Repetitive combined doses of bacteriophages and gentamicin protect against *Staphylococcus aureus* implant-related infections in *Galleria mellonella*

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Cite this article:
Bone Joint Res 2024;13(8):
383–391.

DOI: 10.1302/2046-3758.
138.BJR-2023-0340.R1

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Aims

Bacteriophages infect, replicate inside bacteria, and are released from the host through lysis. Here, we evaluate the effects of repetitive doses of the *Staphylococcus aureus* phage 191219 and gentamicin against haematogenous and early-stage biofilm implant-related infections in *Galleria mellonella*.

Methods

For the haematogenous infection, *G. mellonella* larvae were implanted with a Kirschner wire (K-wire), infected with *S. aureus*, and subsequently phages and/or gentamicin were administered. For the early-stage biofilm implant infection, the K-wires were pre-incubated with *S. aureus* suspension before implantation. After 24 hours, the larvae received phages and/or gentamicin. In both models, the larvae also received daily doses of phages and/or gentamicin for up to five days. The effect was determined by survival analysis for five days and quantitative culture of bacteria after two days of repetitive doses.

Results

In the haematogenous infection, a single combined dose of phages and gentamicin, and repetitive injections with gentamicin or in combination with phages, resulted in significantly improved survival rates. In the early-stage biofilm infection, only repetitive combined administration of phages and gentamicin led to a significantly increased survival. Additionally, a significant reduction in number of bacteria was observed in the larvae after receiving repetitive doses of phages and/or gentamicin in both infection models.

Conclusion

Based on our results, a single dose of the combination of phages and gentamicin is sufficient to prevent a haematogenous *S. aureus* implant-related infection, whereas gentamicin needs to be administered daily for the same effect. To treat early-stage *S. aureus* implant-related infection, repetitive doses of the combination of phages and gentamicin are required.

Article focus

- Evaluation of repetitive doses of phages or antibiotics alone, or combination of both, against haematogenous and early-stage biofilm implant-related infections in *Galleria mellonella*.

Key messages

- A single combined dose of phages and gentamicin, and repetitive doses with gentamicin alone or in combination with phages, were able to prevent haematogenous infection.
- Only repetitive combined administration of phages and gentamicin was effective

against early-stage biofilm implant-related infection in *G. mellonella*.
- A significant reduction in number of bacteria was observed in the larval tissue as well as on the implant surface, with repetitive doses of phages and/or gentamicin against haematogenous and biofilm *Staphylococcus aureus* infections.

Strengths and limitations

- Repetitive administration of the combination of phages and antibiotics, such as gentamicin, is a potential approach in the fight against implant-associated infections in a clinical setting.
- This model has the limitation that the larvae lack an adaptive immune system and musculoskeletal system, which may limit conclusions on bone and joint infections.

Introduction

Orthopaedic implants such as joint prostheses and fracture fixation devices have substantially improved the quality of life of patients.[1] However, implant-associated bone and joint infections (BJIs), such as periprosthetic joint infections (PJIs) and fracture-related infections (FRIs), can pose life-threatening complications in orthopaedic and trauma surgery.[2] *Staphylococcus aureus* is a predominant bacterium involved in the colonization of implants and is responsible for 28% of PJIs and 37% of FRIs.[3] Additionally, most *S. aureus* strains isolated from BJIs have the ability to form biofilms.[4] In biofilms, bacteria are embedded in an extracellular matrix composed of self-produced extracellular polysaccharides, DNA, and proteins, or host-derived matrices such as fibrin.[5] Further, *S. aureus* can enter a 'persister' state, e.g. when invading osteoblasts and osteoclasts, characterized by reduced metabolic activity and increased tolerance to higher antibiotic concentrations.[6] The efficacy of antimicrobial drugs in biofilms is hindered by several factors such as the physical barrier of the biofilm matrix, increased resistance due to enhanced exchange of antibiotic resistance genes, and slower growth rates.[7] Hence, bacteria in the biofilm growth stage are up to 1,000 times more resistant to antimicrobial agents than their planktonic counterparts.[8] The treatment of infections critically depends on the duration of infection, e.g. biofilm maturation time, identified pathogens, and the status of the implant, such as whether the osteosynthesis material or endoprosthesis is loose.[9]

Implant-related infections are caused either by bacterial contamination during surgery or bacterial entry via the wound post-surgery (i.e. early-stage biofilm infection), or by seeding of the implant with bacteria from the bloodstream from another source of infection (i.e. haematogenous infection). A haematogenous infection, often caused by *S. aureus*, originates from a secondary infection, most often from the skin, gums/teeth, or urinary tract, and has travelled through the blood to the bone and implant.[10,11] Therefore, preventing bacterial attachment and colonization of bacteria on implants is a primary objective.

