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Wax moth larva (*Galleria mellonella*): an *in vivo* model for assessing the efficacy of antistaphylococcal agents

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Objectives: To investigate whether the wax moth larva, *Galleria mellonella*, is a suitable host for assessing the *in vivo* efficacy of antistaphylococcal agents against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) infections.

Methods: Wax moth larvae were infected with increasing doses of *S. aureus* to investigate the effect of inoculum size on larval survival. In addition, infected wax moth larvae were treated with daptomycin, penicillin or vancomycin to examine whether these agents were effective against *S. aureus* and MRSA infections *in vivo*.

Results: Increasing inoculum doses of live *S. aureus* cells resulted in greater larval mortality, but heat-killed bacteria and cell-free culture filtrates had no detrimental effects on survival. Larval mortality rate also depended on the post-inoculation incubation temperature. After larvae were infected with *S. aureus*, larval survival was enhanced by administering the antistaphylococcal antibiotics daptomycin or vancomycin. Larval survival increased with increasing doses of the antibiotics. Moreover, penicillin improved survival of larvae infected with a penicillin-susceptible methicillin-susceptible *S. aureus* (MSSA) strain, but it was ineffective at similar doses in larvae infected with MRSA (penicillin resistant). Daptomycin and vancomycin were also effective when administered to the larvae prior to infection with bacteria.

Conclusions: This is the first report to demonstrate that antibiotics are effective in the wax moth larva model for the treatment of infections caused by Gram-positive bacteria. The new wax moth larva model is a useful preliminary model for assessing the *in vivo* efficacy of candidate antistaphylococcal agents before proceeding to mammalian studies, which may reduce animal experimentation and expense.

Keywords: antibacterial, antimicrobial, daptomycin, drug discovery, drug resistance, insect infection model, MRSA, *Staphylococcus aureus*, vancomycin

Introduction

Staphylococcus aureus is a major cause of community and nosocomial infections. Strains with resistance to antibiotics, including methicillin-resistant *S. aureus* (MRSA), are particularly problematic, as infections caused by these microbes are difficult and expensive to treat.[1,2] As resistance to conventional and newly introduced agents is commonly encountered[3,4] new treatment options are under active investigation.[5–8] After identifying an antibacterial compound with potent antistaphylococcal activity *in vitro*, evaluations are performed to determine its efficacy in complex biological fluids (human serum for instance) and toxicity. Compounds deemed to be non-toxic but effective in sera must then be assessed for efficacy *in vivo*. Typically a mammalian system is used (e.g. a murine infection model), but these experiments are time-consuming, expensive and require full ethical consideration. Moreover, many compounds prove to be ineffective *in vivo* for various reasons, including binding to immune components or enzymatic degradation. Hence there is a need for preliminary and alternative infection models that generate *in vivo* data quickly and inexpensively and that do not require the same ethical considerations. Such new models can reduce animal experimentation by highlighting compounds that are unlikely to be effective in mammalian systems.

The introduction of a wax moth model of systemic *S. aureus* infection may prove useful to this end. Like other non-mammalian infection models,[9–12] microbial virulence is similar in the wax moth larva and mammals, and this model has already been used to determine the virulence of various human pathogens, including *S. aureus*.[13–20] *S. aureus* mutants lacking the accessory gene regulator (*agr*) gene, which controls the global expression of multiple virulence factors, have attenuated virulence in mammalian models and these strains are similarly less virulent in the wax moth larva.[16] This model has also

been used to identify novel virulence genes in *S. aureus*. For example, strains with point mutations in *relA* that result in a permanently activated stringent response are less virulent in the wax moth larva.[19] Further, Purves *et al.*[20] demonstrated that two glyceraldehyde-3-phosphate dehydrogenase homologues have reduced virulence in this model. The benefits of the wax moth larva model compared with some other non-mammalian models of infection are numerous. First, the insect immune response is similar to the innate immune response of mammals and pathogen killing occurs by similar mechanisms.[10,21] Second, the wax moth model can be run at the human core body temperature of 37°C, which can be important for the expression of certain microbial virulence factors.[22] Third, an accurate inoculum of the pathogen can be delivered directly into the host's body. Finally, and crucially, the wax moth model is also amenable for assessing the efficacy of antimicrobial agents, and this model has been used to study therapeutics for infections, including those caused by *Acinetobacter baumannii* and fungi.[17,23–25]

The aim of this study was to determine the usefulness of the wax moth larva model of systemic *S. aureus* infection for evaluating the efficacy of antistaphylococcal agents.

