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In vitro analysis of interactions between *Pseudomonas aeruginosa* and *Candida albicans* treated with silver sulfadiazine in wound infections

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Background: Microorganisms tend to rely on close relationships with other species to survive. Consequently, biofilms formed by interactions of different species have been shown to delay the wound healing process. Studies suggest these mixed-population infections contribute to the development of drug resistance and inhibition of host immune response. Silver sulfadiazine (SSD) has been shown to effectively decrease the risk of infection in an open wound. Typically, these are bacterial wound infections; however, the role of fungal species needs further attention.

Objectives: The purpose of this *in vitro* study was to determine the effect of SSD on interactions between *Pseudomonas aeruginosa* 09-009 (PA1) or *P. aeruginosa* 09-010 (PA2) and *Candida albicans* ATTC 64550 (CA).

Methods: A mixture of 4 mL of tryptic soy broth (TSB) and 100 µL of CA and/or PA1 or PA2 (~10^6 log cfu/mL) inoculums were deposited into either wells or vials. The wells or vials were then sonicated (50 W for 10 s) to separate microorganisms attached to the walls. After incubation, cell counts were performed at 24 and 48 h for each microorganism using specific media.

Results: Our results show that without SSD treatment, *P. aeruginosa* exhibits an inhibitory effect on *C. albicans*. Treatment with SSD demonstrated significant reduction of *P. aeruginosa*; however, *C. albicans* persisted. This experiment demonstrates that SSD was effective in reducing the bioburden of both *P. aeruginosa* strains after 24 and 48 h; however, it was not as effective in reducing *C. albicans*.

Conclusions: The data suggest that for polymicrobial mixed infections containing *Pseudomonas* spp. and *C. albicans*, treatment with SSD may be beneficial but does not provide adequate microorganism eradication. As such, added treatments that provide coverage for *Candida* infection are necessary. Additional *in vivo* studies are needed to obtain a better understanding of the complex interactions between these organisms.

Introduction

Skin infections are a major concern in patients with open wounds, particularly in burn victims. Burn wound infections are often caused by Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. However, fungal pathogens, particularly *Candida* spp., are increasingly being reported in the literature, especially after use of broad-spectrum topical antimicrobial agents.[1–3] Fungal burn wound infections are typically observed with extensive, deep burns and are associated with both increased morbidity and mortality.[4] *Candida albicans*, the most prevalent human fungal pathogen, has the capacity to infect a variety of host niches and can cause significant disease in immunocompetent as well as immunocompromised individuals.[5] In a large study of burn patients, Gupta *et al.*[6] revealed that as many as 59% of burn wound cases may be coinfected with *Candida* species.

Colonization with more than one microorganism is quite common and makes the wound very difficult to manage. Biofilms consisting of microbial colonies encased in a polysaccharide matrix at wound surfaces play an important role in the pathogenesis of polymicrobial wound infections.[7,8] Initially, bacteria and fungi originating from the host's endogenous skin, gastrointestinal, respiratory flora or sometimes the external environment, colonize burn wound surfaces. Over time, some of these organisms grow and delay wound healing by producing destructive

enzymes and toxins, leading to a chronic inflammatory state.[8] Studies have reported the synergistic interactions between multiple microorganisms in coinfected wounds, which have been implicated in delayed wound healing and antimicrobial tolerance when compared with monospecies wound infections.[9,10] Specifically, *P. aeruginosa* and *C. albicans* are two major opportunistic pathogens that have been found to form polymicrobial biofilms, which significantly impede the wound healing process and contribute to drug resistance.[11,12]

Understanding the interactions occurring in these polymicrobial wound infections is of great importance given their ubiquitous nature and ability to delay wound healing. A study performed by Pastar *et al.*[13] found that wound re-epithelialization is significantly delayed by *S. aureus* and *P. aeruginosa* biofilms consisting of multiple species through suppression of keratinocyte growth factor 1. Furthermore, polymicrobial wound infections containing *P. aeruginosa* were found to increase expression of virulence factors such as Panton–Valentine leucocidin and α-haemolysin, which are formed by MRSA. Similarly, studies have shown that in mixed biofilms, both *P. aeruginosa* and *C. albicans* exhibit increased virulence and mutability. In these settings of interspecies competition, *P. aeruginosa* has been found to increase production of virulence factors such as pyoverdine, rhamnolipids and pyocyanin, which further enhance its ability to cause more severe disease.[11]