In the past three decades, the understanding of biofilm pathophysiology has led to optimized therapies of implant-associated BJI.[12] Substantial contributions have been made to the development of therapeutic strategies for systemic antibiotic treatment targeting biofilm.[13,14] For example, the combination of local and systemic administration of antibiotics for effective antibiofilm treatment has been recognized.[15,16] Against the background of the increase in antimicrobial-resistant bacterial strains, alternative approaches for antibiotics must be established to effectively prevent and treat BJI. This has prompted extensive research into implant modifications, such as coating of implants with antimicrobial compounds[17,18] and surface modifications,[19] and into combining administration of different antimicrobial agents.[20] Among the latter, one promising approach involves the use of bacteriophages, also known as phages, which are ubiquitous in the environment and the most abundant biological agent on earth.[21] Phages bind to bacteria through a specific receptor, inject its DNA into the host, replicate inside the bacterial cells, and ultimately cause the host cell to lyse, resulting in the death of the bacteria.[22] They have been considered as potential antimicrobial agents since their discovery by Felix d'Herelle,[23] and in recent years they have gained renewed attention as a therapeutic option against implant-associated BJI, particularly in light of emerging infections caused by multidrug-resistant bacteria.[23,24]

Whereas first preclinical studies on the efficacy of phages in the treatment of implant-associated infections are limited, further research is required to translate phage therapy into clinical practice.[25,26] Further, implant-associated infection models are required to evaluate the in vivo activity of phages. The insect larva model *Galleria mellonella*, which reduces the usage of higher mammalian models, has shown promise for studying implant-associated *S. aureus* biofilm infections.[27] In a recent study, Mannala et al[28] demonstrated the efficacy of the *S. aureus*-specific phage 191219 against a wide range of methicillin-sensitive (MSSA) and methicillin-resistant *S. aureus* (MRSA) bacteria in planktonic state, in biofilms, and even located intracellularly, and subsequently tested the effectiveness of the phage in a *G. mellonella* larva implant infection model. In this model, a single administration of phages failed to improve the survival rate, but there appeared to be a non-statistically significant enhanced effect when combined with gentamicin, whereas rifampicin showed a significant enhanced effect on the survival both in the absence and presence of phages.[28]

Therefore, our aim was to evaluate the repetitive administration of phages or gentamicin, either alone or in combination to improve the survival of *G. mellonella* larvae with a *S. aureus* implant-associated infection. In this study, we have assessed the efficacy of either a single dose or repetitive doses of phages, gentamicin, or their combination against a haematogenous infection following Kirschner wire (K-wire) implantation and against an early-stage biofilm infection on K-wires. Furthermore, we have analyzed the bacterial burden in the tissue of the larvae and on the surface of the K-wires to demonstrate the effect of the different (combined) antimicrobials on the number of bacteria.

Methods

Bacterial strain

In this study, the MSSA EDCC 5055 strain was used. This strain was isolated from a wound infection and is known for its high capacity for biofilm formation and sensitivity to gentamicin (minimum inhibitory concentration (MIC): ≤

2 µg/ml, determined by VITEK (bioMérieux, Germany)).[29] The whole genome sequence of this strain is currently available.[30,31] Brain heart infusion (BHI; Merck, Germany) broth was used to culture the bacteria aerobically at 37°C by constant shaking at 180 rpm.

Phages

The *S. aureus* lytic virulent phage 191219, which is specific for *S. aureus* and active against a wide range of *S. aureus* (MSSA and MRSA) strains,[28] was provided by D&D Pharma (Germany). At the concentrations of phages used in this study, gentamicin does not interfere with the replication of phage 191219 in *S. aureus* EDCC 5055. These phages were propagated in the laboratory using the *S. aureus* EDCC 5055 strain, as previously described.[28] Briefly, overnight cultures of *S. aureus* bacteria in BHI broth were subcultured into fresh broth and incubated on a shaker at 37°C until reaching an optical density of 1.0 at 600 nm. Subsequently, 5 ml of the phage solution, containing approximately 5×10^8 plaque-forming units (PFUs)/ml, was added to 25 ml of bacterial solution and further incubated at 37°C overnight. The phages present in the bacterial suspension were obtained by centrifugation at 13,000 rpm for ten minutes, followed by filtration of the supernatant through 0.45 µm and 0.2 µm filters. The concentration of the phage solution was determined using a plaque assay after serial-diluting the phage solution,[32] resulting in a final concentration of 2×10^{12} PFUs/ml.

Implants

Stainless steel K-wires with a diameter of 0.8 mm (Synthes, Switzerland) were cut into pieces with a length of 4 to 5 mm using a cable cutter, sharpened on one side, and sterilized in 70% ethanol before the experiment.

G. mellonella infection models

G. mellonella larvae were ordered from Evergreen (Germany) and maintained on wheat germ (Tropic Shop, Germany) at room temperature during the entire experiment. For each survival experiment, ten larvae in the last instar stage weighing around 400 to 450 mg were used per group, and each experiment was repeated three times (total of n = 30 per group). To determine the number of bacteria at the implant surface and in the tissue of the larvae, five larvae per group were used, and each experiment was repeated three times (total of n = 15 per group).

