Evaluation of a silver-impregnated coating to inhibit colonization of orthopedic implants by biofilm forming Methicillin Resistant *Staphylococcus pseudintermedius*

M.A. Azab[1, 3]; M.J. Allen[1, 2]; J.B. Daniels[1]

[1]Department of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH, USA; [2]Department of Veterinary Medicine, University of Cambridge, England; [3]Department of Surgery, Faculty of Veterinary Medicine, Damanhour University, Egypt

Correspondence to

Joshua B. Daniels, DVM, PhD, DACVM

The Ohio State University College of Veterinary Medicine

601 Vernon Tharp Dr.

Columbus, OH, USA 43210

Phone: 614-247-1725

Fax: 614-292-0895

Email: daniels.384@osu.edu

Acknowledgements: The authors thank Bio-Gate AG for providing the Ag/SiOxCy plasma coating on the test discs.

Conflict of interest:

Dr. Allen is a paid consultant to BioMedtrix, LLC, who provided the samples for the study. However, no compensation was provided for performing the study.

Introduction

Surgical site infection (SSI) related to orthopedic procedures is a major complication that is associated with increased morbidity, mortality, and financial expenses. Implant-related SSI can be difficult or impossible to resolve with routine antimicrobial therapy alone due to the formation of biofilms on the orthopedic implants (1). Staphylococci are the most frequent causes of biofilm-associated infections as they are common opportunistic bacteria that reside on the skin and mucous surfaces (2). The most clinically relevant Staphylococci are the coagulase positive Staphylococci, and in dogs specifically: *Staphylococcus pseudintermedius*. Recently, methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has emerged as an important cause of SSI in dogs (3). MRSP isolates are often not only resistant to β-lactam antibiotics, but also to several other classes of antimicrobial drugs (4). The large increase in antimicrobial-resistant microorganisms clearly shows that new control strategies are required. Antimicrobial coatings of implant surfaces have a great potential in this context, such as coatings containing or releasing antimicrobial agents. Coatings containing inorganic antimicrobial agents are very attractive alternatives from the perspective of doping of biomaterials, which have advantages including good antibacterial activity, biocompatibility, and stability (5). Silver coated orthopedic implants have widely been used to prevent the growth of bacterial biofilms (6, 7) due to silver's broad-spectrum of activity against gram-positive and gram-negative bacteria. (8).

As the use of silver and silver-based products increases, it is becoming important to clarify the efficacy and efficiency of silver against different microorganisms and biofilms. Accordingly, the objective of this study is to evaluate the *in vitro* antibacterial activity of new ultrathin plasma coating with polysiloxan embedded silver particles against a strong biofilm-forming MRSP strain.

Methods, Results & Discussion

Additional details are furnished in the supplemental materials.

An MRSP isolate (OSU 12-2910), originally sourced from an infected canine total knee replacement in a 9 year old male neutered Kuvasz was evaluated for biofilm production using a microtitre plate assay (MPA) described by Stepanovich et al (9). The average OD_{570} of the triplicates of isolate and negative controls and the cut-off value (ODc) were established where ODc = average OD_{570} of the negative control + 3×SD of the negative control. According to the scheme of Stepanovich et al., the tested clinical MRSP isolate was classified as a strong biofilm producer where the OD_{570} of the eluted crystal violet was greater than 4 times of the cut off value ($4\times OD_C$), consistent with an earlier study of *S. pseudintermedius*, which showed that the majority of isolates produced biofilm, and 96% were classified as either strong or moderate biofilm producers (10).

Silver/Siloxane chemistry (Ag/SiO_xC_y) plasma polymer-coated circular discs (10 mm diameter and 1 mm thickness) were manufactured from commercially pure titanium (ASTM F67), and had previously undergone cytotoxicity testing in L-929 mouse fibroblast cells with a method compliant with ISO 10993-5; 2009, with no cytotoxicity evident by 72 h (unpublished data). Uncoated titanium discs were used as negative controls. All discs were sterilized by gamma irradiation prior to laboratory testing. The *in vitro* antimicrobial activity assay was performed according to the standard test method, ASTM E-2180-07, with the modification of using one log step higher inoculum (11). The antimicrobial efficacies of silver-coated titanium specimens (n =12), and controls (n =12) were evaluated at two times: 5 minutes after inoculation of the specimens (T_0) and after 24 hours of incubation (T_{24}). The numbers of recovered organisms were averaged as the mean CFU/ml. The averaged means were then transformed and

expressed as mean $\log_{10}$ CFU. The statistical significance between the mean $\log_{10}$ CFU counts of silver-coated and control specimens at T_0 and T_{24} was evaluated using an unpaired *t*-test; a value of $P < 0.05$ was considered statistically significant.

