Sinus culture poorly predicts resident microbiota

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Background: **Chronic rhinosinusitis (CRS) is an inflammatory disorder of the paranasal sinuses in which bacteria are implicated. Culture-based assays are commonly used in clinical and research practice; however, culture conditions may not accurately detect the full range of microorganisms present in a sample. The objective of this study was to determine the accuracy of clinical culture of CRS specimens compared with DNA-based molecular techniques.**

Methods: **Ethmoid samples from 54 CRS patients collected during endoscopic sinus surgery were analyzed by both clinical culture and 16S ribosomal RNA (rRNA) gene sequencing. The association between 16S relative abundance and detection by culture was determined using logistic regression.**

Results: **Each subject had an average of 3 isolates identified by bacterial culture and 21.5 ± 12.5 species identified by 16S sequencing. On average, 1.6 dominant taxa (>10% abundance) per subject were identified using molecular techniques, but only 47.7% of these taxa were identified by culture. Low abundance taxa (abundance <1%) were detected in only 4.5% of cultures. The odds that any organism would be detected by culture were 2.3 times higher with each 10% increase in relative abundance ($p < 0.01$). Conversely, only 29.5% of isolates identified by culture represented the dominant species, whereas 40% accounted for species with 1% to 10% abundance. Interestingly, 12% of isolates detected by culture were not identified by 16S pyrosequencing.**

Conclusion: **Standard clinical culture is a poor representation of resident microbiota. The incorporation of modern culture-independent techniques into clinical and research practices provides additional information that may be relevant for CRS.** © 2014 ARS-AAOA, LLC.

Key Words:
sinusitis; chronic rhinosinusitis; bacteria; microbiome; pyrosequencing

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Chronic rhinosinusitis (CRS) is a chronic inflammatory disorder of the paranasal sinuses, in which bacteria are thought to play a role in disease etiology and pathogenesis.[1,2] Antibiotics have long been a mainstay in the treatment of CRS despite the lack of proven efficacy, with associated major concerns including the potential for medication side effects, antibiotic resistance, and healthcare expenditures. CRS patients are approximately 5 to 7 times more likely than healthy persons to be prescribed an oral antibiotic.[3,4] In addition, more than 90% of otolaryngologists use a prolonged course of oral antibiotics as a part of "maximal medical therapy" prior to surgical intervention.[5] Though evidence supporting the utility of antibiotic therapy is debatable, short courses of culture-directed antibiotics are routinely recommended in the literature and guidelines for acute exacerbations of CRS.[1,2,6,7]

To date, the majority of studies that have examined bacterial presence in CRS have used clinical microbiological culture to enumerate bacteria.[8–12] It has become apparent with the incorporation of new microbiologic techniques that clinical culture is likely insufficient for the detection of organisms,[13] and that the community of organisms may be of more interest than a single isolate grown in a given culture medium.[14] We, and others, have previously shown that use of culture-independent molecular methods for bacterial

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sinus investigation allows for greater detection of bacterial biodiversity than standard culture, but is also able to elucidate the relative quantities of bacterial species present in a specimen.[15,16]

Clearly, there are many questions about the role of bacteria in CRS, and culture-based identification is the current "gold standard" in clinical practice and research examination of the disease. The goal of this study was to determine the accuracy of clinical culture when compared to advanced molecular techniques.

