

## Assessment of *in-vivo* safety and antibacterial activity of phages against methicillin resistant *S. aureus* in mouse model

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Traditionally Ganges water that contains bacteriophages, are used in Hindu rituals in India. Bacteriophages are bacterial-specific viruses and present abundantly on Earth. They are present most widely in the biosphere, soil and the intestine of animals. Specificity of infection makes them a powerful alternative for the control of bacterial infection which is normally showing resistant to antibiotics. In present study, isolation of bacteriophage was carried out from sewage of piggery and dairy farms to find out antimicrobial property. Isolated bacteriophages were showing an icosahedral symmetry with long tail of 109 nm and head of 52.20 nm in diameter under electron microscopic observation. These phages were considered as member of Myoviridae family. Presently, antimicrobial resistance is an acute problem across the globe, and therefore alternate for antimicrobials should be searched on priority. Primary aim of this study was to isolate and evaluate the efficacy of a bacteriophage against Multi drug resistance pathogen like Methicillin resistance *Staphylococcus aureus*. A total 150 sewage samples were processed for bacteriophage isolation out of them 27 were found to be positive for bacteriophages. Four isolates of bacteriophage lysate ØVS1, ØVS5, ØVS9 and ØVS 27 were used as a Cocktail in trail. Therapeutic and safety profile of the purified phage lysate cocktail were performed in well-defined mouse model at laboratory animal house of College of Veterinary Science & A.H., Jabalpur (M.P.). Results showed 100% in mice G-III (challenged with MRSA) was treated with phage Cocktail with in ten days whereas G-IV was showing approx. 40% in abscess (challenged with MRSA). Our findings reported that the bacteriophage therapy is safe and better than traditional antibiotics therapy especially in multidrug resistance cases.

**Keywords:** Bacteriophage, Methicillin resistance, Mice, Phage lysate, *Staphylococcus aureus*

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In the recent time antimicrobial resistance is very common and huge in many numbers of pathogens. List of some bacteria published by WHO to make awareness about degree of antimicrobial resistance of pathogens with critical, high and medium priority<sup>1</sup>. The current rate of antibiotic resistance development exceeds with the level of antibiotic discovery and represents a global public health challenge. It highlights the serious problem regarding therapeutic options for multi-drug resistant (MDR) bacterial infections<sup>2</sup>. Several pathogens which are showing antimicrobial resistance are: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas* spp, *Klebsiella* spp, *Enterobacter* spp and *Mycobacterium* spp etc. Among these pathogens *Staphylococcus aureus* is showing huge amount of multiple drug resistance. *Staphylococcus aureus* is causing severe fatal

infections in both humans and also associated with food poisoning<sup>3</sup>. *Staphylococcus aureus* is mainly present as commensal on the skin and nasal tract of healthy individuals which can cause a severe infections<sup>4</sup>. *S. aureus* is showing very high morbidity and mortality in surgical wound infections<sup>5</sup>. *S. aureus* is having huge multiple drug resistance which leads to the large economic loss in dairy industry<sup>6</sup>. Latest studies also suggested the collateral damage of antibiotics to the microbial flora of animals<sup>7</sup>. Multiple Drug resistance in microbial pathogen is a concern not only in veterinary, but also in general worldwide<sup>8</sup>. In recent time Methicillin-resistant *Staphylococcus aureus* (MRSA) is most difficult to control amongst antimicrobial resistant pathogens. Normally, whenever antimicrobial resistance emerged, scientist developed the new antibiotics to treat resistant bacterial pathogens. However, it is tough to develop effective new drugs every time as a new resistance

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mechanism emerges. In these circumstances market is hoping to develop novel strategies to control multidrug resistance bacterial infections, such as AMPs, Phage therapy and Phage endolysins. Among all the strategies, phage therapy is very promising as it involves a phage lysate that specifically infects and kill the pathogen<sup>9</sup>. Development of multiple drug resistance is also a big threat to human population due to the residual effect of antibiotics in livestock products like milk, meat and poultry etc. This is an alarming sign for livestock product has rejuvenated interest in phage therapy. Bacteriophage is mainly present in soil, water and gastrointestinal tract of animals<sup>10</sup>. Phages are potential antibacterial therapeutic agents against multiple drug resistance pathogens<sup>11</sup>. Phages lysate used against methicillin resistant *Staphylococcus aureus* (MRSA) are a new, potential alternate agent. They can destroy bacteria very effectively. Phages cannot penetrate the mammalian cells which protect the eukaryotic cells from lytic activity and have no side effect on cells<sup>12</sup>.