Materials and methods

Reagents, microorganisms and insects

All reagents and culture media were purchased from Sigma–Aldrich Ltd (Poole, UK) except daptomycin (Novartis Pharmaceuticals, Horsham, UK), penicillin G (Duchefa Biochemie, Ipswich, UK) and vancomycin (Duchefa Biochemie B.V., Haarlem, The Netherlands). All solutions were made with sterile ultrapure deionized water (Maxima; Elga, High Wycombe, UK). Water, PBS (for 1 L: 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4 and 0.2 g of KH_2PO_4; pH 7.3) and media were sterilized by autoclaving at 121°C for 15 min. *S. aureus* Newman (methicillin-susceptible; MSSA) and *S. aureus* BB270 (MRSA) were sourced and cultured for inoculum preparation as described previously.[7] MICs of daptomycin, penicillin and vancomycin were determined for each *S. aureus* strain according to the CLSI protocol.[26] For daptomycin MICs, Mueller–Hinton broth was supplemented with $CaCl_2$ to 50 mg/L. Batches of wax moth larvae (75 g; Livefood UK Ltd, Rooks Bridge, UK) in their final instar stage were stored in the dark at 4°C and used within 7 days of receipt. Larval masses varied slightly but were typically 250 mg, and this value was used to calculate treatment doses. Unless stated, all experimentation used groups containing 15 larvae and each experiment was repeated using larvae from a different batch. In all experiments there were two negative control groups; one group that underwent no manipulation whatsoever, while the other group (uninfected control) was injected with PBS only, which controlled for the impact of physical trauma. Typically there were no deaths in these control groups and there were never more than two deaths per control group per experiment. During the following experimentation, larvae were stored in Petri dishes in the dark at 37°C for 120 h. Larvae were inspected every 24 h and were considered dead if they did not move in response to touch.

Inoculation of larvae with culture filtrate, heat-killed or live S. aureus

S. aureus Newman cells were harvested in mid- to late-exponential phase by centrifugation at 2500 **g** for 10 min. To examine the effect of culture filtrates on larval survival, the supernatant was passed through a sterile polyethersulfone 0.22 μm filter (Millipore, Watford, Herts, UK). Meanwhile, the cell pellet was washed by re-suspension in PBS and then centrifugation was performed as before. This wash was repeated before cell density of the resultant bacterial suspension was determined by measuring optical density at 570 nm (OD_{570}). The bacterial suspension was diluted with PBS to give the desired cfu/mL. Larvae were inoculated with 10 μL of bacterial suspension (containing 1×10^5–1×10^7 cfu total) in the last left proleg using a 50 μL Hamilton syringe. For heat-killed inoculum experiments, the bacterial suspension was kept in a water bath at 90°C for 30 min. Heat killing was confirmed by plating 100 μL of bacterial suspension across tryptone soya agar (TSA) and incubating this plate at 37°C for 48 h. The heat-killed bacterial suspension was diluted with PBS and inocula equivalent to 1×10^6–5×10^7 cfu were used for infecting the larvae, as above.

Effect of post-inoculation temperature on larval survival

To investigate the effect of post-inoculation incubation temperature on the survival of infected larvae, larvae were inoculated with 2.5×10^6 cfu of live bacteria and incubated at 25, 30 or 37°C. Negative control groups were set-up for each incubation temperature.