Patients and methods

Study design and population

This cross-sectional study was approved by the Institutional Review Board of the University of Colorado (COMIRB protocol number 11-1442). The diagnosis of CRS was made according to the 2007 Adult Sinusitis Guidelines, and accordingly, CRS patients were initially managed medically with a minimum trial of saline rinses, oral antibiotics, and topical intranasal steroids.[17] Those with continued evidence of disease who elected to undergo endoscopic sinus surgery (ESS) between January 2011 and February 2012 were enrolled in the study. At the time of surgery, approximately 75% of patients were using nasal saline rinses and intranasal corticosteroids. Patients less than 18 years of age, antibiotic use within 1 month of surgery (systemic or topical), or those with cystic fibrosis or autoimmune diseases were excluded from the study. Clinical data were recorded at the time of surgery. 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. Culture swabs were endoscopically guided to the area of interest with care taken to avoid contamination from the nasal cavity. The mucosal surface and overlying mucus of the ethmoid and/or maxillary sinus was aggressively swabbed for at least 5 full rotations until fully saturated. CultureSwabs for DNA extraction were placed on ice upon collection and frozen at −80°C until DNA extraction. Because swabs were placed immediately on ice and frozen within 1 hour of collection, no stabilization solution was added prior to freezing.[18–20] ESwabs sent to the hospital clinical microbiology laboratory were aerobically and anaerobically cultured for bacterial growth and isolation. For aerobic cultures, specimens were plated and streaked for isolation onto blood, chocolate, and MacConkey agars (Remel, Lenexa, KS) and incubated at 35°C and 5% CO_2 for 2 days. For anaerobic cultures, prereduced Brucella agar with 5% sheep blood, anaerobic centers for disease control and prevention (CDC) phenethyl alcohol (PEA) blood agar with vitamin K, and *Bacteroides fragilis* isolation agar/anaerobic reducible Laked Blood with Kanamycin and Vancomycin (LKV) blood agar biplate (Remel, Lenexa, KS) were inoculated and incubated at 35°C in a Whitley A45 anaerobic workstation (Don Whitley Scientific Limited, West Yorkshire, UK) with 9.5% H_2, 10% CO_2, and N_2 as the balance for 7 days.

DNA extraction, polymerase chain reaction, and DNA sequencing

DNA was extracted from clinical specimens and normalized by 16S template counts (measured by pan-bacterial quantitative polymerase chain reaction [PCR]).[21] PCR was amplified and prepared for sequencing as previously described.[15,22] Amplicons of the V1V3 variable region 16S rRNA gene (~500 bp; primers 27FYM+3 and 515R)[23,24] were generated via broad-range PCR (30 to 36 cycles) using 5′ barcoded reverse primers.[25] Pooled amplicons were provided to the Center for Applied Genomics at the University of Toronto for pyrosequencing on a 454/Roche Life Sciences GS-GLX instrument using titanium chemistry (Roche Life Sciences, Indianapolis, IN). Pyrosequences were screened for nucleotide quality: bases at 5′ and 3′ ends with mean Q < 20 over a 10-nucleotide window, sequences with less than 200 nucleotides, and sequences with more than 1 ambiguous nucleotide were discarded.[24,25] Mean trimmed sequence length was ~340 bp. Potential chimeras were identified and excluded from analysis using Uchime (usearch6.0.203_i86linux32)[26] and the Schloss SILVA reference sequences and were removed from subsequent analyses.[27] We generated a median of 1068.5 high-quality sequences/specimen (interquartile range [IQR], 297.5 to 1783.5). Sequences were aligned and classified with SINA 1.2.11 (Pruesse et al.[28]) using the 418497 bacterial sequences in Silva 111NR (Quast et al.[29]). Operational taxonomic units (OTUs) were produced by clustering sequences with identical taxonomic assignments, and these taxa groups were used in further community analysis. Species-level taxonomy precision was obtained via Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi.)[30] against a database of sequences obtained from Silva 111NR (Quast et al.[29]) tagged as isolates and reported results demanded at least 99% sequence identity over 95% of sequence length. All DNA sequence data were deposited in the NCBI short read archive (Project PRJNA221204). The Good's coverage index[31] of each sequence library, which estimates the percent of species identified within the sample, had a median value of 98.5% (IQR, 95.5% to 99.2%), indicating that nearly all of the biodiversity in a sample was represented in its 16S sequence dataset.[32]

Statistical analysis

Descriptive statistics include mean, standard deviation, and range for clinical data. Species-level information was used when possible, because it represents the most consistent information for comparison to the culture results. In cases where the organism could not be identified at the species level using the 99% identity cutoff, then the taxa was labeled with the genus name and "unclassified" for the species. The genus level information was used

TABLE 1. Patient demographics and clinical characteristics (n = 54)

Age, years, mean ± SD	49.1 ± 14.8
Gender, male:female, n	31:23
Allergic rhinitis, n (%)	37 (69)
Prior endoscopic sinus surgery, n (%)	30 (56)
Purulent secretions, n (%)	29 (54)
Nasal polyposis, n (%)	21 (39)

SD = standard deviation.

