

Identifying intracellular *Staphylococcus aureus* in chronic rhinosinusitis: A direct comparison of techniques

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ABSTRACT

Background: The emerging concept of intracellular pathogens such as *Staphylococcus aureus* playing a role in chronic rhinosinusitis (CRS) has led to the development of numerous imaging techniques for their identification. Traditional methods of bacterial culture are not effective at localizing bacteria to the surface or within tissue samples. The aim of this study was to develop and validate a novel imaging technique using confocal scanning laser microscopy (CSLM) coupled with a fluorescence in situ hybridization (FISH) probe and nucleic acid counterstain (propidium iodide [PI]) that allows for simultaneous analysis of *S. aureus* intracellular status and surface biofilm within whole mucosal samples.

Methods: A prospective study was performed including 17 patients undergoing endoscopic sinus surgery for CRS. Tissue samples were analyzed with both CSLM-FISH/PI and immunohistochemistry (IHC) for intracellular *S. aureus* status.

Results: Using CSLM-FISH/PI intracellular *S. aureus* was identified in 9/17 (47%) patients and in 7/17 (39%) using IHC. Surface biofilm can be identified with CSLM-FISH/PI in the same piece of tissue; however, deeper imaging to the submucosa is impossible. IHC showed submucosal bacteria in three patients.

Conclusion: Both CSLM-FISH/PI and IHC are complementary techniques that can be used to identify intracellular *S. aureus*. CSLM-FISH/PI allows for the simultaneous detection of intracellular status and surface biofilm within the tissue analyzed. IHC has a role in the identification of intracellular and submucosal *S. aureus* within these tissues. Additional investigation is required to identify the true pathogenic nature of intracellular organisms as well as any relationship to surface biofilm status.

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Chronic rhinosinusitis (CRS) remains a complex, poorly understood disease characterized by relapsing episodes of inflammation within the sinonasal cavities. Although medical treatment and endoscopic sinus surgery (ESS) can be successful in eradicating the disease burden,¹ a significant number of cases fail such treatment and develop recalcitrant disease. *Staphylococcus aureus* has been identified as a prominent pathogen in the disease process.² In particular, patients with *S. aureus* biofilms at the time of ESS have significantly worse preoperative symptoms, intraoperative surgical findings, and a higher rate of recalcitrant disease.³ Identification techniques for *S. aureus* biofilm status has been optimized within our department by using confocal scanning laser microscopy (CSLM) coupled with peptide nucleic acid–fluorescence in situ hybridization (PNA-FISH) probes in both the CRS patients⁴ and in an animal model of sinusitis.⁵

Despite recent interest in the role of bacterial biofilms in CRS, it is but one virulence mechanism adopted by organisms such as *S. aureus* in chronic disease. Intracellular detection of *S. aureus* has also been reported in CRS patients and may represent a bacterial phenotype with clinical relevance. It is recognized that nonprofessional phagocytic cells such as epithelium can internalize live *S. aureus*,⁶ and recent studies have identified its role in certain chronic disease processes including osteomyelitis,⁷ mastitis,⁸ and CRS.⁹ The first study identifying intracellular *S. aureus* in CRS patients used immunohistochemistry (IHC) on paraffin-embedded tissue sections⁹ with intracellular bacteria were found in 17/27 (63%) CRS patients. The authors fol-

lowed a cohort of intracellular *S. aureus*-positive patients who also cultured the organism from the middle meatus at the time of surgery. They noted a higher clinical relapse rate with persistent infection at 1 year, which was confirmed to be the same bacteria by clonal typing. PNA-FISH probes have been used in paraffin-embedded tissue sections reporting intracellular-positive rates of between 32 and 57%.^{10,11} Despite this evidence for intracellular organisms, the true pathogenic nature still remains to be proven and should be a focus for further research.

Both IHC and FISH techniques provide useful information on intracellular bacterial localization when analyzed; however, a major limitation is that it is difficult to accurately assess the biofilm status of a patient from single tissue slices. This is in comparison with whole mucosal samples taken at the time of surgery where biofilm status and potentially intracellular detection can be simultaneously achieved. In achieving a holistic understanding of microbial interactions with the host in CRS it is likely that the interplay of surface biofilm and intracellular residence will hold pathogenic importance. Their codetection in sinus mucosal specimens will be an important step in understanding the link between the two.

Therefore, the aim of this study was to validate a new method of intracellular *S. aureus* detection that has the capability for simultaneous biofilm analysis using CSLM coupled with PNA-FISH and a nucleic acid counterstain, propidium iodide (PI), and compare it with a recognized technique of IHC in tissue sections within the same cohort of patients as well as highlight the technical aspects of each modality.

