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PRELIMINARY COMMUNICATION

Mucosal Biofilm Formation on Middle-Ear Mucosa in the Chinchilla Model of Otitis Media

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Context Chronic otitis media with effusion (OME) has long been considered to be a sterile inflammatory process. The previous application of molecular diagnostic technologies to OME suggests that viable bacteria are present in complex communities known as mucosal biofilms; however, direct imaging evidence of mucosal biofilms associated with OM is lacking.

Objective To determine whether biofilm formation occurs in middle-ear mucosa in an experimental model of otitis media.

Design and Materials A total of 48 research-grade, young adult chinchillas weighing 500 g were used for 2 series of animal experiments: one to obtain specimens for scanning electron microscopy and the other to obtain specimens for confocal laser scanning microscopy using vital dyes. In each series, 21 animals were bilaterally injected with viable *Haemophilus influenzae* bacteria and 1 was inoculated to account for expected mortality. Three served as negative controls. Effusions and mucosal specimens were collected from 2 infected animals that were euthanized at 3, 6, 12, and 24 hours and at days 2, 4, 5, 10, 16, and 22 after inoculation.

Main Outcome Measures Images were analyzed for biofilm morphology, including presence of microcolony formation and for presence of bacteria on tissue surfaces.

Results Scanning electron microscopy demonstrated that biofilm formation was evident in all specimens from animals beginning 1 day after infection and was present through 21 days. Confocal laser scanning microscopy indicated that bacteria within the biofilms are viable.

Conclusion These preliminary findings provide evidence that mucosal biofilms form in an experimental model of otitis media and suggest that biofilm formation may be an important factor in the pathogenesis of chronic otitis media with effusion.

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OTITIS MEDIA (OM) IS THE most common reason for an ill child to visit a physician or other health care professional and is the most common reason for a child in the United States to receive antibiotics or undergo a general anesthetic.[1] The underlying pathophysiology of OM is poorly understood although it is clear that OM results from an interplay of infectious, environmental, and host genetics factors.[2,3] Although most effusions from acute OM are culture-positive for bacteria (predominantly *Haemophilus influenzae, Streptococcus pneumoniae,* and *Moraxella catarrhalis*), the majority of chronic effusions are culturalnegative, refractory to antibiotic treatment, and positive for a variety of inflammatory mediators.[4-6] These observations led to the concept that chronic OM effusion (OME) is not a bacterial process but rather represents a sterile, inflammatory process directed against residual bacterial metabolites.[7]

However, several recent observations cannot be explained by a sterile, inflammatory process. These include that (1) the majority of pediatric sterile effusions contain bacterial DNA[7]; (2) in the chinchilla model of OM, pasteurized bacteria and purified bacterial DNA are cleared within hours from the middle-ear space while the DNA from live infectious bacteria persists in sterile effusions for up to 4 weeks after antibiotic treatment[8]; (3) bacterial mRNA is present in culturally sterile, DNA-positive pediatric effusions, demonstrating that the bacteria are intact and metabolically active[9]; and

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(4) bacteria synthesize proteins in sterile effusions.[10]

In an attempt to reconcile these disparate observations, we previously advanced the mucosal biofilm hypothesis of chronic OME,[11] which suggests that chronic OME is the result of a bacterial biofilm forming directly on the mucosal surface of the middle ear. Bacteria growing as a biofilm display a different phenotype than free-living bacteria; they have greatly reduced metabolic rates that render them nearly impervious to antimicrobial treatment[12]; they have an exopolysaccharide matrix that provides protection from phagocytosis and other host defense mechanisms; they demonstrate reliance on complex intercellular communication systems that provide for organized growth characteristics[13]; and they are recalcitrant to standard culture techniques because of altered metabolism.

The reduced metabolic and divisional rates of biofilm bacteria largely explain the failure of antibiotics to eliminate infections in patients who have biofilm-colonized indwelling medical devices, primarily because nondividing bacteria largely escape antibiotic killing.[14] Antibiotic treatment of biofilms kills bacteria on the periphery, but deep organisms persist and act as a nidus for regrowth and periodic planktonic showers, that can result in systemic infection.

Although our previous studies have provided circumstantial evidence that supports the biofilm concept in OM, only through imaging is it possible to demonstrate unequivocally biofilm formation. In this investigation, we sought to determine whether bacteria are growing as a biofilm on the middle-ear mucosa in an experimental model of OM.

