**Objective:** To determine the incidence of biofilm on the vocal fold epithelium of patients with chronic laryngitis. Bacteria can grow in biofilm protected by a glycoprotein mass. Recent studies have shown the importance of biofilm in chronic otolaryngologic infections. Because chronic laryngitis is often recurrent and occasionally purulent, we hypothesized that it is biofilm related.

**Design:** Prospective, controlled, blinded study. Epithelial biopsy specimens from true vocal folds from patients with chronic laryngitis undergoing diagnostic laryngomicroscopy were prepared for confocal scanning laser microscopy (CSLM) and polymerase chain reaction (PCR) microarray: CSLM images were evaluated for bacterial biofilm morphologic characteristics; PCR with microarray-based diagnostic assay was used to identify the bacterial species involved. Patients with vocal fold polyp served as control cases.

**Setting:** Tertiary care university hospital.

**Patients:** Eighteen consecutive patients were enrolled in the study. Thirteen of them had chronic laryngitis, and 5 had vocal fold polyps.

**Results:** In 9 cases, the CSLM revealed bacterial growth in biofilm form, and most of these specimens (8 out of 9) were from patients with chronic laryngitis. The PCR results were positive in 13 cases, including all 9 biofilm-positive cases.

**Conclusions:** Direct detection of biofilm in laryngeal biopsy specimens from patients with chronic laryngitis supports the hypothesis that chronic laryngitis may be biofilm related. Biofilm was found in 62% of the cases of chronic laryngitis. To our knowledge, this is the first report of bacterial biofilm associated with chronic laryngitis; however, further investigation is warranted before a clear conclusion can be drawn.


Bacteriology is based on studying free-floating planktonic bacteria. However, bacterial growth can also occur in biofilm form where large communities of bacteria, protected by self-produced glycoprotein mass, live attached to a surface. Several mechanisms protect the bacteria in biofilm against antibiotics and host defense, and biofilm tends to act as a chronic reservoir for bacterial pathogens. Acute symptoms often recur shortly after antibiotic treatment, and long-term treatments and/or high doses of antimicrobial agents may be needed to eradicate the biofilm. In several otolaryngologic diseases, bacterial biofilm plays an important role. Biofilm has been associated with chronic infections of the upper respiratory tract such as chronic rhinosinusitis, chronic tonsillitis and chronically infected adenoid tissue. Typically, biofilm-related infections are recalcitrant and require intensive treatment, often including revision of chronically infected tissue, especially when implant materials are involved. Biofilm diagnosis is based on morphologic studies of the infected tissue using mainly confocal scanning laser microscopy (CSLM) and scanning electronic microscopy (SEM). Fluorescent in situ hybridization is often combined in the study, but this technique requires knowledge of the pathogen. The sensitivity and specificity of CSLM in biofilm detection make it perhaps the most objective technique available.

Acute laryngitis is a common and typically self-limiting laryngeal inflammation lasting a couple of weeks, and a purulent exudate is often seen. In chronic laryngitis, which is seldom considered an infectious process, signs and symptoms of laryngitis last for months. In cases where a purulent exudate is seen, bacterial samples are taken and antibiotic treatment is administered. In chronic laryngitis without pus, bacterial involvement should also be suspected when symptomatic treatment fails. In these cases, seemingly effective antibiotic treatment often leads to frustrating relapses. As laryngeal
malignancy and chronic infection cause similar symptoms, all measures should be taken to unravel the origin of the symptoms. Thus, laryngomicroscopy for biopsy specimens, bacterial and fungal samples, and samples for tuberculosis is recommended.

Although it is well known that biofilm causes problems in laryngologic biomaterials such as voice prostheses and tracheostomy tubes, we could not find any research articles concerning biofilm in laryngitis itself. Based on the nature of chronic laryngitis, we hypothesized that it is related to biofilm. To test the hypothesis, we investigated the incidence of biofilm in vocal fold epithelium in patients with chronic laryngitis resistant to antibiotic treatment. Patients with laryngeal polyps served as control cases.

METHODS

The study protocol was approved by the ethics committee of Helsinki University Central Hospital. Thirteen consecutive patients with longstanding chronic laryngitis with at least occasional suppuration who underwent laryngomicroscopy between May 2009 and February 2011 were invited to participate in the study. All of them accepted. Furthermore, 5 control cases with laryngeal polyps were enrolled in the study. The study protocol was approved by the ethics committee of Helsinki University Central Hospital. Before each study procedure, patients signed an informed consent. The patients had hoarseness with findings of laryngitis including edema, hyperemia, and occasional suppuration for a minimum of 12 months. They had all undergone at least 2 courses of antibiotic treatment with only short-term relief from their symptoms. The antibiotic treatments were mostly doxycycline, amoxicillin, or cephalaxin, often combined with fluconazole. Two patients with gastroesophageal reflux symptoms had also used proton-pump inhibitors without any notable effect on symptoms.