TABLE 2. Clinical culture results for 54 samples*

Coagulase-negative staphylococci	39 (72)
P. acnes	19 (35)
Mixed anaerobes	14 (26)
S. aureus	11 (20)
S. viridans	9 (17)
Corynebacterium	4 (7.4)
Hemophilus	4 (7.4)
P. aeruginosa	3 (5.6)
S. pneumoniae	3 (5.6)
S. milleri	3 (5.6)
Negative culture	1 (2)

*Values are n (%). Other culture results include: usual respiratory flora (9); rare GNR (2); diphtheroids (2). Each of the following were detected in a single sample: GNR, rare diphtheroids, GPCs, *Staphylococcus lugdunensis*, *Eikenella* spp., and non-hemolytic streptococci.
GNR = Gram-negative rod; GPC = Gram-positive coccus.

for the classification of aerobic and anaerobic bacteria as described.[15] Taxa were categorized as dominant, major, or minor in terms of their relative abundance within a specimen (>10%, 1% to 10%, and <1%, respectively). To account for differences in sequencing depth, the relative abundance (RA) of each taxa was calculated (number of sequences for specific taxa/total number of sequencing *100) (McMurdie and Holmes[33]). 16S sequencing data were described using number and percent of samples in which a taxon was detected and the median and range for its relative abundance. Logistic regression was used to determine the association between relative abundance and detection by culture. Demographic variables were not included in the model, because they would not affect whether an organism would be identified. All tests of null hypotheses were evaluated at $\alpha = 0.05$. All analyses were performed using SAS version 9.3 software (SAS Institute Inc., Cary, NC).

Results

Fifty-four subjects met criteria for inclusion in the study. Patient demographics and clinical characteristics are listed in Table 1. Each subject had an average of 3 ± 1.3 isolates identified by bacterial culture and 21.5 ± 12.5 species identified by 16S sequencing. A description of the culture results is presented in Table 2, and the most commonly identified taxa (species and genus for those samples not able to be identified to the species level) are listed in Table 3. On average, 1.6 aerotolerant-dominant bacterial taxa were identified per subject by molecular technique (defined as >10% abundance of 16S sequences), but only 47.7% of these taxa were identified by culture (Fig. 1). Low-abundance taxa (abundance <1%) were only detected in 4.5% of cultures (Fig. 1). Conversely, of the organisms that were cultured, only 29.5% of the isolates represented the dominant taxa,

TABLE 3. Top 10 taxa identified by 16S rRNA pyrosequencing

				Relative abundance		
	Genus	Species	Prevalence (%)	Median	lIQR	uIQR
Aerobe	*Corynebacterium*	*Corynebacterium*	98.2	3.62	0.68	10.42
Anaerobe	*Propionibacterium*	*Propionibacterium acnes*	92.6	4.53	0.79	13.85
Aerobe	*Staphylococcus*	Coagulase-negative *Staphylococcus*	87.0	5.23	1.38	12.34
Other	*Ralstonia*	*Ralstonia* unclassified	83.3	2.68	0.49	7.62
Anaerobe	*Propionibacterium*	*Propionibacterium* unclassified	83.3	1.95	0.24	5.53
Aerobe	*Staphylococcus*	*Staphylococcus* unclassified	83.3	1.70	0.27	5.84
Other	*Ralstonia*	*Ralstonia pickettii*	72.2	0.70	0.00	1.58
Aerobe	*Stenotrophomonas*	*Stenotrophomonas* unclassified	66.7	0.33	0.00	1.52
Anaerobe	*Anaerococcus*	*Anaerococcus* unclassified	63.0	0.57	0.00	2.32
Aerobe	*Streptococcus*	*Streptococcus* unclassified	53.7	0.24	0.00	1.96

lIQR = lower interquartile range, rRNA = ribosomal RNA; uIQR = upper interquartile range.

