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An *in vitro* biofilm model to examine the effect of antibiotic ointments on biofilms produced by burn wound bacterial isolates

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Abstract

Purpose—Topical treatment of burn wounds is essential as reduced blood supply in the burned tissues restricts the effect of systemic antibiotics. On the burn surface, microorganisms exist within a complex structure termed a biofilm, which enhances bacterial resistance to antimicrobial agents significantly. Since bacteria differ in their ability to develop biofilms, the susceptibility of these biofilms to topically-applied antibiotics varies, making it essential to identify which topical antibiotics efficiently disrupt or prevent biofilms produced by these pathogens. Yet, a simple *in vitro* assay to compare the susceptibility of biofilms produced by burn wound isolates to different topical antibiotics has not been reported.

Methods—Biofilms were developed by inoculating cellulose disks on agar plates with burn wound isolates and incubating for 24 h. The biofilms were then covered for 24 h with untreated gauze or gauze coated with antibiotic ointment and remaining microorganisms were quantified and visualized microscopically.

Results—Mupirocin and triple antibiotic ointments significantly reduced biofilms produced by the *Staphylococcus aureus* and *Pseudomonas aeruginosa* burn wound isolates tested, as did gentamicin ointment, with the exception of one *P. aeruginosa* clinical isolate.

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Conflict of interest statement

There are no conflicts of interest in this manuscript.

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Conclusions—The described assay is a practical and reproducible approach to identify topical antibiotics most effective in eliminating biofilms produced by burn wound isolates.

1. Introduction

Second degree burns partially destroy the epidermis and extend into the dermis while full thickness (third degree) burns cause more extensive damage that extends into the underlying connective tissues [1]. Both types of burns compromise the physical and chemical protective mechanisms within the skin, which facilitates the colonization of burn wounds by different microorganisms [1,2]. These microorganisms may come from the host's skin, respiratory, or gastrointestinal flora; or, they may be transferred to the patient through contact with other environmental sources, including the hands of healthcare workers [2–4]. Following the injury, burn wounds are initially colonized with predominantly gram-positive bacteria such as staphylococci and enterococci [1,5–7]. Within a week of the burn, the wound surface becomes colonized with gram-negative bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* and *Serratia* species [8–11]. In critically injured patients, local wound colonization may lead to invasive burn wound infection and the possibility of sepsis and multi-organ failure [7]. The presence of coagulated proteins as well as the avascular nature of the eschar increases the susceptibility of burn wounds to infections [3,7,8,12]. The eschar significantly limits the delivery of host immune cells and systemically-administered antibiotics [1,7]. Thus, topical antimicrobial ointments play a critical role in reducing the wound bioburden.

Within different sites (including burn wounds), infecting microorganisms exist in specific structures termed biofilms [13]. The presence of bacterial biofilms has been described at a number of infection sites including the lung during chronic lung infections in cystic fibrosis patients, the ear during otitis media, and the infected tooth [13–15]. James *et al.* [16] described the development of polymicrobial biofilms in chronic wounds such as foot ulcers, pressure ulcers, and venous leg ulcers. Kennedy *et al.* [17] recently examined multiple biopsies from eleven burn wounds. Results revealed the presence of biofilms in the ulcerated areas of the burn wounds [17].

Mature biofilms are usually described as mushroom shaped multi-cellular structures with channels that presumably function in delivering nutrients and remove the wastes [13,15]. Within the biofilm, the microorganisms are surrounded by a glycocalyx composed of a combination of an extracellular matrix (EPS) that is produced by the microorganisms and the host surrounding tissues [13,15]. The glycocalyx contributes to the enhanced resistance of microorganisms to the host response as well as to various antibiotic treatments [18–22].

Due to increased antimicrobial resistance in general, and within the biofilm specifically, it is essential to determine which antibiotic is most effective in eliminating the biofilm produced by a specific wound pathogen. In this study, we describe a simple and quick *in vitro* assay designed to address this need.