METHODS

Study Design and Patient Population

This prospective, blinded study was completed in the tertiary rhinologic practice of the senior author (P.J.W.). The institution's Human Research Ethics Committee gave their approval for the study to be performed and all subjects provided informed consent to participate in the study. The study group consisted of 17 patients undergoing ESS with a diagnosis of CRS as per the Rhinosinusitis Task Force^{12,13}

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criteria. Exclusion criteria included age of <18 years; those who suffered from any form of immunodeficiency such as cystic fibrosis; Kartagener's syndrome, and primary ciliary dyskinesia; or those who had clinical or radiological evidence of invasive disease.

Data Collection

Preoperative data were collected on the day of surgery including age, gender, number of previous sinus surgeries, history of asthma or smoking, allergy status, and preoperative CT scores (Lund-McKay). Clinical symptom scores for five major symptoms of CRS (nasal obstruction, rhinorrhea, postnasal drip, headache or facial pain, and sense of smell) were also recorded on a 0–5 scale and a total out of 25 recorded. No patient had taken any antibiotic, antifungal, systemic steroids, or other immunosuppressant medication in the 3 weeks before surgery.

Perioperative data were collected during the subject's surgery for polyp status, presence of mucopus or eosinophilic mucous, and condition of mucosa (normal, edematous, or polypoid). All patients had mucosal samples sent for histological analysis as well as samples for routine microbiological and fungal culture analysis. Bacterial culture was performed using standard laboratory techniques (Adelaide Pathology Partners, Mile End, SA).

Tissue Collection and Transport

All sinus mucosa specimens were collected at the time of surgery and immediately placed into Dulbecco's modified eagle's medium (Gibco, Invitrogen Corp., Carlsbad, CA) and transported to our laboratory. Under sterile conditions, samples were washed thoroughly in MilliQ water (Millipore, Billerica, MA) and dissected free of any underlying bone. Mucosal samples for CSLM-FISH/PI analysis were frozen to -80°C , and samples for IHC were placed into 5 mL of 10% buffered formalin (Sigma-Aldrich, St. Louis, MI) and embedded in paraffin.

Confocal Scanning Laser Microscopy–Fluorescence *in situ* Hybridization/Propidium Iodide

The bacterial species-specific FISH probe used in this study has been validated by our department as an effective method of *S. aureus* biofilm detection.⁴ Briefly, approximately single 4×4 -mm whole mucosal samples were washed in MilliQ water and heat fixed to glass microscope slides (Superfrost; Thermo Fisher, Waltham, MA) for 20 minutes in a 55°C incubator, dehydrated in 90% ethanol and air-dried. The *S. aureus* FISH probe (AvanDX, Woburn, MA) was applied to the mucosa and hybridization occurred for 90 minutes at 55°C . Slides were washed in the manufacturer's wash solution for 30 minutes. Subsequently, tissue was counterstained with 70 μL of PI (Sigma-Aldrich) in glycerol at a concentration of 50 $\mu\text{g}/\text{mL}$ to clearly identify cellular nuclei and was prepared for microscope analysis.

Immunohistochemistry

Paraffin-embedded tissue was prepared into 5- μm sections and mounted on glass slides (Superfrost). The sections were deparaffinized and rehydrated using graded xylene and ethanol. Slides were immersed in 10-mM sodium citrate and antigen retrieval was performed using a microwave technique. Once the slides had cooled they were transferred into PBS, marked with a PAP pen (Abcam, Cambridge, U.K.) and subsequently into a Tris-buffered saline and Tween-20 buffer (TBST). Serum-free protein block (Dako, Glostrup, Denmark) was applied for 1 hour at room temperature and slides were incubated simultaneously with mouse anti-*S. aureus* (Millipore) at a concentration of 1/50 and rabbit anticytokeratin pan (PAN) antibodies (Invitrogen Corp.) at a concentration of 1/100 overnight at 4°C . Slides were washed in TBST four times on a horizontal shaker and secondary antibodies (goat anti-mouse IgM-Alexa488 [1/100; Invitrogen Corp.]; sheep anti-rabbit IgG-Cy3 [1/250; Sigma-Aldrich])

were applied for 1 hour at room temperature in a darkened moisture chamber. Finally, slides were washed in TBST five times on a horizontal shaker and coverslips were mounted in 90% glycerol for microscope analysis. Positive control sections were prepared by incubating 5×5 -mm pieces of nasal mucosa harvested from non-CRS patients with *S. aureus* ATCC 25923 for various time points and fixed in formalin before being embedded in paraffin and sectioned as per the protocol. Negative control slides were prepared identically using normal control rabbit IgG (Sigma-Aldrich).

