Microbiome Complexity and *Staphylococcus aureus* in Chronic Rhinosinusitis

Leah M. Feazel, M.S.1, Charles E. Robertson, Ph.D.2, Vijay R. Ramakrishnan, M.D.3, and Daniel N. Frank, Ph.D.1,4
1Division of Infectious Diseases, University of Colorado School of Medicine, Aurora, CO 80045, United States
2Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO 80304, United States
3Department of Otolaryngology-Head & Neck Surgery, University of Colorado School of Medicine, Aurora, CO 80045, United States
4Microbiome Research Consortium, University of Colorado School of Medicine, Aurora, CO 80045, United States

**Abstract**

**Objectives/Hypothesis**—The aim of this study was to compare microbiological culture-based and culture-independent (16S rRNA gene sequencing) methodologies for pathogen identification in chronic rhinosinusitis (CRS) patients. We hypothesized that (a) bacterial culture and DNA sequencing would yield largely concurrent results, though sequencing would detect greater bacterial diversity, and (b) the sinonasal microbiomes of CRS patients would differ in composition and diversity compared with non-CRS controls.

**Study Design**—Cross-sectional, observational study.

**Methods**—Middle meatus swabs of CRS patients collected during endoscopic sinus surgery were analyzed by both clinical culture and broad-range analysis of 16S rRNA gene pyrosequences.

**Results**—21 swab samples from 15 CRS patients and 5 non-CRS controls were analyzed. One CRS patient was also swabbed three weeks post-operatively due to evidence of purulence during a clinical visit. All subjects had positive bacterial cultures, with a mean of 2.8 isolates per subject. The most prevalent cultivars were coagulase-negative staphylococci (15/20 specimens, 75%), *Staphylococcus aureus* (10/20, 50%), and *Propionibacterium acnes* (6/20, 30%). Among 57,407 pyrosequences generated, the most prevalent were from coagulase-negative staphylococci (21/21 specimens, 100%), *Corynebacterium spp.* (18/21, 85.7%), *P. acnes* (16/21, 76.2%), and *S. aureus* (14/21, 66.7%). Bacterial diversity correlated with recent antibiotic use, asthma, prior sinus surgery, and relative abundance of *S. aureus*.

**Conclusions**—DNA pyrosequencing revealed greater biodiversity than culture, although in most cases culture results represented a sub-set of the abundant DNA sequence types. CRS patients were characterized by altered microbial composition (p=0.02), and greater abundance of *S. aureus* (p=0.03).
Keywords
bacteria; bacterial sinusitis; human microbiome; sinus culture; middle meatus

Introduction

Sinus infections in chronic rhinosinusitis (CRS) cause significant patient discomfort and increased treatment costs. CRS is characterized by >12-weeks of signs and symptoms resulting from inflammation of the paranasal sinuses.1 The occurrence of bacterial infection in CRS can be either a primary cause or resulting factor of the inflammatory disease.2 In primary bacterial CRS, pathogen colonization causes chronic immune up-regulation and inflammation. Alternatively, an otherwise inflamed or blocked sinus, resulting from anatomical obstruction, allergies, or mucosal dysfunction, can promote the ideal environmental conditions for secondary pathogenic bacterial growth. Microbial colonization of the sinuses in CRS often is characterized by the presence of polymicrobial bacterial biofilms, possibly due to abnormal innate immune function in the patient.2–4 Biofilms can protect pathogen subpopulations from both host immune systems and antibiotic therapies, and may thus result in poor outcomes with conventional treatments.5

Previous studies suggest that a small suite of bacteria is common in the paranasal sinuses of subjects with CRS, whereas the sinuses of healthy individuals are lightly colonized by commensal microorganisms. Commonly observed species include aerobes and anaerobes typical of the nares, oropharynx, and mouth.6–8 However, conventional culture methods may under-represent the bacterial diversity of sinonasal specimens, particularly those that harbor complex microbial communities. In contrast, molecular methods for bacterial identification that do not require axenic cultivation, such as polymerase chain reaction (PCR), DNA sequencing, and microarrays, have the potential to elucidate a broader understanding of the complex microbial ecology that is typical of the human body.9 The goal of this pilot study was to characterize the composition and diversity of microbial communities that colonize the paranasal sinuses of CRS patients via both conventional culture and culture-independent molecular methodology. We hypothesized that (a) bacterial culture and DNA pyrosequencing would yield largely concurrent results, though more diversity would be observed through sequencing, and (b) the sinonasal microbiomes of CRS patients would differ markedly in both composition and diversity compared with non-CRS controls.

