bacteria culture were inoculated into 2 ml of LB broth media and incubated at 37°C for 1-2 h. Ten-fold serial dilutions of phage stock were prepared. After the preparation of phage serial dilutions, 250 μl of bacterial culture were incubated with 20 μl of each phage dilution for 10 min. Then, they were gently mixed with 3 ml of LB top agar at 45°C and poured onto the LB bottom agar plate. Finally, the plates were left at room temperature to solidify. After that, they were inverted and incubated overnight at 37°C. Single plaques were picked using sterile toothpicks by touching the plaque surface, and the toothpick was placed in 0.3 ml of SM buffer, mixed well, and vortexed for 30 seconds. This process was repeated for six rounds to obtain a pure phage.

2.4. Liquid Media/PEG Phage Propagation

The phage was produced in Liquid medium by incubating 20 ml of LB broth with 2 ml of overnight bacterial culture and incubated overnight at 37°C for 1 h. Then, 100 μl of high titer phage (> 108 PFU/1 ml) were added to the bacterial culture and incubated at 37°C while shaking at 50 rpm. After that, the infected bacterial culture was centrifuged at 6000×g at room temperature for 10 min. The supernatant was transferred to a new sterile tube and filtrated using a 0.22 μm Sterile Syringe filter [15]. The phage was precipitated by mixing 20 ml of PEG- 6000 (20%) solution with 20 ml of each phage supernatant and

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leaving it overnight at 4ºC to allow the phage to precipitate. The next day, the mixture was centrifuged at 4500×g for 30 min at 4°C and the supernatants were gently poured off without disturbing the pellet. The pellets were dissolved in 5 ml of SM buffer and stored at 4°C until use [16]. The titer of the phage was estimated by Ten-fold dilutions of phage stock and double layer agar method, the titer of the phage was calculated according to the following formula: plaque forming units per milliliter (PFU/ml) = number of plaques/dilution factor x volume of diluted phage added to the plate (ml) [17].

2.5. Biological Characterization of Bacteriophage

2.5.1. Temperature Effect

In order to study the thermal stability of the purified phage, it was incubated for 1 h at various temperatures (20, 37, 45, 50, 60, and 70 °C). And the phage titer was confirmed using the double-layer plate method.

2.5.2. pH Effect

To investigate the effect of the pH on the phage activity, each 10 µl of phage suspension (>10^9 PFU/mL) was added to 990 µl of SM buffer. At different pH values, ranging from 3 to 11, with pH 7.2 being used as a control. They were incubated at 37°C for 18 h. After that, the phage titers were determined based on double-layer agar method.

2.5.3. Phage Host Range

The host range of the phage on *S. aureus* isolates and other gram-positive and negative bacterial isolates was tested using the plaque assay.

2.5.4. Transmission Electron Microscopy (TEM)

Purified phage (>10^9 PFU/mL) was pipetted onto the surface of three hundred-mesh Cu grids with formvar coating and washed twice with sterilized dH2O. The grids were then stained with 1% uranyl acetate for approximately 2 min. The excess stain was removed using a wedge of filter paper and left to air dry. The grid was imaged at 80 kV in a Tecnai Bio-Twin Transmission Electron Microscope (Thermo Fisher Scientific, USA).

2.6. Molecular Characterization of Bacteriophage

2.6.1. Phage Genomic DNA Extraction

Phage genomic DNA was extracted according to the phenol, chlorophorm, and isoamyl alcohol/SDS (PCI/SDS) method. The main stages of extraction were: (1) In a 1.5 ml microfuge tube, 1 ml of phage stock was added and mixed gently with 12.5 µl $MgCl_2$ (1M); (2) Then, 2 µl DNase I (2000 U/mL) and 2.5 µl RNase A (50 mg/mL) were added and mixed by vortex and incubated at room temperature for 30 minutes; (3) After that, 40 µl of 0.5 M EDTA, 5 µl of Proteinase K (10 mg/ml), and 50 µl of 10% SDS were added and mixed vigorously by vortex and incubated at 55°C for 60 min; (4) Each 500 µl of the mixture in (step 3) was pipetted in new 1.5 ml microfuge tubes, an equal volume of phenol: chloroform: isoamyl alcohol (in a 25:24:1 ratio) was added, and the tubes were inverted several times; (5) The mixture was centrifuged at 13,000 rpm for 5 min. The top aqueous layer was transferred to the new tube. Steps 4 and 5 were repeated several times to get rid of any contaminated proteins; (6) After that, the top aqueous layer was transferred into a new tube and 1 ml of ethanol (95%) and 50 µl sodium acetate (3 M) were added. The tubes were placed on ice for 5 minutes; (7) Then, it was centrifuged at 13,000 rpm for 10 min at room temperature. The supernatant was carefully decanted and the pellet was washed with 70% ethanol and centrifuged at 13,000 rpm for 10 min at room temperature; (8) The supernatant was decanted and the tubes were left at room temperature for 10–20 min. Finally, the DNA pellets were dissolved in 50 µl of dH2O and stored at -20°C; (9) The concentration and purity of the extracted DNA were determined using the NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, USA).