Tissue Analysis

Slides prepared for CSLM-FISH/PI were analyzed with a Leica TCS SP5 Confocal Laser Microscope (Leica Microsystems, Wetzlar, Germany). Multiple consecutive images were taken in the axial z-axis of tissue samples with a slice thickness of 0.5 μm . The tissue was assessed for evidence of *S. aureus* visualized as bright green coccal structures fluorescing on stimulation with a 488-nm laser.¹⁴ PI was stimulated with a 561-nm laser and epithelial nuclei were identified as bright red spheric areas with a size of roughly 10 μm . Furthermore, PI also stains *S. aureus* and thus when the two channels were merged, true *S. aureus* colocalized between the two stains and appeared bright yellow. Samples were categorized as intracellular positive if *S. aureus* could be identified at least one z-plane and closely localized adjacent to intraepithelial nuclei. Analysis for biofilm status was also made as per a validated protocol¹⁴ using the same tissue sample. Once the intracellular plane was identified through the PI stain, the microscope stage could be digitally manipulated to bring the field of focus into a z-plane that is located more superficially to the cells to identify biofilm as clusters and towers of immobile, irreversibly attached coccal shaped bacteria, ~ 0.5 – $2 \mu\text{m}$ in size.¹⁵ IHC slides were analyzed with a Zeiss Axio Vert.A1 (Carl Zeiss AG, Jena, Germany). Positive *S. aureus* cocci could be visualized as bright green fluorescing dots within nasal epithelium outlined by cytokeratin stained in red. Although nuclei were not counterstained, a cytokeratin void could clearly be seen in the correct morphology and dimensions of an epithelial nucleus. Microscope images were collated, deidentified, and analyzed by three independent, blinded observers for their surface biofilm and intracellular *S. aureus* status.

Statistical Analysis

Results of this study were analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA) with nonparametric data were displayed as a median with interquartile ranges (IQRs). Dichotomous data were analyzed using Fischer's exact test with statistical significance accepted when $p < 0.05$. Interrater agreement was assessed using Minitab 16 Statistical Software (Minitab, Inc., State College, PA) with a Fleiss's κ calculated.

RESULTS

Demographics and Preoperative Data

Seventeen patients undergoing ESS for CRS were enrolled in this study (nine men and eight women). Mean age was 45.5 years (IQR, 30–57). Six patients had a history of asthma and none were current smokers. Median symptom score was 18 (IQR, 13.5–20) and median Lund-Mackay score was 10 (IQR, 4–15).

Intraoperative Data

Five patients underwent minor surgery in the form of mini-ESS (middle meatal antrostomy and anterior ethmoidectomy) and 12 underwent major surgery classified as either modified endoscopic Lothrop/Draf 3/frontal drillout procedure or complete sphenoid ethmoidectomy and frontal sinusotomy. Seven patients had nasal polypoidosis.