Methods

Study design and Population

This cross-sectional study was approved by the Institutional Review Board of the University of Colorado (protocol number 10-1437). CRS was diagnosed in patients according to the 2007 Adult Sinusitis Guidelines, and was initially managed medically with a minimum of three weeks of antibiotics and topical intranasal steroids prior to the decision for surgery.10 Asthma was diagnosed either clinically based on history of wheeze, cough, shortness of breath and chest tightness, or on spirometry response with bronchodilator and methacholine administration.11 The diagnosis of allergic rhinitis was made by positive skin prick testing or clinically by the classic constellation of symptoms of nasal congestion, sneezing, rhinorrhea, and pruritis. Swabs were collected through convenience sampling of patients undergoing outpatient endoscopic sinus surgery (ESS) at University of Colorado Hospital between January and April 2011. Swab specimens were obtained during ESS with ESwabs (COPAN Diagnostics, Inc., Murrieta, CA) for hospital laboratory culture and CultureSwabs™ (BD, Franklin Lakes, NJ) for DNA extraction. Swabs were endoscopically guided to sample areas of mucopurulence, when present, or into the middle meatus when no gross disease was
encountered. Samples were sent for routine aerobic/anaerobic culture at the hospital laboratory and placed on ice and frozen at −80°C until DNA extraction.

**DNA-based Techniques**

Total genomic DNA was purified from swab heads by a phenol:chloroform, bead-beating protocol (complete details for molecular methods are described in the Supplemental Methods). All DNA extraction and PCR steps were performed in a HEPA-filtered laminar flow hood that was decontaminated by UV light. Quantitative PCR (QPCR) assay used previously published oligonucleotide primers for Total Bacteria (16S rRNA gene, FAM reporter)\(^{12}\) and *S. aureus* (femA gene, TET reporter)\(^{13}\). A multiplex PCR assay for *S. aureus* specific methicillin-resistance genes (mecORF)\(^{14}\) was utilized to test for MRSA in specimens that were positive for *S. aureus* by QPCR and sequence.

Amplicons of the 16S rRNA gene (~500 b.p.; primers 27FYM+3 and 515R)\(^{15,16}\) were generated via broad-range PCR (30–36 cycles) using 5'-barcoded reverse primers\(^{17}\). Pooled amplicons were provided to the Center for Applied Genomics at the University of Toronto for pyrosequencing on a 454/Roche Life Sciences GS-FLX instrument using Titanium chemistry (Roche Life Sciences, Indianapolis, IN). The software used for sequence polishing and 16S RNA gene sequence analysis consisted of the function specific tools that were considered to be the standards in the academic community at the onset of this project. The mean trimmed sequence length was ~340 b.p. Genus-level taxonomic calls were produced by the *RDP Classifier*, which performs naïve Bayesian taxonomic classification versus a training set.\(^{18}\) Species level taxonomy precision was obtained via BLAST against a database of sequences obtained from Silva 104\(^{20}\) tagged as isolates and reported results demanded at least 99% sequence identity over 95% of sequence length. Standard ecological indices of diversity, richness, and evenness were conducted with software tool *biodiv* embedded within the sequence analysis pipeline.\(^{21}\)

**Statistical Analysis**

The R-statistical package was used for all statistical analyses.\(^{22}\) Differences in the compositions of microbiomes (i.e., OTU distributions taken as a whole) between CRS patients and non-CRS controls were measured by the Morisita-Horn index using the adonis function of the R package “vegan”, which performs a non-parametric multiple analysis of variance with adjustment for covariates.\(^{22,23}\) A label-permutation test with 10,000 replicates was used to assign a p-value to the difference in OTU counts between groups of samples. Demographic characteristics were compared using ANOVA for continuous outcomes and Fisher exact tests for categorical outcomes including OTU prevalence. Percent OTU abundance data were logit-transformed to a linear scale for ANOVAs. All tests of null hypotheses were evaluated at \(\alpha = 0.05\).