METHODS

Animal Model

Research-grade, young adult chinchillas (R and R Chinchilla Ranch, Jenera, Ohio) weighing approximately 500 g were placed under general anesthesia on day 0 using intramuscular administration of 0.1 mL of a mixture consisting of ketamine, 20 mg/mL; xylazine, 20 mg/mL; and acepromazine, 1 mg/mL. After a suitable level of anesthesia (determined by the abolishment of the eye-blink reflex) was obtained, 0.1 mL of an inoculum containing 10^4 colony-forming units per milliliter of viable *H influenzae* bacteria were injected bilaterally via a transbullar approach using a tuberculin syringe with a 0.5-inch 27-gauge needle. The strain of *H influenzae* chosen, 1128, was a low-passage clinical isolate obtained from the middle ear of a child with otitis media. This strain was previously determined to be sensitive to ampicillin under planktonic growth conditions.[15] At 72 hours after inoculation, all animals were treated with ampicillin, 150 mg/kg twice daily for a maximum of 4 days, to avoid the complications of systemic infections and to render the middle-ear effusion culturally sterile.[5,6,8-10,16] This dosage regimen has been consistently demonstrated to render all *H influenzae* culture-negative in the chinchilla model of OM.[8,15]

Two series of animal experiments were performed: one to obtain specimens for scanning electron microscopy (SEM); the other to obtain specimens for confocal laser scanning microscopy (CLSM). For each series of experiments a cohort of 21 animals was inoculated at time 0 and another 3 animals served as negative controls. Two infected animals at each time point (3, 6, 12, and 24 hours and 2, 4, 5, 10, 16, and 22 days) following inoculation were killed for specimen collection. One additional animal for each set of experiments was inoculated to account for expected mortality, for a total of 48 chinchillas in these studies.

Specimen Collection

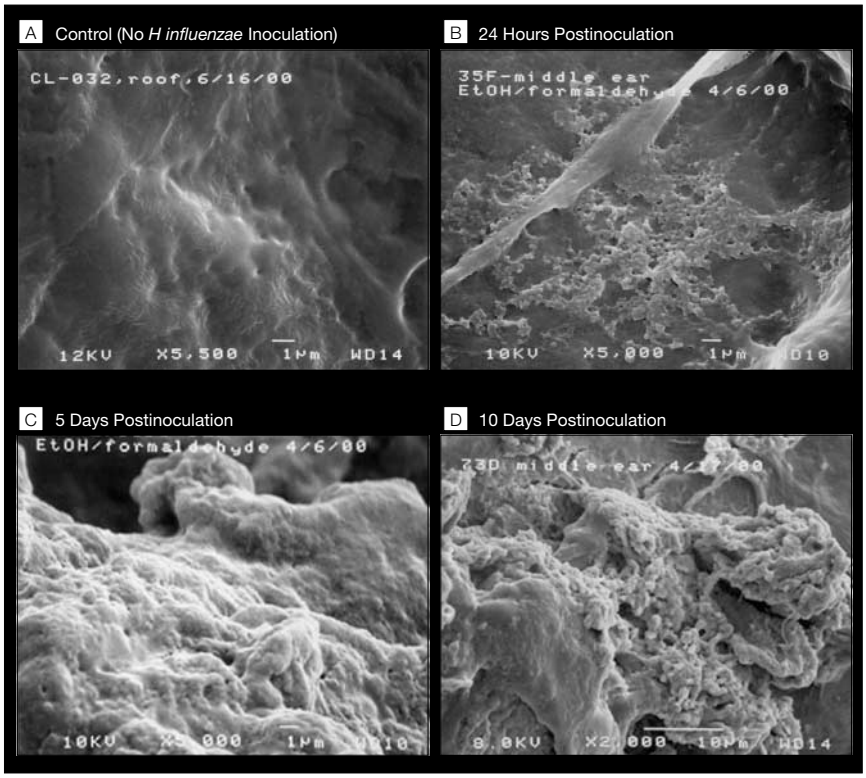
Bilateral middle-ear effusions (if present) and mucosal specimens were collected from all animals in each cohort at the time of death. Animals were placed under a deep general anesthesia by the intramuscular administration of 0.2 mL of the above anesthetic mixture and then killed with a 2-mL intracardiac mixture of potassium chloride. The tympanic bullae, the bony structure that encompasses the middle-ear mucosa, were harvested by carrying a midline vertical incision down to bone and exposing the dorsal surface of the skull. For the SEM series representative mucosal specimens were sharply dissected and either frozen or fixed in formalin and sent for imaging to the Center for Biofilm Engineering (Bozeman, Mont). For the CLSM series, the bullae were exposed and removed en bloc. The bullae were then split longitudinally into dorsal and ventral halves and placed in transport medium and shipped via overnight carrier to the imaging center.

