Methods for removing bacterial biofilms: *In vitro* study using clinical chronic rhinosinusitis specimens

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ABSTRACT

Background: *Bacterial biofilms may be involved in refractory chronic rhinosinusitis (CRS).* In vitro*, we studied methods for removing biofilms formed by* Staphylococcus aureus *and* Pseudomonas aeruginosa.

Methods: *Bacterial isolates were obtained from patients with refractory CRS and were plated and treated with either static administration of citric acid/zwitterionic surfactant (CAZS), saline delivered with hydrodynamic force, or CAZS delivered hydrodynamically. Results were assessed by counting colony-forming units (CFUs) and by confocal scanning laser microscopy (CSLM).*

Results: *All treatments produced significant reductions in CFU counts ($p \geq 0.002$). Hydrodynamic CAZS provided the greatest reduction, decreasing CFU counts from control values by 3.9 ± 0.3 logs and 5.2 ± 0.5 logs for* S. aureus *and* P. aeruginosa*, respectively (99.9% reduction; $p = 0.001$). CSLM showed decreases in biofilm coverage.*

Conclusion: *Hydrodynamic delivery of a soap-like surfactant and a calcium-ion sequestering agent may disrupt biofilms associated with CRS. Our results may be relevant to a new approach to refractory CRS.*

(Am J Rhinol 21, 527–532, 2007; doi: 10.2500/ajr.2007.21.3069)

Key words: Bacterial biofilm, chronic rhinosinusitis, citric acid, confocal scanning laser microscopy, hydrodynamic force, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, zwitterionic surfactant

A biofilm is a community of bacteria attached to a nonliving surface or viable tissue, with the organisms in the community contained within an extracellular polymeric substance (EPS) matrix produced by the bacteria.[1] The EPS matrix allows the biofilm to stick to the surface and also protects the embedded organisms; thus, bacteria in biofilms are ~100–1000 times more resistant to the effects of antibiotics than planktonic (suspended) bacteria.[2] Biofilms have been found on the surfaces of numerous medical devices and in a wide range of human diseases, including dental caries, periodontal disease, musculoskeletal infections, necrotizing fascitis, endocarditis, cystic fibrosis pneumonia, otitis media (OM), and chronic rhinosinusitis (CRS).[1,3–6] The most commonly observed biofilms are those formed by *Streptococcus* species, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Haemophilus influenzae*. All of these organisms are important in otorhinolaryngologic disease.[7] Recent research has provided substantial evidence that many patients with OM or CRS refractory to standard antibiotic therapy may harbor a biofilm-related infection.[3,6,8–10]

A comprehensive consensus document produced in 2004 by representatives of five professional otorhinolaryngologic societies (The American Academy of Allergy, Asthma and Immunology; The American Academy of Otolaryngic Allergy; The American Academy of Otolaryngology–Head and Neck Surgery [AAO-HNS]; The American College of Allergy, Asthma and Immunology; and the American Rhinologic Society) suggested that elimination of a biofilm in patients with CRS may require mechanical removal of the biofilm.[11] Mechanical disruption of biofilm has a long history of success in, *e.g.*, dentistry, in which it is used to remove dental plaque (a known biofilm). Such disruption may occur during functional endoscopic sinus surgery (FESS),[5] which produces symptomatic relief in ~69% to >90% of patients with CRS refractory to medical therapy.[12,13] However, in patients in whom neither medical therapy nor FESS alone is effective, a different approach to mechanical removal of a biofilm may be required.

We hypothesized that an effective biofilm-removal method should include two principal components: a nontoxic, water-soluble, low-viscosity chemical solution (surfactant) capable of breaking up the biofilm and allowing the debris to be flushed from the sinuses; and a device for delivering this solution with pressure (or shear force, similar to that provided by a Waterpik dental water jet system [Waterpik Technologies, Fort Collins, CO]). We further posited that for this technique to be effective, the solution would have to interfere with the calcium-ion bridges that produce gelling and "cross-links" in the polymeric chains in the EPS structure and thereby prevent it from dissolving. Thus, the solution should include both a sequestering agent to address the calcium-ion bridge and a surfactant molecule to bond with the unbound polymer chains and dissolve them.