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2.6.2. Whole Genome Phage DNA Sequencing and analysis

The phage DNA genome was sequenced using Illumina Next Generation Sequencing (NGS) by Macrogen Company. The TruSeq Nano DNA Kit was used to construct the sequencing library by randomly fragmenting the DNA sample, followed by 5' and 3' adaptor ligation. The library was fed into a flow cell for cluster production, where fragments were collected on a lawn of surface-bound oligos complementary to the library adapters. Through bridge amplification, each fragment was then amplified into independent clonal clusters. The templates were ready for sequencing after the cluster creation was completed. Illumina Sequencing by Synthesis (SBS) technology recognizes single bases since they were integrated reversible terminator-based into DNA template strands in each cycle. Sequence data was transformed to raw data. SPAdes software was used to perform de novo assembly [18]. After the complete genome was assembled, Prokka [19] and RASTtk [20] were used to determine the location of the genes, and the NCBI- BLASTn database was used to learn more about the predicted genes in the assembled sequence.

To phylogenetic tree analysis, the nucleotide sequence of the genome was uploaded into ViPTree [21] using default settings; dsDNA nucleic acid type and Prokaryote host.

3. Results

3.1. Isolation of Staphylococcus aureus

Only 45 isolates were given the culture characteristics and biochemical tests that are specific to *Staphylococcus aureus*. The round and golden colonies was recognized on Mannitol Salt Agar (MSA) as well as shift the color of media from light red to yellow [22]. On blood agar, it formed yellow, round, moderate-sized colonies surrounded by a clear zone, indicating the ability of bacteria to secrete β-hemolysin and complete lysis of the blood cells in the media. The appearance of bubbles after the addition of hydrogen peroxide reagent to bacterial samples indicated that the catalase reaction was successful. The coagulase positive reaction was given, which was used to differentiate *S. aureus* species from other *Staphylococcus* species. a negative result of the oxidase reaction was determined [23]. Also, a confirmation of *S. aureus* isolates was done by molecular detection of nuc gene encoded for the thermo-stable nuclease enzyme by using specific primers. The PCR product of the nuc gene appears in the form of a single band of DNA, with a molecular size of 279 bp [13] compared with the DNA ladder (Figure 1).

All *S. aureus* isolates (45 isolates) was resistance to cefoxitin, the cefoxitin resistance test is used for the identification of methicillin (oxacillin) resistant *S. aureus* (MRSA) as a phenotypic detection for PBP2a protein or mecA gene [24, 25]. Consequently, all isolates that were tested in this study are categorized as MRSA.

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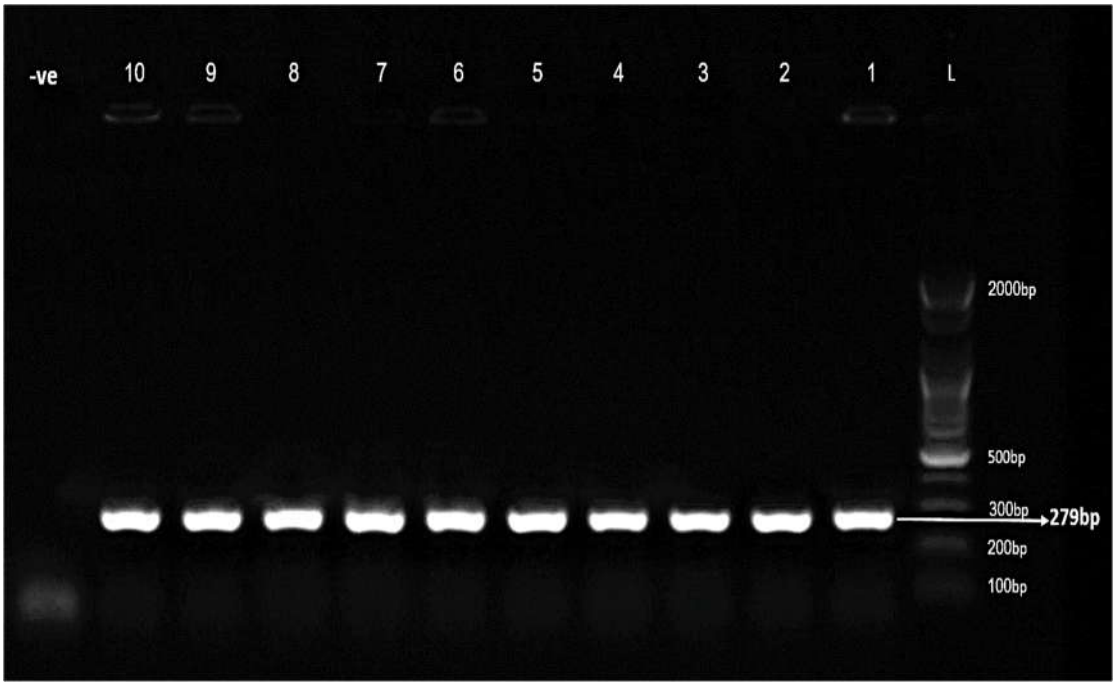

