

# Could bacteriophages isolated from the sewage be the solution to methicillin-resistant *Staphylococcus aureus*?

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## ABSTRACT

**Introductions:** The emergence of multidrug-resistant bacteria such as Methicillin-Resistant *Staphylococcus aureus* (MRSA) complicates the treatment of the simplest infection. Although glycopeptides such as vancomycin still proves to be effective in treating MRSA infections, the emergence of vancomycin-resistant strains limits the long term use of this antibiotic. Bacteriophages are ubiquitous bacterial viruses which is capable of infecting and killing bacteria including its antibiotic-resistant strains. Bactericidal bacteriophages use mechanisms that is distinct from antibiotics and is not affected by the antibiotic-resistant phenotypes.

**Objectives:** The study was undertaken to evaluate the possibility to isolate bacteriolytic bacteriophages against *S.aureus* from raw sewage water and examine their efficacy as antimicrobial agents *in vitro*.

**Methods:** Bacteriophages were isolated from the raw sewage using the agar overlay method. Isolated bacteriophages were plaque purified to obtain homogenous bacteriophage isolates. The host range of the bacteriophages was determined using the spot test assay against the 25 MRSA and 36 MSSA isolates obtained from the Sarawak General Hospital. *Staphylococcus saprophyticus*, *Staphylococcus sciuri* and *Staphylococcus xylosus* were included as non-SA controls. The identity of the bacteriophages was identified via Transmission Electron Microscopy and genomic size analysis. Their stability at different pH and temperature were elucidated.

**Results:** A total of 10 lytic bacteriophages infecting *S.aureus* were isolated and two of them namely  $\Phi$ NUSA-1 and  $\Phi$ NUSA-10 from the family of *Myoviridae* and *Siphoviridae* respectively exhibited exceptionally broad host range against >80% of MRSA and MSSA tested. Both bacteriophages were specific to *S.aureus* and stable at both physiologic pH and temperature.

**Conclusion:** This study demonstrated the abundance of *S.aureus* specific bacteriophages in raw sewage. Their high virulence against both MSSA and MRSA is an excellent antimicrobial characteristic which can be exploited for bacteriophage therapy against MRSA.

## KEY WORDS:

*Staphylococcus aureus*, bacteriophages, Methicillin-resistant

## INTRODUCTION

*Staphylococcus aureus* is a Gram-positive bacterium linked to a multitude of diseases in both humans and animals.<sup>1</sup> *S.aureus* is known to cause mild diseases to life-threatening diseases. *S.aureus* is known to be present as a commensal in human and animals such as livestock, wildlife and domestic animals with human-to-animal, animal-to-animal transmission or vice versa and these have been reported and validated via molecular genotyping.<sup>2-10</sup>

Staphylococcal infections can be treated with beta-lactam antibiotics until the emergence of the Methicillin-resistant *S.aureus* (MRSA) in 1960, less than a year after the introduction of the second-generation beta-lactam antibiotic.<sup>11</sup> Since 1999, nine antibiotics against MRSA have been approved namely linezolid, daptomycin, tigecycline, ceftobiprole, telavancin, ceftaroline, dalbavancin, oritavancin and tedizolid but only oxazolidinones belongs to a new class of antibiotics.<sup>12,13</sup> Human is at risk to fall back into the pre-antibiotics era without the discovery of new classes of antibiotics.

Currently, the antibiotics of choice for MRSA infection are glycopeptides such as vancomycin but the emergence rate of vancomycin-resistant and vancomycin-intermediate *S.aureus* (VRSA and VISA) has been increasing in various parts of the world.<sup>14</sup> Neither VRSA nor VISA has been reported in Malaysia to date but Vancomycin 'MIC creep' phenomenon has been observed since the last decade<sup>15</sup> suggesting the continuous selective pressure for the emergence of Vancomycin-resistant strains. Nevertheless, the nephrotoxic nature of glycopeptides and other adverse side effects limit its use in clinical settings.<sup>1,16</sup>

As a solution to MRSA, we envisaged to revisit the potential use of bacteriophages, viruses that infect and kill susceptible bacterial host including the multidrug-resistant variants. Bacteriophages are known to be ubiquitous in nature, especially where the host can be found but some bacteriophages are globally distributed while the rest be geographically unique.<sup>17-19</sup> In the absence of commercially

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available therapeutic bacteriophages against MRSA, local laboratories which are interested to venture into bacteriophage therapy are required to develop their own in-house bacteriophage collection against the host bacteria of interest.

