**Objective:** To demonstrate that hematoxylin-eosin staining can be used to detect the presence of bacterial biofilm in patients with chronic rhinosinusitis (CRS).

**Design:** A prospective study.

**Setting:** The University of Southern California University Hospital and the Department of Otolaryngology–Head and Neck Surgery, University of Southern California, Keck School of Medicine, Los Angeles.

**Patients:** A total of 34 patients: 24 undergoing endoscopic sinus surgery for CRS and 10 undergoing septoplasty with or without turbinate reduction with no history of sinusitis, were enrolled in the study.

**Main Outcome Measures:** Contiguous sections from patient samples were examined by both hematoxylin-eosin staining and fluorescence in situ hybridization (FISH) with the bacterial-specific probe EUB338 for evidence of bacterial biofilm.

**Results:** Biofilm was detected by hematoxylin-eosin staining in 15 of 24 patients with CRS and 1 of 10 control patients. In all cases, hematoxylin-eosin staining was found to be an accurate predictor of the presence or absence of biofilm using FISH as a control standard.

**Conclusion:** Hematoxylin-eosin staining of surgical specimens is a reliable and available method for the detection of bacterial biofilm in chronic infectious disease.


We describe for the first time, to our knowledge, how to detect biofilm on routine hematoxylin-eosin (H-E) preparations. The Centers for Disease Control and Prevention estimate that at least 65% of human bacterial infectious processes involve biofilms.1 A biofilm is a group of bacteria associated with a surface, such as a mucous membrane, and enclosed in a matrix of extracellular polysaccharide material. Bacterial biofilm has been implicated in many human diseases.2 These include cystic fibrosis, otitis media, periodontitis, chronic prostatitis, and artificial joint infections, among others. Despite this, biofilm has been difficult to identify and study. Multiple types of bacteria can be living in a biofilm structure. They are frequently difficult to culture.3 Therefore, diagnosis is often difficult. Bacteria in biofilms have numerous defense mechanisms, and therefore their response to antibiotics is usually incomplete.4

Bacteria in the biofilm state live in a community protected by a 3-dimensional extracellular polymeric substance that constitutes 90% of the volume and can account for more than 50% of the total carbon content. Biofilms may be composed of mixed species of bacteria surrounded by this extracellular polymeric substance, which is a physical, noncellular barrier. Staphylococcus and Pseudomonas species are frequently implicated in sinonasal disease biofilms.

Traditionally, biofilm has been detected by scanning electron microscopy5 or confocal scanning laser microscopy.6 More recently, fluorescence in situ hybridization (FISH) has been used.7 In the course of our ongoing institutional review board–approved study of biofilm in patients with chronic rhinosinusitis (CRS), we discovered that biofilm can be reliably detected with ordinary H-E preparations. This can be performed on fresh tissue as well as archival material. Histologically, biofilm appears as clusters of basophilic bacteria and host cells entrapped in a layer of extracellular polymeric substance. To our knowledge, this has not been previously described. To verify this, we performed the following prospective study.

**METHODS**

Biopsy specimens were obtained for analysis from patients undergoing either endoscopic si-
chloride [pH 8], 5mM EDTA, and 0.01% SDS). Slides were treated with 10 mg/mL of lysozyme in Tris EDTA buffer and then washed in PBS and mounted with Vectashield (Vector Laboratories) mounting medium with DAPI.

**LIVE/DEAD BACLIGHT STAINING**

Fresh tissue samples were obtained in parallel from selected cases stained with SYTO9 (Invitrogen) and propidium iodide using the LIVE/DEAD Baclight kit, as previously described.3 These samples were then prepared as whole mounts with Vectashield and coverslips. The SYTO9 green stains only live cells in a fresh sample, whereas the propidium iodide red stains only dead or dying cells. The SYTO9 was used to verify live bacteria in the biofilm, which are smaller in size than host cells.

**MICROSCOPY**

Hematoxylin-eosin–stained sections were analyzed by light microscopy using a Leica DM LB2 epifluorescence microscope (Wetzlar, Germany). The FISH and stained samples were analyzed using the same microscope with UV and Cy3-, DAPI-, and fluorescein isothiocyanate–specific filters. Images that were original magnification ×20 and ×40 of H-E staining and FISH were acquired with a CCD digital camera (model 7.2; Diagnostic Instruments, Sterling Heights, Michigan). A Zeiss 510 laser scanning confocal microscope (Göttingen, Germany) was used to acquire images that were original magnification ×40 and ×63 of LIVE/DEAD Baclight–stained samples.

**RESULTS**

Of the 24 patients with CRS, 15 had biofilm detected by H-E (62%). In the 10 control patients, only 1 had biofilm (10%). This difference was statistically significant (P = .008, Fisher exact test). The amount of biofilm present in each sample was categorized as extensive, present, or absent for both groups (Table). Patient samples classified as having extensive biofilm had involvement of 50% or more of the mucosal surface analyzed, whereas any amount of biofilm less than this was classified as present. All biofilms detected by H-E were also detected by FISH. Furthermore, all patient samples classified as negative for biofilm by H-E were also classified as negative for biofilm by FISH, which was considered positive for biofilm when a significant quantity of staining was observed either covering the epithelial surface or in clusters along the surface. Therefore, in this study, H-E correctly identified the presence or absence of biofilm in all cases, using FISH as a control standard. In selected cases, fresh samples of patient tissue were collected in parallel for Baclight LIVE/DEAD staining and examination by confocal laser scanning microscopy (CLSM). The detection of biofilm by CLSM was consistent with that of H-E staining and FISH (Figure 1).

