

Original Article

Efficacy of lytic *Staphylococcus aureus* bacteriophage against multidrug-resistant *Staphylococcus aureus* in mice

Joseph Michael Ochieng' Oduor^{1,2}, Nyamongo Onkoba³, Fredrick Maloba⁴, Washington Ouma Arodi², Atunga Nyachieo^{1,5}

¹ Reproductive Health Biology Department, Institute of Primate Research (IPR), Nairobi, Kenya

² Medical Laboratory Science Department, School of Medicine, Kenyatta University, Nairobi, Kenya

³ Tropical Infectious Diseases Department, Institute of Primate Research (IPR), Nairobi, Kenya

⁴ Department of Zoological Sciences, School of Pure and Applied Sciences, Kenyatta University, Nairobi, Kenya

⁵ Department of Biochemistry, School of Medicine, University of Nairobi, Kenya

Abstract

Introduction: The use of bacteriophages as an alternative treatment method against multidrug-resistant bacteria has not been explored in Kenya. This study sought to determine the efficacy of environmentally obtained lytic bacteriophage against multidrug-resistant *Staphylococcus aureus* (MDRSA) bacterium in mice.

Methodology: *Staphylococcus aureus* bacterium and *S. aureus*-specific lytic phage were isolated from sewage and wastewater collected within Nairobi County, Kenya. Thirty mice were randomly assigned into three groups: MDRSA infection group ($n = 20$), phage-infection group ($n = 5$), and non-infection group ($n = 5$). The MDRSA infection group was further subdivided into three groups: clindamycin treatment (8 mg/kg; $n = 5$), lytic phage treatment (10^8 PFU/mL ($n = 5$), and a combination treatment of clindamycin and lytic phage ($n = 5$). Treatments were done at either 24 or 72 hours post-infection (p.i.), and data on efficacy, bacterial load, and animal physical health were collected.

Results: Treatment with phage was more effective (100%) than with clindamycin (62.25% at 24 hours p.i. and 87.5% at 72 hours p.i.) or combination treatment (75% at 24 hours p.i. and 90% at 72 hours p.i.) ($p < 0.001$).

Conclusions: The results show that the environmentally obtained *S. aureus* lytic bacteriophage has therapeutic potential against MDRSA bacterium in mice.

Key words: MDRSA; efficacy; phage therapy; waste and sewage water.

J Infect Dev Ctries 2016; 10(11):1208-1213. doi:10.3855/jidc.7931

(Received 20 November 2015 – Accepted 19 May 2016)

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Introduction

Globally, antimicrobial resistance is a public health concern [1]. Most pathogenic bacteria such as *Staphylococcus aureus* have been reported to be resistant against multiple classes of antibiotics [2]. Antimicrobial resistance is a consequence of unmonitored antibiotic use in hospitals, homes, and farms [3]. The exposed bacteria gets sensitized to antibiotics and acquires resistance genes (e.g., *S. aureus* acquires the *mecA* gene that makes it resistant to methicillin) [4]. However, strains of methicillin-resistant *S. aureus* (MRSA) have also been documented to have resistance against glycopeptides, macrolides, oxalinezolid, daptomycin, and dalfopristin [5,6]. This means that *S. aureus* possesses resistance against an array of antibiotic classes, which is referred to as multidrug-resistant *S. aureus* (MDRSA). In humans and animals, MDRSA infections are acquired through

inhalation [7], direct contact with infected objects, persons, or animals, and consumption of contaminated animals products [8] or water [9].

Infections due to MDRSA are destructive and often lead to amputations and multi-organ pathologies [10,11]. There are concerted efforts in antibiotic discovery and development, but the process is slow and expensive [12]. Therefore, there is a need for an alternative method of treating infections caused by MDRSA. The method should be cheap, versatile, feasible, as well as effective. The use of bacteriophages is one such method and its applicability in sub-Saharan Africa should be explored. Phage therapy in some regions of Eastern Europe has been reported to possess some potential in treating bacterial infections [13].