2. Materials and Methods

2.1. Bacterial strains and antibiotics

The bacterial strains utilized in this study are described in Table 1. Clinical isolates were obtained under an approved Institutional Review Board ethical protocol. Bacterial strains were grown in Luria Bertani (LB) broth or on LB agar plates [23]. Three types of antibiotic ointments were utilized; gentamicin sulfate ointment USP, 0.1% (Fougera, Melville, NY), mupirocin ointment USP, 2% (Fougera), and triple antibiotic ointment containing 400 U bacitracin, 3.5 mg neomycin, and 5,000 U polymixin B per gram (Perrigo Company,

Allegan, MI). Four hundred mg of the different antibiotic ointments were applied evenly to 1×1 " sterile gauze squares with a sterile spatula to provide 400 μ g gentamicin, 8 mg mupirocin, or 160 U/1.4 mg/2000 U of bacitracin, neomycin, and polymyxin B, respectively, for the triple antibiotic ointment. The 1×1 " gauze squares were cut from sterile 2×2 " 100% cotton 8-ply gauze sponges (Kendall Curity®; Covidien, Mansfield, MA) using sterile scissors and forceps. Gauze without any ointment served as the control for each experiment.

2.2. *In vitro* wound biofilm model for assessing effectiveness of antibiotic ointments

A 1-mL aliquot of an overnight LB broth culture of the tested strain was pelleted, washed, resuspended in 1 mL of fresh LB broth, and serially diluted (10 fold). Preliminary experiments determined that a 10^{-5} dilution produces 10^2 – 10^3 colony forming units (CFU)/10 μ L (data not shown). Five sterile 6 mm cellulose disks (Becton Dickinson and Company, Sparks, MD) were placed on the surface of LB agar plates and 10- μ L aliquots from the 10^{-5} dilution were spotted each disk (Fig. 1a) for each antibiotic to be assessed and five for the control. We previously showed that *P. aeruginosa* and *S. aureus* form mature biofilms on cellulose disks [24].

To examine the effectiveness of antibiotics in preventing development of biofilm in a colonized but non-infected burn wound (biofilm prevention model), either untreated gauze (control) or gauze spread with the antibiotic ointment was placed over each set of five disks immediately after inoculation with the strain (Fig. 1a). To examine the ability of the antibiotic ointments to disrupt an established biofilm (biofilm disruption model), the inoculated disks were incubated for 24 h at 37 °C to allow formation of a mature biofilm and then the disks were covered with treated or untreated gauze (Fig. 1a). In both models, to maintain direct contact between the antibiotic ointment and the inoculated disks, light-weight stir bars were placed on the gauze and held in place with adhesive tape attached to the sides of the plate (Fig. 1a). The plates were then incubated at 37 °C for 24 h. Twenty-four h after application, the gauzes were removed and the disks were analyzed for the presence of biofilm by quantitative and qualitative methods.

To examine the effect of antibiotic ointments on a dual-organism biofilm, the disks were inoculated with $\sim 10^{1.5}$ CFU each of the clinical isolates PACI-2 and SACI-1 (total of $\sim 10^3$ CFU/disk) and incubated for 24 h at 37 °C. The disks were then covered with the antibiotic-treated or control gauze as above and incubated for 24 h at 37 °C. Following this incubation, the disks were analyzed for the presence of each organism by fluorescence *in situ* hybridization (FISH).

2.3. Quantitative analysis of the biofilms

Following the removal of the gauze, three of the cellulose disks were rinsed gently in phosphate buffered saline (PBS) [23] to remove any cells that had detached from the biofilm or that were loosely attached. Each disk was then transferred to a 1.5 mL microcentrifuge tube containing 1 mL of PBS (Fig. 1b). The tubes were vigorously vortexed three times for 2–3 minutes each time to mechanically disrupt the biofilms and detach the bacteria from the disks. Suspended cells were then serially diluted (10 fold) in PBS and 10- μ L aliquots of each dilution were spotted on LB agar plates (Fig 1b). The plates were incubated at 37 °C for 14–16 h and the numbers of CFU were counted. The number of CFU/disk was determined using the following formula: CFU counted $\times$ dilution factor $\times$ 100.

