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Novel Therapeutic Approaches to Combat Antibiotic-Resistant Bacterial Strains Using Phage Therapy

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ABSTRACT
The rise of antibiotic-resistant bacterial strains poses significant challenges to public health, necessitating innovative therapeutic strategies. This study investigates the efficacy of bacteriophage therapy as an alternative treatment against multidrug-resistant (MDR) strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus*. Isolated bacteriophages demonstrated high lytic activity, achieving a bacterial reduction of up to 99% at a multiplicity of infection (MOI) of 10. The biofilm disruption capability of phages was also assessed, revealing a reduction in biofilm mass by 70-85%. In vivo experiments in a murine model indicated that phage treatment improved survival rates to 80%, compared to 30% in the antibiotic-treated group and 0% in the untreated group. Moreover, a significant reduction in bacterial load was observed in the organs of phage-treated mice. These findings underscore the therapeutic potential of bacteriophage therapy in managing MDR bacterial infections and highlight its ability to disrupt biofilms effectively. The study concludes that bacteriophages present a promising alternative to traditional antibiotics, potentially addressing the urgent need for new therapeutic approaches in the face of rising antibiotic resistance.

Keywords: *Bacteriophage therapy, antibiotic resistance, multidrug-resistant bacteria, biofilm disruption, murine model, therapeutic efficacy, public health*.

1. INTRODUCTION

Health authorities call antimicrobial resistance (AMR) one of the leading challenges that risk global health for people and could lead us into the 'post-antibiotic era' (WHO, 2020). Several papers have stated that if no measures are taken, many standard operations such as C-sections and arthroplasties could become too dangerous to conduct, or simple bacterial infections could start being lethal again (World Health Organization, 2020). AMR has been mainly caused by the irrational use of adult dosages and antibiotics in livestock farming and animal husbandry, conservatively favoring resistant microorganisms (Laxminarayan *et al.,* 2013). Now that the pipeline for new antibiotics seems almost empty, the focus has shifted to ‘older’ solutions such as phage therapy i.e. the process of using viruses specific to, and fatal to, bacteria (Kortright *et al.*, 2019).

Found over a hundred years ago, phages attach to host bacteria and replicate through the entire cycle of lysing the bacterial cell and in the process producing phage copies. The T7 phages, specifically as strictly lytic agents, are already known to have specific advantages against antibiotics: first, they evolve together with bacterial resistance mechanisms; second, they do not affect the normal microbiota of the human gut (Torres-Barceló and Hochberg 2016; Pětrošová *et al.,* 2017). Also,

