Evidence of Bacterial Metabolic Activity in Culture-Negative Otitis Media With Effusion

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Context.—Otitis media with effusion (OME) can lead to significant hearing loss in children. Although previous studies have shown that bacterial DNA is present in a significant percentage of effusions sterile by culture, whether the DNA represents viable organisms or “fossilized remains” is unknown.

Objective.—To determine if bacterial messenger RNA (mRNA), as detected by a reverse transcriptase–polymerase chain reaction (RT-PCR)–based assay, is present in chronic pediatric middle ear effusions that contain bacterial DNA but are sterile by standard cultural methods. Bacterial mRNAs have a half-life measured in seconds to minutes; therefore, detection of bacteria-specific mRNAs would be evidence that metabolically active organisms are present.

Design.—Blinded comparative study.

Patients.—A total of 93 effusions from pediatric outpatients seen for myringotomy and tube placement for chronic (>3 months) OME (median age of children, 17 months).

Setting.—Tertiary care pediatric hospital.

Main Outcome Measures.—Percentage of positive test results for RT-PCR–based assays compared with culture for Haemophilus influenzae and concordance between RT-PCR and PCR-based findings for bacterial nucleic acids.

Results.—Eleven (11.8%) of the 93 specimens tested positive by culture, PCR, and RT-PCR for H influenzae. A total of 29 specimens (31.2%) were positive by PCR but negative by culture for H influenzae. All 29 specimens were positive by RT-PCR for H influenzae–specific mRNA.

Conclusions.—The RT-PCR–based assay system can detect the presence of bacterial mRNA in a significant percentage of culturally sterile middle ear effusions, establishing the presence of viable, metabolically active, intact organisms in some culture-negative OME.
All patients were diagnosed by otoscopy and tympanometric evaluation. Criteria for myringotomy and tube placement included the presence of middle ear fluid for at least 3 months despite multiple courses of antimicrobial therapy, which included agents effective against β-lactamase-producing organisms. Treatment was not influenced by the results of this study. This study was deemed exempt by the Human Rights Committee of CHP.

**Clinical Specimen Acquisition and Microbial Culture**

The external ear canal was disinfected and desquamated by placing 70% isopropyl alcohol in the ear canal for 1 minute, after which it was suctioned out before myringotomy. Immediately after myringotomy, the effusion was removed from the middle ear cleft with a surgical suctioning device (a sterile 14-gauge Baxter Quik-Cath cover attached to a Senturia trap). An aliquot of the effusion was removed from the trap for culture using a small rayon-tipped swab on an aluminum shaft and immediately placed in transport medium in the operating room. All cultures were performed by the Clinical Microbiological Laboratories of CHP. Culture for H influenzae was performed using chocolate agar medium. Culture results were recorded for each specimen. The remainder of the sample was snap frozen on dry ice and then transported to the University of Pittsburgh Center for Genomic Sciences, where it was stored at −80°C before molecular analyses. The specimen set analyzed in this study was collected specifically to minimize RNA degradation and was, therefore, collected separately from the specimens analyzed in our previous study.7

**Clinical Specimen Preparation for RT-PCR**

To establish the sensitivity and specificity of the RT-PCR–based assays, pure H influenzae cultures obtained from CHF were lyzed, and the RNA was extracted using a commercial chaotropic agent (Trizol LS) according to the manufacturer’s recommendations (Life Technologies, Gaithersburg, Md). After thawing of the effusion, the specimen mRNA was extracted using the Trizol LS protocol. The effusion was kept on ice and combined with Trizol LS reagent at a ratio of 3:1. Effusions of less than 100 µL were brought up to volume with 0.1% diethyl-pyrocarbonate (DEPC)–treated water. No sample volume greater than 250 µL was used. The mixture was subjected to sonication at 0°C for 1 hour in a Branson waterbath sonicator (Fisher Scientific, Pittsburgh, Pa) to lyse the bacteria. Two hundred microliters per 250 µL of sample of RNase-free chloroform was added. The sample was vortexed briefly and then centrifuged for 15 minutes at 12,000 × g at 4°C. The aqueous layer was removed, and the phenol layer was reserved for a DNA extraction using the Trizol protocol. The mRNA was precipitated using RNase-free isopropanol, pelleted, washed in 75% ethanol, pelleted again, and suspended in DEPC-treated water. The extraction was then kept at −80°C until use.

