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# Mucosal Biofilm Formation on Middle-Ear Mucosa in a Nonhuman Primate Model of Chronic Suppurative Otitis Media

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***Background:*** **An increased awareness of bacterial biofilms and their formation has led to a better understanding of bacterial infections that occur in the middle ear. Perhaps the best studied pathogen for its propensity toward biofilm formation is *Pseudomonas aeruginosa*, also the primary pathogen in chronic suppurative otitis media (CSOM).** ***Objective:*** **The aim of this study was to determine whether *P. aeruginosa* forms a biofilm in the middle ear in the setting of CSOM in a nonhuman primate model.** ***Methods:*** **Cynomolgus monkeys underwent perforation of the tympanic membrane and inoculation of the middle ear with a known biofilm-forming strain of *P. aeruginosa*. The contralateral ear was used as an internal control and was neither perforated nor infected. At the end of the study period, both ears were irrigated to remove planktonic bacteria, and the middle ear mucosa was removed and examined ultrastructurally using scanning electron microscopy (SEM) for determination of the presence or absence of biofilm formation.** ***Main Outcome Measure:*** **The identification of middle ear biofilm containing rod-shaped bacteria.** ***Results:*** **SEM revealed that *P. aeruginosa* formed bacterial biofilm in vivo on the middle ear mucosal surface, seen only in the infected ear. Interestingly, biofilm formation caused by cocci was also seen in both the experimental as well as the control ear.** ***Conclusion: P. aeruginosa*** **forms biofilms in the middle ear in CSOM in primates. To our knowledge, this is the first report of disease-associated bacterial biofilm in a nonhuman primate model of CSOM. Such a model lays a foundation for much needed study into the role of biofilms in the pathophysiology of CSOM. Should CSOM be caused by biofilms, which is uncertain at this time, development of novel strategies for treatment and prevention may be possible. The finding of both rods and cocci forming biofilms also warrants further investigation.** ***Key Words:*** **Bacteria, biofilms, pseudomonas aeruginosa, otitis media, middle ear, mucosa, nonhuman primates, animal model.**

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## INTRODUCTION

A bacterial biofilm is a complex association of attached bacteria coexisting in organized structures composed of bacterial cells and an exopolysaccharide matrix. Bacteria reside in a unique and protected microenvironment in the biofilm that facilitates bacterial communication and survival.[1] An increased awareness of bacterial biofilms and their formation may lead to a better understanding of human disease involving bacterial infections, including those that occur in the middle ear.[2] Biofilms can be composed of multiple bacterial species living within distinct microenvironments. Confocal laser scanning microscopy of single species biofilms[3] revealed that the bacteria live in cellular towers composed of exopolysaccharide-enclosed microcolonies separated by open water channels that act as a primitive circulatory system for the delivery of nutrients and removal of metabolic waste products. These microcolonies and open water channels were soon found to be structural features of many single and mixed species biofilms grown in vitro and of all natural mixed species examined in their natural environments. Although the majority of published studies have focused on planktonic (free floating) bacteria, it is now recognized that most bacteria exist in nature in the biofilm state. Perhaps the best studied pathogen for its propensity toward biofilm formation is *Pseudomonas aeruginosa*.[4] *P. aeruginosa*

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is the primary pathogen in chronic suppurative otitis media (CSOM).[5]

CSOM is the stage of ear disease in which there is chronic inflammation of the middle ear cleft (middle ear cleft is a term frequently used for the middle ear, eustachian tube, and mastoid gas cells) and chronic perforation (or tympanostomy tube) of the tympanic membrane. CSOM is the stage of ear disease defined as the drainage of fluid (otorrhea) from the middle ear space through an opening in the tympanic membrane. Mastoiditis is invariably a part of the pathologic process. Otorrhea is almost always present. There is no consensus regarding the duration of otitis media to be designated CSOM, but it can be even as short as 3 weeks, especially when the causative organism is *P. aeruginosa*. CSOM is a major health problem in many populations around the world, affecting diverse racial and cultural groups living not only in temperate climates but also in climate extremes ranging from polar to equatorial. The etiology and pathogenesis of CSOM are multifactorial, but they usually begin with an episode of acute otitis media. Acute otorrhea is the most common complication of tympanostomy tube placement, occurring in approximately 12% to 30% of cases[6] and is the acute precursor of CSOM. Acute otitis media with perforation may also precede CSOM.