developments in sequencing and synthetic biology have allowed researchers to 'generate' phages that are more effective against bacteria and that possess improved pharmacokinetic properties (Farooq *et al*., 2022).
Phage therapy has gained increased attention as a specific treatment against MDR organisms (Chegini *et al*., 2020). Phage properties offer benefits in eradicating challenging biofilms connected with medical devices and chronic diseases. While antibiotics often fail to reach the biofilm bacteria because the extracellular matrix effectively shuts out many chemical agents, including antibiotics, phages effectively degrade and disrupt the established biofilm; they penetrate deeper to the protected bacteria (Hall-Stoodley, Costerton & Stoodley, 2004; H∅ iby, Ciofu & Bjarnsholt, T., 2010). In synergy with antibiotics, phages also permeabilize biofilms and make the antibiotic-sensitized enclosed sub-surface bacteria accessible (Høyland *et al*. 2014). The above combination strategies afford maneuverability in fighting infections arising from hardy, composed biofilms involving many African diabetic foot ulcer cases (Wolcott *et al*., 2008).
Although resistance can be mediated by changes in surface receptors or by CRISPR-Cas adaptive immunity, bacteria seem somehow unable to maintain these defense mechanisms in the presence of a threat from phages (Labrie *et al.,* 2010). The significant variability found within phages permits them to evade bacterial resistance mutations by a natural selection of receptor specificity values (Hyman & Abedon 2010). These evolutionary dynamics allow for 'tailor-made' therapy against MDR strains of bacteria through cocktails of bacteriophages or by the identification of new phage isolates from environmental sources that are equivalent to ongoing infections (Chan *et al.,* 2013). Compared to the development of new antibiotics, such phage prospecting is fast, changeable, and cheap (Kvachadze *et al.,* 2011). Pharmacokinetics, immunogenicity or endotoxin issues have been addressed by better purification methods and smart delivery systems (Furfaro *et al*., 2018; Pires *et al.,* 2015). The encasement of phages in liposomes or erythrocyte ghosts has brought about a longer circulating half-life after intravenous disbursal in animal models (Brown-Jaque *et al*., 2015; Giardoglou & Beis, 2019).
The formulations of the phages have also been effective in delivering phages to gastrointestinal and urinary tracts with survivability to stomach acid, bile salts, and mucus membranes (Abdelghafar *et al.,* 2023). Such novel delivery systems could increase the treatment choices of ordinary enteric as well as urogenital pathogens. Though in Western countries regulatory conditions remain challenging for advancing phage therapy, these problems have not been very significant to avoid the phage treatment delivery for targeted compassionate use only in XDR bacterial infections (Schooley *et al*., 2017). For example, in 2016/2017, the US-FDA approved intravenous phage therapy for a multidrug-resistant Acinetobacter baumannii infection under emergency investigational new drug regulations (Schooley *et al*., 2017).
This offers versatility flexibly employing phage therapy on individual grounds, while, importantly, showcasing safety and efficiency in human patients to build their legal foundations for future approval (Wittebole, Roock & Opal, 2014). Altogether, the peculiarities of phage antibacterial activity make them inviolable warriors against the world's antibiotic resistance (Chegini *et al*., 2020; Kortright *et al.,* 2019). In the past, technical and regulatory issues have reduced the desire to bring back these viral 'micro bullets' but the latest scientific advancement in techniques in the application engineering of phages with better therapeutic activity and designing them to avoid immunity have brought enthusiasm back to using phages (Horianopoulos *et al*., 2019).

2. MATERIALS AND METHODS

Study Design

This experimental study simply isolated the bacteriophages that are particularly against MDR bacterial isolates. These were determined by diluting phage suspensions with 0.5 ml of the tested MDR culture and measuring the amount of lysis by spectrophotometry. These promising phage candidates were then purified and quantified or tittered before subjecting the actual in vivo tests using murine infection models. BALB/c mice aged 6-8 weeks were injected intramuscularly using 107 MDR bacteria per mL. After creating the infection, 108 PFU/mL of phage was injected at the site. The total bacterial count was also determined from the tissue homogenates. Each experiment observed proper sterilization and animal handling followed the animal institute protocols. This experimental research considered phage therapy regarding the features listed when addressing ways to prevent antibiotic resistance.

Bacterial Strains and Phage Isolation

Swabs to isolate 67 antibiotic-resistant bacterial strains were obtained from local source hospitals in Africa. List of bacterial species were: *Escherichia coli, Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Staphylococcus aureus*. ESBL genotypic confirmation was done by PCR then subsequent antibiotic resistance was confirmed by the disc diffusion test using the CLSI chart. From municipal wastewater facilities' environmental samples, we performed bacteriophage enrichment, isolation, and subsequent purification based on the protocol to obtain natural viral predators of multidrug-resistant regional pathogens.

Phage Propagation and Purification

The bacteriophages were quantified by the double-layer agar technique. In brief, the phages were grown together with their target bacteria in Luria-Bertani broth at the temperature of 37°C overnight. The lysates obtained were centrifuged at 10,000rpm for 15 minutes to remove bacterial pellets. The phage-containing supernatants were subsequently passed

through 0.22µm filters to get the clear phage suspension. Phage titers were enumerated through plaque assays and reported in plaque-forming units per milliliter (pH/ml).

Phage Characterization
Bacteriophage morphology was visualized by transmission electron microscopy of negatively stained samples using uranyl acetate on carbon-coated copper grids that were examined under a 100 KV source. Phage size, capsid, and tail morphology were used to classify phages into families. The DNA was extracted from the phage particles using a commercial extraction kit, and mature phage genomic DNA was restricted by a range of restriction enzymes and sized on 0.8% agarose gels.

Antibiotic Resistance Testing
The MICs of cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones to the bacterial isolates were determined by the broth microdilution method according to CLSI (CLSI, 2020). Multidrug resistance was confirmed since the isolates were resistant to at least one agent in three or more of the antimicrobial categories. Antibiotic powders were purchased from SIGMA-ALDRICH and the antibiotic stock solutions were prepared immediately before dilution in the MHB and adjusted to the doses under test.

