Location of Bacterial Biofilm in the Mucus Overlying the Adenoid by Light Microscopy

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Objective: To determine the location of bacteria and biofilm in adenoid tissue and in mucus overlying the adenoid.

Design: Adenoids removed in 1 piece were oriented to the cephalic and caudal ends. Mucus was fixed by the gradual addition of Carnoy fluid. Consecutive histologic sections were stained with periodic acid–Schiff for visualization of the exopolysaccharide matrix, Giemsa for visualization of bacteria and cells, and fluorescent in situ hybridization with a universal probe for visualization of bacteria.

Setting: Department of Otolaryngology–Head and Neck Surgery, University of Virginia.

Participants: We obtained adenoids from children 10 years or younger who had chronic adenotonsillitis or obstructive sleep apnea. Twenty-seven adenoids were used to develop the fixation method. We examined histologic sections from 9 of 10 adenoids fixed using the final fixation protocol. One adenoid that was missing the surface epithelium was excluded from further evaluation.

Main Outcome Measure: Identification of bacteria by light microscopy.

Results: Bacteria in large numbers were present in the mucus overlying the surface of all 9 adenoids; bacteria were not found in the parenchyma of the adenoids below the epithelial surface. Bacterial biofilms were present on 8 of the 9 adenoids. Sessile (attached) biofilm was present on the caudal end of only 1 adenoid. Multiple planktonic (unattached) biofilms were present on 7 adenoids, always in areas not subject to mucus flow. Biofilms were most common on the caudal portions of adenoids.

Conclusions: Bacteria of the adenoid reside in secretions on the surface and in crypts. Biofilms, predominantly planktonic, were present on 8 of 9 adenoids excised because of hypertrophy. Whether biofilms have a role in the causation of adenoid hypertrophy is not known.

lost during formalin fixation. In the present work, we used Carnoy fluid to fix surface mucus in place to allow visualization of bacteria on the adenoid. The definitive means for identification of bacteria consisted of FISH with a universal bacterial probe.

Bacterial biofilms are defined as aggregates of bacteria “embedded in a matrix of extracellular polymeric substances.” The 2 types are sessile biofilm, which is adherent to a surface, and planktonic biofilm (sludge flocs), which is roughly spherical in shape and is not attached to a surface. The community of bacteria in biofilm is surrounded by an extracellular substance commonly composed of polysaccharides, protein, and nucleic acid. For the purpose of locating bacteria and biofilm in this study, consecutive histologic sections from each paraffin block of adenoid tissue were stained with periodic acid–Schiff (PAS) for visualization of the exopolysaccharide matrix, Giemsa for visualization of bacteria and cells, and FISH for specific detection of bacteria.

### METHODS

#### ADENOID SPECIMENS

Thirty-seven adenoids removed in 1 piece (Figure 1A) were used. The indications for adenoid removal were chronic adenotonsillitis or obstructive sleep apnea. The use of the specimens was exempt from the need for informed consent because they were considered discarded material by the Institutional Review Board for Health Sciences Research at the University of Virginia; no identifying information on the patients was available other than age (≤10 years).

We used 27 adenoids to develop a protocol for the retention of bacteria and mucus on the adenoid surface so that the relationship of bacteria to the adenoid surface could be assessed. The histologic findings on 9 of 10 adenoids fixed using the final protocol are described in the “Results” section. One adenoid that was missing the surface epithelium was excluded from further evaluation.

#### DEVELOPMENT OF FIXATION PROTOCOL

**Fixative**

We used Carnoy fluid as a fixative throughout. Carnoy fluid contains ethanol, chloroform, and acetic acid. The ethanol concentration is 60%, but the acetic acid concentration may be 30% with 10% chloroform or 10% with 30% chloroform. Three adenoids were cut in half; half was fixed with the 10% acetic acid formulation and the other half with the 30% acetic acid formulation. Fixation with the 30% acetic acid version resulted in better preservation of the mucus on the surface. Therefore, Carnoy fluid with 60% ethanol, 30% acetic acid, and 10% chloroform was adopted as the standard fixative; tissue was fixed for 2 hours.