Since burns are associated with hypermetabolism, chronic inflammation and weakened immune status, wound infections can lead to several complications such as sepsis.[14] Over the last several decades, significant advances have been made in the management of burn wounds as a result of improved fluid resuscitation, nutritional support, surgical excision techniques, infection control and wound care. Local management of infected burn wounds often includes cleansing, debridement, routine wound dressing changes and topical antimicrobial agents.[15] Silver sulfadiazine (SSD) is a thick white cream that is widely available and relatively inexpensive.[16] It is a commonly used burn wound dressing with antimicrobial activity.[17] While it is used to reduce wound infections, there are limited studies that demonstrate its utility in treating polymicrobial wounds.[18]

The purpose of this *in vitro* study was to investigate the relationship between bacteria and fungi within burn wound infections and to determine the effect of SSD on interactions between either *P. aeruginosa* 09-009 (PA1) or *P. aeruginosa* 09-010 (PA2) and *C. albicans* ATTC 64550 (CA). Many other studies have been performed to challenge microorganisms and have demonstrated the treatment's efficacy.[19] We hypothesize that when bacteria and fungi coexist in a burn wound infection, they influence each other's ability to proliferate and act as pathogens. In order to test this hypothesis, we performed *in vitro* proliferation assays involving *P. aeruginosa* and *C. albicans*. The role of biofilm formation in such polymicrobial infections was investigated.

Materials and methods

Microorganism strains and growth conditions

CA and two different strains of *P. aeruginosa* isolated from clinical combat wounds (PA1 and PA2, obtained from the US Army Institute of Surgical Research, Fort Sam Houston, TX, USA) were used. Tryptic soy broth (TSB) was used as the growth medium for both microorganisms. For quantification, Pseudomonas Agar Base with CN supplement (Oxoid) was used to isolate *P. aeruginosa* and BBL™ CHROMagar™ Candida was used to isolate *C. albicans*.

In vitro biofilm assay to investigate the interactions between P. aeruginosa and C. albicans

Two different sets of experiments, one with SSD treatment and one without treatment, were performed to investigate the interactions between the two different strains of *P. aeruginosa* and *C. albicans*, namely PA1 versus CA and PA2 versus CA. All inoculum suspensions were quantified to obtain the exact concentration of viable organisms prior to the experiment. For the *in vitro* study without treatment, the *P. aeruginosa* and *C. albicans* inoculums were prepared with 100 µL (~10^6 log cfu/mL) of microorganisms inoculated in 4 mL of TSB medium in each well (9 out of 12) in a 12-well polystyrene plate. There were nine replicates of each microorganism (or a combination of both species) along with three empty wells with only TSB medium serving as negative controls in each plate. These plates were incubated at 37°C for 24 and 48 h. For the first set of experiments, PA1, CA and a combination of the two species grew in TSB under the same conditions. For the second set of experiments, PA2, CA and a combination of the two species also grew in TSB under the same conditions. In order to quantify the number of microorganisms viable on the biofilm, microorganisms were recovered through sonication at 50 W for 10 s to separate the microorganisms attached to the wells. Serial dilutions were made and the microorganisms were plated on the selective media Pseudomonas Agar Base with CN supplement and CHROMagar™ Candida to isolate and quantify the bacterial counts.

In vitro biofilm assay to investigate effect of SSD treatment on interactions between P. aeruginosa and C. albicans

Two different sets of experiments were performed to investigate the effect of treatment with SSD on the interactions between strains of *P. aeruginosa* and *C. albicans*, specifically PA1 and CA, as well as PA2 and CA. For each experiment, the *P. aeruginosa* and *C. albicans* inoculums were prepared and 100 µL (~10^6 log cfu/mL) of microorganisms were inoculated in 4 mL of TSB medium contained in a glass test tube. There were a total of 12 vials, of which 9 contained replicates of each microorganism (or a combination of both species). Following these steps, 100 mg of SSD was added to each test tube and vortexed to mix the samples. For negative controls, three test tubes containing only 4 mL of TSB and 100 mg of SSD was used. For the positive control, a test tube with 4 mL of TSB and 100 µL (~10^6 log cfu/mL) of one microorganism was used without any SSD. These tubes were incubated at 37°C for 24 and 48 h. For the first set of experiments, PA1, CA and a combination of both species were grown in TSB under the conditions listed above with the SSD treatment. For the second set of experiments, PA2, CA and a combination of both species were grown in TSB under the same conditions, along with SSD. After 24 and 48 h, in order to quantify the number of viable microorganisms, serial dilutions were made and the microorganisms were plated on the appropriate selective medium, Pseudomonas Agar Base with CN supplement or CHROMagar™ Candida, to isolate and quantify the organisms.

