Blinded Multiplex PCR Analyses of Middle Ear and Nasopharyngeal Fluids from Chinchilla Models of Single- and Mixed-Pathogen-Induced Otitis Media

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Multiplex PCR analyses for both bacterial and viral pathogens were conducted in a blinded manner on 33 archival specimens, of known culture status, procured from chinchilla models of both single- and mixed-pathogen-induced otitis media and from a pediatric patient. These specimens had been maintained at −70°C for up to 6 years. Experimental specimens evaluated included middle-ear effusions, nasopharyngeal lavage fluids and middle-ear lavage fluids from animals which were immunologically naive, sham-immunized or actively immunized with nontypeable Haemophilus influenzae and Moraxella catarrhalis, Streptococcus pneumoniae, or adenovirus as the causative agent. A PCR-positive signal for the microbe(s) inoculated was also obtained in four animal model specimens (12%) which were culture negative. One of two culture-negative human effusions was also PCR positive. Thus, overall, results obtained by blinded PCR were 85% concordant with traditional culture methods or correctly indicated the specific pathogen introduced in four specimens that were sterile. In no instance was a false-positive signal obtained for any of the five etiologic agents being evaluated. We conclude that the multiplex PCR analyses are rapid and accurate methodologies when they are used to retrospectively evaluate diverse archival specimens of limited volume from experimental models of otitis media.

Otitis media (OM), or middle-ear infection, presents as a spectrum of clinical entities from acute to chronic, with effusion, that are caused by one or more bacterial agents (30). Several upper respiratory tract viruses can predispose individuals to develop bacterial infections of the middle ear, resulting in a truly mixed etiology (3–5, 7, 12, 13, 25–27, 45–47, 50). Because OM and the medical and surgical management of this highly prevalent pediatric disease are of global socioeconomic importance (30), there is substantial interest in the development of novel treatments and intervention strategies, particularly in prevention via vaccine development (19, 24, 26, 32). To attain these goals, however, it is essential that there be a clear and complete understanding of the cascade of events which occur in the pathogenesis of OM in its multiple forms. Therefore, one area of enormous interest is the complex microbial etiology of OM, which was at one time considered to be a noninfectious disorder (34). Recently, the focus has been on the dynamic interrelationship between microbes and their human hosts throughout the evolution of OM and its resolution.

The bacterial species associated with OM are benign commensals in the microenvironment of the nasopharynx. However, following retrograde ascension of the Eustachian tube and invasion of the tympanum, these species behave like opportunistic pathogens. Once in the middle-ear space, they are likely capable of sensing these unique environmental cues and of responding in kind with altered expression of bacterial surface components (36). Likewise, the human host modifies its tolerance of the bacteria colonizing the upper airway to an active immunological response after this retrograde movement. Antecedent viral infections can reduce the effectiveness of the immune response to the bacteria. Depending on the nature of the predisposing viral infection and the cognate bacterial species, the host response will vary, resulting in the distinct presentations that characterize the spectrum of OM (53).

Attempts to simply culture viral or bacterial agents from chronic middle-ear effusions (MEEs) have often provided misleading and inadequate information about the microbial etiology of OM. The application of culture techniques in a prospective manner to both human studies of OM and experimental animal models has furthered researchers’ understanding of the microbiology of OM (8, 14–18, 21, 23, 26, 28, 38). With the advent of PCR, this area of study advanced rapidly and showed clearly that the incidence of both bacterial (29, 33, 52, 54) and viral (39, 40, 48) involvement in all forms of OM has been grossly underestimated.

We wanted to assess both the sensitivity and specificity of the nucleic acid amplification-based assays that have been specifically designed to target the bacterial and viral pathogens associated with OM through a retrospective, blinded evaluation of microbiologically characterized archival specimens. This study was undertaken to examine the potential of PCR-
based assays to provide information from both past studies and planned future experimental models.

The goal of the present study was to subject various archival effusions and lavages to multiplex PCR analyses for comparison with traditional culture data. Thus we blindly evaluated 31 experimental specimens and two human effusions via multiplex PCR techniques (43).