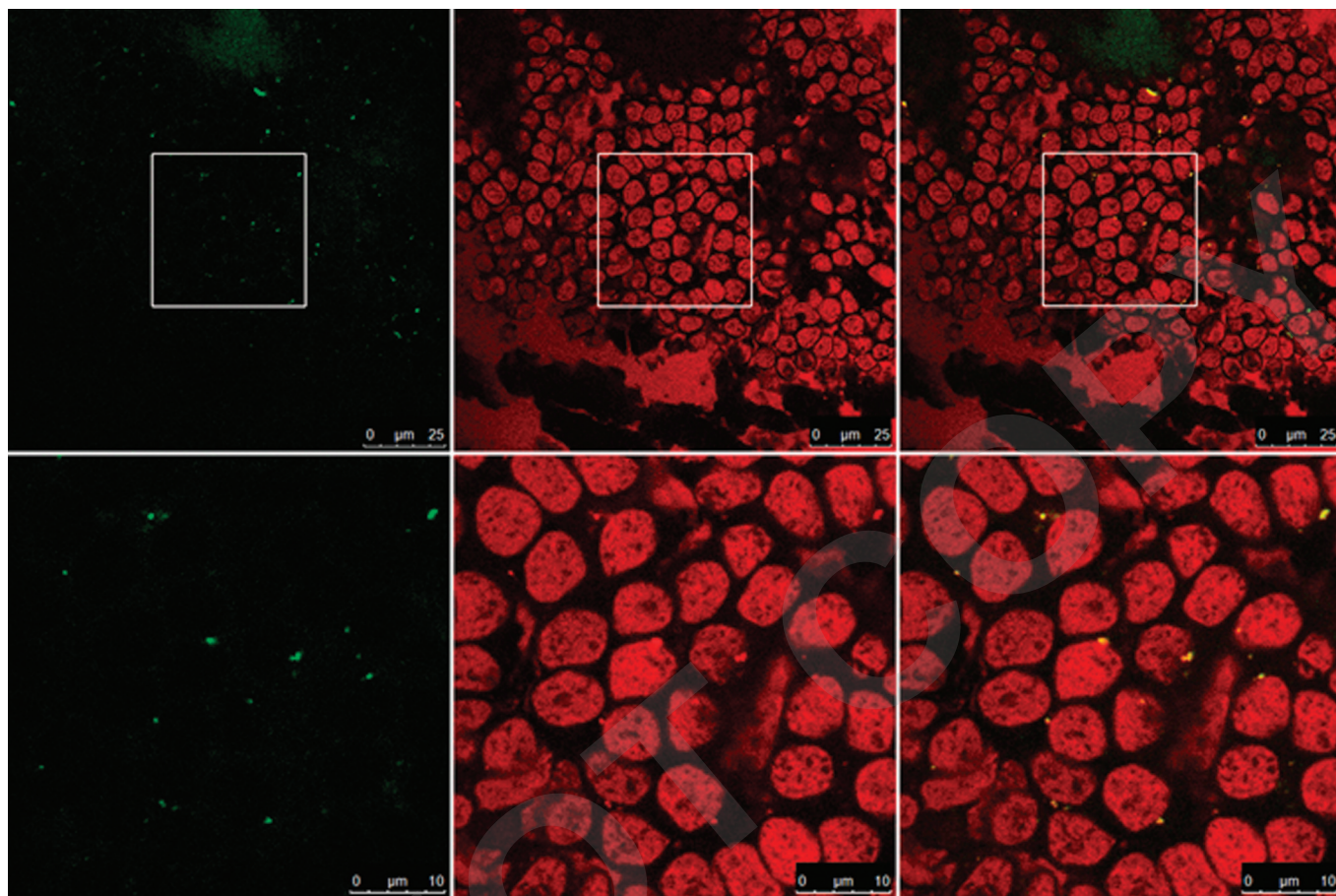


Figure 1. Detection of intracellular *Staphylococcus aureus* by confocal scanning laser microscopy–fluorescence in situ hybridization–propidium iodide (CSLM-FISH/PI; green, left column) in conjunction with PI (red, middle column). The organism is identified by colocalization of peptide nucleic acid (PNA)–FISH with PI (yellow, merged channels). High magnification of the boxed area (bottom) reveals cocci closely related to cellular nuclei (red only).

Microscope Analysis

Images of all patients were deidentified and assessed by blinded observers who categorized the sample as either intracellular positive or negative. Interobserver variability in both CSLM-FISH/PI and IHC was assessed using Fleiss's κ , which gave results of 0.72 and 0.63, respectively, which showed substantial agreement between the three observers.¹⁶

Using the CSLM-FISH/PI technique there were 9/17 (53%) patients that showed evidence of intracellular *S. aureus*. In these cases, *S. aureus* could be seen fluorescing on both the green FISH and the red PI channels, colocalizing to show bright yellow cocci closely related to red counterstained nuclei when viewed in the merged channel (Fig. 1). Consecutive z-plane images show the cellular nuclei with *S. aureus* fluorescing only at certain planes of focus, indicating that the organism is at a z-plane within the cell itself (Fig. 2). Three-dimensional reconstruction of highly magnified z-stacks is possible using the CSLM. This gives an even more vivid presentation of the presence of bacteria closely localized to the nuclei of epithelial cells (online supplemental Video E1).

Using IHC, intracellular *S. aureus* was identified in 7/17 (41%) patients. IHC gives excellent cellular morphology and the ability to clearly identify green fluorescing *S. aureus* located within the boundaries of epithelial cells as outlined by the red cytokeratin stain (Fig. 3). In three cases it was possible to identify *S. aureus* within the subepithelial layer. IHC analysis of tissue explants incubated with *S. aureus* *ex vivo* showed evidence of organisms within an hour of infection and by 13 hours there were cocci within both the epithelium and the subepithelial glandular tissue (Fig. 4).

Comparing both techniques, agreement in intracellular *S. aureus* status (either positive or negative) was found in 13/17 (76%) patients (Table 1). There were three patients deemed intracellular-positive on FISH but negative on IHC and one patient positive on IHC but not FISH. Fischer's exact test indicated no significant difference in the detection rates of *S. aureus* ($p = 0.73$).