Toxicity of antistaphylococcal agents to larvae

The toxicities of the antistaphylococcal agents (daptomycin, penicillin and vancomycin) were tested by administering repeat doses of 64 mg/kg at 0, 24 and 48 h to groups of 10 larvae. This treatment dose was in excess of those used in subsequent experimentation (see below). Treatments were administered similar to the initial inoculation, but these were given into alternate prolegs moving up the body toward the head such that each proleg was injected only once.[23] A non-significant difference in larval survival between the treated group and the control group (injected with PBS only) would indicate a lack of toxicity up to the dose and regimen under examination.

Treatment of infected larvae with antistaphylococcal agents

Groups of larvae were inoculated with live *S. aureus* (2.5×10^6 cfu; Newman or BB270) as above. All larvae were confirmed to be alive at 2 h post-inoculation (here designated 0 h). Then, the first treatment doses of daptomycin (2, 10 and 50 mg/kg), penicillin (0.5, 2 and 5 mg/kg) or vancomycin (1, 10 and 50 mg/kg) were administered in PBS. In addition, one group of larvae that had been inoculated with live bacteria received PBS only as treatment. Repeat treatment doses of daptomycin, penicillin, vancomycin or PBS were given at 24 and 48 h. In addition, the uninfected control group received PBS treatments to control for multiple injections.

Pre-treatment of larvae with antistaphylococcal agents

To assess the ability of daptomycin and vancomycin to protect against subsequent *S. aureus* Newman infection, single treatment doses of daptomycin (2, 10 and 50 mg/kg) and vancomycin (1, 10 and 50 mg/kg) were given 2 h prior to inoculation with live bacteria (2.5×10^6 cfu), as described above. For this experiment the time of inoculation was designated 0 h.

Statistical analyses

Statistical tests were performed using SPSS v17.0 for Windows (SPSS Inc., Chicago, IL, USA). For statistical testing and the preparation of figures, data from duplicate experiments were pooled to give $n=30$, except the toxicity determinations, which were performed once only and used a

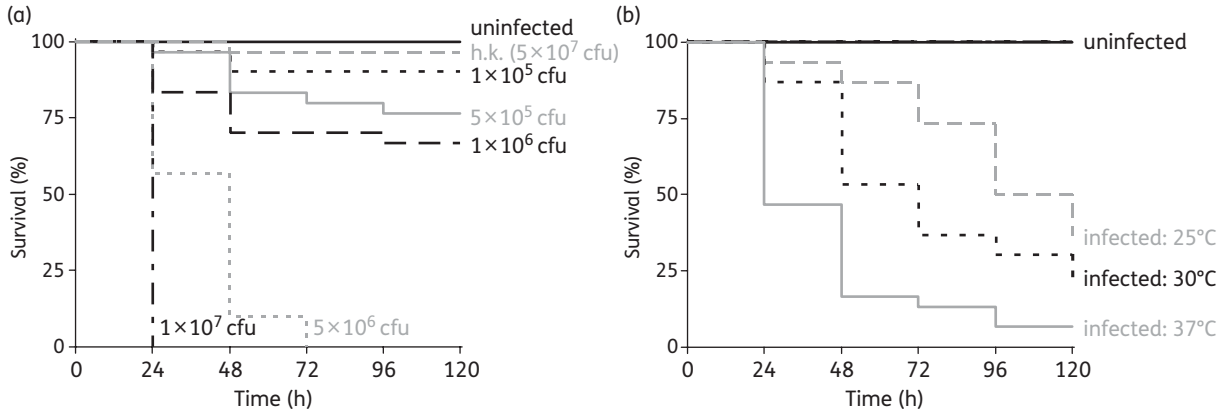

Figure 1. (a) Effect of varying inoculum dose of live *S. aureus* Newman on the survival of *G. mellonella* larvae during incubation at 37°C showing that survival is reduced with increasing doses of bacteria in a dose-dependent manner. Data from the greatest dose of heat-killed (h.k.) bacterial inoculum is also given showing that heat-killed *S. aureus* had no significant effect on larval survival. (b) Effect of post-inoculation temperature on the survival of *G. mellonella* larvae after infection with 2.5×10^6 cfu of live *S. aureus* Newman showing that survival is reduced with increasing post-inoculation temperature. There were no deaths in each of the uninfected control groups (incubated at 25, 30 and 37°C; $n=30$).