Bacteriophages are ubiquitous viruses that parasitize on bacteria [14], regulating their density in humans, animals, and the environment [13,14]. Phages

possess specificity and are bactericidal in nature [16]. In applied medicine, the use of phages is feasible due to their auto-dosing, where only a single dose is required for phage multiplication at the infection site compared to antibiotics that require several doses [17]. Despite these promising findings, the application of phages has not been explored in most developing and developed countries. Therefore, the present study was designed to isolate *S. aureus*-specific lytic phage and to determine its efficacy against MDRSA in mice.

Methodology

Bacterial isolation

A *Staphylococcus aureus* bacterial strain was isolated from sewage and wastewater samples collected from within Nairobi County, Kenya. The waste and sewage water samples were collected from 20 sites within the county and transported to the microbiology laboratories of the Institute of Primate Research, Kenya. The water samples were decanted and a loopful of the supernatant was streaked on selective mannitol salt agar (Liofilchem, Roseto degli Abruzzi, Italy) supplemented with 4 µg of ciprofloxacin (Liofilchem, Roseto degli Abruzzi, Italy) and incubated at 37°C overnight in aerobic conditions. Prior to *in vitro* and *in vivo* assays, the bacterial isolates were sub-cultured in nutrient broth (NB) (HiMedia, Mumbai, India) at 37°C for 18 hours, centrifuged at 2,000 g for 5 minutes, and washed and diluted in saline to 10⁸ colony-forming units per milliliter (CFU/mL). Strains of *Staphylococcus* were identified using microscopy, physiological tests, and the analytical profile index of *Staphylococcus* (API STAPH) system (Bio-Mérieux, Marcy l'Etoile, France). Antimicrobial resistance and susceptibility profiles of the isolated bacterium against multiple antibiotics was determined according to the Clinical and Laboratory Standards Institute (CLSI) protocol [18]. The antibiotics used were ceftazidime, oxacillin, vancomycin, netilmicin, gentamicin, erythromycin, cefuroxime, and trimethoprim-sulfamethoxazole (Liofilchem, Roseto degli Abruzzi, Italy).

Phage isolation

Sewage and wastewater samples were ultra-filtered, and a mixture of the ultra filtrate in NB and 18-hours-old MDRSA culture was added prior to overnight incubation at 37°C while shaking the culture at 120 rpm (Lab-Line Incubator-Shaker, Waltham, USA). After 18 hours of incubation, the culture was centrifuged at 10,000 g for 10 minutes (Fisher Centrifuge, Waltham, USA), and the supernatant was filtered

through a 0.22 µm filtration unit (µStar LB, ref. no. 8110) for phage screening using a double-layer plaque assay [19]. The resultant plaques were sub-cultured in 2 mL of NB containing sensitive bacterial host (10⁶ CFU).

In vitro screening for phage anti-MDRSA activity

A lawn of MDRSA isolate of McFarland standard 2 (6.0 × 10⁸ CFU/mL) was made on a dry nutrient agar (1.5%) using the spread inoculation method and 5 µL of pure lysate spotted on the lawn. Sterile normal saline of equivalent volume was used as a control and the plates were incubated at 37°C overnight.

Study animals

Thirty BALB/c mice of mixed sexes, between 6 and 8 weeks of age, were sourced from the rodent facility of the Institute of Primate Research (IPR), Kenya, and used in the study. The mice were fed with antibiotic-free food rations (Unga Feeds, Nairobi, Kenya), and water was provided *ad libitum*.

Ethical statement

All experimental protocols and procedures used in this study were reviewed and approved by the Institutional Review Committee (IRC) on Animals Ethics of the Institute of Primate Research (ref. no. IRC/02/14) in accordance with the international guidelines on animal care, handling, and use for biomedical research. The experiments are reported in accordance with Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines [20].

Experimental design

The study animals were randomly assigned into three groups: the MDRSA infection group (n = 20), the non-infection group (naïve) (n = 5), and the phage infection group (control phage) (n = 5). The 20 mice in the infection group were infected with 10⁸ CFU/mL of MDRSA isolate intravenously via tail vein, and the mice were subdivided into four subgroups (n = 5): MDRSA treated with clindamycin (8 mg/kg body weight), MDRSA treated with phage (10⁸ PFU/mL), MDRSA treated with a combination (clindamycin [8 mg/kg body weight] and phage [10⁸ PFU/mL]), and MDRSA with no treatment (MDRSA only). Mice were either treated at 24 or 72 hours post-infection (p.i.). The experiments were repeated three times.