At T_0, there was no significant difference in MRSP growth between control uncoated (3.83 ± 0.51 $\log_{10}$ CFU/ml, mean ± SD) and silver-coated discs (3.59 ± 0.33 $\log_{10}$ CFU/ml) ($P = 0.36$) (Figure 1). This demonstrates that the initial bacterial challenge was similar in test and control specimens. At T_{24}, the silver coated discs had significantly reduced growth (0.64 ± 0.99 $\log_{10}$ CFU/ml) which resulted in a difference of more than four log steps as compared to the non-coated discs (4.60 ± 0.91 $\log_{10}$ CFU/ml) ($P < 0.0001$). Uncoated discs did not show any reduction in the number of bacteria while the silver coating demonstrated a significant antimicrobial efficacy and showed more than 99.98 % reduction in the number of CFU/ml after 24-hour incubation.

Previous studies that have evaluated clinically relevant *Staphylococcus spp.* affecting human beings rather than dogs, have shown excellent *in vitro* antimicrobial activity of different formulations of silver coatings against *Staphylococcus aureus*, *Staphylococcus epidermidis*, methicillin-resistant *Staphylococcus aureus* (MRSA), and methicillin-resistant *Staphylococcus epidermidis* (MRSE) (6, 12). Khalilpour et al. (6) reported that Ag/SiO_xC_y coating showed a significant *in vitro* antimicrobial activity against MRSA and *ex vivo* suppression of more than 99.9% of bacterial growth by the coating compared to non-coated samples after 28 days. Furkert et al. (7) observed similar results during a study on *Staphylococcus epidermidis* where they demonstrated that fixation pins coated with silver showed a 3-log step reduction in the number of biofilm-forming bacteria compared to a non-coated stainless steel or titanium implant. Similarly, the present study demonstrated that the new silver plasma coating was highly effective against

biofilm-forming MRSP and showed more than 99.98 % reduction in the number of CFU compared with the non-coated specimens. This work is the first report of successful application of silver coating technology to a MSRP isolate that is of direct relevance to canine orthopedics.

The antimicrobial activity of silver is dependent on the availability of free silver ions (SI). In the presence of moisture, the embedded metallic silver particles (Ag^0) generate silver ions (SI) which diffuse through the siloxane top layer to create an antimicrobial surface. The pure metallic silver particles act as a depot of silver and provides a continuous and long term generation of silver ions. SI strongly bind to cellular components such as enzymes and structural proteins leading to altered function (8, 13, 14). Free SI interfere with bacterial cell metabolism and disturb the integrity of the bacterial cell membrane (13, 15). Furthermore, SI can interact with the DNA of bacteria, preventing bacterial replication (15). Antimicrobial coating of surfaces with silver seems to reveal differences based on the size of the silver particles, which are used. Meyer et al. (16) reported that the use of colloidal silver for coating of fixation pins caused deficient antimicrobial effect. In contrast, nanoparticulate silver provides a larger active surface area and a more homogeneous distribution of silver on biomaterials. The titanium specimens used in this study were coated with a plasma polymer in which silver nanoparticles (5–50 nm) were embedded. Our findings are similar to those demonstrated by Panácek et al. (13) who reported that the smaller particles with a larger surface area available for interaction provided a more efficient means of antibacterial activity than larger particles. It has been reported that impregnation of silver into a coating can be more effective than direct surface coating alone as surface silver can be deactivated by protein anions (14).

In vitro antibacterial efficiency of the silver coating and biofilm structure was secondarily evaluated by scanning electron microscopy (SEM). Three titanium specimens (one silver-coated

and two control uncoated) were incubated separately, each in a petri dish containing 10 ml of MRSP suspension in tryptic soy broth of OD_{600} = 0.5 for 24 hours aerobically at 37°C to initiate biofilm formation. Following incubation, each specimen was washed by immersion in 10 ml of PBS and then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer; pH 7.4 at 4°C until time of SEM imaging. The results obtained by SEM revealed no bacterial growth or biofilm formation on the silver-coated specimen (*n* =1) after 24 hours of incubation in strong biofilm forming MRSP suspension, while biofilm formation was observed on the control uncoated specimens (*n* =2). The biofilm was characterized by micro colonies of bacteria along with large amounts of irregularly extracellular polymeric substances (EPS) (Figure 2). Similar findings were observed by Singh et al. (10). These SEM images correlated with the lower number of CFU recovered on the silver-coated specimens after 24 hours of incubation compared to uncoated specimens.

