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Major Article

The *in vitro* efficacy of betadine antiseptic solution and colloidal silver gel combination in inhibiting the growth of bacterial biofilms

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Introduction: Betadine (Povidone-Iodine) solution is a topically applied antiseptic, which has been used routinely used in wound care and general surgery to prevent skin and wound infections. However, several studies have documented the ineffectiveness of betadine. Other topical antimicrobial dressings, including those that contain silver, have been used in the management of infected wounds. The present study was undertaken to determine if the combination of 5% betadine solution and silver colloidal gel (Ag-gel) is more effective than either substance alone in inhibiting the growth gram-negative and gram-positive bacteria.
Methods: The effectiveness of 5% betadine solution and Ag-gel as anti-microbial agents were assessed using both colony forming unit (CFU) assay and confocal laser scanning microscopy (CLSM).
Results: Ag-gel showed complete inhibition on all the bacteria species examined except the *Klebsiella pneumoniae* clinical isolate (CL) strain while 5% betadine concentrations did not completely kill any of the tested bacteria. In contrast, *K. pneumoniae* was completely eliminated in the presence of both 5% betadine solution and Ag-gel together. The CLSM showed similar findings to the CFU results examining the 5% betadine solution and Ag-gel combination.
Conclusions: This study demonstrated that while the individual treatments using either 5% betadine solution and Ag-gel alone were infective antimicrobial agents, the combination of 5% betadine solution and Ag-gel was superior at eliminating all tested bacteria, including *K. pneumoniae* CL.
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The skin is the human body's biggest organ and a powerful first line of defense against infection. Both the innate and adaptive immune systems use the skin as a physical barrier to infections as well as a mechanism of surveillance and reactive reaction.[1] When the barrier function of the skin is damaged, the immune response is harmed. Although microbial colonization of wounds is common, the immune system plays an important role in avoiding active infection. Large wounds, such as serious burns, are more vulnerable to microbial invasion and the production of biofilms. As such, wound infections are a serious problem. Despite substantial advancements in burn wound treatment and supportive care, infection is still a major concern for burn patients.[1] Pseudomonas aeruginosa and Staphylococcus aureus are the predominant infective organisms in the clinically significant microbiome of large, acute burns.[1]

Due to the difficulties of antibiotic diffusion through inflamed wound tissue, systemic antibiotic treatment is frequently of limited efficacy in burn wounds and other skin injuries. Furthermore, the emergence of acquired resistance to frequently administered systemic antibiotics has created a greater push for antimicrobial stewardship. As a result, antiseptics and modifications to wound dressings have garnered greater interest for preventing bacterial infections.[1] Among the current antiseptics, betadine (Povidone-Iodine) is a broad spectrum antiseptic for topical application in the treatment and prevention of infection in wounds.[2-5] Currently, it is generally accepted to use 5% betadine solutions for preparation of different surfaces such as in wound preparation.[3,4] The reason concentrations below 5% for

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povidone-iodine are not used is that concentrations below 5% are not effective bactericidal agents.[6,7] Moreover, 10% povidone-iodine solution is commonly used as a surgical scrub pre- and post-op to surgical sites.[8]

Topical antimicrobial dressings, including those that contain silver, have long been used to assist with management of infected wounds and those at risk of infection in a wide range of wounds. As a result, dressings are increasingly extensively utilized, particularly ones containing silver. Silver dressings suppress microbial development in wounds through a variety of mechanisms and may speed up healing.[9,10] Silver is inert in its metallic condition. However, when silver comes into contact with moisture from the skin or fluid from a wound bed, it dissolves and ionizes. Silver ions (Ag+) are extremely reactive, inducing structural changes in bacterial cell walls, intracellular and nuclear membranes, and cell lysis.[1] Silver ions also have bacteriostatic qualities because they attach to and denaturize bacterial DNA and RNA, preventing protein synthesis and reproduction.[1] Silver containing dressings (Silvazine, Aquacel Ag, and Acticoat) studied so far have shown promise in preventing and eliminating bacterial infections in human patients.[1] *In vitro* studies have shown that silver can kill gram-positive and gram-negative bacteria, in addition to Methicillin Resistant *S. aureus* and Vancomycin Resistant *Enterococci*.