Therefore, we performed preliminary studies with a wide variety of surfactants, including acetone, isopropanol, toluene, and several other agents. These investigations allowed identification of the optimal agent (a combination of citric acid and zwitterionic surfactant [CAZS; caprylyl sulfobetaine]). This agent was used subsquently for a controlled *in vitro* study of its effectiveness in eradicating *S. aureus* and *P. aeruginosa* biofilms formed by bacteria isolated from sinus samples

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Presented at the American Rhinologic Society meeting, San Diego, California, April 2007

Financial support for this research and the preparation of the article was provided by Medtronic ENT, Jacksonville, Florida; Dr. Myntti is an employee of Medtronic ENT; Dr. Derosiers is a paid consultant to Medtronic ENT

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from patients with CRS. Here, we describe the results achieved with CAZS delivered under static conditions and with use of hydrodynamic shear force.

METHODS

This study was approved by the Ethical Review Board for Human Subjects of the Centre Hospitalier de l'Université de Montréal.

Sinus Sample Collection and Initial Processing

Bacterial isolates were recovered from the sinuses of a consecutive series of patients with sinus disorders. Patients with cystic fibrosis or an underlying immunosuppressive disease (human immunodeficiency virus [HIV] infection, insulin-dependent diabetes mellitus, or renal disease) and patients who had taken antibiotics or oral prednisone in the previous month were excluded from the study. All patients had refractory sinusitis, *i.e.*, persistent symptoms resistant to medical therapy despite having undergone technically successful FESS for CRS (with or without nasal polyposis) diagnosed in accordance with the 2003 AAO-HNS guidelines[14] and refractory to medical therapy for >12 months before sample collection. In all patients enrolled in the study, the failure of FESS was judged not to be associated with technical factors such as obstructive synechiae, frontal sinus obstruction, or a retained uncinate process. Samples were collected consecutively until 10 specimens each of *S. aureus* and *P. aeruginosa* were obtained.

All samples were obtained under direct endoscopic guidance, using the procedure described by Nadel *et al.*[15] Briefly, a topical anesthetic agent was administered, the nasal ala was retracted, and an endoscope was used to visualize the middle meatus and sinus cavities. A thin, flexible calcium alginate swab (Starwab Collection and Transport System; Starplex Scientific, Etobicoke, Ontario, Canada) was inserted and directed to the site with the most purulence. If no purulence was observed, the surface of the maxillary sinus was swabbed for 15 seconds. Care was taken to avoid contact with the lateral nasal wall or nasal vestibule.

Samples were plated and incubated using standard procedures. Bacteria were identified with the Vitek 2 system (Biomérieux, Durham, NC). Crystal violet staining to confirm the presence of biofilms was performed according to the method described by Stepanovic *et al.*[16] This method was previously used to assess biofilms in sinus isolates and provided consistent results.[17] For incubation and culture, previously frozen strains were inoculated on trypticase soy agar (TSA) with 0.5% sheep blood. After 24 hours, one to four colonies per strain were cultured on TSA. Cultures were incubated at 37°C for 24 hours to condition them to the trypticase soy broth (TSB)–TSA medium and ensure noncontamination. Colonies grown on TSA solid medium were then amplified in 5 mL of TSB medium with 0.5% glucose[18] and incubated at 37°C for 24 hours. For the biofilm-removal experiments, previously frozen strains of *S. aureus* and *P. aeruginosa* were inoculated on TSA in glass tubes and transported by overnight courier to the Medical Biofilm Laboratory, Center for Biofilm Engineering, Montana State University (Bozeman, MT).

Laboratory Model System

An *in vitro* system, the drip-flow reactor (DFR) containing microscope slides (designated "coupon" in Fig. 1), was used to determine the effectiveness of the test solutions, delivered with and without hydrodynamic force, in removing *S. aureus* and *P. aeruginosa* biofilms. The DFR models a low shear environment. In this study, bacteria were inoculated on hydroxyapatite (HA)-coated glass slides because previous investigations showed that biofilms are better retained on HA slides than on other surfaces during processing for microscopical analysis.

The DFR experiments were conducted in an incubator at 37°C and under aerobic conditions. Approximately 20 minutes before bacterial inoculation, sterile medium (10% TSB for *S. aureus*; 1% TSB for *P. aeruginosa*) was dripped on the slides in the DFR and allowed to collect over the slides to form a conditioning layer. The slides were then inoculated with 1 mL of a culture of either *S. aureus* or *P. aeruginosa*. The DFR was placed in a horizontal position for 4 hours to allow bacterial attachment to the substrate. Subsequently, the device was placed at a 10° angle, with sterile medium dripping on the slides at a rate of 10 mL/hour. After 3 days, the biofilm-removal experiments were performed.

