Confocal scanning laser microscopy in the study of biofilm formation in tissues of the upper airway in otolaryngologic diseases

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The full significance of bacterial biofilms in human chronic rhinosinusitis has yet to be determined. However, the corroboration of biofilms in vivo and the demonstration of the high capacity to form biofilms in vitro for all isolated bacteria, suggest a nexus between chronic rhinosinusitis and chronic inflammation or infection. Confocal scanning laser microscopy has proved a useful tool to analyze the structure of both the extracellular matrix exopolysaccharide and the cells that form the biofilms immersed in the tissue of the upper airway. Nevertheless, the possibility of viewing biofilms by an optical technique may also be helpful in ordinary practice in a global approach to these patients.

Keywords: biofilms; rhinosinusitis; optical microscopy; fluorescence microscopy; confocal scanning laser microscopy.

1. Introduction

Bacteria in nature exist in two states, as free-floating planktonic bacteria or as matrix-enclosed bacteria attached to a surface (biofilm). Since the first report on biofilms was published in 1943 by Zobell, a great amount of research has been carried out, principally in three broad areas: food, environmental and biomedical fields. Biofilms exist from a natural tendency to form a complex association of bacteria attached to an inert or living surface. These organized communities of bacteria collaborate among themselves and are composed of bacterial cells and an exopolysaccharide matrix. Consequently, the bacteria reside in a unique and protected microenvironment in the biofilm, which facilitates bacterial communication and survival [1]. The dynamic process of biofilm formation comprises two stages, with the first one involving the adhesion of free-living or planktonic bacteria to a surface. The second stage includes cell multiplication which subsequently causes microcolonies to develop and the formation of a mature structure consisting of many cell layers embedded in a self-produced extracellular polymeric matrix, whose main component is glycosaminoglycans [2].

The planktonic form of the bacteria has been very useful in the study of acute infections and is generally susceptible to host defense mechanisms and to antibiotic treatment whereas bacteria in biofilms are substantially resistant [3, 4]. Usually the chronic forms are found in the presence of biofilms, with current research indicating an important role for bacterial biofilms in recurrent or chronic infection, including those which are not responsive to a culture-appropriate antibiotic therapy [5]. Thus, biofilm formation represents a serious clinical problem, and it has been estimated that more than 65% of all human bacterial infections involve biofilms, which can be up to 1000 times more resistant to antimicrobial treatment than planktonic bacteria of the same species [6]. Based on recent studies that showed the presence of biofilms in common sites of chronic infections, it has become clear that bacteria may persist on mucosal surfaces due to biofilm formation [7]. In particular, biofilms are involved in chronic otitis, chronic tonsillitis, cholesteatomas and other inflammatory and infectious disorders [8, 9, 10, 11, 12], with the link between the concept of biofilms and chronic infectious disease being an ongoing subject of investigation.

The presence of biofilms on airway surfaces can only be observed using a limited number of techniques. The reason why the demonstration of mucosal biofilms is challenging is because it is difficult to stain both the bacteria and glyocalyx in human tissue. Furthermore, light and electron microscopy techniques require a dehydration process that reduces the total volume of the matrix and alters its architecture [13]. In order to achieve a fundamental understanding of the formation and presence of bacterial biofilms, the analysis should include detection of the bacteria and the matrix. The most common methods of assessing biofilm heterogeneity are direct microscopic imaging of local biofilm morphology or microscopic measurement of the local biofilm thickness. For many applications, time lapse microscopy using confocal scanning laser microscopy (CLSM) is an ideal tool for monitoring at a spatial resolution of the order of micrometers, and allows the non-destructive study of biofilms through an examination of all the layers at different depths, thus making it possible to reconstruct a three-dimensional structure [14, 15]. The detection of the matrix can be achieved using a double-staining technique in combination with CLSM, which allows the simultaneous imaging of the structural elements of mucosal biofilms (i.e., cells and glyocalyx) [11, 16].
2. Objective
The aim of this work was to determine and to characterize the presence of biofilms in tissues of the upper airway, using CSLM in a comparative form together with optical and fluorescence microscopy. Quantification of the in vitro biofilm development of the isolated bacteria was also carried out.