**Results**

Twenty consecutive patients undergoing ESS between January and April 2011 were included in this study (Table 1, Figure 1). Fifteen patients were diagnosed with CRS and five patients served as non-CRS controls. One patient was swabbed both at the time of surgery and three weeks post-operatively during a clinic visit due to evidence of continued purulence (specimens 03A and 03D respectively). Therefore, a total of 21 specimens from 20 subjects were analyzed. Frank pus and/or thick mucus was present in 5 CRS patients (33%) and 1 control (20%). Out of the fifteen patients with CRS, only two (13%) had polyps noted on endoscopy (mean Lund-Mackay preoperative CT score 9.5 ± 4.9). With so few polyp patients this clinical feature was not included in statistical analyses. The controls included patients undergoing ESS for tumor (N=1), fungal ball (N=2), silent sinus syndrome...
and septoplasty (N=1). None were immunocompromised or known to have cystic fibrosis. Swabs in control patients were taken from the side opposite of disease, from a radiographically and endoscopically normal appearing middle meatus. All subjects and controls used intranasal steroid sprays for at least one month prior to surgery.

Standard hospital cultures were positive for all subjects and controls, with an average of 2.8 isolates reported per subject (range 1–5). One specimen from a control subject (Patient 17) was not sent for culture, so culture results were available for 19 of 20 subjects, and 20 of 21 specimens. There was no statistical difference between the number of isolates obtained from CRS patients and controls (not shown). The most common cultivars were coagulase-negative streptococci (15/20 cultures, 75%), Staphylococcus aureus (10/20, 50%), and Propionibacterium acnes (6/20, 30%). For many specimens (9/20, 45%), culture results were incompletely speciated, with broad phenotypic groups reported, e.g. “mixed anaerobes” or “Gram-negative rods.”

Microbes present in middle meatus specimens were comprehensively surveyed through pyrosequencing and phylogenetic analysis of bacterial 16S rRNA genes. A total of 57,407 high-quality pyrosequencing reads were generated for all 21 specimens (median 1,485 reads per specimen, IQR 682–3,296). The most prevalent DNA sequence types detected were coagulase-negative staphylococci (21/21 specimens, 100%), Corynebacterium spp. (18/21, 85.7%), P. acnes (16/21, 76.2%), and S. aureus (14/21, 66.7%). For 20 of 21 specimens (95.2%) the majority of sequences (>50%) belonged to known or suspected upper respiratory bacterial genera (Figure 1).

In the majority of specimens, bacteria identified by clinical culture also were identified in the sequence dataset (Figure 1). In 13 of 20 specimens analyzed by culture (65%), more prevalent sequence types (>15% of sequences) corresponded to the organisms identified by culture. For example, the sequence library from Patient 05 contained more than 52% S. aureus and 34% S. epidermidis sequences, which were both identified by culture. However, non-specific culture reports such as “mixed GNR” were difficult to correlate with sequence data in a meaningful way. Some of the un-resolved culture results may, however, represent species detected by sequence; e.g. the “moderate mixed anaerobes” of Patient 12 could include Prevotella spp., which comprised 75% of the total sequences from that specimen. Although the dominant sequence types frequently corresponded to culture results, in 7 of 20 (35%) of the specimens, the cultured organisms were rare components of the corresponding sequence libraries (<15% of sequences). For example, the specimen from Patient 08 cultured Streptococcus, Neisseria and Propionibacterium (each of which were <1% of sequences), but did not culture Stenotrophomonas (>78% of sequences).