Biofilm-Removal Experiments

Three methods were used to treat the biofilms formed by each bacterial species. The first involved applying a solution of 0.02 M of CAZS (constituent chemicals from Sigma Aldrich, St. Louis, MO) on slides in the DFR (static treatment). The other two treatments were delivery of 0.09% saline and delivery of 0.02 M of CAZS with use of hydrodynamic shearing force. For all treatments, preliminary runs were done to ensure that variations among slides were within acceptable limits. In addition, multiple plates of both bacterial species were produced to determine the within-run and run-to-run variations. A control slide was made for each DFR run. Subsequently, the experiments included three runs for each treatment of each type of bacteria.

For the static treatments, flow to the DFR was halted, the device was placed in a horizontal position, and the cover was removed. Either 25 mL of 0.02 M of CAZS (active treatment) or 25 mL of 0.9% saline was applied to one slide. Control

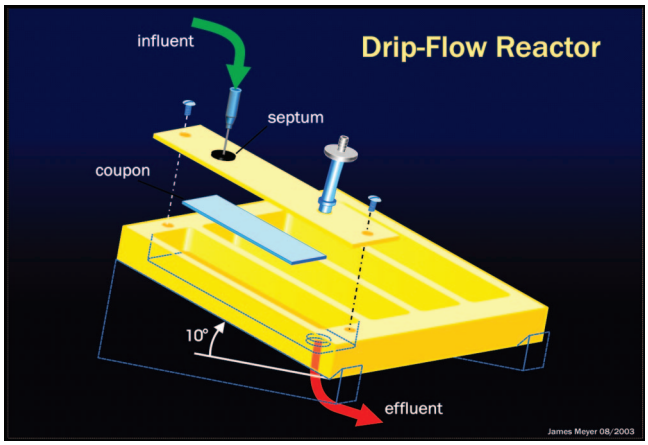

Figure 1. *DFR used in biofilm-removal experiments.*

slides were not treated with either saline or CAZS. After 10 minutes, the slides were rinsed with saline (25 mL). The DFR was then disconnected from the inflow tube, and each slide was removed under a laminar flow hood and placed in a sterile 50-mL tube. After another saline rinse (2 mL), the surface of the slide was scraped repeatedly, and the scrapings and saline were collected in the tube. The tube was then vortexed for 10 seconds, sonicated for 2 minutes, and vortexed again for 10 seconds to disperse the bacteria into suspension.

Next, the suspensions were serially diluted and 100-μL aliquots were applied to three plates containing TSA. After incubation at 37°C for 24 hours, colony-forming units (CFUs) were counted manually, and the number of CFUs per square centimeter was calculated. The resulting plate counts were log (10) transformed and expressed as the mean (±SD) value derived from plate counts from two DFR runs of three slides each.

For the hydrodynamic treatments, the slides were removed from the DFR and placed in a glove box. The slides were placed in a holder and sprayed for ~20 seconds with either saline or 0.02 M of CAZS (~150 mL of fluid) by using a device that provided pressurized jet lavage. Applied pressure was 31.4 psi with a flow rate of 5.5 mL/s, delivered *via* a nozzle with a 0.03-in. diameter. The resulting force on the tissue from the spray was $10 \times g$, applied over a 0.03-in. diameter (or 0.000706 in.^{2}), remaining constant at distances from 0.1 inch to 1 inch. The spraying was done with both a side-to-side and an up-and-down sweeping motion so that all areas were sprayed twice, once in each axis. Then, the slides were placed in sterile 50-mL tubes and scraped as described previously. The scrapings were processed and plate counts were obtained as described previously.

Confocal Scanning Laser Microscopy (CSLM)

CSLM was performed on three slides (for each treatment and bacteria species) not subjected to plate counts to allow imaging of the biofilm architecture in control and treated samples. The CSLM method has been found to be especially effective in assessing bacterial biofilms, including those observed in patients with otorhinolaryngologic diseases such as OM and CRS.[3,19] Staining of slides for CSLM was done with the BacLight Live/Dead kit (Molecular Probes, Invitrogen, Carlsbad, CA). This kit consists of two nucleic acid stains: SYTO 9, which detects living cells by fluorescing green, and propidium iodide, which detects dead cells by fluorescing red.