3. Materials and methods

Study Design and Population
The Ethics and Research Committees of Private Health Centers and the Faculty of Chemical Sciences, National University of Córdoba approved this study, with patients giving their written consent before donating tissue for the study. This work is a prospective observational study from patients with chronic rhinosinusitis (CRS), divided into two groups: adult patients with nasal polyps (No. 1-8) and children with chronic inflammation of the tonsils with obstructive hypertrophy, persistent cervical adenopathy, and recurrent upper airway pathology, with an age range between 1-6 (No. 9-20). Ten patients, scheduled for septoplasty for nasal obstruction and without a history or physical evidence of recurrent sinus infections or nasal discharge, served as the control group. Tissue samples were obtained from the inferior turbinate mucosa. None of these patients had received antibiotic therapy for at least one month prior to surgery.

Bacterial Isolation and Biofilm Quantification
The bacteriological study was performed by first scraping the surface of the tissue with a scalpel. This sample was then seeded in blood agar and thioglycollate broth before being incubated for 72 hours. Identification of the bacteria was determined using with classical biochemical tests and antimicrobial susceptibility testing by the disk diffusion method of Kirby-Bower.

The biofilm-forming ability was measured by evaluating their adhesion to polystyrene microtiter plates. The assay for biofilm formation used for this study was adapted from the method of O’Toole and Kolter [17], which is based on the ability of bacteria to form biofilms on solid surfaces and uses crystal violet (CV) to stain biofilms. Due to CV binding to negatively charged molecules, including nucleic acids and acidic polysaccharides, it thereby serves as an overall measure of the whole biofilm. Briefly, a final cell concentration of approximately $1 \times 10^6$ colony forming units (cfu) per ml was put with 200 µl of tryptic soy broth (TSB) into each well of flat-bottomed microtiter plates (96-well Greiner Bio-One, Germany), and incubated at 37 °C without agitation for 24 h. Negative control wells contained only TSB. The non-adherent bacteria were decanted from the wells, and any remaining planktonic cells were removed by three rinses with sterile distilled water (dH2O). After drying, staining for adherent biofilms was performed using CV (1%). Then, the CV was removed and cells were rinsed three times with 300 µl of phosphate buffer solution (PBS-pH 7.2) before drying for 24 h at room temperature. A quantitative assessment of the biofilm formation was obtained by extracting the CV with 200 µl/well of the bleaching solution ethanol/glacial acetone (70:30), and the intensity of the coloration was determined at 595 nm using a microplate reader (Model 680 BioRad, CA, USA) [18]. All strains were tested in three independent experiments on different days, and the average optical density (O.D.) at 595 nm was determined by four replicates and interpreted using the following scale: Positive (>0.24); weak (>0.12 and <0.24) or negative: (<0.12) [19].

Biofilm Research
The criteria for classifying bacterial biofilms in vivo by microscopy were: the presence of the characteristic bacterial morphology, micro and macro- colonies (for all three techniques: optical, fluorescence and CSLM) and surrounding polysaccharide blush with tower formation (only for CSLM) [17].

The biofilms present in the tissue obtained by surgery were identified by Gram staining with optical microscopy, by acridine orange with fluorescent microscopy, and with two fluorescence stainings (propidium iodide/ concanavalin A - conjugated fluorescein isothiocyanate) by CSLM.

The material was cut using a microtome, then Gram staining was carried out and an optical microscope (Carl Zeiss Axiosvert, Germany) was used for observation. To assess biofilms by fluorescence microscopy, 10 mg/mL of acridine orange solution (Molecular Probes, Eugene, Oregon) was used for 5 min at 37°C [20]. Stained cells were observed using an Axioplant-fluorescent microscope equipped with a digital camera (dxm 1200, Nikon, Japan).