**Application of Fixative**

Adenoids placed in fixative in a bottle did not have mucus with bacteria on the surface because the mucus had washed off before fixation. Subsequently, adenoids were placed with the cut (posterior) surface on filter paper in a Petri dish; fixative was added to the dish so that the mucus on the nasopharyngeal surface might be fixed in place by acetic acid fumes before the level of fixative was high enough to cover the tissue. Adenoids fixed in this way for 2 hours before being cut into smaller pieces for

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**Figure 1.** Adenoid removed from a child 10 years or younger with chronic adenotonsillitis or obstructive sleep apnea. A, Gross appearance of the adenoid removed in 1 piece and oriented to the cephalic/caudal ends. Lines indicate 3 cross-section cuts (cephalic, middle, and caudal). B, Whole mount of a cross section, stained with periodic acid–Schiff (PAS). The adenoid has opened up like a fan, revealing interfold spaces. C, Schematic of a cross section of adenoid that was missing the surface epithelium was excluded from further evaluation.
embedding had flakes of fixed mucus, but the fixed mucus was often dislodged during cutting. Sectioning the adenoids before fixation caused cells from the interior of the adenoid to be squeezed out onto the surface.

Finally, Carnoy fluid was added gradually during a 30-minute period to the adenoid on the filter paper. At 30 minutes, the surface with overlying mucus was fixed, but the interior of the adenoid was not. The tissue was cut into smaller pieces that were placed in cassettes without sponges before being returned to Carnoy fluid for the remaining 90 minutes required for thorough fixation. The cassettes were then processed for embedding.

Adenoids examined with this technique demonstrated that flocs (planktonic biofilm) were present in the fixed mucus of some adenoids, but the location on the adenoid and the number of areas involved were not apparent. This led to formulation of a final protocol in which the surgeon at the time of removal (S.V.E.) placed the adenoid on the filter paper with the cephalic/caudal orientation noted on the paper.

**FINAL STUDY PROTOCOL**

Ten adenoids oriented to the cephalic/caudal direction (Figure 1) were placed on filter paper (labeled cephalic/caudal) in a Petri dish. Specimens were transported to the laboratory within 30 minutes; Carnoy fluid (with 30% acetic acid) was gradually added. After 30 minutes of fixation, the adenoid was cut into 3 horizontal cross sections (cephalic, middle, and caudal) that were placed in cassettes for fixation for another 90 minutes. After fixation, cassettes were transferred to 70% ethanol and processed for paraffin embedding. Serial 4-µm sections were cut, and histologic sections from the cephalic, middle, and caudal portions of the adenoid were each placed on a poly-L-lysine–coated slide.

**STAINING**

Sections on slides were stained with hematoxylin-eosin (HE) and PAS using routine methods. The PAS stains proteoglycans and polysaccharides; biofilm could be readily located by the blue-purple color of the exopolysaccharide matrix. Bacteria were stained with Giemsa to allow visualization of cells in addition to the bacteria. Brown and Hopps staining for bacteria applied to some sections was not as useful as Giemsa because cells were difficult to identify. Fluorescent in situ hybridization with a universal bacterial probe was used as the definitive method for identifying bacteria.

**FISH FOR BACTERIA**

A previously described method for staining bacteria in paraffin-embedded tissue sections was followed exactly except for omission of the digestion of sections with proteinase K. The antisense oligonucleotide probe 5’-F-ACTGCTGCCTCCCGTGAGGATTTATCCCTT-3’ with 1 molecule of fluorescein isothiocyanate per probe (Life Technologies, Carlsbad, California) is generic for bacterial 16S ribosomal RNA. The histologic sections were dewaxed, rehydrated, air dried, and then hybridized overnight at 37°C with the probe (10 ng/µL) in hybridization buffer. Sections were mounted in a commercially available medium with 4’,6-diamidino-2-phenylindole (DAPI) (Vectorshied; Vector Laboratories, Burlingame, California) and viewed with UV light with a triple bandpass filter to identify blue staining (DAPI) of the cell nuclei and fluorescent green bacteria. Representative images were captured with the 3 color channels (red, green, and blue) by using a digital camera, and a composite was produced. Identification of single bacteria required viewing under oil immersion at a magnification of ×1000.

**RESULTS**

Each adenoid specimen was removed as 1 piece and placed on filter paper with the cephalic/caudal orientation (Figure 1A). After gradual fixation in Carnoy fluid for 30 minutes to retain the surface mucus, 3 horizontal cuts through the specimen yielded cephalic, middle, and caudal sections. The horizontal cut through the adenoid folds allowed the adenoid to spread (open up) like a fan, revealing interfold spaces lined by adenoid epithelium that were hidden in the intact specimen (Figure 1B and C). Adenoid epithelium on top of the folds faces the nasopharyngeal lumen; most of the adenoid epithelium lines the interfold spaces. Each fold has a central core containing blood vessels. Between the vascular core and the epithelium are primary and secondary lymphoid follicles with the germinal zone facing specialized adenoid epithelium (Figure 1B). In the fold base are abundant mucoid-secreting glands containing PAS-positive material (Figure 1B). Narrow adenoid crypts open onto the nasopharyngeal surface and the interfold spaces.

