Combinations of maggot excretions/secretions and antibiotics are effective against *Staphylococcus aureus* biofilms and the bacteria derived therefrom

Mariena J. A. van der Plas¹ ², Cheryl Dambrot¹, Heleen C. M. Dogterom-Ballering¹, Simone Kruijthof¹, Jaap T. van Dissel¹ and Peter H. Nibbering¹*

¹Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands; ²Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands

*Corresponding author. Tel: +31-71-526-2204; Fax: +31-71-526-6758; E-mail: p.h.nibbering@lumc.nl

Received 1 October 2009; returned 20 October 2009; revised 26 January 2010; accepted 26 January 2010

Objectives: Maggots of the blowfly *Lucilia sericata* are used for the treatment of chronic wounds. Previously we reported that maggot excretions/secretions (ES) break down *Staphylococcus aureus* biofilms but do not kill the bacteria. As many antibiotics are not effective against biofilms we assessed the effect of combinations of ES and antibiotics on *S. aureus* biofilms and on the survival of the bacteria released from the biofilms.

Methods: Effects of ES, antibiotics (vancomycin, daptomycin or clindamycin) and combinations thereof on *S. aureus* biofilms and bacterial viability were determined using microtitre plates and *in vitro* killing assays.

Results: Vancomycin and daptomycin dose-dependently enhanced biofilm formation, whereas clindamycin reduced *S. aureus* biofilm size. Adding ES to antibiotic incubations caused a complete biofilm breakdown. After a lag time the bacteria derived from biofilms became susceptible to vancomycin and clindamycin, provided that the medium was refreshed. Daptomycin dose-dependently eliminated the biofilm-derived bacteria immediately. Furthermore, it was significantly more effective against bacteria derived from ES-exposed biofilms than those from control biofilms. ES did not affect the activity of the antibiotics against log-phase *S. aureus*.

Conclusions: Combinations of maggot ES and antibiotics eliminate *S. aureus* biofilms and the bacteria derived therefrom.

Keywords: *Lucilia sericata*, clindamycin, vancomycin, daptomycin, bacterial killing

Introduction

Chronic wounds are common in patients with vascular insufficiencies and underlying chronic conditions such as diabetes mellitus, as well as in patients suffering from acute, extended trauma.¹ ² These wounds and associated amputations result in decreased physical, emotional and social function of patients, a reduced quality of life and major economic costs for patients, their families and society as a whole.³ ⁴ A severe complication of the healing process is bacterial colonization and subsequent infection of the wound surface,⁵ ⁶ ⁷ especially when the bacteria are residing in biofilms.⁸ These latter bacteria exhibit altered growth characteristics and gene expression profiles as compared with those present free in the environment, the so-called planktonic bacteria.⁹ Importantly, biofilm formation and the consequences thereof for bacterial growth characteristics render microorganisms resistant to the action of many antibiotics,¹⁰ ¹¹ as well as cells and effector molecules of the host’s immune system.⁷ ¹² Bacterial fragments/products released from biofilms continuously attract host cells to the wound. As phagocytes cannot ingest the biofilm-associated bacteria and therefore are unable to eliminate the cause of infection, the subsequent accumulation of inflammatory cells and enhanced release of proinflammatory cytokines, proteases and reactive oxygen species eventually leads to inactivation of growth factors and tissue destruction,¹³ ¹⁴ thereby contributing to the establishment and maintenance of chronic wounds.

Sterile larvae—maggots—of the green bottle blowfly *Lucilia sericata* are used as a treatment for various types of chronic wounds.¹⁵ ¹⁶ ¹⁷ Previously we reported the use of maggot excretions/secretions (ES) to break down *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms.¹⁸ However, the bacteria released from these biofilms were not killed by ES. On the other hand, many antibiotics cannot break down bacterial
biofilms but effectively kill planktonic bacteria. Therefore, we assessed the effect of combinations of maggot ES and antibiotics on S. aureus biofilms and on the survival of the bacteria released from these biofilms.

Materials and methods
Maggots and maggot ES
ES of sterile second- and third-instar larvae of L. sericata (a gift from Bio-Monde GmbH, Borsbüttel, Germany) were collected as described.\(^{19}\) Larvae were incubated in H\(_2\)O (5 μL/larva) for 60 min at ambient temperature in the dark. Next, ES were checked for sterility and stored at −20°C. Prior to use, ES preparations were pooled and centrifuged at 1300 g for 5 min at 4°C to remove particulate material. ES protein concentration was determined using the Pierce BCA Protein Assay kit according to the manufacturer’s instructions.

