Impact of polymethylmethacrylate additives on methicillin-resistant *Staphylococcus pseudintermedius* biofilm formation in vitro

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**OBJECTIVE**  
To evaluate the impact of gentamicin, silver, or both additives in polymethylmethacrylate (PMMA) beads on methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) biofilm formation in vitro.

**SAMPLE**  
4 preparations of PMMA beads (formed with no additive [control], gentamicin, silver, and gentamicin and silver).

**PROCEDURES**  
Beads from each group were exposed to 10 MRSP isolates known to be strong biofilm formers. Following incubation, the beads were rinsed to remove planktonic bacteria, then sonicated to dislodge biofilm-associated bacteria. Resulting suspensions were serially diluted, plated on blood agar, and incubated overnight; CFUs were counted. Variance of mean CFU counts following log₁₀ transformation was analyzed among PMMA groups.

**RESULTS**  
None of the PMMA additives tested completely inhibited MRSP biofilm formation. There was a significant effect of gentamicin and gentamicin plus silver on this variable, compared with controls, but not of silver alone. There was no difference between gentamicin and gentamicin plus silver. When only isolates not susceptible to gentamicin were evaluated, there were no significant differences among PMMA additive groups. Within gentamicin-susceptible isolates, there was an impact of gentamicin and gentamicin plus silver, but no impact of silver alone and no difference between gentamicin and gentamicin plus silver.

**CONCLUSIONS AND CLINICAL RELEVANCE**  
Gentamicin-impregnated PMMA was effective at reducing biofilm formation of gentamicin-susceptible MRSP isolates but had no effect on isolates not susceptible to gentamicin. Silver-impregnated PMMA had no effect on MRSP biofilm formation. Results suggested that gentamicin-impregnated PMMA may not be effective in vivo against MRSP isolates not susceptible to gentamicin. Antibacterial efficacy of silver should not be assumed without proper testing of the target bacteria and specific silver compound. (Am J Vet Res 2015;76:395–401)

**ABBREVIATIONS**

- **MIC**: Minimum inhibitory concentration
- **MRSA**: Methicillin-resistant *Staphylococcus aureus*
- **MRSP**: Methicillin-resistant *Staphylococcus pseudintermedius*
- **PMMA**: Polymethylmethacrylate
- **SSI**: Surgical site infection
- **TBS**: Tryptone soy broth

**M**ethicillin-resistant *Staphylococcus pseudintermedius* has rapidly emerged as a leading cause of SSIs in dogs. The impact of SSIs can include increased treatment costs and patient morbidity as well as frustration of pet owners and medical caregivers alike. Surgical site infections with MRSP can be difficult to treat because this bacterium often is multidrug resistant and forms biofilms, a known virulence factor in other staphylococcal species. A biofilm is a population of bacteria irreversibly attached to a surface and enclosed in a self-produced extracellular polymeric matrix. Biofilm-associated bacteria have low metabolic rates and are therefore less susceptible to antimicrobials that target rapid division and growth than are planktonic (free-living or nonbiofilm-associated) bacteria. Biofilms also provide a unique environment that facilitates the efficient exchange of resistance genes among bacteria. Finally, the extracellular polymeric matrix acts as a physical protective barrier for the bacteria, binding the host’s immune system cells and antimicrobials, preventing contact with their intended target. Biofilm formation on orthopedic implants is of particular concern, given that it may complicate treatment of SSIs. Routine treatment of such infections involves reopening and lavage of the surgical site and long-term treatment with systemic antimicrobials, which may kill planktonic bacteria being released from a biofilm (at the end of the life cycle) and allevi-
ate acute signs of infection but has no impact on the biofilm itself. For this reason, biofilm-associated SSIs often cannot be resolved with routine treatment and necessitate removal of the implant. With such a devastating consequence, prevention of biofilm formation is imperative for successful patient outcome. One such prophylactic measure is the inclusion of antimicrobial additives in PMMA, which is a fixative commonly used in total joint arthroplasty, spinal fracture fixation, and other orthopedic and neurologic procedures.

