Zuragen™ Injection, an antimicrobial/antithrombotic solution, is effective against mature *Staphylococcus aureus* biofilms

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ABSTRACT

Background. *Staphylococcus aureus*, a versatile human and animal pathogen, is commonly associated with catheter-related bloodstream infections and has been demonstrated to frequently colonize catheters as biofilms. Since catheter lumens are frequently filled in-between uses with catheter lock solutions, we tested the effect of the anticoagulant catheter lock solution Heparin (containing preservatives), a novel antimicrobial/antithrombotic solution Zuragen™, and saline on already established *S. aureus* biofilms. **Method**. *S. aureus* biofilms were grown in flow cells for 3 days, followed by treatment with Heparin (containing benzoyl alcohol), Zuragen™ or saline for 48 h under static conditions. The biofilm architecture was subsequently analyzed by confocal microscopy and COMSTAT. Biofilm viability was determined by Live/Dead® BacLight™ stain and CFU counts. **Results**. Compared to untreated biofilms, treatment with Zuragen™ for 1 h resulted in a 27-fold reduction in the biofilm biomass. After 48 h, Zuragen™ treatment resulted in a 240-fold reduction in the biofilm biomass. In contrast, treatment with Heparin for 1 and 24 h only resulted in a ~2.4 fold and 1.5-fold reduction in biofilm biomass, respectively. Saline treatment did not affect the biofilm biomass. The findings were confirmed by viability determinations. Treatment with saline resulted in less than 0.1 log reduction while treatment with heparin resulted in a <3-log reduction of the biofilm biomass. However, more than 10^5 cells were detected in the bulk liquid (lumen). In contrast, no viable cells were detected in the bulk liquid after Zuragen™ treatment which resulted in a 5-log reduction of the biofilm biomass. **Conclusion**. Compared to Heparin and saline, Zuragen™ is effective in reducing the *S. aureus* biofilm biomass and in preventing biofilm cells from dislodging from the surface as well as from growing planktonically in the bulk liquid.

INTRODUCTION

In today's practice, central venous catheters (CVC's) are frequently used as a path for delivering nutritional solutions, blood products, medications, and for hemodynamic monitoring. For renal patients they provide vascular access for dialysis often for many months after starting dialysis. With the increasing use of CVC's it has become evident that these catheters are often associated with a high risk of infection, greater than any other transcutaneous or indwelling medical device (Klevens et al., 2005), resulting in drastically increased risks for nosocomial and catheter-related bloodstream infections.

CVC's are frequently contaminated at the time of placement, by the skin microflora at the insertion site (organisms migrate along the catheter's external surface); through the catheter hub (organisms enter and move up the lumen), or subsequent catheter handling. Once planktonic bacteria have contaminated the device, they colonize the device surface and quickly begin to form resistant biofilm structures which are the source of catheter-related infections and contribute to catheter clotting and poor flow, complications that limit the utility and safety of catheters. Biofilms may be identified within 3 days after catheter placement (Anassi et al., 1995). It is estimated that 200,000 to 400,000 episodes of CRBSI occur annually in the United States with an attributable mortality ranging from 12% to 25% and average prolongation of hospital stay of 7 days (Hanna and Raad, 2005).

Taken together, the findings indicate that there is a need for a novel and effective treatment preventing microbial colonization and biofilm in catheters. Zuragen™ is an antimicrobial catheter lock solution (CLS) capable of eliminating planktonic bacteria from indwelling devices. Zuragen™ is a mixture of several compounds: 0.24 M citrate buffer with pH around 6.2, 0.05% methylene blue (MB), 0.15% methyl paraben (MP) and 0.015% propyl paraben (PP). Zuragen™ is also an effective anticoagulant, through the chelating functions of 7% sodium citrate.

Here, we present the efficacy of Zuragen™ on *S. aureus* biofilm developed in flow cell bioreactor. The biofilm architecture before and after treatment with tested lock solutions was studied and analyzed using the COMSTAT image analysis program. The effect of Zuragen™ was compared with several types and concentrations of heparin solution. Our findings indicate that Zuragen™ is capable of preventing and eliminating bacterial colonization of indwelling devices.

MATERIAL and METHODS

Bacterial strains and medium used. *S. aureus* ALC2085 (strain RN6390 containing pALC2084) was used for this study. The strain chosen expressed GFP-when exposed to 50ng/mL tetracycline under flowing conditions. LB medium was used for all experiments.

Biofilm Formation. Biofilms were grown in flow cells as previously described (Sauer et al., 2002, Southey-Pillig et al., 2005, Allegrucci and Sauer, 2007). The reactor was inoculated with 4 mL of *S. aureus* (~3x10^7 CFU/ml, obtained by dilution with saline of a stationary phase culture). Bacteria were allowed to attach to the glass substratum for 1 hour prior to initiating flow. The flow rate of the system was adjusted to 0.1 ml/min, resulting in a fluid residence time of 60 minutes. Biofilms were grown under flowing conditions at 37°C in 5% CO_2.