### Methodology

Research work was performed in the Department of Veterinary Microbiology, College of Veterinary Science and A.H., N.D.V.S.U., Jabalpur, Madhya Pradesh, India. One hundred fifty sewage samples were collected from various collection tanks and storage pits of livestock farm, adhartal, N.D.V.S.U., Jabalpur (M.P.) for isolation of bacteriophage. Recovered bacteriophage lysate identified by transmission electron microscopy. Further, therapeutic study was performed in mice to know *in vivo* antibacterial activity as well as the safety profile of phage lysate. Cocktail of phage lysate and *S. aureus* were inoculated in various groups of mice by subcutaneous route. This study was carried out to evaluate *in-vivo* therapeutic activity of phage lysate against methicillin resistant *S. aureus* (MRSA).

### Materials and Methods

This research work was done in the year 2020 in the Department of Microbiology, College of Veterinary Sci. and A.H., N.D.V.S.U., Jabalpur, Madhya Pradesh, India. Primary aim of study was to find out the alternative of antimicrobial agent. Bacteriophage was isolated from animal waste (sewage) of livestock farms. Phage lysate could be an effective alternate to control multi drug resistance pathogens.

### Phage cocktail preparation

Isolation of bacteriophages was performed by soft agar overlay method as described by Synnot *et al.*<sup>13</sup> with slight modification from sewage samples collected from dairy piggery and goaterly farms. *Staphylococcus aureus* (ATCC 25923) was used as a primary host for the isolation of bacteriophage. Four good-scoring phages lysate were used for the preparation of therapeutic cocktail in the study (Fig. 1). Isolated bacteriophages were titrated to obtain the effective phage lysate cocktail. Tenfold serial dilution ( $10^{-1}$  to  $10^{-12}$ ) was prepared in normal saline for each phage lysate. Now each dilution of bacteriophage was subjected to the plaque formation by propagating through double agar overlay method. PFU of the phage was calculated as per Chandra *et al.*<sup>14</sup>. PFU count = number of plaques × dilution/volume of phage preparation used (Table 1).

### Experimental design for *in-vivo* assessment of antimicrobial activity of phage lysate

*In vivo* experiment was conducted in mouse model to assess the safety as well as antimicrobial activity of phage lysate against methicillin resistant *S. aureus* (Fig. 2). These mice strains (C57B1/6) were used in experimental design for the study of bacteriophage safety and efficacy against the methicillin resistant *S. aureus* microorganism which were received from ICMR-National Institute of Research in Tribal Health,

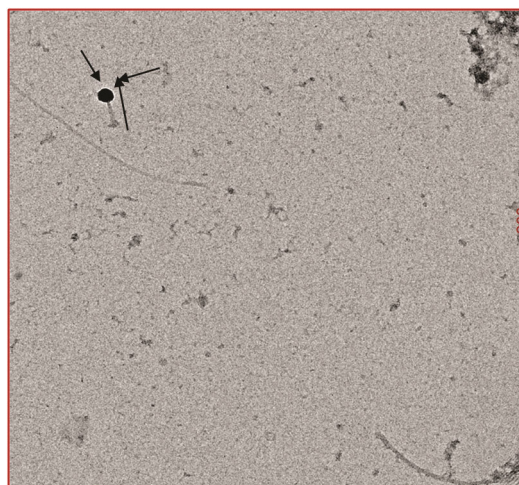


Fig. 1 — TEM image of *S. aureus* phage with tail fiber

Table 1 — Titration of phage lysate (PFU/ml) prepared for trial

S. No.	Phages used for titration	Average titer of phage (PFU/mL)
1	ØVS 1	$1 \times 10^{11}$
2	ØVS 5	$1 \times 10^{10}$
3	ØVS 9	$1 \times 10^{12}$
4	ØVS 27	$1 \times 10^9$

Jabalpur (M.P.). Cocktail of bacteriophage lysate and methicillin resistant *S. aureus* (MRSA, ATCC 43300) were inoculated in different mice groups by subcutaneous route. The observations were recorded daily up to ten days. Experiment was conducted according to Capparelli *et al.*<sup>15</sup> with slight modifications (Table 2).

## Results and Discussion

A total 150 sewage samples were collected from lives tock farms out of them 27 samples were found positive for bacteriophage isolation. Isolation percentage of bacteriophages was maximum from sewage of cattle farm (30%) followed by sewage of buffalo farm (20%) and sewage of piggery (17.50%). There were no any bacteriophages recovered from goatery. Isolated phages were having hexagonal structure with an icosahedral symmetry observed under transmission electron microscopy. The size of head of bacteriophage was 52.20 nm with 109 nm long tail. Phage lysates ØVS1 ( $1 \times 10^{11}$ ), ØVS5 ( $1 \times 10^{10}$ ), ØVS9 ( $1 \times 10^{12}$ ) & ØVS 27 ( $1 \times 10^9$ ) were used for the preparation of Phage “Cocktail”.