In conclusion, the results from this laboratory confirm the *in vitro* antimicrobial activity of the silver impregnated coating against a strong biofilm-forming MRSP strain that was isolated from a dog with an infected total knee replacement. Our findings suggest that this silver plasma coating may represent a potentially valuable strategy for reducing adhesion of MRSP and preventing implant-associated infections in dogs undergoing orthopedic surgery.

References

1. **DiCicco M, Neethirajan S, Singh A, et al**. Efficacy of clarithromycin on biofilm formation of methicillin-resistant *Staphylococcus pseudintermedius*. BMC Vet Res 2012; 8:225-232.
2. **Vuong C, Otto M**. *Staphylococcus epidermidis* infections. Microbes Infect 2002; 4:481–489.

3. **Eugster S, Schawalder P, Gaschen F, et al**. A prospective study of postoperative surgical site infections in dogs and cats. Vet Surg 2004; 33:542-550.

4. **Wettstein K, Descloux S, Rossano A, et al**. Emergence of methicillin-resistant *Staphylococcus pseudintermedius* in Switzerland: three cases of urinary tract infections in cats. Schweiz Arch Tierheilkd 2008; 150: 339-343.

5. **Zhao L, Chu PK, Zhang Y, et al**. Antibacterial coatings on titanium implants. J Biomed Mat Res B 2009; 91:470-480.

6. **Khalilpour P, Lampe K, Wagener M, et al**. Ag/SiOxCy plasma polymer coating for antimicrobial protection of fracture fixation devices. J *Biomed* Mater Res B 2010; 94(1):196-202.

7. **Furkert FH, Sörensen JH, Arnoldi J, et al**. Antimicrobial Efficacy of Surface-Coated External Fixation Pins. Curr Microbiol 2011; 62:1743-1751.

8. **Melaiye A, Youngs WJ**. Silver and its application as an antimicrobial agent. Expert Opin Ther Pat 2005; 15:125-130.

9. **Stepanovich S, Vukovic D, Hola V, et al**. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS 2007; 115:891-899.

10. **Singh A, Walker M, Rousseau J, et al.** Characterization of the biofilm forming ability of *Staphylococcus pseudintermedius* from dogs. BMC Vet Res 2013; 9(1): 93-98.

11. **ASTM Standard E2180-07, 2012**, "Standard test method for determining the activity of incorporated antimicrobial agent(s) in polymeric or hydrophobic materials," ASTM International, West Conshohocken, PA, 2012, *DOI*: 10.1520/*E2180*-07R12, www.astm.org.

12. **Alt V, Bechert T, Steinrücke P, et al**. An in vitro assessment of the antibacterial properties and cytotoxicity of nanoparticulate silver bone cement. Biomaterials 2004; 25:4383-4391.

13. **Panácek A, Kvítek L, Prucek R, et al.** Silver colloid nanoparticles: synthesis, characterization, and their antibacterial activity. J Phys Chem B 2006; 110:16248-53.

14. **Furno F, Morley KS, Wong B, et al.** Silver nanoparticles and polymeric medical devices: a new approach to prevention of infection? J Antimicrob Chemother 2004; 54:1019-1024.

15. **Damm C, Münstedt H, Rösch A**. The antimicrobial efficacy of polyamide 6/silver-nano- and microcomposites. Mater Chem Phys 2008; 108:61-66.

16. **Meyer Ch, Keßler J, Alt V, et al.** Antimicrobial effect of silver-coated external fixator pins. Osteo Trauma Care 2004; 12:81-84.

Figure legends

Figure 1

Antimicrobial efficiency of control uncoated and silver-coated specimens against biofilm forming MRSP at T_0 and T_{24} ($\log_{10}$ CFU/ml). (*) significant difference at $p < 0.0001$.

Figure 2

Scanning electron microscopy (SEM) of non-coated (left) and silver-coated (right) titanium specimens inoculated with MRSP. The SEM images were taken at three different magnifications, 1000x, 2500x, 10000x (from top to bottom).