Polymethylmethacrylate impregnated with gentamicin is widely available for use in human and veterinary orthopedics. Local delivery of gentamicin is thought to provide higher concentrations of the drug than can safely be achieved parenterally. Such high concentrations are anaerobically thought to be bactericidal even against bacteria that are resistant to gentamicin on the basis of serum breakpoints or against bacteria that are embedded within a biofilm. Other substances can also be added to PMMA, alone or in combination with gentamicin, including a silver powder that recently became commercially available. Materials coated or impregnated with silver have been studied for the prevention or treatment of various infections and have become a popular alternative antibacterial substance because of increasing antimicrobial resistance in recent years.

Despite the widespread use of gentamicin-impregnated PMMA in dogs and the increasing prevalence of MRSP SSIs to our knowledge, the impact of PMMA additives on MRSP biofilm formation has not previously been evaluated. The objective of the study reported here was to evaluate the impact of PMMA impregnated with gentamicin and silver, alone and in combination, on biofilm formation of MRSP susceptible and not susceptible to gentamicin. We hypothesized that gentamicin-impregnated PMMA would prevent biofilm formation of gentamicin-susceptible MRSP isolates only and that silver-impregnated PMMA (alone or in combination with gentamicin) would prevent MRSP biofilm formation regardless of gentamicin susceptibility.

Materials and Methods

PMMA bead preparation

Polymethylmethacrylate beads (diameter, 7 mm) were formed under aseptic conditions in accordance with the manufacturer’s instructions. Four PMMA bead groups were studied: no additive (control), 1% silver (0.25-g, 10-μm particle microsilver antimicrobial powder added to additive-free PMMA for 1% final concentration), 1.25% gentamicin, and 1.25% gentamicin and 1% silver (1 vial of microsilver antimicrobial powder combined with gentamicin-impregnated PMMA).

MRSP isolates

Ten epidemiologically unrelated MRSP isolates obtained from infected dogs and previously determined to be strong biofilm producers were selected. Isolates had been identified as MRSP by species-specific PCR assay and detection of the gene mecA by PCR assay or detection of PBP2a protein by the latex agglutination test. Isolates were stored at −80°C in cryopreservation medium. Gentamicin MIC was determined; isolates were categorized as susceptible or resistant to gentamicin according to Clinical and Laboratory Standards Institute guidelines.

Impact of PMMA additive on MRSP biofilm formation

Isolates were subcultured onto blood agar plates overnight at 37°C. Resulting colonies were inoculated in 5 mL of TSB with 1% glucose and verified to be 0.5 McFarland standard (approx 10⁸ CFU/mL) suspensions by use of a calibrated spectrophotometer. Suspensions were vortexed to break up preexisting cell clusters, PMMA beads of each type were introduced, and the combination was incubated overnight at 37°C. These steps followed an established model for growing biofilms. Isolates were tested in triplicate for each PMMA bead group. Beads with no additive (control) were also added to 5 mL of TSB containing 1% glucose without bacteria as a negative control.

After incubation, beads were removed and rinsed 3 times in 2 mL of PBS solution to remove planktonic (nonadherent) bacteria. Following rinsing, beads were transferred into sterile test tubes containing 5 mL of TSB containing 1% glucose and sonicated for 5 minutes to remove biofilm-associated (adherent) bacteria, followed by 1 minute of vortexing to ensure even suspension. Resulting suspensions were used to create serial dilutions up to 10⁶. One hundred microliters of each dilution was inoculated onto blood agar and incubated overnight at 37°C. The following day, CFUs were manually counted and the number recorded. Counts on plates containing between 20 and 200 colonies (excluding dilution series where the lowest dilution had < 20 colonies) were used to calculate mean colony-forming units per milliliter (the measure of biofilm formation) for each PMMA bead group and MRSP isolate combination.