After staining, the slides were examined by using CSLM (magnification, ×630; Leica acoustic-optical beam splitter SP2 with a 2-photon Mai Tai attachment; Leica Microsystems, Bannockburn, IL) with fluorescence excitation and detection in both the green and the red spectra. Each slide area was divided into 10 equally sized segments. A microscopical field was selected at random from each segment, and images were obtained at 1-μm intervals from the top of the biofilm to the substrate, thereby creating an image stack for each location.

Statistical Analysis

The mean (±SD) percentage of reduction from control values in the quantity of *S. aureus* and *P. aeruginosa* bacteria (*i.e.*, number of CFUs on plates) after each treatment was calculated. Results were assessed by using two-sample *t*-tests (Minitab version 14; Minitab, State College, PA). A value of $p < 0.05$ was considered a significant difference from the control value.

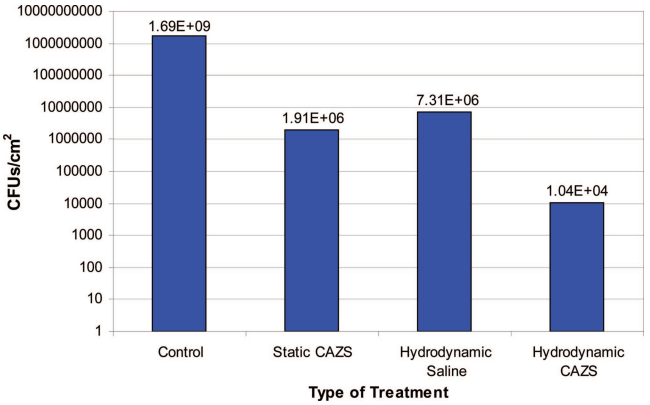

Figure 2. *Log and CFUs per squared centimeter representations of mean plate counts for samples of* S. aureus *for controls and after treatment with static 0.02 M of CAZS, saline delivered with hydrodynamic force (hydrodynamic saline), and 0.02 M of CAZS solution delivered with hydrodynamic force (hydrodynamic CAZS).*

RESULTS

Plate Counts

Figures 2 and 3 and Table 1 show quantitative results from the biofilm-removal experiments. Before treatment, the biofilms formed in the DFR cultures of both *S. aureus* and *P. aeruginosa* from patients with CRS were extensive, with CFU counts for these controls ranging from 7.8 to 9.5 log/cm^2. For both bacterial species, all three treatment methods significantly reduced the quantity of CFUs from control values. Static administration of CAZS resulted in a 2.5-log reduction (5.11×10^8 to 1.65×10^6; $p = 0.001$) in the number of *S. aureus*

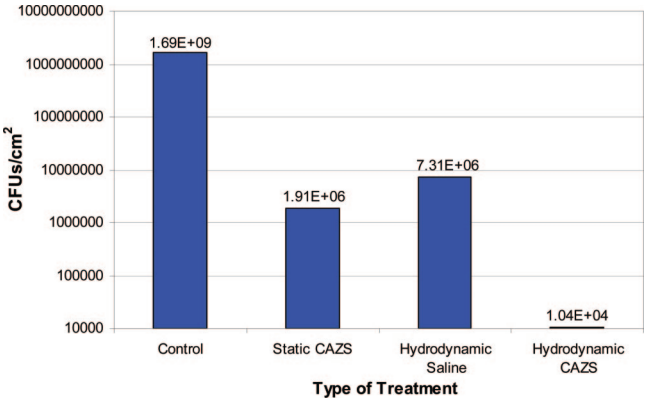

Figure 3. *Log and CFUs per squared centimeter representations of mean plate counts for samples of* P. aeruginosa *biofilms for controls and after treatment with static 0.02 M of CAZS, saline delivered with hydrodynamic force (hydrodynamic saline), and 0.02 M of CAZS solution delivered with hydrodynamic force (hydrodynamic CAZS).*

Table 1 **Bacterial plate counts according to type of treatment**

Treatment	Type of bacteria*	
	S. aureus	*P. aeruginosa*
None (no saline)	8.7 ± 0.4	9.2 ± 0.2
Static delivery of CAZS	6.2 ± 0.3	6.3 ± 1.3
Hydrodynamic delivery of saline	6.4 ± 0.2	6.9 ± 0.1
Hydrodynamic delivery of CAZS	4.8 ± 0.3	4.0 ± 0.5