Statistical analysis

A *t*-test was used to perform statistical comparisons of cfu, which were presented as means and standard deviations. Statistically significant differences were defined as $P < 0.05$.

Results

Results showed for the experiment where CA and PA1 were combined without treatment that both microorganisms co-existed in

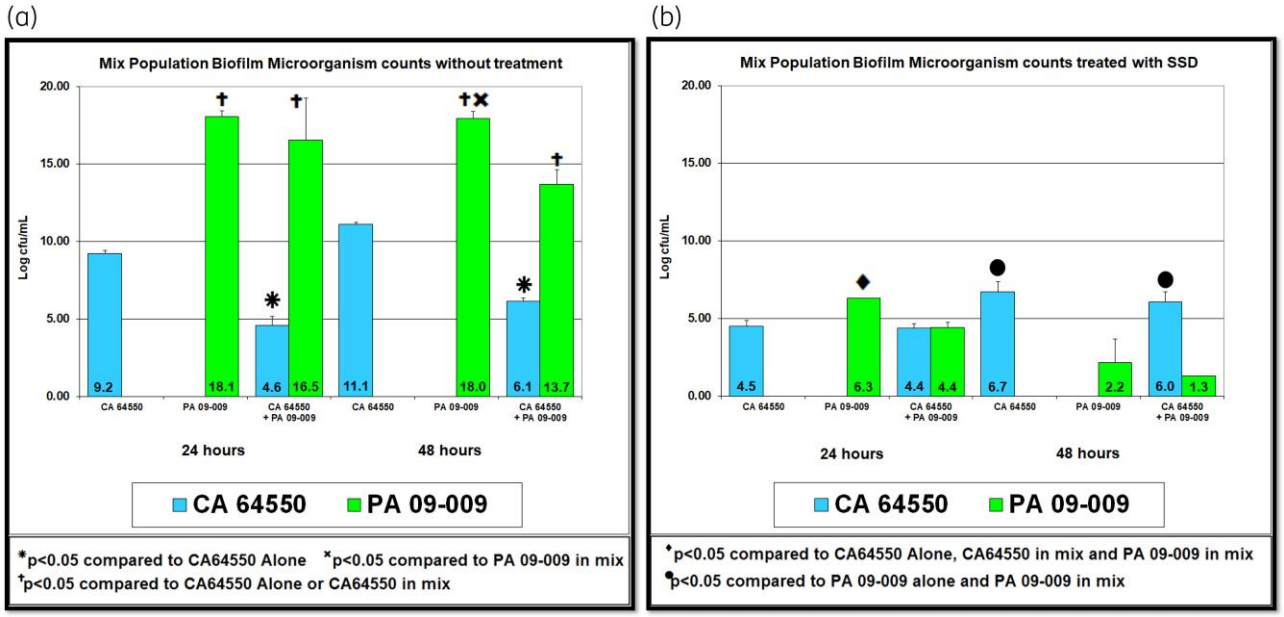

Figure 1. Growth interactions between PA1 and CA *in vitro*, without and with SSD treatment. (a) In cultures without treatment, CA and PA1 cfu were quantified based on growth of single-species or mixed-species biofilms *in vitro* on wells ($n=9$). At 24 and 48 h, PA1 reduced the growth of CA by approximately 5 logs. (b) When treated with SSD, CA and PA1 cfu were quantified based on growth of single-species or mixed-species biofilms in test-tube vials ($n=9$). After 24 h, CA had lower total bacterial counts but PA1 in treated mixture showed reduced growth by 2 logs. After 48 h, PA1 alone and in mixed culture was reduced by 4 and 5 logs, respectively, with a 99.99% bacterial reduction ($P<0.05$), while CA continued to grow.