To mimic the haematogenous implant infection route, a K-wire was implanted at the rear end of the larvae by piercing their cuticle with the sharp end of the K-wire and incubated at 37°C for 24 hrs (Figure 1a). The next day, the larvae received an injection with 10 µl of *S. aureus* (5×10^5 colony forming units (CFU)/larva). The early-stage biofilm infection was modelled as previously published.[27] In short, K-wires were pre-incubated in a solution of *S. aureus* EDCC 5055 (5×10^6 CFU/ml) for 30 minutes at 150 rpm, washed with 10 ml phosphate-buffered saline (PBS; Thermo Fisher Scientific, USA) and subsequently implanted in the larvae as stated above (Figure 1b). Before implantation, the number of adhered bacteria was determined by sonicating additional K-wires (n = 4) at 45 kHz for two minutes (Ultrasonic Cleaner USAC-T; VWR, Germany), followed by serial dilutions and quantitative culture on LB agar plates (Carl Roth, Germany). After implantation, the *G. mellonella* larvae were maintained at 37°C.

Injection of gentamicin, phages, or the combination

Either one hour after receiving the injection with the bacterial inoculum (i.e. haematogenous implant infection model) or one day after implantation of the pre-inoculated implant (i.e. early-stage biofilm implant infection model), the larvae received an injection with 10 µl of phages (10^9 PFUs/larva) or PBS. After 30 minutes, 5 µl of gentamicin (60 mg/kg) or PBS was administered, to assess the effect of phages and gentamicin alone or in their combination (Table I). The larvae were incubated at 37°C and the survival was monitored for five days. To assess the effect of administration of repetitive doses, the injections were repeated daily for five days following the same procedure as described before (Table I). For the non-infected control group ('control') and the untreated control group ('*S. aureus*'), the same procedures were followed, this time implanting a (sterile) K-wire and injecting PBS instead of the antimicrobial agents.

Quantitative culture

The antimicrobial effect of repetitive doses of phages, gentamicin, or their combination was determined by retrieving bacteria from the implant surface and in the tissue of the larvae. Therefore, the implants were explanted from the larvae two days after the first injection with antimicrobial solutions, so having received two repetitive injections. Both the implants and the tissue of the larvae were collected in 2 ml PBS and processed for CFU analysis by sonication at 45 kHz for two minutes and homogenization using a micro tissue homogenizer (Fisher Scientific, Germany), respectively. The sonicates and homogenates were serially diluted and plated on LB agar plates supplemented with the antibiotic ampicillin to suppress growth of the skin flora of the larvae. The numbers of CFUs/sample were determined after overnight incubation at 37°C, and expressed as $\log_{10}$ CFUs per implant or $\log_{10}$ CFUs per 100 mg of larval tissue.

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism 9.5 (GraphPad Software, USA). For the analysis of bacterial numbers, the Mann-Whitney U test was applied. Differences between pairs of survival curves of the *G. mellonella* larvae were analyzed using the log-rank test. The data were represented as means (D) from three independent experiments with five or ten technical replicates each for the quantitative culture and survival experiments, respectively. The data were considered statistically significant if the p-value was ≤ 0.05.

Results

Combined administration of single dose of phages and gentamicin

To assess the ability of phages, gentamicin, or their combination in preventing haematogenous implant infection, the larvae first received an implant, and the following day they were infected with *S. aureus*. Subsequently, the larvae were administered either PBS, phages, gentamicin, or the combination of both. The larvae were incubated at 37°C, and their survival was monitored for five days. At five days