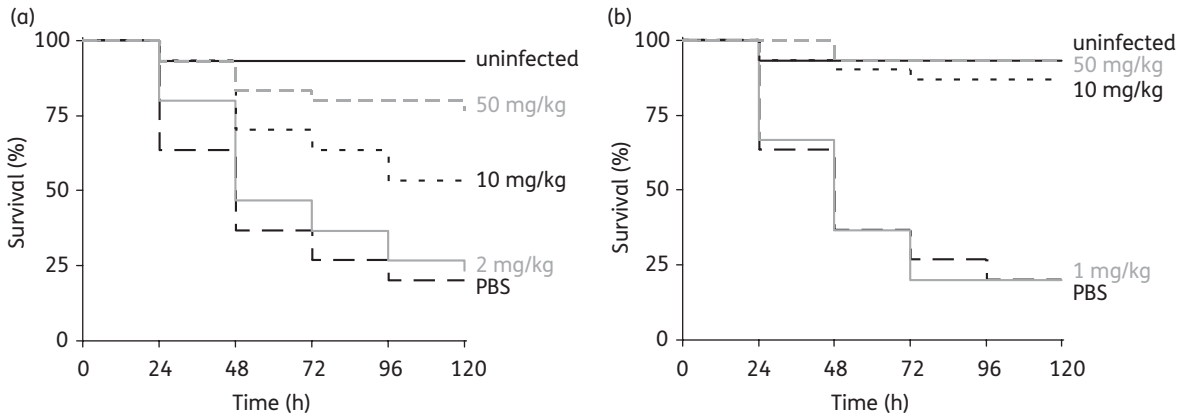

Figure 2. Effect of treatment of *G. mellonella* larvae (inoculated with 2.5×10^6 cfu of *S. aureus* Newman; $n=30$) with (a) daptomycin (2, 10 and 50 mg/kg) and (b) vancomycin (1, 10 and 50 mg/kg) on survival at 37°C. First treatments were administered at 0 h (i.e. 2 h after inoculation), with subsequent treatments at 24 and 48 h. These data indicate that antistaphylococcal agents are efficacious against an MSSA strain in the wax moth larvae in a dose-dependent manner.

group size of 10. These pooled survival data were plotted using the Kaplan–Meier method and comparisons made between groups using the log rank test. In all cases $P\leq0.05$ was considered significant and Holm's correction[27] was applied to account for multiple comparisons. In all comparisons to the negative control it was the uninfected control (rather than the 'no manipulation' control) that was used.

Results

Effect of inoculum dose on larval survival

The heat-killed *S. aureus* Newman inoculum (up to the maximum inoculation dose of 5×10^7 cfu) and cell-free culture filtrate had no significant effect on larval survival ($P>0.05$; Figure 1 and data not shown). With the live bacterial inocula, larval survival was affected by the inoculum dose and larger doses of bacteria gave reduced larval survival in a dose-dependent manner during 120 h incubation (Figure 1). Even the lowest inoculum (1×10^5 cfu) reduced larval survival compared with the uninfected control, though this just failed to achieve statistical significance ($P=0.078$) (Figure 1). Further, post-inoculation temperature affected larval survival after injection of live *S. aureus* Newman (Figure 1). During 120 h of incubation, larval survival was lowest in the group kept at 37°C, while percentage survival was significantly greater ($P<0.05$) for the groups kept at 25 and 30°C (Figure 1).