To cause otorrhea, *P. aeruginosa* must attach to the epithelium of the middle ear and induce a suppurative host response. However, the source of *P. aeruginosa* in posttympanostomy acute otorrhea and CSOM is not known with certainty and may not be the same in all cases. Or, most often, it is present in the external auditory canal (to which it can readily adhere) and may enter the middle ear by "twitching motility" or other travel methods once the tympanic membrane is perforated. As Kenna et al.[7] suggest, *P. aeruginosa* may be incorporated into the existing discharge and essentially "swim upstream" into the middle ear. Finally, if bacteria readily adhere to a tympanostomy tube, a biofilm formed there may serve as a reservoir of *P. aeruginosa*, tending to cause recurrent infection and otorrhea while the tube remains in place. The attachment of bacteria to a surface initiates the expression of a cascade of gene cassettes that culminates in the "biofilm phenotype."[1] The aim of this study was to determine whether the pathogenic bacteria *P. aeruginosa* forms a biofilm in the middle ear in the setting of CSOM in a nonhuman primate model. The methods of the study were approved by the Institutional Animal Care and Use Committee of The Children's Hospital of Pittsburgh.

## METHODS

The monkey model of CSOM has been established by the primary investigator of this study, and the method has been described in detail in previous publications.[8,9] A cynomolgus monkey species (*Macaca fascicularis*) was used. This species has been found to be a reliable model and anatomically similar to humans, for the establishment of CSOM.[9] CSOM was induced by perforating the tympanic membrane and then inoculating the middle ear with a known biofilm-forming strain of *P. aeruginosa* (strain-PITT27853). The contralateral ear was used as an internal negative control. It was neither perforated nor infected. Reinoculation at 1 week for subsequent time-points was performed routinely, as previously described for the model.[8,9]

Ears were examined weekly for persistence of infection using otoscopy and cultured for the pathogen. A sample of the otorrhea was cultured onto standard LB plates to ensure the presence of *P. aeruginosa*. Otorrhea quality was evaluated using a scoring system previously established by our laboratory in the nonhuman primate model with CSOM.[8,9] At the end of the study period, both ears were irrigated to remove planktonic bacteria.

The middle ear mucosa was removed and examined ultrastructurally using scanning electron microscopy (SEM) for determination of the presence or absence of biofilm formation. SEM was performed at the Center for Biofilm Engineering, Montana State University–Bozeman. Samples of frozen middle ear mucosa were affixed to a beveled brass coon using OCT compound in a cold room. Liquid carbon was then applied to two to three points along the edges of the sample to provide a conductive bridge between sample and coon. These were attached to a threaded rod and dipped in liquid nitrogen for approximately 2 minutes. The samples were then introduced into the first dovetailed cryostage for coating: first, at -80° C under high vacuum to remove any surface water for 3 to 5 minutes and then at -140° C with the chamber back-filled with argon, coated with gold, and the argon shut off. The samples were then introduced into the second dovetailed cryostage within the SEM chamber for imaging.

## RESULTS

All infected ear cultures were positive for *P. aeruginosa*. The average drainage scores were consistent with those previously published by our laboratory in this nonhuman primate model of CSOM.[8,9]

Representative examples of the mucosal surface of the middle ear from the nonperforated, noninfected control ears are shown in Figures 1 and 2 below. SEM revealed that *P. aeruginosa* formed bacterial biofilm in vivo on the middle ear mucosal surface, seen only in the infected ear. Figures 3 and 4 show biofilm formation at 1 week and Figures 5 and 6 show biofilm formation at 4 weeks. Interestingly, some microscopic evidence of adherence to the middle ear mucosa caused by cocci was also seen sporadically in both the experimental as well as the control ears. This biofilm or phenotypic variant (small

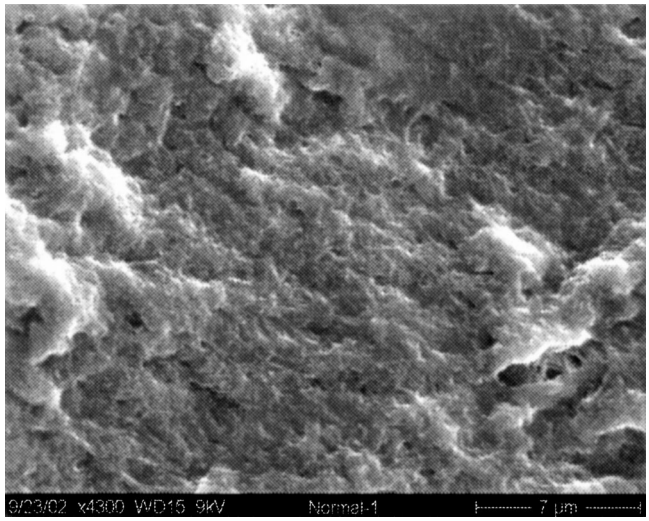


Fig. 1. Scanning electron micrographs show representative examples of the mucosal surface of the middle ear from the nonperforated, noninfected control ears.

