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ARTICLE OPEN

Removal of *Staphylococcus aureus* from skin using a combination antibiofilm approach

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Staphylococcus aureus (*S. aureus*) including methicillin resistant *S. aureus* (MRSA) is one of the primary microorganisms responsible for surgical site infection (SSI). Since *S. aureus* contamination is known to originate from the skin, eradicating it on the skin surface at surgical sites is an important intervention to reduce the chance of SSIs. Here we developed and evaluated the efficacy of a combination probiotic/brush sonication strategy for skin preparation at surgical, injection and insertion sites in medicine. A 24 h biofilm on porcine skin explants was used as a worst-case scenario for the evaluation of preparation strategies. Conventional ethanol wipes achieved 0.8~2 log reduction in viable bacteria depending on how many times wiped (x4 or x6). Brush sonication or probiotic supernatant pre-treatment alone achieved a similar reduction as ethanol wipes (1.4 and 0.7~1.4 log reduction, respectively). Notably, combining sonication and probiotic pre-treatment achieved a 4 log reduction in viable bacteria. In addition, probiotic supernatant incubation times as short as 2 h achieved the full effect of this reduction in the combined strategy. These findings suggest the promising potential of combination-format skin preparation strategies that can be developed to more effectively penetrate cracks and folds in the skin to remove biofilms.

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INTRODUCTION

Surgical site infection (SSI) is the most common (160,000~300,000 per year) and most costly healthcare-associated infection[1] in the United States and ranges from superficial skin infection to life-threatening postoperative complication. Foreign materials such as indwelling and implanted medical devices increase the risk of SSI significantly because less bioburden—as low as 100 CFU—is needed to cause infection.[2] According to the 1999 CDC Guideline for Prevention of SSI, the endogenous microbes of a patient's skin and mucous membrane are the primary source of pathogen contamination for most SSIs.[3] Preventing initial bioburden transfer from the skin to foreign materials and adjacent tissue is thought to be an important intervention to prevent medical device associated SSI.[4,5] However, current research on preventing medical device associated infections has focused more on antimicrobial biomaterials and sterile practices (such as handwashing) than on understanding how bioburden is transferred from the skin surrounding a penetration site. Therefore, understanding this aspect of the pathogenesis process can help inform the development of skin preparation countermeasures. By preventing contamination of normally sterile internal compartments, we can target the critical first step before bacterial colonization, multiplication and biofilm entrenchment. This could improve antimicrobial stewardship by reducing the use of antibiotics and antimicrobials.[6]

The human skin microbiota is diverse and includes numerous pathogenic bacteria.[7] *Staphylococcus aureus* (*S. aureus*) are the most commonly isolated pathogen,[8] accounting for 20–30% of SSI occurring in hospitals.[9] This prevalence is related to the carriage of *S. aureus* in the healthy population (~20% persistent, ~60% intermittent).[10] While topical antibiotics and antiseptics are often employed to reduce *S.aureus* colonization, these treatments may alter skin microbiota and reduce colonization by *S.aureus* competitors.[11] Current patient-focused interventions to reduce contamination of surgical sites with pathogenic bioburden are limited to skin preparation and antibiotic prophylaxis. For surgical procedures at high risk of infection (contaminated wounds or dirty wounds), the use of prophylactic antibiotics has markedly reduced SSIs.[12] However, the increasing spread of antibiotic resistant organisms makes prophylaxis more challenging and necessitates rethinking current approaches to improve stewardship of existing antibiotic resources. Considering that about 30% of infectious pathogens may be resistant to standard prophylactic antibiotics in the United States, as many as 120,000 SSIs and 6,300 deaths each year may be due to resistant organisms.[13] The proportion of infections with resistant organisms is also on the increase.[14]

