

ORIGINAL RESEARCH—SINONASAL DISORDERS

Biofilms with fungi in chronic rhinosinusitis

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OBJECTIVES: Demonstrate that bacterial biofilm in sinus mucosal samples from patients with eosinophilic mucin chronic rhinosinusitis (EMCRS) and allergic fungal rhinosinusitis (AFRS) contains fungal elements; identify specific organisms in the biofilm.

METHODS: Mucosa samples from 11 patients undergoing sinus surgery were collected. Patients were classified as having AFRS, EMCRS, or chronic rhinosinusitis (CRS) based on histopathologic findings. Three mucosal samples from controls were also collected. Samples were stained with specific bacterial fluorescent in situ hybridization (FISH) DNA probes (*Haemophilus influenzae*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) and a general pan-fungal FISH probe. The samples were analyzed for bacterial biofilm ultrastructure and fungal elements using epifluorescent microscopy.

RESULTS: Bacterial biofilm was demonstrated in 9/11 samples and 2/3 controls. *H. influenzae* was the predominant biofilm present. There was a trend showing more fungal elements in AFRS and EMCRS biofilms than in CRS and controls.

CONCLUSION: This is a preliminary study demonstrating fungal elements within sinus mucosal biofilm and demonstrating biofilm in AFRS.

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Chronic rhinosinusitis (CRS) is a common condition that is refractory to medical and surgical treatment. Many pathophysiologic mechanisms have been proposed to explain CRS, including environmental factors, anatomic obstruction, compromised mucociliary clearance, altered immune response, chronic bacterial infection or colonization, and fungal colonization.¹ All patients with CRS have sinus mucosal inflammation, which in turn produces increased sinonasal drainage, facial pressure and pain, loss of smell, and other symptoms.

The underlying mechanisms of chronic sinusitis in the immunocompetent host remain poorly understood. It is theorized that chronic bacterial infection may play an important role, since infection is clearly involved in acute cases, and CRS patients often improve transiently with antibiotic treatment. Clinical studies attempting to elucidate the microbiology of CRS have shown remarkably heterogeneous results and do not conclusively correlate to the disease state.²

Fungal species are also theorized to contribute to CRS and are clearly involved in the pathogenesis of allergic fungal rhinosinusitis (AFRS).^{3,4} AFRS is a clinically distinct subset of CRS characterized by gross production of eosinophilic mucin with fungal hyphae in an immunocompetent host who demonstrates IgE-mediated fungal allergy.⁵ In classic cases of AFRS, the clinical findings are not subtle: Patients present with robust polyposis obstructing the diseased sinuses. CT imaging reveals sinuses impacted with mucin demonstrating serpiginous patterns and calcifications; this mucin shows a thick, tenacious, “peanut butter” consistency that is difficult to remove.^{1,2} Histologic examination of the mucin must demonstrate fungal hyphae, Charcot-Leyden crystals, and many eosinophils to clinch the diagnosis.⁶

AFRS can be considered as only one extreme of a spectrum of CRS subtypes based on the presence or absence of eosinophil and fungal elements. The presence of eosinophils in the mucin of CRS patients (allergic mucin) has been associated with increasing severity of disease.⁷ As a result, the subtype “eosinophilic mucin chronic rhinosinusitis” (EMCRS) has been used to describe this histologic finding. The presence of eosinophils in the mucin suggests a basic difference exists in the mechanism of mucosal inflammation from CRS without eosinophilia (dominated by neutrophils and macrophages) and has been proposed to be a marker of fungal presence in the sinuses.⁸ The true importance of eosinophils has yet to be elucidated.