the wells and that the growth of CA was reduced by PA1 *in vitro* (Figure 1a). CA mixed with PA1 at both 24 and 48 h resulted in a significantly lower PA1 bacterial count than fungal count of CA alone ($P<0.05$). PA1 alone and in mixed cultures had significantly higher bacterial counts when compared with CA alone or in mixed cultures ($P<0.05$). CA was found to have a 99.99% reduction in mixed cultures after 24 and 48 h. At 48 h, PA1 in the mixed culture had a statistically significant lower count when compared with PA1 alone ($P<0.05$). When those strains were treated in combination with SSD treatment, CA and PA1 co-existed in the vials, and the growth of both CA and PA1 was reduced *in vitro* (Figure 1b). After 24 h, we found that CA, either alone or mixed, and PA1 in the mixed culture had lower counts than PA1 alone ($P<0.05$). Interestingly, after 48 h, counts of PA1, alone or in mixed culture, were lower than CA alone or in the mixed culture ($P<0.05$). In fact, in the mixed cultures treated with SSD, at both 24 and 48 h, PA1 counts were lower than those of CA, with a microbial reduction of 99.99% ($P<0.05$).

When the other *P. aeruginosa* (PA2) was combined with CA, the results showed that CA and PA2 co-existed in the wells and that the growth of CA was reduced by PA2 *in vitro* (Figure 2a). After both 24 and 48 h, the CA count in the mixed culture was lower than for CA alone, as well as for PA2 found alone or in mixed cultures ($P<0.05$). At both assessment points, CA had lower microorganism counts than PA2 in the mixture, with microbial reductions of 99.99% ($P<0.05$). Those mentioned organisms in combination and treated with SSD showed that when CA and PA2 co-existed in the vials, the microorganism growth was reduced *in vitro* compared with those without treatment (Figure 2b). After 24 and 48 h, the PA2 count in the mixed culture was lower than for PA2 alone ($P<0.05$). At both timepoints, PA2 bacterial counts in mixture were found to be lower than for CA either alone or mixed ($P<0.05$). Interestingly, at 48 h, CA levels were slightly higher in the mixed culture than alone.

Overall, the results show that when calculating the microbial reduction percentage between each *P. aeruginosa* microorganism against CA without treatment, CA had a statistically significant reduction at both 24 and 48 h. When examining the mixed cultures of *P. aeruginosa* strains with CA treated with SSD after 24 h, both strains of *P. aeruginosa* had a significant microbial reduction when compared against *P. aeruginosa* strains cultured alone.

Discussion

The complex polymicrobial nature of biofilms in chronic non-healing wounds has been studied and is well documented in the literature.[20–24] However the majority of microbiome research focuses on the diverse bacterial component of chronic wounds, as fungi in wounds often go undetected.[25,26] More recent studies are examining the interactions between fungi and bacteria in wounds.[27,28] Clinical and research biofilm experts are in agreement that *in vitro* methods can be useful in screening treatments for their efficacy against biofilms.[29–33] Yet there are a limited number of *in vitro* studies that have examined the synergistic relationship between the commonly encountered pathogens *C. albicans* and *P. aeruginosa* or the impact of SSD on their growth. To further investigate the interactions of *C. albicans* with *P. aeruginosa* strains and the effects of SSD on the wound environment, we utilized an *in vitro* model focusing on PA1 versus CA and PA2 versus CA. In this study we demonstrate that