Detailed genomic and bioinformatic characterisation of the bacteriophages are beyond the scope of this paper.

## MATERIALS AND METHODS

### *Bacterial strains*

A total of 61 non-repeat *S.aureus* clinical isolates were obtained from the Pathology laboratory, Sarawak General Hospital (SGH), Kuching, Sarawak from 2015-2016. Twenty-five of them were MRSA while the remaining 36 were MSSA. *S.aureus* ATCC 25923 was obtained from the American Type Culture Collection (ATCC). The representative of other coagulase-negative Staphylococcal strains namely *Staphylococcus saprophyticus*, *Staphylococcus sciuri* and *Staphylococcus xylosus* were included as non-*S.aureus* controls.

### *Bacteriophage enrichment, isolation and host range*

The raw sewage water was obtained from the Kuching Centralised Sewage Treatment Plant, Sewerage Services Department Sarawak, Kuching, Sarawak. Sewage water was collected during the dry season which is defined as no rain for five consecutive days in order to eliminate potential dilution factor as stormwater is also fed into the centralised sewerage system. The bacteriophages were isolated against host bacterium (either *S.aureus* ATCC strain 25923 or *S.aureus* strain 995).

### *Bacteriophage enrichment*

Briefly, 40mL of raw sewage water was mixed with 10mL of 5X LB medium and seeded with 100µL of the overnight host bacterium. The mixture was incubated at 37°C and shaken 225rpm overnight in an incubator shaker. The bacteria were killed with the addition of chloroform, releasing free bacteriophages into the medium. The enriched medium was clarified by centrifugation at 3000g at 4°C for 30 min (Eppendorf centrifuge 5702R) followed by sequential filtration through 0.45µm and 0.22µm using Minisart PES syringe filter (Sartorius Stedim Biotech, Germany). The potential bacteriophages in the bacteria-free enrichment were transferred into a sterile 50ml centrifuge tube and stored at 4°C for bacteriophage isolation.

### *Bacteriophage isolation*

One hundred microlitre of the potential bacteriophages was plated on a LB-agar containing Petri dish along with 300µL of overnight host-bacterium and 3mL of melted 0.7% LB agar. The plate was incubated overnight at 37°C. The resulting plaques of different visual morphologies were randomly picked and transferred into 500µL of LB broth. Plaque-purification was repeated six times for all discovered bacteriophages in order to isolate a single homogenous bacteriophage from the potentially heterogeneous bacteriophage mixture in the initial enrichment. All bacteriophages isolated against *S.aureus* are termed staphyphages in this paper.

### *Host-range Assay*

The staphyphage host range was performed on the *S.aureus* isolates using the spot test method.<sup>20</sup>