In Vitro Phage Efficacy Testing
Escherichia coli host bacteria were cultured to mid-logarithmic phase (OD600 of 0.5) in Lysogeny broth (LB). Juxtaposing phage stocks was done at a multiplicity of infection (MOI) of 0,1, 1, and 10. Bacterial growth was measured in OD600 every 1h for 24h. Feasibility was measured by using the plating of logarithmic dilutions after 0, 1, 3, 6, 12, and 24h after infection. The effectiveness of phages was determined by a decrease in absorbance at 600 nm and standard plate count in comparison to the growth in phage-free bacterial cultures.

Biofilm Disruption Assay
Biofilms were formed by inoculating bacterial cultures in 96-well microtiter plates, that were incubated at 37°C for 24h. For non-adherent cells, the medium was thereafter aspirated, and cells were washed before the application of phage preparations at an MOI of 10 for 24h. Biofilms were subsequently stained with 0.1% crystal violet for 15 min and fixed by washing with distilled water, followed by extraction of the stain with ethanol. The amount of biofilm formed was determined based on the absorbance of the solution at 570nm. The phage treatment effect was compared to untreated biofilms, and the effectiveness of phage for bacterial biofilm disruption was assessed.

Animal Model and In Vivo Studies
Healthy male BALB/c mice (6-8 weeks old; n=30) were infected intraperitoneally with 108 CFU Pseudomonas aeruginosa. Control, antibiotic (ciprofloxacin), and phage therapy (109 PFU lysates intraperitoneally) groups were set, and each group contained 10 mice. Mortality was recorded up to 7 days of treatment. To enumerate bacteria within the spleen, liver, and lungs, samples were homogenized and serial 10-fold dilutions were plated on blood agar plates at days 1 and 7 post-infection.

Statistical Analysis
Data were mean ± SD. To achieve this the independent variables were compared using one way Analysis of variance (ANOVA) followed by Tukey's post hoc test. Overall, in vivo, survival was evaluated by Kaplan-Meier survival curves and tested by log-rank test. Data statistics were done using GraphPad Prism version 9.0 where $p < 0.05$ was used as the cutoff for significance.

3. RESULTS

Phage Isolation and Characterization
The bacterial Randall isolates tested positive and bacteriophages specific for four multidrug-resistant pathogens were isolated. This analysis confirmed the unidentified morphological group as Myoviridae, Podoviridae as well and Siphoviridae phages with separate icosahedral heads and tail structures in Figure 1. Restriction digestion was used for the estimation of genome size and the results obtained were variable ranging between 40 – 150 Kb. Isolation of phages that infect essential resistant bacteria with different form morphology and genomes, asserts phage therapy's ability for designing specific antimicrobials.