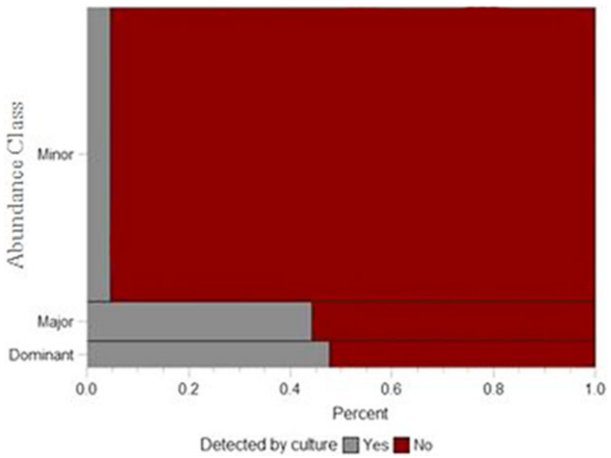

FIGURE 1. Mosaic plot of the number of times a culture was positive (shown in gray) and negative (shown in red) compared to 16S sequencing. Comparison was made at the species or less commonly the genus level, based on the most specific phylogenic level reported by the culture results. Bacterial taxa identified by pyrosequencing were divided into dominant (>10%), major (1% to 10%), and minor (<1%) according to relative abundance of each sample. Dominant taxa were cultured 47.7% (31/65) of the time. Major taxa were cultured 44.2% (42/95) of the time. Minor taxa were cultured 4.5% (32/704) of the time.

whereas 40% accounted for taxa with 1% to 10% abundance. It appears that the culture result may potentially be more likely to reveal a low-abundance organism than the dominant taxon.

Logistic regressions were used to determine whether bacterial taxa were more likely to be detected by culture with increasing relative sequence abundance. The odds that a culturable organism would be detected by culture were 2.3 times higher with each 10% increase in relative abundance ($p < 0.01$) (Fig. 2). One sample had a completely negative culture, but had 25 taxa identified by 16S pyrosequencing. Interestingly, 12% of isolates detected by culture were not identified by DNA pyrosequencing. These were from 10 samples with 4 isolates of *Streptococcus viridans*, 4 isolates of *Staphylococcus aureus*, 2 isolates of *Hemophilus influenza*, 2 isolates of coagulase-negative staphylococci, 1 isolate of *Streptococcus pneumonia*e, and 1 isolate of *Pseudomonas aeruginosa*.

Discussion

Prior studies directly comparing standard culture with broad-range 16S rRNA amplification and sequencing in the detection of bacteria in chronic wounds have documented the increased sensitivity of molecular methods over standard cultures and called for the increased use of culture-free methods in clinical practice with the hope of improving clinical outcomes.[34–37] However, this comparison has not previously been examined in head and neck diseases. Our data also suggest that standard clinical cultures for sinus bacteria are poorly representative of the actual bacteria present as detected by 16S sequencing. Despite the growing evidence that standard cultures may not adequately detect the range of bacterial species present in the sinonasal cavities, the use of cultures continues to be recommended in the management of CRS. Recent guidelines recommend that purulent secretions and acute exacerbations of CRS be treated with culture-directed antibiotics.[1,7] Cultures are also recommended to monitor for the development of antibiotic resistance in those patients being treated with long courses of antibiotics.[2] Additionally, culture results have been demonstrated to change antibiotic therapy in about one-half of CRS patients with purulent secretions, suggesting that the use of cultures is widespread in clinical treatment algorithms.[6]

Previous studies have reported that between 10% and 45% of bacterial cultures in CRS fail to grow any bacteria.[38,39] In the current study, only 1 of 54 subjects had a completely negative culture; however, in this case 16S sequencing detected 1 dominant *Acinetobacter* species, 4 major bacterial species (1 *Acinetobacter*, 1 *Ralstonia*, and 2 *Comamonas*), and 20 minor bacterial species. This supports the idea that DNA sequencing techniques are more sensitive, as all specimens had bacteria based on 16S. Standard cultures identified several bacteria in our cohort that were not present based on 16S sequencing. This included bacteria that are often thought to be pathologic in CRS and the focus of many culture-directed antibiotics (*S. aureus*, *S. viridans*, *S. pneumonia*, *H. influenza*, and *P. aeruginosa*). Although deeper sequencing of specimens may have increased the sensitivity for molecular detection of these organisms, we were unable to find any relationship between sequencing effort and incongruent detection between the molecular approach and culture. All cultures were processed in the standard fashion by the hospital laboratory and no special requests were made for culture media, handling, or processing, though this may have allowed for better bacterial detection by cultures. Culture results are limited by the routine practices of the clinical laboratory and as such, different institutions that follow different culture parameters may produce varied results. Previous authors have conjectured that discrepancies between cultures and molecular sequencing are more likely explained by the limitations and inadequacies of cultures.[35,37] Multiple explanations have been proposed to explain why cultures may be negative even when bacteria are present and these may also explain why cultures detect organisms that may not be present. These include the inability of culture media to replicate the sinus conditions, presence of biofilms, overgrowth of contaminants, selection for fast-growing bacteria over pathogens, poor specimen handling, and simple misidentification of bacteria,[13,35,37] thus suggesting that incomplete information may be obtained from the routine use of cultures in clinical practice.