Of interest, 3 of the patients with CRS had samples with detectable fungal elements on H-E (Figure 2), whereas no fungal elements were present in any control patient samples. Two of the 3 patients with CRS with fungal elements had no bacterial biofilms. In the 1 patient with both bacterial biofilm and fungal elements, there was no apparent overlap of these entities, which were identified in separate regions of the sample.

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**Table. Amount of Bacterial Biofilm Present on Hematoxylin-Eosin–Stained Sections**

<table>
<thead>
<tr>
<th>Group</th>
<th>Extensive Amount</th>
<th>Biofilm Present</th>
<th>Biofilm Absent</th>
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</thead>
<tbody>
<tr>
<td>Patients with CRS (n=24)</td>
<td>7 (29)</td>
<td>8 (33)</td>
<td>9 (38)</td>
</tr>
<tr>
<td>Controls (n=10)</td>
<td>0</td>
<td>1 (10)</td>
<td>9 (90)</td>
</tr>
</tbody>
</table>

Abbreviation: CRS, chronic rhinosinusitis.

*Biofilms were classified as extensive (≥50% of mucosal surface involved), present (≥50% involved), or absent. The percentage of patients with CRS with detectable biofilm was significantly greater than that of control patients (62% vs 10%; P = .008, Fisher exact test).*

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Hematoxylin and eosin–stained sections were analyzed by routine anatomic microscopy. FISH was used to verify live bacteria in the biofilm, which are smaller in size than host cells.
Biofilm can be detected on routine H-E. It can be seen as irregularly shaped groupings of small basophilic bacteria, exopolymERIC substance, and entrapped erythrocytes and leukocytes resting on the surface epithelium (Figure 1 and Figure 2). Depending on fixation and processing, it may be tightly adherent to the surface epithelium or pulled away slightly. Precise identification of the bacterial species involved in biofilms still requires culturing, following physical disruption of the biofilm, or molecular methods, such as FISH, with bacterial species-specific probes. Because there is increasing evidence that biofilm plays an important role in many chronic diseases, it is important to identify easier and cheaper methods to study biofilm in clinical samples. In particular, the wide availability of H-E staining of surgical specimens through clinical pathology laboratories makes this a highly practical method for detecting biofilm in clinical practice. Gram staining is another simple histological technique that has been previously used in conjunction with other methods to describe biofilms. We are currently investigating the efficacy of Gram staining in detecting biofilms in CRS compared with H-E staining and FISH. Although specific treatments are not currently available to target biofilm, awareness of the presence of biofilm by physicians may affect overall patient treatment and follow-up, given the association between biofilm and potential for treatment failure and persistent symptoms. Therefore, the presence of biofilm in tissue samples from patients with CRS should be reported by pathology laboratories when noted. Additional studies of patient outcomes will be needed to determine whether the actual amount of biofilm detected is also an important factor correlating with risk of persistent symptoms or whether merely reporting the presence or absence of biofilm is sufficient.

Figure 1. Detection of bacterial biofilm by hematoxylin-eosin stain (H-E), fluorescent in situ hybridization (FISH), and confocal laser scanning microscopy (CLSM). Representative images of a patient with chronic rhinosinusitis (CRS) with bacterial biofilm (A, B, C) and a control patient without bacterial biofilm (D, E, F). A and D, Hematoxylin-eosin–stained sections reveal small basophilic bacterial clusters (arrow) on the surface epithelium of the sample of patients with CRS (A), whereas the control patient sample (D) shows normal eosinophilic ciliated respiratory epithelium. B and E, FISH with the bacterial-specific probe EUB338 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, California) reveals positive staining of bacterial clusters (arrow) at the epithelial surface in the CRS sample (B), whereas there is no staining in the control (E). Panels B and E are contiguous with A and D, respectively. C and F, CSLM reveals SYTO9 (Invitrogen, Carlsbad, California) positive live (PL) bacterial elements (arrows) in the CRS sample (C), whereas only much larger epithelial cellular staining is observed in the control sample (D). Panels A, B, D, and E are original magnification ×20, whereas panels C and F are original magnification ×63.

Figure 2. Appearance of bacterial biofilms on hematoxylin-eosin (H-E)–stained sections. Images of surface epithelium from H-E–stained sections of patients with chronic rhinosinusitis with varying appearance of biofilm. A, Basophilic bacterial clusters (arrows) line the epithelial surface. B, Extracellular polysaccharide substance (EPS) material with embedded bacteria and entrapped erythrocytes and leukocytes, partially sheared from the epithelial surface. C, Dense EPS material with embedded basophilic bacteria coating the epithelial surface. D, Image from a sample from a patient without bacterial biofilm but in which fungal elements were identified (arrowhead). All images are original magnification ×20.
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Author Contributions: All authors 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: Hochstim, Choi, and Rice. Acquisition of data: Hochstim, Choi, Lowe, Masood, and Rice. Analysis and interpretation of data: Hochstim, Choi, and Masood. Drafting of the manuscript: Hochstim, Choi, and Rice. Critical revision of the manuscript for important intellectual content: Hochstim, Lowe, Masood, and Rice. Statistical analysis: Hochstim. Administrative, technical, and material support: Choi, Lowe, and Rice. Study supervision: Choi, Masood, and Rice.

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REFERENCES