**RT-PCR Assay**

An RT-PCR–based assay was developed for H influenzae. Primers and probe were designed from online DNA sequence information provided by the The Institute for Genomic Research (TIGR) World Wide Web genome database (http://www.tigr.org). The Oligo primer analysis software program (National Biosciences, Plymouth, Minn) was used as an aid in the design of primers and probes. All oligonucleotides were synthesized on-site using standard β-cyanoethyl phosphoramidite chemistry on a DNA synthesizer (model 392, Applied Biosystems, Inc, Foster City, Calif). Oligonucleotides were purified using Oligo purification cartridges (Applied Biosystems, Inc), vacuum centrifuged to dryness, resuspended in 1× TE (10-mmol/L TRIS buffer [pH 7.5] and 1-mmol/L sodium EDTA), and quantitated by absorption spectrophotometry at 260 nm.

Reverse transcription was performed in an 11-µL volume; 3.75 µL of clinical specimen was denatured for 3 minutes at 72°C and then added to 7.25 µL of a master mix. Reverse transcriptase reaction conditions consisted of 2.2 µL of 5× buffer (250-mmol/L TRIS buffer [pH 8.3], 375-mmol/L potassium chloride, 15-mmol/L magnesium chloride, 4-mmol/L each of the deoxycyriebonucleoside triphosphates [deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, and deoxythymidine triphosphate]), 50 U of Moloney murine leukemia virus (MLMV) reverse transcriptase (Life Technologies, Gaithersburg, Md), 10-mmol/L dithiothreitol, and 900-nmol/L primer). The mixture was incubated for 1 hour at 37°C. The complementary DNA generated from the RT reaction was used as a substrate for PCR. The PCR amplifications were performed as described.7 The GAPDH primers were evaluated for specificity against a battery of human pathogens and commensal flora, as well as against DNA isolated from human peripheral blood mononuclear cells.

Controls were included for each set of assays; 1 negative control was included for every 7 clinical specimens. The study was designed such that if any of the negative controls tested positive, the entire run would be discarded and then repeated; however, in this study, none of the runs contained false positives. All RT-PCR amplifications were performed in the University of Pittsburgh’s Core PCR Facility, which was specifically designed and staffed to eliminate false-positive PCR results attributable to end-product contamination.12,13 The Core PCR Facility serves as a clinical reference laboratory and has been certified by the College of American Pathologists for molecular diagnostic testing. To eliminate the possibility of bias, the individuals responsible for the microbiological isolation and identification of the bacteria were blinded to the results of the study. Autoradiographs were interpreted by one of us, a certified molecular diagnostician (G.D.E.), who was blinded as to the culture results.

**Labeling of Oligonucleotide Probes and Liquid Hybridization**

The PCR products were assayed by liquid hybridization and gel retardation analysis as described.7,10,11

**Deoxiribonuclease Digest**

Specimens that tested positive in the RT-PCR–based assay were designated as genetically positive for H influenzae nucleic acids (RNA, DNA, or both), as no effort had been made to remove contaminating DNA moieties from the extracted nucleic acid. Therefore, a second aliquot of each clinical specimen that tested positive in the generic nucleic acid assay was then subjected to a deoxiribonuclease (DNase) digestion procedure prior to the RT-PCR amplification to ensure that positive results were attributable only to the presence of mRNA and not to contaminating bacterial DNA. DNase digestion was accomplished by adding 5 µL of the clinical specimen to 5 µL of a 2× DNAse master mix. Reaction conditions for the digest consisted of 1 unit of DNAse I (Gibco BRL), 1 µL of 10× buffer (200-mmol/L TRIS buffer [pH 8.4], 20-mmol/L magnesium chloride, 500-mmol/L potassium chloride), and 3 µL of DEPC-treated water. Samples were incubated at room temperature (25°C) for 15 minutes. The digest was then heated for 10 minutes at 72°C to destroy the remaining DNAse I activity. One 3.75-µL aliquot was immediately used as the substrate in the GAPDH RT-PCR reaction, while another 3.75-µL aliquot was reserved on ice and subjected to PCR without RT as control for effective DNA digestion.
RESULTS

Development of an RT-PCR-Based Assay for H influenzae

A PCR primer-probe set, corresponding to the GAPDH gene of H influenzae, was developed from DNA sequence information obtained from the TIGR database and was used to develop a PCR-based assay and an RT-PCR-based assay (Table 1). Sensitivity testing demonstrated the ability of PCR assay to detect 100 fg of H influenzae DNA (50 genomic equivalents).

Specificity analyses detected no amplification in the presence of 100 ng (in excess of 30 million genomic equivalents) of DNA prepared from pure cultures of a panel of highly related bacterial species and other upper respiratory pathogens and commensal flora including Neisseria mucosa, Neisseria spp., Escherichia coli, Acinetobacter spp., Pseudomonas aeruginosa, Moraxella catarrhalis, Haemophilus parainfluenzae, Streptococcus pyogenes, the viridans group of streptococci, Streptococcus pneumoniae, streptococcus group C, Enterococcus spp., Staphylococcus epidermidis, and Staphylococcus aureus. In addition, DNA isolated from Candida albicans and from human peripheral blood mononuclear cells did not support amplification. Similarly, no amplification was evidenced from reagent blanks containing no exogenous template DNA.