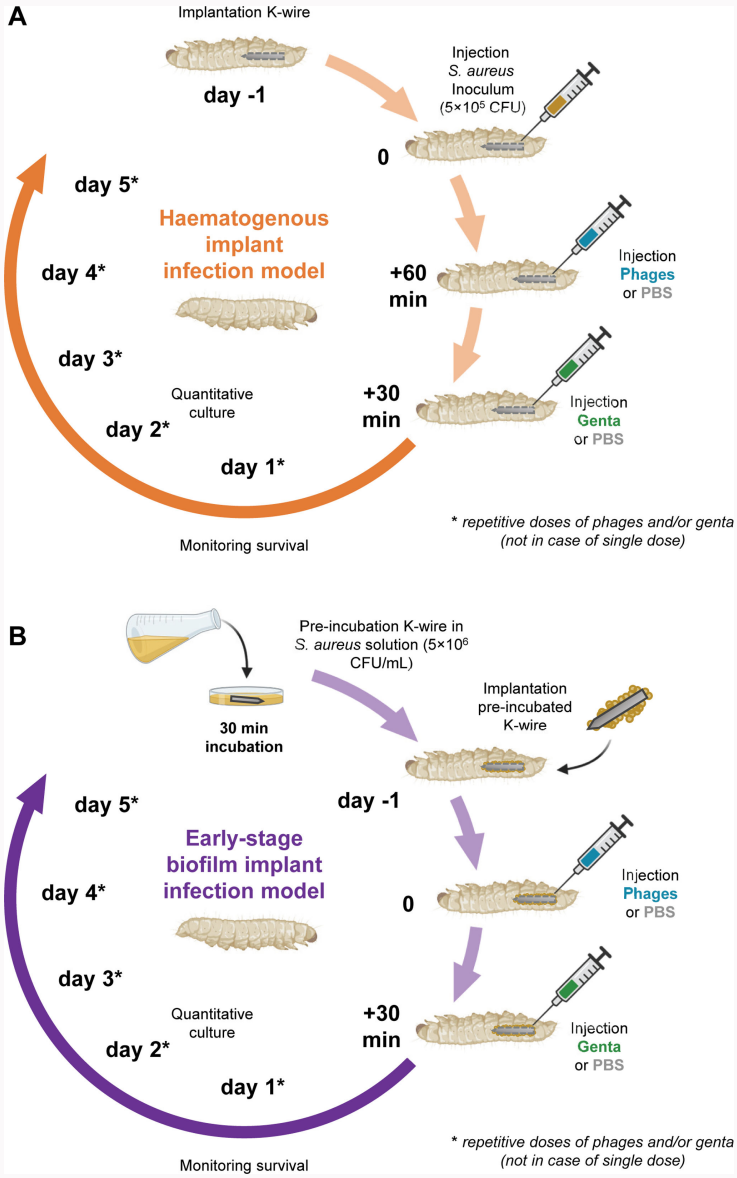

Fig. 1

Schematic overview of the *Galleria mellonella* implant-infection models used in this study. a) Haematogenous implant infection model: a sterile stainless steel Kirschner wire (K-wire) was implanted in the larva, and 10 µl *Staphylococcus aureus* inoculum (5×10^5 colony forming units (CFUs)/larva) was injected the following day. b) Early-stage biofilm implant infection model: a K-wire was incubated in a *S. aureus* solution (5×10^6 CFUs/ml) for 30 minutes before implantation in the larva. Either at 60 minutes after infection (haematogenous implant infection model) or 24 hours after implantation of the pre-incubated implant (early-stage biofilm implant model), the larvae received an injection of 10 µl bacteriophages ('Phages') or phosphate-buffered saline (PBS), followed by an injection of 5 µl gentamicin ('Genta') or PBS 30 minutes later. The survival of the larvae was monitored for five days. In the case of repetitive doses, the injections with antimicrobial solutions or PBS were repeated daily following the same procedure. Each experimental group contained 30 larvae. At two days after infection, the number of CFUs at the implant surface and in the tissue of the larvae was quantitatively determined (additional larvae, n = 15 per group).

after infection, the survival analysis revealed that injection of phages alone (46%; p = 0.274, log-rank test) or gentamicin alone (48%; p = 0.070, log-rank test) led to a non-significant improvement in larval survival, when compared to the control group receiving PBS injections (27%; Figure 2a). Importantly, the combination of phages and gentamicin resulted in a significantly improved survival (72%; p = 0.002, log-rank test). Thus, these results showed that combined administration of a single dose of phages and gentamicin is effective in preventing haematogenous *S. aureus* infection, whereas a single injection of either gentamicin or phages alone did not show a significant effect.

Repetitive administration of gentamicin alone or in combination with phages

To improve the survival of the larvae receiving phages and/or gentamicin, the larvae were administered daily doses of the respective treatments, both individually and in combination, for a period of five days. The survival of the larvae was monitored throughout this duration. The results revealed that repetitive administration of gentamicin alone significantly improved larval survival (68%; p = 0.039, log-rank test), whereas repetitive treatment with phages alone showed a non-significant increased survival rate (54%; p = 0.109, log-rank test) when compared to the control group receiving PBS (33%; Figure 2b). The combination of phages

Table I. Experimental conditions used in current study. Per experiment, ten larvae were used per condition, and the experiment was repeated three times (i.e. n = 30 per condition, per infection model).

Condition	Abbreviation	*S. aureus* infection	K-wire implant	First injection (10 µl)*	Second injection (5 µl)*
Non-infected control	Control	N/A	Yes	PBS	PBS
Control injection	*S. aureus*	Yes	Yes	PBS	PBS
Phage injection	*S. aureus* (P)	Yes	Yes	Phages	PBS
Gentamicin injection	*S. aureus* (G)	Yes	Yes	PBS	Gentamicin
Combined injection	*S. aureus* (P + G)	Yes	Yes	Phages	Gentamicin

*In case of repetitive doses, the injections with antimicrobial solutions or phosphate-buffered saline were administered daily.
K-wire, Kirschner wire; N/A, not applicable; PBS, phosphate-buffered saline; *S. aureus*, *Staphylococcus aureus*.

and gentamicin resulted in even higher survival rates (80%; $p \leq 0.001$, log-rank test). Moreover, the combination also performed significantly better than phage alone ($\Delta = 27\%$; $p = 0.042$, log-rank test). These findings clearly indicate the added value of repetitive administration of gentamicin alone or in combination with phages in preventing *S. aureus* haematogenous implant infection in the *G. mellonella* larvae model.