2.4. Qualitative analysis of the biofilms using confocal laser scanning microscopy

The remaining two disks were examined for the presence of biofilm by confocal laser scanning microscopy (CLSM). Biofilms formed by the *P. aeruginosa* strain PAO1/pMRP9-1 and the *S. aureus* strain AH133 were stained with the red fluorescent nucleic acid

stain SYTO 61 (Invitrogen, Carlsbad, CA) as previously described [25]. Biofilms formed by the *P. aeruginosa* and *S. aureus* clinical isolates were stained using the BacLight™ Bacterial Viability kit (Molecular Probes, Eugene, OR), which contains two fluorescent dyes; Syto-9™ that stains live bacteria green and propidium iodide, which displaces Syto-9™ in dead bacteria staining them red. Staining procedures were conducted according to the manufacturers' recommendations.

The FISH was done using primer 5'/5Cy3/GCTGGCCTAGCCTTCC-3' for the *P. aeruginosa* strain (stains green) and primer 5'/5Cy5/GAAGCAAGCTTCCTCGTCCG-3' for the *S. aureus* strain (stains pink) (Integrated DNA Technologies, Inc., Coralville, IA). Disks were rinsed with PBS to remove loosely attached cells, placed in 24-well plates, and covered with 350 µL hybridization buffer (0.9M NaCl, 0.02M Tris-HCl [pH 7.2], 0.1% SDS, and 30% formamide) [26]. Primers were added at a concentration of 6.25 ng/µL (1:8 dilution of 50 ng/µL stock solution) and the plates were covered and incubated for 3 hours at 46 °C [26]. Following incubation, the disks were moved to fresh wells containing 1 mL pre-warmed (46 °C) washing buffer (0.1M NaCl, 0.02 M Tris-HCl [pH7.2], and 0.01% SDS) and incubated for 5 min [26]. The wash step was repeated twice more using fresh wells and washing buffer. Disks were then rinsed gently with purified water, drained by touching the edge of the disk to blotting tissue, and examined microscopically.

Visualization of the biofilms was accomplished using an Olympus IX71 Fluo-view 300 confocal laser scanning microscope (Olympus America, Melville, NY).

2.5. Statistical analyses

Statistical analyses of the results of the experiments were done using GraphPad InStat 3.06 (GraphPad Software, San Diego, CA). One-way ANOVA with the Dunnett's Multiple Comparison Test was used to determine significant differences between control and each treatment. Unpaired, two-tailed *t*-tests were also used to compare treatments to the control.

3. Results

3.1. Biofilm prevention model – gentamicin prevents formation of *P. aeruginosa* biofilm

Debridement and irrigation of an infected wound removes most of the bacteria. However, any remaining bacteria may replicate and produce a new biofilm. As *P. aeruginosa* is a major cause of morbidity and mortality in burn patients, we developed our model using PAO1, a well-characterized strain that was originally isolated from a wound. For ease of visualization, we utilized PAO1 carrying pMRP9-1, which encodes green fluorescent protein. Therefore, we determined if 0.1% gentamicin ointment prevents or inhibits the development of PAO1/pMRP9-1 biofilm. To mimic the situation of a freshly debrided burn, we applied 10²–10³ CFU per disk and immediately applied either untreated gauze or gauze spread with 400 mg 0.1% gentamicin ointment (400 µg/gauze gentamicin) directly on the disk. Quantitative analysis showed that this treatment prevented the growth of *P. aeruginosa* (no CFU were recovered from any of three disks; data not shown). Qualitative analysis by CLSM showed that PAO1/pMRP9-1 produced a well-developed mature biofilm on disks covered with untreated gauze, while disks covered with gentamicin-containing gauze produced no biofilm (Fig. 2).