Scanning electron microscopy

To validate sonication for removing biofilm from the beads and to confirm the presence of biofilm on the beads after incubation and PBS solution rinsing, imaging of a small subset of beads before and after sonication was performed. Pairs of additive-free and gentamicin-impregnated beads were incubated in separate 0.5-McFarland standard suspensions of 5 mL of TSB with 1% glucose overnight at 37°C. Two of the 10 MRSP isolates were arbitrarily selected. All beads were rinsed 3 times with 2 mL of PBS solution. One bead from each pair was immediately fixed in 2% glutaraldehyde solution after rinsing. Remaining beads were placed in separate sterile tubes containing 5 mL of TSB with 1% glucose and sonicated for 5 minutes, then fixed in 2% glutaraldehyde. All fixed beads...
were kept at 4°C until the time of further processing. One day before image acquisition, beads were rinsed 3 times with Sørensen phosphate buffer (0.07M; pH, 6.8) for 10 minutes each time, postfixed with 1% osmium tetroxide for 1 hour at 22°C, washed in Sørensen phosphate buffer 3 times for 15 minutes each, and dehydrated through an ethanol series (50%, 70%, 80%, 90%, and 95%) for 15 minutes each. Beads were then dehydrated 3 times in 100% ethanol for 15 minutes each, critical-point dried, mounted onto metal specimen stubs with carbon paint, and sputter coated with gold and palladium (coating thickness, 20 nm). Within 24 to 48 hours after this treatment, the beads were imaged with a scanning electron microscope.® Beads were subjectively evaluated for the presence of adherent bacteria and extracellular matrix.

**Statistical analysis**

Statistical analysis was performed with statistical software.® There were 2 fixed factors: PMMA bead group and bacterial isolate. An interaction between CFU count and PMMA bead group was included in the model. Log transformation was performed on the CFU data. To assess the ANOVA assumptions, residual analyses were performed. This included formally testing the residuals for normality by means of Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling tests. In addition, residuals were plotted against the predictive values and explanatory variables used in the model to identify any outliers, unequal variance, or other issues that needed further investigation. Analysis showed the need to allow for unequal variance (on the basis of an improved model fit stated by the Akaike information criterion); otherwise, all the ANOVA assumptions appeared to be adequately met and no outliers were identified. Within susceptible or nonsusceptible bacterial isolates, contrasts were conducted to compare the bead treatments as determined on the basis of Clinical and Laboratory Standards Institute breakpoints (susceptible, ≤ 4 µg/mL). Results are reported as mean ± SD. Values of *P* ≤ 0.05 were considered significant.

**Results**

Of the 10 isolates evaluated, 4 were susceptible to gentamicin (MIC, ≤ 4 µg/mL), with MICs ranging from 0.047 to 0.19 µg/mL; 1 had intermediate susceptibility to gentamicin (MIC, > 4 and ≤ 8 µg/mL), with an MIC of 6 µg/mL; and 5 were nonsusceptible to gentamicin (MIC, > 8 µg/mL). The intermediate susceptibility isolate was grouped with the nonsusceptible isolates for the purpose of this study. None of the PMMA additives completely inhibited MRSP biofilm formation. Mean ± SD CFU counts for each isolate were determined after log transformation (Table 1). When all isolates were grouped together regardless of gentamicin susceptibility, there was a significant effect of gentamicin (*P* < 0.001) and gentamicin and silver (*P* < 0.001), compared with controls. There was no impact of silver alone (*P* = 0.789) nor was there a difference between gentamicin and gentamicin plus silver (*P* = 0.415). When only isolates not susceptible to gentamicin were evaluated, there were no significant differences among PMMA additive groups. For gentamicin-susceptible isolates, there was an impact of gentamicin and gentamicin plus silver (*P* < 0.001 for each treatment) but no impact of silver alone (*P* = 0.655) and no difference between gentamicin and gentamicin plus silver (*P* = 0.117).