Systemic bacterial blood load

Whole blood (50 µL) was sampled daily from the tail vein of each mouse for 10 days, serially diluted with

normal saline (1:20). This mixture was plated on nutrient agar containing 7.5% sodium chloride and was incubated at 37°C for 18 to 20 hours for MDRSA selection. The blood samples of the phage-infected non-treated group were processed to determine phage titer by double-layer plaque assay.

Statistical analysis

Bacterial and phage counts were represented as mean ± standard error of the mean. A two-way analysis of variance (ANOVA) was used to compare differences between groups, and Bonferroni post-hoc analysis was done to determine the levels of statistical significance between groups by Graph Pad Prism 5.0.1 (GraphPad Software, San Diego, USA). A p value < 0.05 was considered statistically significant.

Results

Bacteria isolation

The isolated *S. aureus* bacterium showed resistance to ceftazidime, oxacillin, vancomycin, netilmicin, gentamicin, cefuroxime, and erythromycin, but was susceptible to trimethroprim-sulfamethoxazole (Table 1).

Phage isolation and in vitro screening for phage anti-MDRSA activity

Table 1. *Staphylococcus aureus* isolate resistance to various antibiotics compared with ATCC 43300 (MRSA) isolate.

ID Number	Antibiotic disk	ATCC 43300 (MRSA) inhibition zone diameter (mm) (QC)	<i>S. aureus</i> environmental isolate inhibition zone diameter (mm)	Results interpretation
1	Vancomycin (VAN) 30 µg	13	12	Resistant
2	Ceftazidime (CAZ) 30 µg	11	8	Resistant
3	Oxacillin (OX) 1 µg	9	6	Resistant
4	Trimethroprim-sulfamethoxazole (SXT) 25 µg	24	30	Susceptible
5	Cefuroxime (CXM) 30 µg	23	32	Resistant
6	Netilmicin (NET) 30 µg	14	10	Resistant
7	Erythromycin (E) 15 µg	12	9	Resistant
8	Gentamicin (CN) 10 µg	9	7	Resistant

MRSA: methicillin-resistant *Staphylococcus aureus*; QC: quality control.

Table 2. In vitro activity of the isolated lytic phage strains against the MDRSA isolate on nutrient agar media.

Phage strains	Phage plaque diameter size (mm)	
	MDRSA lawn	<i>Escherichia coli</i> lawn
A	12	0
B	14	0
C	20	0
D	40	0
E	15	0
F	10	0

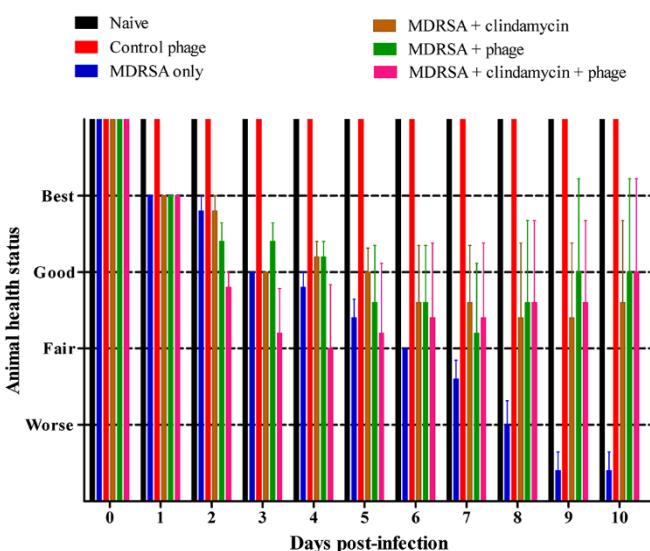
MDRSA: multidrug-resistant *Staphylococcus aureus*

The lytic phage obtained from the wastewater and sewage samples showed bacterial specific virulence towards MDRSA. The virulent phages had plaque diameter of more than 20 mm compared to non-virulent ones that had a diameter of less than 14 mm (Table 2).

Health status and survivorship

There were no adverse events observed in animals infected with lytic phages (Figure 1). Mice in the phage infection and the non-infection groups had a 100%

Figure 1. Animal health status scores of mice.