**Values are mean (±SD) numbers of CFUs per centimeter (log) derived from three plates assessed twice.*

CFUs and a 2.9-log reduction (1.69×10^9 to 1.91×10^6; $p = 0.002$) in the number of *P. aeruginosa* CFUs. Mechanical disruption alone (hydrodynamic saline–only treatment) decreased the number of *S. aureus* CFUs by 2.3 logs (5.11×10^8 to 2.38×10^6; $p = 0.001$) and the number of *P. aeruginosa* CFUs by 2.4 logs (1.69×10^9 to 7.31×10^6; $p = 0.001$). Clearly, however, the delivery of CAZS with hydrodynamic force had the greatest effect on both bacterial species, decreasing the *S. aureus* CFU count by 3.9 logs (5.11×10^8 to 6.37×10^4; $p = 0.001$) and the *P. aeruginosa* CFU count by 5.2 logs (1.69×10^9 to 1.04×10^4; $p = 0.001$)

CSLM Imaging

Figure 4 shows representative CSLM images of control and treated *S. aureus* biofilms. The control (Fig. 4 *A*) shows a thick biofilm carpeting the slides. Hydrodynamic treatment with saline and static treatment with CAZS (Figs. 4, *B* and *C*) decreased the amount of biofilm coverage markedly and reduced the organization of the remaining biofilm. Hydrodynamic treatment with CAZS (Fig. 4 *D*) produced a greater reduction both in biofilm coverage and in the amount of order in the biofilm community. These results reflect those of the plate count assessments with respect to the relative reductions in the amount of biofilm achieved with each treatment.

DISCUSSION

This *in vitro* study assessed the effect of three biofilm-removal treatments on *S. aureus* and *P. aeruginosa* biofilms that formed in specimens of purulence obtained from patients