Treatment of S. aureus-infected larvae with daptomycin, penicillin or vancomycin

Daptomycin, penicillin or vancomycin at 64 mg/kg had no effect on larval survival compared with the PBS-treated controls ($P>0.05$) and were therefore deemed to be non-toxic at these doses (data not shown). Treatment of larvae that had been infected with 2.5×10^6 cfu *S. aureus* Newman showed that the percentage larval survival increased with increasing concentrations of daptomycin and vancomycin in a dose-dependent manner (Figure 2). However, only those groups that received

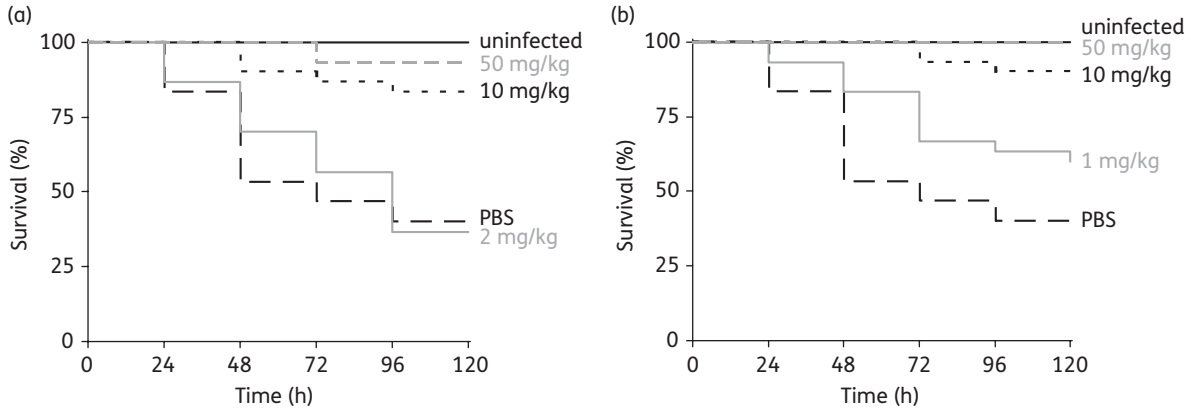

Figure 3. Effect of treatment of *G. mellonella* larvae (inoculated with 2.5×10^6 cfu of *S. aureus* BB270; $n=30$) with (a) daptomycin (2, 10 and 50 mg/kg) or (b) vancomycin (1, 10 and 50 mg/kg) on survival at 37°C. First treatments were administered at 0 h (i.e. 2 h after inoculation), with subsequent treatments at 24 and 48 h. These data indicate that antistaphylococcal agents are efficacious against an MRSA strain in the wax moth larvae in a dose-dependent manner.