Microbiological Outcomes

Of the patients who were categorized as intracellular positive by CSLM-FISH/PI, 7/9 (78%) cultured *S. aureus* from middle meatal swabs taken at the time of surgery. In the IHC-positive group 6/7 (86%) were culture positive (Table 1).

DISCUSSION

To our knowledge, this is the first study that reports the use of CSLM coupled with a dual probe technique of PNA-FISH and PI for intracellular *S. aureus* detection in whole mucosal samples of sinonasal epithelium, in addition to using a recognized technique of IHC within the same cohort of CRS patients. Intracellular localization of *S. aureus* was consistently detected by both methods with an equivalent efficacy. The sets of results obtained by the two independent methods are in a relative accordance with each other and this study allows for comparative highlighting of technical issues associated with detection and imaging of the organism infection by these two modalities. The significance of this study is in highlighting a modality of intracellular *S. aureus* detection that allows for simultaneous biofilm detection colocalized to the same piece of tissue and may further our under-

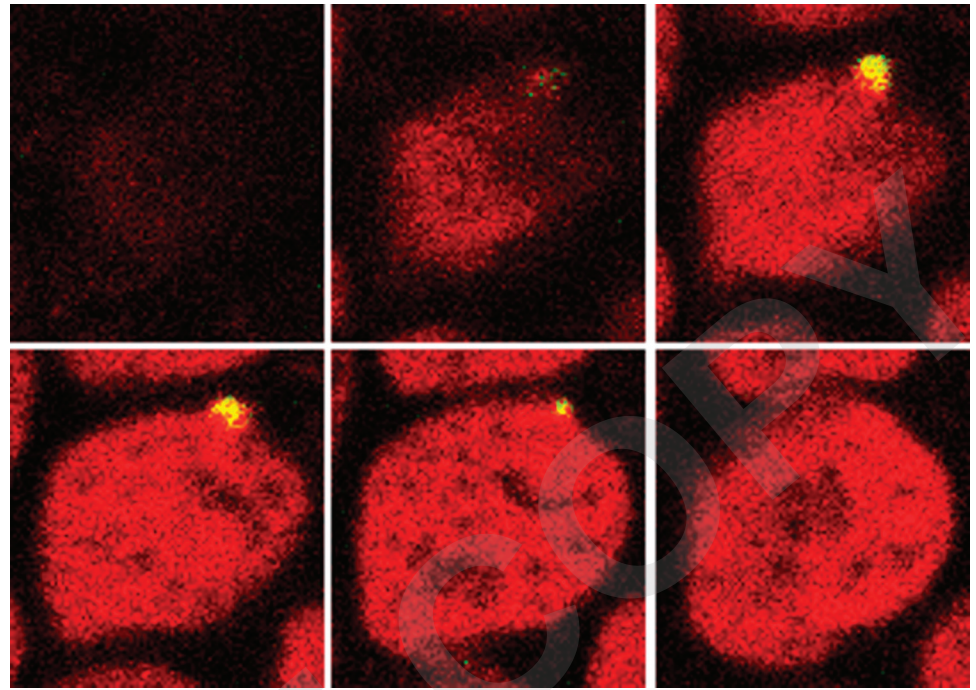


Figure 2. Confocal scanning laser microscopy-fluorescence in situ hybridization-propidium iodide (CSLM-FISH/PI) appearance of consecutive z-plane images showing a single cellular nucleus (red) with a focus of intracellular *Staphylococcus aureus* (yellow) at consecutive planes of focus suggesting intracellular presence.

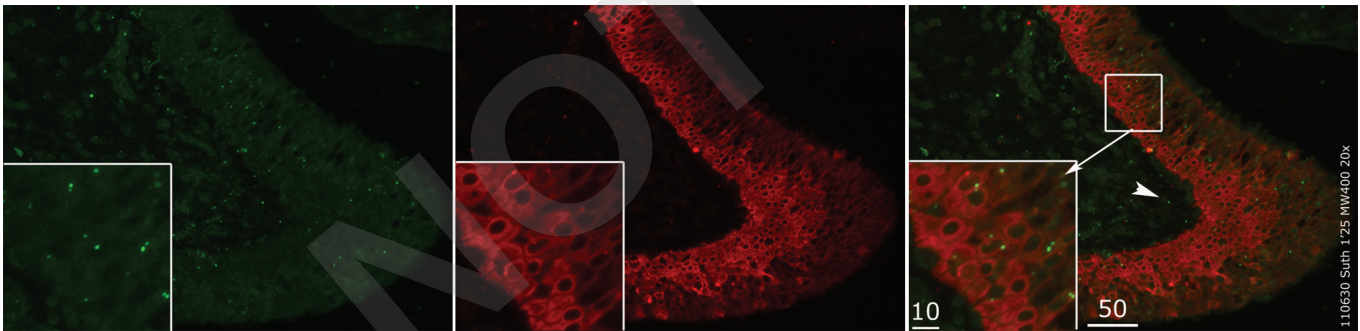


Figure 3. Detection of *Staphylococcus aureus* in paraffin sections of sinus mucosa tissue by immunohistochemistry (IHC); green fluorescence label of *S. aureus*; Middle: red fluorescence of epithelium-specific cytokeratin (middle); merged channels (right). Scales are in micrometers and applied for all panels.

