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Use of Multiplex PCR for Simultaneous Detection of Four Bacterial Species in Middle Ear Effusions

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A multiplex PCR procedure was developed for the simultaneous detection of *Alloiococcus otitidis*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* in middle ear effusions (MEEs) from patients with chronic otitis media with effusion. The bacterial 16S rRNA gene was chosen as the target, and the procedure used one common lower primer and four species-specific upper primers. The reaction was optimized by changing the primer concentrations to yield equal amounts of amplification products. The specificity of the reaction was verified with various bacterial species found in the nasopharynx. The performance of the procedure was examined with 25 MEE specimens, and the results were compared to those obtained by conventional culture methods. A detection level of 10 bacterial cells/reaction for each of the study organisms was achieved. By conventional culture methods, 8 (32%) of the specimens showed growth of one of the study organisms. In contrast, 21 (84%) of the specimens tested positive by the multiplex PCR. None of the culture-positive specimens were PCR negative, whereas three (12%) of the PCR-positive specimens tested positive for two of the four study organisms. Thus, the multiplex PCR method improves the detection rate significantly compared to that of the conventional culture method.

Otitis media with effusion (OME) is characterized by the presence of persistent fluid in the middle-ear cavity without any symptoms of acute infection, such as fever and otalgia (3). Despite intensive research during the past three decades, the etiology and pathogenesis of OME have remained unclear. Multiple infectious agents have been implicated in this disease (16), and the three most commonly encountered bacterial species by culture are *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* (8). However, in chronic OME (duration, >3 months), middle ear effusion (MEE) cultures yield positive results for only 20 to 30% of patients (11), although the great majority of MEEs show leukocytes by Gram staining, indicating an infectious etiology (15). The conventional culture methods have not been able to make clear the pathogenic cascade underlying OME (16). Therefore, PCR has been applied to the detection of bacterial DNA in the absence of culturable organisms.

Hotomi et al. (10) detected *H. influenzae* in 13 of 20 culture-negative MEE specimens by PCR combined with Southern blotting. Ueyama and coworkers (18) examined 80 MEEs, and found 46 *H. influenzae*-positive subjects by a PCR-dot blot setup but only 9 culture-positive subjects. However, there is only one report containing PCR data for all three species (16). Post et al. (16) analyzed 97 MEEs from pediatric patients and found 45 *M. catarrhalis*-, 53 *H. influenzae*-, and 29 *S. pneumoniae*-positive specimens, whereas culture resulted in 5, 21, and 5 positive specimens, respectively. In their study, PCR was carried out with two primer pairs for each bacterial species, and the detection of amplified products was done by liquid hybridization. Moreover, an algorithm was applied to interpret the results of replicate PCRs (16). These features make the system less feasible for analysis of a large number of samples, such as those needed for epidemiological studies. In addition, the presence of *Alloiococcus otitidis*, a potential pathogen found solely in OME (4, 7), has been ignored.