One way to reduce dependence on the use of antibiotics could be improved skin preparation to remove microbial counts to sub-pathogenic levels.[15] Conventional skin preparation methods are widely accepted (alcohol, chlorhexidine, povidone-iodine and their combinations),[16,17] and there are relatively few studies focused on improved approaches. Skin preparation strategies may benefit from other areas of infection control research, where an emerging approach to the treatment of biofilm involves the combination of physical forces—such as sonic energy or electric field--with antimicrobial treatment.[18–20] These combined approaches are synergistic because the physical field helps break up biofilm structure while the antimicrobial component helps to kill segregated bacterial cells. In particular, the use of non-chemical antimicrobial approaches such as probiotics[21] and

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phage[22] is being explored to improve performance over conventional antimicrobials while benefiting antimicrobial stewardship. Since skin is colonized by endogenous bacteria, pretreatment with beneficial bacteria that already exist in healthy skin is potentially a safe and effective option.[23] Beneficial probiotics compete with pathogens for adhesion and nutrients, weakening their ability to survive and proliferate. The supernatant produced by probiotic bacteria is rich in metabolites that are the likely source of antimicrobial activity against existing biofilms. For example, *Lactobacillus spp*. and *Bifidobacteria spp*. supernatants have been reported to reduce biofilm.[24,25] Preliminary clinical studies have suggested that probiotic ingestion and nasal spray may be effective in eradicating persistent carriage of MRSA in the throat and nose.[26,27] However, less has been studied about how probiotic strains might prevent biofilm associated infections, especially at surgical incision/injection sites.

In this work, we developed and assessed a combination approach for skin preparation. We used a recently developed porcine skin explant model[28] to study the effectiveness of several alternative skin preparation approaches alone and in combination. The porcine skin model simulates a physiological tissue environment where pathogens may be more persistent than on abiotic materials.[29] For example, *S. aureus* biofilm with host fibrin as part of the matrix have been shown to be more robust than on abiotic surfaces which are often used in in vitro testing.[30] Since clinical testing is not possible with virulent pathogens, this approach provides a rapid, reproducible, and cost-effective way to test skin preparation strategies. We evaluated conventional alcohol-based skin wipes, a sonication brush, and probiotic bacteria, along with combinations of these approaches, for the potential to remove *S. aureus* growing in biofilm on the skin surface.

RESULTS

Effect of alcohol wipe and brush sonication on skin *S. aureus* biofilm removal

Porcine skin surface was inoculated with *S. aureus* (10^5 CFU mL^{-1}) and cultured 24 h for biofilm formation. The established bioburden was characterized with both CLSM imaging and plating. Before skin preparation (Fig. 1a), heterogeneous skin biofilm structure was observed. Both alcohol wipes (Fig. 1c) and brush sonication (Fig. 1d) were found to significantly reduce bioburden levels of *S. aureus* AH2547 24 h biofilm. To assess potential contamination from other microorganisms, plain porcine skin incubated with growth media was imaged after 24 h, and showed no bacterial growth (Fig. 1b). There were between 10^8 to 10^{10} CFU cm^{-2} surface viable bacteria (PC) from different cultures and skin surfaces. After normalizing the viable number of PC to 10^6 CFU cm^{-2} (Fig. 2), the 4 × alcohol wipe (A4), 6 × alcohol wipe (A6), sonication brush (B), and sonication brush with alcohol (BA) bioburden levels resulted in surface bacterial densities of $(13.2 \pm 2.7) \times 10^6$, $(1.00 \pm 0.27) \times 10^6$, $(4.36 \pm 1.8) \times 10^6$, and $(0.0630 \pm 0.011) \times 10^6$ CFU cm^{-2}, respectively. A significant difference ($p < 0.005$) was seen when comparing A4 with A6, B, and BA. The three skin preparation methods (A6, B, and BA) were statistically different ($p < 0.05$), and BA removed 3 logs of bioburden.