Biofilms were observed by CSLM as described below [7]. Prior to imaging, the samples were rinsed with sterile 50 mM potassium phosphate buffer (pH 7.2; no autofluorescence detected) for 10 min and were then stained with 15µM propidium iodide (Sigma) for 5 minutes at room temperature in order to detect red bacterial cells. After being washed in PBS, the sections were incubated with 50 µg/ml of concanavalin A -conjugated fluorescein isothiocyanate (Con A-FITC) (Sigma) for 5 minutes at room temperature to stain the glyocalyx matrix green. The propidium iodide was excited at 520 nm, the emission was monitored at 620 nm, and Con A-FITC was excited and monitored at 495 nm and 525 nm, respectively. Intact biofilms were examined non-destructively using a Fluoview FV1000 Espectral Olympus.
CSLM (Olympus Latin America, Miami, FL, USA) equipped with a UPlanS Apo 100X/1.40 oil UIS2 Olympus oil immersion lens. Optical sections of 0.87 µm were collected from the complete thickness of the biofilms. Then, for each sample, images from three randomly selected positions were obtained and analyzed using an Olympus Fluoview FV 1000 [17].

**Statistical analysis**
All experiments were performed in triplicate, and numerical data are presented as means with error bars representing the standard deviations. The data were statistically analyzed by using a one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. Differences between means were assessed with a **p** value < 0.05 being considered statistically significant.

### 4. Results

**Biofilm Quantification in vitro**
The isolated microorganisms were *Staphylococcus aureus* in four patients, *Streptococcus viridians* in three, *Staphylococcus* coagulase negative and *Haemophilus influenzae* in two patients, and *Pseudomonas aeruginosa* and *Enterococcus faecalis* in one patient. Biofilms were also present as mixed complexes formed by combinations of *Streptococcus viridians-Corinebacterium*, *Haemophilus influenzae*, *Streptococcus viridians*, *Haemophilus influenzae*, *S. pneumoniae*, *Haemophilus influenzae-S. pyogenes*, *Haemophilus influenzae-S. coagulase negative*, *Haemophilus influenzae* and *Staphylococcus aureus*, with the latter being found in two patients.

In vitro studies of the isolated species demonstrated a biofilm-forming capacity in all of the bacteria isolates from patients with CRS. Table 1 shows that the UV-quantification by spectrophotometer of the isolated strains observed when stained with CV was generally positive, with a high capacity to form biofilms in vitro. Values of absorption ranged from 0.248 to 4.764 of O.D.\textsubscript{590nm} in 17 cases, with two on the border line between “Positive” and “Weak”, and one being “Weak”.

**Biofilm Research for optical, fluorescence microscopy and CSLM**
Biofilms were identified by Gram staining for optical microscopy (Fig. 1A), by fluorescence microscopy with orange acridine (Fig. 1B) and with two fluorescence staining (propidium iodide/ Con A-FITC) by CSLM (Fig. 1C). Immobile, irreversibly attached live bacteria in characteristic clusters and towers of micro and macrocolonies were found at varying degrees of density throughout the slides for all three methodologies. Some structures were present on the surface of the tonsils, whereas in others, they were located on the inner part of the invaginations.

![Fig. 1](image)

**Fig. 1** Comparison of the microscopy techniques: (A) optical and (B) fluorescence microscopy were used for the study of bacterial biofilms present in tissues of the upper airway.

Figure 2 shows bacterial cells and the surrounding glycocalyx matrix. Bacterial biofilm formation was present on the surface of crypts of tonsillar tissue of the patients, with some biofilms being mixed bacterial species.
Fig. 2 Mixed bacterial biofilm in tonsil tissue. (A) Optical microscopy and (B) CSLM. Bacterial cells stained red (propidium iodide), observed encased in a glycocalyx matrix, with green fluorescent staining (Con A-FITC) around the bacteria indicating the presence of glycocalyx (X 1000).

In Figure 3, it is possible to visualize bacterial cells and the surrounding glycocalyx matrix (which is indicative of bacterial biofilm formation) present on the surface of tissue by CSLM. Double staining was performed using propidium iodide and Con A-FITC. Bacterial cells and the nuclei of tonsil and polyp cells stained red and were easily identified by their size and morphologic features, despite the fact that the nuclei of tonsils and polyp cells also stained when using

TABLE 1: Bacterial Biofilm Results “in vitro”