Although the antimicrobial efficacy of betadine is controversial, the present study was undertaken to measure the effectiveness of 5% betadine solution by itself and in combination of silver colloidal gel in inhibiting the growth of both gram-negative and gram-positive bacteria. The silver colloidal gel (Ag-gel) is silver in the Ag° form, which doesn't react with the iodine in the betadine. The present study was undertaken to determine if the combination of 5% betadine solution and silver colloidal gel (Ag-gel) is more effective than either substance alone in inhibiting the growth gram-negative and gram-positive bacteria.

EXPERIMENTAL SECTION

Povidone-Iodine solution preparation

About 10% povidone-iodine or betadine topical solution (#NDC-67618-150-04, Betadine Microbicides, Purdue Products LP, Stanford, CT, USA) was purchased from Betadine Microbicides. Betadine solution was diluted to 5% in sterile 1X PBS (pH = 7.4). The solution was stored at room temperature until use.

Silver dressing

The dressing evaluated contained colloidal silver (Ag^o) in a gel form (Ag-gel). The Ag-gel was obtained from Viridis Biopharma Pvt., Ltd, Mumbai, India. It was tested as 50 mg evenly spread on a 6 mm diameter BBL blank paper discs (#B31039, Fisher Scientific, Waltham, MA, USA).

Bacterial strains, media, and growth conditions

The laboratory strains of bacteria tested were *Staphylococcus aureus* GFP strain AH133 [20] and *Pseudomonas aeruginosa* PAO1 GFP strain MM294, which constitutively expresses green fluorescent protein from plasmids pCM11 and pMRP9-1 respectively. These strains were used for all studies performed in this study. The strains were routinely grown in Luria-Bertani (LB) broth at 37°C, with shaking (250 rpm). To maintain pCM11 in AH133, Luria Broth (LB) was supplemented with 1 μg/mL erythromycin. To maintain pMRP9-1 in PAO1, LB was supplemented with 300 μg/ml carbenicillin. In addition, clinical isolates of the following bacteria were studied: *Klebsiella penumoniae* (*K. penumoniae* clinical isolate (CL)), *Staphylococcus epidermidis* (*S. epidermidis* CL), and Methicillin-resistant *Staphylococcus aureus* (MRSA CL). The efficacy of 5% betadine solution, Ag-gel, and combination of 5% betadine solution with Ag-gel was examined using LB medium (#113002022, MP Biomedicals, Solon, OH) and LB-Agar medium (#113002222, MP Biomedicals, Solon, OH, USA) as the growth medium. The clinical isolates were obtained from the Clinical lab at Texas Tech University Health Sciences Center under an approved Institutional Review Board protocol, Texas Tech University Medical center and/or Lubbock, Texas, USA.

Colony forming unit assay

We analyzed the remaining bacteria on the discs using the Colony Forming Unit (CFU) assay.[11] The CFU assay is a well-standardized, reliable susceptibility testing techniques. Briefly, bacteria were grown overnight in LB medium. The following day, the bacterial culture was washed in Mueller Hinton (MH) broth (#70192, Sigma-Aldrich, St. Louis, MO, USA), and the bacterial suspension was adjusted to an OD_{600} of 0.5 in MH broth according to the standard guidelines of the National Committee for Clinical Laboratory Standards.[12] Following this, a sterile cotton swap was dipped into the adjusted bacterial culture, and a lawn of bacteria was made on a LB Agar plate using the dipped swab. The bacteria were then allowed to grow and form a biofilm for 24 hours at 37°C. The betadine discs were prepared by adding 20 μl of 5% betadine solution onto 6 mm diameter BBL blank paper discs. In the Ag-gel studies, 50 mg of Ag-gel was applied over the entire discs. In the betadine and Ag-gel combination studies, 50 mg of Ag-gel was applied over discs containing 20 ul of betadine. Three discs were distributed evenly onto the LB Agar surface. Following incubation, each disc piece was transferred to a sterile 1.5-mL micro centrifuge tube containing 1 mL of PBS (pH = 7.4) for enumeration of bacteria. The tubes were placed in a water bath sonicator for 10 minutes to loosen the cells within the disc and then vigorously vortexed 3 times for 1 min to detach the cells. Suspended cells were serially diluted (10-fold) in PBS, and 10-μL aliquots of each dilution were spotted onto LB Agar plates. In addition, the remained 900 μL zero dilution sample was also plated on a different LB agar plate. Thus, the equation for back calculating the bacterial concentration was CFU $\times$ dilution factor and/of inoculum size in mL. This means that if only 1 bacterial cell was originally in the tube, we would have a 90% chance of detecting it. All experiments were repeated 3 times with 3 discs each time. Thus, there were 9 separate determinations for each combination tested.