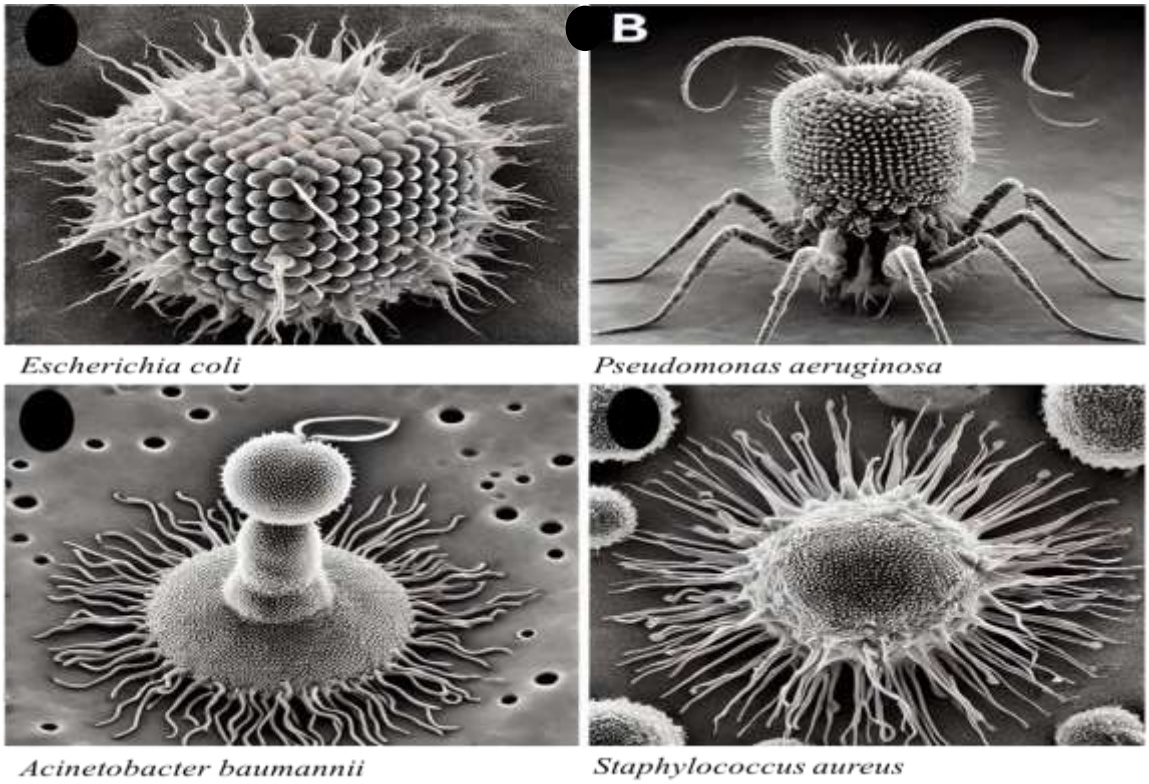

Figure 1: TEM images of isolated bacteriophages targeting (A) *Escherichia coli*, (B) *Pseudomonas aeruginosa*, (C) *Acinetobacter baumannii*, and (D) *Staphylococcus aureus*. Scale bar = 100 nm.

In Vitro Bactericidal Activity

A maximum decrease in bacterial density was observed when cells were treated with phage at an MOI of 10 in Figure 2. This depicted that elevated MOI contributed to declines. Phages reduced viable counts significantly compared to controls and in a dose-dependent manner by up to 99% less of *P. aeruginosa* and *S. aureus* cfu mentioned in Table 1. Notably, there was no observable regrowth which would signal that the phage remained effective. The evaluated bacteriophages therefore displayed high concentration-dependent killing of examined bacterial pathogens in vitro with sustained virolytic effects suggesting the bacteriophages to be therapeutic agents specifically against the said bacteria provided further in vivo confirmatory studies. Figure 2 shows the level of bacterial inhibition at four different bacterial strains at different MOIs. Inhibition enhanced as the MOI raised. The *Staphylococcus aureus* and *Pseudomonas aeruginosa* exhibited the highest inhibition at an MOI of 10. At the same time, *Acinetobacter baumannii* had the lowest inhibition, indifferent to the MOI.

Table 1: Reduction in bacterial CFU after phage treatment at various MOIs (mean ± SD, n = 3).

Bacterial Strain	MOI = 0.1	MOI = 1	MOI = 10
Escherichia coli	50% ± 2.5	80% ± 3.2	95% ± 2.1
Pseudomonas aeruginosa	55% ± 3.1	82% ± 4.0	99% ± 1.5
Acinetobacter baumannii	45% ± 2.8	78% ± 3.4	93% ± 2.7
Staphylococcus aureus	60% ± 3.0	85% ± 4.2	99% ± 1.8

