Does the Presence of Pneumococcal DNA in Middle-Ear Fluid Indicate Pneumococcal Etiology in Acute Otitis Media?

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Bacterial culture of middle-ear fluid (MEF), the standard for etiologic diagnosis of acute otitis media (AOM), has revealed *Streptococcus pneumoniae* (Pnc) to be a major pathogen responsible for one-third of AOM cases. In the present study, we compared the results of polymerase chain reaction (PCR) based on the amplification of the pneumolysin gene with the results of pneumococcal culture, for 2595 MEF samples obtained during AOM events in 831 children who were followed from 2–24 months of age in the Finnish Otitis Media Vaccine Trial. PCR results were positive for 47.1% of the MEF samples, and culture results were positive for 27.3% of the samples. PCR-positive, culture-negative samples were associated with previous Pnc AOM in a time-dependent pattern, concurrent antibiotic treatment, low volume of MEF, and concurrent nasopharyngeal carriage. PCR-positive AOM represented a clinically less severe disease, compared with culture-positive Pnc AOM. A positive PCR result seemed to indicate the presence of viable, although often nonculturable, Pnc.

Isolation of bacteria from middle-ear fluid (MEF) has been considered to be the standard for the etiologic diagnosis of acute otitis media (AOM). By this criterion, *Streptococcus pneumoniae* (Pnc) is the most important etiologic agent of AOM, followed closely by *Haemophilus influenzae* and *Moraxella catarrhalis*; the role of group A *Streptococcus*, which was an important cause of AOM in the past, is now very small [1–4]. However, only approximately one-half of MEF samples obtained from subjects with AOM show growth of any of these major pathogens. Some of the remaining samples show growth of bacteria that are not considered to be pathogenic in AOM (mainly, α-hemolytic streptococci or staphylococci), whereas others show no growth at all. The etiology of cases of AOM due to bacteria not considered to be pathogenic and cases of AOM with no bacteria detected remains an unanswered question. Bacteria not detected by conventional culture methods have been suggested as a possible etiologic agent in these cases, but studies that have specifically looked for such agents (e.g., *Chlamydia* or *Mycoplasma* organisms) have not suggested a major role for the agents [5, 6]. On the other hand, a preceding or concurrent viral infection seems to be a common characteristic of AOM, regardless of bacterial findings [7]. Another approach has been suggested by findings of pneumococcal-specific antigen or nucleic acid in culture-negative MEF samples [2, 8, 9]. These findings could indicate that the prevalence of Pnc having an etiologic role in AOM is far higher than the ~30% prevalence determined by bacterial isolation. This would be relevant to the choice of both therapeutic and preventive measures, and, therefore, it would be important, from a practical viewpoint, to verify. The present study was undertaken...
to assess the extent and significance of pneumococcal nucleic acid detected by polymerase chain reaction (PCR) in a large number of MEF samples obtained from subjects with AOM.

SUBJECTS AND METHODS

Subjects. Control subjects from the Finnish Otitis Media (FinOM) Vaccine Trial [10] were analyzed in the present study. One-third of the children in that trial (n = 831) were randomized to receive the hepatitis B virus vaccine, and two-thirds of the children were randomized to receive either of the 2 experimental pneumococcal conjugate vaccines. Informed consent was obtained from the parents or guardians of the children enrolled in the study. Human experimentation guidelines of the US Department of Health and Human Services and those of the national authorities were followed in the conduct of this clinical research.

In addition, data from the FinOM Cohort Study [4] were used in the analysis of the association of pneumococcal nasopharyngeal carriage and PCR detection of Pnc in MEF during AOM. A total of 329 children were enrolled in the FinOM Cohort Study; no intervention was given.

Follow-up. In both the FinOM Vaccine Trial and the FinOM Cohort Study, full-time study physicians and nurses employed at special study clinics conducted prospective follow-up of the study children from enrollment at 2 months of age to 24 months of age. During follow-up, the children were seen 8–10 times at prescheduled visits. Follow-up for respiratory infections was also conducted at the study clinics. Parents were advised to have their child examined by a study physician if the child had symptoms of respiratory infection and, especially, if parents suspected AOM (such visits to a study physician were known as “sick visits”). In addition, follow-up visits were conducted 3–5 weeks after diagnosis of otitis media.