Confocal laser scanning microscopy (CLSM)

This was prepared as described above in CFU assay. Following the growth of the bacteria on the discs with and without antimicrobial material, some of the discs were removed for the CLSM study. The discs from the *S. aureus* GFP AH133/pCM11 and *P. aeruginosa* GFP PAO1/pMRP9-1 plates were examined under the Confocal Laser Scanning Microscopy (CLSM). *S. aureus* GFP and *Pseudomonas aeruginosa* GFP are lab strains which constitutively express green fluorescent protein from plasmids pCM11 or pMRP9-1 when grown in the presence of 1 μg/ml erythromycin or 300 μg/ml carbenicillin respectively. Three discs for each plate were examined for the presence of bacteria remaining on the discs by the confocal laser scanning microscopy (CLSM). Visualization of the *S. aureus* GFP AH133 and *P. aeruginosa* PAO1 GFP bacteria was accomplished with a Nikon Eclipse Ti upright confocal laser scanning microscope (Nikon, Melville, NY, USA). Samples were examined under 10X objective lens, FITC fluorescence laser (488.0 nm), 4 channel confocal, and 512 scan size. The images were processed and analyzed using NIS-Elements AR Imaging Software as previously described.[11] All experiments were done in triplicate, and all measurements were repeated 3 times.

Statistical analyses

Results of the CFU assays were statistically analyzed using GraphPad InStat 3.06 (GraphPad Software, San Diego, CA, USA). Significance between pairs of values was calculated using an unpaired 2 tailed t test. Differences were considered significant when the *P* value was < .05.

RESULTS

Effect of 5% betadine treatment on bacterial biofilm formation for in vitro *CFU studies*

The results for the 24 hour *in vitro* CFU studies in the presence of betadine using *Staphylococcus aureus* GFP strain AH133 and *Pseudomonas aeruginosa* PAO1 GFP strain MM294, and clinical isolates of *Klebsiella pneumoniae* (*K. pneumoniae* CL), *Staphylococcus epidermidis* (*S. epidermidis* CL), and Methicillin-resistant *Staphylococcus aureus* (MRSA CL) are illustrated in Figure 1 (A, B, C) and Figure 2 (A,B). The 5% betadine in the placebo gel showed at best approximately 1 log of inhibition of growth for *Staphylococcus aureus* GFP strain AH133, and clinical isolates of *K. pneumoniae* CL, *S. epidermidis* CL), and MRSA CL. However, 5% betadine showed little or no inhibition of *Pseudomonas aeruginosa* PAO1 GFP strain MM294.

Effect of Ag-gel treatment on bacterial biofilm formation for in vitro *CFU studies*

The results for the 24 hour *in vitro* CFU studies in the presence of Ag-gel using *Staphylococcus aureus* GFP strain AH133 and *Pseudomonas aeruginosa* PAO1 GFP strain MM294, and clinical isolates of *K. pneumoniae* CL, *S. epidermidis* CL, and MRSA CL are illustrated in Figure 1 (A, B, C) and Figure 2 (A,B). As seen, the Ag-gel by itself showed inhibition of over 7 logs of the bacterial growth (100%) in each case when compared to the untreated samples and to the placebo gel, which did not contain Ag, with all the bacterial strains except *K. pneumoniae*. With *K. pneumoniae* (Fig 2 B) it only showed approximately 5 logs of inhibition when compared to the placebo