SEM Imaging

Excised chinchilla bullae were either fixed in formaldehyde (4% in phosphate-buffered saline) or immediately frozen in liquid nitrogen. Samples of the formaldehyde-fixed bullae were dehydrated in aqueous mixtures of 25%, 50%, 75%, and 95% ethanol for 20 minutes, each at room temperature; affixed to aluminum stubs using carbon tape and/or colloidal carbon; and they were coated with 15 to 20 nm of gold and palladium using a Hummer VII Sputtering System (Anatech Ltd, Alexandria, Va) prior to imaging.

Samples of frozen bullae were maintained in a frozen state throughout the SEM imaging process. In a cold room (–20°C) samples were affixed to a beveled brass coupon (≈13 mm × 28 mm × 3 mm thickness) using Tissue-Tek O.C.T. compound (Sakura Finetek USA Inc, Torrance, Calif). Liquid carbon was then applied to 2 to 3 points along the edges of the sample to provide a conductive bridge between sample and coupon. The brass coupons with mounted samples were then attached to a threaded rod and dipped in liquid nitrogen. After approximately 2 minutes, the coupon and samples were quickly removed from the liquid nitrogen and, via the rod-and-cap assembly, introduced to the first of 2 dovetailed cryostages for coating. The

Figure 1. Scanning Electron Microscopy of the Chinchilla Middle-Ear Mucosa Following Formaldehyde Fixation and Dehydration



A, Control at ×5500 magnification. This specimen was obtained at time 0 in an animal that was not inoculated with *Haemophilus influenzae*; B, *H influenzae* microcolonies on middle-ear mucosa 24 hours after inoculation at ×5000; C, mature *H influenzae* biofilm on middle-ear mucosa 5 days after inoculation at ×5000; D, mature biofilm 10 days after inoculation at ×2000. Scale indicated by the 1- or 10-µm bars.

cryostage was first adjusted to a temperature of approximately –80°C for 3 to 5 minutes under high vacuum ($<10^{-2}$ millbars) to sublimate any surface water. The cryostage temperature was then decreased to lower than –140°C and the chamber was back-filled with argon to a pressure of between 0.1 and 0.2 millibar. Samples were gold coated to a thickness of 10 to 20 nm. The argon gas was then shut off, and the pressure was again allowed to equilibrate to lower than 10^{-2} millibars. All of the above processes were conducted using an Oxford CT1500 Cryostation System (Oxford Instruments, Oxford, England).

The sample-bearing coupon was then introduced into the second dovetailed cryostage within the SEM chamber for imaging using a JEOL JSM-6100 scanning microscope. All images were collected at an accelerator voltage of ~12.0 kV, a filament current of ~3.2 A, a working distance of ~13 to 14 mm, and recorded using Polaroid Type 665 (pos/neg B&W Instant Pack, ISO 80/20) film. All images were digitized as high-resolution TIFF computer files (resolution 635 dpi) with a personal computer platform and Hewlett-Packard Scan Jet 4c (Boise, Idaho) and then converted to high-quality JPEG files using Photoshop 5.0 software (Adobe Systems, San Jose, Calif).

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy was used in conjunction with differential staining to assess the presence of bacteria on bullae tissue surfaces. Mucosal specimens were fluorescently stained using the Live/Dead BacLight bacterial viability kit (Molecular Probes, Eugene, Ore) that uses the 2 dyes: SYTO 9 (green) and propidium iodide (red). This 2-component stain is based on differential cell membrane permeability of 1 component compared with the other. Green indicates uncompromised bacterial cell membranes (ie, live cells), whereas red indicates dead bacterial cells and is also taken up by the host cell nuclei.