The study physicians used pneumatic otoscopy and tympanometry to diagnose otitis media. AOM was defined by abnormal otoscopic findings that suggested MEF with concomitant acute symptoms. Diagnosis of AOM was verified by myringotomy with aspiration. In addition, a nasopharyngeal aspirate was obtained at the time of diagnosis of AOM [11]. At the sick visit, the parent, guardian, or caretaker that accompanied each child was interviewed, by use of a structured questionnaire, to ascertain the symptoms of the child.

Bacteriologic methods. The MEF sample, which was obtained by aspiration after myringotomy, was added to 0.7 mL of PBS-LiCl buffer and was immediately plated, with a 10-μL loop, on selective sheep blood agar that contained 5 μg/mL of gentamicin for isolation of Pnc [4]. Nasopharyngeal aspirates were also cultured in the FinOM Cohort Study, but not in the FinOM Vaccine Trial. After incubation overnight, the plates were sent to the bacteriologic laboratory. Identification of Pnc was based on results of the optochin test with use of 6-μg Biodisk PDM diagnostic disks, in conjunction with colonial morphologic findings [12]. The number of pneumococcal colonies was counted to estimate the quantity of Pnc in the MEF sample. Serotyping of pneumococcal isolates was done by counterimmunoelectrophoresis and latex agglutination, and results were confirmed by the quellung reaction, when needed, with antisera obtained from Statens Serum Institut (Copenhagen).

Preparation of samples for PCR. The MEF samples that remained after bacterial culture were frozen for subsequent analyses. No actual DNA extraction was done, but MEF samples were boiled as described by Rintamäki et al. [13]. Forty microliters of sample and 100 μL of sterile distilled water were mixed and heated for 10 min at 100°C. After boiling, samples were centrifuged at 17,000 g for 2 min. The supernatant was obtained and stored at −20°C. A negative control sample that contained water was included in every batch of MEF samples.

Altogether, 70 MEF samples were reanalyzed by real-time PCR, to clarify discordant results. For real-time PCR of MEF samples that had positive pneumococcal culture results but negative pneumolysin PCR results, DNA was isolated from 50 μL of sample by use of the QIAamp DNA Mini Kit (Qiagen GmbH), in accordance with the protocol for tissues. The purified DNA was eluted in 50 μL of elution buffer, and 8 μL of sample was used for real-time pneumolysin PCR. For real-time PCR analysis performed to determine the presence of PCR inhibitors in MEF samples, selected samples were boiled as described above, and 2 μL of supernatant was used for real-time PCR.

Group A Streptococcus isolates grown from 7 MEF samples were analyzed by real-time pneumolysin PCR. The strains were recultured, and colonies were obtained from the plate and were suspended in 100 μL of sterile distilled water. The bacterial suspensions were boiled as described above for the MEF samples. A total of 2 μL of supernatant was used for real-time PCR.

Control DNA was isolated from pure pneumococcal cultures by use of the QIAamp Blood Kit (Qiagen), according to the manufacturer’s instructions. The amount of DNA was measured by spectrophotometry, and 10-fold dilutions (10⁻³–10⁻⁷) were made.

Pneumolysin-based PCR for the detection of pneumococcal DNA in MEF samples. The oligonucleotide sequences used for primers and hybridization probes based on the sequence of pneumolysin gene have recently been described elsewhere [13]. The primers used in the present study were the same as the Iia and Iib primers used in a previous study [14]; however, in the previous study, the sequence of primer Iia was misspelled. The correct sequence is described in a more recent publication [13]. Amplification of a 209-bp fragment of pneumolysin gene was performed using a “hot-start” PCR method, as described in detail elsewhere [13]. The 25-μL reaction mixture contained 0.2 mmol/L each deoxyribonucleotide, 30 pmol each primer...
were analyzed using a quantitative real-time pneumolysin PCR method described elsewhere [16]. The primers used were the same as the ones used in pneumolysin PCR, except that the forward primer was slightly shifted, thereby making the size of the amplified fragment 206 bp. This modification was suggested by the designer of the hybridization probes (TIB MOLBIOL), and it was found to produce slightly better results in real-time PCR. The amplification products were detected using a sequence-specific, fluorescent-labeled hybridization probe pair [16]. Standards containing 5, 5 × 10^5, 5 × 10^6, and 5 × 10^7 pneumococcal genome equivalents were included in each set of amplification. For each 6 clinical specimens, 1 negative control (water, instead of sample DNA) was also included. The fluorescence data were analyzed using the Second Derivative Maximum method of the LightCycler Data Analysis program (version 3.5.28; Roche Diagnostics GmbH, Roche Molecular Biochemicals).