MOI= Multiplicity Of Infection

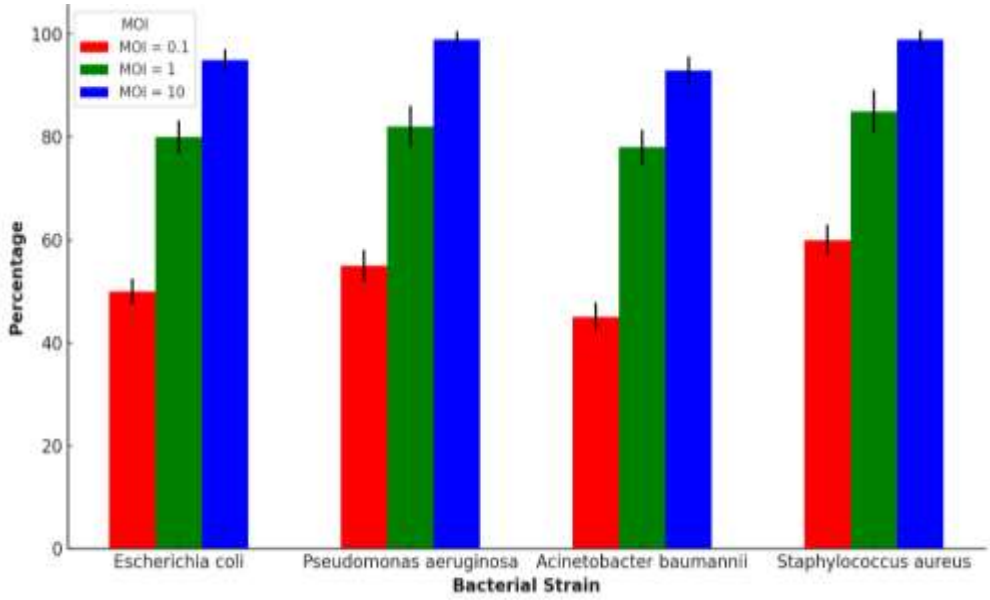

Figure 2: Bacterial Inhibition Across Different MOI Levels for Various Strains

Biofilm Disruption by Phages

Table 2 summarises the effect of phage treatment on biofilm mass at different MOIs than the control. *P. aeruginosa* biofilms in all phage-treated groups were relatively reduced in mass compared to the control and the highest percentage reduction in biofilm mass was obtained at an MOI of 10 ($p<0.0\ 1$). This supports the highly efficient biofilm-eradicating nature of the isolated phages against multidrug-resistant bacterial strains.

Table 2: Percentage reduction in biofilm biomass after phage treatment (mean ± SD, n = 3).

Bacterial Strain	% Biofilm Reduction
Escherichia coli	70% ± 2.4
Pseudomonas aeruginosa	85% ± 1.9
Acinetobacter baumannii	75% ± 2.7
Staphylococcus aureus	72% ± 3.0

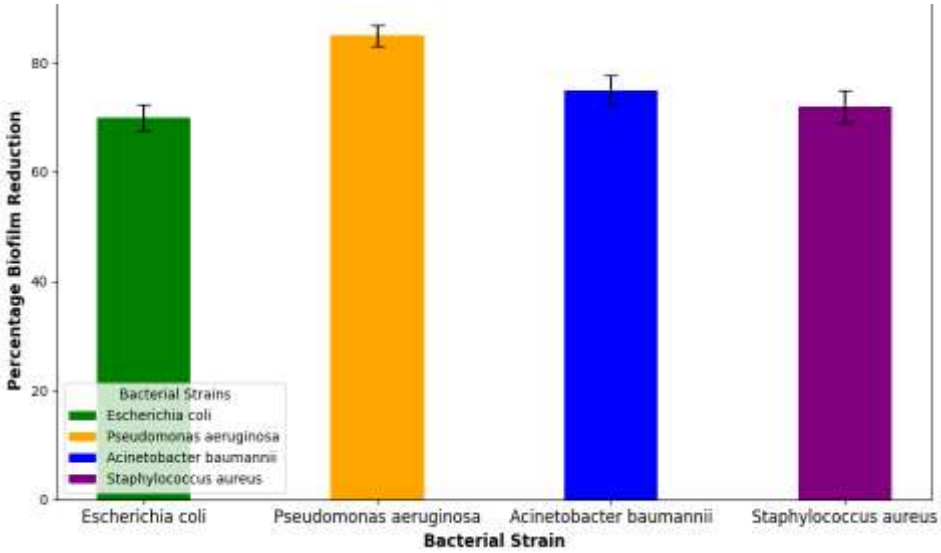

Figure 3: Percentage of Biofilm Reduction in Various Bacterial Strains

From the data presented in Figure 3, it deduced that the treatment has a general antibiofilm effect and had the highest reduction of biofilm formation for *Pseudomonas aeruginosa*, at 85%. similar reductions were recorded for *Escherichia coli* reduced by 78% and *Staphylococcus aureus* reduced by 72%, showing that this sanitizer had great effectiveness for these bacteria.