In all patients, the laryngomicroscopy was scheduled primarily to rule out a malignant process and to collect representative samples for microbiological diagnosis. A standard laryngomicroscopy with cold instruments was performed. One or several biopsy specimens were taken for histopathologic examination. In each case, bacterial, fungal, and tuberculous samples were taken along with 2 biopsy specimens of vocal fold epithelium for bacterial biofilm detection. One biofilm specimen was examined by CSLM for evidence of bacterial growth in the biofilm using BacLight LiveDead stain (Molecular Probes) to rinse away any planktonic bacteria and then stained with SYTO 9; Life Technologies) and stains dead or damaged cells red (propidium iodide).

To diagnose the biofilm, we carried out a morphologic analysis of the samples. Characteristic for biofilm is bacterial microcolony formation on the epithelial surface or even deep within the epithelium, exopolysaccharide formation, and mushroom-shaped tower formation. The entire depth of the epithelial sample was scanned using the CSLM tomographic z-stack technique. The bacteria were recognized by size (0.5-2.0 µm) and shape as well as by green fluorescent staining with SYTO 9 under CSLM. The epithelial sample was considered biofilm positive when a colony of immobile live bacteria was seen lying in thick colonies within the epithelium. The image analysis was performed by 3 independent observers blinded to the disease state of the patient (T.J.K., H.L., and T.H.).

DNA EXTRACTION

The samples were pretreated by adding 10 to 30 µL of 100 mM DTT (dithiothreitol) (Fluka Chemie GmbH) and incubating for 30 minutes at 37°C with 300 rpm agitation. Thereafter, 20 µL of proteinase K (Roche) was added to the samples and incubated for 1 hour and 50 minutes at 60°C with 300 rpm agitation. To enhance cell lysis, 20 µL of the lysis buffer (Arrow VIRAL NA kit) was mixed with the samples and incubated for 10 minutes at 60°C with 300 rpm agitation. After the lysis step, the initial sample volume was brought up to a volume of 200 µL with phosphate-buffered saline solution. DNA extraction was conducted using the Arrow VIRAL NA kit, the NorDiag Arrow device, and the Viral 010 program according to the instructions of the manufacturer (NorDiag). Elution volume was 100 µL. One negative extraction control was included in the test series.

ANALYSIS WITH THE PCR AND MICROARRAY-BASED PROVE-IT ASSAY

DNA samples were analyzed with the modified Prove-it Sepsis assay in conjunction with the StripArray Reader and the Prove-it Advisor analysis software (Mobidiag). The PCR reactions were conducted according to the instructions of the Prove-it Sepsis assay using the application of bone and joint infections. The amplified PCR products were hybridized onto the microarray, and the hybridization protocol was adapted from the instructions of the Prove-it Sepsis StripArray with slight modifications. To evaluate the reliability and success of the analysis and before accepting the result of a particular test series, we verified that the negative DNA extraction and negative PCR controls tested negative, and that the positive PCR control (DNA from methicillin-resistant Staphylococcus aureus) tested positive for both S. aureus and the mecA gene, a methicillin resistance marker.

DNA SEQUENCING

DNA sequencing was conducted on the PCR products that were amplified in the Prove-it assay, but which tested negative in the microarray analysis. The PCR products of 300 bp were run and extracted from the 2% agarose gel using the QiAquick Gel Extraction Kit (Qiagen). Sequencing was performed using cycle sequencing with Big Dye Terminator kit (version 3.1) supplied by Applied Biosystems (ABI), and the reactions were run on an ABI 3130XL capillary sequencer according to the manufacturer’s instructions. Sequences were edited and analyzed with the Vector NTI Advance (Invitrogen) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) programs using the ClustalW alignment algorithm version 1.4. The BLAST algorithm was used to search for homologous sequences in the European Bioinformatics database and the National Centre for Biotechnology Information database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
Eighteen patients were enrolled in the study. In 9 patients (8 with chronic laryngitis and 1 with polyps), the CSLM revealed bacterial growth in biofilm form. Bacterial cells were found either densely packed, demonstrating a typical 3-dimensional architecture of biofilm (Figure 1A), or in smaller amounts dividing (Figure 1B). No free-floating bacteria were found in the samples. However, biofilm growth was not uniform, and normal epithelium without biofilm was also found in all cases. Eight of thirteen patients with chronic laryngitis (62%) were found to be biofilm positive. The PCR-based assay found a pathogen in 13 cases, including all 9 biofilm-positive cases found to be biofilm positive. The microbiological analysis showed that all biofilm-positive cases tested positive by PCR. Also 3 biofilm-negative cases were PCR positive (38%). The bacterial population found was similar to the earlier findings in a study of bacterial flora of healthy larynx and chronic laryngitis. The amount of PCR-positive cases among controls is not surprising because it is known that a diverse flora of commensal bacteria resides in normal laryngeal surface. The fact that the bacteria of the aerodigestive tract can live in biofilm form without causing symptoms could explain the finding of bacterial biofilm in 1 of our laryngeal polyp cases with no history or findings of laryngeal infection.

