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Original article

Direct confocal microscopy studies of the bacterial colonization in vitro of a silver-coated heart valve sewing cuff

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Abstract

The antimicrobial coating of prosthetic heart valve sewing cuffs has been considered a potentially effective method for preventing prosthetic valve endocarditis. Although traditional in vitro bacterial adherence studies are often useful as screening tools, they can be inadequate in examining the antiinfective efficacy of antimicrobial-coated devices. We conducted a pilot in vitro study to directly assess the antimicrobial activity of a silver-coated sewing cuff versus uncoated cuff using confocal scanning laser microscopy. *Staphylococcus epidermidis* adhered more to the surfaces of the silver-coated sewing cuff compared with the uncoated cuff. These pilot in vitro results cast a doubt on the antiinfective efficacy of silver-coated prosthetic heart valve sewing cuffs and suggest further assessment should be carried out using animal studies.

Keywords: Confocal microscopy studies; Bacterial colonization; Silver-coated

1. Introduction

Although many methods exist for the in vitro examination of the antiinfective efficacy of antimicrobial-coated materials, some of the traditional adherence tests suffer from inherent limitations. For instance, the direct inoculation test involves the exposure of coated material to a suspension of planktonic organisms and the subsequent enumeration of the bacteria that have colonized the surface by scraping, swabbing, and/or sonication. Although the enumeration process usually involves dilution and plating to determine the number of 'replicating units', it may not necessarily reflect the actual number of living bacterial cells because of clumping of bacterial cells. Moreover, the accuracy of traditional adherence methods is affected by our habitual use of laboratory-adapted bacterial strains, many of which have been repeatedly subcultured, and may have consequently lost both their ability to adhere to surfaces and their inherent resistance to antibacterial agents. In addition, such traditional in vitro studies allow for rather 'blind' assessment of the consequences of microbial challenge without directly visualizing the surfaces of devices that have been exposed to bacterial suspension.

Therefore, it seems appropriate to rely more on direct observations of bacterial adhesion and colonization, and less on extrapolation from 'blind' in vitro data. This approach is vindicated by the use of confocal scanning laser microscopy (CSLM) to visualize and count bacterial cells directly on transparent or opaque surfaces. The CSLM allows the examination of living fully hydrated biofilms in real time, and the simultaneous use of specific molecular probes allows us to determine the identity (oligonucleotide probes) and the physiological state (live versus dead) of the adherent bacterial cells [1]. This CSLM-based method may be used to accurately assess the antibacterial properties of putative biofilm-resistant biomaterials.

The use of prosthetic heart valves has become an important component of modern medical practice. De-

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spite adherence to sterile guidelines for insertion of prosthetic heart valves, infection remains the most drastic complication of such potentially life saving devices. For instance, although millions of mechanical heart valves have been implanted with rather acceptable low rates of mechanical failure, catastrophic infections affect 3.2–5.7% of patients over a 5 period after valve implantation [2–4]. Because device-related infections can impose the greatest limitation on the successful use of medical devices, there has been a great interest in searching for truly antiinfective coatings. To accurately assess the antimicrobial activity of antimicrobial-coated mechanical heart valve sewing cuffs, we conducted a pilot in vitro study to compare bacterial adherence to the surfaces of silver-coated with uncoated sewing cuffs by relying on direct confocal microscopic observations.

2. Materials and methods

2.1. Tested devices

Two types of prosthetic heart valve sewing cuffs were evaluated: (1) the St. Jude Medical (SJM) Masters Series Mitral Valve, standard cuff-polyester (Model 23MJ-501; St. Jude Medical, Inc., St. Paul, MN, USA) referred to in this report as the 'uncoated sewing cuff', and (2) the SJM Masters Series Mitral Valve, expanded cuff-polyester with Silzone™ (Model 27MECS-602; St. Jude Medical, Inc.) referred to in this report as the 'silver-coated sewing cuff'.