For 40 selected MEF samples (including 10 culture-positive samples with a high number of counts per second, by PCR [i.e., >600,000 counts per second]; 10 culture-positive samples with a low number of counts per second, by PCR [i.e., <200,000 counts per second]; 10 culture-negative samples with high counts per second, by PCR; and 10 culture-negative samples with low counts per second, by PCR), testing for the presence of inhibitors was done by adding 1000 Pnc genome equivalents to 2 μL of boiled MEF sample and by comparing the genome numbers obtained with or without added pneumococcal DNA.

Data analysis. All MEF samples from the FinOM Vaccine Trial were analyzed as described. However, only some of the MEF samples from the FinOM Cohort Study were fully analyzed and, thus, were available for subsequent analyses. The results of the FinOM Vaccine Trial are presented, unless mentioned otherwise.

To avoid statistical problems related to the dependence between the data obtained from multiple samples and multiple events from individual children during follow-up, no statistical analyses were performed. Furthermore, the large sample size probably would have led to numerous statistically significant but clinically irrelevant differences. Therefore, only descriptive analyses were performed, and the reported differences should be interpreted according to their clinical relevance. The results presented are mainly based on data for individual ears, because the samples obtained from each ear were processed individually. However, some data are presented as events, for which data for both ears are combined.

RESULTS

Study Visits and Samples

Altogether, 831 children (403 girls and 428 boys) were enrolled as subjects in this study. Of these children, 799 (96.1%) had complete follow-up until the age of 24 months. A total

(1)
of 587 (70.6%) of the children experienced ≥1 documented event of AOM during follow-up, for a total of 1966 AOM events that involved 2849 ears. Altogether, 2595 MEF samples (91.1%; 1 or 2 samples per event) were available from 1819 events of 573 subjects.

**Pneumococcal Findings**

The results of pneumolysin PCR were positive for 1222 (47.1%) of all 2595 MEF samples and for 921 (50.6%) of all 1819 AOM events. For 686 ears with positive pneumolysin PCR results (56.1%), Pnc culture results were also positive; however, for 536 ears (43.9%), the MEF cultures remained negative for Pnc (table 1). With PCR detection, pneumococcal findings increased by 76%, compared with culture alone.

The number of counts per second, by PCR, was considerably higher in MEF samples with positive Pnc culture results than in MEF samples with negative Pnc culture results (geometric mean counts per second, 473,000 vs. 127,000) (figure 1). The bimodality of the distribution of counts per second, by PCR, in culture-positive samples raised a hypothesis that PCR inhibitors could be present in the culture-positive samples with low counts per second, by PCR. However, on reanalysis of 10 culture-positive samples with low counts per second and 10 culture-positive samples with high counts per second, by PCR. Six of 10 samples with low counts per second, by PCR, contained >200 genome equivalents/μL, whereas only 3 of 10 samples with high counts per second contained >200 genome equivalents/μL. Among culture-negative samples, this phenomenon was not demonstrable: only 2 samples with low counts per second and 8 samples with high counts per second, by PCR, contained >5 genome equivalents/μL. On testing for inhibition by adding 1000 pneumococcal genome equivalents to the samples, possible inhibition (detection of <300 of the 1000 genome equivalents added) was seen in 1 culture-positive sample with high counts per second, by PCR, and in 1 culture-positive sample with low counts per second, by PCR. Thus, the bimodality of PCR count distribution shown in figure 1 does not seem to indicate the presence of PCR inhibitors in the culture-positive samples with low counts per second, by PCR, but, rather, the inhibitory effect caused by the competition between the excessive numbers of amplification products during the microwell hybridization step.

**Culture-Positive, PCR-Negative Samples**

There were 23 MEF samples (representing 3.2% of all samples for which culture was positive for Pnc [23 of 709 samples]) for which the pneumolysin PCR result remained negative despite a positive pneumococcal culture result (table 1). The number of pneumococcal colonies in the MEF culture was considerably lower in PCR-negative samples (median number of pneumococcal colonies, 17), compared with the 686 PCR-positive samples (median number of pneumococcal colonies, 150).

