**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.

nus surgery for CRS (n=24 study patients) or septoplasty with or without turbinectomy with no history of sinusitis (n=10 controls). All patients gave informed consent, and the study was approved by the institutional review board.

TISSUES

Samples of nasal mucosa were taken from the ethmoid sinus in study patients and from the middle turbinate in controls. These mucosal specimens were fixed in 4% formalin for 48 hours and processed and embedded into paraffin blocks according to routine procedures. Fresh samples were collected in phosphate-buffered saline (PBS) on ice for analysis by FISH or BacLight staining (DEAD BacLight kit; Invitrogen, Molecular Probes, Carlsbad, California), and confocal microscopy.

HISTOLOGIC EXAMINATION

Contiguous sections were taken for H-E preparation and for FISH. Sections were stained with H-E using standard pathologic procedures. Briefly, sections were deparaffinized in xylene (2 x 5 minutes) and rehydrated with successive 1-minute washes in 100%, 96%, 80%, and 70% ethanol. They were then stained with hematoxylin (2 minutes), rinsed with distilled water, rinsed with 0.1% hydrochloric acid in 50% ethanol, rinsed with tap water for 15 minutes, stained with eosin for 1 minute, and rinsed again with distilled water. The slides were then dehydrated with 95% and 100% ethanol successively followed by xylene (2 x 5 minutes) and mounted with coverslips.

All paraffin-embedded samples analyzed by H-E staining in this study were also subjected to FISH using the bacterial-specific probe EUB338 (5'-Cy3-GCTGCTCCCTCCTGAGTAGT-3') 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, California), which is hybridized to the conserved 16S ribosomal RNA sequence found in nearly all bacteria. Thus, FISH with the EUB338 probe will specifically label bacteria but not eukaryotic cells within a tissue sample. The protocol was performed as previously described. Briefly, sections were deparaffinized, rehydrated, and postfixed in 4% paraformaldehyde for 5 minutes. Sections were washed in PBS and then treated with 10 mg/ml of lysozyme in Tris EDTA buffer for 10 minutes at room temperature. Slides were preincubated in hybridization buffer (0.9M of sodium chloride; 20mM of Tris hydrochloric acid, pH 8; 0.01% sodium dodecyl sulfate [SDS]; 30% formamide) for 5 minutes at 35°C, and then hybridized overnight (12-18 hours) at 35°C with a 5 μg/ml final concentration of 5’Cy3-labeled EUB338 probe in hybridization buffer. Next, 2 x 15-minute stringent washes were performed at 37°C in washing buffer (65mM sodium chloride, 20mM Tris hydrochloric acid [pH 8], 5mM EDTA, and 0.01% SDS). Slides were 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. 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.

<p>| Table. Amount of Bacterial Biofilm Present on Hematoxylin-Eosin–Stained Sections a |
|--------------------------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>Extensive Amount</th>
<th>Biofilm Present</th>
<th>Biofilm Absent</th>
</tr>
</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.

a 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).

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).
Biofilm can be detected on routine H-E. It can be seen as irregularly shaped groupings of small basophilic bacteria, exopolymere 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.
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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