Mucus carried by mucociliary clearance from the nasal cavity is deposited on the nasopharyngeal surface of the adenoid. The mucus from the nose may enter and traverse the interfold spaces of the adenoid and exit from the caudal portion.

**LOCATION OF BACTERIA ON ADENOID SPECIMENS**

Three horizontal sections (cephalic, middle, and caudal) from 9 adenoids were studied for a total of 27 samples. The surface epithelium of 1 adenoid was not intact, and it was omitted from further evaluation. To locate bacteria, consecutive 4-µm histologic sections from each sample were stained with PAS, Giemsa, and FISH with the universal bacterial probe. Clusters of bacteria were easily visualized at a magnification of ×400 with FISH; single bacteria were sought by examination under oil immersion (magnification ×1000).

**Submucosa and Nasopharyngeal Surface**

No bacteria, single or in clusters, were detected below the adenoid epithelium despite extensive search under a magnification of ×1000. Single and/or small clusters of bacteria were found on the epithelial surface facing the nasopharyngeal lumen of all 9 adenoids. Rows of bacteria embedded in a thin film of mucus on the epithelial surface of the top of folds were common (Figure 2). In addition, many bacteria were found dispersed in mucus on indentations of the nasopharyngeal surface overlying the mouths of crypts and at the openings of interfold spaces onto the nasopharyngeal surface. Few, if any, single bacteria were present in the deeper half (farthest from the nasopharyngeal lumen) of the interfold spaces.
The thin layer of mucus covering the adenoid epithelium on the nasopharyngeal surface of a fold. A, With Giemsa staining, cells and bacteria are shown embedded in the mucus layer (original magnification ×400). B, In the contiguous section, bacteria are stained with universal bacterial fluorescent in situ hybridization probe (original magnification ×1000).

**Table. Biofilm on Cross Sections of the 8 Adenoids With Positive Staining Results**

<table>
<thead>
<tr>
<th>Adenoid No.</th>
<th>Cephalic</th>
<th>Middle</th>
<th>Caudal</th>
<th>Biofilm Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Small flocs</td>
</tr>
<tr>
<td>36</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>Small flocs</td>
</tr>
<tr>
<td>37</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>Large and small flocs</td>
</tr>
<tr>
<td>34</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>Large flocs</td>
</tr>
<tr>
<td>28</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>Large and small flocs</td>
</tr>
<tr>
<td>29</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>Large flocs</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>Small flocs</td>
</tr>
<tr>
<td>33</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>2 Areas of sessile biofilm in shallow indentations on nasopharyngeal surface</td>
</tr>
</tbody>
</table>

Total 3 2 7

Abbreviations: flocs, planktonic biofilm; +, positive staining results.

*Biofilm identified with a combination of periodic acid–Schiff and Giemsa stains and fluorescent in situ hybridization with a universal bacterial probe.

With Giemsa staining, PMNs with ingested bacteria were frequently found in mucus at the mouths of interfold spaces and on the nasopharyngeal surface of folds. However, the ingested bacteria did not stain with the FISH probe for ribosomal RNA of bacteria, suggesting that they were no longer metabolically active.

**Biofilm**

Eight of the 9 adenoids were found to have biofilm on the adenoid surface, which was readily detected by PAS staining of the exopolysaccharide matrix in which the community of bacteria was located (Table). The bacteria in biofilm stained with Giemsa and with FISH. Biofilm was found on the caudal sections of 7 adenoids, on the middle sections of 2, and on the cephalic sections of 3. Five adenoids had biofilm on 1 section, 2 had biofilm on 2 sections, and 1 had biofilm on all 3 horizontal sections.

Sessile biofilm was present on only 1 adenoid; planktonic biofilm (flocs) was present on the other 7. Sessile biofilm, attached to a surface to resist being washed away by mucus flow, was found on the caudal section of adenoid number 33 (Table). Two areas on the nasopharyngeal surface of that section had the exopolysaccharide matrix with embedded bacteria in an indentation at the opening of a crypt (Figure 3). This sessile biofilm was present at the same site on the surface through multiple histologic sections, confirming that it was attached.

