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Comparison of PCR Assay with Bacterial Culture for Detecting *Streptococcus pneumoniae* in Middle Ear Fluid of Children with Acute Otitis Media

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We have studied etiological diagnosis of acute otitis media (AOM) by comparing a newly developed pneumococcal PCR for *Streptococcus pneumoniae* to bacterial culture with 180 middle ear fluid (MEF) samples of 125 children with 125 episodes of AOM. For pneumococcal PCR assay, DNA from MEF samples was extracted by phenol-chloroform. The outer primers used amplified a 348-bp region of the pneumolysin gene, and the inner primers amplified a 208-bp region. *S. pneumoniae* was cultured in 33 (18%) samples, and pneumolysin PCR was positive for 51 (28%) of 180 MEF samples. Only 2 of 21 PCR-positive, *S. pneumoniae* culture-negative samples were positive for other otitis pathogens. By combining MEF culture and PCR results, 54 (30%) of 180 MEF samples had evidence of pneumococcal etiology. In conclusion, pneumolysin PCR is a sensitive and specific new method to study pneumococcal involvement in MEF samples of children with AOM.

The microbial diagnosis of acute otitis media (AOM) is currently based on bacterial culture of a middle ear fluid (MEF) sample. *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* are cultured in 30 to 40 and 10 to 20% of cases, respectively (7). Other bacteria that are isolated less often include *Branhamella catarrhalis*, group A streptococcus, and *Staphylococcus aureus*. By using both bacterial culture and antigen detection methods, *S. pneumoniae* has been found in 40 to 60% of all cases of AOM (4, 12). However, large numbers of MEF samples still remain negative for bacterial antigens or by culture, although polymorphonuclear leukocytes present in MEF samples (17) suggest bacterial etiology in these cases as well.

Gene amplification methods like PCR are increasingly being used in the etiological diagnosis of microbial infections. The presence of several common respiratory viruses (2, 3, 14, 15) and the outer membrane protein P6 of *H. influenzae* (6) in nasopharyngeal secretions and MEF samples has been demonstrated by PCR. The detection of pneumococcal DNA in the blood of adults with pneumonia has been described by Rudolph et al. (18) and, with another set of pneumolysin-specific primers, by Salo et al. (19).

Pneumolysin is a species-specific protein toxin produced intracellularly by all clinically relevant pneumococcal strains (16). A demonstration of pneumolysin DNA is thus expected to indicate pneumococcal involvement in the disease, regardless of the pneumococcal serotype. We used the newly developed PCR assay (19) for the first time to detect pneumolysin DNA in secretions, MEF samples of children with AOM. The aim of this study was to improve etiological diagnosis of AOM.

MATERIALS AND METHODS

Patients. Altogether, 135 children with AOM, aged 3 months to 7 years 5 months (median, 2 years 6 months), were enrolled in the study during 1990 to 1992 in a private otolaryngological center (15 children) or the Department of Otolaryngology, University of Helsinki, Helsinki, Finland (120 children). AOM was defined by pneumatic otoscopic examination suggesting MEF and at least one of the following symptoms: otalgia, tugging at or rubbing of the ear, rectal or axillary temperature of at least 38.0°C, irritability, restless sleep, acute gastrointestinal symptoms (vomiting or diarrhea), or other simultaneous respiratory infection. Patients with secretory otitis media, tympanostomy tubes, spontaneous perforation of the tympanic membrane, or antibiotic treatment within 1 week of enrollment were excluded. Written informed consent was obtained from the parents of all children before enrollment. The study protocol was approved by the ethics committees of the National Public Health Institute and University of Helsinki.

Samples. MEF samples and nasopharyngeal aspirates (NPA) were obtained at each patient's initial visit. After mechanical cleaning of the external ear canal, the tympanic membrane was anesthetized with 90% liquified phenol. A paracentesis needle was inserted through the anteroinferior or posteroinferior part of the tympanic membrane, and MEF was aspirated with an electric suction device into the sterile glass suction tip. Thereafter, the secretion was rinsed out into a polypropylene microtube with 0.5 ml of phosphate-buffered saline (PBS). NPA samples were collected by inserting a suction catheter through a nostril to a depth of 4 to 8 cm and then applying gentle suction with an electric suction device. The secretion was rinsed out as described for MEF samples. If the amount of secretion, MEF or NPA, was ample, a cotton-tipped swab was dipped directly in the secretion; if not, a swab was dipped in the PBS-diluted rinse. Swabs were immediately placed in modified Stuart transport media (Transpocult; Orion Diagnostica, Espoo, Finland) and transported at 4°C to the bacteriological laboratory.