Similar to prior studies comparing cultures and 16S sequencing, we found that bacteria with higher abundance are significantly more likely to be identified by culture than bacteria with lower abundance.[35] Though this finding suggests that culture would be likely to detect the bacteria of highest abundance, the dominant species was still not

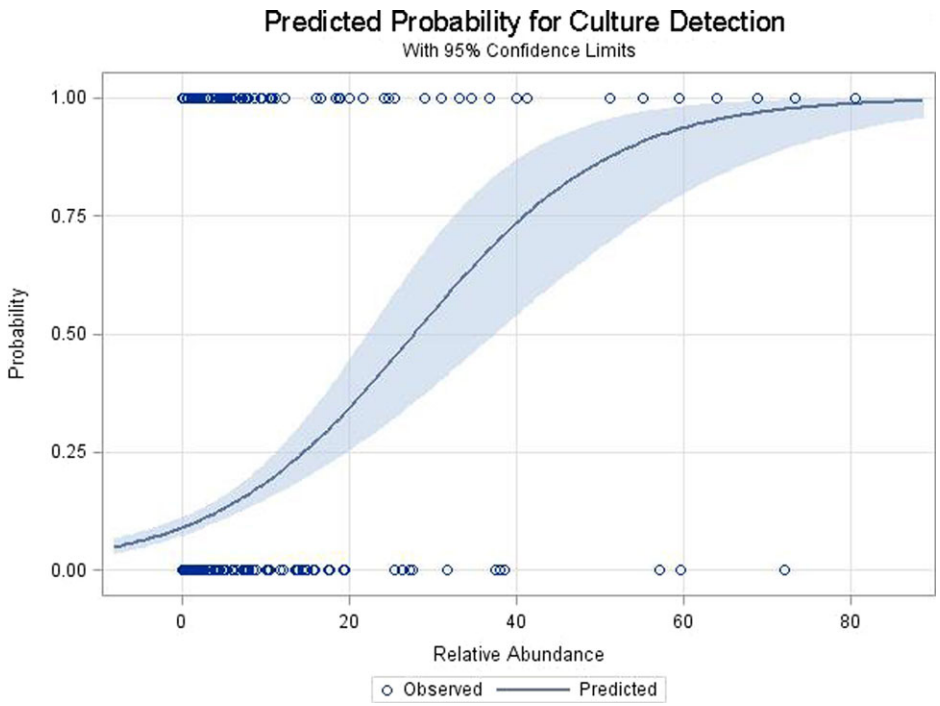

FIGURE 2. Predicted probabilities for detection by culture (value of 1 indicates detected and 0 not detected) from a logistic regression by the relative abundance value from 16S sequencing. The odds that an organism will be detected by culture is 2.3 times higher with each 10% increase in relative abundance ($p < 0.01$).

detected by culture in nearly 50% of cases. The concept that lower abundance bacteria may play a significant role in the disease process has been demonstrated,[40] and we have noted that culture is even less able to detect these lower-abundance bacteria (>95% of these bacteria were missed). Additionally, culture results are often too vague to be of clinical value. Though our cultures did show many bacteria that are commonly associated with CRS, including coagulase-negative staphylococcus, *Staphylococcus aureus*, and *Corynebacterium*,[8,9,41] other results included "Gram-negative rods" and "usual respiratory flora."