Analysis of Pediatric Middle Ear Effusions for H influenzae

A comparative analysis for the detection of H influenzae was performed on 93 chronic middle ear effusions between traditional microbiological culture methods and an RT-PCR/PCR-based assay for the H influenzae GAPDH gene. Fifty-three specimens (57.6%) were culture negative and RT-PCR and PCR positive. Thus, the total number of specimens testing positive for H influenzae was 40 (43.0%).

Using our algorithm for RNA positivity (DNAase treated, RT-PCR positive, and PCR negative), of the 29 RT-PCR-positive, PCR-positive, culture-negative specimens, 28 (96.6%) tested positive for H influenzae mRNA (Figure). One (3.4%) of these 29 specimens tested RT-PCR positive and PCR negative, indicating that the DNAse treatment of this specimen was not totally effective. There was a highly significant tendency for H influenzae culture-negative specimens to test positive by RT-PCR (P<.001).

COMMENT

Comparison of RT-PCR and Culture Results

The percentage of culture-positive effusions (11.8%) for H influenzae is within the range normally reported for children with OME. The RT-PCR-based detection of H influenzae resulted in a 264% increase in the number of OME specimens testing positive compared with standard culture results. This number is in keeping with previous studies involving PCR-based detection systems for DNA.

Metabolically Active Pathogens in Culture-Negative Effusions

We have developed an assay to detect the existence of H influenzae mRNA and have achieved the successful amplification of H influenzae mRNA from the GAPDH gene in culture-negative, PCR-positive specimens. It has been shown that mRNA is generally a very rapidly degraded molecule, with most bacterial mRNAs having a half-life measured in seconds to minutes and most eukaryotic mRNA having a half-life measured in hours. Haemophilus influenzae is an obligate aerobe, and the glycolytic pathway is the organism’s main source of energy. Our choice of the GAPDH gene and the fact that mRNA synthesis requires an intact organism demonstrate that H influenzae organisms detected in culture-negative effusions are intact and are metabolically active. The finding that nearly all DNA-positive effusions in the study are also RNA positive suggests that DNA detection assays will be sufficient for diagnostic purposes.

Statistical Methods

The final RT-PCR with the DNAse I digest results were compared with standard culture results for H influenzae using \( \chi^2 \) analysis with 93 specimens as the total sample.

OME as a Possible Biofilm Disease

The relationship between bacteria and OME has long been a subject of research. Recent advances in the field of biofilm physiology may help to reconcile the conflicting observations that OME generally does not resolve despite the use of antimicrobial agents and our current findings that metabolically active bacteria are present in culture-negative effusions. In most natural environments the vast majority of bacteria exist as members of a biofilm. Quantitative bacterial enumeration experiments have demonstrated that less than 0.1% of the bacteria exist in the planktonic state; however, the majority of microbiological studies have focused on the planktonic population. Planktonic, or free-floating, bacteria are exquisitely sensitive to antimicrobial agents unless they possess specific antibiotic resistance genes that immobilize, deactivate, or cause efflux of the antibiotic from the bacterium. This sensitivity is largely attributable to their very rapid metabolic and divisional rates. Biofilm bacteria are characterized by a sessile lifestyle, reduced metabolic activity, greatly reduced rates of cell division, and tolerance to very high concentrations of antibiotics. The reasons behind this extraordinary resistance of bacteria in biofilms to antibiotics are unclear; however, it is most likely attributable to their greatly reduced metabolic rates. In addition, they extrude a glynocalyx polymer that acts as a protective exoskeleton that may serve as a physical barrier or adsorbent, thereby limiting the effective antimicrobial concentrations within the biofilm microenvironment. The bacteria within a biofilm create multiple microenvironments; these provide for the growth of both aerobic and anaerobic bacterial species that develop complex mutualistic relationships, analogous to a simple multicellular organism. The matrix of polysaccharide fibers produced by the bacteria serve as conduits that can act as a primitive circulatory system for the delivery of nutrients and removal of metabolic waste products. In essence, the bio-

Table 1.—Primers and Probe Used for the Detection of Haemophilus influenzae—Specific Messenger RNA in Middle Ear Effusions From Pediatric Patients With Chronic Otitis Media With Effusion

