

Characterization and lytic activity of methicillin-resistant *Staphylococcus aureus*(MRSA) phages isolated from NICU

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RESEARCH

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ABSTRACT

Background

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a well-known pathogen that causes serious diseases in humans. As part of the efforts to control this pathogen, an isolated bacteriophage, *Siphoviridae*, which specifically targets Methicillin-resistant *Staphylococcus aureus* (MRSA), was characterized.

Aims

The objective of this study was to characterize of a virulent bacteriophage (*Siphoviridae*) isolated from a NICU bathroom sink.

Methods

The MRSA strain was isolated from patient blood. The isolated strain was confirmed as MRSA using conventional methods. Phages were isolated from a NICU bathroom sink and activity was lytic as determined by spot test. Titer phage lysate was measured by the Double Layer Agar (DLA)

technique. The morphology was found with electron microscopy. The single-step growth curve was plotted.

Results

Electron microscopy showed the phage as a member of the family *Siphoviridae*, serogroup A and F. The isolated phage was capable of lytic activity against methicillin-resistant *Staphylococcus aureus* (MRSA) strain as shown by spot test. By DLA, the titre of the phages was determined to be 10×10^8 PFU/ml. The single-step growth curve showed that the latent period of the isolated bacteriophage was 30 min and the total number of viable progeny per infected host, burst size, was 2600 PFU/infected host.

Conclusion

In this study, two phages were isolated and characterized from a NICU bathroom sink, from the *Siphoviridae* family, which specifically targets methicillin-resistant *Staphylococcus aureus* (MRSA).

Key Words

MRSA, bacteriophage, lytic activity, NICU

What this study adds:

1. What is known about this subject?

The presence of numerous outbreaks of antibiotic resistance MRSA have been reported in the NICU. Prevalence of MRSA colonization and infection in the NICU are irregularly distributed worldwide.

2. What new information is offered in this study?

In previous studies investigators have not isolated phages against MRSA from NICU. Presence of phages in this section can be transferred of antibiotic resistance factors.

3. What are the implications for research, policy, or practice?

Isolated phages from improper structured NICU bathroom

sink caused sludge production and will be participation of phages. This should be appropriate structured NICU bathroom sink.

Background

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first isolated in hospitals in the United Kingdom in 1961. Twenty years after the first MRSA case was described, the first MRSA infection in a neonate receiving treatment in a neonatal intensive care unit (NICU) in the United States was reported in 1981.¹

Prevalence of MRSA colonization and infection in the NICU is irregularly distributed worldwide because of many concurrent factors, such as the different prevention and local MRSA epidemiology. Despite these variations in prevalence measurements, previous studies have reported anywhere between 0.6 per cent – 8.4 per cent of NICU patients being colonized or infected with MRSA during their study periods.²⁻⁶ Infection rates as high as 75 per cent have been reported during outbreaks.⁷

Resistance to vancomycin has also been reported in MRSA although the *vanA* and *vanB* gene clusters noted in *Enterococci* have, thus far, rarely been noted in *Staphylococci*.⁸ The presence of numerous outbreaks of antibiotic resistance MRSA have been reported in the NICU.^{9,10}

For the treatment of infections caused by these MDR bacterial strains a novel alternative to antimicrobial agents needs to be developed. Bacteriophages are the viruses that infect and kill bacteria and they or their lysins may be used as an alternative antimicrobial therapy.^{11,12} *S. aureus* phages belong to the order *Caudovirales* which are composed of an icosahedral capsid filled with double-stranded DNA and a thin filamentous tail. Based on the tail morphology, they can be further classified into three major families: *Podoviridae*, *Siphoviridae* and *Myoviridae*.^{13,14} Since the phages identify their specific host, so any region has specific phages.¹⁵⁻¹⁷ Therefore in every region to be studied, there is comprehensive information about the bank phage and their structure.¹⁸⁻²¹ Up to now, methicillin-resistant *Staphylococcus aureus* (MRSA) phages have not been characterized from a NICU in Iran. The objective of this study was to characterize the structure and morphology of the methicillin-resistant *Staphylococcus aureus* (MRSA) phages from the NICU.

Method

1. Sampling and phage isolation method:

A liquid sample of 200cc from a NICU bathroom sink was collected. The sample was transported to the laboratory and stored at 4°C. Then, 10ml of sample was centrifuged (11,100xg, 5 min). The obtained supernatant mixed with chloroform, centrifuged (11,100xg, 5 min), then filtered through a 0.22µm filter. An equal volume of 2xLB broth (QUELAB, United States) was added to sample with an overnight culture of a MRSA strain^{22,23} that was previously isolated from the blood of a six-month-old newborn with septicaemia at the NICU at tertiary Bouali Sina Hospital in Sari, Mazandaran province in northern Iran. The isolated strain was confirmed as *S. aureus* with conventional methods for the identification of the organism.