Bacterial biofilms have been demonstrated in mucosal samples of patients with chronic rhinosinusitis in studies by the current authors and other researchers.⁹ A biofilm is a microbially derived sessile community characterized by cells that are attached to a substratum and/or to each other, are embedded in a matrix of extracellular glycopolysaccharide, and exhibit an altered phenotype with respect to growth rate and gene transcription.^{10,11}

It is crucial to differentiate a bacterial biofilm from a bacterium’s planktonic form when evaluating CRS because the effective treatment may be different. Evidence suggests that most bacteria can live in a biofilm state and will assume this phenotype under the correct conditions. A biofilm can survive with significantly lower metabolic demands and in a shear environment. In a human host, biofilms demonstrate

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great resistance to the host's immune response and to antibiotics, both topical and systemic. Additionally, biofilms have greater ability than planktonic forms to defend against attacks from other microbes and environmental stressors. In human disease, a pathogen that has assumed a biofilm phenotype will be highly resistant to both host immune responses and antimicrobial agents.

The purpose of this study was to demonstrate the presence of bacterial biofilm on the sinus mucosa of patients with AFRS and CRS and to identify the presence of fungal elements within the biofilm. The authors differentiate between mucosal samples with and without eosinophilic mucin (EMCRS), since this distinction may represent a gradient between AFRS and CRS.

METHODS

Study Design and Population

This was a prospective controlled study of sinus mucosa samples from CRS and AFRS patients undergoing endoscopic sinus surgery. The protocol was approved by the Institutional Review Board (IRB). Patients and controls signed consent forms approved by the IRB. All study patients met the criteria for chronic rhinosinusitis of the AAO-HNS Committee for Definition of Chronic Rhinosinusitis. All patients underwent CT scan before surgery.¹ Controls were drawn from patients with isolated nasal obstruction, without history or evidence of infection, undergoing septoplasty in a tertiary medical center.

Patients scheduled for endoscopic sinus surgery fulfilling the criteria after at least three months of medical treatment, including at least one course of antibiotics in the previous 30 days, were asked to participate in the study. Exclusion criteria included pregnancy and inability to safely obtain a sample specimen. Patients scheduled for septoplasty for nasal obstruction or orbital decompression surgery without history or physical evidence of recurrent sinus infections or nasal discharge served as control samples. Because of the lack of clinical history of sinus disease, no sinus CT scans were performed. At the time of surgery, sample specimens were taken from the anterior ethmoid bulla.

Data Sources and Collection

Patient information collected included symptoms, duration of symptoms, presence of allergy symptoms, treatment history, and diagnostic results (RAST, IGE, previous cultures, and sinus computed tomography results). At surgery, the sinuses were cultured for aerobic and anaerobic bacteria using standard endoscopically guided transnasal culture techniques. Sinus mucosa and mucin were collected for histopathologic characterization. Patients who satisfied the Bent and Kuhn diagnostic criteria for AFRS were classified as having AFRS.¹² Patients who did not satisfy these criteria, but who had eosinophilic mucin (without fungal ele-

ments), were classified as having EMCRS. Patients without eosinophilic mucin were classified as having CRS.

Biopsies of the sinus mucosa were taken during surgery. Every attempt was made to obtain a specimen that was truly representative of sinus mucosa. Commonly, the lateral portion of the uncinate process or mucosa removed from the maxillary sinus or its ostia was used for samples. For control samples, a portion of the anterior ethmoid bulla was removed. Specimens were enclosed in aluminum foil, immediately preserved in liquid nitrogen, and stored in a freezer at less than 70°C, then forwarded on dry ice to the Biofilm Laboratory at Northern Arizona University for fluorescent in situ hybridization (FISH) testing using DNA probes for *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and a general fungus probe.¹¹ The presence of biofilms was determined using FISH and epifluorescent microscopy.