3.2. Biofilm disruption model – various antibiotic ointments disrupt biofilms formed by *P. aeruginosa* or *S. aureus*

One desirable goal in using topical ointments to treat burn wound infections is to reduce the wound bioburden by killing the bacteria within the biofilm. The success in inhibiting the

development of a PAO1 biofilm by gentamicin prompted us to examine the possibility that gentamicin and other topical ointments may significantly reduce an already established biofilm. Thus, we modified our system to examine the effectiveness of antibiotic ointments on a mature (24-h) biofilm as described in Materials and Methods. Compared with the control biofilms, 0.1% gentamicin ointment, 2% mupirocin ointment, and triple antibiotic (400 U bacitracin, 3.5 mg neomycin, and 5,000 U polymixin B per gram) ointment reduced the PAO1/pMRP9-1 mature biofilms on the cellulose disks significantly (Fig. 3a). Image analysis by CLSM supported these results. Untreated disks produced well-developed mature biofilms at 24 h (Fig. 4a). In contrast, no PAO1/pMRP9-1 biofilms were detected on cellulose disks that were treated with any of the three antibiotic ointments (Fig. 4b, c, d), indicating that these three antibiotic ointments are effective in disrupting a 24-h PAO1 biofilm.

We then tested the biofilm disruption model using two *P. aeruginosa* isolates, PACI-1 and PACI-2 (Table 1). Unlike PAO1/pMRP9-1, gentamicin ointment did not disrupt the PACI-1 biofilm (Fig. 3b). Only mupirocin and triple antibiotic ointments produced a significant reduction in the 24-h biofilm formed by PACI-1 (Fig. 3b). However, all three topical antibiotics significantly reduced biofilms produced by PACI-2 (Fig. 3c). Confocal LSM visualization of the biofilms produced by PACI-1 and PACI-2 using BacLight™ confirmed that PACI-1 inoculated control disks and disks covered with gentamicin ointment-treated gauze produced well-developed biofilms (Fig. 4e, f), while mupirocin and triple antibiotic ointments reduced the biofilm (4g, h). Compared with the control biofilm, all three antibiotic ointments reduced biofilms produced by PACI-2 (Fig. 4i-l).

The gram-positive opportunistic pathogen *S. aureus* is commonly isolated from infected burned tissues. Thus, we next used our model to examine the effect of the three antibiotic ointments against biofilms formed by a *S. aureus* laboratory strain AH133 [27] and two clinical isolates, SACI-1 and SACI-2 (Table 1). The analysis of the biofilms was done as described above. Similar to PAO1/pMRP9-1, the *S. aureus* strain AH133 carries the gene that codes for the gene fluorescent protein (pCM11). Isolates SACI-1 and SACI-2 were visualized using the BacLight™. Compared with the control, the three antibiotic ointments reduced the biofilms produced by AH133, SACI-1 and SACI-2 significantly (Fig. 5). Image analysis revealed similar results (Fig. 6). Using the biofilm prevention model, we showed that, similarly to *P. aeruginosa*, the three ointments inhibited biofilm development by the tested *S. aureus* strains (data not shown).

The mechanisms by which the different antibiotics eliminate bacteria within the biofilm is most likely the same as their mechanisms against planktonic bacteria. However, disrupting the biofilms by eliminating the bacteria within them requires concentrations of antibiotics hundreds to thousands times greater than that required to kill susceptible planktonic forms [18,20,22].

3.3. Effects of antibiotic ointments on a dual-microbial biofilm

Polymicrobial infections are not unusual in burn wounds [17]. Therefore, we tested whether the biofilm disruption model would determine the efficacy of the antibiotic ointments against a dual biofilm containing the clinical isolates PACI-2 and SACI-1 using FISH experiments (Materials and Methods). The untreated biofilm (Fig. 7a) showed the presence of approximately equal numbers of each strain. Gentamicin ointment reduced the numbers of both strains, with only scattered presence of either seen (Fig. 7b). Mupirocin ointment appears to have had a greater effect on *S. aureus* in the dual biofilm, while triple antibiotic ointment appears to have practically eliminated *P. aeruginosa* from the dual biofilm (Fig. 7c, d). These results also suggest that the effectiveness of certain antibiotics differs between single and dual biofilms. For example, triple antibiotic ointment is very effective in

eliminating SACI-1 biofilm (Figs. 5 & 6). However, triple antibiotic ointment appears to be less effective against SACI-1 when PACI-2 is present in the dual biofilm (Fig. 7d).