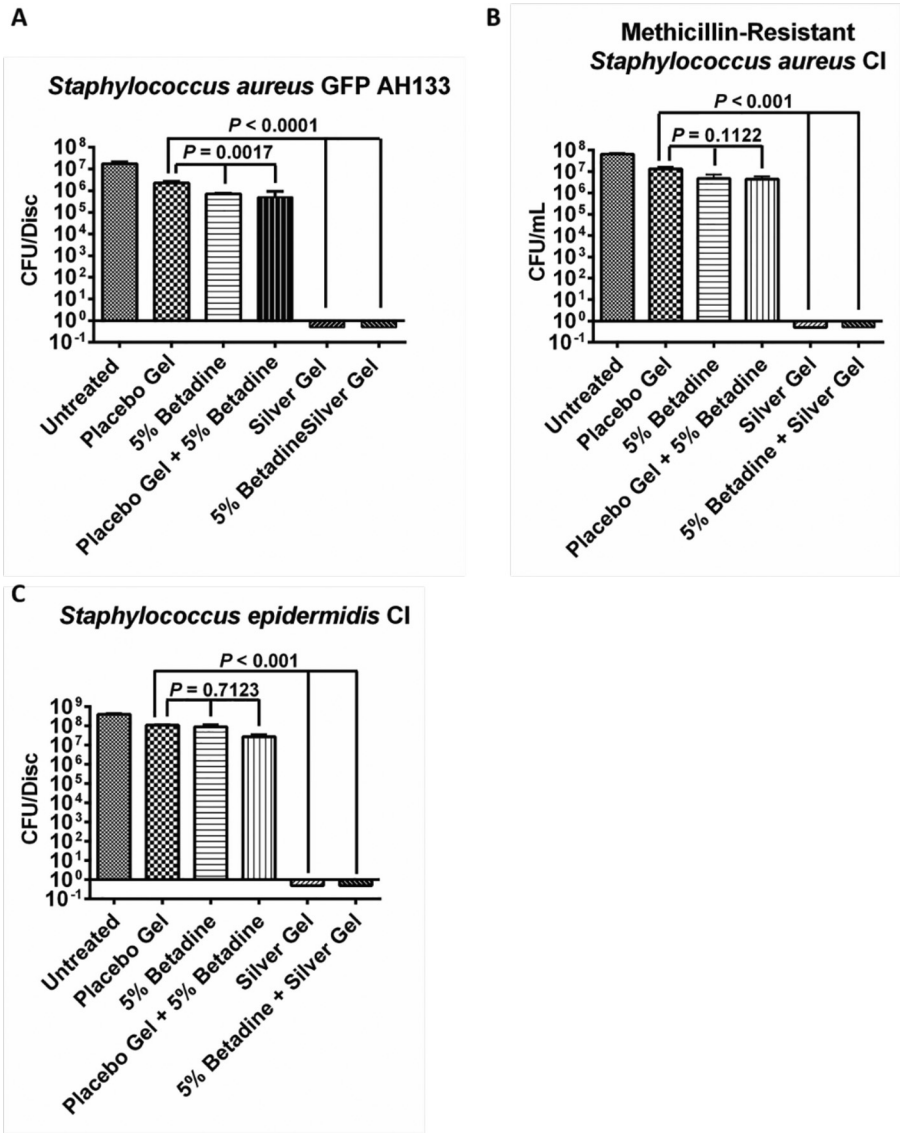


Fig 1. The CFU results for the Gram positive bacteria, S. aureus GFP AH133 (A) MRSA CL (B), and S. epidermidis (C), on cellulose discs. The treatments were, none, placebo gel alone, placebo gel plus 5% Betadine, Ag-gel, and a combination of the 5% betadine and Ag-gel. Results were after 24 hours at 37°C. All experiments were done at least in triplicate and were repeated 3 times.