Antibiotics
Stock solutions of vancomycin (Pharmachemie B.V., Haarlem, The Netherlands), daptomycin (Cubicin, Chiron Corporation Limited, Uxbridge, UK) and clindamycin (Upjohn GmbH, Heppenheim, Germany) were dissolved in distilled water to a final concentration of 10 g/L.

S. aureus cultures
S. aureus ATCC 29213 (Manassas, VA, USA) were grown in tryptone soya broth (TSB) at 37°C under vigorous shaking. The MIC values for this strain are 0.5–2 mg/L for vancomycin, 0.25–1 mg/L for daptomycin and 0.06–0.25 mg/L for clindamycin.\(^{20}\)

Biofilm assay
Biofilm formation of S. aureus in 96-well polystyrene plates was conducted as described.\(^{18}\) In short, bacteria from overnight cultures were diluted 1:1000 and 5 μL of these bacterial suspensions were added to each well containing 100 μL of ‘biofilm medium’ consisting of 0.5x TSB supplemented with 0.2% (w/v) glucose. After 24 h, planktonic cells were removed and 100 μL of biofilm medium with or without antibiotics (1–400 mg/L) and/or ES (20–200 mg/mL) were added to the biofilms. At the indicated time intervals, planktonic cells were harvested from these wells and the numbers of viable bacteria were determined microbiologically using serial dilutions of these suspensions plated six times each onto agar plates. The lower detection limit of this method is 100 cfu/well. In addition, after washing the wells with tap water, biofilms were exposed to a 1% (w/v) Crystal Violet solution for 15 min, washed and then incubated in absolute ethanol for 15 min to extract the Crystal Violet retained by the cells. Next, this solution was used to quantify the amount of biofilm by measuring the absorbance at 590 nm.

Furthermore, we investigated the effect of antibiotics on bacteria derived from the biofilms and subsequently transferred to fresh biofilm medium. For this purpose, the planktonic cells were removed from 24-h-old biofilms, and fresh biofilm medium was added to the wells containing ES (20–200 mg/mL) or H\(_2\)O as a control. After an additional 24 h, the bacteria released from the biofilms were harvested and 25 μL of these bacterial suspensions were transferred to wells of a PVC plate containing 75 μL of TSB medium supplemented with antibiotics; the final concentration of the medium was 0.5x TSB and 0.2% glucose. After 3 and 24 h, the numbers of surviving bacteria were determined microbiologically as described above.

Concentration–effect relationship for antibiotics on exponentially growing S. aureus
To further determine the concentration–effect relationship for antibiotics on planktonic S. aureus in the presence or absence of ES, in vitro killing assays were conducted as described\(^{19}\) with minor modifications. Bacteria in mid-log phase were centrifuged at 2000 g for 10 min, washed twice with PBS and resuspended to a concentration of 1x10\(^7\) bacteria/mL of biofilm medium supplemented with antibiotics (0.005–500 mg/L) and/or ES (20–200 mg/mL). Subsequently, 100 μL aliquots of these bacterial suspensions were transferred to wells of a 96-well PVC plate and incubated at 37°C. After 1, 2 and 3 h, the numbers of surviving bacteria were determined microbiologically as described above.

Next, the differences between the logarithms (base 10) of the numbers of cfu in the absence and presence of antibiotics and/or ES were calculated for each timepoint.\(^{22}\) For further calculations, the highest value of the net killing rate during the 3 h of exposure was used (\(E_0\)). The concentration–effect relationship was established by using the Hill equation:

\[
ER = \frac{ER_{\text{max}} \times C}{EC_{50} + C}
\]

where \(ER_{\text{max}}\) is the estimated maximal killing rate, \(C\) the antibiotic concentration (mg/L) and \(EC_{50}\) the estimated antibiotic concentration at which 50% of the maximal killing is reached. The parameters of this pharmacodynamic model were calculated in SPSS using non-linear regression analysis.

Statistical analysis
Statistical analyses were performed using Graphpad Prism version 4.02. Statistical differences between the values for ES-incubated and control-incubated bacteria were analysed using a paired t-test. The level of significance was set at \(P\) values <0.05.