MATERIALS AND METHODS

Bacterial and viral agents. Clinical isolates of nontypeable Haemophilus influenzae (NTHI) (1885, 1128, 1712, and 86-028NP) and Moraxella (Brumallella) catarrhalis (1857) were obtained from children undergoing tympanostomy and tube insertion at Columbus Children’s Hospital for chronic OM; these isolates have been described previously (10, 11, 13, 49, 51). Streptococcus pneumoniae 6A was obtained originally from B. Anderson, University of Göteborg, Göteborg, Sweden, and has been described previously (35). All isolates were minimally passed and stored frozen in liquid nitrogen in skim milk plus 10% glycerol until needed. Adenovirus serotype 1 was also isolated from a pediatric patient at Columbus Children’s Hospital and has been characterized in chinchilla models (8, 10, 37, 51).

Experimental models of OM. Chinchillas (Chinchilla lanigera), free of OM as determined by otoscopy and tympanometry, were used for all studies. The inoculation of OM was either transbursular (TB) (31, 49) or intranasal (i.n.) (9, 10, 35, 49, 51) and is indicated in Tables 1 to 3. Specimens were obtained by epitympanic tap of the cephalad bulla or by lavage of the nasopharynx or tympanum with prewarmed sterile saline or culture medium. We included specimens which were culture positive and negative for the inoculated bacteria and/or virus. No specimens were sent from influenza A virus-inoculated animals, which thus served as a negative control in this study. Specimens were selected from animals that were immunologically naive, sham immunized, or actively immunized with NTHI antigens. Other specimens were from animals which had been treated with antibiotics. The two human specimens from the right and left ears of a child with chronic OM (E. and E.) were obtained with informed consent (Human Subjects Approval no. 94HS047) at the time of tympanoscopy and tube insertion. The child had not received antibiotics for the 2 weeks prior to surgery. Specimens were subjected to traditional culture, as detailed below, prior to freezing.

All archival specimens had been maintained at ~70°C without dilution or addition of a preservative. Specimens (approximately 50 to 100 µl) were shipped by Ohio State University on dry ice to the University of Pittsburgh Medical Center’s core PCR facility for analysis and were identified only by a code letter.

Viral and bacterial culture. MEIs and bulla lavage (BL) or nasopharyngeal lavage (NL) fluids were serially diluted and cultured on chocolate (Choc II; BBL Microbiology Systems, Cockeysville, Md.) or sheep’s blood agar (Fischer Scientific, Franklin, Ky.). Plates were incubated at 37°C in a humidified atmosphere with 5% CO2 for 48 h to detect and count the number of colonies. NTHI, M. catarrhalis, or S. pneumoniae per milliliter. Culture was also used to rule out bacterial contaminants in animals receiving only a viral pathogen.

Virus identification was performed on specimens processed within 2 to 3 h of collection at the Viral Identification Laboratory, The Ohio State University Medical Center (8, 51). Adenovirus (AV) was preliminarily identified on the basis of characteristic cytopathic effects (cell rounding and degeneration) on A549 and NHDFF cells for up to 21 days. Confirmatory identification was performed by the fluorescence-activated monoclonal antibody (Adenoclonal; Cambridge Biosciences Corp., Worcester, Mass.).

Specimen processing and analysis. Frozen specimens were processed for DNA by clarification and sonification as described previously (42). All specimens were analyzed by both a multiplex PCR-based assay for H. influenzae, S. pneumoniae, and M. catarrhalis (42, 43) and a simplex assay with a different primer set for each bacterial target. A duplex PCR-based assay for influenza virus (IV) and AV was used for some specimens (2). For other specimens, an individual AV assay was used. The oligonucleotide primers and probes for the three bacterial pathogens of OM have been described previously (42); those for the two viral pathogens are described below. Six data points were therefore generated for each specimen, discriminating a positive signal by signal intensity.

Specimens analyzed for bacteria with three or more positive data points were considered positive. Specimens with one or two positive data points were considered weakly positive. Any positive signal was considered indicative of the true presence of the target template in the specimen, as comprehensive anticontamination measures were employed (42). In addition, all the primer pairs utilized had been extensively characterized (42) and do not support amplification of DNA templates other than the intended targets. Weak-positive signals are indicative of sampling error wherein the specimen analyzed does not contain any target template, as opposed to an intrinsic lack of sensitivity of the assay. Specimens analyzed for AV with two or three positive data points were considered positive. Negative data point was considered to be weakly positive. After the initial comparison of the blinded PCR results with cultural and clinical data, additional aliquots of the discordant specimens were retested, reextracted, and reanalyzed.