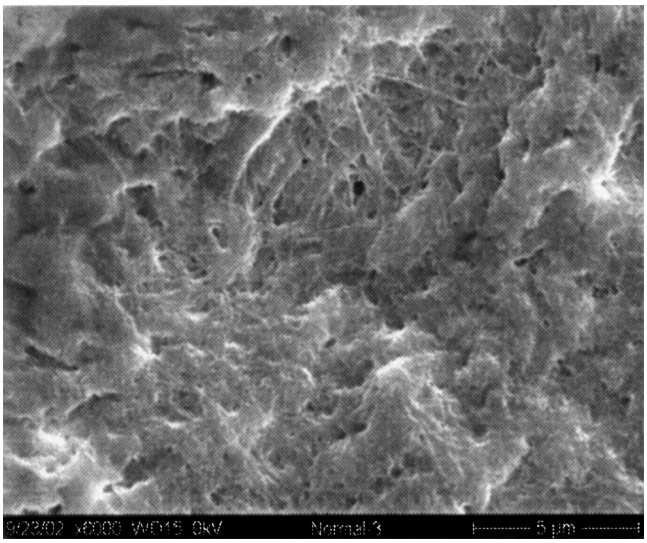


Fig. 2. Scanning electron micrographs show representative examples of the mucosal surface of the middle ear from the nonperforated, noninfected control ears.

colony variant [SCV]) was clearly caused by a microorganism other than *P. aeruginosa*.

## DISCUSSION

To our knowledge, this is the first report of biofilm formation in a nonhuman primate model of CSOM. Such a model is important because the pathophysiology of CSOM is so complex and multifactorial that the more commonly used rodent models such as chinchilla would be less applicable to humans. This is especially true if a cause-and-effect relationship between biofilm formation and CSOM is demonstrated. Novel treatment and prevention studies are best performed in such a model because of anatomic similarities of the middle ear cleft between humans and nonhuman primates and because safety, evidenced by lack of ototoxicity, cannot be as accurately tested in the far more vulnerable rodent models.

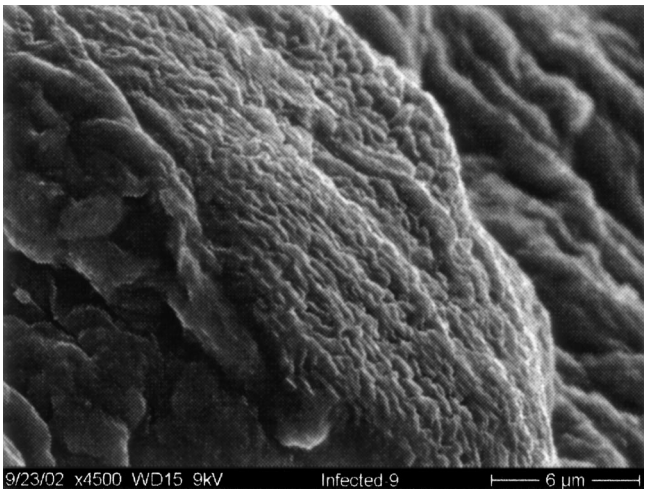


Fig. 3. One week samples. Biofilm formation (central structure) was observed on the mucosal surface by scanning electron microscopy.

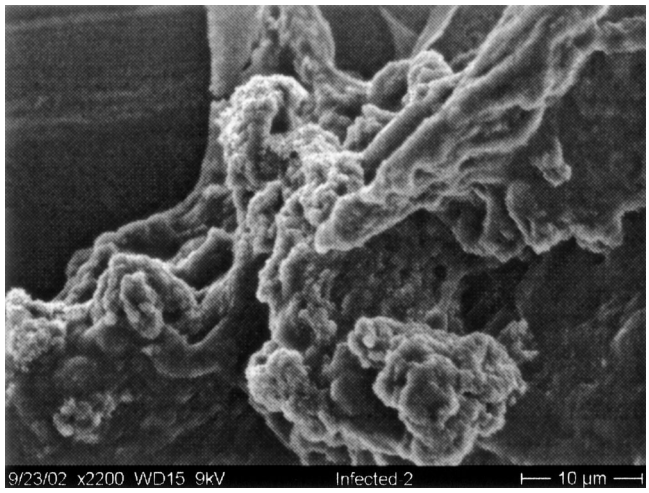


Fig. 4. One week samples. Biofilm formation (central structure) was observed on the mucosal surface by scanning electron microscopy.