In the case of four isolates, cultivation of microorganisms identified species not detected in the sequence dataset. In one specimen (Patient 13), the culture result of Peptostreptococcus was not identified by sequence. However, the related genera Finegoldia, Peptinophilus, and Anaerococcus were present in the sequence data, and these organisms have morphologies similar to peptostreptococci. The other discordant results included two P. acnes isolates (Patients 9 and 18). In these specimens, sequences of other anaerobic bacilli were detected including Actinomyces, Rothia, and Prevotella. The final case of discordance was the cultivation of a Citrobacter freundii isolate (Patient 16). No sequences of other citrate-utilizing genera were identified from the specimen, though numerous other members of the Enterobacteraceae family were present in the sequence library. Thus, DNA pyrosequencing provided a broader, more incisive, and oftentimes more sensitive, catalog of microbes resident in the middle meatus, than did culture.
Different kinds and quantities of bacteria inhabited the middle meatuses of CRS and non-CRS patients (Figure 1). A non-parametric multiple ANOVA test with label permutation (see methods) identified significantly different overall distributions of 16S rRNA sequences recovered from the two groups of subjects (p = 0.02). Although the total bacterial composition varied significantly, neither richness nor evenness indices reached statistical significance between cases and controls (Table 2, Supplemental Figure 1). CRS patients were, on average, characterized by fewer bacterial types (34 vs. 49 genus-level operational taxonomic units, OTUs, in CRS vs. non-CRS; p = 0.23) and less even distributions of genus-level sequence types (p = 0.09) compared with controls (Supplemental Figure 1).

In addition to community-level disparities between CRS patients and controls, one species in particular, *S. aureus*, was selectively enriched in CRS. Cultivation and pyrosequencing methods for *S. aureus* detection gave broadly concordant results. Culture results identified *S. aureus* in 10 of 16 CRS specimens (63% prevalence) and 0 of 5 non-CRS controls, whereas DNA sequences representative of *S. aureus* were detected in 11 of 16 CRS patient specimens (69% prevalence) and 3 of 5 (60%) non-CRS controls. Although the prevalence of *S. aureus* sequence detection in the cases and controls was similar, the abundance of *S. aureus* (when present) in CRS patients was higher than in non-CRS patients (mean 38% vs. 4.6% *S. aureus* sequences, range 0.7–95.7% vs. 0.2–13.3%). This trend did not reach statistical significance (p = 0.07), due at least in part to the small population size (15 cases and 5 controls).

In order to better understand the distribution and abundance of *S. aureus* in the middle meatus of CRS and non-CRS populations, swabs from 22 additional patients and controls, along with the initial sample set, were screened using a quantitative PCR method specific for *S. aureus* DNA (i.e., enumeration of femA genes). Nine samples (from 6 CRS cases and 3 controls) were excluded due to failure of PCR amplification with pan-bacterial primers, indicating low biomass. A total of 34 PCR positive specimens were analyzed: 26 CRS and 8 non-CRS. *S. aureus* DNA was detected in 19 of 26 (73.1%) CRS cases (including all 10 culture-positive samples) with an average of 14.8% abundance (range 0.003–100%), when normalized to the total bacterial signal. In control patients, 2 of 8 (25%) had *S. aureus* amplification, with an average of 0.4% abundance (range 0.06–0.75%). This larger patient population resulted in significant statistical differences between CRS patients and controls for both prevalence (p = 0.03) and mean abundance (p = 0.03) of *S. aureus* in middle meatus specimens.

For cases in which *S. aureus* was detected by DNA sequencing, a further non-quantitative PCR assay for a *S. aureus*-specific methicillin-resistance gene (mecA) was conducted. This assay detected mecA in one specimen (Patient 02), the only patient diagnosed with MRSA by culture.

In order to identify clinical factors influencing the observed differences in microbial community diversity and *S. aureus* abundance between CRS cases and controls, we performed univariate ANOVA for several candidate demographic factors (Table 2). In this analysis, antibiotic history and asthma were the most significant factors associated with biodiversity (both richness and evenness) and *S. aureus* abundance. Both antibiotic exposure and asthma were associated with reduced microbial diversity and increased *S. aureus* abundance, as measured by specific Q-PCR. Because *S. aureus* abundance also was strongly correlated with both richness and evenness, it is likely that the effects of antibiotics and asthma on biodiversity were mediated by their promotion of higher *S. aureus* abundances in the middle meatus.
Discussion