### *Staphyphage genome extraction and restriction analyses*

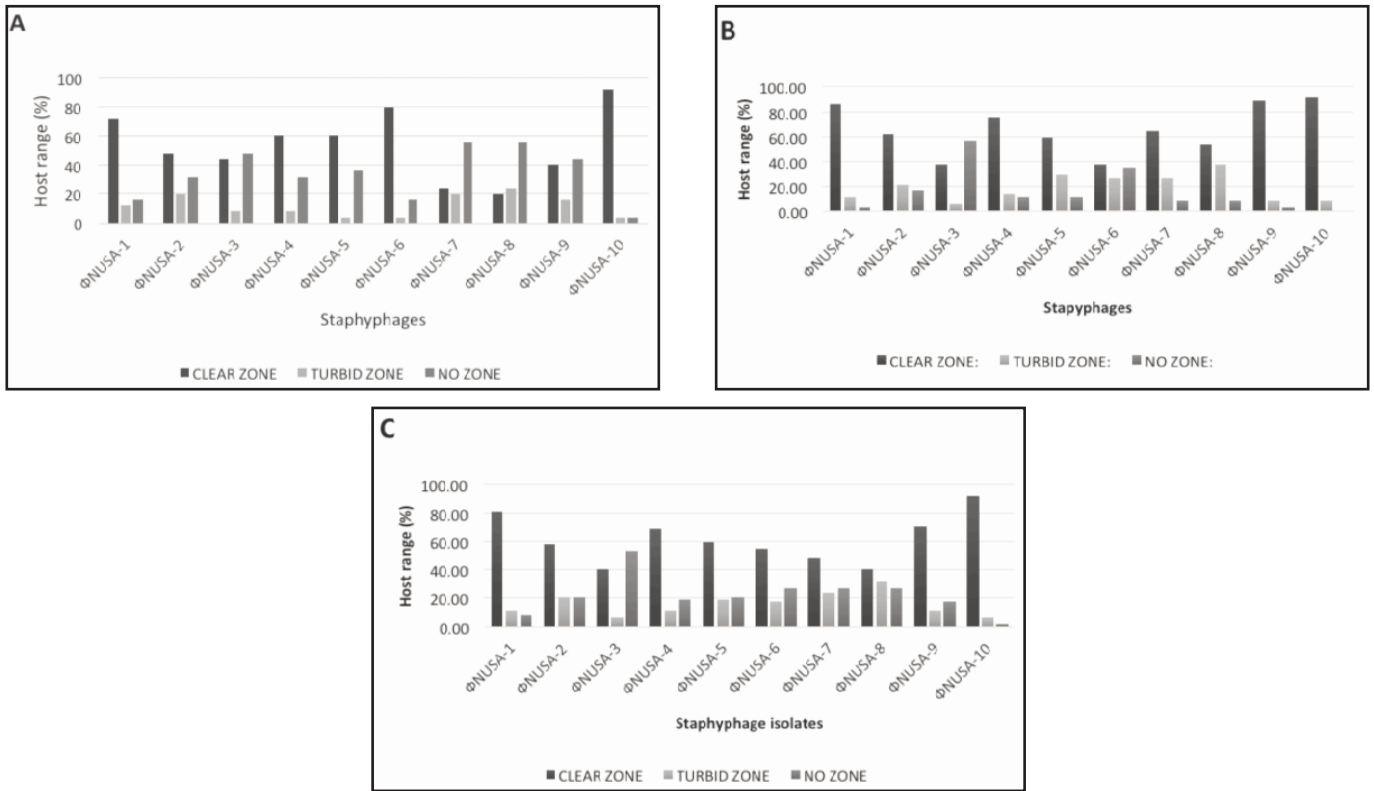
The staphyphages' nucleic acid was extracted using High Pure Total Nucleic Acid Extraction Kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. The extracted nucleic acid was digested with FastDigest restriction endonucleases (*EcoRI*, *HindIII*, *BamHI*, *PstI*, *Sall*, *EcoRV*, *NdeI* and *SacI*) according to the supplier's recommendations (Thermo Scientific, USA). The DNA fragments were then separated by 0.8 % agarose gel electrophoresis stained with Ethidium bromide in Tris-acetate-EDTA buffer at 60 V for 2 h. The digestion profiles of the digested DNA fragments were analyzed using GelAnalyzer (<http://www.gelanalyzer.com/index.html>) to calculate the size of the staphyphage genome.

### *Staphyphage stability*

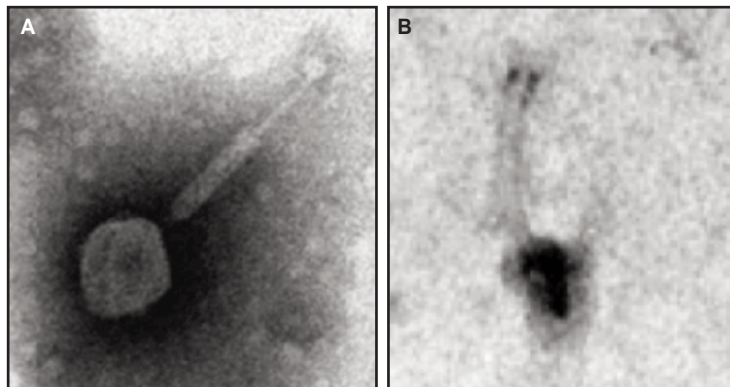
Five temperatures (40, 45, 50, 55 and 60°C) were used in this study to identify the thermal tolerance of staphyphage in LB broth. The method was adapted from Li & Zhang with slight modification.<sup>21</sup> Briefly, the staphyphage lysate was incubated in a water bath at the selected temperature and an aliquot of the phage was taken every 10 min for one hour and titrated immediately using the agar overlay method by plating a dilution series ( $10^{-2}$ - $10^{-8}$ ). On the other hand, pH stability tests were carried out with pH values ranging from 2 to 12 (adjusted with 1M NaOH or 1M HCl) where 100µl of staphyphage was mixed in a series of tubes containing 900µl of LB liquid medium and incubated for 3h at 37°C. They were titrated immediately by using double-agar overlay method as described earlier.