In the present study, we describe a multiplex PCR detection method for the simultaneous detection of *H. influenzae*, *M. catarrhalis*, *S. pneumoniae*, and *A. otitidis*. The 16S rRNA gene, which contains highly variable as well as conserved sequences, was chosen as the target. One common lower primer and four species-specific upper primers were used to generate four PCR products with different sizes. This study demonstrates the feasibility of our multiplex PCR system for the rapid and simple analysis of MEE specimens from children with OME.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains used in this study were isolated from clinical specimens. The strains were grown on standard bacterial culture plates and were identified by standard microbiological methods (14). The strains were as follows: *Arcanobacterium haemolyticum*, *Candida albicans*, *Enterobacter cloacae*, *Escherichia coli*, *H. influenzae*, *Haemophilus parainfluenzae*, *Lactobacillus* sp., *M. catarrhalis*, *Neisseria* sp., *Pseudomonas aeruginosa* (two isolates), *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *S. pneumoniae*, *Streptococcus pyogenes* (two isolates), viridans group streptococci, group C (Voges-Proskauer positive; three isolates) and group F streptococci, beta-hemolytic streptococci, non-group ACGF, and one yeast isolate (not characterized). For each PCR sample, a small amount of the culture was taken from the culture plates and was suspended by vortexing in ice-cold sterile water. The number of bacterial cells was estimated by making a 1:100 or 1:1,000 dilution in a 0.001% aqueous solution of crystal violet and counting at least 13 squares from duplicate samples on a Bürker 100-μm counting chamber (Paul Marienhof GmbH, Bad Mergentheim, Germany). Two dilutions containing 10^5 and 10^8 cells/ml were made in sterile water for PCR analysis. The number of bacterial cells in positive controls was determined by culturing freshly prepared serial dilutions of overnight cultures of *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae* suspended in sterile phosphate-buffered saline. The samples were boiled for 10 min to lyse the bacterial cells, aliquoted, and stored frozen at −20°C until use. The samples were thawed quickly at 37°C, kept on ice, and mixed thoroughly by pipetting immediately prior to removing a 1-μl sample for each PCR. The samples were thawed no more than 10 times, since repeated freeze-thaw cycles cause weakening of the PCR signal (20). Each sample was analyzed two or three times, and each PCR analysis included a positive control and at least two blanks with reagents only (all components except target DNA were included).

PCR primers. Various regions of the 16S rRNA gene sequences obtained from the GenBank database were studied to find sequences unique to as well as common among *A. otitidis*, *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae*. The potential primer sequences were analyzed for secondary structure formation,

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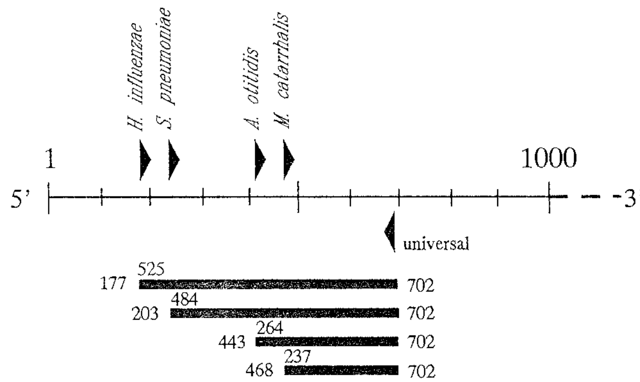

FIG. 1. Map of amplification by the multiplex PCR. Triangles indicate the relative location of the primers in the 16S rRNA gene sequence, and bars represent the amplification products. The numbers above the bars denote the exact lengths of the products (in base pairs), and the numbers at the ends of the bars indicate the product location in the *E. coli* 16S rRNA gene sequence (GenBank accession no. M24836).

G+C content, and primer-dimer formation with the OLIGO primer analysis software (National Biosciences, Plymouth, Minn.). A search for homology to known DNA sequences in the GenBank database was made with the BLAST algorithm (2). To select compatible primers for a multiplex PCR amplification, a simulated test PCR was run with AMPLIFY software (Genetics Department, University of Wisconsin, Madison, Wis.). Five primers were chosen following the homology searches and simulations.

The common lower primer is a 21-mer (5′-CTA CGC ATT TCA CCG CTA CAC-3′). It is located at positions 676 to 696 in the *A. otitidis* sequence (EMBL accession no. 59765), positions 679 to 699 in the *H. influenzae* sequence (GenBank accession no. M35019), positions 630 to 650 in the *M. catarrhalis* sequence (GenBank accession no. L13736), and positions 567 to 587 in the *S. pneumoniae* sequence (GenBank accession no. X58312). The specific upper primers were as follows: *A. otitidis* primer, a 20-mer (5′-GGG GAA GAA CAC GGA TAG GA-3′) located at positions 437 to 456; *H. influenzae* primer, a 24-mer (5′-CGT ATT ATC GGA AGA TGA AAG TGC-3′) located at positions 177 to 200; *M. catarrhalis* primer, a 20-mer (5′-CCC ATA AGC CCT GAC GTT AC-3′) located at positions 416 to 435; and the *S. pneumoniae* primer, a 22-mer (5′-AAG GTG CAC TTG CAT CAC TAC C-3′) located at positions 106 to 127. The relative locations of the primers in the *E. coli* 16S rRNA gene sequence are indicated in Fig. 1.