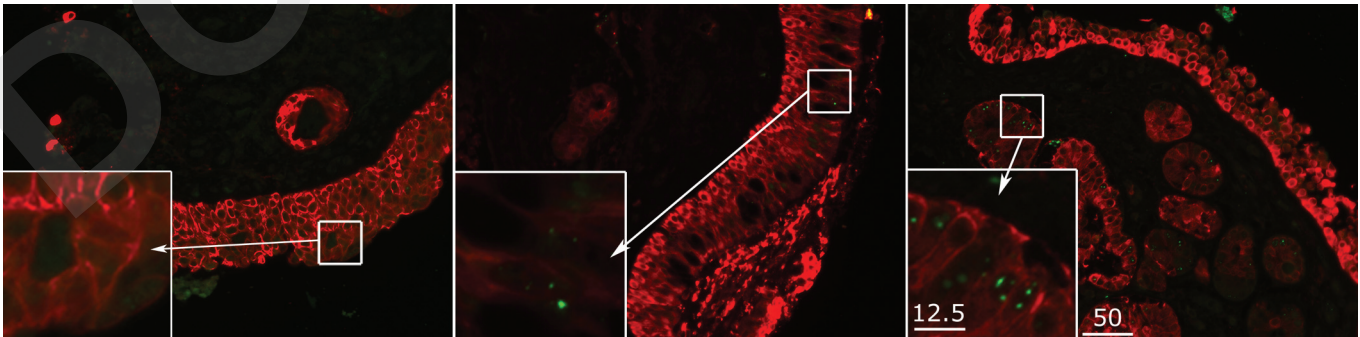


Figure 4. Immunofluorescence of tissue explants experimentally infected with *Staphylococcus aureus*. Green fluorescence bound to the IgM monoclonal antibody to *S. aureus*; red fluorescence of epithelia-specific cytokeratin. Prior to infection (left); 1-hour postinfection (middle); 13-hour postinfection (right). Scales are in micrometers and applied for all panels.

standing of the interplay between surface biofilm and intracellular residence in the chronic inflammatory state present in CRS sufferers. In terms of technical ease, the protocol for FISH/PI analysis takes 4 hours to complete with image analysis immediately after this. Whole

mucosal samples can be frozen on the day of harvest for later analysis, a process validated within our department¹⁴ where a side-by-side analysis of frozen versus immediately processed tissue was performed, showing no difference in reported outcomes. For IHC, tissue

Table 1 CSLM-FISH/PI vs IHC for identification of intracellular *Staphylococcus aureus*

Patient No.	CSLM-FISH/PI	IHC	Microbiological Culture
1	✓	x	<i>S. aureus</i>
2	x	x	No growth
3	✓	✓	<i>S. aureus</i>
4	✓	✓	<i>S. aureus</i>
5	x	✓	<i>S. aureus</i>
6	x	x	<i>S. aureus</i>
7	x	x	No growth
8	✓	x	<i>S. aureus</i>
9	✓	✓	MRSA
10	x	x	<i>S. aureus</i>
11	✓	x	No growth
12	x	x	No growth
13	✓	✓	No growth
14	✓	✓	<i>S. aureus</i>
15	✓	✓	<i>S. aureus</i>
16	x	x	Respiratory flora
17	x	x	<i>Moraxella catarrhalis</i>

CSLM = confocal scanning laser microscopy; IHC = immunohistochemistry; FISH = fluorescence in situ hybridization; PI = propidium iodide.

harvested on the day of surgery is soaked in 10% formaldehyde overnight, paraffin blocked, and sectioned, followed by our protocol that takes 24 hours to complete. Multiple tissue sections can be processed simultaneously with both techniques and although the FISH protocol is faster, both can be performed in the laboratory setting with relative ease. Indeed, it should be noted that our IHC protocol was optimized to include an overnight incubation with the primary antibody; however, it is entirely feasible that this could be altered to a shorter primary incubation step at room temperature or 37°C.