2.2. Experimental system

All experiments were performed using Nutrient Broth (Difco # 0001-17-0; Difco laboratories, Sparks, MD, USA) medium that contained bacto beef extract (3 g/l), Bacto Peptone (5 g/l), and Bact Agar (15 g/l). A continuous-culture bioreactor was configured as a once-flow-through system [5]. The broth was pumped through the bioreactor at a flow rate of 2.2 ml/h using a syringe pump (model 341B; Sage Instruments, Boston, MA, USA). The flow was laminar with a Reynolds number of 0.34 and a fluid resistance time of 0.43 min. The bioreactor was composed of polycarbonate with a glass coverslip affixed to its top.

2.3. Bacterial inoculation

A clinical isolate of *Staphylococcus epidermidis* was obtained from Bozeman Deaconess Hospital in Bozeman, Montana. Cultures were grown in Nutrient Broth media at 37°C and examined hourly by optical density spectroscopy (Spectronic 601; Milton Roy, New York, NY, USA). Cell doubling time was determined to be 2.8 h, and mid-log phase growth was achieved 6 h after inoculation. Filtration of 1 ml of batch culture on 0.2 micron filtration membranes, and stained by epifluorescent acridine orange direct counts (AODC) resulted in an average cell count of 3.2×10^6 cells. AODC counts provide the necessary data for confirming the dilution rate needed to achieve an appropriate challenge. A 20-ml aliquot of the batch culture was diluted in 20 ml of sterile media 6 h after inoculation at ambient temperature in a 30cc syringe, resulting in a bacterial challenge equivalent to 3.2×10^3 cells. The seeding phase consisted of pumping the diluted bacterial suspension through the once-flow-through system for a period of 2 h. This was followed by the growth phase during which sterile broth was pumped for the following 22 h.

2.4. Bacterial staining

The tested devices were then removed and stained with Molecular Probes Live/Dead BacLight Bacterial Viability Kit (Part #: L-7012, Molecular Probes, Eugene, OR, USA). The viability kit consists of two nucleic acid stains: (1) SYTO 9 (excitation maximum 508 nm, emission maximum 527 nm), a lipophilic membrane permeant cationic stain that labels live bacteria with green fluorescence, and (2) propidium iodide (excitation maximum 536 nm, emission maximum 620 nm), a membrane impermeant anionic stain that labels membrane-compromised bacteria with red fluorescence [1,6]. The stains were prepared in autoclaved double-filtered nanopure water at a concentration of 0.1% (v/v). The tested material was directly stained with 0.4 ml of propidium iodide, then 0.4 ml of SYTO 9, and allowed to react for 5 min. The sample was then gently washed with autoclaved nanopure water to remove excess stain and minimize background fluorescence. The material was then directly imaged by CSLM.

2.5. Confocal scanning laser microscopy (CSLM)

An Olympus BH-2 microscope with a 50X ultra long working distance objective was used for epifluorescence microscopy. Images were collected with an Optronics charge coupled device (Optronics Engineering, Goleta, CA, USA) and the imaging program Image Pro-Plus 3.0 for Windows 95 (Media Cybernetics, Silver Spring, MD, USA). CSLM was performed with a Leica TCS-NT confocal microscope (Leica, Heidelberg, Germany). A 100X1.4 N.A. Oil Plan Apo and 63X0.7 N.A. Plan Fluotar objectives were used for confocal laser microscope imaging. The confocal microscope was optimally configured for SYTO 9/propidium iodide analysis by using the 488 nm excitation laser with a 488 nm/568 nm/633 nm dichroic mirror and relative short pass filter of 580 nm in the first beam splitter position. A band filter allowing wavelengths of 525 to 550 nm to pass to

the first PMT was used for imaging the SYTO 9 stain. A long pass filter of 645 nm was used for imaging the propidium iodide. The number of bacteria, relative surface area of each device covered with bacteria, viability of bacteria, and mucoid appearance of biofilm upon the sewing cuff fibers were compared between the uncoated and silver-coated sewing cuffs. Cuffs were was submerged in 50 ml buffered saline, put on ice, and sonicated at power setting 5 for 1 min to determine if adherent bacteria and the biofilm in which they reside could be removed. Consistent with standards traceable to NIST, 20 fields were examined.