PCR protocol. The multiplex PCR mixture contained 1.6 μM *A. otitidis* primer, 1.4 μM *H. influenzae* primer, 0.2 μM *M. catarrhalis* primer, 0.04 μM *S. pneumoniae* primer, 0.4 μM common lower primer, 200 μM (each) the four deoxyribonucleoside triphosphates, and 1× Dynazyme buffer (10 mM Tris-HCl [pH 8.8] at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100; FinnZymes, Espoo, Finland). For each reaction, 3 U of *Taq* polymerase (FinnZymes) was used in a 50-μl reaction volume. The reaction profile was 3 min of initial denaturation prior to the addition of enzyme and 38 cycles of 94°C for 30 s, 66°C for 45 s, and 72°C for 1 min, followed by a 5-min final extension at 72°C. The amplification products were separated in 3% NuSieve 3:1 agarose containing 3 μg of ethidium bromide per ml at 6.5 V/cm for 2 h and were visualized by UV light illumination. The sensitivity of the PCR was studied with a dilution series of bacterial cells.

MEE specimen acquisition. The study included 16 children with OME (nine boys and seven girls; ages, 1 to 7 years; median age, 3 years) who underwent therapeutical myringotomy during the year 1996 at the Department of Otorhinolaryngology at Kuopio University Hospital, Kuopio, Finland. All the patients were diagnosed by validated otoscopy and tympanometric evaluation. Criteria for myringotomy included the presence of MEE for at least 1 month, despite ongoing or multiple courses of antimicrobial therapy. The external ear canal was mechanically cleaned. Immediately after myringotomy, the effusion was suctioned from the middle ear cavity with an electronic suction device into a sterile suction tip. An aliquot of the effusion was removed from the tip and instantly placed into a bacterial transport tube (Amies Charcoal Transport Swab System; Technical Service, Lancashire, Great Britain), and the tube was sent to the Department of Clinical Microbiology of the Kuopio University Hospital for culture of the sample. The remainder of the sample was stored frozen at −70°C before specimen preparation for PCR. A summary of the patient data is presented in Table 1.

Specimen preparation for PCR. The thawed effusion was transferred into a sterile Eppendorf tube for sample processing. The sample was boiled for 10 min,

TABLE 1. Summary of sample data and results of bacterial culture and PCR analysis

Specimen[a]	MEE type[b]	Duration of effusion (mo)	Antimicrobial therapy		Bacterial culture[c]	Multiplex PCR result
			Previous month	Ongoing		
0	Muc	3.5	Yes	No	−	*Hi*+++
1	Muc., pur.	4	Yes	No	−	*Ao*++
2	Muc.	2.5	No	No	−	*Ao*++
142.1	Muc.	7.5	Yes	Yes	−	*Hi*+++
142.2	Muc.	7.5	Yes	Yes	−	*Hi*++
143	Muc.	2.5	No	No	*Mc*	*Mc*+++
144	Muc.	2	NK[d]	No	*Sp*+++	*Sp*+++
145.1	Muc.	1	NK	No	−	*Hi*++
145.2	Muc.	1	NK	No	−	*Hi*+
148	Seromuc.	NK	No	No	*Mc*+	*Mc*+
153.1	Muc.	NK	No	No	−	*Ao*+, *Hi*++
153.2	Muc.	NK	No	No	−	*Ao*+++, *Hi*+
156.1	Muc.	4	No	No	−	*Hi*+
156.2	Muc.	4	No	No	−	−
159.1	Seropur.	3	No	No	*Sp*+++	*Hi*+++, *Sp*+++
159.2	Seropur.	3	No	No	−	*Hi*++
162.1	Muc.	5	No	No	*Mc*+	*Mc*+
162.2	Muc.	5	No	No	*Mc*+	*Mc*+++
165.1	Muc.	1.5	No	No	*Hi*+++	*Hi*++++
165.2	Muc.	1.5	No	No	*Hi*+++	*Hi*++++
166.1	Muc.	1	No	No	−	−
166.2	Muc.	1	No	No	−	*Ao*+
167	Muc.	1	No	No	−	*Hi*+++
1000.1	Muc.	NK	Yes	Yes	−	−
1000.2	Pur.	NK	Yes	Yes	−	−