When examining our images and technical protocols, the benefits and drawbacks of each method can be identified. IHC gives more accurate cellular morphology and an advantage of this technique is the ability to image a whole section of tissue from surface epithelium to submucosa, thereby allowing deeper structures and organisms to be identified. We noted subepithelial fluorescence in three patients, mirroring the findings of Corriveau *et al.*¹¹ who first recognized submucosal bacteria in CRS. The same group went on to report that macrophages derived from CRS patients were less capable of phagocytosing *S. aureus*¹⁷, a factor that may well play a part in persistent disease. It remains unknown whether submucosal bacteria are a continuum of intracellular infection or a different pathogenic entity and, clearly, this is an area of research that would benefit from further scrutiny. A significant weakness of this technique, however, is the inability to obtain accurate biofilm data using the same tissue section, potentially caused by tissue processing techniques or sampling error. It is not possible to examine whole mucosal samples using an upright microscope because of the inability of the illuminating source to penetrate large pieces of tissue and produce an accurate in-focus view. Furthermore, the biofilm represents a three-dimensional structure on the exterior surface of mucosa, and paraffin blocking and sectioning appeared to disrupt the biofilm such that we were unable to identify extramucosal surface *S. aureus* in any patient. Because sections of tissue are only 5 µm in thickness, there is a significant chance that truly adherent biofilm may be missed within the examined section.

A key feature of CSLM is its ability to select depth within a sample *via* a process known as optical selection. Whole mucosal samples can be imaged using specialized optical filters to block out light from areas that are out of the ultrafine focal z-plane. Thus, multiple slices

from adjacent levels within a sample can be imaged with a slice thickness of as little as 0.3 µm and a true three-dimensional appreciation of the tissue obtained. Therefore, it is possible to identify whether fluorescent *S. aureus* cocci are superficial to the intraepithelial z-plane or at an equivalent plane closely localized to epithelial nuclei. Although CSLM-FISH/PI does not give as accurate tissue morphology as IHC, it does allow for examination of larger pieces of tissue, as much as 5 × 5 mm. Under low-power magnification, this allows a significantly larger area to be assessed for biofilm presence in addition to the identification of intracellular *S. aureus*. There still remains the possibility for sampling error; however, in our opinion this is greatly reduced because of the ability of CSLM-FISH/PI to examine mucosal samples with wider surface areas. The use of CSLM has already been established as an effective method of detecting biofilms from multiple pathogenic species including *S. aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, or fungal sources^{4,15,18} and, importantly, it allows for the simultaneous analysis and detection of surface biofilm attached to the same piece of tissue in which the intracellular bacteria are identified. A major weakness of the CSLM is that the laser beam is unable to penetrate deeper than the epithelial layer and thus can not identify the presence or absence of submucosal bacteria. As previously discussed, this may be an important pathogenic factor that use of this technique will overlook.

Both methods appear capable of identifying intracellular *S. aureus* with neither seemingly better than the other. In our patient cohort we found agreement between the two techniques in 13/17 (76%) samples tested. In a number of cases we were able to identify organisms using CSLM-FISH/PI but not IHC. Our IHC protocol uses formalin-fixed tissue specimens, and although we have confirmed our protocol with positive controls, there does remain the possibility that the antigen retrieval process does not wholly allow binding of the primary antibody. A benefit of CSLM-FISH/PI is the ability of the PNA-FISH probe to accurately target the 16S sequence of *S. aureus*. When used in combination with nonspecific DNA targeting PI, this means that the two fluorescing channels can be merged and true *S. aureus* can be identified by colocalization of both stains within the images. It should also be noted that our analysis was performed in CRS patients only; therefore, further validation of CSLM-FISH/PI would be beneficial in undiseased mucosal samples. However, the fact that in some patient samples no organisms were identified with either technique coupled with negative microbiological swabs suggests that these patients were truly negative of *S. aureus*. An additional weakness is that although both techniques are able to identify *S. aureus* within the tissue of these patients, they are unable to answer an important question as to whether they represent live or dead bacteria. This question can only be concluded with further investigation. Recent literature has recognized that *S. aureus* infection can be responsible for an altered TH2 cytokine response, as shown *ex vivo* by Wang *et al.*¹⁹ and *in vivo* by Foreman *et al.*²⁰ Whether biofilm or intracellular infection is responsible for these complex immunologic changes is still unknown. In addition to their active pathogenic role, these bacteria may represent a reservoir of infection that exists within the tissue of CRS patients that is resistant to medical and surgical treatment. An important step will be to apply these techniques to a cohort of CRS and control patients and ascertain whether biofilm plays a role in intracellular infection, as well as establish the prevalence of intracellular *S. aureus* and identify its true pathogenic nature.