Bacterial culture of MEF and NPA samples. Culture was done within 24 h on nonselective chocolate and blood agar media and selective blood agar media with 5 μg of gentamicin per ml. Plates were incubated at 37°C in a 5% CO_2 atmosphere

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for 1 to 3 days. Isolated bacteria were identified by standard methods (1): *S. pneumoniae* by optochin sensitivity, *H. influenzae* by requirement of X and V factors, and *B. catarrhalis* by microscopic morphology, positive oxidase and tributyrin reactions, and negative carbohydrate utilization tests. *H. influenzae* was screened for the presence of encapsulated strains with Levinthal agar containing antiserum. Pneumococcal strains were serotyped or grouped by Quellung, counterimmunoelectrophoresis, or latex agglutination (groups 7 and 14) (10) with type- or group-specific antiserum pools from Statens Serum-institut, Copenhagen, Denmark. All identified strains were stored at −70°C for future reference.

DNA extraction of MEF samples. Immediately after culture, MEF swabs were placed back in transport media and frozen at −20°C until they were studied by PCR. After thawing of transport media for other purposes, swabs were frozen again at −20°C in a sterile polypropylene tube with 0.5 ml of PBS. DNA extraction was begun by rubbing each swab against the tube wall to release bacteria into PBS and then by following the method previously published (20). Briefly, each sample was centrifuged at 12,000 × *g* and 4°C for 10 min, and the pellet was suspended in 300 μl of solution containing 0.1 M NaOH, 2 M NaCl, and 0.5% sodium dodecyl sulfate. The suspension was incubated at 95°C for 15 min, and 200 μl of Tris-HCl (pH 8.0) was added. Lysate was extracted twice with saturated phenol-chloroform-isoamyl alcohol (25:24:1; Sigma, St. Louis, Mo.). Finally, DNA was precipitated with cold ethanol, dried under vacuum, and dissolved in 100 μl of sterile distilled water. Purified DNA was stored at −20°C.

PCR analysis. The selection of oligonucleotide primers was based on the pneumolysin gene sequence previously published (21). The outer primers were 23-mers and amplified a 348-bp region of the pneumolysin gene, and the inner primers were 23- and 21-mers and amplified a 208-bp region (19). Amplification and detection were performed by the method described by Salo et al. (19) with a few modifications. Briefly, in the reaction mixture of 50 μl, 20 μl of dissolved DNA extract from MEF swab samples was used. For nested PCR, 1.5 μl of the sample from the first PCR round was transferred to a new tube containing a reaction mixture with the inner pair of primers and amplification was repeated. Purified pneumococcal DNA (30 ng/ml) (19) was used as a positive control, and sterile distilled water was used as a negative control for each reaction series. Generally recommended procedures were used to avoid contamination (9). To detect the amplified product, 10 μl of nested PCR mixture was run on a 1.5% agarose gel stained with ethidium bromide and bands were identified by fluorescence and photographed under UV light. A clear-cut band of the expected size was regarded as a positive result.

Samples that were positive by culture but negative by PCR were subjected to two control procedures. First, they were tested for the presence of inhibitors of DNA synthesis by adding 3 μl (equivalent to 90 pg of DNA) of the positive control to each sample prior to amplification. Second, the pneumococcal strains isolated from these samples were tested for the presence of the pneumolysin gene by extracting DNA from the pure culture of each isolate and performing the PCR assay as described above.

Statistics. The carriage rates of *S. pneumoniae* in the nasopharynges of children with PCR-positive and -negative findings for MEF samples were compared by using the chi-square test with the Yates correction. The numbers of positive findings detected by *S. pneumoniae* culture and/or PCR were compared by using the McNemar test.

TABLE 1. Comparison of culture and pneumolysin PCR results for 180 MEF samples from 125 children with AOM

Bacterial culture isolate	No. of samples	No. of samples with pneumolysin PCR result	
		Positive	Negative
S. pneumoniae	33	30	3
H. influenzae	28	0	28
B. catarrhalis	17	2	15
Group A streptococcus	1	0	1
Other bacteria[a]	1	0	1
Negative	100	19	81
Total	180	51	129

[a] *K. pneumoniae* and *P. fluorescens* in the same sample.