Multiplex PCR analysis for bacterial pathogens. All amplifications were performed in a volume of 100 µl. A 2× PCR master mix was prepared (1× PCR buffer [50 mM Tris-HCl [pH 8], 50 mM [each] deoxynucleoside triphosphate, 2 U of Taq polymerase [Perkin-Elmer], and primers [100 nM each]). A 50-µl aliquot of the 2× master mix was then combined with specimen DNA adjusted to a volume of 50 µl for each amplification.

Thermal cycling was conducted with the Perkin-Elmer 9600 system. Standard cycling parameters included an initial 10-min denaturation step at 94°C, followed by 30 cycles with a 30-s denaturation step at 94°C, a 30-s primer annealing step at 50°C, and an extension step of 1 min at 72°C. After the 10-min extension at 72°C was included, followed by storage at 4°C.

RNA extraction and RT. RNA was extracted from specimens with RNAzol B (Cinna Biotech; Houston, Tex.) in accordance with the manufacturer’s instructions at 55°C for 30 min. Aliquots of the cDNA reaction products were then used in the duplex viral PCR assay.

Duplex PCR for viral pathogen analysis. The duplex PCR was performed on coded OM specimens adjusted to 50 µl with sterile distilled H2O. All amplifications were performed in triplicate in a volume of 100 µl with 50 µl of the RT reaction mixture for each assay (the RNA preparation contains sufficient residual DNA to permit sufficient amplification of the AV genomic sequence). Prior to cycling, a 10-min denaturation step at 94°C was included. The amplification parameters consisted of denaturation at 94°C for 10 min, followed by 30 cycles, each consisting of a 30-s denaturation step at 94°C, a 30-s primer annealing step at 55°C, and an extension step of 1 min for 35 PCR cycles. A 10-min final extension step at 72°C was included following cycling. Each amplification run included a positive control dilution series for each of the individual viruses with cDNA (IV) or DNA (AV). Multiple negative controls consisting of reagent blanks with no exogenous template DNA were also included with each assay.

The primers and probes used in the duplex PCR assay for AV and IV were as follows. For AV, the PCR-amplified length was 301 bp and the primers were 5′-GCC GCA GTG TGT GTA CAT GCA CAT C′-3′ (ADVHP5, upstream primer) and 5′-CAG CAC GCC GCG GAT GAT AAA TCA′-3′ (ADVHP3, downstream primer). The sequence of the probe was 5′-GGG CTT GTG CTT TGT CCC GGC GCC ACC GAC ACG TA′-3′ (ADVHPXPR2). For IV, the PCR-amplified product length was 212 bp and the primers were 5′-CAG CAT GAT GTA TTA CTA′-3′ (INFPR1, upstream primer) and 5′-CAG CAT GAT GTA TTA CTA′-3′ (INFPR2, downstream primer). The amplification parameters were the same as those described previously (22a, 42, 43). Briefly, following amplification of DNA and cDNA templates, the resultant PCR and cDNA products were subjected to liquid hybridization with a 32P-5′-end-labeled oligonucleotide probe. All probes were selected to be complementary to one of the amplified strands and were located internally to the primers to ensure that there was no chance of the probe hybridizing to the primer sequences. The liquid hybridization was carried out for 15 min at 55°C following denaturation of the amplified DNA in the presence of the probe for 5 min at 95°C. The liquid hybridization products were then subjected to gel retardation analysis through a 16-cm, 8% polyacrylamide gel, run at 225 V for 60 to 90 min. Detection of retarded probe, indicative of the specific amplified DNAs, was accomplished by autoradiography. Retarded bands for experimental specimens demonstrating equal migration with those from positive control specimens were scored as positive bases.