CONCLUSION

CSLM-FISH/PI and IHC are complementary techniques, capable of detecting intracellular *S. aureus* within sinonasal epithelial tissue samples with neither one appearing superior to the other. CSLM-FISH/PI allows for the simultaneous detection of surface biofilm within the tissue analyzed with a protocol that is less time-consuming and labor intensive. IHC has a role in the identification of intracellular and submucosal *S. aureus* within these tissues. Additional investigation is

required to identify the true pathogenic nature of intracellular *S. aureus* as well as its relationship to surface biofilm status.

REFERENCES

1. Fokkens WJ, Lund VJ, Mullol J, et al. European position paper on nasal polyps 2007. *Rhinology Suppl* 45:1–136, 2007.
2. Foreman A, Jervis-Bardy J, and Wormald PJ. Do biofilms contribute to the initiation and recalcitrance of chronic rhinosinusitis? *Laryngoscope* 121:1085–1091, 2011.
3. Singhal D, Foreman A, Bardy JJ, and Wormald PJ. *Staphylococcus aureus* biofilms: Nemesis of endoscopic sinus surgery. *Laryngoscope* 121:1578–1583, 2011.
4. Foreman A, Singhal D, Psaltis AJ, and Wormald PJ. Targeted imaging modality selection for bacterial biofilms in chronic rhinosinusitis. *Laryngoscope* 120:427–431, 2010.
5. Le T, Psaltis A, Tan LW, and Wormald PJ. The efficacy of topical antibiofilm agents in a sheep model of rhinosinusitis. *Am J Rhinol* 22:560–567, 2008.
6. Garzoni C, and Kelley WL. *Staphylococcus aureus*: New evidence for intracellular persistence. *Trends Microbiol* 17:59–65, 2009.
7. Ellington JK, Harris M, Webb L, et al. Intracellular *Staphylococcus aureus*. A mechanism for the indolence of osteomyelitis. *J Bone Joint Surg Br* 85:918–921, 2003.
8. Brouillette E, Grondin G, Shkreta L, et al. In vivo and in vitro demonstration that *Staphylococcus aureus* is an intracellular pathogen in the presence or absence of fibronectin-binding proteins. *Microb Pathog* 35:159–168, 2003.
9. Clement S, Vaudaux P, Francois P, et al. Evidence of an intracellular reservoir in the nasal mucosa of patients with recurrent *Staphylococcus aureus* rhinosinusitis. *J Infect Dis* 192:1023–1028, 2005.
10. Sachse F, Becker K, von Eiff C, et al. *Staphylococcus aureus* invades the epithelium in nasal polyposis and induces IL-6 in nasal epithelial cells in vitro. *Allergy* 65:1430–1437, 2010.
11. Corriveau MN, Zhang N, Holtappels G, et al. Detection of *Staphylococcus aureus* in nasal tissue with peptide nucleic acid-fluorescence in situ hybridization. *Am J Rhinol Allergy* 23:461–465, 2009.
12. Lanza DC, and Kennedy DW. Adult rhinosinusitis defined. *Otolaryngol Head Neck Surg* 117:S1–S7, 1997.
13. Benninger MS, Ferguson BJ, Hadley JA, et al. Adult chronic rhinosinusitis: Definitions, diagnosis, epidemiology, and pathophysiology. *Otolaryngol Head Neck Surg* 129:S1–S32, 2003.
14. Foreman A, Psaltis AJ, Tan LW, and Wormald PJ. Characterization of bacterial and fungal biofilms in chronic rhinosinusitis. *Am J Rhinol Allergy* 23:556–561, 2009.
15. Psaltis AJ, Ha KR, Beule AG, et al. Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis. *Laryngoscope* 117:1302–1306, 2007.
16. Landis JR, and Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 33:159–174, 1977.
17. Krysko O, Holtappels G, Zhang N, et al. Alternatively activated macrophages and impaired phagocytosis of *S. aureus* in chronic rhinosinusitis. *Allergy* 66:396–403, 2011.
18. Foreman A, and Wormald PJ. Different biofilms, different disease? A clinical outcomes study. *Laryngoscope* 120:1701–1706, 2010.
19. Wang JH, Kwon HJ, and Jang YJ. *Staphylococcus aureus* increases cytokine and matrix metalloproteinase expression in nasal mucosae of patients with chronic rhinosinusitis and nasal polyps. *Am J Rhinol Allergy* 24:422–427, 2010.
20. Foreman A, Holtappels G, Psaltis AJ, et al. Adaptive immune responses in *Staphylococcus aureus* biofilm-associated chronic rhinosinusitis. *Allergy* 66:1449–1456, 2011. □

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