RESULTS

Of the 135 children initially enrolled, 125 children with 125 episodes of AOM and 180 MEF samples were included in this study since they had both bacterial culture and pneumolysin PCR results for MEF samples available. Unilateral AOM was diagnosed for 70 children; bilateral AOM was diagnosed for 55 children. The age range of the 125 children was from 3 months to 7 years 5 months (median, 2 years 6 months); 54 (43%) were under 2 years of age.

Bacterial culture. Bacteria were cultured in 80 (44%) of 180 MEF samples. Three samples contained more than one bacterial strain; the remaining 77 contained pure cultures. *S. pneumoniae* was found in 33 (18%) of 180 samples; *H. influenzae*, in all cases nontypeable, was found in 29 (16%) samples; *B. catarrhalis* was found in 17 (9%); group A streptococcus was found in 1; *Klebsiella pneumoniae* and *Pseudomonas fluorescens* were found in 1. Bacterial growth was found in 97 (78%) of 125 NPA samples, and two or more otitis pathogens were present in 30 (24%) samples. *S. pneumoniae* was cultured from the nasopharynges of 25 (89%) of 28 children who had *S. pneumoniae* cultured from at least one ear. Serogroups 19, 23, and 6, in this order, were the most frequently found pneumococcal isolates in both MEF and NPA samples. When *S. pneumoniae* was found in both MEF and NPA, the serotype was always the same.

PCR analysis. Of 33 *S. pneumoniae* culture-positive MEF samples, 30 (91%) were positive by PCR (Table 1). There was no evidence of inhibitors in the three samples positive by culture but negative by PCR, since added pneumococcal DNA was amplified without problems. The pneumococcal strains isolated from these three samples were tested for the presence of pneumolysin DNA and found positive by PCR. Of the 21 PCR-positive but *S. pneumoniae* culture-negative MEF samples, 4 came from children from whom *S. pneumoniae* was found by culture in the MEF of the other ear and in the nasopharynx, and an additional 14 came from children from whom *S. pneumoniae* was found in NPA. One of the two PCR-positive, *S. pneumoniae* culture-negative MEF samples with a positive MEF culture for *B. catarrhalis* came from a child with *S. pneumoniae* cultured in NPA. By combining MEF culture and PCR results, 54 (30%) of 180 MEF samples, instead of the 33 (18%) found by culture alone, had evidence of pneumococcal involvement.

The number of children with *S. pneumoniae* in MEF samples was 28 (22%) by MEF culture alone and 43 (34%) by MEF culture and PCR (Table 2). Twelve (80%) of 15 children with PCR-positive but *S. pneumoniae* culture-negative MEF samples had *S. pneumoniae* in the nasopharynx, whereas 28 (34%)

TABLE 2. Comparison of *S. pneumoniae* culture results for MEF samples and NPA to pneumolysin PCR results for MEF samples

S. pneumoniae culture	No. of children	No. of children with pneumolysin PCR result	
		Positive	Negative
MEF+ NPA+	25	23	2
MEF+ NPA−	3	3	0
MEF− NPA+	40	12[a]	28[a]
MEF− NPA−	57	3	54
Total	125	41	84

[a] 12 of 15 versus 28 of 82 ($P = 0.002$, chi-square test with Yates correction).

of 82 children with MEF samples negative for *S. pneumoniae* by both PCR and culture harbored *S. pneumoniae* in the nasopharynx ($P = 0.002$).

When the results were analyzed by the age of the child, 15 (28%) of 54 children under 2 years of age had *S. pneumoniae* cultured from at least one ear and 16 (30%) had positive PCR findings. Of 71 children 2 years or older, only 13 (18%) had a positive *S. pneumoniae* culture but 25 (35%) had positive PCR results. Thus, *S. pneumoniae* culture and PCR findings were similar for children under 2 years of age, whereas in children over 2 years of age, PCR was positive more often than culture ($P < 0.003$).

DISCUSSION

Sensitive and reliable etiological diagnosis of AOM is needed for epidemiological and immunological studies aimed at prevention of the disease. We used bacterial culture and a newly developed pneumolysin PCR assay to study the pneumococcal etiology of AOM in 125 children. The use of PCR in addition to culture increased the number of possible pneumococcal otitis episodes from 28 (22%) to 43 (34%).

