An *In vitro* Analysis of the Effects of Various Topical Antimicrobial Agents on Methicillin-resistant and Methicillin-sensitive Strains of *Staphylococcus aureus*

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**Abstract**

Infections of acute and chronic wounds have a substantial negative impact on patient outcomes. Because bacterial resistance to traditional antimicrobials continues to increase, an *in vitro* study was conducted to examine current sensitivities of various methicillin-resistant and methicillin-sensitive strains of *Staphylococcus aureus* (MRSA and MSSA) to commonly used topical antimicrobial agents. Using fresh cultures of eight strains of MRSA and MSSA, the area of the zone of inhibition produced by various topical antimicrobials, including an aminoglycoside antibiotic, monocarboxylic acid antibiotic, pleuromutilin antibiotic, triple antibiotic ointment, and petrolatum ointment, was examined. Six culture plates per antimicrobial were prepared using the Kirby Bauer method; soy blood culture plates were inoculated with the bacteria, incubated for 24 hours at 37˚C, and their zones of inhibition measured and calculated. Data were analyzed using ANOVA testing. Mupirocin treatment was the most effective antimicrobial, with areas of inhibition ranging from 30.34 cm² to 61.70 cm² (*P* <0.05), as compared to the next most effective, retapamulin, with areas of inhibition ranging from 11.97 cm² to 23.54 cm². This study provides current scientific data to help the development of a thoughtful rationale for the use of topical antimicrobials in wounds. Additional *in vivo* studies to substantiate these findings are needed.

**Keywords:** Staphylococcal skin infection, methicillin-resistant *Staphylococcus aureus*, drug resistance, bacteria, wound

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**Potential Conflicts of Interest:** none disclosed

Aacute and chronic wounds have an enormous economic and social impact and are a substantial and increasing burden on healthcare systems. Chronic wounds affect 6.5 million individuals in the United States, and the incidence of these wounds is expected to increase rapidly with an aging population. The costs associated with the treatment of chronic wounds are equally as staggering. In the United Kingdom, the costs to the National Health Service are estimated to be £1 billion per year, while in the US expenditures for the treatment of chronic wounds are approximately $25 billion per year.

A plethora of research has been conducted to find methods that will facilitate healing of chronic wounds with the aim of decreasing mortality, morbidity, and cost. One of the most common factors implicated in delayed wound healing is the presence of bacterial infection. A prospective study of leg ulcer microbiology and a review article on the role of microbes in chronic wounds show polymicrobial flora contributes to nonhealing. The tissue in chronic wounds is at increased risk for bacterial invasion due to poor vascular supply and systemic, patient-specific factors that include advancing age, diabetes mellitus, obesity, malnutrition, and the use of steroid and other immunosuppressant therapy.

Multiple retrospective and prospective studies encompassing more than three decades of research have demonstrated *Staphylococcus aureus* is the dominant species isolated from skin infections, including chronic and acute wounds. Colsky et al retrospectively reviewed the bacterial wound cultures obtained from hospitalized dermatology patients; *S. aureus* accounted for 77% of the isolates in superficial skin...
wounds and 43% (not a majority, but still the most common) among bacteria in chronic leg ulcers.

For the past 60 years, the incidence of *S. aureus* infection has been increasing. Along with this increase has come biochemical changes in this bacterial pathogen, leading to increased resistance to antimicrobial agents. According to an extensive review,\(^1\) this was noted as early as 1944 when the first isolates resistant to penicillin appeared. Additional reports of resistance to methicillin as well as other antimicrobials followed in the 1960s.\(^{14,15}\) The first and original report of methicillin (celbenin) resistance was a letter to the editor\(^1\); an associated letter\(^1\) that discussed the degree of resistance of single colony strains and the original cultures quickly followed. Subsequent testing revealed an extremely high degree of resistance with prolonged incubation. An *in vitro* analysis\(^1\) of more than 60,000 cultures documented the number of methicillin-resistant *S. aureus* (MRSA) cultures and their slow increase over the course of a 3.5-year study and analyzed the sources and screening methods for methicillin resistance.