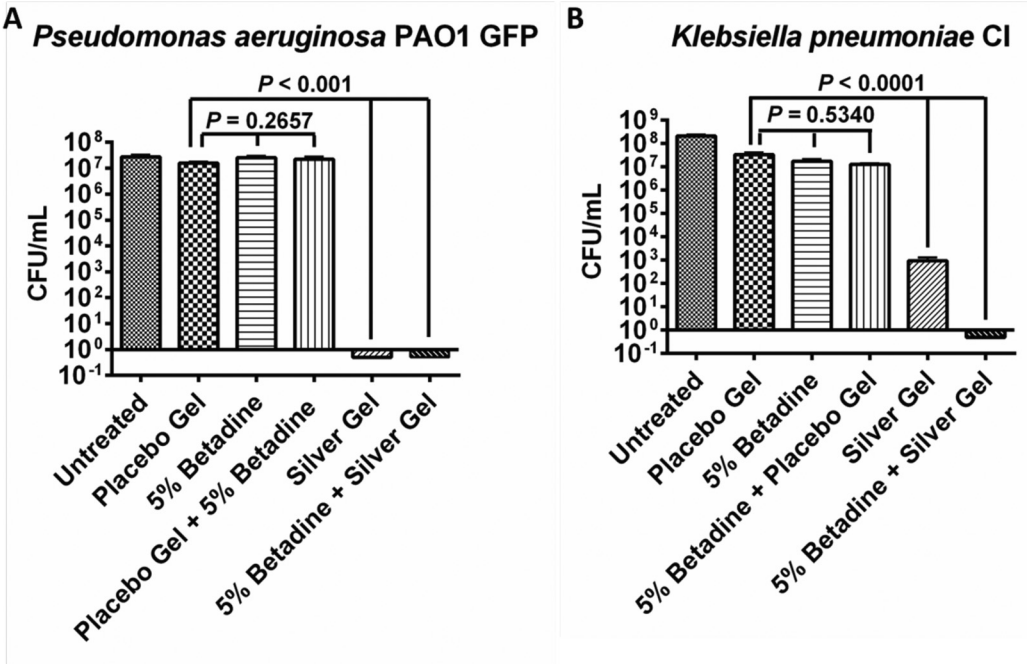


Fig 2. The CFU results for the Gram negative bacteria, P. aeruginosa PAO1 GFP (A) K. pneumoniae CL (B) on cellulose discs. The treatments were, none, placebo gel alone, placebo gel plus 5% Betadine, Ag-gel, and a combination of the 5% betadine and Ag-gel. Results were after 24 hours at 37°C. All experiments were done at least in triplicate and were repeated 3 times.

gel. These results are in distinct contrast to those of 5% betadine shown above.

Effect of the combination of 5% betadine and Ag-gel treatment on bacterial biofilm formation for in vitro *CFU studies*

The results for the 24 hour *in vitro* CFU studies in the presence of the combination of 5% betadine and Ag-gel using *Staphylococcus aureus* GFP strain AH133 and *Pseudomonas aeruginosa* PAO1 GFP strain MM294, and clinical isolates of *K. pneumoniae* CL, *S. epidermidis* CL, and MRSA CL are illustrated in Figure 1 (A, B, C) and Figure 2 (A,B). The difference between these results and those of either the 5% betadine and the Ag-gel alone are that the combination of the 2 antimicrobials showed compete (100%) inhibition to all the bacteria tested.

Effect of 5% Betadine, Ag-gel, and the combination of 5% betadine and Ag-gel treatments on bacterial biofilm formation from *in vitro* Confocal laser scanning microscopy (CLSM) studies

To confirm the results of the previous experiments, the biofilm formation by *Staphylococcus aureus* GFP strain AH133 and *Pseudomonas aeruginosa* PAO1 GFP strain MM294 strains was studied on cellulose discs CLSM. For this study, we were investigating the ability of bacteria to grow on the discs. The bacterial strains were inoculated onto the discs in the same manner as for the CFU biofilm assay. Untreated discs were coated with placebo gel only. As above the discs were incubated for 24 hour 37°C. As seen in Figures 3 and 4, *S. aureus and P. aeruginosa* formed typical biofilms characterized by the presence of significant biofilm formation on the cellulose discs receiving no treatment, treated with the placebo gel, or with 5% betadine alone. However, no bacteria or biofilm were seen on the cellulose discs treated with Ag-gel. These results confirm those obtained previously with the CFU assay.

DISCUSSION

Betadine is a widely used antiseptic because it has particularly broad spectrum of antimicrobial activity that includes gram-positive and gram-negative bacteria, bacterial spores, fungi, protozoa, and viruses.[7,13] However, many studies have shown the inefficacy of Betadine solution against different bacteria.[5,14,15] Anderson et al. documented the prolonged survival of *Burkholderia* cepacia in commercially manufactured povidone-iodine.[16] In contrast, Cichos et al. showed that intraoperative povidone-iodine reduced *Burkholderia* cepacia and other bacterial species to prevent periprosthetic joint infection during primary total joint arthroplasty.[17] This study suggests that povidone-iodine kills all bacteria tested immediately on contact and that the exposure time is not the key factor.[17] In addition, there are several other studies have documented intrinsic contamination of a povidone-iodine solution led to both infections and pseudomonas infections. Cadexomer iodine (Iodosorb or Iodoflex) is an effective form of iodine against bacteria. Since Cadexomer iodine is in a paste, ointment or gel form, we used Betadine as it would mix easier with our Ag-gel. Our results, as seen in Figures 1–4, confirm that betadine is very poor at inhibiting biofilm formation on cellulose pads *in vitro*. At best, betadine showed 1 log of inhibition of biofilm formation in in vitro CFU and confocal laser scanning microscope studies, and in the case of *P. aeruginosa*, it did not appear to show any inhibition.