The results of our microbiological analysis of middle meatus specimens from CRS and non-CRS patients indicate that clinical culture and culture-independent analysis of 16S rRNA pyrosequences are broadly consistent, but that the sequence-based approach both provides finer phylogenetic resolution to identify microorganisms and detects greater biodiversity than does culture. Although sequencing generally identified the same pathogens as culture, the converse was not the case, as culture failed to detect potentially pathogenic members of many genera (Figure 1). Most notably, the genus Stenotrophomonas, a group of difficult-to-treat, biofilm-forming microbes related to Pseudomonas spp., was observed at high relative abundance in specimens from both of the fungal non-CRS cases (Patients 08 and 16), but was not cultivated from either individual. Additionally, the diversity of anaerobic genera (e.g., Propionibacterium, Prevotella), in particular, was not demonstrated by cultivation, with most specimens reported as containing “mixed anaerobes.” Thus, these cases demonstrate the utility of DNA detection in conjunction with cultivation for the identification of difficult-to-culture bacterial pathogens.

In 3 of the 4 discordant events in which cultured isolates were not detected by pyrosequencing, the cause may have been inaccurate identification of isolates, because other morphologically and/or physiologically similar species were identified by sequence. However, in the case of one isolate, Citrobacter freundii, failure of detection/identification by DNA sequencing is possible, because no other citrate-utilizing genera were detected in the sequence library. This could occur if the isolate were a relatively rare member of the sinonasal microbiota and the depth of pyrosequencing was insufficient to detect such rare microbes. Alternatively, this could be a case of contamination during cultivation. Despite these few discordant isolates, sequencing generally identified the cultured species in addition to many species not detected by cultivation.

Comparison of rRNA gene sequences from CRS cases and non-CRS controls revealed that middle meatus microbiomes differed significantly in composition. These community-level differences could result from repeated antibiotic therapies or pathogen-induced alterations in the normal microbiota of the middle meatus (i.e., dysbiosis). Antibiotic treatment in the twelve weeks prior to surgery reduced genus-level richness significantly, and specimens from these patients had more uneven communities, suggesting that a few organisms were numerically dominant in the community. Because of the chronic nature of CRS, antibiotic therapy is likely to be a significant driver of change in the sinonasal microbiomes of CRS patients. One species in particular, S. aureus, was highly prevalent and abundant in CRS patients, whereas it was a minor component in non-CRS controls. These results clearly warrant further investigation in order to assess their generalizability to a wider patient population. The presence of S. aureus is noteworthy in CRS due to the increasing incidence and awareness of methicillin-resistant S. aureus (MRSA) carriage in the nares, which is a significant contributor to surgical site infections and slow mucosal healing following ESS as a result of biofilm formation. This study population had only one MRSA case (confirmed by PCR), which was successfully treated in a culture-directed manner of combined oral and topical antibiotic therapy.

The use of middle-meatus swabs for DNA-based bacterial assays is appropriate for detection of multiple bacterial species, including anaerobes, which may be undetected when swabs are used solely for culture. Based on available cultivation-based studies, the microbiology of the middle meatus correlates well with that of the sinuses, however these experiments have not been repeated with pyrosequencing technology. This is a potential weakness of the current study, and sequence-based analyses currently are underway. Furthermore, the bacterial communities observed in this study of the middle meatus, both by culture and DNA
sequence, differed from those previously reported in studies of the anterior nares. For example, representatives of the genus *Corynebacterium*, a common microbe of the skin and anterior nares, were cultivated only once from the middle meatus, and comprised only ~3% of total sequences versus 8–20% reported in our previous study of the nares. This indicates that (a) swabs of the anterior nares would not be appropriate as a replacement of middle meatus swabs in research studies or clinical investigations of CRS microbiology, as has been noted in the literature previously, and (b) swabs were not contaminated by microbiota of the anterior nares during insertion/retraction from the middle meatus or sinuses.

Although sequencing may be superior to clinical culture in some respects, the method currently requires more time and labor than does microbial culture, thus limiting its clinical applicability. In contrast, quantitative-PCR assay of specific pathogens is very rapid (several hours) and comparably sensitive to culture, but is limited to detecting known species. A potential solution to this problem would be the design of a PCR panel assay or microarray for all suspected CRS pathogens, thus giving the benefits of broad-range DNA detection with the necessary rapidity for clinical applications. Finally, because antibiotic sensitivity testing in most practical situations currently requires testing of axenic cultivars, clinical culture is unlikely to be replaced by purely molecular methods in the near term.