Figure 1. Amplification of nuc gene of *S. aureus* by PCR. Amplificated product of 279 bp was electrophoresis on a 1% agarose gel at 70 volts for 90 min. Line (L) for DNA ladder (100 bp), Line (-ve) for Negative control.

3.2. Isolation of Staphylococcus aureus Phage

Only one type of phage showed high lytic activity (The biggest and clearest plaques formed) against *S. aureus* isolates, (Figure 2), which was obtained from the wastewater of Al-Kadhimiya public Hospital.

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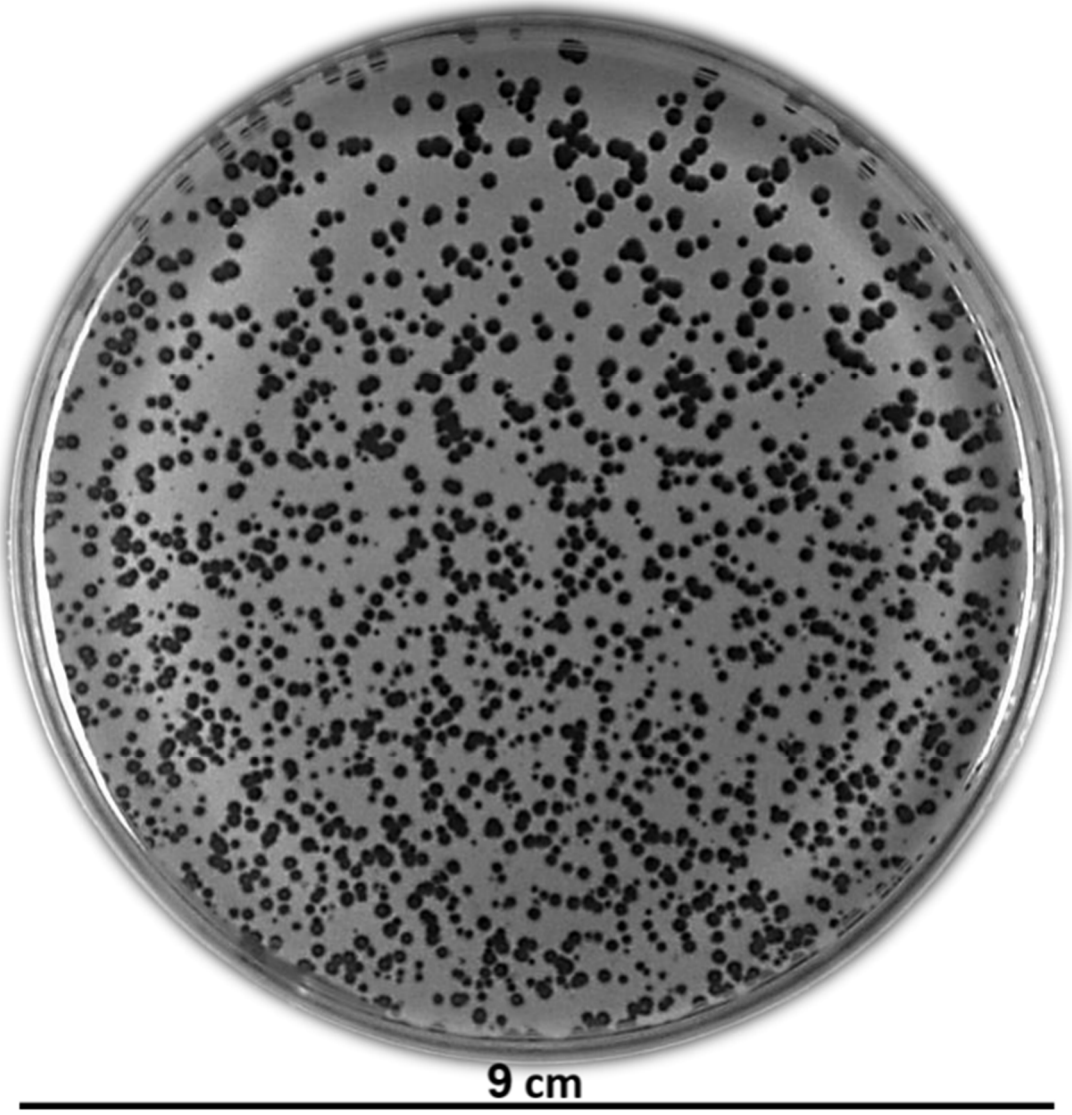