### *Transmission Electron Microscopy*

The staphyphages were prepared and their images were taken via Transmission Electron Microscopy (TEM) using the method as described by Ackermann, (2009) with modifications. Briefly, 1 ml of sterile high-titer lysate ( $10^8$  of viable phages/ml or better) was centrifuged at 18,000g at 4°C for 1 hour. The supernatant was discarded and the pellet was re-suspended in cold 0.1M of ammonium acetate solution (pH 7.0). The solution was centrifuged again at 18,000g at 4°C for 1 hour and the procedure was repeated another time. Nine hundred and fifty µl of the supernatant was carefully aspirated out of the tube leaving behind approximately 50µl of solution and left overnight at 4°C to allow the pelleted phage to diffuse into the liquid. For image preparation of the phage, 10µl of the staphyphages suspension was spotted on top of a Formvar carbon-coated copper 300-mesh grid and allowed to adsorb for 30 min. The excess amount of staphyphages was removed by carefully touching the side of the copper grid with filter paper. Then, 5µl of distilled water was spotted on the copper grid and removed shortly, followed by negative staining with phosphotungstate (2%, w/v) for 10 min. The excess stain was removed and the copper-grid is left to air-dry for 30 min. The copper grid was finally examined using TEM (Tecnai G2, Electron Microscopy Unit, Institute for Medical Research, Malaysia) operating at 75kV. Average phage dimensions were calculated by measuring the head diameter, head length, tail diameter and tail length. The results were compared to a known dimension of T4 phage.



**Fig. 1:** Host range of bacteriophages against *Staphylococcus aureus*. A. MRSA; B. MSSA, C. MRSA and MSSA. Clear zone indicates complete lysis of the bacteria, turbid zone indicates partial lysis of bacteria and no zone indicates the resistance of *Staphylococcus aureus* against bacteriophages. Host range data is depicted in Figure 5.



**Fig. 2:** Transmission electron micrograph of staphyphages . Micrograph of negatively stained sample with 2% phosphotungstate with magnification of 80,000x. A. ΦNUSA-1 micrograph showing the contractile tail of the staphyphage consistent with the family of Myoviridae; B. ΦNUSA-10 micrograph showing the long non-contracting tail consistent with the family of Siphoviridae.