FISH analysis on sinus mucosa was performed as described by Thurnheer et al,¹³ with modifications. Briefly, oligonucleotide DNA probes, labeled at the 5' end with either Cy3 (red fluorescence) or 6-FAM (green fluorescence), were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). These probes were designed specifically for the organisms predetermined as described above, as well as a pan-fungal DNA probe (5'-156-FAM/TCC GTA GGT GAA CCT GCG G-3'). Each probe was individually validated by the following protocol: Distinct microorganisms were grown in broth culture, aliquots heat-fixed on glass slides, and the appropriate FISH stains utilized for microbial visualization. For example, cultures of *H. influenzae*, heat-fixed to the slides, were stained by the *H. (influenzae)* flu-specific probe. As a negative control, each FISH probe was incubated with cultures of bacteria that were not specific to the individual stains. For these experiments, broth cultures were heat-fixed as above and then challenged with FISH probes that were not directed against that organism. For example, *H. influenzae* cultures were challenged with the other FISH probes, including the fungal probes, and no staining was observed. These controls ensured that the FISH probes utilized in this study were specific to each organism. Biofilm samples were heat-fixed on glass slides, and a solution of hybridization buffer, DAPI, and the specific FISH probes was added to each sample. The mucosal sinus samples were then incubated either at 45°C for 2 hours or 37°C overnight in a humidified hybridization chamber. The slides were removed after incubation and washed 3× with 1× SET buffer at the hybridization temperature, then allowed to air dry. The slides were next examined for expression of FISH fluorescence by a Leica DMIRE2 (Wetzler, Germany) inverted automated Z-scanning epifluorescent microscope. Species orientation within the biofilm was determined, and relative abundance from the fluorescent signals was noted. Interpretation of the FISH slides with the epifluorescent microscope was based on fluorescence of tagged segments of bacterial DNA; the

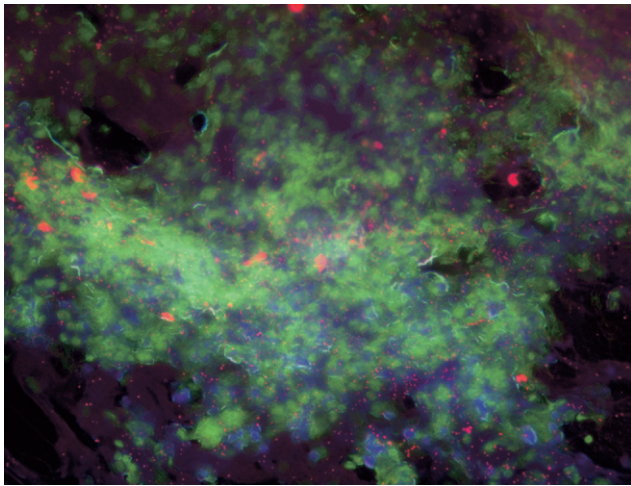


Figure 1 Pseudocolor overlay of three fluorescent in situ hybridization (FISH) epifluorescent (40X) images from Patient 7 (EMCRS). *Haemophilus influenzae* biofilm is stained red (Cy-3 probe); fungal elements invested within this biofilm are stained green (6-FAM); epithelial cells and inflammatory cells are stained with a blue DAPI stain.

bacteria fluoresced intensely, showing individual bacteria and clusters as seen in biofilm formation. The pan-fungal FISH probe tagged fungus-specific DNA and brightly fluoresced in areas of high fungus density. It did not, however, show the characteristic morphology of fungus. The glycopolysaccharide of the biofilm also contained a more dilute amount of the specific DNA, producing a halo or blush of fluorescence, which defined the form of the biofilm containing the protected bacteria. This established the presence of biofilm specific to the stained bacteria.

Outcome

The primary outcome was the presence of biofilm on the sinus mucosa samples. The criteria for classifying a biofilm

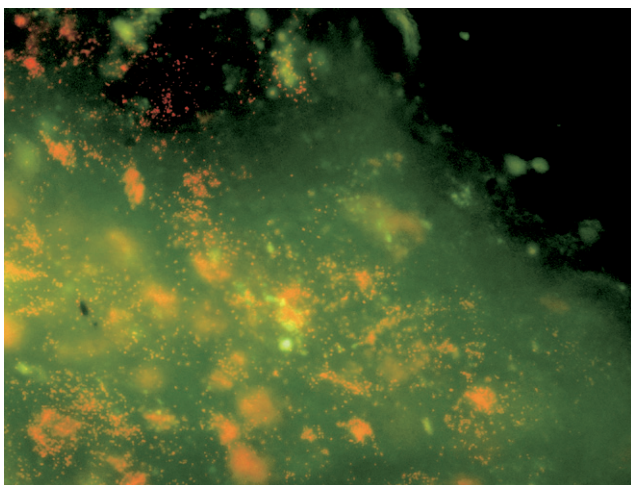


Figure 2 Pseudocolor overlay of two images from patient 6 (LK) (EMCRS). *H. influenzae* biofilm (red) with robust fungal elements (green) at the mucosal interface.