2. Bacterial Cells Preparation:

The isolate was streaked for purity growth on Blood Agar plates and incubated overnight at 37°C. Microscopic examination, Gram stain smears from a Staph Culture, tube coagulase, catalase, DNase, and mannitol fermentation tests, were performed on the specimen. For detection of MRSA the Kirby–Bauer technique of disk diffusion method was done using the antibiotic discs methicillin, and oxacillin. Determination of MIC with E test was done by using E-test packages vancomycin, and linezolid (HI Media, India).^{24,25}

Then the isolate was incubated at 37°C with shaking overnight. The culture was centrifuged at 4°C (10,000xg; 10 min); the supernatant was filtered through Millipore filters 0.22µm. Spot test was used to detect the presence of phage. The filtered phage lysate was titrated by DLA Technique.^{22,26}

3. Determine host range:

3. 1. Spot test:

Mixed in a sterile tube, 100µl of an overnight culture of the potential indicator MRSA with 3ml of soft agar 0.4 per cent and pour the contents on to Petri dishes containing 15 ml of bottom agar 1 per cent. Left the plate on the bench until the soft agar has solidified then poured 5µl of the sterile supernatant over the solidified soft agar. After it was absorbed the plate was incubated upside up overnight at the 37°C. The following day, the plate was checked for zones of clearing.²⁶

4. Determine titer of bacteriophage:

4. 1. Double-Layer Plaque Assay (DLA assay):

Set up a row of 11 sterile capped tubes and aseptically added 900µl Luria Bertani (LB) broth to each tube, Phage diluted in the LB broth medium. Added 100µl of phage lysate to the first tube mixed and transferred 100µl to the

second tube in the series (“1” to “9”). Mixed 100µl of an overnight culture of the indicator MRSA with 100µl of the decimal dilution of the phage lysate suspension (“1” to “10”) and CaCl₂ is added to a final concentration of 10mM. The tubes were preincubated for 8 min at 30°C or 37°C to allow adsorption of the phages. Cells were added to 3 ml of molten soft agar (0.4 per cent) at 45°C and the poured on the Petri dishes containing 25ml of bottom agar (1 per cent). The dishes were left on the bench until the soft agar had solidified and incubated upside down, overnight at 37°C. The following day, the number of plaques was counted. Plaque-forming units per millilitre calculated which was equal to the number of plaques x10x inverse of the dilution factor.²⁶

5. Electron microscope observation of phage:

Phages were concentrated by centrifugation at 25,000×g for 60 min using a high-speed centrifuge, followed by two washes in 0.1M neutral ammonium acetate. Purified phages were deposited on carbon-coated copper grids and stained with 2 per cent uranyl acetate (pH=4-4.5). After staining, phages were observed on a Philips CM 300 electron microscope at 150Kv.²⁶

6. The Single-Step Growth Curve:

The phage lysate (100µl) was added to 100µl LB broth containing MRSA, then preincubated 10 min at 37°C to allow adsorption of the phages. After 10 min, a row of 16 sterile capped tubes divided into four groups of four and aseptically added 900µl LB broth to each tube. Phage was diluted in the LB broth medium by adding 100µl to the first tube, mixed and 100µl transferred to the second tube in the series (“1” to “4”), mixed 100µl of an overnight culture of the indicator MRSA with 100µl of the decimal dilution of the phage lysate suspension (“1” to “4”). The tubes preincubate for 30 min to 5 h at 37°C to allow adsorption of the phages. At intervals, a sample can be removed from the mixture and the number of free phage counted using a plaque assay. Petri dishes were incubated upside down overnight at 37°C. The following day, the number of plaques was counted and plaque-forming units per millilitre was calculated, which is equal to the number of plaques x10x inverse of the dilution factor.²⁶

Results

The following results were shown to confirm isolation of MRSA from the blood of a six-month-old new-born with septicemia at the NICU (Table 1) (Figures 1 and 2).

To demonstrate phage isolation and its effect on MRSA, a spot test was done and clear plaques observed wherever

phage lysate was spotted onto LB agar plates covered with a bacterial lawn of MRSA. This indicated that there was phage in the lysate (Figure 3). Using the DLA technique, the phage formed plaques with a 9-14µm diameter; the results indicated that isolated bacteriophages in this study had a titre of 10×10⁸ PFU/ml (Figures 4 and 5).