In this study, the culture positivity, 80 (44%) of 180 MEF samples, was somewhat lower than that generally reported (7), probably because of some features of our patient population: these children were a bit older and had a higher percentage of recurrent attacks than reported for a previous group. Because of a suspicion that MEF cultures underestimate the role of *S. pneumoniae* in AOM, we evaluated PCR with MEF samples as a possible means of obtaining evidence of pneumococcal involvement. The nested pneumolysin PCR assay we used is capable of detecting 24 fg of purified DNA, corresponding to 10 bacteria (19). However, a major obstacle in detecting microbial DNA in body fluids is the presence of polymerase inhibitors, like heme (5) and urea (8), which may result in false-negative findings. Extracting DNA from crude samples by phenol-chloroform is one of the best ways to remove such inhibitors, but small quantities of target DNA may be lost during this procedure (13). In this study, all samples were treated with phenol-chloroform. The three MEF samples which remained negative by PCR, although they were culture-positive for *S. pneumoniae*, were tested for their ability to inhibit the amplification reaction; no inhibition was found. The pneumococcal strains isolated from these MEF samples all contained pneumolysin DNA. Therefore, the negative PCR results for these samples were most likely due to a small number of bacteria initially present in the swab and/or the loss of DNA during the extraction procedure.

Another problem with PCR is the occurrence of false-positive reactions caused by contamination. To avoid this, we followed carefully the generally approved laboratory practices for reliable PCR analysis (9). Of 147 MEF samples culture-negative for *S. pneumoniae*, 126 remained negative by PCR but 21 gave clear-cut positive results. Our hypothesis that PCR findings for culture-negative MEF samples are diagnostic for pneumococcal involvement in otitis is supported by several sorts of evidence. First, 13 of 21 PCR-positive, *S. pneumoniae* culture-negative MEF samples came from 11 children with bilateral otitis; in 4 (36%) of them, *S. pneumoniae* was found in the other ear and nasopharynx by culture. The remaining 7 children had *S. pneumoniae* cultured from the nasopharynx. Second, 12 (80%) of 15 children with PCR-positive, *S. pneumoniae* culture-negative MEF samples had *S. pneumoniae* in NPA samples, compared with 28 (34%) of 82 children with PCR-negative, *S. pneumoniae* culture-negative MEF samples (Table 2). Moreover, samples from children over 2 years of age were positive more often by PCR than by culture, which might be due to preexisting immunity to *S. pneumoniae* in this older age group. In relation to clinical data, children with PCR-positive, *S. pneumoniae* culture-negative MEF samples did not differ from *S. pneumoniae* culture-positive ones. Therefore, we suggest that a positive PCR finding for an MEF sample really indicates pneumococcal involvement in the present episode of AOM and is not due to the persistence of DNA from previous episodes. Overall, the increase in the number of pneumococcal cases diagnosed by pneumolysin PCR in addition to culture is close to the increase obtained by combining antigen detection methods with culture (4, 11, 12).

In conclusion, pneumolysin PCR is a sensitive and specific new method to study pneumococcal involvement in MEF samples of children with AOM. Even with DNA extraction and two rounds of PCR, the result is available the following day, 1 day earlier than by routine culture of *S. pneumoniae*. However, this PCR assay is more expensive, and culture is still needed to obtain strains both for antimicrobial susceptibility testing and for serotyping of *S. pneumoniae*. Therefore, at this stage, pneumolysin PCR cannot be recommended for routine use in patient care but rather as an aid in research when a more accurate and sensitive pneumococcal diagnosis is needed, for instance, in clinical efficacy trials with new pneumococcal conjugate vaccines.

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REFERENCES

1. **Balows, A., W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.).** 1991. Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
2. **Donofrio, J. C., J. D. Coonrod, J. N. Davidson, and R. F. Betts.** 1992. Detection of influenza A and B in respiratory secretions with the polymerase chain reaction. PCR Methods Applications **1**:263–268.
3. **He, Q., J. Mertsola, H. Soini, M. Skurnik, O. Ruuskanen, and M. K. Viljanen.** 1993. Comparison of polymerase chain reaction with culture and enzyme immunoassay for diagnosis of pertussis. J. Clin. Microbiol. **31**:642–645.
4. **Herva, E., V. M. Häivä, M. Koskela, et al.** 1984. Pneumococci and their capsular polysaccharide antigens in middle ear effusion in acute otitis media, p. 120–122. *In* D. J. Lim, C. D. Bluestone, J. O. Klein, and J. D. Nelson (ed.), Recent advances in otitis media with effusion. B. C. Decker, Inc., Toronto.
5. **Higuchi, R.** 1989. Simple and rapid preparation of samples for PCR, p. 31–38. *In* H. A. Erlich (ed.), PCR technology: principles and applications for DNA amplification. Stockton Press, New York.
6. **Hotomi, M., T. Tabata, H. Kakiuchi, and M. Kunimoto.** 1993.