Because no universal biofilm markers exist, the diagnosis of a biofilm infection is based on morphologic examination of the infected tissue. In this study, clinical diagnosis of biofilm was made using CSLM, which is the most reliable method to diagnose biofilm growth in live tissue samples. Owing to its tomographic characteristics, CSLM can provide an image for reliable 3-dimensional morphologic analysis of the sample, and the presence of biofilm even within deep layers of the tissue can be detected. Samples can be studied fully hydrated without excessive preparation, which is an advantage over electron microscopy. Furthermore, CSLM preserves the diagnostic 3-dimensional architecture of the biofilm because it avoids the dehydration that occurs in SEM. A major challenge in biofilm diagnostics in laryngitis is collecting the samples without using general anesthesia.

Our findings indicate that the inflamed epithelium itself can provide a substrate for chronic biofilm infection. Biofilm works as a bacterial reservoir and results in relapses of acute symptoms after an adequate antimicrobial treatment. Because chronic laryngitis is often resistant to antibiotic treatment and seems to be associated with bacterial growth in biofilm, consultation with an infectious disease specialist should be considered. One treatment option is a long-term broad-spectrum antibiotic regimen combined with mucolytic agents. To help the mucolysis, all measures to avoid tissue dehydration should be applied. In our opinion, CSLM for biofilm imaging should be considered whenever a patient with chronic laryngitis is scheduled for laryngomicroscopy.

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<th>Table. Isolated Pathogens in Biofilm-Positive and Biofilm-Negative Cases</th>
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<td><strong>Biofilm Positive</strong></td>
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<td>Candida albicans</td>
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<td>Moraxella nonliquefata</td>
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<td>Neisseria meningitidis</td>
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<td>Haemophilus influenzae</td>
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<td>Staphylococcus aureus</td>
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<td>Streptococcus pneumonia</td>
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In this study, CSLM revealed bacterial biofilm of the vocal fold epithelium in 8 patients with chronic laryngitis (62%) and in 1 of 5 controls (20%). The biofilm findings of the chronically infected tissue were typically not homogenous, and biofilm-negative areas were found. Thus false negative findings can occur. The incidence of biofilm in this study was lower than that found in other chronic respiratory infections, such as chronic otitis media using SEM. However, it seems that SEM leads to a considerable risk of overdetection of the biofilm.

Figure 1. Confocal scanning laser microscopy images of biofilm formation in vocal fold epithelium. The samples were stained with BacLight LiveDead stain (Molecular Probes), a combination of propidium iodide and SYTO 9 (Life Technologies) dyes. The live bacteria in biofilm are stained with fluoro green, and the host cells with red. The bacteria are seen densely packed (A) or in division (B).

Figure 2. Association between chronic laryngitis, biofilm findings, and polymerase chain reaction (PCR) findings.
In conclusion, this study provides evidence that epithelial specimens from patients with chronic laryngitis may harbor bacterial biofilms. This supports the hypothesis that biofilms may play a role in the pathogenesis of chronic laryngitis. The clinical course of chronic laryngitis, and of biofilm infections in general, is often recurrent, and resistance to antibiotic treatment is evident. However, the true relationship between biofilm growth demonstrated on biopsy and the clinical symptoms clearly requires further investigation.

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Author Contributions: Dr Kinnari had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Kinnari and Aarnisalo. Acquisition of data: Kinnari, Lampikoski, Hyyrynen, and Aarnisalo. Analysis and interpretation of data: Kinnari, Lampikoski, Hyyrynen, and Aarnisalo. Drafting of the manuscript: Kinnari, Lampikoski, Hyyrynen, and Aarnisalo. Critical revision of the manuscript for important intellectual content: Kinnari, Lampikoski, Hyyrynen, and Aarnisalo. Statistical analysis: Kinnari. Obtained funding: Kinnari and Aarnisalo. Administrative, technical, and material support: Kinnari and Aarnisalo. Study supervision: Kinnari and Aarnisalo.

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