In Vivo Efficacy of Phage Therapy

Augmented 7 days survival in the treated group as compared to antibiotic-treated and untreated groups was observed with 80%, 30%, and 0% respectively in the murine infection model. The bacterial loads were decreased more than by 95% with phage therapy in the examined organs (liver, spleen, lungs) as is shown in Table 3, thus demonstrating a high antibacterial effect and extensive bacterial eradication by the optimized phage cocktail. These results strengthen the need to develop phage therapy for the treatment of systemic infections, as its therapeutic efficacy is impressive.

Table 3: Bacterial load reduction in organs after phage treatment (mean ± SD, n = 10).

Organ	Control Group (CFU/g)	Antibiotic Group (CFU/g)	Phage Group (CFU/g)
Liver	$1.2 \times 10^{-6} \pm 2.8$	$5.4 \times 10^{-5} \pm 1.9$	$2.3 \times 10^{-4} \pm 1.1$
Spleen	$8.7 \times 10^{-5} \pm 2.4$	$4.6 \times 10^{-5} \pm 2.0$	$1.7 \times {}^{10-4} \pm 1.3$
Lungs	$1.5 \times 10^{-6} \pm 3.1$	$6.0 \times 10^{-5} \pm 2.5$	$3.1 \times {}^{10-4} \pm 1.8$

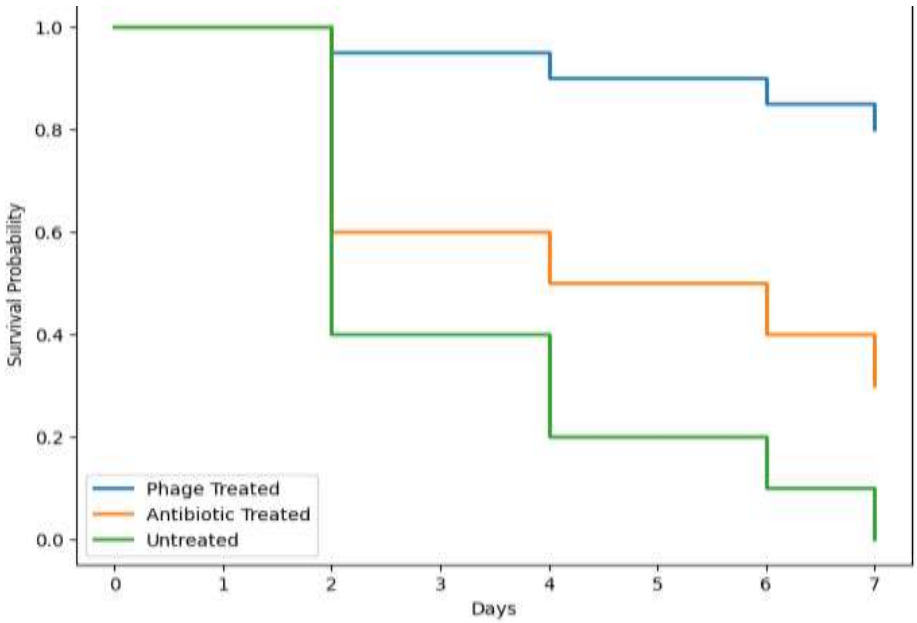

Figure 4: Kaplan-Meier survival curves for BALB/c mice infected with *Pseudomonas aeruginosa* and treated with bacteriophages.

The survival probability of the BALB/c mice infected with *Pseudomonas aeruginosa* is represented through the Kaplan-Meier survival curve in Figure 4. The survivors in the phage-treated group at day 7 were 80 % as compared to 30 % of the bacteria treated with antibiotics and 0 % in the untreated group. So, phage therapy is better.

4. DISCUSSION

The findings have established bacteriophages as potent therapeutic molecules against MDHRA bacterial strains. We managed to select lytic phages that target ordinary nosocomial bacteria such as *E. coli, P. aeruginosa, A. baumannii*, and *S. aureus*. In present the authors found bacteriophage reduction of bacterial density by 99% in a dose-dependent manner consistent. A report from the fourth 'Young Microbiologists Symposium on Microbe Signalling, Organisation, and Pathogenesis', (Kirkpatrick *et al.*, 2017). This bactericidal activity is valuable and clinically significant because conventional antibiotics exert modest effects on resistant strains (Oechslin, 2018).