Statistical analyses. The PCR results were compared to standard culture results for NTHI, M. catarrhalis, S. pneumoniae, and AV with 37 pairs as the total sample for bacterial pathogens and 15 pairs as the total sample for viral pathogens. McNemer’s test, with correction for continuity, was used throughout to test the null hypothesis of nonequivalent PCR and culture results. Conservative two-tailed P values are reported. The intraclass correlation coefficient kappa (κ) is also reported as a measure of interrater reliability.
### RESULTS

To summarize, of the 33 original specimens (all of which were diluted during processing and prior to amplification), 24 (73%) yielded PCR results which were concordant with traditional culture data for bacterial pathogens (Table 1). A positive PCR signal for *M. catarrhalis* was obtained only in the two specimens procured from animals inoculated with this organism (specimens A and B). Positive signals for NTHI were obtained from seven middle-ear and nasopharyngeal specimens. A positive signal for NTHI was obtained from two NL specimens (L and N) and two MEE specimens (P and Q) (15%) despite the culture-negative status of the microbe inoculated (100, 85, and 100%, respectively). A positive PCR signal was obtained in five specimens (L, N, P, Q, and EE2) (15%) despite the culture-negative status of the microbe inoculated (100, 85, and 100%, respectively). A positive PCR signal was obtained in five specimens (L, N, P, Q, and EE2) (15%) despite the culture-negative status of the microbe inoculated (100, 85, and 100%, respectively). A positive PCR signal was obtained in five specimens (L, N, P, Q, and EE2) (15%) despite the culture-negative status of the microbe inoculated (100, 85, and 100%, respectively). A positive PCR signal was obtained in five specimens (L, N, P, Q, and EE2) (15%) despite the culture-negative status of the microbe inoculated (100, 85, and 100%, respectively). A positive PCR signal was obtained in five specimens (L, N, P, Q, and EE2) (15%) despite the culture-negative status of the microbe inoculated (100, 85, and 100%, respectively).

Immune status of the chinchilla host had no effect on PCR amplification of bacterial or viral nucleic acid, as sham-immunized (K, L, and S), actively immunized (C to F, I, J, M to R, and T [reanalyzed specimens Z, AA, CC, and DD]), and naive animals (A, B, and U1 to W3) all yielded equivalent percentages of PCR results which matched with either traditional culture or the microbe inoculated (100, 85, and 100%, respectively).

A positive PCR signal was obtained in five specimens (L, N, P, Q, and EE2) (15%) despite the culture-negative status of the microbe inoculated. A positive signal for *S. pneumoniae* was obtained from one culture-negative human effusion (EE2) collected from a child with chronic OM of unknown etiology. In no instance was a false-negative signal obtained for any of the three bacterial pathogens in any specimen recovered from an experimental model.