Figure 2. Plaque assay of high lytic activity (The biggest and clearest plaques formed) of isolated of phage against *S. aureus* clinical isolate.

3.3. Staphylococcus aureus Phage Propagation

The phage titer after propagation by the Liquid media method and concentrated by PEG-6000 was estimated at 2.35×10^{11} PFU/ml. The concentration of the phage by using PEG-6000 precipitation is an easy method to obtain a large number of active phage particles compared with the standard bacteriophage method such as cesium chloride (CsCl) density gradients [15].

3.4. Characterization of Staphylococcus aureus Phage

3.4.1. Sensitivity of Phage to Temperature Treatment

The phage stock was incubated at different temperatures to assess the effect of temperature on the stability of the phage. The results revealed the phage titer did not change when it was incubated at 20°C and 37°C. While the phage titers started to decrease at 45°C, and 50°C, until the phage completely lost its activity at 60°C (Figure 3).

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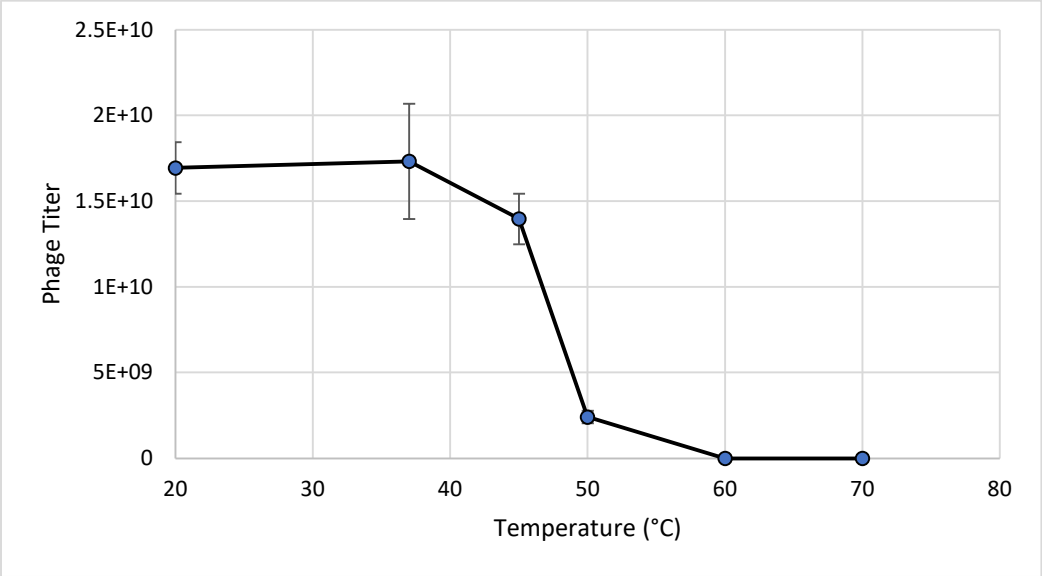

Figure 3. The effect of various temperatures on phage titer. The optimum temperature was 37°C and the phage activity decreased at 45°C, until lost completely activity at 60°C.