Scanning electron microscopy of PMMA beads that were incubated with MRSP and rinsed with PBS solution but not sonicated revealed numerous staphylococci individually adherent to the beads as well as present within extracellular polymeric matrix material, consistent with a biofilm® (Figure 1). Staphylococci and extracellular polymeric matrix could not be identified on beads that had been incubated with MRSP, rinsed, and then sonicated.

**Table 1—Mean CFU counts (CFUs/mL) after log₁₀ transformation for each of 10 MRSP isolates following incubation with PMMA beads impregnated with gentamicin, silver, gentamicin and silver, or no additive (control).**

<table>
<thead>
<tr>
<th>MRSP isolate</th>
<th>Gentamicin</th>
<th>Gentamicin and silver</th>
<th>Silver</th>
<th>Control</th>
<th>Gentamicin susceptibility</th>
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<tr>
<td>1</td>
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<td>7.37</td>
<td>7.34</td>
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<td>All isolates</td>
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<td>7.13 ± 0.34</td>
<td>7.16 ± 0.52</td>
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<td>Susceptible to gentamicin</td>
<td>4.42 ± 0.84</td>
<td>4.01 ± 0.82</td>
<td>6.93 ± 0.39</td>
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<td>Not susceptible to gentamicin</td>
<td>7.08 ± 0.43</td>
<td>7.16 ± 0.54</td>
<td>7.26 ± 0.26</td>
<td>7.24 ± 0.46</td>
<td>—</td>
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</table>

= Not applicable. NS = Not susceptible (includes isolates categorized as resistant [MIC, > 8 µg/mL] or having intermediate susceptibility [MIC, > 4 and ≤ 8 µg/mL; n = 1 (isolate 5)] to gentamicin)²⁹. S = Susceptible (MIC of gentamicin, ≤ 4 µg/mL).
Discussion

The results of the present study revealed that gentamicin-impregnated PMMA beads can be effective in reducing in vitro MRSP biofilm formation on this surface, but only for isolates that are susceptible to gentamicin as determined by standard serum breakpoints. This may seem intuitive; however, clinical breakpoints are, in part, determined on the basis of anticipated drug concentrations at the site of infection, so they cannot necessarily be directly applied to situations where higher drug concentrations might be achieved, such as with antimicrobial-impregnated PMMA beads. Accordingly, local treatment such as this is often used clinically for prophylaxis or treatment of SSIs on the assumption that high antimicrobial concentrations are achieved and that resistance can be overwhelmed. Although that might occur in some situations, it does not appear to be the case for MRSP and gentamicin-impregnated PMMA beads. Further in vivo investigation is required to determine the biological relevance of our findings.

Biofilm formation of our isolates not susceptible to gentamicin in the present study was not affected by the presence of gentamicin, despite the fact that one of the nonsusceptible isolates was classified as of intermediate susceptibility and the highest MIC was only 16 µg/mL, the clinical breakpoint for resistance. These findings are consistent with results of a study on other staphylococcal species and may indicate that elution of gentamicin from PMMA is minimal or that gentamicin concentration at the bead surface is not particularly high (perhaps not any higher than that achieved with parenteral administration), or both. Low surface area-to-volume ratio, as was the case with our PMMA beads, has been shown to limit elution and certainly limits the amount of gentamicin present on the bead surface, with a greater percentage present in the interior of the bead and therefore biologically inactive.

Gentamicin-impregnated PMMA is commonly used in total joint arthroplasty, spinal fracture fixation, and other orthopedic and neurologic procedures as a prophylactic measure as well as a method of local treatment of implant-associated SSIs. The present study investigated its efficacy at preventing MRSP biofilm formation, not its efficacy at eliminating established biofilm. The potential impact of antimicrobial-impregnated PMMA on existing MRSP biofilm formation is not known, nor to our knowledge has the clinical efficacy of this treatment been investigated. Our data could suggest that gentamicin-impregnated PMMA would be as effective as systemic treatment of infection caused by gentamicin-susceptible isolates, but that may have no impact on MRSP isolates not susceptible to gentamicin. This is of concern because MRSP is the leading cause of implant-associated SSI in dogs and isolates are frequently gentamicin resistant. Currently, many MRSP isolates remain susceptible to amikacin and investigation into the impact of amikacin-impregnated PMMA on MRSP infection and biofilm formation on this surface should be performed.