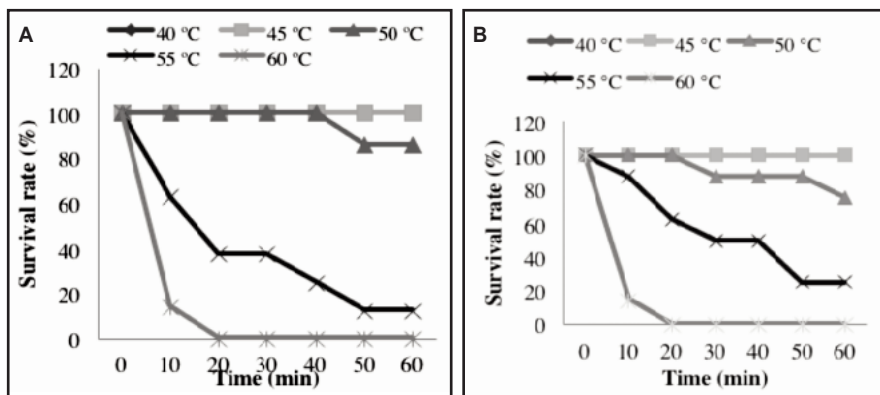
**RESULTS**

*Staphyphages*

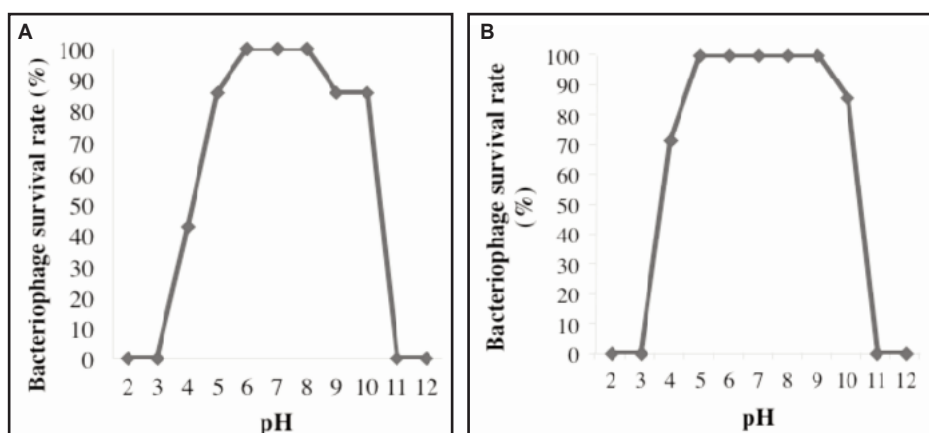
A total of 10 homogenous staphyphages were isolated and purified. They were named ΦNUSA-1 to ΦNUSA-10. The first 5 were isolated against MSSA strain 995 and the rest against *S.aureus* ATCC strain 25923 respectively.

*Host range*

Host range experiments indicated that all bacteriophages were not able to form plaques on representative strains of *S.xylosus*, *S.sciuri* and *S.saprophyticus* suggesting its species-specificity against *S.aureus*. Staphyphages ΦNUSA-6 and ΦNUSA-10 exhibited high virulence against 80% or more of the MRSA tested (1A) while ΦNUSA-1, ΦNUSA-9 and ΦNUSA-10 exhibited high virulence against MSSA isolates tested



**Fig. 3:** Thermostability test for staphyphages. A. ΦNUSA-1 B. ΦNUSA-10. Staphyphages were incubated at various temperatures as indicated. Samples were collected at 10 min intervals for 60 min and immediately tested using the agar overlay method. The test was carried out in triplicates and the mean graphs were plotted. A. ΦNUSA-1 B. ΦNUSA-10.



**Fig. 4:** pH stability test for staphyphages A. ΦNUSA-1 B. ΦNUSA-10. Staphyphages was incubated under different pH conditions as indicated for 3 hours and immediately tested using the agar overlay method. The tests were carried out in triplicates.

(Figure 1B). When analysed together, ΦNUSA-1 and ΦNUSA-10 showed the combined virulence against most *S.aureus* isolates tested (Figure 1C). cross-infectivity test data is shown in Figure 5.

#### Transmission Electron Microscopy and genome size

The morphology of the two staphyphages namely ΦNUSA-1 and ΦNUSA-10 were analysed via TEM (Figure 2). Both staphyphages belong to the order of *Caudovirales*. ΦNUSA-1 possessed contractive tail with hexagonal symmetrical capsid with a head length of 84x84nm<sup>2</sup> attached to a rod-like body of 167nm long alongside tail fibres identifying it within the family of *Myoviridae* (Figure 2A). The calculated genomic size is 100-134 bp. The electron micrograph of ΦNUSA-10 (Figure 2B) was not clear enough for ΦNUSA-10 to distinguish between the family of *Myoviridae* or *Siphoviridae* but the calculated genomic size of 60-68 kb suggests that it belonged to the family of *Siphoviridae* which is known to have a smaller genomic size compared to *Myoviridae*.

#### Thermal and pH stability of two bacteriophages

The stability of ΦNUSA-1 and ΦNUSA-10 was investigated under different thermal and pH conditions. Both

bacteriophages exhibited high thermos-stability at the temperature <45°C for at least 60 min (Figure 3) while ΦNUSA-1 was stable at pH range between pH 6-8 without any loss of viability. However, ΦNUSA-10 has a wider pH tolerance between pH 5-9 (Figure 4).