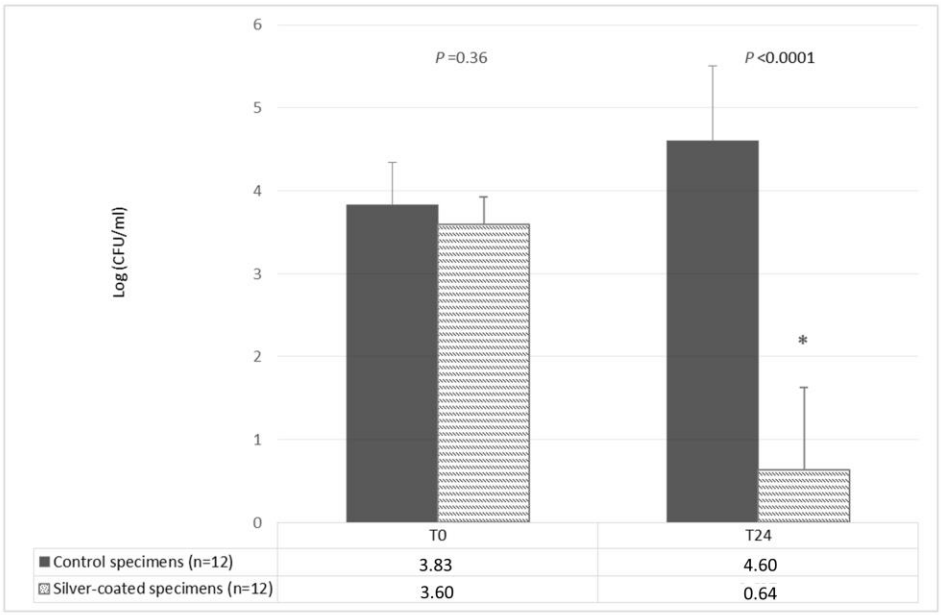

Figure 1. Antimicrobial efficiency of control uncoated and silver-coated specimens against biofilm forming MRSP at T_0 and T_{24} (log_{10} CFU/ml). (*) significant difference at $p < 0.0001$.

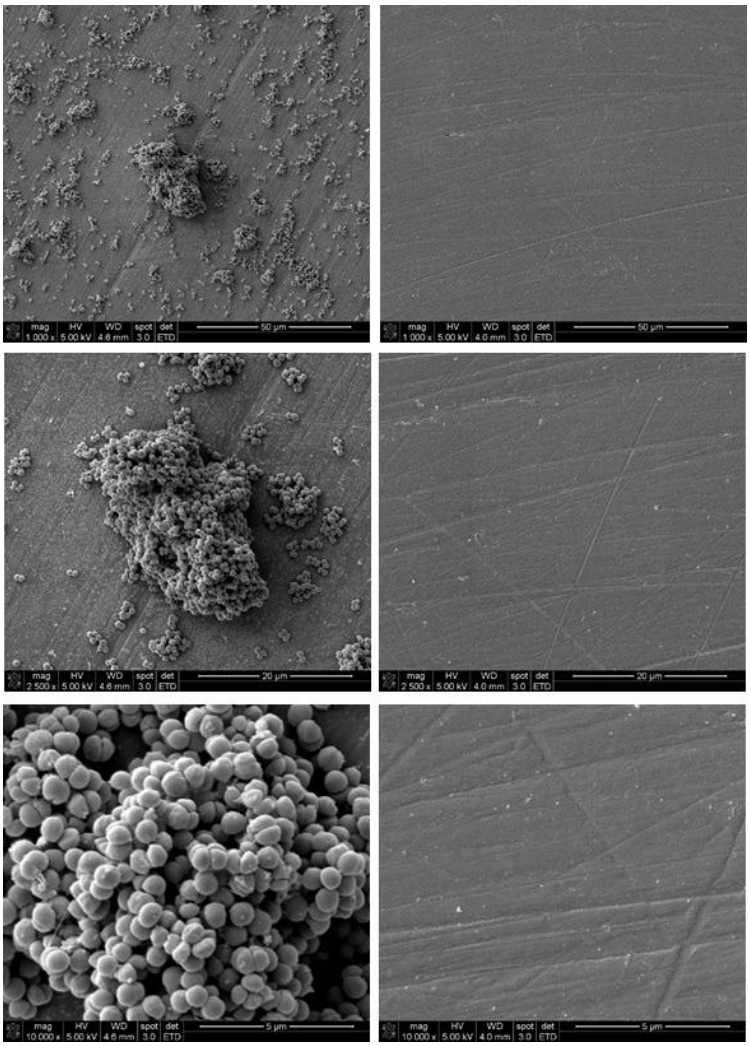

Figure 2. Scanning electron microscopy (SEM) of non-coated (left) and silver-coated (right) titanium specimens inoculated with MRSP. The SEM images were taken at three different magnifications, 1000x, 2500x, 10000x (from top to bottom).