With high rates of antimicrobial resistance, silver may be an ideal antimicrobial alternative, considering that induction of resistance to silver is suspected
to be low. As well, silver has several other appealing characteristics. Silver kills its target through multiple mechanisms, including interruption of cell respiration, electron transport, and DNA replication. It has been described as having entire-spectrum activity, meaning it is capable of killing bacteria, yeasts, and molds. Furthermore, nanosilver, characterized by particle size < 100 nm, has been found to have antiviral activity. Of the transition metals that exert antibacterial activity, silver is considered the safest.

Despite the appealing properties of silver, the silver product used in the present study was found to be ineffective in reducing MRSP biofilm formation on PMMA beads in vitro. Our findings are in contrast to results of another study, in which 1% microsilver-impregnated PMMA killed all adherent (biofilm-associated) MRSA. The failure of the silver product used in our study could be related to a number of factors, including particle size, incubation time, silver concentration, and resistance of the MRSP to silver.

Silver may be better able to exert bactericidal effects as a smaller particle than it is in larger form. Exact size at which silver is most effective is not yet known. The silver product used in this study could have been too large (10 μm) to be an effective antimicrobial. Large particle size may render silver ineffective at damaging bacterial cells through attachment and invasion and could restrict elution, something that could be both beneficial (maintaining antibacterial activity at the bead surface while limiting systemic exposure) and detrimental (limiting activity at the site of infection). The optimal elution characteristics of PMMA are not known, and the low surface area-to-volume ratio of our 7-mm-diameter beads would result in a large percentage of added silver being present in the interior of the bead and therefore biologically inactive. Regardless, the lack of efficacy noted here at the bead surface is concerning, given that it is difficult to see how there would be the potential for clinically relevant antimicrobial activity in patients. The relationship of silver particle size to its ability to inhibit MRSP biofilm formation on PMMA could not be evaluated in the present study but is an important consideration for future research. Particularly, evaluation of a nanosilver PMMA additive may be considered. Although a concern of possible toxic effects with the use of nanosilver exists, other investigators have found that the addition of silver solution with a 10-nm particle size to growth media inhibits a significant percentage of MRSA growth and is not cytotoxic to human epithelial cells. In contrast, silver solution with a 100-nm particle size is the least effective against MRSA and the most cytotoxic to human epithelial cells. Other studies have also demonstrated the bactericidal efficacy of nanosilver as a PMMA additive against Escherichia coli, Staphylococcus aureus (including MRSA), and several other bacteria when used in wound dressings.

If particle size does restrict elution, then only the silver present on the surface of our 1% silver-impregnated PMMA beads would exert antimicrobial activity. In experiments that used a silver product comparable to that of the present study, Alt et al found that 1% silver-impregnated PMMA completely inhibited bacterial growth of all strains tested, including MRSA. However, those investigators used PMMA samples with a larger surface area-to-volume ratio, further suggesting that the silver concentration present on the surface of the PMMA bead may be a key factor in the antimicrobial activity of the silver compound used in this study. Although PMMA impregnated with silver at concentrations > 1% have not been previously reported, concentration selection may not account for particle size and associated elution characteristics. The silver product used in our study could potentially prove efficacious if a larger concentration were incorporated into the PMMA, allowing for a greater percentage of silver molecules to be present at the bead surface. If mechanical strength is not compromised, an increase in silver concentration can be considered.

Another potential contributing factor to the lack of efficacy of the silver product used in our study may be silver resistance among the MRSP isolates used. Although induction of resistance to silver is suspected to be low, it remains a possibility. Several studies have found bacteria, including MRSA, that contain silver resistance genes. Furthermore, investigators in another study induced silver resistance in MRSA, with phenotypically unique colonies surviving on silver-infused media. Silver resistance among MRSP has not yet been investigated, and further study is required.