Because cell fixation using aldehydes or other chemical treatments alters bacterial cell membranes, we stained only frozen samples based on our previous findings that freezing of bacterial cells does not materially affect viability[15] nor significantly alter bacterial membrane permeability. An approximate 0.5-mL tissue sample was immersed in 1 mL of phosphate-buffered saline. A 1.5-µL aliquot of both components A and B was then added to the immersed tissue, mixed thoroughly, and incubated at room temperature in the dark for 15 minutes. The tissue specimen was then rinsed sequentially in 1 mL of phosphate-buffered saline and 1 mL of sterile deionized water prior to mounting on a slide for imaging.

Imaging was performed using a Leica DM RXE microscope with a Leica TCS NT confocal scanning laser system and laser capabilities at 488 (primary), 568, and 633 nm. Images were collected using the Leica TCS NT software (Leica Camera AG, Solms, Germany), primarily using a Leica PL APO 63×/1.20 W CORR lens (∞/0.14-0.18/0). Red-and-green image stacks were combined into single TIFF and/or JPEG images using Metamorph software (Version 4.6r6, Universal Imaging Corp, Downingtown, Pa).

Image Analysis and Interpretation

Images were analyzed for characteristic biofilm morphology, including presence of microcolony formation, elaboration of extruded exopolysaccharide, and tower formation. No attempt was made to quantify biofilm formation. Image analyses were performed on representative specimens from each time point by 4 of the authors (G.D.E., R.V., J.W.C., and J.C.P.). Interpretations were

made independently and consensus of all observers was required.

RESULTS

Scanning Electron Microscopy

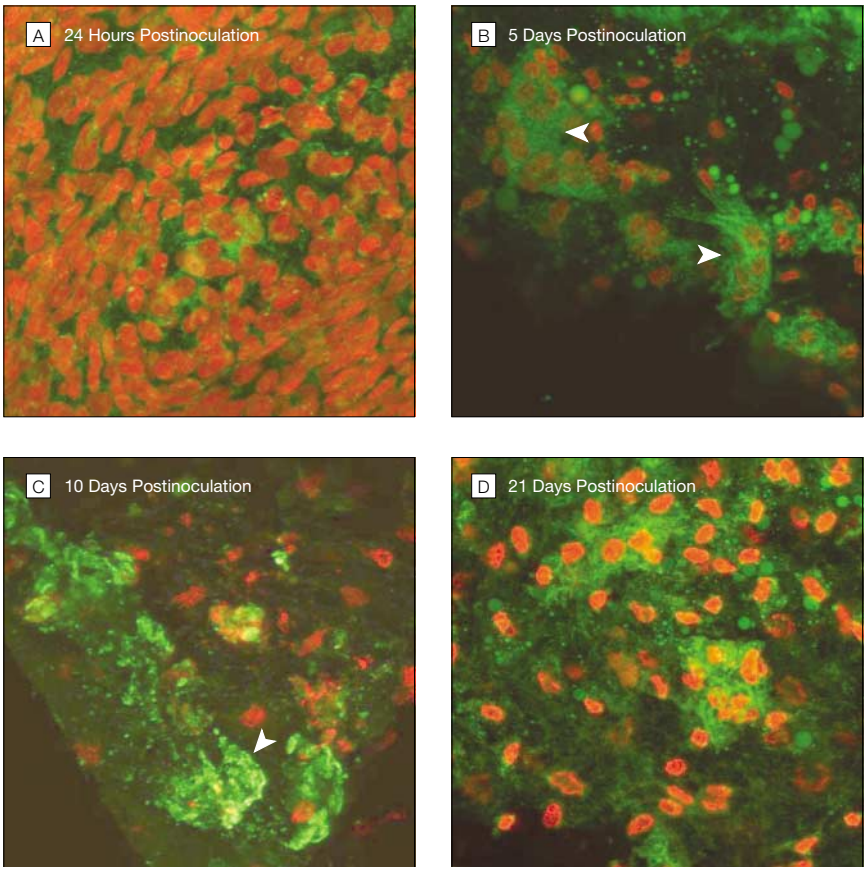
Scanning of representative specimens during microscopy demonstrated that biofilm formation was evident in all specimens from animals that had developed effusions (about 50%). In addition, some of the specimens from inoculated animals that did not evidence frank effusions (about 50%) also contained biofilms. Biofilm formation was not uniform over the entire mucosal surface but rather was present in patches.