Effect of probiotic supernatant on skin *S. aureus* biofilm development and removal

LAB supernatant from multiple culture times was initially tested for inhibition of *S. aureus* growth to determine the optimal time (Supplementary Materials, Figure S1). Supernatants obtained at 16 and 24 h time points inhibited *S. aureus* growth for 24 h. We then tested how supernatants collected with different initial probiotic cell concentrations would inhibit *S. aureus* growth with the pig skin model (Fig. 3, hollow square). The results showed that inhibition of *S. aureus* growth increased (27, 70, 81, and 84%) with the starting inoculum of probiotic supernatants (10^4, 10^6, 10^8, and 10^{10} CFU mL^{-1}, respectively). The supernatants were also used to pre-treat well-established *S. aureus* biofilms (24 h) on skin (Fig. 3, solid circle). Compared to the control, supernatants collected from 10^4 to 10^{10} CFU mL^{-1} *L. rhamnosus* inoculum reduced 65–89% of well-established skin surface bioburden.

Combination treatment on skin *S. aureus* biofilm removal

Probiotic supernatant pretreatment of *S. aureus* biofilms for both 2 h (PB2 + 2) and 24 h (PB2 + 24) was evaluated (Fig. 4). After normalizing the viable number of PC to 10^8 CFU cm^{-2}, the PB2 + 2, PB2 + 24, and combined with brush sonication PB2 + 2 + B, PB2 + 24 + B bioburden levels resulted in surface bacterial densities of $(24.6 \pm 14) \times 10^8$, $(2.97 \pm 2.7) \times 10^8$, $(0.00674 \pm 0.0015) \times 10^8$, and $(0.00742 \pm 0.0055) \times 10^8$ CFU cm^{-2}, respectively. With only probiotic pretreatment, the PB2 + 2 bioburden level is statistically different from the others ($p < 0.05$). While PB2 + 24 is significantly different from the other two combination treatments ($p < 0.005$), the difference in bioburden within the combination treatments (PB2 + 2 + B and PB2 + 24 + B) is not significant.

The bioburden removal efficacy (%) and log reduction for all tested skin preparation treatments on *S. aureus* 24 h biofilm were summarized in Table 1. Wiping more thoroughly with alcohol pads (A6 vs. A4) improved the removal efficacy from 86.8 to 98.9%, greater than one log increase. For skin preparation with the sonication brush, spraying the brush head with alcohol greatly helped biofilm removal compared to saline (~2 log improvement). Probiotic pretreatment alone reduced viable bacteria 75.4–97% (2–24 h). When combined with sonication brush/alcohol, the difference between probiotic pretreatment was minimized. Over 4 log reduction was achieved with combination treatment for well-established *S. aureus* biofilm (24 h) on porcine skin surfaces.

DISCUSSION

There is a need for better models to study how to improve skin preparation before penetrating procedures, to help reduce the chance of microbial contamination/infection in transcutaneous medical procedures. For infections associated with medical devices, the skin is a significant source of potential bioburden and could benefit from more effective preparation procedures. Due to our increasing understanding of the persistence of biofilm and its presence on human skin, it is important to test potential preparation strategies specifically against biofilm.

In this work, we used both plating and confocal microscopy (CLSM) to characterize bioburden after preparation of pig skin. Although CLSM is not ideal for quantifying large amounts of bacteria on the skin, it was valuable to show the distribution of bacteria after various cleaning procedures (Fig. 1). Unlike biofilms on smooth abiotic surfaces often used for in vitro effectiveness testing (plastic, silicone, etc.), biofilm on the rough topography of skin were heterogeneous and were made even more heterogeneous by the cleaning process. Bacteria remaining after alcohol wipes (3c) tend to be clustered in certain areas of the skin, likely at folds and ridges where the wipe was not able to make good contact. For the alternative skin preparation methods (3d–f) bacteria was left more homogenously scattered on the surface.

The roughness of skin and heterogeneous nature of cleaning are primary reasons why we tested sonication as an alternative to wiping as a physical removal method. Since increasing alcohol wipe steps from 4 × (A4) to 6 × (A6) significantly improved the reduction of bioburden (Fig. 2), we hypothesized that sonication might further help break up the biofilm matrix and work synergistically with other approaches to remove *S. aureus* contamination from skin. We know from previous results that there is little difference in skin wipes (alcohol, povidone-iodine and chlorhexidine) against *S. aureus* biofilm even among different