The repetitive administration of either phages or gentamicin alone to prevent haematogenous infection resulted in a significant reduction in bacterial colonization of larval tissue at two days after infection, with a 1.3-log ($p = 0.009$) and 2-log ($p \leq 0.001$, both Mann-Whitney rank-sum test) reduction, respectively, when compared to control group receiving PBS (log 5.8 CFU in the tissue; Figure 2c). The combined administration of phages and gentamicin exhibited an ever larger effect, leading to a 2.8-log ($p \leq 0.001$, Mann-Whitney rank-sum test) reduction in bacterial burden. Similarly, the bacterial burden on the K-wires exhibited a reduction of more than 1.7-log ($p \leq 0.001$, Mann-Whitney rank-sum test) in all cases, when compared to control receiving PBS (log 5.2 CFU/implant; Figure 2d). Administration of gentamicin in combination with phages improved the bacterial clearance on the implant surface ($p = 0.005$) and in the tissue ($p \leq 0.001$, both Mann-Whitney rank-sum test), when compared to administration of phages alone.

Single dose treatment with phages and/or gentamicin

In order to assess the effectiveness of a single dose treatment using phages and/or gentamicin, we developed an early-stage *S. aureus* biofilm implant model by implanting a pre-incubated K-wire in the *G. mellonella* larvae. After pre-incubation in the *S. aureus* solution, the mean total number of bacteria adhered to the stainless steel implants before implantation was 2,854 CFU (SD 364) per implant. At one day after implantation, the larvae were treated with either phages, gentamicin, or combination of both. There was no significant improvement in larval survival with any of the treatments against the early-stage biofilm implant infections, i.e. phages (43%; $p = 0.358$) or gentamicin (40%; $p = 0.426$) alone, or combination of both (45%; $p = 0.143$, all log-rank test), when compared to the control group receiving PBS (28%; Figure 3a). Although the combined administration of a single dose of phages and gentamicin was able to prevent a haematogenous infection, it does not prevent an early-biofilm *S. aureus* infection in *G. mellonella*, indicating the great difficulties when dealing with bacteria in biofilm mode of growth.

Repetitive administration of the combination of phages and gentamicin

To evaluate the impact of multiple-dose treatments on early-stage biofilm infections, the larvae received pre-incubated K-wires, containing a mean of 3,276 CFUs (SD 578)/implant. After 24 hours, the larvae received daily injections of phages, gentamicin, or combination of both. Repetitive treatments with phages alone (44%; $p = 0.339$) or gentamicin alone (55%; $p = 0.169$, both log-rank test) showed only slight improvements in larval survival without reaching statistical significance, when compared to the control receiving PBS (33%; Figure 3b). However, a significant improvement in survival of the larvae was observed when the phages and gentamicin were used in combination and administered repetitively (72%; $p = 0.003$, log-rank test), and also performed better than phages alone ($\Delta = 28\%$; $p = 0.044$, log-rank test). These findings underscore the potential of combining phages with antibiotics to enhance the efficacy of therapies against biofilm-associated implant infections.

After two days of repetitive treatment, there was significant reduction of more than 1.1-log ($p \leq 0.01$ (Mann-Whitney U test) in all cases) in bacterial burden on the K-wire as well as in larval tissue with both repetitive phages and gentamicin treatments when administered alone. The repetitive treatment with the combination of phages and gentamicin exhibited the largest reduction in numbers of CFUs, resulting, in both cases, in a reduction of more than 2.4-log ($p \leq 0.001$, Mann-Whitney U test) in bacterial burden compared to the control group receiving PBS (log 5.6 CFU in the tissue (Figure 3c) and log 5.2 CFUs/implant (Figure 3d)). Also in this model, the combined treatment of phages with gentamicin improved the bacterial clearance on the implant surface ($p \leq 0.001$) and in the tissue ($p \leq 0.001$, both Mann-Whitney U test), when compared to administration of phages alone. These findings highlight the potential of the combined administration of phages and gentamicin in effectively reducing bacterial colonization in both haematogenous and early-stage biofilm infections both on the implant as well as in the surrounding tissue.