[a] The specimen number extension (.1 and .2) refer to different ears of the same individual.
[b] Muc., mucoid; Pur., purulent; Seromuc., seromucinous.
[c] *Ao*, *A. otitidis*; *Mc*, *M. catarrhalis*; *Hi*, *H. influenzae*; *Sp*, *S. pneumoniae*; −, negative; +, a few CFU on culture plate and weak signal in PCR; ++, 10 to 100 CFU and clear signal; +++, crowded culture plates and intense signal; ++++, very intense signal in PCR; no sign, identification after enrichment culture.
[d] NK, not known.

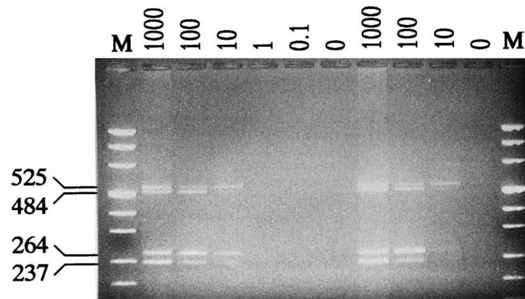

FIG. 2. Sensitivity of the multiplex PCR. Numbers above the lanes indicate the amount (in bacterial cells per reaction) of bacterial cells of each of the four study organisms used in the reaction. Lanes M, molecular size markers (*Msp*I-*Dra*I-digested pUC19 and *Hin*dIII-digested pUC57 yielding the following fragments 1,116, 883, 692, 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, 26, and 19 bp), and the sizes of the specific amplification products (in base pairs) are indicated on the left.

and after cooling, the resulting pellet was crushed in an equal volume of Tris-buffered phenol (pH 6.4). Sterile water was added to the tubes, and the mixture was vortexed. After centrifugation, the aqueous phase was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and then with chloroform-isoamyl alcohol (24:1). The samples were stored at −20°C.

Cloning and sequencing of the *A. otitidis* PCR product. At the time that the PCR procedure was designed, no culture data were available for *A. otitidis*. Therefore, the presumed *A. otitidis* PCR product was cloned into a plasmid vector for sequencing. The *A. otitidis*-positive samples were amplified with the specific primer and the common primer at equal concentrations (0.4 μM) and an annealing temperature of 63°C. The other conditions and the amplification profile were identical to those for the multiplex reaction. The amplification products were run in a 1.0% SeaKem GTG agarose and recovered from the gel for separation with the GeneClean purification kit (Bio 101, Inc., Vista, Calif.). The purified products were cloned into the pUC57 plasmid with the T-cloning kit (MBI Fermentas, Vilnius, Lithuania) and sequenced by the Sanger dideoxynucleotide chain termination method. Sequencing was performed in duplicate, and the sequences were aligned by using Dnasis software (Hitachi Software Engineering Co., Ltd.).