Fig. 2 — Growth of methicillin resistant *S. aureus* on MeReSa agar plate

Cocktail of four phages lysate was used for the *in-vivo* assessment of antimicrobial activity. Result of *in vivo* experiment was conducted in mouse model to assess the safety as well as antimicrobial activity of phage lysate against methicillin resistant *S. aureus* showed that the mice of group-I which were inoculated with *S. aureus* developed the closed abscess and act as positive control to compare with other phage treated and antibiotic treated groups (Fig. 3). Mice of group-VI were inoculated with normal saline did not show the formation of abscess; act as negative control group to compare with other phage treated and antibiotic treated groups (Fig. 4).

The Mice of group-II was treated with bacteriophage cocktail just before the inoculation of *S. aureus* did not show the formation of abscess indicated that bacteriophage can an effective antimicrobial agent as prophylaxis against *S. aureus*. Our findings were supported by findings of other workers like Wills *et al.*<sup>16</sup>; Furusawa *et al.*<sup>17</sup>; Saglam *et al.*<sup>18</sup> and Ding *et al.*<sup>19</sup> who reported that the bacteriophage therapy prevented the formation of abscesses and reduce the bacterial load. Results of the study suggest that bacteriophage lysate would be promising prophylactic agent. Phage

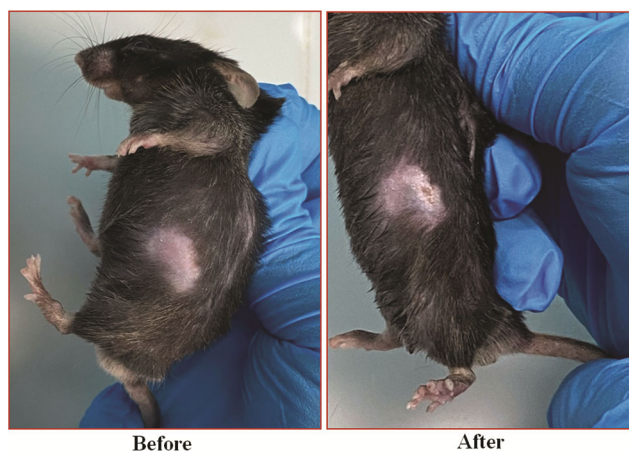


Fig. 3 — Abscess formation in mice (G-I) after administration of *S. aureus* positive control

Table 2 — *In-vivo* assessment of antimicrobial activity of phage lysate

S. No.	Group	Number of animals	Administration of agent (s/c)	Parameter
1.	G-I	06	0.1 mL of <i>S. aureus</i> ( $10^8$ CFU) was given to each mice.	Abscess formation
2.	G-II	06	0.1 mL of <i>S. aureus</i> ( $10^8$ CFU) was given to each mice immediately after phage lysate application ( $10^9$ PFU).	Prevention of abscess Reduction in bacterial load
3.	G-III	06	0.1 mL of phage lysate ( $10^9$ PFU) was given on 5 <sup>th</sup> day after the infection with <i>S. aureus</i> ( $10^8$ CFU).	Reduction in abscess Reduction in bacterial load
4.	G-IV	06	0.1 mL of antibiotic was given on 5 <sup>th</sup> day after the infection with <i>S. aureus</i> ( $10^8$ CFU).	Reduction in abscess Reduction in bacterial load
5.	G-V	06	0.1 mL Phage lysate was given in each mice	Abscess formation
6.	G-VI	06	0.1 mL normal saline was given in each mice.	Abscess formation



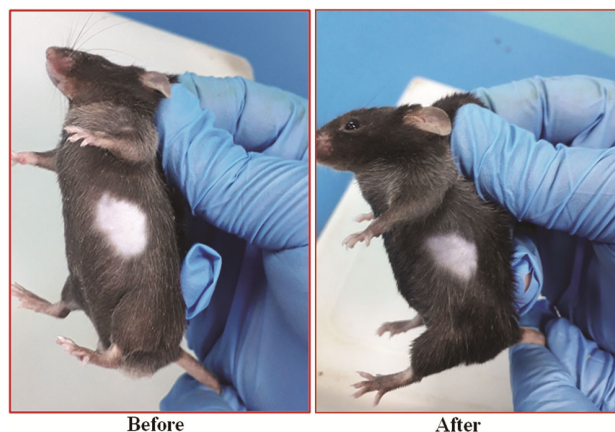


Fig. 4 — No abscess formation in mice (G-VI) after administration of normal saline-negative control

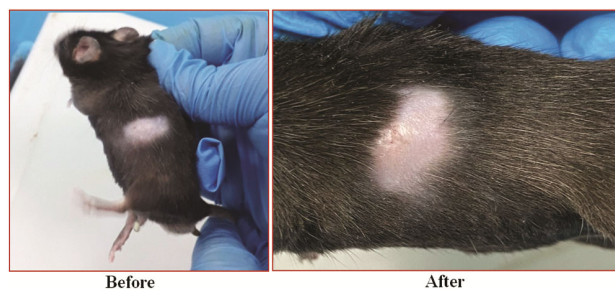


Fig. 5 — No abscess formation in mice (G-II) after administration of phage lysate just before *S. aureus*

therapy can provide effective solution of prophylactic and therapeutic alternative treatment especially in chronic cases where pathogens were not responding towards conventional therapy due to multiple resistance problems (Fig. 5).