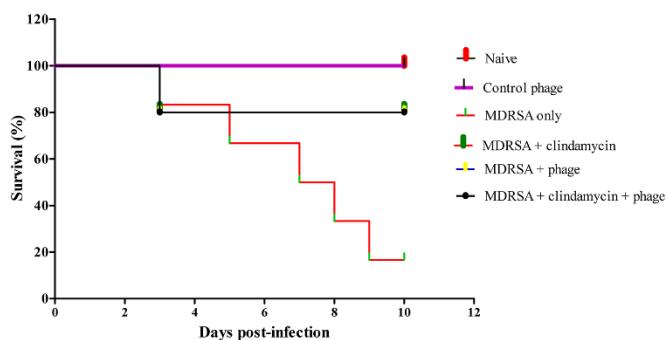
survival rate compared to those of the MDRSA infection group, while those that were infected and did not receive treatment had a 20% survival rate by day 10. However, when treated either at 24 hours or 72 hours p.i., survival rates were 80% and 60%, respectively, within the same period (Figure 2).

Efficacy study of phage therapy

Clindamycin or combination treatment at 24 hours or 72 hours p.i. did not clear MDRSA bacterial load in the blood of the infected mice by day 10 p.i. However, phage treatment was effective, as the mice had 0 CFU/mL bacteria by day 9 p.i. ($p < 0.001$) (Figure 3).

Brain, lung, and liver homogenate cultures of mice treated with phage subsequently had 0 CFU/gm of MDRSA, while those that were treated with clindamycin and a combination treatment had an average of 2.0 CFU/gm each (Table 3).

Figure 2. Survival rates.



Persistence of the phage in the animal system

There was no observed phage circulating in the blood of the phage-infected non-treated mice 96 hours p.i (Figure 1). On the contrary, mice from the MDRSA-infected phage-treated group showed a presence of phage in the liver tissue. MDRSA-infected mice that received a combination treatment had lower phage PFU/mL counts than those treated with phage only (Table 4).

Figure 3. Blood bacteremia and viremia levels of mice.

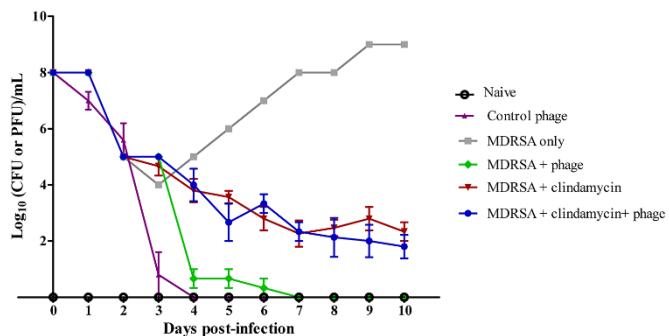


Table 3. End point (day 10) bacteria count (mean \log_{10} CFU/g \pm SE) isolate from organs.

Groups	Mean \log_{10} CFU/mL \pm SE at day 10 p.i		Mean \log_{10} CFU/g \pm SE at day 10 p.i		
	Treatment at 24 hours post infection	Treatment at 72 hours post infection	Brain	Lungs	Liver
Naive	0.0	0.0	0.0	0.0	0.0
Control phage	0.0	0.0	0.0	0.0	0.0
MDRSA only	8.0 \pm 0.2	9.0 \pm 0.2	7.2 \pm 0.2	7.0 \pm 0.2	9.0 \pm 0.2
MDRSA + clindamycin	3.0 \pm 0.2 (62.25%)	1.0 \pm 0.2 (87.5%)	3.0 \pm 0.2	1.4 \pm 0.2	1.6 \pm 0.2
MDRSA + phage	0.0 (100%)	0.0 (100%)	0.0	0.0	0.0
MDRSA + clindamycin + phage	2.0 \pm 0.2 (75%)	0.0 (100%)	0.0	4.0 \pm 0.2	2.0 \pm 0.2

MDRSA: multidrug-resistant *Staphylococcus aureus*; p.i.: post-infection.

Table 4. End point phage count (mean \log_{10} PFU/ml \pm SE) from the liver.