3.4.2. Sensitivity of phage to different pH

Phage sensitivity to pH was determined by incubating the phage with different pH for 18 h. The results showed that the phage was completely inactivated at highly acidic (3, 4) and basic (10, 11) pH values. On the other hand, the phage lost part of its activity at pH 5, 6, 8, and 9. At the same time, the maximum phage titer was recorded at pH 7.2 (Figure 4).

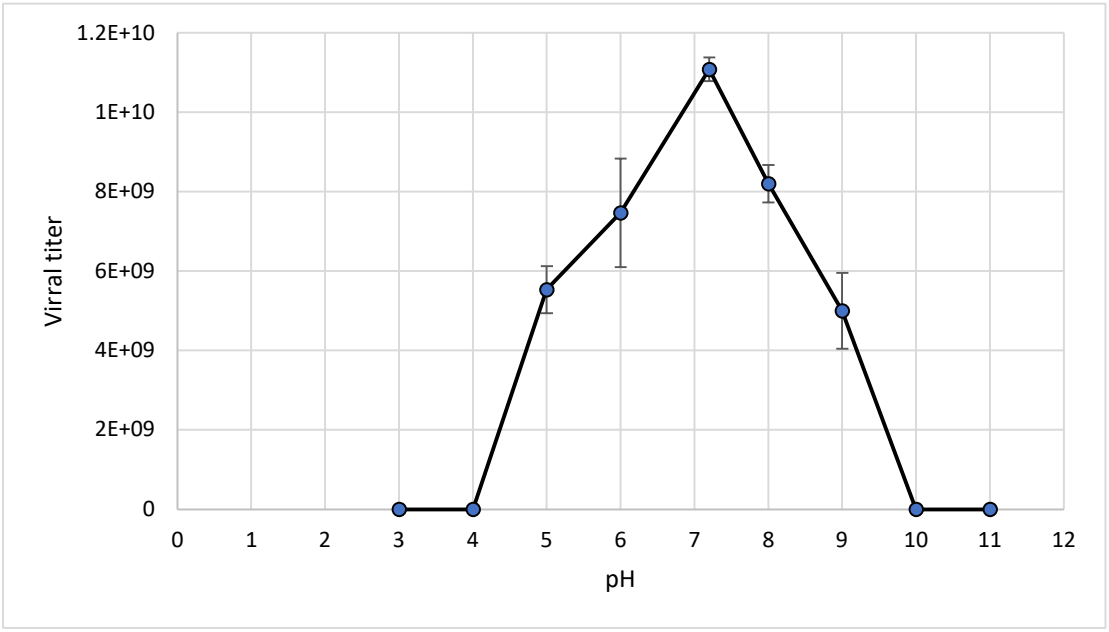

Figure 4. Effect of pH treatment on phage titer incubated for 18 h. the The optimum phage titer was at 7.2 pH and lost complete activity under high acidic (3, 4 pH) and at high basic (10, 11 pH) condition.

3.4.3. Host range of phage

The host range of phage on the *S. aureus* isolates and other gram-positive and negative bacteria was determined using the double-layer agar method (Table 1). The results revealed that 50% of the *S. aureus* isolates were sensitive to the phage and had no effect on either gram-positive or negative bacteria tested in this study.

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Table 1. Host range of *S. aureus* phage testing against a panel of bacteria.

***S. aureus* isolate**	**Clinical Source**	**Phage lytic activity**
S. aureus **isolate-29**	Oral	+
S. aureus **isolate-23**	Ear	+
S. aureus **isolate-27**	Ear	-
S. aureus **isolate-6**	Blood	-
S. aureus **isolate-22**	Oral	+
S. aureus **isolate-12**	Nasal	-
S. aureus **isolate-2**	Blood	+
S. aureus **isolate-38**	Wound	-
S. aureus **isolate-7**	Blood	+
S. aureus **isolate-10**	Blood	-
Gram positive bacteria		**Phage lytic activity**
Staphylococcus sciuri		-
Bacillus licheniformis		-
Micrococcus yunnanensis		-
Gram negative bacteria		**Phage lytic activity**
Proteus mirabilis		-
pseudomonas aeruginosa		-
Klebsiella pneumoniae		-

No.: number; +: lytic effect; - = no lytic effect

3.4.4. Phage Morphology

By using Transmission Electron Microscopy (TEM), a unique nonenveloped phage was detected that has a rectangular head (length of 100 nm, width 50 nm) and a long, flexible, noncontractile tail (300 nm) (Figure 5). These morphological features were fitted to order: Caudovirales, family: Siphoviridae, genus: Triavirus.