We also tested silver because it appears to use a different killing mechanism from betadine. It is believed that the killing mechanism is due to the binding of silver ions to bacterial proteins causing structural changes in the bacterial cellular membrane.[1] Silver ions also can bind and denature DNA and RNA, inhibiting bacterial replication.[1] Silver is biologically active when it is soluble which is the Ag^{+} and Ag° forms. Specifically, Ag^{o} is the form present in nanocrystalline silver.[1] A comparison of silver ions vs nanocrystalline silver was carried out by Wright et al.[18] They found that the nanocrystalline form was more efficacious than the ionic one. The use of nanocrystalline silver dressing has been recently reviewed by Fong & Wood.[19] Another study by Cox et al. showed that silver-coated nanocrystalline dressing led to a decreased number of dressings and shorter hospital stays.[20]

We found that Ag-gel is fairly effective in inhibiting bacteria from forming biofilms on cellulose. As seen above (Fig. 1-4), the Ag-gel was 100% effective in blocking bacterial attachment of *Staphylococcus aureus* GFP strain AH133 *Pseudomonas aeruginosa* PAO1 GFP strain MM294, and clinical isolates of *S. epidermidis* CL, and MRSA CL.

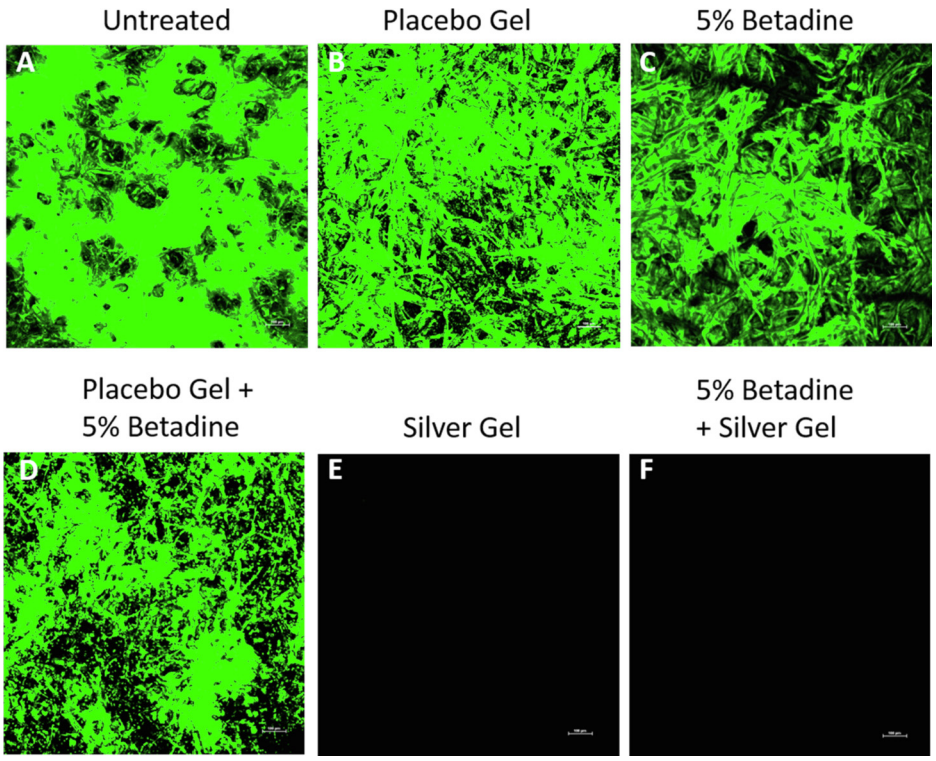


Fig 3. Representative visualization with a Nikon Eclipse Ti upright confocal laser scanning microscope of S. aureus GFP AH133 (A-F), remaining on cellulose discs after 24 hours at 37°C in LB medium. The treatments were, none, placebo gel alone, placebo gel plus 5% Betadine, Ag-gel, and a combination of the 5% betadine and Ag-gel. The images were processed and analyzed using NIS-Elements AR Imaging Software as previously described. The green color shows the presence of bacteria from GFP while the black color shows no bacteria present. Bar scale equals 100 μm. (Color version of figure is available online.).