Flocs (planktonic biofilm) were not attached to the adenoid surface. Flocs were present in interfold spaces (Figure 4A) and in large cryptlike passages opening into the interfold spaces (Figure 4B); flocs were not found on the nasopharyngeal surface where they would be washed away by mucus flow. Large flocs (Figure 4A) were different in appearance from small flocs (Figure 4B). A representative large floc (Figure 5) was spherical. The central portion appeared to consist of a matrix with few bacteria. The density of bacteria increased toward the periphery in a somewhat radiating formation (Figure 5C and D). The sphere was surrounded by a layer of PMNs that appeared to be unable to enter the matrix where the bacteria were embedded (Figure 5A). Staining of bacteria by FISH with the probe for ribosomal RNA was intense in the periphery of the sphere with diminished staining in the center (Figure 5E). Other large flocs had FISH-positive bacteria distributed throughout the matrix (Figure 5F).

In contrast to large flocs, small flocs had exopolysaccharide matrix with embedded bacteria that did not appear to be organized, and they usually were not spherical (Figure 6). Small flocs were frequently found close together in interfold spaces or in crypts emptying into interfold spaces (Figure 4B) and were sometimes found in the mouth of interfold spaces close to the junction with the nasopharyngeal surface (not shown).
This study has demonstrated 2 major findings about the relationship of bacteria to the adenoid using Carnoy fluid for the fixation of overlying mucus and FISH staining of bacteria. First, bacteria were not found in the adenoid parenchyma despite extensive search under a magnification of $\times1000$. Bacteria that had been ingested by phagocytes (PMNs and macrophages) could have been inside cells below the adenoid surface but were not detected because they were metabolically inactive as they were being killed. Second, the bacteria of the adenoid were located in the overlying surface secretion, which confirms our hypothesis that preserving mucus during the fixation process is required for detection of the bacterial biofilm by light microscopy. Staining of consecutive histologic sections with PAS, Giemsa, and FISH allowed definitive localization of the bacteria in relation to the adenoid epithelium. All 9 adenoids had bacteria in mucus on the epithelium on the top of folds facing the lumen of the nasopharynx; all but 1 also had bacteria in biofilm. The location of the 2 types of biofilm differed: sessile biofilm was attached to the nasopharyngeal surface on top of a fold, whereas the more common planktonic biofilm (flocs) was invariably in areas not subject to the mucus flow across the nasopharyngeal surface. Biofilms were more common on caudal sections than on cephalic and middle sections, perhaps related to being at the terminus of mucociliary clearance or to proximity of the caudal section to the oropharynx.

This study provides a context for viewing the findings reported by other investigators who have examined the relationship of bacteria to the adenoid. In 1994, Forsgren and coworkers used FISH and transmission electron microscopy of frozen sections to demonstrate Hemophilus influenzae in macrophagelike cells that they found in the subepithelial layers of 10 adenoids. They speculated that the intracellular bacteria had entered by way of the specialized epithelium of crypts, had been ingested by macrophages where they resided, and might constitute a “cellular reservoir.” We did not detect bacteria in phagocytes in the subepithelial tissue associated with crypts, but our FISH probe for bacterial ribosomal RNA would be unlikely to stain metabolically inactive bacteria that had been ingested by phagocytes.

Ivarsson and Lundberg used touch preparations to characterize cells and bacteria on the surface of adenoids from 24 children before excision; formalin-fixed histologic sections from the same adenoids were used to examine the adenoid parenchyma. They demonstrated

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Figure 5. Representative large planktonic biofilm. An omega-shaped fixation artifact is seen in all sections of adenoid 37. A and B, Periodic acid–Schiff stain of adenoid 37 (original magnification $\times20$ and $\times400$, respectively). C and D, Giemsa stain of adenoid 37 (original magnification $\times200$ and $\times400$, respectively). E, Fluorescent in situ hybridization (FISH) stain for bacteria in adenoid 37 (original magnification $\times400$). F, FISH stain of a floc from adenoid 31 (original magnification $\times400$).