Electron microscopy showed that two phages groups were isolated and classified: Group (I) had an icosahedral head and a long non-contractile tail (400nm) (Figure 6). Group (II) had a distinct prolate head and a long non-contractile tail (300nm) (Figure7). Phages Group (I), belonged to the family *Siphoviridae* (order *Caudovirales*) serogroup F. Phages Group (II), was belonged to the family *Siphoviridae* (order *Caudovirales*), serogroup A.

The proliferation rate of bacteriophages is usually determined by the latent period and the burst size. Both parameters can be calculated from the one-step growth curves. The single step growth curve was shown that latent period of isolated bacteriophage was 30 min and the total number of viable progeny per infected host, burst size, was 2600 PFU/infected host (Figure 8).

Discussion

In the current study we isolated bacteriophages against MRSA from a liquid sample from the NICU bathroom sink at tertiary Bouali Sina Hospital in Sari, Mazandaran province in northern Iran.

In previous studies investigators have not isolated phages against MRSA from the NICU and due to an improper structured bathroom sink that caused sludge production and the result of sludge formation will be participation of phages. Presence of phages in this section can be one of the reasons to be justified the transfer of antibiotic resistance or virulence factors via the transduction.^{18,27} Pathogenic *S. aureus* strains are dependent on the presence of virulence factors encoded mainly by mobile genetic elements. Microarray studies have shown that prophages integrated in the bacterial chromosomes are the most widespread mobile genetic elements in *S. aureus* strains.²⁸ Bacteriophages can, through horizontal gene transfer and lysogenic phage conversion, convert a non-virulent strain of staphylococcus to a virulent one.²⁹ Many transduction experiments have been conducted, intending to either test the transduction ability of staphylococcal phages or prove the mobility of variable genetic elements with genes encoding antibiotic resistance or toxins.³⁰ For the reasons mentioned above, NICU bathroom sink was chosen to confirm the presence and characterization of phages in sludge produced in

bathroom sink.

In a study by Aidan et al in 2009, to prove the presence of phages in sludge produced in bathroom sink, the sample was taken and phages were isolated by the method described previously for isolating phages of *S. aureus*.¹⁹ The isolated phages were characterised with the electron microscopy. The result of electron micrograph showed that two isolated phages belonging to the family *Siphoviridae* (order *Caudovirales*) serogroup A and F with lytic activity against MRSA that was similar to the results of our study. The single step growth curve was shown Latent period of isolated bacteriophage was 30 min and burst size was 2600 PFU/ infected host. A study by Matsuzaki et al in 2013 detected a short latent period (~25 min) for ØMR11 phage. The efficient host-cell lysis characteristics of ØMR11 are reminiscent of typical virulent phages, such as the T-even coliphages.^{31,32}

To determine the characterization, the molecular studies were ignored due to lack of financial support. As the vast majority of phages, the *S. aureus* phages known so far are double-stranded DNA phages belonging to the *Siphoviridae* family of the *Caudovirales* order.^{20,21} In general, they are temperate phages detected as a prophage inserted in the chromosome, some of them being lytic due to mutations in the lysogeny functions.²³

According to the morphological classification previously proposed by Ackermann, staphylococcal *Siphoviridae* are composed of an icosahedral capsid and a non-contractile tail ended by a base-plate structure. Capsids may adopt elongated or isometric shapes, and tail length varies from short (130 nm) to long (400 nm).³²

In previous studies, investigators have isolated phages against MRSA from different sources, for examples milk from a cow with mastitis, faecal samples, sewage/pond water, skin using the strain *S. aureus* RN4220 as a bacterial host, soil samples collected from poultry and livestock farms that isolated phages belonged to the family *Myoviridae*, *Siphoviridae* and *Podoviridae* that have lytic activity against MRSA.^{17,20,33,34}

Conclusion

The results obtained in this study showed that the improper structured sink caused the formation of sludge and therefore the presence of phages. The presence of phages in NICU will be raised on antibiotic resistance or convert a non-virulent strain of *Staphylococcus* to a virulent one. Therefore it is recommended, if elsewhere the structure of sink in NICU is improper, presence of phages should be

investigated because of their key role in antibiotic resistance or pathogenesis.