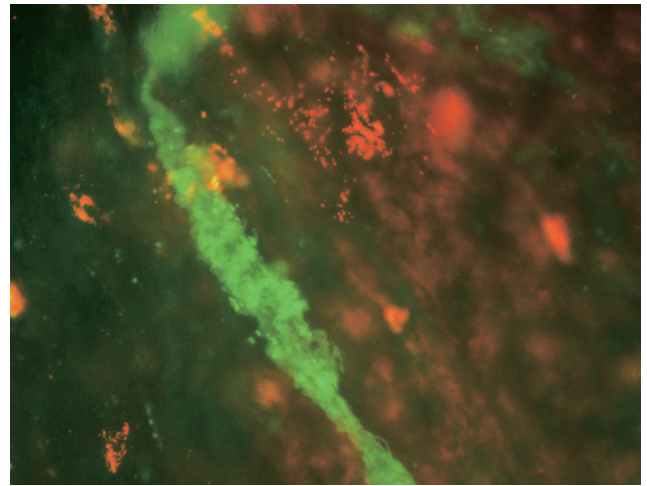


Figure 3 Pseudocolor overlay of two images from patient 5 (LH) (EMCRS). *H. influenzae* biofilm (red) with streak of fungal elements invested with biofilm.

by FISH/epifluorescent microscopy were the presence of the characteristic bacteria morphology, microcolonies, surrounding polysaccharide blush, and tower formation. Fungal elements that were structurally invested in high concentration in bacterial biofilm were graded as “+++,” but it cannot be claimed that the fungus represented was an independent biofilm.

RESULTS

Most chronic rhinosinusitis samples demonstrated bacterial biofilm with fungal elements (Figs 1-3). Abundant bacterial biofilm formation was evident on all three of the AFRS samples. Three out of four of the EMCRS samples showed

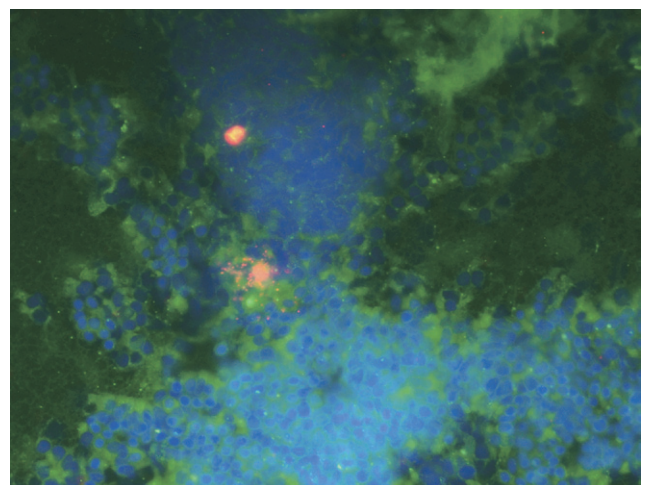


Figure 4 Pseudocolor overlay of different mucosal area of Patient 13 (CRS). *H. influenzae* microcolonies (red) with fungal elements (green). This is an example of what would be classified as “microcolonies without biofilm ultrastructure.”

abundant bacterial biofilm, and one out of four CRS (without eosinophilia) samples showed abundant biofilm. Of the 11 patients sampled, nine demonstrated bacterial biofilm. Smaller arrays of bacterial microcolonies were demonstrated on the remaining one EMCRS sample and on two out of four CRS samples (Fig 4). Only one specimen in this study (CRS sample) demonstrated zero evidence of biofilm formation (Table 1).

The majority of biofilm was *H. influenzae* (eight of nine). Some samples demonstrated a heterogeneous, patchy array of biofilm, with one type of bacteria represented in one area and another represented elsewhere in the same sample. A close relationship between microcolonies of different bacteria was

not shown. One sample demonstrated a biofilm without any *H. influenzae* (Patient 11 had a *P. aeruginosa* biofilm only; Fig 5).