Results
Effect of antibiotics and ES on S. aureus biofilms
The results showed a dose-dependent increase in biofilm size by vancomycin and daptomycin already within 3 h (Table 1). This effect persisted over the next 21 h. In contrast, clindamycin dose-dependently decreased the amount of biofilm; after 3 h of incubation the biofilm diminished by ~28% (Table 1). As reported previously,\(^{18}\) within 3 h ES degraded the S. aureus biofilms completely and this effect was not counteracted by the antibiotics (data not shown).

Effect of combining ES and antibiotics on the viability of S. aureus released from biofilms
To find out if the biofilm-derived bacteria are susceptible to antibiotics, we incubated the biofilms with various concentrations of antibiotics and/or ES and determined the number of viable bacteria at different intervals.

Preliminary experiments revealed no reduction in the number of viable bacteria when using ≤10 mg/L of vancomycin and daptomycin. Vancomycin at concentrations of ≥50 mg/L slightly but significantly reduced the number of viable bacteria at 24 h (Figure 1a), but not after 3 h (data not shown). Daptomycin dose-dependently reduced the number of biofilm-derived bacteria within 3 h; a 3 log reduction was seen for 400 mg/L (data not shown). This reduction in bacterial numbers continued for
the next 21 h (Figure 1b). After 3 h of incubation, the number of viable bacteria was 1 log lower in the presence of clindamycin compared with control incubations of bacteria derived from either ES-treated or control biofilms (data not shown). Over the following 21 h, no increase in bacterial numbers was observed in the presence of clindamycin (Figure 1c). Furthermore, a dose-dependent effect of clindamycin was observed at the lowest concentrations used in the experiments (i.e. 1, 5 and 10 mg/L, resulting in a reduction in the number of viable bacteria by 53%±9%, 78%±6% and 80%±14%, respectively), whereas maximal inhibition was reached with clindamycin concentrations >10 mg/L. Of note, ES (200 mg/L) did not affect the antibacterial activity of the antibiotics (Figure 1a–c); 20 mg/L ES yielded similar results (data not shown).

Effect of ES and antibiotics on biofilm-derived bacteria transferred to fresh biofilm medium

As large numbers of bacteria derived from the biofilms remained viable in the presence of the antibiotics, we considered the possibility that these biofilm-derived bacteria were in a dormant state making them resistant to these antibiotics. Therefore, bacteria derived from ES-incubated or control-incubated biofilms were transferred to fresh biofilm medium supplemented with antibiotics.

Vancomycin failed to affect the number of viable bacteria at 3 h but induced a 2 log reduction in bacterial counts at 24 h. This effect was independent of the dose of antibiotics or whether the biofilms had been exposed to ES (Figure 2a). Daptomycin dose-dependently reduced the number of bacteria within 3 h. Moreover, the bactericidal effect of daptomycin against bacteria derived from biofilms exposed to 200 mg/L ES (Figure 2b), but not to 20 mg/L ES (data not shown), was higher than that against bacteria from control biofilms. After 24 h, all bacteria were killed by the various concentrations of daptomycin (data not shown). Clindamycin prevented an increase in the number of bacteria at 3 h of incubation (data not shown) and the number of bacteria remained constant during the following 21 h (Figure 2c); the activity of clindamycin against bacteria derived from ES-exposed biofilms was similar to that from control biofilms.

Effect of ES on the concentration–effect relationship of antibiotics on exponentially growing S. aureus

To investigate the effect of ES on the activity of the antibiotics, we determined the killing curves for the various antibiotics using log-phase bacteria. The results showed a dose-dependent reduction in the number of viable S. aureus by all three antibiotics; the maximum effect of daptomycin was higher than that of clindamycin and vancomycin, which were equally effective against the bacteria (Figure 3a–c). The estimated EC_{50} and E_{max} max values are given in Table 2. The activity of the antibiotics was not affected by 20 or 200 mg/L ES. Of note, 500 mg/L daptomycin was sufficient to kill all bacteria within 1 h in four out of five experiments under all conditions. Furthermore, the maximum effect of clindamycin was observed at 1 mg/L, the maximum effect of vancomycin was observed at ~10 mg/L and 500 mg/L daptomycin was required to reach a maximal effect.