Groups	Mean \log_{10} PFU/gm \pm SE	
	24 hours p.i. treatment	72 hours p.i. treatment
Non-infected, non-treated	0.0	0.0
Phage infected, non-treated	3.0 \pm 0.2	4.0 \pm 0.2
MDRSA infected, non-treated	0.0	0.0
MDRSA infected + clindamycin treated	0.0	0.0
MDRSA infected + phage treated	7.0 \pm 0.2	8.0 \pm 0.2
MDRSA infected + clindamycin + phage	2.0 \pm 0.2	3.0 \pm 0.2

MDRSA: multidrug-resistant *Staphylococcus aureus*; p.i.: post-infection

Discussion

The findings show, for the first time, that multidrug-resistant *Staphylococcus aureus* is present in sewage and wastewater collected from within Nairobi County, Kenya. The isolated bacterium showed antimicrobial resistance to ceftazidime, oxacillin, vancomycin, netilmicin, gentamicin, cefuroxime, and erythromycin, but was susceptible to trimethoprim-sulfamethoxazole. The observed multidrug resistance can be attributed to the abundance of antibiotics in the community due to easy accessibility through over-the-counter purchases, over-prescription, unmonitored use in local hospitals, and abuse of these drugs at home and in farms as animal additives [21] that later get dumped into the environment.

Phage was non-pathogenic in mice since it did not show any evidence of adverse side effects. Similarly, the lytic phage isolated was able to prevent growth of *S. aureus* on nutrient agar and establishment of infection in mice. Furthermore, the phage was 100% effective against MDRSA infection, even at the fatal sepsis stage (72 hours p.i.). The absence of circulating bacteria in the blood of phage-treated mice clearly showed the efficacy of phage therapy. Clindamycin and phage-clindamycin achieved 62.5% and 75% efficacy, respectively, at 24 hours p.i. treatment. Administration of a single dose of clindamycin at 72 hrs p.i. only achieved 75% efficacy, as there were still bacteria circulating in mice blood by day 10. On the contrary, a similar dose of phage-clindamycin was 85% effective when it was administered within the same time frame.

The efficacy of clindamycin was dependent on dosage and administration time. Phage-clindamycin efficacy was dependent on time, as there were a few bacteria circulating in the blood of the mice by day 10. This finding has, for the first time, shown that clindamycin antagonizes the phage activities against targeted pathogenic bacteria (MDRSA). However, phage therapy efficacy was independent of dosage and time. The phage-treated MDRSA infection group had 0 CFU/mL bacteremia level by day 9 when the mice were treated at either 24 hours or 72 hours p.i. This can be attributed to the capability of phages to auto-dose at the infection site, thus clearing the bacteria. This is evident where a single dose of phage (10^8 PFU/mL) reduced 8 log cycles of bacterial load to 0 CFU/mL [17]. The study shows that the auto-dosing ability of phages is advantageous over antibiotics, as the efficacy of the antibiotic is dependent on multiple or intermittent administration. These findings corroborate results observed in others studies [20,21].

Conclusions

The study shows that *S. aureus*-specific lytic phage available in waste and sewage water of Nairobi County can act as an alternative treatment option for bacterial infections caused by MDRSA bacterium. In addition, the study offers proof of the concept that phage therapy can be used to combat antimicrobial resistance in sub-Saharan Africa.

Acknowledgements

We acknowledge IPR staff for providing logistical support during experimentation, Nairobi city water and sewage company management for providing us with samples from their sewage treatment plants, Dr. Geoffrey Omuse and Dr. Allan Njoroge (Aga Khan University Hospital, Nairobi, Kenya) for donating ATCC 43300, and Prof. Andrzej Górski and Mr. Marzena Lusiak-Szelachowska (Institute of Immunology and Experimental Therapy, Wrocław, Poland) for providing the protocols on phage isolation.

Authors' contributions

OJMO, AWO, and NA designed the study and the experimental protocols. OJMO conducted the experiments and collected and analyzed the data. MF assisted in reading the histology slides. OJMO and ON wrote the article. All authors read and approved the final article for publication.

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Corresponding author

Joseph Michael Ochieng' Oduor
 Department of Reproductive Biology
 Institute of Primate Research
 End of Karen Road
 00502 Nairobi, Kenya
 Phone: +254-202606235/6
 Fax: +254-202606231
 Email: josemisredo@gmail.com

Conflict of interests: No conflict of interests is declared.