Fungal elements were also richly demonstrated in this study. Seven of 11 patients demonstrated significant fungal FISH staining within bacterial biofilms. Just one sample showed no evidence of fungus. The allergic mucin of one of the AFRS patients showed robust fungal and bacterial elements (Fig 6). Two polyp samples from patients with AFRS showed only sparse fungal elements and robust bacterial biofilm formation. There was a trend to greater fungal element presence in the EMCRS samples over the CRS samples. Because of the small sample size, a statistical difference was not sought.

Table 1
Tissue FISH/epifluorescent microscopy results with culture results

Patient	Histologic subtype	Polyps	Fungal elements	<i>H. influenzae</i>	<i>S. pneumoniae</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	Culture
1a	AFRS	yes	+++	+++	0	+	0	2+ <i>Staph aureus</i> / <i>Aspergillus niger</i>
1b	AFRS	yes	+++	+++	NC	0	NC	3+ <i>Staph aureus</i> /4+ <i>Enterobacter cloacae</i> /no fungus
2	AFRS	yes	+	+++	+	0	++	2+ <i>Citrobacter koseri</i> /no fungus
3	AFRS	yes	+	+++	0	0	++	4+ <i>Staph aureus</i> /no fungus
4	EMCRS	yes	++	0	0	+	0	1+ coag neg <i>Staph sp.</i> /no fungus
5	EMCRS	yes	+++	+++	0	++	0	2+ coag neg <i>Staph sp.</i> /2+ alpha strep/no fungus
6	EMCRS	no	+++	+++	0	0	0	4+ <i>Pseudomonas/Fusarium sp.</i>
7	EMCRS	yes	+++	+++	+++	0	0	2+ <i>Diphtheroides</i> /2+ <i>Staph sp.</i> /1+ <i>Enterobacter aerogenes</i> /1+ <i>Citrobacter freundii</i> /no fungus
8	CRS	no	0	0	0	0	0	None
9	CRS	yes	++	+++	0	+	0	None
10	CRS	no	++	+	0	0	0	4+ <i>Staph aureus</i> /no fungus
11	CRS	yes	+	+	+	0	++	2+ normal nasal flora/no fungus
12	CRS*	no	+++	+++	0	+++	0	3+ coag neg <i>Staph sp.</i> /2+ <i>Acinetobacter spp.</i> /2+ <i>Pseudomonas spp.</i> /no fungus
13	Control	no	0	++	0	0	0	None
14	Control	no	0	++	0	0	0	None
15	Control	no	+	0	++	0	0	None

Fungal Elements in Biofilms
 +++ Abundant fungal elements
 ++ Scattered fungal elements
 + Sparing fungal elements
 0 No fungal elements

Bacterial Biofilms on Sinus Mucosal Samples
 +++ Abundant microcolonies with biofilm ultrastructure
 ++ Scattered microcolonies with biofilm ultrastructure
 + Sparing microcolonies of bacteria without biofilm ultrastructure
 0 No bacteria present
 NC Not counted

*Histologic subtype not available.

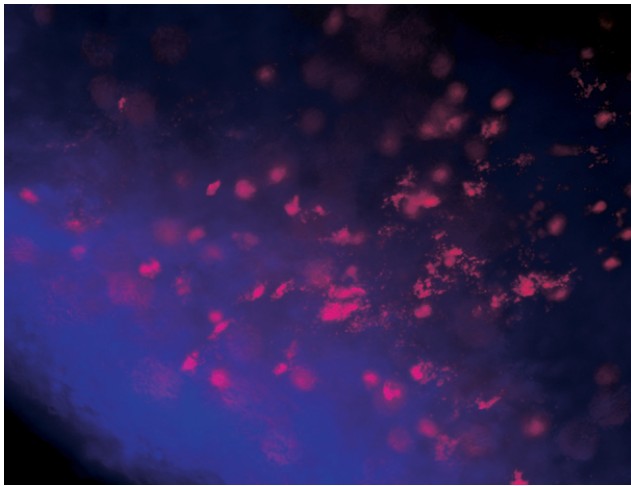


Figure 5 Pseudocolor overlay of Patient 13 (CRS). *Pseudomonas aeruginosa* biofilm without fungal elements closely associated with epithelial layer of mucosa. When found, *P. aeruginosa* biofilm had this characteristic layered appearance.