### Table 1. Results of traditional culture versus multiplex PCR analysis of OM specimens for bacterial pathogens

| Blinding code | Bacterium (strain) | Inoculation route | Bacterial culture result<sup>a</sup> | Specimen<sup>b</sup> | PCR result<sup>d</sup> |
|--------------|-------------------|------------------|-------------------------------|----------------|$\text{NTHI} \quad S. \text{pneumoniae} \quad M. \text{catarrhalis}$ |
| A            | *M. catarrhalis*   | i.n.             | 5E6                           | NL              | –                     | +                     |
| B            | *M. catarrhalis*   | i.n.             | 1E7                           | NL              | 1/6+                  | –                     |
| C            | NTHI (1128)        | TB               | 9E5                           | MEE (serous)    | +                     | –                     |
| D            | NTHI (1885)        | TB               | 2E4                           | MEE (serous)    | 1/6+                  | –                     |
| E            | NTHI (1128)        | TB               | 8E3                           | MEE (serosanguinous) | + 1/6+   | –                     |
| F            | NTHI (1885)        | TB               | NG                             | MEE (serosanguinous) | –   | –                     |
| G            | —                  | i.n.             | NA                             | BL              | 1/6+                  | –                     |
| H            | —                  | i.n.             | NA                             | NL              | 1/6+                  | –                     |
| I            | NTHI (86-028)      | TB               | 2E7                           | MEE (mucopurulent) | –   | –                     |
| J            | NTHI (1712)        | TB               | 5E7                           | MEE (mucopurulent) | –   | –                     |
| K            | NTHI (86-028)      | TB               | 3E7                           | MEE (purulent)  | +                     | –                     |
| L            | NTHI (1712)        | TB               | NG                             | NL              | 1/6+                  | –                     |
| M            | NTHI (1712)        | TB               | 2E1                           | NL              | –                     | –                     |
| N            | NTHI (1712)        | TB               | NG                             | NL              | –                     | –                     |
| O            | NTHI (86-028)      | TB               | 2E10                          | MEE (mucopurulent) | +   | –                     |
| P            | NTHI (1712)        | TB               | NG                             | MEE (mucopurulent) | + 2/6+   | –                     |
| Q            | NTHI (86-028)      | TB               | 3E2                           | MEE (highly mucoid, purulent) | +   | –                     |
| R            | NTHI (86-028)      | TB               | 3E2                           | NL              | 1/6+                  | –                     |
| S            | NTHI (1712)        | TB               | 1E3                           | NL              | –                     | –                     |
| T            | NTHI (1712)        | TB               | 5E3                           | NL              | 1/6+                  | –                     |
| U<sub>1</sub> | *S. pneumoniae*    | i.n.             | + (not available)             | NL              | –                     | –                     |
| U<sub>2</sub> | *S. pneumoniae*    | i.n.             | 3E3                           | BL (right)      | –                     | +                     |
| U<sub>3</sub> | *S. pneumoniae*    | i.n.             | NG                             | BL (left)       | –                     | –                     |
| V<sub>1</sub> | *S. pneumoniae*    | i.n.             | + (not available)             | NL              | –                     | –                     |
| V<sub>2</sub> | *S. pneumoniae*    | i.n.             | 6E4                           | BL (right)      | –                     | +                     |
| V<sub>3</sub> | *S. pneumoniae*    | i.n.             | 3E2                           | BL (left)       | –                     | –                     |
| W<sub>1</sub> | *S. pneumoniae*    | i.n.             | + (not available)             | NL              | –                     | –                     |
| W<sub>2</sub> | *S. pneumoniae*    | i.n.             | NG                             | BL (right)      | –                     | –                     |
| W<sub>3</sub> | *S. pneumoniae*    | i.n.             | NG                             | BL (left)       | –                     | –                     |
| X            | NTHI (1128)        | TB               | NG                             | MEE (purulent, sanguinous) | –   | –                     |
| Y            | NTHI (1128)        | TB               | NG                             | MEE (purulent, sanguinous) | –   | –                     |
| EE<sub>1</sub> | Unknown<sup>c</sup> | TB               | NG                             | MEE (sanguinous) | –   | –                     |
| EE<sub>2</sub> | Unknown<sup>c</sup> | TB               | NG                             | MEE (purulent)  | –   | –                     |

<sup>a</sup> BL, bulla lavage (right or left ear).

<sup>b</sup> 1/6+, one positive out of six.

<sup>c</sup> Human; sterile effusion.

<sup>d</sup> 8E6, 8 \times 10^6 CFU/ml; NG, no growth; NA, not applicable (no bacterium inoculated).
sults (D, I, R, and T) were reanalyzed with a second blinded aliquot (specimens Z, AA, CC, and DD) (Table 2). When available, an identical aliquot was sent; however, if there was none remaining, an aliquot from the same site of the same animal but collected 3 to 7 days earlier was sent. Reanalysis was performed without dilution of these specimens prior to amplification. Two of these specimens were consistently PCR negative, but the other two became positive upon reanalysis. The two negative specimens (Z and CC) were procured from chinchillas inoculated with two different NTHI strains (1885 and 86-028NP) both known to support amplification by the multiplex PCR (specimens K and O, for example; and additional data not shown). Overall, however, there was excellent agreement between bacterial culture results and PCR results (McNemar’s test, $\chi^2 = 0, P = 1.0; k = 0.4, P = 0.02$).