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Isolated bacteria</th>
<th>Biofilm Quantification</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Streptococcus viridians</em></td>
<td>3.337 ±0.029</td>
<td>Positive</td>
</tr>
<tr>
<td>2.</td>
<td><em>Staphylococcus aureus</em></td>
<td>1.237 ±0.014</td>
<td>Positive</td>
</tr>
<tr>
<td>3.</td>
<td><em>Staphylococcus aureus</em></td>
<td>0.332 ±0.003</td>
<td>Positive</td>
</tr>
<tr>
<td>4.</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>0.956 ±0.062</td>
<td>Positive</td>
</tr>
<tr>
<td>5.</td>
<td><em>Streptococcus viridians-Corinebacterium</em></td>
<td>0.312 ±0.019</td>
<td>Positive</td>
</tr>
<tr>
<td>6.</td>
<td><em>Staphylococcus coagulasa negativa</em></td>
<td>0.316 ±0.019</td>
<td>Positive</td>
</tr>
<tr>
<td>7.</td>
<td><em>Staphylococcus aureus</em></td>
<td>0.428 ±0.012</td>
<td>Positive</td>
</tr>
<tr>
<td>8.</td>
<td><em>Enterococcus faecalis</em></td>
<td>0.428 ±0.017</td>
<td>Positive</td>
</tr>
<tr>
<td>9.</td>
<td><em>Staphylococcus aureus</em></td>
<td>1.552 ±0.055</td>
<td>Positive</td>
</tr>
<tr>
<td>10.</td>
<td><em>Staphylococcus coagulasa negativa</em></td>
<td>1.825 ±0.124</td>
<td>Positive</td>
</tr>
<tr>
<td>11.</td>
<td><em>Streptococcus viridians</em></td>
<td>0.872 ±0.146</td>
<td>Positive</td>
</tr>
<tr>
<td>12.</td>
<td><em>Streptococcus viridians – H. influenza</em></td>
<td>0.503 ±0.165</td>
<td>Positive</td>
</tr>
<tr>
<td>13.</td>
<td><em>Streptococcus viridians</em></td>
<td>0.173 ±0.038</td>
<td>Weak</td>
</tr>
<tr>
<td>14.</td>
<td><em>H. influenzae – S. pneumoniae</em></td>
<td>0.248 ±0.001</td>
<td>Positive / Weak</td>
</tr>
<tr>
<td>15.</td>
<td><em>H. influenzae</em></td>
<td>0.912 ±0.356</td>
<td>Positive</td>
</tr>
<tr>
<td>16.</td>
<td><em>H. influenzae</em></td>
<td>4.764 ±0.453</td>
<td>Positive</td>
</tr>
<tr>
<td>17.</td>
<td><em>H. influenzae – S. pyogenes</em></td>
<td>0.248 ±0.043</td>
<td>Positive / Weak</td>
</tr>
<tr>
<td>18.</td>
<td><em>H. influenzae – S. coagulasa negativa</em></td>
<td>4.600 ±0.388</td>
<td>Positive</td>
</tr>
<tr>
<td>19.</td>
<td><em>H. influenzae – Staphylococcus aureus</em></td>
<td>1.053 ±0.085</td>
<td>Positive</td>
</tr>
<tr>
<td>20.</td>
<td><em>H. influenzae – Staphylococcus aureus</em></td>
<td>0.785 ±0.001</td>
<td>Positive</td>
</tr>
</tbody>
</table>
propidium iodide. We used Con A to detect sugar residue present in the glycocalyx. Con A-FITC binds to mannose residues, resulting in green staining and indicating the presence of a bacterial glycocalyx. Although, we observed the marked colocalization of green Con A-FITC staining with clusters of bacterial cells, the staining of the matrix was not homogeneously distributed. The presence of dark areas within the biofilm can be explained by the existing water channels, the heterogeneous production of the matrix and the types of exopolysaccharides within the biofilm, as well as the absence of Con A-FITC binding to the matrix. Isolated planktonic bacteria were found in the controls, but no biofilm structures were evident in any of these. Taken together, our data show that the CLSM analysis of stained tissue sections provided a good technique to demonstrate the presence of mucosal biofilms in human tissue of the upper airway.

Fig. 3 Confocal laser scanning microscopy image sequences demonstrating bacterial biofilms on human tonsils, from the surface to the base of the biofilms. Single bacterial cells are stained red (propidium iodide). Green fluorescent staining (Con A-FITC) around bacteria indicates the presence of glycocalyx. Most of the bacteria were cocci shaped, with interconnected bacteria being encased in a scaffolding network composed of an extracellular matrix, suggesting a 3-dimensional architecture of biofilm formations. (X 1000).