We often rely on the accuracy of cultures when choosing treatment for recalcitrant disease or persistent purulent secretions and are perplexed when these antibiotics fail or are unsure of the next step to escalate treatment. This often leads to more prolonged courses of stronger antibiotics with the potential for many side effects or the emergence of resistance, sometimes still with no clinical response. Our data suggest that cultures agree with molecular detection only 50% of the time, are only able to detect 3 of the 20 or more organisms that are present, and 12% of the time identify bacteria that are not detected by sequencing. Because we frequently use culture results to guide therapy, it is possible that limitations of standard clinical cultures may contribute to difficulties in the treatment of culture-negative and refractory CRS. Additionally, cultures are the foundation of almost all research to date on the role of bacteria in CRS. This prior understanding can be greatly increased with the incorporation of additional bacterial community information with the use of molecular techniques. Our understanding of the disease process may need to be reexamined as our ability to accurately define the microbiome of CRS improves with these culture-independent techniques.

One potential limitation of the current study is that different swabs were used for clinical and research sample processing. The ESwab (COPAN Diagnostics, Inc., Murrieta, CA) is used in our hospital for clinical culture as it may have a 10-fold greater recovery of organisms when compared to similar swabs.[42] For 16S sequencing, we used the CultureSwab (BD, Franklin Lakes, NJ) for ease of use, cost considerations, and prior experience with DNA extraction using these swabs. Given that our results show inferior bacterial detection by culture despite the potential advantage of the ESwab, we do not feel that differences due to the swabs would have improved the culture results.

Culture-independent molecular techniques are still being developed and improved, but recently many authors have considered 16S pyrosequencing to be the gold standard in identifying bacterial populations.[13,35,36] Despite its increasing popularity, there are still a number of potential limitations to the use of molecular techniques. With such high specificity of identification by 16S sequencing, comparing 16S and culture data can be challenging. Our clinical microbiology laboratory does not routinely identify anaerobic bacteria to the species level and may report their presence as "mixed anaerobic bacteria." To compare these culture results to our pyrosequencing data, we classified all bacteria as either "aerobic" or "anaerobic" as

described.[15] Similar to other studies, we classified only strict anaerobic bacteria as "anaerobes," leaving facultative anaerobes to be considered "aerotolerant" and classified as "aerobic."[35] Though it is possible that the "mixed anaerobic bacteria" culture results may include some bacteria that we identified as "aerotolerant," this is not likely a significant proportion of our samples and would not change our results statistically or clinically, particularly given these culture results do not provide sufficient detail to guide clinical decisions.

In the current study >99% of 16S sequences were reliably categorized to the phylum level, about 90% were identified to the genus level, and 54.5% were classified to the species level. Though bacteria identified to both the genus and species level were compared to the culture results and were used to create the mosaic plot of Figure 1, these comparisons are valid given a Good's coverage of >95%. As sequencing databases and techniques continue to improve (eg, increased sequence lengths permit deeper classifications), these sequences will more routinely be able to be classified to the species level.[43]

The cost of sequencing has previously been considered prohibitive for use in everyday clinical practice. The reagents and equipment costs per sample are less for cultures than DNA sequencing, approximately $5 vs $30 to $50 per sample, respectively, if a PCR machine and sequencer are available for use. However, clinical laboratory charges are added for labor and often samples are charged per isolate identified or varied by method of identification. In our experience, these charges can amount to unexpectedly high culture costs, in the range of >$500 per organism recovered. In comparison, data analysis and reporting for sequencing is not dependent on the number of isolates identified and, at the current time, is estimated at approximately $500 for 100 to 150 samples. Given that the cost of DNA sequencing and turnaround time will continue to decline with increased access, improving technology, and more efficient data analysis pipelines, we agree with other authors who consider the costs of molecular sequencing and cultures to be at least comparable, especially when considering that the use of molecular techniques may improve outcomes and lower overall treatment costs.[13,35,37]

Conclusion

In the current study, we demonstrated that bacterial detection using 16S rRNA gene sequencing allows for greater sensitivity and provides more information on bacterial biodiversity than standard clinical culture. Though cultures may offer some potentially useful information, this technique misses bacteria that are present in disease. The true clinical significance of this remains unknown; however, the ability to more accurately detect the bacteria that are present may allow for more effective, tailored treatment regimens and allow for an improved basis for clinical and laboratory research into CRS. We support the incorporation of culture-independent microbial detection techniques into both clinical and research practices as the assays improve, costs become less prohibitive, and turnaround times shorten. Translational study and technologic development are needed to help delineate the role of DNA-based identification in clinical use.

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