Biofilm treatment. Biofilms (3 day old) were treated with the following lock solutions for a period of 48h: Zuragen™, Saline, Heparin (5,000 U/mL, 5K) containing 1.5% benzyl alcohol as a preservative, heparin (10,000 U/mL, 10K) containing 0.15% methyl paraben and 0.015% propyl paraben, heparin (5,000 U/mL, 5K) containing 0.075% methyl paraben and 0.0075% propyl parabens, and heparin (1,000 U/mL, 1K) without preservatives.

Image acquisition. Biofilm images were acquired using a LSM 510 Meta inverted confocal laser scanning microscope (Zeiss, Heidelberg, Germany) equipped with the LSM 510 Meta image acquisition software (Zeiss). An average of 6 image stacks were acquired 1, 2, 6, 24, 48, and 72h post initiating flow. Images were taken at random along the length of the flow cell. Each image stack was analyzed using COMSTAT (Heydorn et al., 2000) as previously described (Southey-Pillig et al., 2005, Allegrucci and Sauer, 2007).

Viability. The bulk liquid was first drained from the flow cells, subsequently washed, and resuspended in saline. Biofilms were scraped from the flow cells. Viability was determined by serial dilution followed by spread-plating.

RESULTS

To test the efficacy of both Heparin and Zuragen™ on established *Staphylococcus aureus* biofilms, we first determined the time necessary for S. aureus biofilms to reach steady-state biofilms with respect to biofilm biomass and biofilm thickness under the condition tested. This was accomplished by monitoring biofilm formation over a period of 4 days using confocal microscopy (Fig. 1) and subsequently analyzing the confocal images with respect to biofilm biomass and biofilm thickness by COMSTAT.

As shown in Fig. 2 the biofilm biomass increased over a period of 3 days after which the biomass appeared to reach a steady-state. The same trend was observed for the average and maximum thickness of *S. aureus* biofilms. The surface area and biomass increased over a period of 3 days after which a plateau was reached. Therefore, all subsequent experiments were carried out using 3-day old, steady-state biofilms.

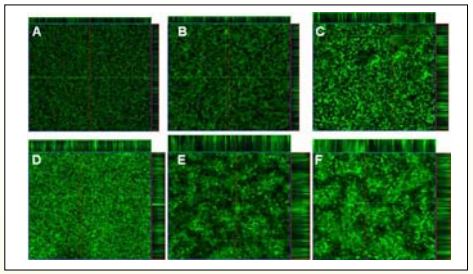

Fig. 1. *S. aureus* biofilm development over the course of 4days growth under flowing conditions. Confocal images were acquired (A) 2 hours, (B) 6 hours, (C) 1 day, (D) 2 days, (E) 3 days, and (F) 4 days post inoculation.

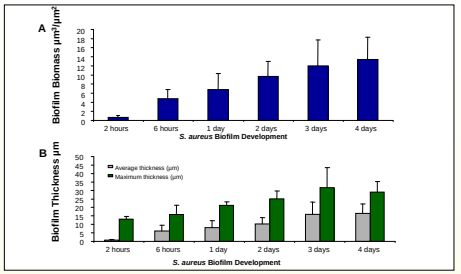

Fig. 2. Biofilm biomass (A) and average and maximum thickness (B) of *S. aureus* biofilms grown for a period of 4 days under flowing conditions. Biofilm variables were analyzed using COMCAST.

Effect of Zuragen™ and Heparin on *S. aureus* Biofilm viability

To determine the efficacy of Zuragen™ and Heparin varying in concentration and preservatives, total CFU from both biofilms and bacteria present in the bulk liquids following 48 of treatment under static conditions was determined. Treatment with saline was used as control. The results are summarized in Figure 3. Our data indicated that increasing Heparin concentrations coincided with reduced biofilm viability and increased log reduction of total biofilm cells. However, all tested Heparin solution tested did not result in the elimination of *S. aureus* cells from the bulk liquid.

Zuragen™ was the most effective of all solutions tested:
- Highest reduction in biofilm viability (Fig. 3A)
- Treatment with Zuragen for 48 h resulted in the total elimination of all *S. aureus* cells from the bulk liquid
  - No viable cells were detected in the bulk liquid (Fig. 3B)
- Treatment with Zuragen™ resulted in a ~5-log reduction in biofilm viability compared to untreated biofilms

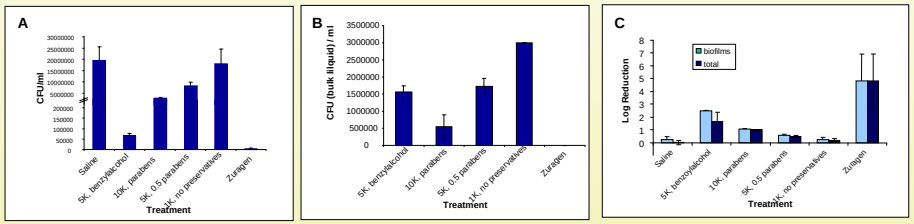

Fig. 3. Effect of catheter lock solutions on *S. aureus* biofilm viability. Viability (CFU/mL) of *S. aureus* biofilms (A) and S. aureus present in the supernatant of biofilms (B) after treatment for 48 hours with different tested solutions under static conditions. (C) Log reduction of *S. aureus* biofilm and total cells (biofilm cells plus S. aureus present in biofilm supernatant) after 48 hours treatment with various tested solutions.