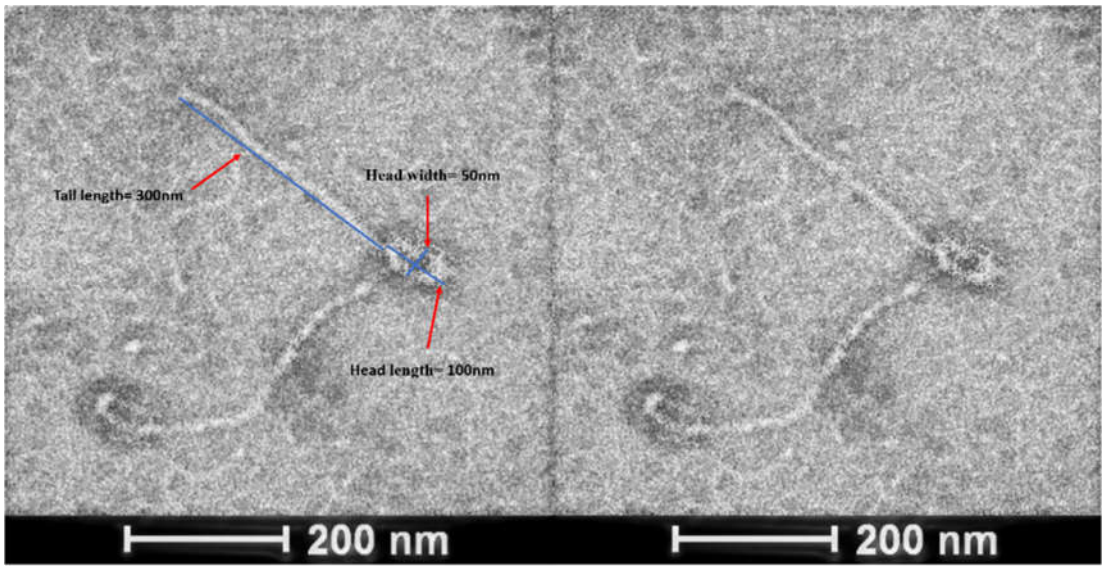

Figure 5. Transmission electron micrographs of *S. aureus* phage which belong to genus of Triavirus. Has a rectangular head (length of 100 nm, width 50 nm) and a long, flexible, noncontractile tail (300 nm).

3.5. Phage Genome

Following the assembly and analysis of the whole phage genome the results revealed; double-stranded DNA genome with a length of 44,789 bp and a G + C content of 33.26%,

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and had 61 predicted genes in the phage genome are deemed coding DNA sequences (CDS), as shown in figure 6. This phage does not encode rRNA or tRNA and doesn't have any virulence factors [26, 27] or antibiotic resistance genes [28]. The results showed the unique phage genome was not similar to any other phage genome published in the NCBI database.

This type of phage does not appear to play a role in the transmission of antibiotic resistance or virulence factors between *S. aureus*. Furthermore, they have unique genes that encode lytic proteins such as the holen and lysin protein, which can be used for specific antibacterial effects [17, 29, 30].

The phylogenetic tree was constructed from *S. aureus* phage genomes that were more closely related to *Staphylococcus* phage Rih21 genome in the NCBI database, by using the ViPTree in GenomeNet [21]; is more closely to *Staphylococcus* phage P240 and *Staphylococcus* phage phiSauS-IPLA35, as shown in figure 7 and 8.