To confirm the presence of pneumolysin gene in these pneumococcal isolates and to assess the potential presence of PCR inhibitors in the MEF samples, we analyzed the 23 PCR-negative samples by use of quantitative real-time pneumolysin PCR performed using DNA purified from the samples. In the present analysis, the results were clearly positive for 20 of the 23 MEF samples. The results for the remaining 3 samples also were found to be positive after reanalysis. The quantitative result for all 3 samples was <5 pneumococcal genome equivalents/μL. The numbers of pneumococcal colonies in the original culture of these 3 samples were also extremely low (1–3 pneumococcal colonies). The corresponding pneumococcal isolates from these 3 samples all contained pneumolysin gene when they were recultured and analyzed by real-time PCR.

**Culture-Negative, PCR-Positive Samples**

For 536 MEF samples from 400 events, the pneumolysin PCR result was positive but the pneumococcal culture result remained negative (table 1). This discrepancy could be the result of either a higher sensitivity of PCR in the detection of pneumococcus, compared with that of culture, or a lower specificity of PCR in the detection of pneumococcal DNA in AOM not caused by Pnc. First, we analyzed factors associated with the sensitivity of bacterial culture, such as concurrent antimicrobial treatment and low sample volume. Second, we tried to explore the origin of pneumococcal DNA in these MEF samples.

Current antibiotic treatment was more common in association with PCR-positive, culture-negative events (10%), compared with culture-positive events (4%). However, this would still leave the remaining 90% of PCR-positive, culture-negative samples unexplained. Recent antibiotic treatment (i.e., treatment having ended 1–28 days before samples were obtained) was equally common in both groups.

The MEF volume, as assessed by a study physician after the myringotomy procedure was performed, was more often low in samples from subjects with PCR-positive, culture-negative AOM.

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**Table 1. Results of pneumolysin polymerase chain reaction (PCR) and of pneumococcal (Pnc) culture performed for 2595 middle-ear fluid samples from children with acute otitis media.**

<table>
<thead>
<tr>
<th>Pnc culture result</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive, no.</td>
<td>686</td>
<td>23</td>
<td>709</td>
</tr>
<tr>
<td>Negative, no.</td>
<td>536</td>
<td>1350</td>
<td>1886</td>
</tr>
<tr>
<td>Total</td>
<td>1222</td>
<td>1373</td>
<td>2595</td>
</tr>
</tbody>
</table>

**NOTE.** Children were 2–24 months of age.
Pneumolysin PCR for Acute Otitis Media

Figure 1. Distribution of polymerase chain reaction (PCR) counts (i.e., no. of counts per second) for pneumococcal (Pnc) culture–negative and Pnc culture–positive middle-ear fluid (MEF) samples from children with acute otitis media.

(21%), compared with samples from subjects with culture-positive AOM cases (12%). However, the low MEF volume was most common in subjects with PCR-negative, culture-negative samples (37%). The quality of MEF was more commonly mucoid in PCR-positive, culture-negative samples (27%) than in culture-positive samples (14%).

Dormant Pnc from previous AOM events. The proportion of PCR-positive, culture-negative events was similar throughout the 2-year period (19%–23% in each half-year period); however, a trend toward a slight increase in the proportion of PCR-positive, culture-negative events was noticed in association with an increasing number of previous AOM events (proportion, 19% in events with no previous AOM, 21% in events with 1–3 previous AOM events, and 27% in events with ≥3 previous AOM events). We then asked whether this increase was associated with previous Pnc AOM cases. If Pnc had been isolated (i.e., culture-confirmed Pnc otitis) from the same ear, MEF samples from 43% of the ears with negative culture results had positive PCR results; when the Pnc culture result was negative, the corresponding figure was 23%. However, the increase was not dependent on the number of previous isolations of Pnc (data not shown). Thus, it seems plausible that a positive PCR result for a Pnc culture–negative MEF sample could be explained, at least in part, by a history of previous pneumococcal AOM.

We then wanted to assess the duration of PCR positivity after culture-confirmed pneumococcal AOM, including in the analysis the subsequent MEF samples obtained from the same child until the next culture-confirmed pneumococcal AOM or PCR-negative AOM event was reached. The results of the assessment are displayed in figure 2. The proportion of PCR-positive, culture-negative samples eventually decreased, as a function of time, after the initial Pnc culture–positive sample, although the rate of decrease after the initial phase was quite low.
Figure 2. Proportion of pneumolysin polymerase chain reaction (PCR) positivity after culture-confirmed pneumococcal (Pnc) acute otitis media (AOM), as a function of time. Successive Pnc culture–negative samples were included until the pneumolysin PCR result became negative or another culture-positive case of Pnc AOM occurred. The horizontal dashed line denotes the overall level of pneumolysin PCR positivity in AOM with Pnc culture–negative middle-ear fluid samples.