Of three controls, two demonstrated *H. influenzae* biofilm and one had *S. pneumoniae* biofilm. One control sample demonstrated sparing fungal elements.

DISCUSSION

In a review of the literature at the time of this submission, this appears to be the first report to identify fungal elements within bacterial biofilms of chronic sinusitis and is also the first to demonstrate biofilm in AFRS. Our research reinforces our previous work and studies done by other researchers that biofilm is present—and may play an important role—in the pathophysiology of CRS.¹⁴⁻¹⁶

The technique used to demonstrate biofilm in this study warrants explanation. The standard method by which we traditionally analyze mucosal samples in CRS is by hematoxylin and eosin staining and special stains for fungal elements (gomori-methamine, PAS, and others). Additionally, the presence of bacteria and fungus is determined by various methods of culture (swab, irrigation, or aspiration of sinus contents). By using these methods, the presence of biofilm will go unnoticed. Staining techniques wash away or destroy a biofilm matrix before adequate staining of the bacteria residing within it. Further, culture techniques are generally inadequate to discover viable bacteria living within a biofilm matrix, as this technique relies on the swab or irrigation to break off sections of biofilm that is attached and then is dependent upon it to grow planktonically. Bacteria in a biofilm usually will not grow in culture: a state called “viable, but not culturable.” This is because to change to a planktonic phenotype, bacteria in a biofilm-producing phenotype require complex and specific environmental factors and signaling that are not available in a culture plate. The technique of tagging specific bacterial DNA with FISH

probes and imaging the samples with epifluorescent microscopy bypasses these difficulties. A strength of this technique is that specific bacteria can be visualized. Scanning electron microscopy cannot do this, and transmission electron microscopy can only suggest certain bacteria by morphologic characteristics. The disadvantage is that specific bacterial probes must be selected in advance, requiring educated guessing. Work is under way to make this selection more objective.

The extracellular polymeric substrate (the “glycocalyx”) that protects the bacteria is not directly visualized using FISH with epifluorescence. Released bacterial DNA can produce a blush of epifluorescence, but this is typically indistinguishable from other elements in a specimen. The true demonstration of biofilm using this technique is the three-dimensional ultrastructure of bacterial microcolonies with towers and water channels, which are visualized by focusing the microscope up and down. These features are better appreciated by confocal laser scanning microscopy (CLSM), which, through computer reconstruction, creates a three-dimensional image of the biofilm structure with the fluorescent staining of specific bacteria and fungi.

As in our previous work, the preponderance of biofilm was *H. influenzae*.⁵ Though the technique does not differentiate between types of *H. influenzae*, it is most likely nontypable *H. influenzae* (NTHI), based on extensive research demonstrating its presence as a nasopharyngeal colonizer and respiratory pathogen. NTHI is one of many bacterial species that have been implicated in CRS, but it is not heavily represented in studies of the microbiology of CRS. Why would *H. influenzae* (NTHI) be so heavily represented in our series and not be commonly cultured? A possible explanation is that NTHI biofilm is difficult to culture, and studies that rely on culture underrepresent its presence.

NTHI biofilm has many features that allow it to thrive in the chronic inflammatory milieu of CRS and AFRS.