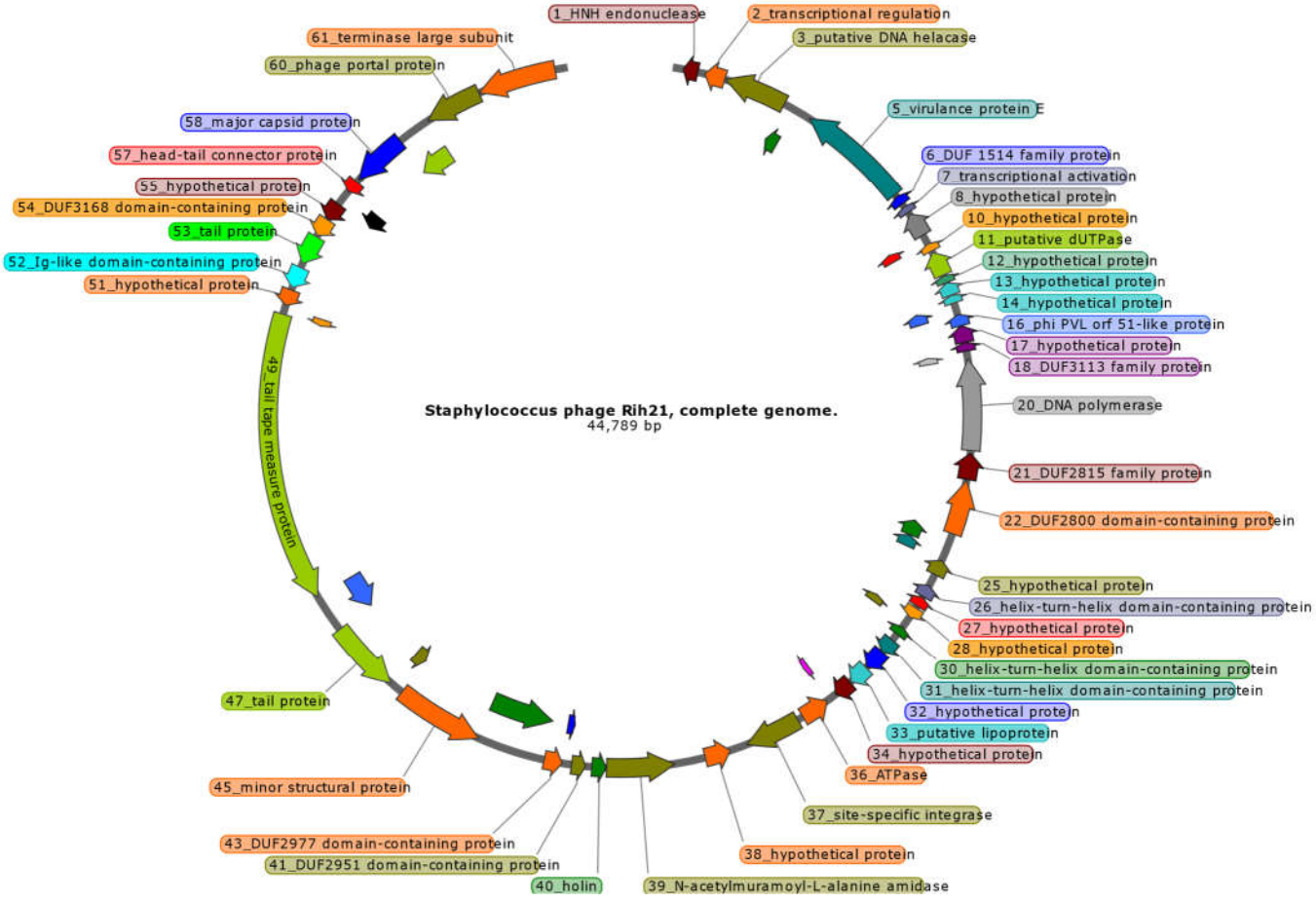

Figure 6. Phage Genome Structure, contained 61 predicted genes are deemed coding DNA sequences (CDS), viewed by SnapGene software (available at snapgene.com).