RESULTS

MEE specimen preparation. The amount of water added to the specimen was found to be critical for the performance of the PCR. If a volume of water corresponding to the specimen weight was added, no more than 1 μl of the resulting sample could be used in the PCR, probably due to the presence of inhibitory substances in the specimen. However, when an amount of water twice the volume of the specimen was added, a 5-μl sample showed no inhibitory effect on the sensitivity of the PCR (data not shown).

Determination of sensitivity of the PCR protocol. The sensitivity of the multiplex PCR procedure for the three pathogens was studied with serial 10-fold dilutions of pure cultures suspended in phosphate-buffered saline. Since cultured *A. otitidis* was not available, a serial dilution was made from a specimen positive for *A. otitidis*. The ability of the multiplex PCR to amplify all four bacterial templates is indicated in Fig. 2. The amounts of all amplification products showed a linear correlation to the amount of bacterial cells added to the reaction. The detection limit of the reaction for the pathogens was found to be 10 bacterial cells. No additional amplification products were observed.

Determination of specificity of the PCR protocol. The specificity of the multiplex PCR procedure was assessed by analyzing 21 patient isolates. The strains were chosen to represent (i) the bacterial species most commonly found in the nasopharynx, (ii) some frequently encountered human pathogens or opportunistic pathogens in clinical specimens, and (iii) bacteria whose 16S rRNA genes showed the highest degree of similarity to the primers used in the study. The first experiment was done with 10^5 bacterial cells (1 μl of a dilution of 10^8/ml) corresponding to nanogram quantities of bacterial DNA, a common amount used in bacterial PCR studies (17). To investigate whether the samples contained inhibitory substances or the DNA was degraded, a preliminary PCR analysis was run at a lower annealing temperature (60°C). As expected, several bacterial strains yielded positive amplification products. However, when the annealing temperature was raised to 66°C, only *H. parainfluenzae* yielded an amplification product. None of the other strains tested yielded amplification products. To validate the results, the experiment at the higher annealing temperature was repeated with three bacterial isolates that yielded the most intense amplification signals at the lower annealing temperature: *E. cloacae*, *E. coli*, and *H. parainfluenzae*. An amount of 100 and 10^5 bacterial cells/reaction mixture was used in this experiment. The results were consistent with those of the second experiment demonstrating the specificity of the method.

Verification of *A. otitidis* PCR signal. A presumed *A. otitidis* amplification product was found in five samples (Table 1). To verify the amplification of *A. otitidis*, products from two samples were sequenced, and the sequences were compared to the *A. otitidis* 16S rRNA gene sequence entry in the GenBank database. The sequences showed a 100% (262 of 262 bp) correspondence, which confirms that *A. otitidis* DNA was present in the MEEs from the pediatric patients.

Detection of bacterial DNA in patient specimens. The applicability of the multiplex PCR protocol to clinical specimens was studied with 25 MEEs from 16 children. An analytical gel run is shown in Fig. 3. Table 1 summarizes the data for all the patients. A relationship between the specimen type and bacterial species detected either by culture methods or by multiplex PCR was not observed. All culture-positive specimens were also PCR positive, and one specimen (specimen 142.1) tested PCR positive for *H. influenzae*, despite ongoing antimicrobial therapy. In addition, the PCR protocol was able to detect two of the study organisms in three samples: *H. influenzae* in specimen 159.1 together with *S. pneumoniae* (also verified by culture) and *A. otitidis* with *H. influenzae* in both ears from one patient (patient 153).