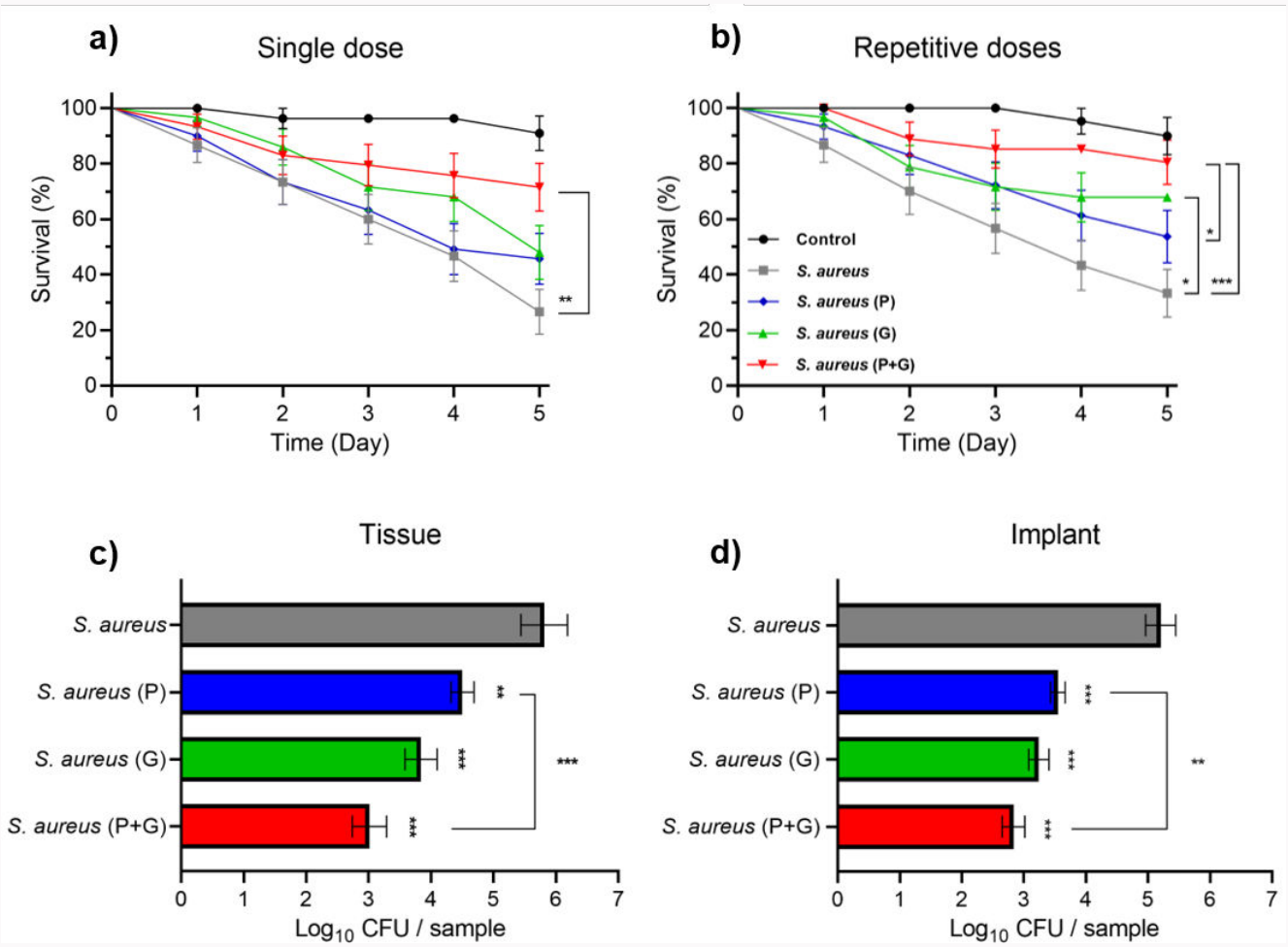

Fig. 2

Prevention of *Staphylococcus aureus* haematogenous implant infection by phages and/or gentamicin in *Galleria mellonella* larvae. First, a stainless steel Kirschner wire (K-wire) was implanted in the larvae, and after 24 hours 10 µl of *S. aureus* inoculum (5×10^5 colony forming units (CFUs)/larva) was injected, followed by a) a single dose or b) repetitive doses of phages (10 µl) and/or gentamicin (5 µl) or the equivalent volume in phosphate-buffered saline (PBS). The percentage survival over time (in days) is displayed after injection(s) with PBS, phages, gentamicin, or their combination. Non-infected larvae served as controls. The numbers of *S. aureus* bacteria c) in the tissue of the larvae ('Tissue') and d) on the K-wire ('Implant') after two days of repetitive injection(s) is shown. The survival data were analyzed from three independent experiments (n = 10 larvae per experiment), and statistical analysis was performed using log-rank test. The quantitative culture data were analyzed from three independent experiments (n = 5 larvae per experiment), and statistical analysis was performed using Mann-Whitney U test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. G, gentamicin; P, phage.

Discussion

With the continuous increase in the number of implant-associated BJIs, there is a substantial burden on public healthcare and the economy.[33] Additionally, the formation of biofilms on implants and the emergence of antimicrobial-resistant bacterial and fungal strains have made it exceedingly difficult to eradicate these infections.[34] Therefore, alternative therapeutic approaches are necessary to effectively treat implant-associated infections. In recent years, due to the rise of antimicrobial resistance, researchers and clinicians have been exploring the use of phages for the treatment of bacterial infections. Several studies have reported that phages can be used as adjuvants to enhance infection treatment.[35,36] In a recent study, Mannala et al[28] showed the efficacy of phages against planktonic, biofilm, and intracellular growth of *S. aureus*. However, when treating early-stage biofilm infection in the *G. mellonella* implant infection model with phages, no significant improvement in larval survival was observed. In the haematogenous implant infection model (prevention), a single combined dose of both phages and gentamicin led to an improvement in larval survival. Furthermore, with repetitive doses, larvae receiving either a combination of phages and gentamicin or gentamicin alone showed a significant improvement in survival. In the early-stage biofilm implant infection model (therapy), only the combined treatment of phages and gentamicin resulted in a significant improvement in larval survival.