Increasing antibiotic resistance also has been seen in chronic wounds and acute wounds. In 2004, a retrospective chart review by Valencia et al\(^1\) found as many as 75% of all *S. aureus* isolates from dermatology inpatients with leg ulcers and 44% of all *S. aureus* isolates from superficial chronic wounds were MRSA. Previously, the rate of MRSA infection in this same inpatient dermatology unit showed MRSA to be present in 26% and 50% of acute and chronic leg ulcers, respectively. Similarly, MRSA was present in 7% and 24% of superficial acute and chronic wounds, respectively.

It is known that with increased rates of infection and required treatment there is an increase in resistance to antimicrobials. With the development of methicillin resistance in *S. aureus* bacteria, other antimicrobial agents should be considered. Mupirocin, a common topical antimicrobial agent, has shown effectiveness against MRSA as well as methicillin-sensitive *S. aureus* (MSSA) in both clinical and animal trials.\(^{17}\) Although some over-the-counter products — eg, Neosporin (Johnson & Johnson, Trenton, NJ) — also have shown efficacy against *S. aureus* in infected porcine wounds, head-to-head studies against both MRSA and MSSA are needed.\(^{17}\)

The purpose of this *in vitro* study was to examine current sensitivities of both MSSA and MRSA to commonly used topical antimicrobial agents. Characterization of antibiotic sensitivities may be useful in developing effective strategies for reducing colonization of, and infection with, these bacteria, which may speed healing of wounds, thereby decreasing morbidity and costs.

### Materials and Methods

**Bacterial strains.** Because *S. aureus* is a genetically sophisticated bacterium notorious for its ability to adapt to almost any antibiotic challenge, multiple different strains of MRSA and MSSA were examined. Commercially available strains were chosen to best represent a broad and realistic spectrum of the cutaneous *S. aureus* clinical experience in the US. This study used fresh cultures of MRSA isolates (USA300, ATCC 33591, 33593, 700699, and AD2A[5]) and MSSA isolates (14776, 6538, and AD4A[7]). Freeze-dried bacterial cultures were recovered according to the standard American Type Culture Collection recovery protocol. All of the challenged inoculum suspensions were prepared by scraping the overnight growth from a culture plate into 20 mL of phosphate buffer solution. Serial dilutions of this suspension were made to create an inoculum suspension of approximately 10^6 colony-forming units per mL (CFU/mL). This concentration was confirmed using historical optical density measurements.

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility testing usually starts with various *in vitro* assays. For this study, a modified Kirby Bauer method was used that consists of an agar well diffusion assay.\(^1\) The antimicrobial agents evaluated in this study were gentamicin sulfate 0.1% ointment (E. Fougera & Co, Melville, NY); Neosporin™ brand triple antibiotic ointment (TAO), containing neomycin, polymyxin, and bacitracin (Johnson & Johnson, Trenton, NJ); mupirocin 2% ointment (Taro Pharmaceuticals, Brampton, Ontario, Canada); and retapamulin (Altabax™ ointment, Glaxo Smith Kline, Research Triangle Park, NC). Target brand Petroleum Ointment (Target Corp, Minneapolis, MN) was used as a control to verify that the changes in bacterial growth were a result of the active antimicrobial agent and not due to the vehicle for the agents.

A 100-μl aliquot of the 10^6 CFU/mL inoculum suspension was plated onto trypticase soy agar with 5% sheep blood (Becton Dickinson and Company, Sparks, MD). Sterile glass beads were used to spread the inoculum evenly over the surface of the agar in order to grow a continuous lawn of bacteria. The topical antimicrobial agent (150 mg) was placed into a round, central, 10-mm well created by removing a portion of the inoculated culture media using a 10-mm skin punch biopsy. A group of six culture plates was created to evaluate
each antimicrobial against all of the eight strains of *S. aureus* in this study. All culture plates were incubated at 37° C for 24 hours.

**Data collection and analysis.** To determine the efficacy of the topical antimicrobial against each *S. aureus* strain, the area of the zone of bacterial growth inhibition was measured using Image J 1.44p software (http://imagej.nih.gov/ij). The average area of growth inhibition for the six plates in each treatment/strain group was calculated. Statistical analysis using an ANOVA test was used to determine significant differences between treatments. Significance was considered when the *P* value was ≤0.05.