A comparison of culture and multiplex PCR methods is presented in Table 2. Culture test results were available for only three of the pathogens most commonly found in MEEs: *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae*. Growth of only one organism was observed in 8 (32%) of the specimens. The multiplex PCR procedure also detected the fastidiously growing organism *A. otitidis* and yielded positive results for 21

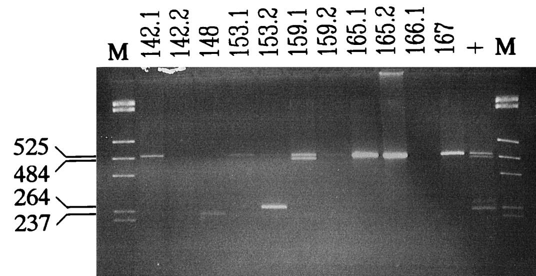

FIG. 3. Example of an analytical agarose gel for MEE specimens. The specimen number (same as in Table 1) is given above each lane. Lane +, positive control; lane M, molecular size markers (*Hin*fI- and *Rsa*I-digested pUC19 yielding the following fragments: 1,769, 1,419, 676, 517, 396, 241, and 214 bp). The sizes of the specific amplification products (in base pairs) are indicated on the left.

TABLE 2. Comparison of culture[a] and multiplex PCR results for 25 MEE from pediatric patients specimens[b]

Bacteria	No. (%) of specimens[b]					
	PCR+, culture+	PCR+, culture−	PCR−, culture+	PCR−, culture−	Total culture+	Total PCR+
A. otitidis	0 (0)	5 (20)	0 (0)	20 (80)	0 (0)	5 (20)
H. influenzae	2 (8)	11 (44)	0 (0)	12 (48)	2 (8)	13 (52)
M. catarrhalis	4 (16)	0 (0)	0 (0)	21 (84)	4 (16)	4 (16)
S. pneumoniae	2 (8)	0 (0)	0 (0)	23 (92)	2 (8)	2 (8)
One or more of the target species	8 (32)	13 (52)	0 (0)	4 (16)	8 (32)	21 (84)

[a] The culture data refer to *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae*. At the time that the PCR procedure was developed, no culture data for *A. otitidis* were available. However, because of the inherent limitations of conventional culture for detecting this fastidiously growing organism, the *A. otitidis* data were presented as culture negative.

[b] +, positive; −, negative.

(84%) specimens. The addition of the PCR data represents an increase of 52% in the total number of specimens identified to be positive for all four test organisms. This represent a 225% increase in the proportion of effusions testing positive for the culturable organisms. The increased sensitivity of the multiplex PCR was due to the detection of *H. influenzae* DNA in an additional 11 specimens (44%) and 5 samples (20%) testing positive for *A. otitidis*. Three (12%) of the PCR-positive samples tested positive for two of the four study organisms.

DISCUSSION

The objective of this study was to determine if a PCR-based procedure was suitable for bacterial DNA detection in pediatric MEEs that are negative by standard culture methods. The PCR procedure is sensitive and is able to identify discrete DNA fragments in femtogram quantities. Moreover, it is able to detect nonviable bacterial pathogens. A multiplex PCR approach was selected for this study, since multiple infectious agents have been implicated in OME. This technique saves considerable amounts of time and reagents, since only a single reaction must be set up and analyzed. Multiplex PCR is also ideal for conserving samples in limited supply (6). The bacterial 16S rRNA gene sequence is a good target choice for multiplex PCR, since it contains conserved as well as variable domains, which can be exploited to generate family- or group-specific amplifications (9, 22). A large number of bacterial species contain up to seven copies of this gene (5), providing further amplification of the signal.

The multiplex PCR procedure was developed to detect the three most common pathogens, *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae*, from OME specimens (8). A specific primer for *A. otitidis*, a gram-positive bacterium with a low G+C content, was included in the reaction mixtures to study the incidence of *A. otitidis* in chronic OME (1, 4, 7, 13). This unique fastidous bacterium was first described by Faden and Dryja (7), who inferred that it was pathogenic due to its occasional intracellular location and the presence of a significant number of inflammatory cells in the MEEs. To our knowledge, no other studies have addressed the presence of *A. otitidis* in MEEs. Although a rare gram-positive coccus was also encountered in other studies (12, 16), its role in OME has not been established.