Preclinical studies in a FRI rabbit model by Onsea et al[24] demonstrated that the intravenous staphylococcal phage (ISP), a monophage targeting *S. aureus*, in saline (without any carrier) was highly effective in the prevention of FRI, when compared to system antibiotic prophylaxis alone. However, treatment of an established infection with phage-loaded hydrogels showed a possible trend of bacterial load reduction, but no superior effect to the antibiotic treatment alone. Furthermore, Materazzi et al[36] demonstrated that treatment with Sb-1 phages prevented *S. aureus* colonization on the K-wire in a *G. mellonella* larvae model, and a more than 3-log

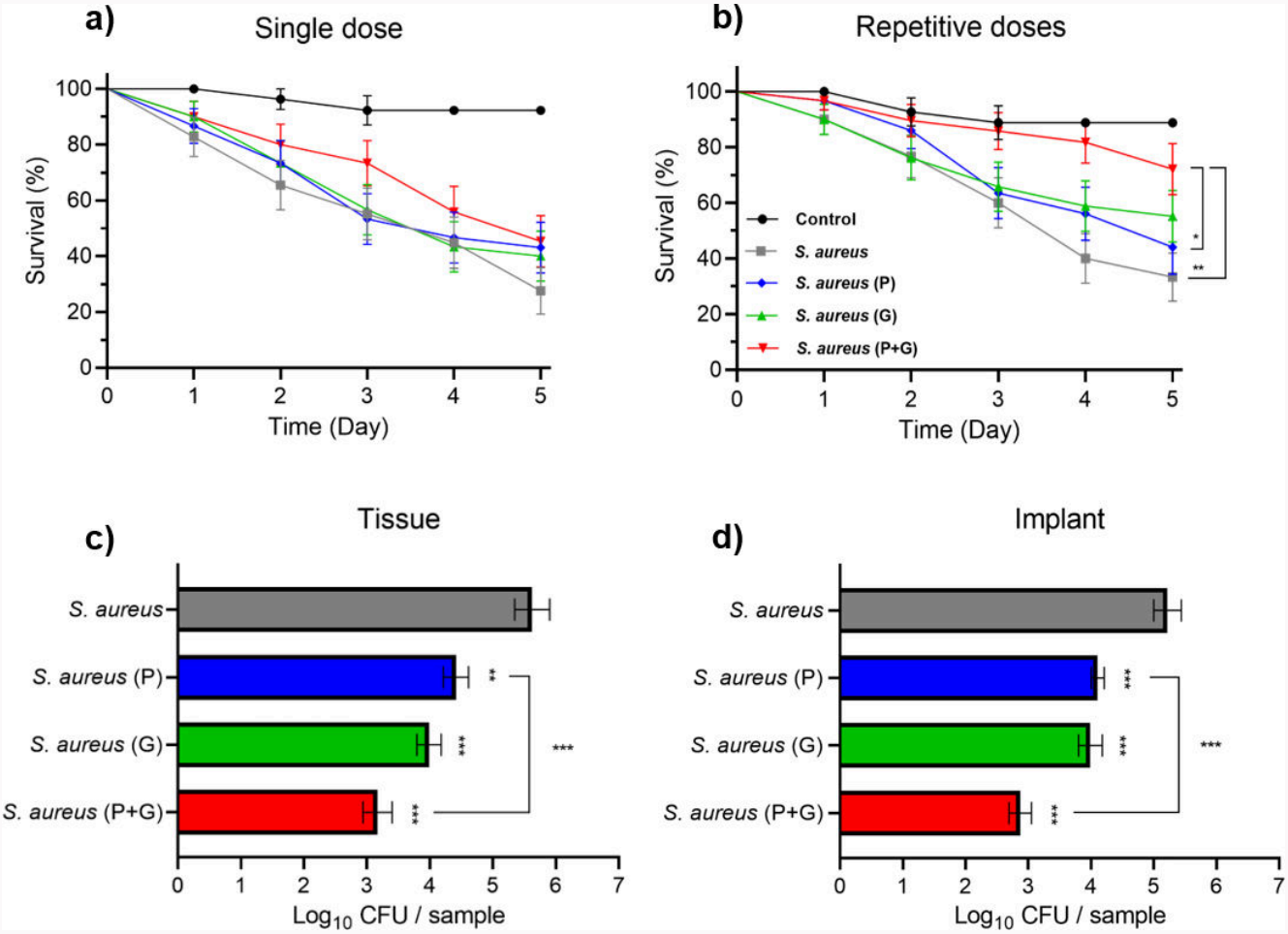

Fig. 3
Treatment of early-stage *Staphylococcus aureus* biofilm implant infection by phages and/or gentamicin in *Galleria mellonella* larvae. First, a stainless steel Kirschner wire (K-wire), pre-incubated for 30 mins in *S. aureus* solution (5×10^6 colony forming units (CFU)/ml), was implanted in the larvae, followed by a) a single dose or b) repetitive doses of phages (10 µl) and/or gentamicin (5 µl) or the equivalent volume in phosphate-buffered saline (PBS) after 24 hrs. The implants contained mean 2,854 (SD 364) and 3,276 (SD 578) CFUs per implant for the single and repetitive injection experiments, respectively. The percentage survival over time (in days) is displayed after injection(s) with PBS, phages, gentamicin, or their combination. Non-infected larvae served as controls. The numbers of *S. aureus* bacteria c) in the tissue of the larvae ('Tissue') and d) on the K-wire ('Implant') after two days of repetitive injection(s) are shown. The survival data were analyzed from three independent experiments (n = 10 larvae per experiment), and statistical analysis was performed using log-rank test. The quantitative culture data were analyzed from three independent experiments (n = 5 larvae per experiment), and statistical analysis was performed using Mann-Whitney rank-sum test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

reduction in numbers of CFUs was observed when larvae were treated with a combination of phages and antibiotics when compared to the phages or antibiotic treatments alone. Currently, antimicrobial prophylaxis is recommended to prevent bacterial colonization during orthopaedic surgeries involving implants.[37] Considering our results, which indicate that the combination of phages and gentamicin prevents K-wire colonization, similar approaches could be employed to prevent surgical site infections in a clinical setting.

Biofilm-associated bone implant infections are disastrous complications in trauma orthopaedic surgery, and occur with colonization of bacteria on the implant. The definitive treatment to eradicate biofilm-related infections is surgical excision of the implant and thorough local debridement. However, removal of prosthesis is not always feasible, especially for the knee and hip location in elderly patients with multiple comorbidities who are at risk of dramatic loss of function, reduction of the bone stock, fracture, or death.[38] Debridement, antibiotics, and implant retention (DAIR) could be used for such patients but the risk of relapse is particularly high due to the bacterial persistence in biofilm on the implant surface. In this context, the use of new adjuvant therapies with phages could increase success rates of antimicrobial treatment.