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PEER REVIEW

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

Table 1: Specifications of MRSA isolated from the blood of six-month-old newborn with septicaemia at the NICU

TEST	RESULT	
Gram staining	Gram-positive cocci	
Catalase Test	Positive	
Coagulase Test	Positive	
DNase Test	Positive	
Mannitol Fermentation Test	Positive	
Kirby-Bauer technique of disk diffusion method	Oxacillin(2µg/mL)	Methicillin(5µg/mL)
	Resistant	Resistant
Determination of MIC with Etest	Vancomycin	Linezolid
	> 0.128 µg/mL	> 0.01 g/mL

Figure 1: Resistant *S. aureus* (MRSA) to: (a) 2µg/mL oxacillin, (b) 5µg/mL methicillin

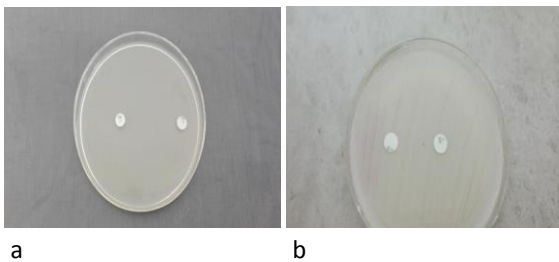


Figure 2: *S. aureus* (MRSA) MICs determined by E test, antibiotics (a) linezolid > 0.01µg/mL and (b) vancomycin, >0.128µg/mL

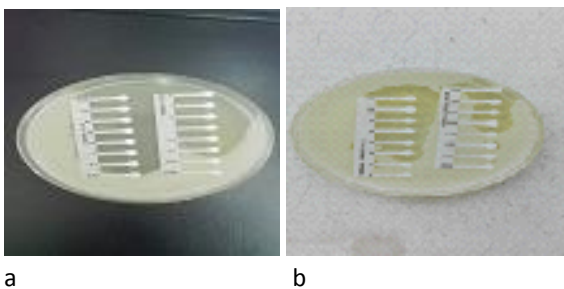


Figure 3: A spot test checking to show the presence of bacteriophage



Figure 4: A DLA Technique showed the titre of bacteriophage



Figure 5: The size of the bacteriophage plaques is marked with a loop

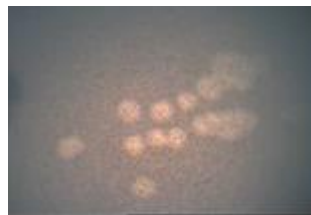


Figure 6: Electron micrographs of the family *Siphoviridae* phage serogroups F. Negatively stained with 2% uranyl acetate (pH=4-4.5). Voltage 150Kv, the scale bar represents 40nm

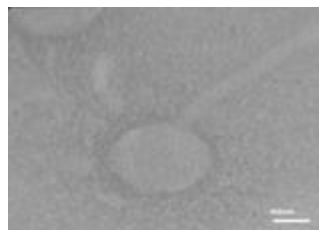


Figure 7: Electron micrographs of the family *Siphoviridae* phage serogroups A. Negatively stained with 2% uranyl acetate (pH=4-4.5), voltage 150Kv, the scale bar represents 60nm

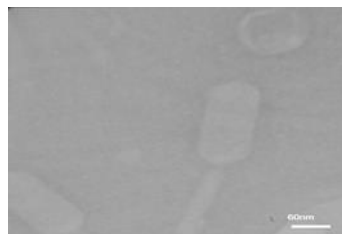
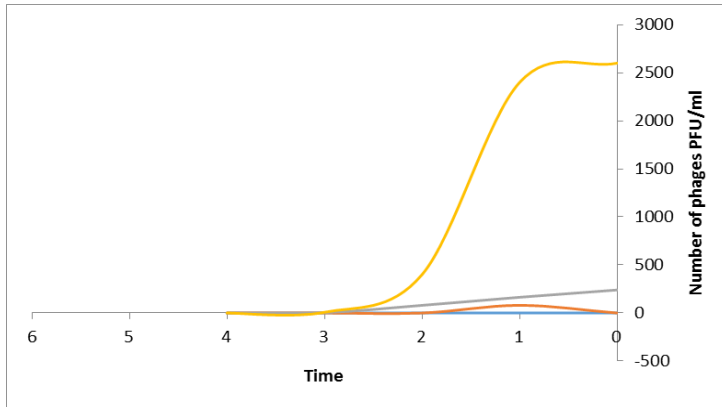


Figure 8: One-step growth curves of isolated phage in *S. aureus*



*Note: Series 4= time 30 min
Series 3= time 60 min
Series 2= time 120 min
Series 1= time 180 min