**Results**

Mupirocin treatment was the most effective antimicrobial, with areas ranging from 30.34 cm² to 61.70 (P <0.05, see Figure 1). Retapamulin, the second most effective antimicrobial, had areas of inhibition ranging from 11.97 cm² to 23.54 cm²; it was significantly more effective at inhibiting bacterial growth than gentamicin sulfate, TAO, and the petrolatum control (P <0.05). The areas of the zone of mupirocin-induced inhibition are more than twice those of retapamulin-induced inhibition. TAO was significantly more effective than the petrolatum control against MSSA 6538 and MSSA AD4A(7) (P <0.05).

The two strains most sensitive to mupirocin compared with all other strains examined were MRSA 700699 and MSSA 14776 (P <0.05, see Figures 2a and 2b). MRSA 700699 and MRSA AD2A(5) were the most sensitive to retapamulin treatment. The MSSA 6538 and MSSA AD4A(7) strains were least affected by mupirocin and retapamulin treatment. Of all the MRSA strains evaluated, MRSA USA300 demonstrated the smallest area of growth inhibition by both mupirocin and retapamulin.

TAO and gentamicin treatments showed relatively small areas of bacterial growth inhibition across all of the strains evaluated, consistent with their significantly lower inhibition of bacterial growth (see Figures 2c and 2d). All strains in the gentamicin group were significantly inhibited relative to MRSA 33593 and MRSA 700699 (P <0.05). These two strains, with areas of growth inhibition near zero, are the only strains in this study that were found to be resistant to gentamicin.

**Discussion**

Wound healing is an intricate process with many factors that can lead to delays in healing. Bacterial presence in wounds can be an important cause of problems in patients with chronic wounds. The presence of bacteria in chronic wounds occurs in varying degrees, from asymptomatic contamination to colonization to local infection, increasing to invasive infection and septicemia. It is the interaction between the patient and the bacteria present in the chronic wound that determines the degree of healing or infection, and therefore the need for treatment.

Numerous studies have shown the most common bacterium present in chronic and acute wounds is *S. aureus*. Over recent past decades, there has been a disturbing trend toward increasing antibiotic resistance of MRSA, accounting for up to 75% of *S. aureus* isolates. MRSA is now in many US healthcare facilities and communities. In 2003, Pan

![Figure 1. Staphylococcus aureus area of Inhibition zone (cm²) with different antibiotics.](http://example.com/figure1.png)
Figure 2.

a. * P < 0.05 compared to MRSA 33593, USA300 and MSSA 6538, AD4A(7)
   † P < 0.05 compared to MRSA USA300 and MSSA 6538, AD4A(7)
   • P < 0.05 compared to MSSA AD4A(7)

b. † P < 0.05 compared to MRSA USA300 and MSSA 6538, AD4A(7)
   * P < 0.05 compared to MSSA 6538, AD4A(7)

c. † P < 0.05 compared to MRDA 700699, USA300, AD2A(5) and MSSA 14776, 6538, AD4A(7)
   x P < 0.05 compared to MRSA 700699

d. * P < 0.05 compared to all strains
   † P < 0.05 compared to MRSA 33593, 700699, USA300, AD2A(5) and MSSA 14776, 6538
   x P < 0.05 compared to MRSA 33593, 700699 and MSSA 14776, 6538
   § P < 0.05 compared to MRSA 33593, 700699 and MSSA 6538
   • P < 0.05 compared to MRSA 33593, 700699
et al. retrospectively reviewed 295 San Francisco County jail inmate health records that showed 74% of *S. aureus* isolates obtained were MRSA. This correlates with Valencia et al’s 2004 retrospective review of patients at a Miami, Florida inpatient dermatology unit, showing MRSA to be present in 36 out of 48 patients’ (75%) *S. aureus* isolates from leg ulcers.

A prospective study in Copenhagen found *S. aureus* to be the most common isolated bacterium (93.5%) in chronic venous ulcers. The current *in vitro* study examined multiple strains of MRSA and MSSA, including the MRSA USA300 strain. Currently, USA300 is the most common strain of MRSA isolated in the US and persists in community populations in at least 16 states. Other strains examined in the current study are commonly used in testing the effectiveness of antimicrobial hand-washing formulations (MRSA 33591, MSSA 6538). MSSA 6538 also is used in a wide variety of commercial testing, including filtration efficiency testing, media testing, and antimicrobial and antiseptic testing. MSSA 14776 is used in World Health Organization International studies to standardize antibiotic sensitivity disks.