**Conclusions**

The results of this preliminary study indicate that bacterial culture and 16S rRNA gene pyrosequencing of specimens from CRS patients and controls yield largely congruent results, although significantly more diversity, particularly of anaerobic groups, was observed by sequencing than culture. Additionally, CRS patients had significantly different microbial communities than non-CRS controls, with more abundant occurrence of *S. aureus*. Prior antibiotic therapy may be a driver of the observed microbiome alterations. Taken together, these results indicate that complex bacterial communities may play a significant role in the etiology of CRS.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

We thank Dr. Sergio Pereira (University of Toronto) for assistance with pyrosequencing and Dr. Todd Kingdom (UC-Denver) for helpful editorial comments, which strengthened the paper.

Financial support: This work was supported in part by NIH grant HG005964 (DNF).

**References**


Figure 1.
Comparison of bacterial culture and 16S rRNA sequencing results in CRS cases and controls. Boxed cells represent genera or species identified by culture. The figure summarizes the distribution of human-commensal and/or airway pathogenic bacterial taxa detected by phylogenetic analysis of pyrosequencing reads, clustered into genus-level groups (because of its potential significance to CRS, Staphylococcus aureus is separated from other staphylococci). Values in the table represent percent abundances of particular taxa for each subject (columns sum to 100%). “Other” refers to bacterial genera not commonly associated with human health or disease and to sequences that could not be assigned to the genus-level of taxonomy. Keys: \( b \) boxed cells with bold numbers indicate positive result by culture, \( m \) “mixed anaerobes” by culture not further identified, \( c \) culture identified Citrobacter freundii, not seen in sequences, \( p \) culture identified Peptostreptococcus, not seen in sequences, * not sent for culture.
<table>
<thead>
<tr>
<th></th>
<th>CRS</th>
<th>Non-CRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects (N=)</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Age (std. dev.)</td>
<td>45 (±15.8)</td>
<td>56 (±20.0)</td>
</tr>
<tr>
<td>Gender (% female)</td>
<td>10 (66.7)</td>
<td>3 (60)</td>
</tr>
<tr>
<td>Allergies</td>
<td>12 (80)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Antibiotics &lt;12 weeks prior (%) *</td>
<td>6/16 (37.5)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Asthma (%)</td>
<td>4 (26.7)</td>
<td>0</td>
</tr>
<tr>
<td>Polyps</td>
<td>2 (13.3)</td>
<td>0</td>
</tr>
<tr>
<td>Prior ESS (%)</td>
<td>7 (46.7)</td>
<td>0</td>
</tr>
<tr>
<td>Pus</td>
<td>5 (33.3)</td>
<td>1 (20)</td>
</tr>
</tbody>
</table>

* 16 total specimens were included because CRS Patient 03 was on antibiotics between the two sampling events.
Clinical and microbiological factors associated with middle meatus biodiversity by univariate analysis.¹

<table>
<thead>
<tr>
<th></th>
<th>Diagnosis²</th>
<th>Allergies</th>
<th>Antibiotics</th>
<th>Asthma</th>
<th>Prior ESS³</th>
<th>Pus</th>
<th>S. aureus⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Richness</td>
<td>0.23</td>
<td>0.71</td>
<td>0.06</td>
<td>0.01</td>
<td>0.05</td>
<td>0.22</td>
<td>0.005</td>
</tr>
<tr>
<td>Evenness</td>
<td>0.09</td>
<td>0.35</td>
<td>0.02</td>
<td>0.01</td>
<td>0.19</td>
<td>0.55</td>
<td>0.0008</td>
</tr>
<tr>
<td>S. aureus⁴</td>
<td>0.15</td>
<td>0.19</td>
<td>0.08</td>
<td>0.001</td>
<td>0.12</td>
<td>0.68</td>
<td>-</td>
</tr>
</tbody>
</table>

¹Table summarizes p-values from univariate ANOVA. Columns are predictor factors and rows are outcome variables.

²CRS vs. Non-CRS.

³Prior endoscopic sinus surgery.

⁴Percent abundance determined by S. aureus 16S rRNA pyrosequencing reads, normalized to total reads per specimen.