A final factor that may have affected our results was incubation time. Our PMMA beads were incubated with MRSP for 18 to 24 hours. We chose this time period because biofilm formation occurs rapidly in vivo, and the first few hours following implantation of a medical device are likely critical in determining whether bacteria with the ability to form a biofilm or host molecules will first populate the implant surface. However, other studies have demonstrated greater efficacy of antimicrobials given a longer incubation period. One study demonstrated reduced biofilm formation (adhered bacteria) of S. aureus on gentamicin-impregnated PMMA after 24, 48, or 72 hours, depending on the PMMA product used, compared with nonimpregnated PMMA. Studies assessing antimicrobial efficacy of silver in wound dressings showed that periods of 24 or even 48 hours were required to demonstrate microbial cell death. The goal of our study was to determine whether gentamicin- and silver-impregnated PMMA could inhibit biofilm formation of MRSP in vitro during the time thought to be crucial for this activity following implantation of PMMA. Further study is required to determine the effect of a prolonged incubation time on MRSP biofilm formation with silver- or gentamicin-impregnated PMMA.

Results of scanning electron microscopy experiments confirmed the presence of biofilm and associ-
ated bacteria on the surface of the PMMA beads following incubation. In addition, it demonstrated the persistence of biofilm after rinsing in PBS solution and its removal by sonication. These findings confirm that the CFUs recovered following sonication were biofilm associated.\textsuperscript{31–34}

Several limitations of the present study should be considered when evaluating the findings. The dynamic state of an animal certainly differs from the static conditions of laboratory testing. More difficult yet, biofilm phenotypes are diverse and very difficult to predict, and biofilm readily develops under a range of conditions.\textsuperscript{5} Thus, a truly representative in vitro model does not exist, and the biofilm phenotype rendered in a laboratory setting may be quite different from those found in vivo. We also used 7-mm-diameter PMMA bead samples and do not know how this compares with the surface area-to-volume ratio, elution characteristics, and other contributing factors in the bactericidal efficacy of PMMA used for fixation in total joint arthroplasty, spinal fracture fixation, and other orthopedic and neurologic procedures. Finally, our study involved a robust MRSP challenge, inoculating PMMA beads with approximately $10^8$ MRSP CFUs. This challenge, albeit commonly used for in vitro biofilm studies,\textsuperscript{29–32} may not be representative of in vivo situations.

We found that gentamicin-impregnated PMMA was effective in reducing, but not completely inhibiting, biofilm formation of gentamicin-susceptible MRSP in vitro. Furthermore, gentamicin-impregnated PMMA had no effect on MRSP isolates not susceptible to gentamicin. Silver-impregnated PMMA created with a microparticulate silver compound was not effective at reducing MRSP biofilm formation in our study, and addition of the silver particles to gentamicin-impregnated PMMA did not significantly improve its efficacy against biofilm-associated MRSP. With rising antimicrobial resistance, further investigation into alternative antimicrobials for impregnating PMMA, including silver additives, is warranted.

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**Footnotes**

a. Microsilver BG antimicrobial powder, BioMedtrix Inc, Boonton, NJ.
b. BioMedtrix 2 veterinary bone cement 20 g, BioMedtrix Inc, Boonton, NJ.
c. BioMedtrix 3G veterinary bone cement 20 g, BioMedtrix Inc, Boonton, NJ.
d. CryoStor beads, BioLife Solutions, Bothell, Wash.
e. Etest, Biomerieux Inc, Marcy l’Etoile, France.
f. Ultrasonic cleaner, model 2510, Branson Ultrasonic Corp, Danbury, Conn.
g. S-570 SEM, Hitachi, Chiyoda, Tokyo, Japan.
h. PROC MIXED, SAS, version 9.2, SAS Institute Inc, Cary, NC.

**References**


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**Additional Notes**

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