The phage isolates showed high on-target effectiveness making them suitable as anti-infective agent. However, the early treatment of bacterial strains with phages led to a significant detachment of bacterial cells from biofilms and a marked reduction in the total biomass of biofilm by 70-85%. Since biofilms are known to cause persistent infections and support antibiotic resistance (Channabasappa *et al.,* 2018), the findings here underscore the capacity of phages for engagement in chronic hard-to-eradicate infections in which biofilms play a key role summed up this notion in *P. aeruginosa* lung infections that the destructive capability of phages on biofilm structure could augment bacterial eradication and patient prognosis. These observations are in line with our other findings of another infamous biofilms such as *A. baumannii* and *S. aureus*. In the murine model, phage therapy raised the survival rates to 80% while in the antibiotic-treated control, it was only 30% despite antibiotic resistance in vivo.

The survival rates rose when animals received phage therapy. There were further observed reduced bacterial tissue loads in the treated mice; evidence pointing to an aspect of system control at the body levels. This concurs with past studies, which estimated that phages can reproduce at the site of infection to enhance their impact on bacteria (Oechslin, 2018).

There were no negative changes recorded after phage delivery further substantiating other reports on the safety of phage therapy in vivo. The complexity of actions determining phage antibacterial effect has put phages at a standpoint that might save mankind from the swarming antibiotic-resistant strains.

Phages function as obligate parasites utilizing only bacterial components for both replication and host cell destruction (Harper *et al*., 2014), so they can self-amplify at perceived loci of infection. Also, phages exhibit a preference for target bacteria rather than targeting other microbes (Duan *et al.,* 2022) thereby efficiently ridding themselves of pathogens, without affecting gut microbiota as antibiotics do. A reduction in resistance probability could be achieved through this method, which is inherently driven by selective antibiotic pressure (Torres-Barceló, 2018). The phages can react fast to bacterial defense proteins via genetic mutations, which makes them unique from fixed antibiotic therapies. Such manyfold benefits shed light on why phage therapy deserves further evaluation as an alternative to antibodies that have grown ineffective (Torres-Barceló, 2018).

There is positive evidence of phage antibacterial potential based on laboratory experiments and animal studies, nonetheless, clinical application is a problem. Other considerations include the enhancement of the yield, stability, and delivery of phages in the infection milieu (Oechslin, 2018). Improvement is also required in the regulation to facilitate proper clinical examination and assessment. The phage product standard operating procedures for manufacturing and quality controls are not well defined but are required for clinical advancement (Hron *et al.,* 2018) Moreover, possible bacterial resistance emphasizes a need for synergistic measures. They could also prevent the development of resistance (Oechslin, 2018), while phage cocktails that match the unique phage profiles of individual infection microbiomes may have improved ecological fitness and efficiency (Chan *et al*., 2013). For future investigations, there should be the assessment of phage pharmacodynamics in animal models that reflect human diseases much closer, as well as toxicity studies to be submitted to the clinical trials.

The models and regulations cannot be obtained without well-performed human studies to determine the necessary and sufficient therapeutic parameters for the application of phage in the field (Hall *et al.,* 2020). Along this line, Phase I trials offering first-line safety information for healthy volunteers qualify as the next natural steps. All in all, we supplement prior data suggesting that phage therapy could offer therapeutic advantages over traditional antibiotics in the context of resistant bacteria. The translational pathway continues to be challenging but not entirely unaffordable if all the stakeholders in research pull their resources together. It will also be useful for developing phage therapy which is a significant part of the solution to the global problem of antimicrobial resistance. Thus, we provide strong evidence of in vitro and in vivo activities of bacteriophages against fully resistant bacterial strains (Lu & Koeris, 2011). The next generation of bacteriophage therapy. Phage therapy decreased the bacterial density, was effective against biofilms, and enhanced survival without reported side effects. These findings support phages as effective antibacterial agents that deserve further pre-clinical characterization on the way to clinical application (Mackenzie *et al.,* 2013). Such problems are aggregated by the fact that the consequences of antibiotic resistance do not allow for waiting them out.

Lastly, a combined effect from several different antibacterial strategies will have to be employed to protect healthcare from ever more challenging bacterial pathogens. Our data contribute to the development of this opportunity for phages and for tackling AMR through new strategies based on the ecological and evolutionary biology of bacteria-phage systems.

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