4. Discussion

Many aspects of the present model resemble the environment within the burned/infected wound. The permeable nature of the cellulose disk allows diffusion of nutrients to the bacteria on the disk, just as nutrients are available to the bacteria within the burn wound. The bioburden of a freshly infected new wound or a debrided wound is likely to be low, but due to the nutritionally-rich environment of the burned tissues, this low number of microorganisms can produce a mature biofilm within 24 h. The biofilm prevention model, in which the number of microorganisms inoculated on the cellulose disks was low (10^2 – 10^3 CFU/disk) reflects the bioburden of the newly infected wound or a freshly debrided established wound, while the mature biofilm of the biofilm disruption model reflects the high bioburden of a burn wound with an established infection. Evidence indicates a number of different pathogenic bacteria, and yeasts, can be found within burn wound biofilms [16,17]. In our model, both laboratory strains and clinical isolates of gram-positive and gram-negative burn wound pathogens (*S. aureus* and *P. aeruginosa*, respectively) formed mature biofilms on cellulose disks within 24 hours of inoculation. Other pathogens, such as *Enterococcus faecalis* or *E. coli*, could be similarly tested in the model. Additionally, it is possible to test dual-microbial biofilms in our model.

Our model also closely mimics the process that is usually followed in treatment of these wounds in the burn unit. The gentle pressure supplied by the light weights and tape over the ointment-treated gauze reprise the role of wound bandaging to maintain close contact between the topical ointment and the wound surface.

The model may be utilized to evaluate the effectiveness of a specific antibiotic ointment in either inhibiting the development of a bacterial biofilm (the biofilm prevention model) or reducing an already established one (biofilm disruption model). Our initial experiments explored the efficacy of gentamicin ointment in interfering with PAO1/pMRP9-1 biofilm development using the biofilm prevention model. For the rest of the study, we utilized the biofilm disruption model to evaluate the efficacy of three different antibiotic ointments in reducing mature biofilms formed by *P. aeruginosa* or *S. aureus* strains. The rationale for continuing with the biofilm disruption model only was that, if an ointment is effective in reducing a mature biofilm, it would be effective in inhibiting the development of a biofilm. However, it is possible that an antibiotic ointment which cannot reduce an established biofilm, may prevent one from becoming established. Among all tested strains, only the 24-h mature biofilm produced by PACI-1 was not significantly reduced by gentamicin. Testing in the biofilm prevention model revealed that gentamicin was also less effective in inhibiting the development of PACI-1 biofilm (data not shown). Future testing of additional antimicrobial ointments may reveal that some antibiotics are effective in preventing biofilm development but not in disrupting an already established one. Thus, for practical purposes, the ability of an ointment to reduce a mature biofilm formed by a wound isolate biofilm may be tested first using the biofilm disruption model. If it fails to do so, its effect in inhibiting the development of a biofilm by the same isolate would then be tested. We were also able to detect variation in the susceptibility or resistance of biofilms formed by different wound isolates to specific levels of antibiotic within ointment in the biofilm prevention model. While residual viable bacteria were present on disks inoculated with PAO1/pMRP9-1 and treated with 400 µg of gentamicin/gauze, no CFU were detected when the gauze was spread with 800 µg or 1600 µg of gentamicin/gauze (data not shown).

The model, in both forms, is simple and provides critical information within a reasonable time frame. Its simple nature is illustrated by the individual components: cellulose disks on which the biofilms were developed, LB agar plates, and gauze on which the antibiotic ointment was spread. The system is also relatively quick, since biofilm prevention testing could be accomplished within 72 hours; 24 h to develop the biofilm, another 24 h to expose the biofilms to the antibiotic ointment, and 24 h to obtain CFU indicative of the effectiveness of the particular antibiotic. The quantitative analysis of the biofilms by determining CFU/disk reflects the practicality of the model. This simple but accurate quantitative assay provides this model with an advantage over the previously reported *in vitro* wound biofilm model in which the biofilms were developed and treated in a manner similar to our model, but analyzed by CLSM only [28]. While we relied on CLSM for some preliminary evidence and to confirm the results of the quantitative assay, the reproducibility of the CFU results indicates that expensive CLSM is not required to analyze the effectiveness of the antibiotic ointments.