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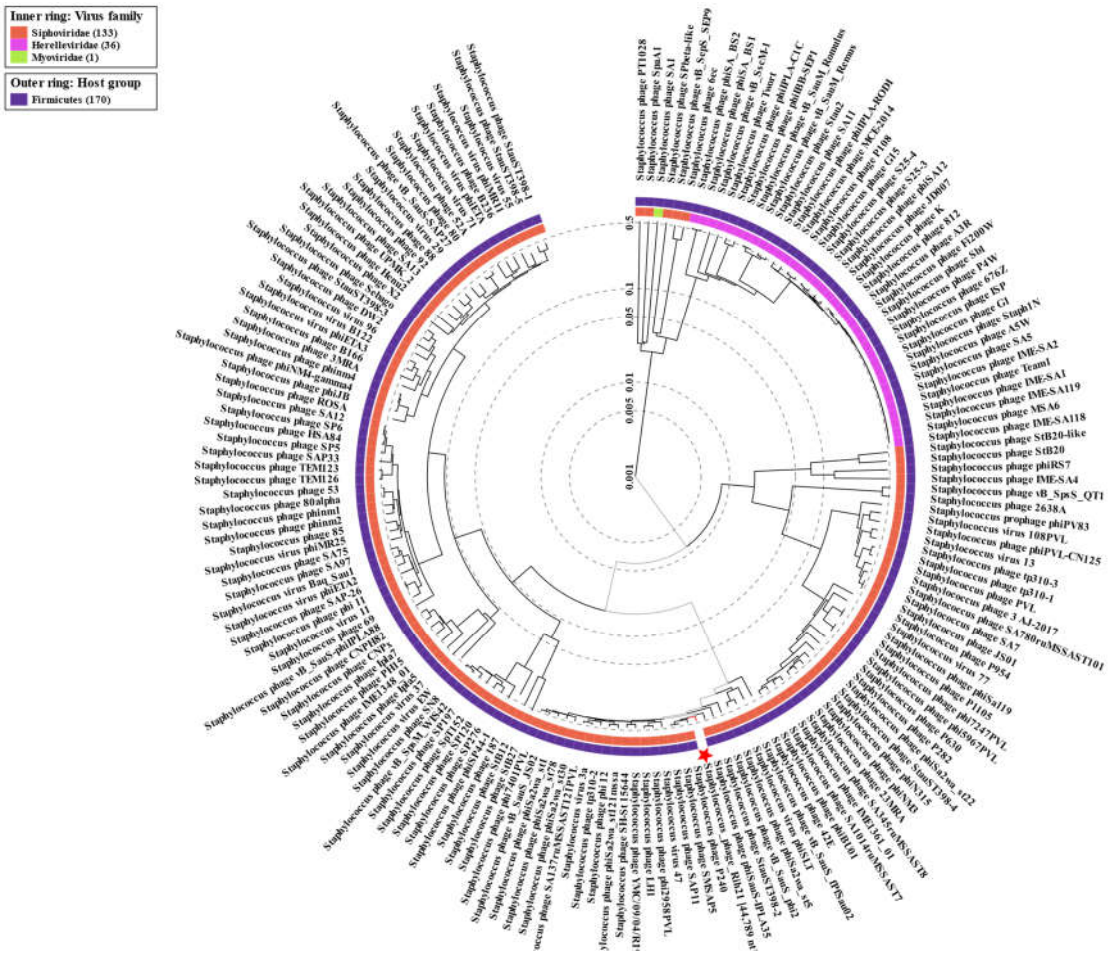

Figure 7. Phage phylogenetic tree. The tree includes 170 related phage genomes, with the phage Rih21 in this study highlighted with red star; showed more closely to *Staphylococcus* phage P240 that classified as Siphoviridae and genus Trivirus.

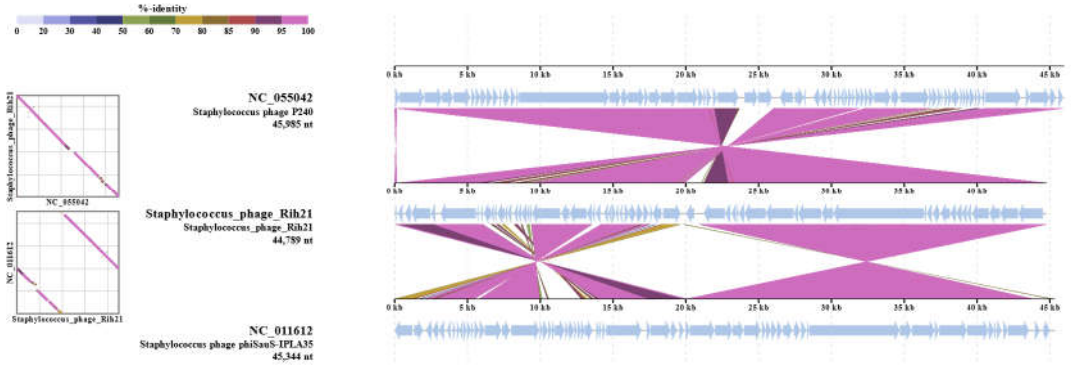

Figure 8. alignment of *Staphylococcus* phage Rih21wihte more related phage genomes (*Staphylococcus* phage P240 and *Staphylococcus* phage phiSauS-IPLA35) according to the results of the ViPTree analysis [21].

3.5.1. Nucleotide sequence accession number

Under the accession number OL840290, the phage genome sequence is found in the NCBI-GenBank database.

4. Discussion

Phage Rih21 has high lytic activity against methicillin-resistant *Staphylococcus aureus* clinical isolates and contains an integrase gene, showed Triavirus morphology, and as

very host species specific phage (targeted some *S. aureus* clinical isolates tested in this study), it's not involved in the transmission of virulence factors or antibiotic resistance genes between bacteria. Phage Rih21 could be a promising and potent biocontrol candidate.

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