Representative images of control mucosa and biofilms are presented in FIGURE 1. The 0 time image (Figure 1A) demonstrates a normal middle-ear mucosal surface. Similar normal-appearing mucosa were also observed interspersed with biofilm-covered regions in animals after infection indicating that biofilm formation is patchy within the middle ear. Regions of normal appearing mucosa were observed at all time points examined after infection.

The image taken at 24 hours after infection (Figure 1B) shows microcolony formation, which is characteristic of early biofilm development wherein the bacteria have attached to the mucosal surface but have not yet propagated into tower-type structures consisting of many layers of bacteria. The bacteria do not completely cover the surface but rather concentrate in certain areas and leave other areas bare even within the emergent biofilm area.

The image at 5 days (Figure 1C) is representative of a mature biofilm. Although the mucosal surface within the field of view is completely occluded by the biofilm the characteristic water channels are evident near the top of the image. At 10 days, the biofilm appeared similar to what it looked like at day 5, suggesting that the biofilm is in a maintenance phase at this point. In all of these images the biofilm matrix is seen collapsed down on top of the bacteria, an artifact of fixation and dehydration.

Figure 2. Confocal Laser Scanning Micrographs of the Chinchilla Middle-Ear Mucosa Following Transbullar Inoculation With *Haemophilus Influenzae*



Unfixed specimens were obtained under general anesthesia, placed in buffer, and shipped via overnight courier from the Center for Genomic Sciences to the Center for Bioengineering for imaging. Specimens were stained using the Live/Dead BacLight bacterial viability kit (Molecular Probes, Eugene, Ore), which uses the 2 dyes, SYTO 9 (green) and propidium iodide (red). Green indicates uncompromised bacterial cell membranes (ie, live cells); red indicates dead cells and is also taken up by the host cell nuclei. Panel A shows an early-stage biofilm, panels B and C show mature biofilms with characteristic tower structure (arrowheads); and panel D shows a biofilm at 21 days after inoculation. Scale: nuclei are approximately 5 µm in diameter; mucosal cells, approximately 15 µm in diameter.

Confocal Laser Scanning Microscopy

FIGURE 2 shows 4 images of the middle ear mucosa that demonstrate biofilm formation. Early stage biofilm formation is present 24 hours after infection (Figure 2A). Bacterial biofilm thickness is increased at days 5 and 10 after infection (Figure 2B, C). The biofilm thickness is manifest by a decreased ability to visualize the host mucosal nuclei concomitant with an increase in the SYTO-9 signal associated with biofilm tower structures. Both of these images display characteristic biofilm towers separated by areas of bacterial effacement. At 21 days, the biofilm is not as robust as it had been (Figure 2D).

COMMENT

A biofilm is a complex organization of bacteria that are anchored to a surface, via a bacterially extruded exopolysaccharide matrix and grow into differentiated towers that can be several hundred bacteria in height.[17] The extruded exopolysaccharide matrix, which comprises more than 90% of the biofilm, envelopes the bacteria and provides protection from phagocytosis and

oxidative burst mechanisms, both in natural environments and by the host.[18] Bacteria within biofilms are also resistant to the host's humoral defense systems because of a lack of accessibility by immunoglobulin and complement.[19]

The mucosal biofilm observed in the chinchilla model of OM in this study is consistent with the biofilm theory initially developed by microbial ecologists studying bacteria growing in natural habitats[20] and then shown to be important in prosthetic infections,[21] dental plaque,[22] and associated periodontal disease.[23] In addition, biofilm-like pseudomonal aggregates[24] are found in the lungs of patients with cystic fibrosis (CF); the tissue damage associated with CF results from host-derived oxidative bursts aimed at the bacteria that are protected by the matrix.[25,26] The findings of our study provide evidence that extends the biofilm concept of chronic bacterial infections to OM and provides a theoretical framework for understanding the interactions of bacteria and mucosal surfaces.

This study has several limitations, including our use of historical data regarding the culturability and DNA positivity of antibiotic-treated middle-ear effusions. We have previously demonstrated in the chinchilla model of middle ear infection that treatment of the animals with ampicillin doses lower than those used in the current study rendered the effusions culture-negative without eliminating DNA positivity.[8,15] However, in the current study, culture and polymerase chain reaction were performed on only a subset of the specimens used in the imaging studies. Thus, although it is likely, it is not possible to determine with certainty that all of the effusions had a culture-negative and polymerase chain reaction–positive result. Another potential concern is that image interpretation with generic dye systems, as used in this study, is a subjective process. Therefore, species-specific fluorescent immunostaining or genome staining would provide additional support for the preliminary results presented herein. Finally, extrapolating any findings from animal models to humans must be done with caution. Thus, future studies using endoscopic confocal laser scanning microscopy are necessary to demonstrate definitively that biofilm formation is associated with middle ear disease in humans.