[39] To simulate the clinical setting, Mannala et al[28] have developed an early-stage biofilm implant model in *G. mellonella* larvae to test the effect of phages in combination with gentamicin on biofilm eradication. Here, the repetitive treatment of larvae with the combination of phages and gentamicin showed significantly improved survival of the larvae and bacterial reduction in the larvae. In general, lower dosages of gentamicin are administered systemically in clinic (e.g. 5 to 7 mg/kg/day; intramuscular (IM) or intravenous (IV)).[40] However, we selected a higher concentration (i.e. 60 mg/kg) for the current study because gentamicin is administered locally and less frequently. Phages are often administered locally, e.g. in the joint during DAIR,

at concentrations comparable to our study (i.e. 1.2×10^9 to 1 to 6×10^{10} PFUs per patient).[26,39,41] In line with our study, Akturk et al[42] showed that gentamicin is an effective adjuvant of phage therapy against chronic wound infections, especially when applied simultaneously and repetitively, in a dual-species biofilm in vitro wound model. Moreover, several clinical studies from the Lyon Bone and Joint Study Group in France highlighted the use of phage therapy as an adjuvant to DAIR to salvage patients with relapsing *S. aureus* and *Pseudomonas aeruginosa* prosthetic knee infection.[26,43]

Thus, the *G. mellonella* model can be used as a preclinical in vivo model to evaluate the efficiency of combinations of antimicrobials against implant-associated bacterial infections. Despite its advantages, the model is limited by the fact that the larvae lack an adaptive immune system and have a short life cycle, which does not allow the study of chronic infections. The production of phage-specific antibodies by the induced imuune system plays a crucial role for the in vivo antimicrobial efficiency of phages, and those are absent in the *G. melonella* larvae. Furthermore, absence of a musculoskeletal system excludes typical bone-associated interactions and may limit conclusions on BJI infections.

In conclusion, our study highlights the translational potential of repetitive administration of phages in combination with antibiotics to be used to prevent haematogenous implant infection and to treat early-stage biofilm implant infection. Furthermore, this small animal model could be used as an alternative in vivo model to evaluate phage and other antimicrobial therapies against implant-related infections.

Social media

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Funding statement

The authors received no financial or material support for the research, authorship, and/or publication of this article.

Data sharing

The data that support the findings for this study are available to other researchers from the corresponding author upon reasonable request.

Acknowledgements

The authors would like to thank Daniela Drenkard, Ruth Schewior, and Tobias Gsottberger for their excellent technical assistance. Figure 1 was created using BioRender.com.

Open access funding

The authors report that they received open access funding for their manuscript from the open access scheme of University Library of Regensburg.