Effect of Zuragen™ and Heparin on *S. aureus* Biofilm architecture

To visualize the effect of Zuragen™ and Heparin varying in concentration and preservatives, confocal images of the *S. aureus* biofilms were acquired 0 to 60 minutes and after, 6 hours, 1 day and 2 days of treatment with various lock solutions (Fig. 4). Several biofilm variables (biofilm biomass, thickness, etc.) indicative of biofilm architecture were subsequently analyzed using COMSTAT. Changes in biofilm biomass following treatment are shown in Fig. 5.

- **Treatment with Heparin**
  - initial reduction in the overall reduction in the biofilm biomass within 6 hours of treatment
  - Continued exposure with Heparin resulted in an increase in biofilm biomass
- **Treatment with Zuragen™**
  - 27-fold reduction of biofilm biomass following 1h of treatment
  - Continued exposure coincided with increasing reduction in biofilm biomass
  - 243-fold reduction in biofilm biomass following 48h of exposure

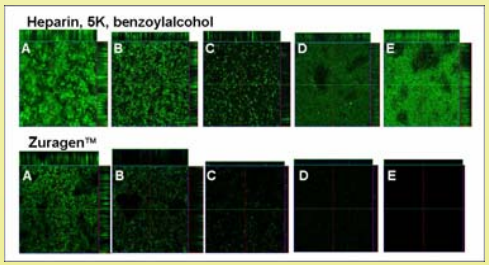

Fig. 4. Visualization of effect of treatment of *S. aureus* biofilms with 5K Heparin/benzoylalcohol and Zuragen™ over a period of 48 hours under static conditions. Confocal images were acquired following (A) 0h, (B) 1 h, 6h, (D) 24h, and (E) 48h following treatment.

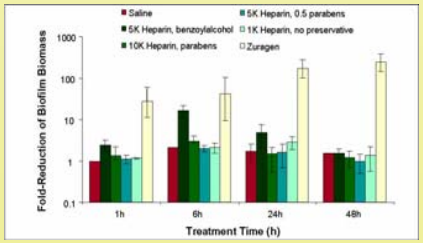

Fig. 5. Fold-reduction of *S. aureus* biofilm biomass following 48 hours of treatment with saline, Heparin (1K-10K), and Zuragen™.

Visualization of Biofilm viability following traetment for 48h

Following 48 h of treatment with various lock solutions, biofilms were stained using a Live/Dead stain to visualize killing of biofilm bacteria (Fig. 6). Bacteria stained in red are dead while bacteria stained in green are viable.

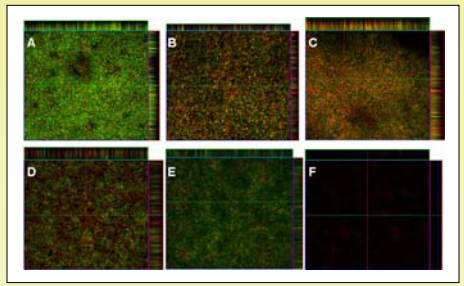

Fig. 6. Visualization of killing efficiency of various lock solutions following 48h of treatment under static conditions. Confocal images of *S. aureus* biofilms obtained after 48 hours treatment with (A) Saline, (B) 5K heparin/benzyl alcohol, (C) 10K heparin/parabens, (D) 5K heparin/0.5 x parabens, (E) 1K heparin/no preservatives, (F) Zuragen™.

Heparin treatment
- Majority of biofilm cells treated with heparin were viable (stained in green)
- Coccoid shaped cells were easily detectable

Zuragen™ treatment
- Majority (>99%) of Zuragen™-treated biofilm was stained in red (Fig. 6F).
  - effective in eradicating *S. aureus* biofilms
- No defined coccoid shaped cells were detectable (except for the few stained in green).
  - Indication of cell lysis
  - Remaining biofilm was very viscous
  - Lysed cells and DNA (binds propidium iodide, red dye)

CONCLUSIONS

Zuragen™:
- **Significantly more effective than Heparin with regard to**
  - **Eliminating bacteria from the bulk liquid**
  - **Preventing growth of biofilms**
  - **Reducing biofilm viability**

Zuragen™ treatment:
- **Results in effective elimination of *S. aureus* from the bulk liquid**
- **Prevents the growth of established *S. aureus* biofilm for several days in a flow cell bioreactor**
- **Results in significant reduction of established *S. aureus* biofilm biomass and overall biofilm structure**

LITERATURE

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