The *in vitro* effectiveness of the topical antimicrobials evaluated in this study correlates with the known antibiotic susceptibilities of the strains evaluated. For example, MRSA 33593 and MRSA 700699, which are known to be resistant to gentamicin, had inhibition zone areas of nearly zero, not significantly different from that of the petrolatum control. Furthermore, the results seen in this *in vitro* study also correlate with the reputation of *S. aureus*’ remarkable ability and history of decreasing susceptibility to antibiotics with increasing exposure. The results from this study are in agreement with those of the *in vitro* study by Suzuki et al., where the USA300 strain was found to be resistant to TAO. The newer antibiotics — mupirocin and retapamulin — used in this study had significantly and far larger areas of bacterial growth inhibition than TAO, which has been in use for many years. Furthermore, the use of mupirocin and retapamulin is not as common as TAO in the US because they are restricted — ie, controlled agents requiring a prescription to acquire and use. Suzuki et al. also concluded that TAO resistance occurs to a greater degree in MRSA strains in the US where TAO is widely used. This is in contrast to MRSA strains in Japan where the use of TAO and its components is not widespread.

The greater sensitivity exhibited in this study by these eight strains of *S. aureus* to mupirocin and retapamulin is consistent with less exposure of these strains to these antimicrobials. Bacterial growth inhibition to these common topical antimicrobials occurred regardless of methicillin susceptibility. This would be expected, given the unique and different mechanisms of action of each antimicrobial agent from that of methicillin.

The ability of infections to slow the healing of both acute and chronic wounds, prolong hospital stays, and increase morbidity and the cost of these wounds is well known. In a retrospective study of 51 patients (17 colonized with MRSA, 34 control), patients with MRSA had longer hospital stays and increased incidence of negative post op outcomes compared to patients without MRSA. A prospective study of 59 venous ulcer patients found *S. aureus* was present in 94.9% of ulcers, compared to 5.1% of patients who were *S. aureus* free, significantly delaying time to healing. A summary of the literature contained in an *in vitro* study of the effectiveness of silver-containing products against bacteria underscores the role of infection on increased patient debilitation and healthcare costs.

Some antimicrobial agents such as iodine in aqueous solution are rapidly consumed in the wound milieu, while the presence of increasingly resistant bacteria may render topical antibiotics in low concentrations ineffective. A review of their role, particularly in diabetic foot ulcers, suggests biofilms pose greater challenges to the effectiveness of topical antimicrobials in chronic wounds; primarily, it is difficult for the antimicrobial to penetrate the biofilm in a sufficient concentration to kill bacteria.

The data from the current *in vitro* study indicate the bacterial growth of both MRSA and MSSA is more affected by mupirocin and retapamulin than by TAO and gentamicin. Judicious use of all of these agents is needed to reduce the likelihood for the development of resistance. Perhaps the use of these common topical antibiotics that can be delivered in a sustained fashion in conjunction with a topical antiseptic, and subsequently have an immediate effect on bacteria, may be more effective against bacteria. This combination may prevent the development of resistance to the antibiotic agent. Furthermore, the antiseptic may disrupt the biofilm and allow greater penetration of the antibiotic, while also having a bactericidal action against a bacterium such as MRSA.

Whether acute or chronic, superficial or deep, skin wounds that are critically colonized with bacteria require diligent wound care to prevent the progression of colonization to varying degrees of infection. Cleansing, debridement, and the use of topical agents and antimicrobials may be needed as important elements in managing acute and chronic wounds.

**Limitations**

Because these evaluations were performed *in vitro*, this study has inherent limitations. In addition to *in vitro* studies, *in vivo* analyses also should be conducted because diffusion assays may only partly reflect the ability of the active agent to diffuse through the agar as opposed to killing bacteria colonized within wounds. *In vivo* evaluations also would facilitate assessment of these active agents against important factors in the wound environment that could influence their activity — eg, pH, temperature, proteases, growth factors, and other characteristics.

**Conclusion**

Bacterial resistance to antimicrobial agents is a constant concern and is continuously evolving. This is evident from
the increasing frequency of multidrug resistance in S. aureus since the early 1990s. Although the increasing prevalence of antibiotic resistance is well known, the interactions between antibiotic resistance and wound microbiology and rationale for therapy are less clear. This study provides additional scientific data to help the development of a thoughtful evidence-based rationale for the treatment of wounds with common topical antimicrobials.

References