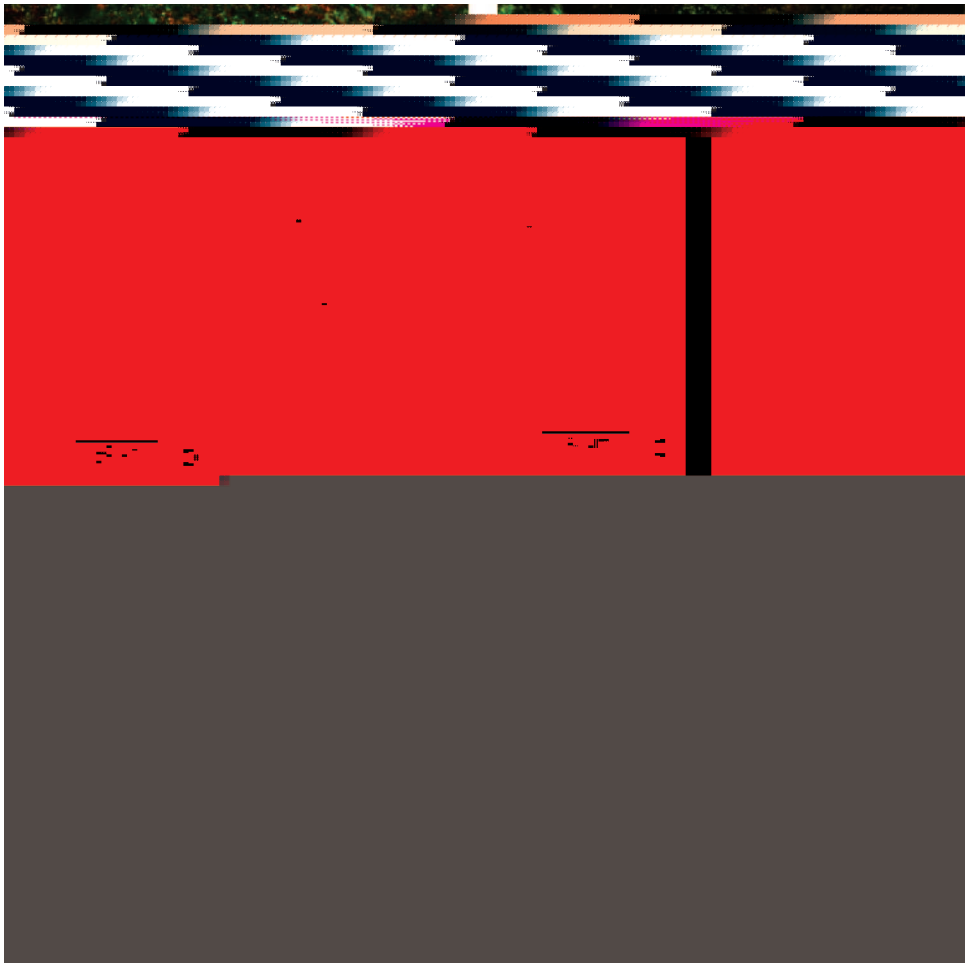

Figure 4. *Representative CSLM images showing* S. aureus *biofilm (A) in a control sample from the DFR and (B) in samples obtained from the DFR after hydrodynamic administration of saline alone, (C) static CAZS treatment, and (D) hydrodynamic CAZS treatment.*

with CRS refractory to both medical therapy and FESS. The DFR used in these experiments allowed growth of the bacteria of interest and administration of the biofilm-removal treatments in the same device. Of the three treatments investigated, the one using delivery of CAZS by means of pressurized jet lavage was the most effective in breaking up biofilms. Although some bacteria remained after this treatment, large, statistically significant reductions occurred, with the mean decreases in bacterial plate counts being 3.9 and 5.2 log (a reduction of 10,000–100,000 times), respectively, for the *S. aureus* and *P. aeruginosa* biofilms. We suggest that a decrease of this magnitude *in vitro* may translate into a similar reduction *in vivo*, with a potentially important clinical impact on biofilms in patients with CRS.

Our findings are in accordance with those of Anglen *et al.*,[20] who investigated methods for removing *S. epidermidis* biofilms from orthopedic stainless steel screws *in vitro*. Their study, which also reported CFU counts as a means of assessing bacterial reduction, showed that compared with bulb-syringe irrigation, power irrigation increased the removal of bacteria by a factor of at least 100, regardless of the type of solution used. The effect of mechanical irrigation on the bacteria was 100-fold that was achieved with neomycin alone and 285-fold that was obtained with polymyxin alone. Adding liquid Castile soap to the irrigation solution as a surfactant dramatically increased the quantity of bacteria removed, whereas adding antibiotics produced results not significantly different from those achieved with saline alone.

In the study reported here, we also found that power irrigation had considerable biofilm-reducing effects. However, we additionally showed that the presence of a surfactant and citric acid in the irrigation solution significantly enhanced the reduction in CFU counts in both *P. aeruginosa* and *Staphylococcus* species biofilms.

The observation in both of these studies that a substance other than an antibiotic was effective in removing biofilms is especially intriguing with respect to possible clinical implications. Currently, orally and topically administered antibiotics are used extensively and, in many cases, unsuccessfully, to treat CRS. This may be because of a reduced effectiveness of antibiotics on bacterial biofilms. In support of this idea, *in vitro* studies have shown that topical application of even high concentration of antibiotics have only a limited effect on bacterial viability in bacterial biofilms from sinus isolates.[21] However, the use of even a topical antibiotic is not without risk because of the possibility that a patient will have an allergic reaction to the agent or a resistant antibiotic strain will develop. Antibiotics also are relatively expensive. In contrast, Castile soap and CAZS are inexpensive and nontoxic. The CAZS solution used in our study was a blend of deionized water and citric acid buffered to 5.4 pH with sodium citrate and containing a low percentage of zwitterionic surfactant. The role of the citric acid is to break the calcium-ion bridges that serve as chemical binding sites connecting the EPS polymeric chains. The surfactant brings the disconnected chains into solution. Because of the relatively low pH of the CAZS solution and the known relatively low toxicity of its constituent chemicals, we suggest that it is unlikely that the solution would adversely affect living tissue exposed to it.

Our study was an *in vitro* investigation, but its results support the idea that delivery of a simple, soap-like surfactant with hydrodynamic force can disrupt biofilms formed by bacteria commonly observed in otorhinolaryngologic disorders, including CRS. In the light of the growing evidence of the presence of bacterial biofilms in patients with refractory CRS,[4–6,8–10] we believe that studies of hydrodynamic treatment of biofilms in living tissue and animal models of CRS are warranted. Such research may provide data that could aid development of a new treatment for CRS that would benefit the many patients in whom the best available medical and surgical therapy has failed to produce a cure.

ACKNOWLEDGMENTS

The authors thank Renée J. Robillard for writing and editorial assistance.

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