2.6. Sensitivity of experimental system

In preliminary experiments, the sensitivity of the BacLight Bacterial Viability Kit for studying this same strain of *S. epidermidis* was initially examined by inoculating one colony that had been grown on blood agar (Difco Laboratories) in 10 ml of nanopure water. A 1-ml aliquot was placed on a 0.20 micron filter (Osmonics, Livermore, CA, USA) and stained with 0.2 ml of SYTO 9 and 0.2 ml of propidium iodide at 0.1% concentration (v/v). The filter was then imaged by CSLM. A 100-μl aliquot of chlorine was then added to the remaining 9 ml of water. Thereafter, samples were collected every 3 h, stained in the same manner and imaged. A gradual progression from 99% viable cells to 100% membrane-compromised cells was observed within a 24 h time period. The confocal microscope was configured in an identical manner for imaging the change in bacteria. These findings were consistent with a previous study demonstrating the positive correlation of staining with the BacLight viability stain and plate counts [6].

3. Results

As Table 1 shows, the silver-coated sewing cuff (Fig. 1) was colonized by a higher number of bacterial

Table 1
Comparison of bacteria colonizing uncoated versus silver-coated sewing cuffs

Variable	Uncoated cuff	Silver-coated cuff
Bacterial count per field		
Mean	0.76	5.80
Range	0.07–3.89	0.02–521.89
Viability, % of adherent bacteria viable	100%	85%
Surface coverage, % of surface area colonized	8.2%	39.3%
Mucoid appearance	No	Yes