Discussion

The main conclusion from the present study is that combinations of maggot ES and antibiotics can break down S. aureus biofilms and subsequently eliminate the bacteria derived therefrom. This conclusion is based on the following observations. First, ES broke down established biofilms within 3 h and this effect was not negatively or positively affected by the antibiotics. In the absence of ES, samples containing vancomycin or daptomycin, antibiotics whose activity depends on their action on the bacterial cell envelope, lacked activity against biofilms; similar findings were observed for the β-lactam antibiotic flucloxacillin (M. J. A. van der Plas, C. Dambret and P. H. Nibbering, unpublished observations).

In contrast, clindamycin and linezolid (M. J. A. van der Plas, C. Dambret and P. H. Nibbering, unpublished observations) decreased the amount of biofilm, albeit that they were unable to completely eradicate it in the 24 h incubations applied here. Secondly, biofilm-derived bacteria became more susceptible to the action of vancomycin and clindamycin after being

Table 1. Effect of antibiotics on established biofilms of S. aureus

<table>
<thead>
<tr>
<th>mg/L</th>
<th>Vancomycin 3 h</th>
<th>Vancomycin 24 h</th>
<th>Daptomycin 3 h</th>
<th>Daptomycin 24 h</th>
<th>Clindamycin 3 h</th>
<th>Clindamycin 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.18±0.02</td>
<td>0.30±0.02</td>
<td>0.21±0.01</td>
<td>0.30±0.02</td>
<td>0.21±0.01</td>
<td>0.29±0.02</td>
</tr>
<tr>
<td>1</td>
<td>0.19±0.03</td>
<td>0.26±0.04</td>
<td>0.20±0.03</td>
<td>0.27±0.03</td>
<td>0.20±0.03</td>
<td>0.24±0.04</td>
</tr>
<tr>
<td>5</td>
<td>0.21±0.03</td>
<td>0.29±0.05</td>
<td>0.20±0.03</td>
<td>0.26±0.05</td>
<td>0.17±0.03</td>
<td>0.24±0.04</td>
</tr>
<tr>
<td>10</td>
<td>0.27±0.04*</td>
<td>0.38±0.06*</td>
<td>0.23±0.04</td>
<td>0.35±0.07</td>
<td>0.17±0.04</td>
<td>0.24±0.04</td>
</tr>
<tr>
<td>50</td>
<td>0.26±0.04*</td>
<td>0.36±0.05*</td>
<td>0.26±0.04*</td>
<td>0.45±0.10*</td>
<td>0.16±0.01*</td>
<td>0.24±0.01*</td>
</tr>
<tr>
<td>100</td>
<td>0.26±0.04*</td>
<td>0.40±0.03*</td>
<td>0.27±0.01*</td>
<td>0.39±0.03*</td>
<td>0.16±0.01*</td>
<td>0.22±0.01*</td>
</tr>
<tr>
<td>200</td>
<td>0.26±0.04*</td>
<td>0.35±0.02*</td>
<td>0.28±0.02*</td>
<td>0.36±0.03*</td>
<td>0.16±0.01*</td>
<td>0.21±0.02*</td>
</tr>
<tr>
<td>400</td>
<td>0.22±0.01*</td>
<td>0.43±0.03*</td>
<td>0.33±0.02*</td>
<td>0.38±0.04*</td>
<td>0.15±0.01*</td>
<td>0.21±0.02*</td>
</tr>
</tbody>
</table>

Results (OD_{590}) are means±SEM of ≥4 experiments. For all samples, the addition of ES resulted in total breakdown of the biofilms (OD<0.10). *Values are significantly different from those for control biofilms (P<0.05).
transferred to fresh medium than when the bacteria remained in the biofilm wells. An explanation for this result could be that the bacteria derived from biofilms are in a static/dormant state and therefore less susceptible to antibiotics that solely target multiplying bacteria. In contrast, daptomycin showed direct activity against biofilm-derived bacteria whether or not they were transferred to fresh medium. Moreover, ES (200 mg/L) enhanced the antibacterial activity of daptomycin, but not of vancomycin and clindamycin, against biofilm-derived S. aureus transferred to fresh medium. Although we cannot explain the latter results, they probably depend on the specific pharmacodynamic mechanisms of daptomycin. Thirdly, ES did not alter the activity of the antibiotics against exponentially multiplying bacteria.