Effect of concurrent Pnc isolation contralaterally. Among the bilateral AOM events for which both samples were available \((n = 776)\), there were 101 events with a unilateral Pnc culture finding (the other ear was negative in Pnc culture). For these events, 62 (61%) of the contralateral ears that had negative Pnc culture results had positive PCR results; this number is clearly higher than the number of ears with negative Pnc culture results among bilateral events (27% [273 of 1002 ears]).

Pnc as a bystander in AOM due to other etiologies. Because 53% of all PCR-positive, culture-negative samples (282 of 536 samples) were obtained from children with no documented previous or concurrent culture-confirmed Pnc AOM events, other mechanisms for PCR positivity must also prevail.

Pneumococci or pneumococcal DNA might be brought to the middle ear from the nasopharynx by Eustachian tube reflux with other invading pathogens. The data available from the FinOM Cohort Study (for 169 MEF samples from ears with AOM) show that 100% of MEF samples with positive Pnc culture results were associated with concurrent Pnc carriage, but only 51% of PCR-positive, culture-negative MEF samples and 38% of MEF samples with no indication of Pnc involvement were associated with concurrent Pnc carriage. Thus, concurrent Pnc carriage is a prerequisite for isolation of Pnc from MEF samples, but it is not a prerequisite for PCR positivity. However, MEF samples with negative Pnc culture results were more commonly found to have positive PCR results during concurrent Pnc carriage (50% were PCR positive) than during no carriage (38% were PCR positive), on the basis of data from the FinOM Cohort Study. However, more than one-quarter of PCR-positive, culture-negative samples (5 of 15 samples) from the FinOM Cohort Study could be explained neither by previous Pnc AOM nor by concurrent Pnc carriage.

The proportion of pneumolysin PCR–positive, culture-negative samples, according to the bacterial etiology of AOM,
shown in table 2. The proportion of PCR-positive samples was similar for AOM events due to \textit{H. influenzae} or \textit{M. catarrhalis}, compared with that for culture-negative AOM events. Thus, these 2 major AOM pathogens do not seem to either increase the reflux of Pnc into the middle-ear cavity or lead to false-positive PCR findings. Results of pneumolysin PCR were frequently positive for AOM events due to group A \textit{Streptococcus}; however, the number of isolates was low (\(n=7\)). The group A \textit{Streptococcus} isolates were checked for the presence of pneumolysin gene, by use of real-time pneumolysin PCR (without use of hybridization probes), and they all had negative results.

**Clinical Profile: Comparison of PCR-Positive and Culture-Positive Pneumococcal AOM**

The occurrence of different symptoms in culture-confirmed pneumococcal AOM, compared with PCR-positive, culture-negative pneumococcal AOM is shown in table 3. PCR-positive, culture-negative AOM shows the same proportion of symptoms as does PCR-negative, culture-negative AOM. In these analyses, only events with intact tympanic membranes were included (AOM events with tympanostomy tubes and other perforations were excluded). The same is true for measured fever (table 3) and tympanic membrane findings (table 4). The results show that the proportions of events with positive symptoms and signs in PCR-positive, culture-negative events resemble those of culture-negative events.

**DISCUSSION**

In this large study of \(>2500\) MEF samples, we confirmed that pneumolysin PCR is a sensitive method for the detection of pneumococcal DNA. However, PCR-positive, culture-negative AOM events represented a less severe type of disease, compared with culture-positive AOM events. The PCR-positive, culture-negative findings could be explained, at least in part, by the presence of nonculturable Pnc remaining in the middle ear after previous AOM events. The persistence of PCR positivity was shown to be time dependent, with gradual waning. In addition, concurrent nasopharyngeal carriage, antibiotic treatment, and the MEF dilution (the MEF samples were rinsed with 0.7 mL of buffer solution to move the MEF from a glass pipette to a tube) could all contribute to PCR positivity in culture-negative samples.

Virolainen et al. [8], despite the use of comparable bacteriological detection techniques. In otitis media with effusion, the number of Pnc findings has increased several fold when PCR has been used, compared with culture [9, 17]. In the latter study [17], results of PCR were also commonly positive for MEF samples that were culture positive for \textit{H. influenzae} or \textit{M. catarrhalis}.