#### DISCUSSION

The isolation of staphyphages against both MSSA host strains from sewage was straightforward. However, Matilla et al., had reported difficulty in isolating staphyphages against MRSA from their local sewage in Finland<sup>22</sup> but Wang et al., found abundance of staphyphages from swine faecal sewage in China.<sup>23</sup> This may be due to the choice of the environmental reservoir used for the *S.aureus* phage isolation.<sup>24</sup> Other factors such as ambient temperature (winter vs summer or drought vs monsoon), source of sewage, amount of disinfectant, sewage flow rate, and exposure to sun UV may play a role in determining the amount of starting material. Nevertheless, new insights in bacteriophage biogeographic study have suggested that not all bacteriophages may not be present in abundance and homogenous at all locations including sewage water.<sup>17</sup>

		ΦNUSA-1	ΦNUSA-2	ΦNUSA-3	ΦNUSA-4	ΦNUSA-5	ΦNUSA-6	ΦNUSA-7	ΦNUSA-8	ΦNUSA-9	ΦNUSA-10
	ATCC 25923*	C	T	N	C	T	C	C	C	C	C
Methicillin-Susceptible <i>Staphylococcus aureus</i>	995*	C	C	C	C	C	N	N	N	T	T
	966910	C	C	T	C	C	T	C	C	C	C
	967304	T	N	N	N	N	N	T	N	C	C
	966915	C	T	N	T	T	C	C	C	C	C
	967297	C	T	N	C	N	T	T	T	C	C
	967299	C	C	C	C	C	C	C	C	C	C
	914260	T	N	N	C	T	T	C	C	C	C
	967291	C	C	N	C	C	N	T	T	C	C
	966917	C	T	N	C	T	N	T	T	C	C
	914272	C	C	C	C	C	N	T	T	C	C
	914263	T	N	N	N	T	T	C	C	C	C
	913842	C	C	C	C	C	C	C	C	C	C
	913824	C	C	C	C	C	C	C	C	C	C
	914266	C	N	N	T	T	C	C	T	N	T
	9774	C	T	N	C	C	C	C	T	T	C
	932100	C	C	N	C	T	T	C	T	T	T
	932378	C	C	N	C	C	C	C	C	C	C
	932565	C	C	T	C	C	C	C	C	C	C
	932556	C	C	C	C	C	C	C	C	C	C
	976391	C	C	N	C	C	T	C	C	C	C
	976390	C	C	C	C	C	N	C	C	C	C
	932387	C	T	N	T	T	N	T	N	C	C
	6789	C	C	N	T	T	N	N	T	C	C
	9772	C	C	N	C	C	C	C	T	C	C
	932373	C	C	C	C	C	N	N	T	C	C
	6484	C	C	C	C	C	C	C	C	C	C
	9763	N	N	N	N	N	N	C	C	C	C
	6781	C	C	C	C	C	C	C	C	C	C
	3885	C	T	N	C	T	C	T	T	C	C
	3854	C	C	C	C	C	C	T	T	C	C
913819	C	C	C	C	C	T	C	C	C	C	
914249	C	C	N	T	C	T	C	C	C	C	
9767	T	N	N	N	T	N	T	T	C	C	
999850	C	T	N	T	N	T	T	T	C	C	
3578	C	C	C	C	C	T	C	C	C	C	
635	C	C	C	C	C	C	C	C	C	C	
Methicillin-Resistant: <i>Staphylococcus aureus</i>	966342	C	T	N	C	C	N	N	N	C	C
	151004	T	N	N	C	T	C	C	C	N	T
	3819	C	C	C	C	C	N	N	N	C	C
	1002587	C	C	C	C	C	N	T	T	C	C
	37	C	C	C	C	C	C	N	N	N	C
	1081255	C	T	T	T	C	C	T	T	C	C
	1084287	N	N	N	N	N	C	N	N	N	C
	1085997	T	N	N	N	N	C	N	N	N	C
	1084043	N	N	N	N	N	C	N	N	N	C
	1093138	C	T	N	N	N	C	N	N	N	C
	6520	C	C	C	C	C	N	N	N	N	N
	1106469	C	C	T	C	C	C	C	T	C	C
	1106482	C	T	N	C	C	C	N	N	T	C
	1070439	T	N	N	N	N	C	N	N	N	C
	850	C	C	C	C	C	C	T	T	C	C
	1070439	C	N	N	N	N	C	T	T	T	C
	587	N	N	N	N	N	C	N	N	N	C
	1109614	C	C	C	C	C	C	T	T	T	C
	1109606	C	C	C	C	C	C	N	N	N	C
	1109587	C	C	C	C	C	C	N	N	N	C
	1109597	N	N	N	N	N	C	N	N	T	C
	1100089	C	C	C	C	C	C	C	C	C	C
	5279	C	C	C	C	C	C	C	C	C	C
	4942	C	C	C	C	C	C	C	C	C	C
	4972	C	T	N	T	N	T	C	C	C	C
Non-SA spp.	<i>S. xylosum</i>	N	N	N	N	N	N	N	N	N	N
	<i>S. sciuri</i>	N	N	N	N	N	N	N	N	N	N
	<i>S. saprophyticus</i>	N	N	N	N	N	N	N	N	N	N