5. Discussion

New evidence shows that biofilms are important in human disease in general, and especially in those of the head and neck. The last decade has seen an increase in the specific (although still relatively scant) literature describing the role of biofilms in otolaryngological disease, with it also becoming increasingly clear that biofilms play an important role in chronic otitis media, chronic tonsillitis and cholesteatoma [7, 9, 10, 11, 21], and also in otolaryngologic devices such as endotracheal tubes, tracheostomy tubes, voice prostheses, and tympanostomy tubes, all of which have been shown to provide surfaces for the rapid formation of biofilms [22, 23, 24].

Several indirect methods for the measurement of biomass or biofilm thickness are reported in the literature. Christensen et al. [25] related O.D., readings obtained from a 96-well plate to concentrations of bacteria in a biofilm covering the well floors. Their method allows for the rapid screening of biofilm formation potential for different bacterial species or environmental conditions. However, the biofilms in well plates are grown under no-flow conditions, which are not representative of most relevant natural and engineered systems. In addition, the well plate reader is unable to provide spatially resolved data. All the O.D. based methods share the advantage that measuring the overall biomass is rapid and relatively simple.

In many research areas, it is the scale of observation that determines what the observer considers to be reality, and biofilm research is no exception to this rule [26]. Several authors [27, 28, 29, 30] have described biofilms in chronic sinusitis using gram staining and transmission electron microscopy, techniques that require a dehydration process, due to the cells present in a biofilm being usually encased in a hydrated (typically 95%-99%) matrix. Therefore, when using
scanning electron microscopy (SEM) or transmission electron microscopy, only the remnants of the original hydrated structure can be visualized. In contrast to electron microscopy, preparation of tissue for CLSM does not require dehydration and thus the structure of a biofilm is better preserved. Related to this, Kania et al [11] demonstrated that CLSM with double staining was superior to SEM for showing the presence of human mucosal biofilms in tonsillitis. In our study, the CLSM revealed live bacteria in cellular towers composed of exopolysaccharide enclosed microcolonies separated by open water channels that acted as a primitive circulatory system for the delivery of nutrients and the removal of metabolic waste products. These microcolonies and open water channels were found to be structural features consisting of many single and mixed species biofilms grown in vitro, observed in the tissues of the upper airway.

The optical microscopy technique is simple, accessible and affordable in terms of reagents and the microscopes, which are present in all microbiology laboratories, but has the drawback that it is very difficult to observe microcolonies of biofilms. Although it is easier to find biofilms using fluorescence microscopy, this method is more expensive. Therefore, CLSM has become the gold standard technique for the study of biofilms, as the combination of fluorescent dyes enables the differential study of sessile cells and the matrix that forms the biofilm exopolysaccharide, making it possible to create a three-dimensional reconstruction. Furthermore, CLSM has proved a useful tool when analyzing the structure of both the extracellular matrix exopolysaccharide, and the cells that form biofilms immersed in the tissue of the upper airway. However, this technique is expensive and requires specialized equipment that is not routinely available. Therefore, the possibility of viewing biofilms with an optical technique may also be helpful in ordinary practice, within the context of attempting to provide a global approach for these patients. Our results have shown that there is strong in situ morphologic evidence for the existence of biofilms in CRS, and that the presence of biofilms offers an explanation for many of the clinical characteristics of infected tissues. Further studies should be carried out focusing principally on the prevention of biofilm development within the pharyngeal associated lymphoid tissues, considering that once the biofilms are installed in the respiratory mucosa they are very difficult to remove.

6. Conclusions

The pathology and symptoms of patients who develop chronic rhinosinusitis might be directly related to the presence of biofilms, but the full significance of bacterial biofilms in the human chronic rhinosinusitis has still to be determined. The corroboration of the presence of biofilms “in vivo” and the demonstration of the high capacity to form biofilms “in vitro” for isolated bacteria suggest a nexus between chronic rhinosinusitis and chronic inflammation or infection. This may indicate that bacteria are protected from host defenses by biofilms, and are able to continue their metabolism and local production of exotoxins, which in turn favors the chronicity of the inflammatory response, as evident by the changes observed in the respiratory mucosa and persistent infections that do not respond to antibiotic treatments.

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