Mice of group-V were administered with s/c injection of bacteriophage lysate and did not show the formation of abscess which indicates that the bacteriophages are safe for animal use. Findings of the study in agreement of findings of Pincus *et al.*<sup>20</sup> they observed that the bacteriophage are safe because they do not infect the animal cells and can be effectively used to treat bacterial infections in animals (Fig. 6).

Mice of G-III were developed the abscess due to administration of *S. aureus*. Cocktail of phage lysate were given to the mice of this group on fifth day after the administration of *S. aureus*. The lesions of abscess gradually subsided like a normal skin after the treatment of cocktail phage lysate within ten days (Fig. 7). Mice of group-IV developed the abscess due to administration of *S. aureus*. These were subjected to administration of conventional antibiotic therapy but did not show the complete recovery within 10 days. Our findings were similar to the findings of Ochieng *et al.*<sup>21</sup> who observed that 100 percent of mice were

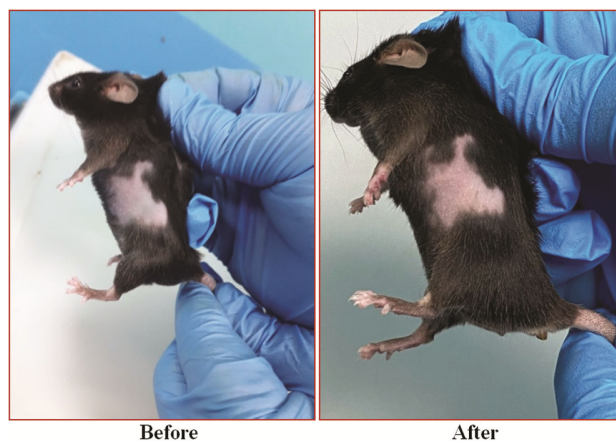


Fig. 6 — No abscess formation in mice (G-V) after administration of phage lysate

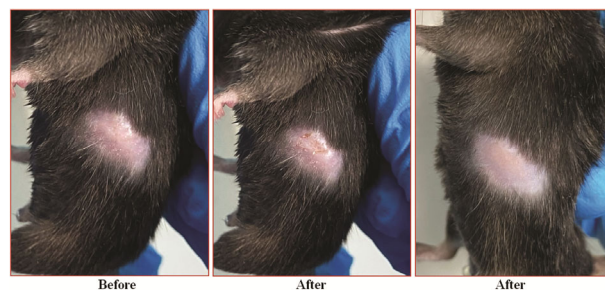


Fig. 7 — Recovery of abscess after phage lysate administration (G-III)



Fig. 8 — No recovery of abscess after antibiotic administration (G-IV)

recovered, treated with bacteriophage as compare to antibiotic treated group which showed approx. 40% recovery. They also reported that the bacteriophage therapy is much better than antibiotics (Fig. 8).

Over all our findings were also in agreement with the findings of other workers like Matsuzaki *et al.*<sup>22</sup>; Duraisamy *et al.*<sup>23</sup> and Lehman *et al.*<sup>24</sup> and supported the potential efficacy of phage therapy against multidrug resistance pathogens and septic infections.

## Conclusions

The present era is showing huge multiple drug resistance all over the globe. This is an alarming sign for pharma industry to search some alternatives therapy.

Our findings are showing potential efficacy against multidrug resistance pathogens as compared to the conventional antimicrobials. Bacteriophage could be an excellent alternative of conventional antimicrobials against multidrug resistance pathogens. There is need to do more research work on the bacteriophage to explore the all aspects related to antimicrobial property to overcome the antimicrobial resistance.

### Ethic Approval

INSTITUTIONAL ANIMAL ETHICAL COMMITTEE has approved the study vide approval no. 95/IAEC/Vety./2018.

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### Conflict of Interest

No potential conflict of interest was reported by the author(s).

### Authors' Contributions

SS and AN conceptualized the idea. SS, RVS and BG drafted the manuscript. RKS and APS Singh checked the manuscript. All authors minutely read and approved the manuscript.

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