Adenoviral DNA was amplified from two NL specimens (specimens A and M) collected 11 and 17 days after i.n. inoculation of AV (Table 3). An indeterminate signal (one of three data points was positive) was also obtained for one additional NL specimen (specimen S) collected 42 days after inoculation, which was negative by traditional culture. None of the seven MEE specimens collected 17 to 42 days after i.n. inoculation of AV yielded a positive signal by PCR (specimens G, I, J, K, O, P, and Q). All of these specimens were also negative for AV by traditional culture, as were an additional five NL specimens collected 17 to 42 days after inoculation, which also were PCR negative (specimens H, L, N, R, and T). In no instance was a false-positive PCR result obtained for AV or for influenza A virus from any specimen procured from an experimental model. There was again excellent agreement between culture data and PCR data for analysis of viral pathogens (McNemar’s test, $\chi^2 = 0, P = 1.0; k = 0.6, P = 0.008$).

**DISCUSSION**

Blinded PCR analyses of specimens collected from chinchillas infected with either a single viral or bacterial pathogen or a combination of infectious agents and maintained frozen for up to 6 years were found to be both highly sensitive and highly specific. On no occasion did the detection signal indicate the presence of bacterial or viral DNA or RNA which had not been experimentally introduced. All three major bacterial pathogens of OM—NTHI, *M. (B.) catarrhalis*, and *S. pneumoniae*—and the viral pathogen AV were correctly identified as being involved in the disease induced. The multiplex and duplex PCR analyses matched our ability to culture the inoculated bacterium or virus by standard methodology. Additionally, in six cases, PCR analyses either correctly indicated the previous involvement of a given pathogen in the induced disease or were able to amplify DNA in the absence of our ability to detect viable microbes by culture.

NTHI DNA from three clinical isolates was amplifiable regardless of the nature or viscosity of the effusion (sanguinous, purulent, mucopurulent, or highly mucoid) or the range of bacterial load determined at the time of collection ($10^2$ to $10^{10}$ CFU/ml). PCR amplification of bacterial or viral genomic fractions was similarly independent of the presence of a coinfected microbe. Immune status of the host was not expected to and in fact did not affect PCR amplification of NTHI DNA.

A persistent PCR-negative result was obtained in two specimens, despite their culture-positive status. Both specimens were from animals inoculated with two different NTHI strains whose DNAs are known to be amplifiable by this methodology. Neither specimen was innately problematic, since other physiologically similar specimens were successfully analyzed; thus,

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### TABLE 2. Results of second PCR analysis of selected specimens

| Blinding code | Bacterium (strain) | Inoculation route | Bacterial culture result | Specimen | PCR result |
|---------------|-------------------|------------------|--------------------------|----------|------------|
| Z             | NTHI (1885)       | TB               | 2E4*                     | MEE (serous) |            |
| AA            | NTHI (86-028)     | TB               | 1E8                      | MEE (mucopurulent) |            |
| CC            | NTHI (86-028)     | TB               | 3E1                      | NL       |            |
| DD            | NTHI (1712)       | TB               | 2E3                      | NL       |            |

* $2E4, 2 \times 10^4$ CFU/ml.

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### TABLE 3. Results of traditional culture versus multiplex PCR analysis of OM specimens for viral pathogens

| Blinding code | Viral pathogen | Viral culture result | Specimen | PCR result |
|---------------|---------------|----------------------|----------|------------|
| A             | AV            | ND                   | ND       |            |
| B             | ND            | NL                   | ND       |            |
| C             | ND            | MEE (serous)         | ND       |            |
| D             | ND            | MEE (serous)         | ND       |            |
| E             | ND            | MEE (serosanguinous) | ND       |            |
| F             | ND            | MEE (serosanguinous) | ND       |            |
| G             | AV            | BL                   | ND       |            |
| H             | AV            | NL                   | ND       |            |
| I             | AV            | MEE (mucopurulent)   | ND       |            |
| J             | AV            | MEE (mucopurulent)   | ND       |            |
| K             | AV            | MEE (purulent)       | ND       |            |
| L             | AV            | NL                   | ND       |            |
| M             | AV            | +                    | ND       |            |
| N             | AV            | NL                   | ND       |            |
| O             | AV            | MEE (mucopurulent)   | ND       |            |
| P             | AV            | MEE (mucopurulent)   | ND       |            |
| Q             | AV            | MEE (highly mucoid, purulent) | ND       |            |
| R             | AV            | NL                   | ND       |            |
| S             | AV            | NL                   | 1/3 + d   | ND         |
| T             | AV            | NL                   | ND       |            |
| U1            | ND            | NL                   | ND       |            |
| U2            | ND            | BL (right)           | ND       |            |
| U3            | ND            | BL (left)            | ND       |            |
| V1            | ND            | NL                   | ND       |            |
| V2            | ND            | BL (right)           | ND       |            |
| V3            | ND            | BL (left)            | ND       |            |
| W1            | ND            | NL                   | ND       |            |
| W2            | ND            | BL (right)           | ND       |            |
| W3            | ND            | BL (left)            | ND       |            |
| X             | ND            | MEE (purulent, sanguinous) | ND       |            |
| Y             | ND            | MEE (purulent, sanguinous) | ND       |            |
| EE1           | Unknown       | ND                   | MEE (sanguinous) |            |
| EE2           | Unknown       | ND                   | MEE (purulent) |            |