<table>
<thead>
<tr>
<th>Primer Sequence (5′-3′)</th>
<th>Primer</th>
<th>Probe</th>
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<tbody>
<tr>
<td>Hflu GAPDH upper 410</td>
<td>AGC CAT ACG CAG GTG AAG ATA</td>
<td>CAC GGA AAG CCA TAG CAC TTA</td>
</tr>
<tr>
<td>Hflu GAPDH lower 677</td>
<td>CAG CAT ACG CAG GTG AAG ATA</td>
<td>CAC GGA AAG CCA TAG CAC TTA</td>
</tr>
<tr>
<td>Hflu 589</td>
<td>GGC CGC GGT GCA TCA CAA ATC</td>
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Table 2.—Comparison of Culture and Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Results From 93 Pediatric Middle Ear Effusions Analyzed for Haemophilus influenzae

<table>
<thead>
<tr>
<th>Culture Results</th>
<th>RT-PCR Positive</th>
<th>RT-PCR Negative</th>
<th>Total</th>
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<tbody>
<tr>
<td>Culture positive</td>
<td>11</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Culture negative</td>
<td>29</td>
<td>53</td>
<td>82</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>53</td>
<td>93</td>
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\( \chi^2 \) Analysis of data for the otitis media with effusion DNAase study. If RT-PCR results were independent of culture results, then the expected numbers for RT-PCR positive would be (11/93) \times 40=4.73 for culture positive and (82/93) \times 40=35.27 for culture negative. The expected numbers for RT-PCR negative would be (11/93) \times 53=5.67 for culture positive and (82/93) \times 53=46.73 for culture negative. From the \( \chi^2 \) table, P<.001.
film serves as the primary habitat, and free-living bacteria occasionally break off in search of new habitats for colonization in a manner analogous to the coral polyp housed within a reef structure and the free-swimming hydra form.

Attachment of planktonic bacteria to a surface triggers the expression of a cassette of genes, which results in the “biofilm phenotype.” These phenotypic changes, analogous to sporulation or starvation survival, occur via the induction of RNA polymerase-associated sigma factors or through sensor-regulator proteins that are activated on attachment.

Technical Considerations

Great effort was used to ensure that the results of the culture and RT-PCR and PCR-based systems would accurately reflect the milieu in the middle ear and that they would not be compromised by external microbial contamination or end-product contamination (carryover). To control for the former, the external ear canal was treated for 1 minute with 70% isopropanol before myringotomy. This procedure renders the skin bacteria nonviable and results in desquamation, which provides for bacterial removal by suction before myringotomy, thus reducing the chance of introducing organisms or nucleic acids from the external environment into the middle ear effusion.

All the RT-PCR and PCR-based analyses were performed in the Core PCR Facility, which is the performance site for RT-PCR and PCR-based clinical diagnostics for the University of Pittsburgh Medical Center. This facility was specifically designed and staffed to eliminate carryover contamination; physical, procedural, and biochemical anticontamination measures were used for all analyses. This facility contains isolated laboratories for specimen receiving and processing, reaction preparation, amplification and analysis, and imaging, each with independent ventilation systems. Reagent controls were included in all experimental runs. Any run registering a positive signal in any of the reagent controls was discarded, and the entire run was repeated with fresh aliquots of the clinical specimens. A specific challenge was in developing a DNase digest that would be rigorous enough to remove all contaminating DNA and be specific enough to preserve the mRNA signal. Several commercial DNases were evaluated prior to use of the Gibco BRL product.

Conclusion

The majority of effusions from chronic OME are culture sterile. The challenge has been to determine, therefore, if OME represents an active bacterial infection, or whether it simply signifies a prolonged inflammatory response to a past insult. The Center for Genomic Sciences has undertaken the development of systems capable of detecting bacterial DNAs and RNAs and has applied these to various patient populations and animal models to address this issue. Hereafter, we have worked with PCR-based assays capable of detecting bacterial DNA. Initial work demonstrated the existence of pathogenic bacterial DNA in culture-sterile effusions from pediatric patients, and subsequent studies using the chinchilla model strongly suggested that viable, intact bacteria were required for detection of bacterial DNA by PCR-based assays. The detection of bacterial mRNA in OME, in the current study, essentially proves that intact, metabolically active H influenzae organisms are present in up to 35% (29 of 82) of culture-negative effusions. Future directions will involve an investigation of mRNAs corresponding to the bacterial genes directly associated with the pathological process and an investigation of de novo protein synthesis using a pulse-chase experimental protocol. In particular, we will focus on determining the biofilm gene expression pattern of H influenzae and then determining if OME bacteria express a biofilm or planktonic pattern of genes. It is our belief that OME will serve as a model for investigating the development of antibiotic resistance in chronic infections and that the demonstration of biofilm communities in vivo will provide a new paradigm for understanding chronic infectious disease.

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References