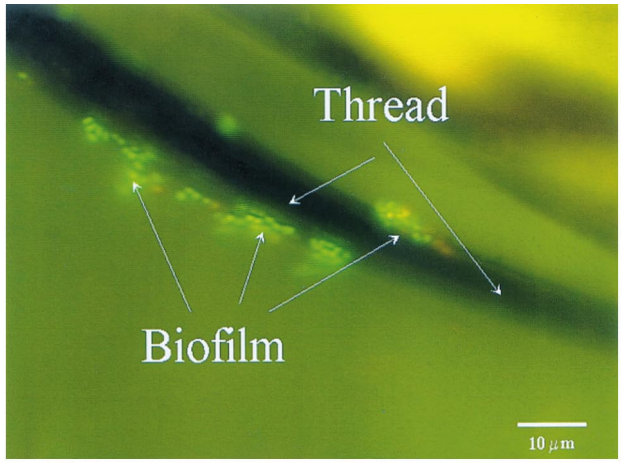

Fig. 1

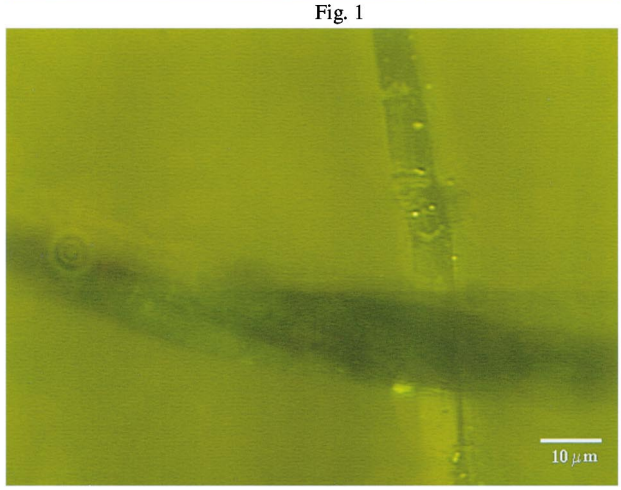

Fig. 2

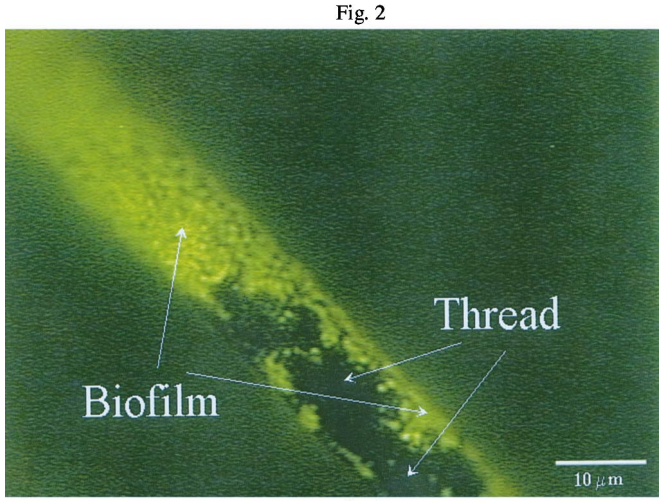

Fig. 3

Fig. 1. CSLM image of silver-coated heart valve sewing cuff fiber 24 h after incubation with *S. epidermidis* (wild strain). Bright green objects are viable biofilm bacteria, orange–red objects are dead bacteria.
Fig. 2. CSLM image of untreated heart valve sewing cuff fiber 24 h after incubation with *S. epidermidis* (wild strain).
Fig. 3. CSLM image of silver-coated heart valve sewing cuff fiber 24 h after incubation with *S. epidermidis* (wild strain) followed by sonication.

organisms than the uncoated cuff (Fig. 2). Although all bacteria adherent to the uncoated sewing cuff were alive, 15% of bacteria adherent to the silver-coated cuff appeared dead. In addition, the surface areas of the silver-coated sewing cuff that became colonized were almost five times larger than those of the uncoated cuff (39.3 versus 8.2%, respectively). Unlike the uncoated sewing cuff, the biofilm surrounding the silver-coated cuff appeared mucoid and could not be removed by sonication (Fig. 3). The data were analyzed using JMP, Statistical Discovery Software, ver. 3.1.6.2. A test for equal variance was performed and determined that the variances were not equal ($P < 0.0001$) using the O'Brien, Brown–Forsythe, Levene, and Bartlett tests for equal variance. A Welch Anova allowing standard deviations which are not the same was used. This resulted in an F ratio of 30.7768 and a t-test value of 5.5477. This showed that there is in fact a significant difference between the data set ($P < 0.0001$). A coefficient of variance was used to verify that there was a difference in the variances and that it was not just due to the extreme differences in the mean value. This verified that the results and test used were acceptable.

4. Discussion

The major morbidity and mortality associated with prosthetic valve endocarditis and the high cost of managing this complication have encouraged a growing interest in making such medical devices antiinfective. Like other types of medical devices, colonization of the prosthetic heart valve is a prerequisite for device-related infection but not all tested antimicrobial-coated devices prove to be clinically beneficial. Therefore, it is important that in vitro investigations of the activity of such devices are done in a fashion that is likely to reflect their antiinfective potential in vivo.