All five primers were designed to have similar PCR kinetics. However, during the reaction optimization, the specific primer concentrations were altered to yield approximately equal amplification products from each of the study organisms. The optimal primer concentration range was wide, varying from 0.04 to 1.4 μM for *S. pneumoniae* and *H. influenzae*, respectively. This suggests that the DNA-releasing properties of the bacterial species may be different, or it may reflect different copy numbers of the 16S rRNA gene. A detection limit of 10 bacterial cells was achieved for each study organism. The sensitivity could not be improved by increases in common lower primer or deoxyribonucleotide concentrations (data not shown). The detection level was consistent with those previously reported for other PCR amplifications of 16S rRNA gene sequences (9, 21).

The specificity of the multiplex PCR for the study organisms was verified by performing the PCR with lysates from different bacteria. Only the *H. parainfluenzae* isolate yielded an amplification product which was similar to that for *H. influenzae*. This result was also validated from a similarity search of the GenBank database with the BLAST algorithm (2). The probability of detecting *H. parainfluenzae* was not considered a potential source of error, since this organism has very rarely been encountered in similar studies. For example, Ueyama et al. (18) and Post et al. (16) analyzed 80 and 97 MEEs from pediatric patients, respectively, and did not detect *H. parainfluenzae* by culture. However, two 16S rRNA gene sequences nearly identical to that of *S. pneumoniae* were found in the GenBank. These are of *Streptococcus mitis* and *Streptococcus oralis*, which have only two nucleotide differences in the 16S rRNA gene sequences compared to the *S. pneumoniae* 16S rRNA gene sequence; these streptococcal species may give identical amplification products in the multiplex PCR. The possibility that and the extent to which these organisms may interfere with the specific detection of *S. pneumoniae* in MEEs require further study. No other sequences in the database showed significant similarity with the specific primers.

The proportion of culture-positive MEEs in this study (32%) is in the range normally reported for children with OME (11, 12, 16). The multiplex PCR-based detection of the three pathogens resulted in a 225% (or 260% with *A. otitidis*) increase in the number of OME specimens testing positive compared with the number testing positive by the culture method. This finding is consistent with the results of comparative analysis of culture methods and PCR in the United States, where PCR-based detection of *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae* yielded a 268% increase in the number of specimens from patients with chronic OME that tested positive (16), and in Japan, where a 511% increase in the incidence of *H. influenzae* alone was detected (18). In pediatric patients with acute otitis media in Finland, PCR for *S. pneumoniae* alone increased the number of positive specimens by 154% compared to the number testing positive by culture (19).

The multiplex PCR procedure detected *A. otitidis* DNA in five specimens (20%). In comparison, *A. otitidis* was detected in 16 (5%) of 320 specimens by the culture method (7). To our knowledge, these are the only studies in which the presence of this organism in clinical specimens has been investigated. In this study, *A. otitidis* was found in two of the five samples together with *H. influenzae*. Faden and Dryja (7) isolated the bacterium in pure culture from 11 MEEs and in mixed culture from 5 specimens. The detection of *A. otitidis* as the sole bacterium in MEEs suggests that *A. otitidis* may be capable of inducing the chronic condition of OME. Our findings provide evidence that this unique bacterium is present in a certain number of MEEs from pediatric patients. The exact role of *A. otitidis* in the pathogenesis of OME will have to be the subject of further research.

In conclusion, these data demonstrate the ability of a PCR-based method to detect bacterial DNA in culture-negative MEE specimens. The methods described here should provide a sensitive and specific means for the eventual identification of four bacterial species in MEEs from pediatric patients with OME. The analysis is simple to perform and it provides results in a fashion that is easy to interpret. The results are available in no more than 7 h, which is much faster than the availability of results by routine culture, and the PCR procedure avoids the isolation and cultivation of the fastidious organism *A. otitidis*. The objective of our future study is to analyze sufficient numbers of specimens to allow for an epidemiologic evaluation of the relative incidence of the four study organisms.

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