Despite the above-described advantages, the current model does not yet meet the criteria for a susceptibility test to determine the effectiveness antimicrobial compounds on biofilm infections of burn wounds. Further experiments are needed to substantiate the clinical relevance of the model. For example, the observed effect of the antibiotic ointments on biofilms formed by the wound isolates should be correlated with relevant clinical data obtained from those patients. These data may include the severity of the wound infection, the response of the patient to the specific antibiotic ointment, and the duration of treatment.

The current model may benefit from one or more of the following modifications. First, in the present system, biofilms are developed on cellulose disks that are placed on LB agar plates, yet many other factors are present in the serous exudate within the infected burn wound. Thus, for a closer resemblance to *in vivo* conditions, LB agar may be replaced with serum-containing medium. Media designed to examine the effect of serum on the virulence of bacterial pathogens already exist, such as SAPI-serum and M9-serum media [25,29,30]. The biofilm would be developed on cellulose disks placed on either medium.

Secondly, instead of developing the biofilms on cellulose disks, they may be developed on *in vivo*-related surfaces such as the commercially available artificial skin (INTEGRA™, Plainsboro, NJ). Currently, we are conducting preliminary experiments to assess the development of *P. aeruginosa* biofilms on artificial skin.

Thirdly, the model may be adapted to examine the success of topical ointments in eliminating biofilm produced by more than one wound pathogen. Polymicrobial biofilms are frequently found within different infection sites, including infected wounds [16,28,31,32]. Using FISH, we evaluated the effect of the three topical ointments on a biofilm formed by *P. aeruginosa* and *S. aureus*. However, these qualitative experiments require specifically-labeled DNA probes for each microorganism and the use of CLSM, a technique not readily available in most clinical laboratories. Therefore, to maintain the simple and practical nature of the model, quantification of different microorganisms may be conducted using commercially available selective media such as mannitol salt agar and *Pseudomonas* selective agar.

In summary, we describe in this report a prototype of a practical model to screen topical antibiotic ointments to identify the ones that are most efficient in eliminating and/or inhibiting the development of biofilms by burn wound pathogens. The model may also be used to determine the efficacy of newly developed antimicrobial ointments. Currently, we are conducting further analyses to confirm the clinical relevance of the model.

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Fig. 1.

Diagram of the *in vitro* biofilm assay. (a) For the biofilm prevention model, inoculated disks (1) were immediately covered with the prepared gauze (2). For the biofilm disruption model, inoculated disks were incubated for 24 h to allow development of biofilm and then covered with prepared gauze (2). Steps 2 through 4 are the same for both models. (b) Following incubation with antibiotic ointment, the biofilms present on the disks were analyzed by enumeration of colony forming units (CFU)/disk and by staining and confocal laser scanning microscopy (CLSM).

Fig. 2. Gentamicin ointment prevents formation of *P. aeruginosa* biofilm. Disks inoculated with 10^{2-3} CFU of PAO1/pMRP9-1 were immediately covered with untreated gauze **(a)** or gauze spread with 400 mg of 0.1% gentamicin sulfate ointment **(b)**. After 24 h, green fluorescent protein (GFP) produced by viable PAO1/pMRP9-1 within the biofilms was visualized by CLSM.

Fig. 3.