Fig. 5: Cross-infectivity of the isolated bacteriophages. White background with the letter 'C' represents clear lysis zone observed; Grey background with the letter 'T' represents dim but visible lysis zone; Black background with the letter "N" represents no lysis zone. Bacterial host strains are indicated with an Asterisk.

We have selected two highly virulent Staphyphages namely,  $\Phi$ NUSA-1 and  $\Phi$ NUSA-10 for further characterisation. Both of them were highly specific to SA. This observation is in sync with Melo et al., (2014) as the authors have reported *Staphylococcus epidermidis* specific bacteriophages only infects *S.epidermidis* and not *S.aureus*.<sup>25</sup> This suggests that staphyphages are generally species-specific. Another observation is the broad host range within the species which has been reported by many researchers.<sup>25-27</sup> The broad host range of these staphyphages may be attributed by the use of more than one highly conserved phage receptor binding proteins for host recognition.<sup>28</sup> Both  $\Phi$ NUSA-1 and  $\Phi$ NUSA-10 were isolated against MSSA host strains but have no problem in killing MRSA. This supports the hypothesis that bacteriophages are using bactericidal mechanisms which is independent from the bactericidal mechanism of antibiotics.

Electron microscopy remains the gold standard in the identification of bacteriophages.<sup>29</sup> This is due to the absence of any highly conserved universal barcoding genes like the 16S rRNA gene in bacteria and the Internal Transcribed Spacer (ITS) region in fungus.  $\Phi$ NUSA-1 and  $\Phi$ NUSA-10 belong to the family *Myoviridae* and *Siphoviridae* respectively under the Order Caudovirales which is the most commonly reported candidate for bacteriophage therapy due to its typical lytic infection cycle, a mandatory property in antimicrobial therapy.<sup>26-28,30-33</sup>

Both  $\Phi$ NUSA-1 and  $\Phi$ NUSA-10 are stable in both physiological pH and temperature. These are crucial conditions required in the selection of a biocontrol agent if it is to be used on a human subject. A biocontrol agent which is administered via oral route must be able to tolerate the acidic gastric juice while surviving the basic bile secretion. Regardless of the route of administration, the staphyphages must also survive the elevated body temperature during pyrexia. Thermo-stability may allow transportation of the staphyphage preparation to locations where the strict adherence to cold-chain is near impossible.

It is worth to mention that this study is the first manuscript in Malaysia describing the isolation of staphyphages as a potential solution to the emergence of MRSA and probably VISA and VRSA. The findings here are supportive of the hypothesis that bacteriophages from sewage possess an antimicrobial potential that is worth to be explored for clinical use.

## CONCLUSION

Bacteriophages can be easily isolated from the environment such as sewage and have a relatively broad host range specific to *S.aureus* regardless of the Methicillin-resistant status. A brief characterisation revealed that these staphyphages hold the characteristics worthy of being explored as the solution to the emergence of MRSA.

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