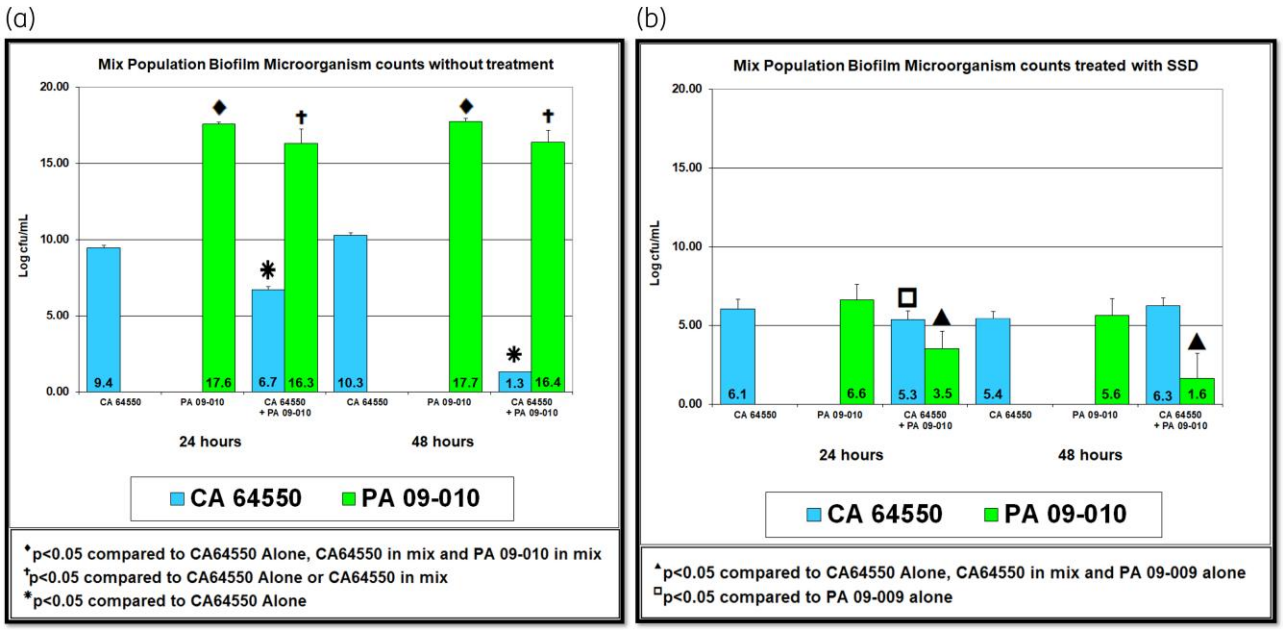

Figure 2. Growth interactions between PA2 and CA *in vitro*, without and with SSD treatment. (a) In cultures without treatment, CA and PA2 cfu were quantified based on growth of single-species or mixed-species biofilms *in vitro* on wells ($n=9$). At 24 and 48 h, PA2 reduced the growth of CA by approximately 3 and 10 logs, respectively. (b) When treated with SSD, CA and PA2 cfu were quantified based on growth of single-species or mixed-species biofilms in test-tube vials ($n=9$). After 24 and 48 h, PA2 in the mixed culture was reduced by 3 and 4 logs, respectively, with a 99.99% bacterial reduction ($P<0.05$), while CA continued to grow.

P. aeruginosa and *C. albicans* co-exist and exhibit growth in biofilms.

Overall, the results show that CA had lower bacterial counts than both PA1 and PA2 strains when cultured alone or without treatment. When calculating the bacterial reduction percentage between each *P. aeruginosa* microorganism against CA without treatment, CA had a 99.99% reduction at both 24 and 48 h. Nonetheless, analysis of these microorganisms combined and treated with SSD showed that bacterial counts for both *P. aeruginosa* strains were lower than those of CA after 48 h. In fact, after 24 h of treatment with SSD, both strains of *P. aeruginosa* mixed with CA had a 98.71% bacterial reduction when compared against isolated *P. aeruginosa*. When examining the treated mixture after 48 h, *P. aeruginosa* had a 99.99% bacterial reduction compared with CA. After both 24 and 48 h, treated *P. aeruginosa* mixtures had a 99.99% bacterial reduction when compared with untreated counterparts. The treatment demonstrated the ability of SSD to efficiently reduce bioburden of both *P. aeruginosa* strains after 24 and 48 h; however, it was not as effective in reducing CA proliferation. CA growth was not significantly affected by either the treatment or competition with *P. aeruginosa*, but when both microorganisms competed against one another without any treatment interference, *P. aeruginosa* colonized at a higher rate.

These data suggest that if a patient has infection with both microorganisms, an additional treatment regimen is needed to eradicate the *C. albicans* infection. Our data highlight the significance of bacterial and fungal interactions in wound infections. As seen in this study, multispecies interactions exhibited in biofilms have the potential to delay healing and may alter microbial susceptibility to therapies. Further *in vivo* studies should be carried out to obtain a better understanding of interspecies interactions for developing new successful therapeutic approaches.

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