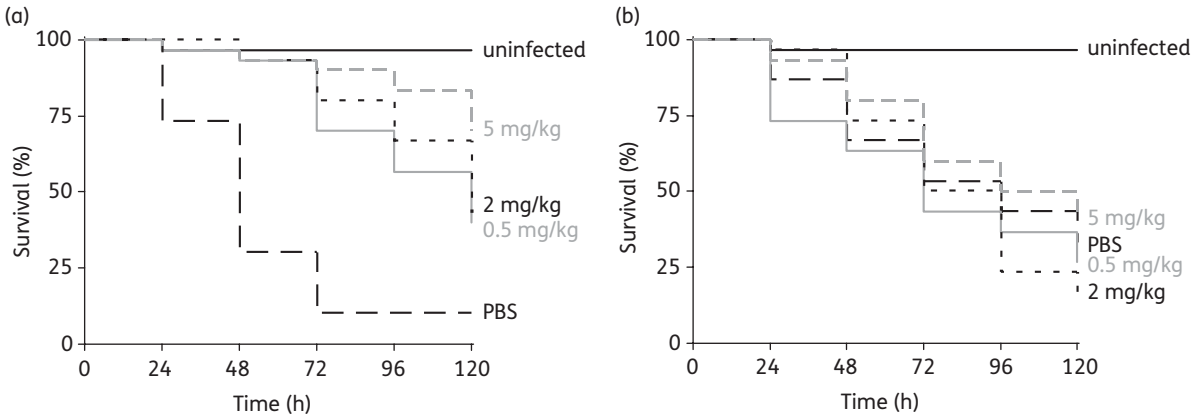

Figure 4. Effect of treatment of *G. mellonella* larvae ($n=30$ for each drug and dose combination) inoculated with 2.5×10^6 cfu of (a) *S. aureus* Newman or (b) *S. aureus* BB270 with penicillin (0.5, 2 and 5 mg/kg) on survival at 37°C. First treatments were administered at 0 h (i.e. 2 h after inoculation), with subsequent treatments at 24 and 48 h. These data confirm that penicillin is effective against the Newman strain (MSSA) but has no effect against the BB270 strain (MRSA).

≥10 mg/kg of either of these antibiotics had a significantly greater percentage survival compared with the larvae treated with PBS only ($P<0.05$). Similarly, for those groups of larvae infected with *S. aureus* BB270, the percentage larval survival increased with increasing concentrations of daptomycin and vancomycin but, again, ≥10 mg/kg of either antibiotic was needed for a significant increase in percentage survival compared with the larvae treated with PBS only ($P<0.05$) (Figure 3). The MICs for *S. aureus* Newman were 2 mg/L for daptomycin and vancomycin, while *S. aureus* BB270 MICs were 4 and 2 mg/L for these agents, respectively.

The use of penicillin (0.5–5 mg/kg) in larvae infected with *S. aureus* Newman (MSSA) showed that survival was significantly greater at each of these doses compared with those larvae treated with PBS only ($P<0.05$) (Figure 4). In contrast, these penicillin treatment regimens had no beneficial effects for larvae infected with the MRSA strain, *S. aureus* BB270 ($P>0.05$ in each case) (Figure 4). The MICs of penicillin for *S. aureus* Newman and *S. aureus* BB270 were 0.031 and 64 mg/L, respectively.

Pre-treatment of larvae with daptomycin and vancomycin

Single treatment doses of daptomycin and vancomycin administered before inoculation with *S. aureus* Newman improved larval survival (Figure 5). This protective effect was dose-dependent, with greater doses of these agents increasing larval survival, though improvements in survival were only significant ($P<0.05$) for those groups receiving ≥10 mg/kg of either antibiotic.

Discussion

Daptomycin and vancomycin are capable of improving the survival of wax moth larvae in a dose-dependent manner after

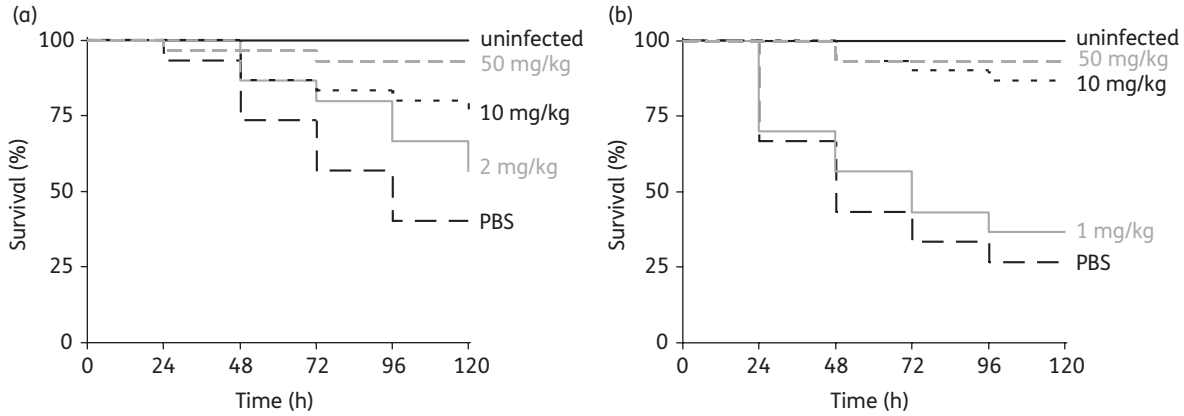

Figure 5. Pre-treatment of *G. mellonella* larvae ($n=30$ for each drug and dose combination) with single doses of (a) daptomycin (2, 10 and 50 mg/kg) or (b) vancomycin (1, 10 and 50 mg/kg) 2 h prior to inoculation with 2.5×10^6 cfu of *S. aureus* Newman, showing that survival at 37°C improved in a dose-dependent manner for both antistaphylococcal agents.