Before this study, most of the focus of biofilm formation in the middle ear had been in the setting of otitis media in rodent models. Post[10] demonstrated that biofilms form on the middle-ear mucosa of chinchillas in experimentally induced otitis media. Our demonstration of biofilm formation on the middle ear mucosa of a nonhuman primate model in CSOM is novel. Fergie et al.[11] reviewed all the existing data suggesting that otitis media with effusion (OME) may be a biofilm disease. Despite a far more robust database available, the authors could not conclude that OME is, in fact, a biofilm disease. The burning question raised by our study is whether CSOM is a biofilm disease. A definitive conclusion cannot be drawn, but there is a greater theoretical reason to believe that CSOM is, at least in part, a biofilm disease. The reasons for this are as follows. First, although neither biofilms nor a unique biofilm phenotype have been demonstrated in OME or seen in bacteria isolated from OME,[11] the likeli-

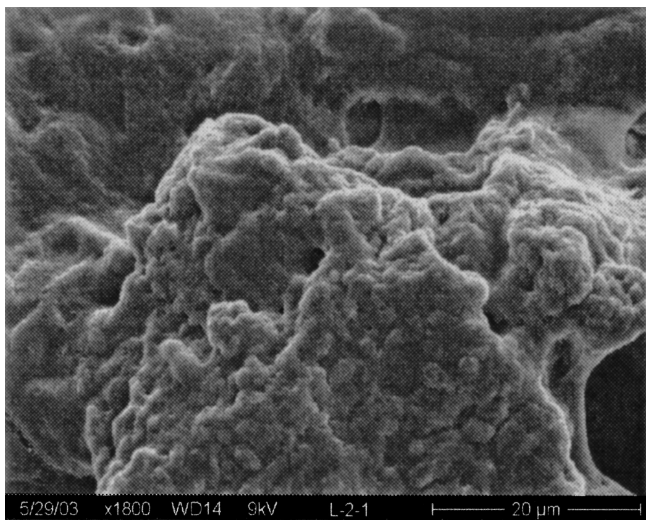


Fig. 5. Four week samples. Biofilm formation was observed on the mucosal surface by scanning electron microscopy.

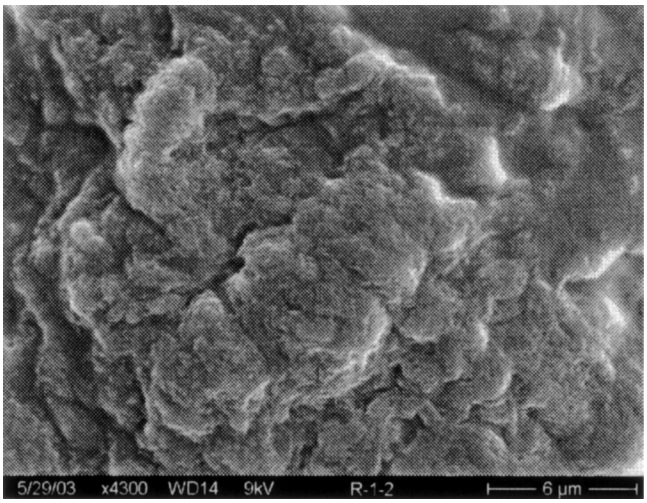


Fig. 6. Four week samples. Biofilm formation was observed on the mucosal surface by scanning electron microscopy.

hood of both is higher in CSOM because of the more central role played by *P. aeruginosa*. Second, although the natural resolution rates seen in OME are not in keeping with other biofilm diseases, resolution seen in CSOM certainly is. Finally, unlike OME, in CSOM and in other biofilm diseases, culture is usually possible (e.g., otorrhea in CSOM and sputum in cystic fibrosis). Whether the presence of mRNA and DNA in culture-negative cases of CSOM will further support a biofilm pathophysiologic link remains to be seen, but such studies are clearly important to perform in the future. This is even more crucial in substantiating CSOM as a biofilm disease because antibiotics do appear to ″cure″ CSOM in a substantial number of cases.[11] The real question given the high recurrence rate is whether the cessation of otorrhea truly represents cure or simply serves as a marker of a bacterial transition between planktonic and biofilm phenotypes. If that is the case, then CSOM is indeed a form of a chronic infective state that is recalcitrant to antibiotic therapy such as that represented by SCV.

Although unanticipated, the microscopic evidence of adherence to the middle ear mucosa by cocci forming SCV was very interesting. More curious was the finding that the SCV was seen sporadically in both the experimental as well as the control ears. This biofilm or phenotypic variant (SCV) was clearly caused by a microorganism other than *P. aeruginosa*. This is not inconsistent with prior reports of biofilms comprising multiple bacterial species. Perhaps the best example of this is the setting of dental plaque, wherein up to 500 different bacterial taxa can be identified.[12] Given, however, that most medical biofilms are monospecies, such a finding requires further study before its significance can be determined. Although as of the writing of this manuscript definitive evidence is lacking to substantiate CSOM as a biofilm disease, if such a mechanism is proven in the future, the door will be opened to exciting new therapies that will allow for the medical treatment of many cases that, at present, are only cured with surgical intervention.

## CONCLUSIONS

- *P. aeruginosa* forms biofilms in the middle ear during CSOM in nonhuman primates.
- Further study is necessary to understand the role of biofilm in the pathophysiology of CSOM.
- The finding of both rods and cocci forming SCV also warrants further investigation.

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