*a ND, not done.
*b BL, bulla lavage (right or left ear).
*c IA, influenza A virus; ND, not done.
*d 1/3 +, one positive out of three.
it is unclear at this time why we obtained a negative PCR result for these two specimens.

Two sterile human MEEs recovered from a single patient, who had not been on antibiotics in the 2 weeks prior to surgery, were also subjected to blinded PCR analyses. Both effusions, one sanguinous, the other purulent, were reported as culture negative, yet one clearly contained amplifiable S. pneumoniae DNA. This type of effusion, culture negative and PCR positive, has stimulated a great deal of discussion recently as to the significance of these results (42). The question has been whether or not PCR methodology, while exquisitely sensitive, simply amplifies residual DNA remnants from an effusion which is truly sterile because the microbes have been killed by antibiotics or the host immune response, rather than indicating the presence of viable, but not culturable, microbes. In the context of the present study, whether or not a specimen is truly culture negative was not our primary focus; however, this same question could legitimately be asked regarding the six culture-negative and PCR-positive results obtained here.

This subject was recently addressed in a series of experiments wherein Post et al. (41) demonstrated that a positive PCR signal was likely due to the presence of viable microbes and not simply to residual DNA fragments. Attenuated bacteria and those which were treated with antimicrobials to which they were sensitive were able to persist in a PCR-amplifiable form in the chinchilla middle ear for periods of 9 days and 3 weeks, even though they were not culturable. Conversely, dead bacteria and purified bacterial DNA were cleared within 1 to 3 days, depending on the species. It is not possible to directly extrapolate from these findings to the data obtained in the present study from archival frozen specimens. It is, however, interesting to speculate as to what PCR-positive signals mean when they are obtained from these culture-negative specimens of varied origins.

Very recently, an RT-PCR methodology was developed and used to detect bacterial mRNA in culture-negative recurrent and chronic pediatric effusions (44). These findings hint at a potentially more complex relationship in the middle-ear milieu between the microbe(s) and an antibiotic-treated or perhaps immune host than traditional culture techniques are capable of. The overriding implications of these data are that metabolically active bacteria persist in the middle ear, despite or perhaps because of antibiotic treatment or elicitation of the host’s immune response that results in a loss in or regression of constitutional disease symptoms.

The culture-negative status of MEEs can be explained by several hypotheses: (i) the titer of organisms is below the limit of detectability, (ii) the microbes have been pressure into a consortium (biofilm) arrangement (20). The latter two mechanisms may be induced by antibiotic treatment and/or the host’s immune response (1). Organisms present in these forms have very low metabolic and cell division rates compared to those of free-floating or planktonic bacteria and thereby could provide the stimulus for the chronic inflammation and prolonged presence of effusions noted in children without causing overt constitutional symptoms. These phenomena are the subject of ongoing investigations.

In conclusion, blinded multiplex PCR analyses were extremely sensitive and specific when they were used to retrospectively analyze experimental effusions and lavage fluids which had been maintained at −70°C, without a cryopreservative, for up to 6 years. The multiplex and duplex PCR assays for bacteria and viruses provide a rapid and accurate mechanism to analyze stored human or animal samples of limited volume and will serve as a powerful adjunct to our future prospective experimental studies.

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