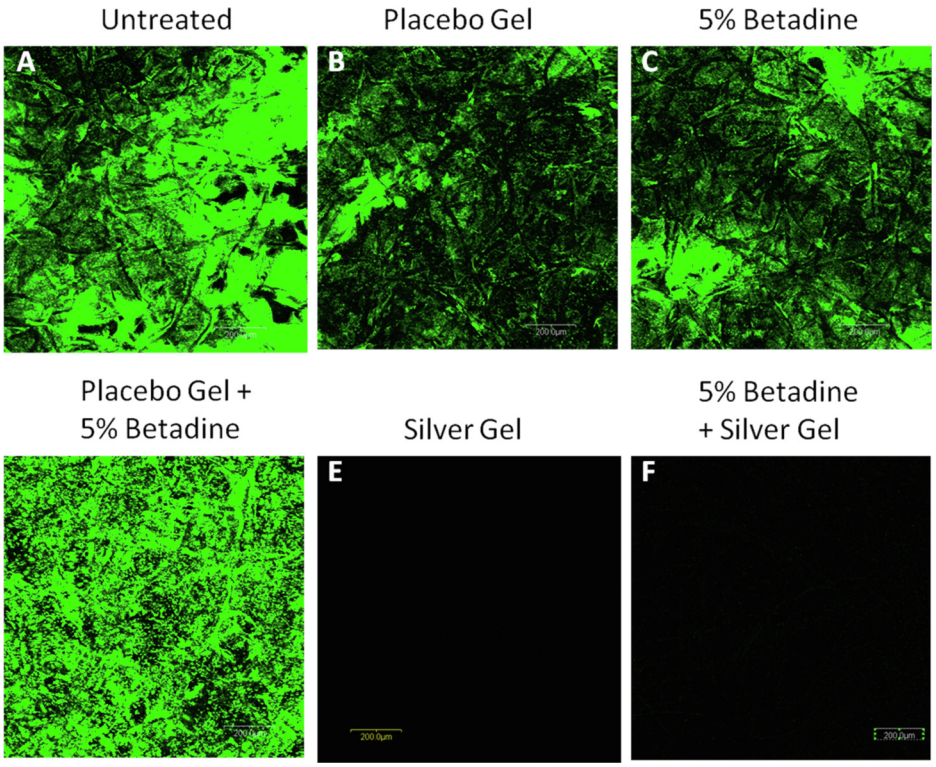


Fig 4. Representative visualization with a Nikon Eclipse Ti upright confocal laser scanning microscope of P. aeruginosa PAO1 GFP (A-F), remaining on cellulose discs after 24 hours at 37°C in LB medium. The treatments were, none, placebo gel alone, placebo gel plus 5% Betadine, Ag-gel, and a combination of the 5% betadine and Ag-gel. The images were processed and analyzed using NIS-Elements AR Imaging Software as previously described. The green color shows the presence of bacteria from GFP while the black color shows no bacteria present. Bar scale equals 200 μm. (Color version of figure is available online.).

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However, it only showed approximately 5 logs of inhibition of *K. pneumoniae* CL in a similar assay. The reason that we feel that 5% betadine uses a different killing mechanism from silver, is that while 5% betadine only showed approximately 1 log of killing of *K. pneumoniae* CL, it was synergistic with the Ag-gel in killing this bacteria. The combination resulted in 100% killing (over 7 logs) when compared to the placebo gel. The same doses, formulations, and isolates were used in all of the experiments for comparisons. It would appear that the combination of these 2 different topical antimicrobials would be a much more effective topical treatment for use as antimicrobial agents. A limitation to the current study is that the experiments were performed on biofilm bacteria, but the biofilm was formed from a single bacterial species. Biofilms formed from more than 1 species of bacterial may be harder to kill.

CONCLUSIONS

The results demonstrate that 5% betadine is not an effective treatment for the prevention of bacterial biofilms grown on a cellulose sponge. A colloidal Ag-gel, however, appears to work quite well for most bacteria. In contrast, 5% betadine was synergistic with Ag-gel in the prevention of growth of *K. pneumoniae* CL biofilms. Thus, the combination would appear to be a better treatment for topical biofilm control.

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