Detection of *Haemophilus influenzae* in middle ear of otitis media with effusion by polymerase chain reaction. Int. J. Ped. Otorhinolaryngol. **27:**119–126.

7. **Karma, P., T. Palva, K. Kouvalainen, et al.** 1987. Finnish approach to the treatment of acute otitis media. Report of the Finnish consensus conference. Ann. Otol. Rhinol. Laryngol. **96:** (Suppl.)**:** 1–19.
8. **Khan, G., H. O. Kangro, P. J. Coates, and R. B. Heath.** 1991. Inhibitory effects of urine on the polymerase chain reaction for cytomegalovirus DNA. J. Clin. Pathol. **44:**360–365.
9. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature (London) **339:**237–238.
10. **Leinonen, M. K.** 1980. Detection of pneumococcal capsular polysaccharide antigens by latex agglutination, counterimmunoelectrophoresis, and radioimmunoassay in middle ear exudates in acute otitis media. J. Clin. Microbiol. **11:**135–140.
11. **Luotonen, J.** 1982. *Streptococcus pneumoniae* and *Haemophilus influenzae* in nasal cultures during acute otitis media. Acta Oto-laryngol. **93:**295–299.
12. **Luotonen, J., E. Herva, P. Karma, M. Timonen, M. Leinonen, and P. H. Mäkelä.** 1981. The bacteriology of acute otitis media in children with special reference to *Streptococcus pneumoniae* as studied by bacteriological and antigen detection methods. Scand. J. Infect. Dis. **13:**177–183.
13. **Nolte, F. S., B. Metchock, J. E. McGowan, Jr., A. Edwards, O. Okwumabug, C. Thurmond, P. S. Mitchell, B. Plikaytis, and T. Shinnick.** 1993. Direct detection of *Mycobacterium tuberculosis* in sputum by polymerase chain reaction and DNA hybridization. J. Clin. Microbiol. **31:**1777–1782.
14. **Okamoto, Y., K. Kudo, K. Ishikawa, et al.** 1993. Presence of respiratory syncytial virus genomic sequences in middle ear fluid and its relationship to expression of cytokines and cell adhesion molecules. J. Infect. Dis. **168:**1277–1281.
15. **Paton, A. W., J. C. Paton, A. J. Lawrence, P. N. Goldwater, and R. J. Harris.** 1992. Rapid detection of respiratory syncytial virus in nasopharyngeal aspirates by reverse transcription and polymerase chain reaction amplification. J. Clin. Microbiol. **30:**901–904.
16. **Paton, J. C., R. A. Lock, and D. J. Hansman.** 1983. Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae*. Infect. Immun. **40:**548–552.
17. **Qvarnberg, Y., E. Holopainen, and T. Palva.** 1984. Aspiration cytology in acute otitis media. Acta Oto-laryngol. **97:**443–449.
18. **Rudolph, K. M., A. J. Parkinson, C. M. Black, and L. W. Mayer.** 1993. Evaluation of polymerase chain reaction for diagnosis of pneumococcal pneumonia. J. Clin. Microbiol. **31:**2661–2666.
19. **Salo, P., Å. Örtqvist, and M. Leinonen.** Diagnosis of pneumococcal pneumonia by amplification of pneumolysin gene fragment in serum. J. Infect. Dis., in press.
20. **Soini, H., M. Skurnik, K. Liippo, E. Tala, and M. K. Viljanen.** 1992. Detection and identification of mycobacteria by amplification of a segment of the gene coding for the 32-kilodalton protein. J. Clin. Microbiol. **30:**2025–2028.
21. **Walker, J. A., R. L. Allen, P. Falmagne, M. K. Johnson, and G. J. Boulnois.** 1987. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. Infect. Immun. **55:** 1184–1189.