Different antibiotic ointments disrupt biofilms formed by *P. aeruginosa*. Disks were inoculated with 10^{2-3} CFU of PAO1/pMRP9-1 (a), PACI-1 (b), or PACI-2 (c) and incubated for 24 h to allow biofilm development. The disks were covered with untreated gauze, or gauze treated with 400 mg 0.1% gentamicin sulfate ointment, 2% mupirocin ointment, or triple antibiotic ointment containing 400 U bacitracin, 3.5 mg neomycin, and 5,000 U polymixin B per gram, and incubated 24 h. Numbers of CFU were determined by plating 10-fold serial dilutions on LB agar as described in Fig. 1b. Values represent the means of three separate disks $\pm$ SEM. *** $P < 0.001$

Fig. 4.

The effect of no treatment (control; **a, e, i**), gentamicin ointment (**b, f, j**), mupirocin ointment (**c, g, k**), or triple antibiotic ointment (**d, h, l**) on 24-h biofilms formed by PAO1/pMRP9-1 (**a–d**), PACI-1 (**e–h**), or PACI-2 (**i–l**) was confirmed by CLSM. PAO1/pMRP9-1 biofilm was visualized using GFP; PACI-1 and PACI-2 biofilms were visualized using BacLight™ (Live/Dead) stain. Except for PACI-1 treated with gentamicin ointment (**f**), the antibiotic ointments disrupted 24-h biofilms produced by all three strains when compared with their untreated controls.

Fig. 5.

Different antibiotic ointments disrupt biofilms formed by *S. aureus*. Disks were inoculated with 10^{2-3} CFU of AH133 (a), SACI-1 (b), or SACI-2 (c) and incubated for 24 h to allow biofilm development. The disks were covered with untreated gauze, or gauze treated with 0.1% gentamicin sulfate ointment, 2% mupirocin ointment, or and triple antibiotic ointment containing 400 U bacitracin, 3.5 mg neomycin, and 5,000 U polymixin B per gram, and incubated 24 h. Numbers of CFU were determined by plating 10-fold serial dilutions on LB agar as described in Fig. 1b. Values represent the means of three separate disks $\pm$ SEM.

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Fig. 6. The effect of no treatment (control; **a, e, i**), gentamicin ointment (**b, f, j**), mupirocin ointment (**c, g, k**), or triple antibiotic ointment (**d, h, l**) on 24-h biofilms formed by AH133 (**a–d**), SACI-1 (**e–h**), or SACI-2 (**i–l**) was confirmed by CLSM. AH133 biofilm was visualized using GFP; SACI-1 and SACI-2 biofilms were visualized using BacLight™ (Live/Dead) stain. Compared with their untreated controls, the antibiotic ointments disrupted 24-h biofilms produced by all three strains.

Fig. 7.

Assessment of dual microbial biofilms in the disruption model. Equal CFU of PACI-2 and SACI-1 were inoculated onto cellulose disks and incubated 24 h at 37 °C for development of biofilm. The disks were then treated with the three antibiotic ointments or covered with untreated gauze for an additional 24 h. The relative presence of PACI-2 (green) and SACI-1 (pink) in the biofilms was analyzed by FISH. **(a)** untreated biofilm; **(b)** gentamicin ointment reduced the numbers of both strains; **(c)** mupirocin ointment affected *S. aureus* more than *P. aeruginosa*; **(d)** triple antibiotic ointment produced the opposite effect.

Table 1**Bacterial strains used in this study**

Strain	Description	Reference/source
<i>Pseudomonas aeruginosa</i>		
PAO1	Prototrophic strain, originally isolated from an infected wound	[25,33–35]
PAO1-GFP	PAO1 carrying plasmid pMRP9-1 in which the green fluorescent protein is constitutively expressed	Mathew Parsek, [36]
PACI-1	Left arm, burn wound isolate,	UMC Burn Center, Lubbock, TX
PACI-2	Left leg, burn wound isolate	UMC Burn Center, Lubbock, TX
<i>Staphylococcus aureus</i>		
AH133	<i>S. aureus</i> strain RN4220 carrying plasmid pCM11 in which the green fluorescent protein is constitutively expressed	Alexander Horswill, [27]
SACI-1	Right foot, burn wound isolate	UMC Burn Center, Lubbock, TX
SACI-2	Head, burn wound isolate	UMC Burn Center, Lubbock, TX