systemic *S. aureus* infection. These agents are similarly effective when administered to the larvae prior to *S. aureus* inoculation. Penicillin treatment improves the survival of larvae infected with MSSA but has no beneficial effect for those larvae infected with MRSA. Lethality of *S. aureus* infection is dependent on the dose of live bacteria given and the incubation temperature after inoculation.

Previously the wax moth model has been used to evaluate the efficacy of antimicrobial agents,[17,23–25] but the present study is the first to show that clinically approved antibiotics are effective in this model against infections caused by Gram-positive bacteria, specifically *S. aureus* and MRSA. Interestingly, daptomycin and vancomycin are efficacious in the wax moth model at doses similar to those recommended for use in humans with *S. aureus* or MRSA infections, notably 4–6 and ~10–20 mg/kg/day of these drugs, respectively.[28] Moreover, both daptomycin and vancomycin enhance larval survival when administered as prophylaxis before infection with bacteria. The beneficial effect of a single prophylactic dose provides similar increases in larval survival compared with three doses administered post-infection. While penicillin improves the survival of larvae infected with the Newman strain (MSSA), it is ineffective in larvae infected with BB270, an MRSA strain. These *in vivo* observations reflect the known differences in susceptibility of these two strains to the action of penicillin *in vitro*. Though penicillin G is not recommended for *S. aureus* infections, its dose for other diseases in humans is ~30–60 mg/kg/day, which is not dissimilar from its effective dose in the wax moth larva against the penicillin-susceptible Newman strain. Taken together, these findings confirm that the wax moth model may prove useful for evaluating the *in vivo* efficacy of new antistaphylococcal agents.

Peleg *et al.*[16] were the first to demonstrate that the wax moth larva model of *S. aureus* infection could prove to be useful for identifying pathogenicity and virulence genes. Our study confirms that live bacteria are needed to kill wax moth larvae, as neither culture filtrate nor heat-killed cells causes significant mortality (even at >500 times the dose of live bacteria that is needed for significant effects on survival). Thus *Galleria mellonella* larvae seem to be actively and specifically killed by *S. aureus*. We also confirm a previous observation that higher post-inoculation temperatures cause greater reductions in larval survival.[16] Some bacteria have thermo-regulated virulence factors, which could account for this observation, and there is some evidence for the existence of such genes in *S. aureus*.[29] However, it is perhaps more likely that the bacterium grows, divides and/or produces virulence factors faster at 37°C compared with 30 or 25°C, thereby killing the larva more quickly.

While the precise mechanisms for wax moth larva killing by *S. aureus* remain enigmatic, it is important to identify the key virulence factors most relevant in this model. In addition, studies are also needed to elucidate the dynamics for *S. aureus* infection of the wax moth larva. Even low doses of live bacteria (1×10^5 cfu) cause significant reductions in larval survival, but it is not clear whether the bacterium is dividing in the host during infection or merely remaining static and producing the necessary virulence factors to cause death. If *S. aureus* does divide in the wax moth larva *in vivo* it would be desirable to establish whether a threshold exists that causes a lethal event. The assessment of bacterial burden in larval haemolymph[19] may also prove useful for increasing the assay's sensitivity, and these values could be compared with blood and tissue burden that are often measured during murine infection models. Finally, the existence of drug-binding immune components and drug degradation pathways warrants investigation in this model.

In conclusion, the new wax moth larva model is a useful preliminary model for assessing the *in vivo* efficacy of candidate anti-*S. aureus* and anti-MRSA agents before proceeding to mammalian studies, which may reduce animal experimentation and costs.

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Transparency declarations

None to declare.

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