The multiple microenvironments within a biofilm vary greatly with respect to oxygen tension, pH level, and nutrient availability.[27] These multiple ecological niches provide for complex biofilms wherein a variety of species can exist mutualistically.[28,29] Adaptation within these diverse niches results in bacteria that display widely varying rates of metabolism and replication,[30] such that bacteria in the core of the biofilm are essentially nondividing, thus there is no single metabolic state that is typical of a biofilm.[31] This metabolic continuum is one of the factors that renders biofilms, as a whole, so resistant to environmental stresses. The greatly reduced rates of replication of bacteria deep within the biofilm also renders the bacterial population as a whole impervious to antibiotic treatment although organisms on the periphery may be susceptible to antibiotic killing.

Biofilm bacteria display a set of markedly different phenotypes compared with the nearly uniform phenotype of planktonic (ie, free floating) bacteria of the same species.[32] One major aspect of this phenotypic difference is a complex interbacterial communication system known as quorum sensing[33,34]; whereby the individual bacteria modify their behavior in response to increased cellular density.[35] Moreover, with the elaboration of the mucosal biofilm paradigm has come the realization that bacteria actually display an entire continuum of growth modes that permit communal growth and survival under diverse environmental conditions.[36] In addition, bacterial biofilms tolerate a much wider range of environmental variation than their planktonic counterparts. This reduced vulnerability to environmental conditions makes the biofilm the optimal phenotype for long-term growth; thus, it is not surprising that quantitative enumeration experiments in natural habitats have demonstrated that up to 99.9% of bacteria exist in the biofilm envirovar.[37] The biofilm phenotypes result from differential gene expression patterns,[38] and the comprehensive analyses of bacterial expressomes and proteomes from various envirovars of a given bacteria is resulting in the discovery of new genes with new functions.[39-41] In infectious disease, the maintenance of a mucosal biofilm provides a selective advantage to the bacteria for survival and propagation at the expense of rapid growth.

The mucosal biofilm concept may help to resolve several questions about the pathogenesis of OME, including (1) why culture results are negative when Gram stain, endotoxin and neuraminidase assays, as well as molecular diagnostics demonstrate the presence of bacteria in sterile effusions, (2) when bacteria are present, why antibiotics fail to effect a clinical cure, and (3) when there are no viable bacteria, how an effusion is maintained for a long time. The mucosal biofilm paradigm of middle-ear disease is a theoretical model that provides a consilience among these conflicting observations. Biofilm bacteria are difficult to culture planktonically, are recalcitrant to antibiotic treatment, and are the preferential bacterial phenotype for indolent, long-term persistence. Furthermore, biofilms in the nasopharynx or other aspects of the upper aerodigestive tract may give rise to recurrent exacerbations of acute OM. Clinical studies have demonstrated that adenoidectomy is effective in the control of OM,[42,43] which may reflect the removal of an environment conducive to biofilm formation.

In summary, this preliminary investigation provides direct imaging evidence of the presence of mucosal biofilm formation in an experimental model of OM. If confirmed in other studies, the biofilm concept could represent a unified theory of bacterial growth that integrates observations made in microbial ecology with infectious disease pathogenesis.

Author Contributions: *Study concept and design:* G. Ehrlich, Veeh, Costerton, Hu, M. Ehrlich, Post. *Acquisition of data:* Veeh, Wang, Hayes, Daigle, Post. *Analysis and interpretation of data:* G. Ehrlich, Veeh, Hayes, M. Ehrlich, Post. *Drafting of the manuscript:* G. Ehrlich, Post. *Critical revision of the manuscript for important intellectual content:* G. Ehrlich, Veeh, Wang, Costerton, Hayes, Hu, Daigle, Post. *Obtained funding:* G. Ehrlich, Costerton, Post. *Administrative, technical, or material support:* G. Ehrlich, Veeh, Wang, Hayes, Hu, M. Ehrlich *Study supervision:* G. Ehrlich, Costerton, Hu, Post. *Literature review:* M. Ehrlich.

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