Figure 6. Two small planktonic biofilms in a narrow space between folds. A. Periodic acid–Schiff stain demonstrates the exopolysaccharide matrix of the biofilm. B. Fluorescent in situ hybridization probe demonstrates bacteria in the biofilm.
abundant bacteria, PMNs with ingested bacteria, and immunoglobulin-secreting cells in the secretion. Bacteria were not seen in the adenoid parenchyma in histologic sections, but PMNs traversed the epithelial layer to reach the surface. Our study confirms and extends the findings of the Swedish study.2

Zuliani et al12 fixed adenoids from 16 patients (7 with chronic rhinosinusitis and 9 with obstructive sleep apnea) with glutaraldehyde for examination with scanning electron microscopy. They reported that an average of 94.9% of the nasopharyngeal surface of adenoids from patients with chronic rhinosinusitis was covered with mature biofilms compared with 1.9% coverage of adenoids from patients with obstructive sleep apnea. Sessile biofilm (but not flocs) could have been visualized with this technique. Our findings are not consistent with those of Zuliani et al12 because we found sessile biofilm at only 2 sites on the caudal section of 1 of the 9 adenoids examined.

Swidsinski et al13 fixed 50 adenoid and 70 tonsil samples with Carnoy fluid and used FISH to characterize bacteria on the adenoid surface by making photographs “consecutively from the entire tissue surface” under a magnification of ×1000. They showed photographs of FISH-stained planktonic biofilms, which they termed microabscesses. They also described bacteria extending into adenoid and tonsil tissue in fissures and diffusely infiltrating subepithelial tissue. Although each tissue sample was treated with another stain (PAS, HE, or Gram stain) in addition to FISH, the precise location of bacteria in fissures and “diffusely infiltrating” adenoid tissue in relation to the complexities of the adenoid surface was not illustrated.

Most recently, Kania and coworkers14 studied 39 excised adenoids with Gram staining of formalin-fixed sections, scanning electron microscopy of glutaraldehyde-fixed tissue, and confocal laser scanning microscopy of frozen sections fixed with acetone. With Gram staining they found microcolonies of mostly cocci on the outer surface of the adenoid, particularly in small depressions between normal-appearing cells. For confocal laser scanning microscopy, bacteria were stained with propidium iodide and glycocalyx was stained with fluorescein-labeled concanavalin A to bind to mannose residues. They reported that 21 of 39 adenoids (54%) showed evidence of biofilm and that bacteria and glycocalyx colocalized by confocal laser scanning microscopy. It is not clear that our findings are comparable to theirs because of differences in fixation technique and different staining specificity in the confocal laser scanning microscopy work.

Summation of the evidence from this and other studies2,11,13,14 allows the following conclusions to be drawn about the relationship between the adenoid and bacteria.

• Viable bacteria are not found in the adenoid parenchyma.
• Bacteria that penetrate the adenoid epithelium are ingested and killed by phagocytes, macrophages, and/or PMNs.
• Bacteria in great profusion are present on the adenoid surface, where they reside in secretions.
• PMNs pass through adenoid epithelium to the surface, where they may ingest and kill bacteria.
• Surface bacteria can form biofilms in which they may be protected from ingestion by PMNs and killing by antibiotics.
• Sessile biofilms may form on the nasopharyngeal surface of the adenoid, where they are attached and resist removal by mucus flow.
• Planktonic biofilms, which are unattached, may form in areas of stagnation, for example, areas with decreased mucus flow.

Phagocytes are important for preventing bacterial penetration into the adenoid parenchyma, and they may limit bacterial growth on the adenoid surface. In the hypertrophied adenoids that are available for examination because they had been removed, biofilm formation is common and is predominantly in the form of flocs rather than sessile biofilm. It may be that biofilms are uncommon on normal adenoids, but adenoids from children without disease of the upper respiratory tract were not available for comparison with the hypertrophied adenoids that have been studied. This limits our ability to conclude that the biofilms are instrumental in producing the hypertrophy.

It appears that the adenoid is like the tonsil in that maintaining homeostasis in the tissue and overlying mucus is an ongoing battle among the bacteria, PMNs, and macrophages.2,15 The effect of biofilm on the adenoid is not clear.

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Author Contributions: Drs Winther, Gross, and Hendley had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Winther, Gross, and Hendley. Acquisition of data: Winther, Gross, Hendley, and Early. Analysis and interpretation of data: Winther, Gross, and Hendley. Drafting of the manuscript: Winther, Gross, and Hendley. Critical revision of the manuscript for important intellectual content: Winther, Gross, Hendley, and Early. Statistical analysis: Hendley. Obtained funding: Winther and Hendley. Administrative, technical, and material support: Winther, Hendley, and Early. Study supervision: Winther and Hendley.

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Additional Contributions: Sheri VanHoose, MLT, embedded, cut, and stained the adenoid tissues per our specifications. Ken Tung, MD, is the director of the Research
Histology Core Laboratory, University of Virginia, and oversees the work of Ms VanHoose.

REFERENCES