Several options exist regarding the potential selection of antimicrobial agents for coating the polyethylene terephthalate (PET) polyester fabric that is used to construct prosthetic heart valve sewing cuffs. Since silver-coated sewing cuffs are the only antimicrobial-coated sewing cuffs that are currently available for clinical use, it was appropriate to examine their antimicrobial activity in comparison with uncoated cuffs. In vitro adherence studies of largely planktonic bacteria demonstrated that such silver-coated sewing cuff fabric significantly reduces microbial adherence [7]. However, this study which used direct observation of bacteria in the biofilm milieu suggested that silver-coated prosthetic heart valve sewing cuffs might not be antiinfective in vivo, where biofilm formation is the most important determinant of the course of device-related infection. It is important to note that this study has two limitations: (1) the CSLM approach for studying antimicrobial-coated medical devices has not yet been standardized (i.e. by comparing CSLM findings using antimicrobial-coated devices that have proven to be effective in vivo with antimicrobial-coated devices that have failed to demonstrate antiinfective efficacy in vivo; and (2) our results represent only pilot findings from examining a single silver-coated sewing cuff versus a single uncoated sewing cuff, and have not been duplicated. However, it should be noted that the findings of this pilot in vitro study support the results of a recent animal study that showed that subcutaneously implanted silver-coated sewing cuff fabric in rabbits did not protect against *S. aureus* device colonization and device-related infection [8].

The layer of biofilm contains a variety of host-derived adhesins to which different organisms variably adhere. For instance, *S. aureus* adheres tightly to fibronectin, fibrinogen, collagen and, to a lesser extent, to laminin, whereas *S. epidermidis* adheres primarily to fibronectin [9,10]. Although the biofilm-embedded organisms play a major role in microbial pathogenesis in vivo and are known to be less susceptible to antibiotic therapy and host immune defense than planktonic organisms [11,12], such biofilm-embedded bacteria are generally not accounted for in traditional microbial adherence studies in vitro. Since the silver-coated sewing cuff does not produce a detectable zone of inhibition around the coated device, it may not inhibit organisms embedded within the biofilm layer around the implanted device [8]. It is also possible that clinical isolates may lose the ability to form biofilm when cultured repeatedly in vitro. These factors combined may help explain, at least in part, the differences in the results of traditional adherence studies in vitro [7] that used ATCC strains versus the current in vitro results that used a recently obtained clinical isolate.

Both prosthetic heart valves and central venous catheters are intravascular devices that are predominantly infected by staphylococci. Since there exists no data on the clinical efficacy of silver-coated prosthetic heart valve sewing cuffs, it may be helpful to review the clinical experience with silver-coated vascular catheters. The clinical efficacy of using silver-impregnated subcutaneous cuffs attached to central venous catheters was demonstrated in some studies [13,14] but not others [15–17] to protect against catheter-related infection. Clinical trials examining the efficacy of central venous catheters with silver coating along the whole length of the catheter have also yielded controversial results. Utilizing data from the same group of 72 catheters studied in a prospective, non-randomized study, a group of investigators reported that silver-coated vascular catheters significantly reduced the rate of catheter colonization when compared with uncoated catheters [18,19]. However, since the catheter materials were different for silver-coated catheters (polyurethane or sili-

cone) in comparison with uncoated catheters (Teflon, polypropylene or polyurethane), there might have been an inherent bias in that investigation towards a higher rate of infection among uncoated catheters which were made of materials that can favor bacterial adherence more so than the materials of the uncoated catheters. Furthermore, a recent prospective, randomized clinical trial demonstrated the failure of silver-coated tunneled hemodialysis catheters to confer clinical protection against catheter colonization and catheter-related infection when compared with uncoated catheters [20]. Notwithstanding the differences in the pathogenesis of heart valve endocarditis and vascular catheter-related infection, those reported clinical studies of silver-coated central venous catheters cast some doubt upon the clinical efficacy of silver-coated heart valve sewing cuffs.

In summary, although traditional microbiological methods can be useful as screening tools, they may be inadequate for examining the antiinfective efficacy of antimicrobial-coated devices. Despite the reported contact kill of bacteria by the silver-coated surface of prosthetic heart valve when tested by traditional adherence studies in vitro, the CSLM-generated results of this pilot in vitro study cast a doubt on the antiinfective capacity of silver-coated sewing cuffs. Because the true antinfective value of using silver-coated prosthetic heart valve sewing cuffs can only be ascertained by clinical trials that are very expensive and require relatively long periods of time for completion, it is important to further assess the antimicrobial activity of such devices in animal studies.

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