In the interpretation of the current findings, the following points need be considered. First, we performed all experiments with a single ATCC reference strain of S. aureus. In addition, our preliminary results show that maggot ES can prevent methicillin-resistant
Nevertheless, we cannot conclude that our findings are generalizable to all S. aureus strains and/or other bacterial species. However, in agreement with our results, several reports have described daptomycin as being one of the most effective antibiotics in the control of biofilm-related S. aureus infections whereas clindamycin and vancomycin were less effective. Secondly, the concentrations of antibiotics used in the in vitro biofilm assay are relatively high compared with the free, active antibiotic concentrations generally achieved in patients (10–40 mg/L vancomycin, 1–15/20 mg/L daptomycin, 1–20 mg/L clindamycin). However, concentrations of antibiotics similar to those used in the current study can be attained in wounds after topical application. Thirdly, at their MIC values vancomycin or daptomycin did not affect the biofilm size, whereas at higher concentrations biofilm formation was enhanced. It should be realized that MIC concentrations of antibiotics did not reduce the number of viable biofilm-derived bacteria whereas the higher, biofilm-enhancing concentrations did. In agreement, supra-MIC concentrations of antibiotics are reported to be effective against killing of bacteria released from biofilms, whereas sub-MIC and MIC levels were not. Fourthly, in contrast to the above-mentioned reports, we did not observe a reduction in biofilm size when using low levels of antibiotics. The explanation for these contradictory results could be the method of quantification. We used Crystal Violet staining to quantify the amount of biomass whereas many reports describe the use of redox indicators to measure the metabolic activity of the bacteria. However, reduced metabolic activity does not exclude similar or even increased biomass. In agreement with this, it is reported that several antibiotics, including vancomycin, reduce the redox potential of bacteria without reducing the matrix. This may lead to bacterial multiplication from the remaining matrix and may even contribute to the development of resistance against the antibiotics. Clearly, more research should be done into the effect of antibiotics on biofilms and the bacteria derived from these structures. Fifthly, previously we reported that the active molecule in ES is heat labile. Our recent studies into the effects of various substances previously we reported that the active molecule in ES is heat labile.18 Our recent studies into the effects of various substances affecting structural features of proteins and inhibiting enzymic activities indicated that the molecule in ES responsible for S. aureus biofilm breakdown may be a serine protease (M. J. A. van der Plas, unpublished observations). Currently, we are purifying the active molecules from maggot ES by activity-guided chromatography. However, more research is required before the identity of the active component of maggot ES is clarified. Obviously, application of purified maggot-derived compounds instead of live maggots will definitely increase the acceptance and use of this very effective therapy for chronic—non-healing—wounds.

What is the clinical relevance of our findings? The failure of antibiotics to affect biofilms and the bacteria derived therefrom parallels their overall lack of activity against bacterial colonization and infection of chronic wounds where biofilm formation may be prominent. Therefore, biofilm matrices and the associated bacteria have to be targeted simultaneously to eradicate chronic infections. Previously we found that maggot ES break down biofilms of S. aureus without killing the released bacteria. Here we report that the released bacteria became susceptible to the actions of antibiotics that fail to affect biofilm-associated microorganisms when they start multiplying. Therefore, combinations of maggot ES and antibiotics would ensure complete breakdown of the biofilms, thereby preventing bacterial re-growth from the remaining matrix, and prompt antibiotic action against the bacteria released from the biofilms. Additionally, these bacteria will be subjected to the effectors
mechanisms of the immune system and ingestion by maggots.28,29 Thus, addition of maggots or maggot ES to antibiotics may become a promising approach for the treatment of chronically colonized/infected surfaces of unresponsive wounds. In this respect, it should be realized that some current treatment modalities, where maggots apparently are used as a replacement for instead of as an adjunct to antibiotics, often overestimate bacterial killing by ES when applied in therapeutically relevant amounts.18 Of note, antibiotics including vancomycin and clindamycin have no detrimental effects on maggot growth and survival.30 Based on our results and other reports,23,24 daptomycin and ES combined appear particularly promising for the treatment of biofilm-related S. aureus wound infections. Daptomycin, in contrast to vancomycin and cationic antimicrobial peptides, kills bacteria without inducing bacterial lysis.31 As chronic wounds often are characterized by prolonged and dysregulated inflammatory responses,13,34–36 decreased bacterial lysis may reduce proinflammatory responses to bacterial products by immune cells, thereby contributing to the healing process.32

Acknowledgements
We thank Emile F. Schippers for technical assistance.

Funding
This study was supported by internal funding.

Transparency declarations
None to declare.

References