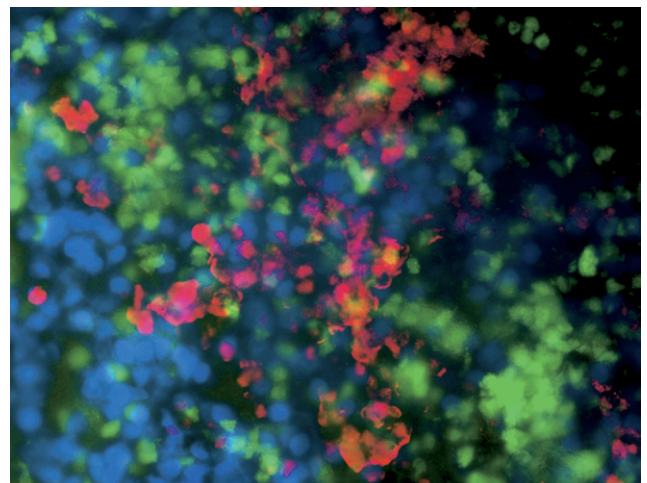


Figure 6 Pseudocolor overlay of allergic mucin from AFRS patient (Patient 1). *H. influenzae* biofilm (red), fungal elements (green), DAPI (blue) staining of host mucosal cells.

NTHI is a well-known colonizer of human respiratory epithelium and a common cause of respiratory illness and middle ear infection. NTHI can apparently live intracellularly in subepithelial mononuclear cells of hypertrophied adenoids¹⁷; it has features that allow it to persist in the airway as a colonizer, including adherence factors, IgA protease, and endotoxin, a complex lipo-oligosaccharide shell. NTHI endotoxin is highly variable and contains glycolipid that mimics host epitopes, blunting the host immune response.¹⁸ Adhesins bind ICAM-1 at the epithelial layer, facilitating NTHI attachment and persistence in epithelial inflammation.¹⁹ Horizontal gene transfer mechanisms account for a “supragenome” of NTHI strains of differing virulence and character that can persist together in a host, allowing for improved defense against environmental changes.²⁰ These highly specialized adaptations allow NTHI to thrive in the otherwise hostile environment of inflamed respiratory mucosa.

Perhaps the most important finding of this study is the common presence of fungal elements in bacterial biofilm; the nearly ubiquitous fungal elements in our series are a testament to the high sensitivity of the presented method of detection. Standard laboratory methods can fail to demonstrate fungus when it is, in fact, present by simply washing it away during processing, failing to stain it, or failing to identify it. By staining fungal-specific DNA, its detection is simplified by bypassing the need to see morphologic characteristics, such as fungal hyphae. The presented technique does not ascertain whether or not the fungal elements are alive and contributing to biofilm formation. It is possible that the mere presence of fungi, alive or dead, could contribute to the host inflammatory response in CRS patients, even when they do not demonstrate the hallmarks of allergic fungal disease.

The possibility of a fungal interaction with a bacterial biofilm may have important clinical implications. Though the imaging technique used in this study cannot definitively show fungal biofilm, fungus is known to create biofilm in both environmental and pathologic models, and mixed fungus-bacteria biofilm is known to exist in the environment. Microbiologists are only beginning to understand the complex interactions of a mixed fungal-bacterial biofilm and its host environment. It is theorized that fungus and bacteria may interact symbiotically to achieve metabolic needs and improve defense mechanisms.²¹ This study suggests this possibility in CRS.

Another interesting trend in this study is the greater preponderance of fungal elements appreciated in the EMCRS samples as compared to the CRS samples. Hypersensitivity to fungus has been theorized to play an important role in the robust eosinophilic response found in patients with EMCRS, even though it is not found in histologic analysis of the eosinophilic mucin. This study provides further evidence that fungus may indeed be an important factor in the pathophysiology of this subtype of CRS.

CONCLUSION

We believe this preliminary study to be the first paper to show fungal elements within bacterial biofilms of chronic rhinosinusitis. It is also the first to demonstrate bacterial biofilms in allergic fungal rhinosinusitis. This leads to several possible explanations for bacterial biofilm containing fungal elements. The fungus may incite a chronic inflammatory response in the host, which is ineffective in clearing the bacterial colonization. The other possibility is that biofilm is simply an opportunistic colonizer of inflamed mucosal surfaces, and it does not participate in the inflammatory response. Fungus and bacteria may interact in a symbiotic way that increases a biofilm's ability to survive host defenses and treatments. Further work needs to be done to determine the causal link between biofilm and the host inflammatory response (Table 1; Figs 1 to 6).

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