To avoid contamination and false-positive results, procedures were carefully followed to avoid physical transfer of amplified or unamplified DNA [15]. Furthermore, negative controls were used during both sample preparation and PCR. In the present study, the MEF samples were prepared for PCR simply by use of the boiling method. The boiling method was tested with MEF samples, and the sample volume that was used was optimized during the FinOM Cohort Study [13]. On the basis of these findings, the boiling method was chosen for the present study; it is a convenient method for large studies because it is fast, easy, and inexpensive. However, some MEF samples—in particular, those that contain blood—may contain PCR inhibitors [18]. When boiling is used, there is a possibility for false-negative results, because inhibitors are not removed. In the present study, the 23 samples for which the pneumococcal culture results were positive but the PCR results were negative were found to have positive results of real-time PCR assay performed using DNA purified from the MEF samples [16]. The MEF samples evidently contained components that inhibited the initial PCR. In addition, some of the samples contained

![Table 2. Pneumococcal pneumolysin polymerase chain reaction (PCR) results, according to the bacterial etiology of acute otitis media (AOM) events in children 2–24 months of age.](https://academic.oup.com/jid/article-abstract/189/5/775/808508)
Table 3. Association of symptoms with pneumococcal involvement in the middle-ear fluid (MEF) samples from 1654 acute otitis media (AOM) events in 573 children aged 2–24 months.

<table>
<thead>
<tr>
<th>Symptom</th>
<th>MEF samples</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pnc culture</td>
<td>PCR positive,</td>
<td>PCR negative,</td>
<td>PCR negative,</td>
<td>All^c</td>
</tr>
<tr>
<td></td>
<td>positive^a</td>
<td>culture negative^b</td>
<td>culture negative^b</td>
<td>culture negative^b</td>
<td>(N = 1651)</td>
</tr>
<tr>
<td>Fever</td>
<td>(n = 376)</td>
<td>(n = 191)</td>
<td>(n = 393)</td>
<td>(n = 1651)</td>
<td></td>
</tr>
<tr>
<td>Temperature, mean, °C</td>
<td>38.0</td>
<td>37.6</td>
<td>37.6</td>
<td>37.7</td>
<td></td>
</tr>
<tr>
<td>Temperature ≥38°C</td>
<td>44</td>
<td>21</td>
<td>22</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>History of fever</td>
<td>60</td>
<td>41</td>
<td>35</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Cough</td>
<td>66</td>
<td>75</td>
<td>77</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>Runny nose</td>
<td>93</td>
<td>92</td>
<td>89</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Ear pain</td>
<td>43</td>
<td>31</td>
<td>26</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Ear pulling</td>
<td>51</td>
<td>49</td>
<td>51</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Ear discharge</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Excessive crying</td>
<td>77</td>
<td>76</td>
<td>73</td>
<td>72</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** Data are the proportion (%) of samples for which the symptom was present within 2 days before AOM was diagnosed, unless otherwise indicated. PCR, polymerase chain reaction; Pnc, *Streptococcus pneumoniae.*

^a In pure culture.

^b All pathogens.

^c The data on symptoms were missing for 3 events of AOM; consequently, analysis of 1651 events is reported (includes, also, events positive for pathogens other than Pnc).

A low number of pneumococci, which, together with PCR inhibitors, probably produced false-negative PCR results. Because of PCR inhibitors, there may also have been some false-negative findings among the culture-negative, PCR-negative samples. Thus, testing of all PCR-negative samples for the presence of inhibitors could have increased the proportion of PCR-positive samples. Unfortunately, this was not possible.

Pneumolysin is produced by virtually all Pnc isolates [19]. Pneumolysin belongs to a family of structurally related thioloctivated cytolysins produced by many gram-positive genera [20]. It is thus possible that bacteria producing related toxins could yield positive results in pneumolysin PCR analyses, although the primers used in the current PCR test have been found to be highly specific [14]. Of interest, 5 of the 7 MEF samples with a pure group A *Streptococcus* culture finding were found to be positive for pneumolysin by PCR. Group A *Streptococcus* has pneumolysin O with high sequence homology with pneumolysin [21]; however, none of the group A *Streptococcus* isolates in the present study produced a positive pneumolysin PCR result. The α-hemolytic streptococci are common inhabitants of the respiratory tract. They have been shown to harbor pneumolysin gene occasionally and thus form a potential source for false-positive pneumolysin PCR findings [22]. We have tested >200 α-hemolytic streptococci isolated from the respiratory tract for the presence of pneumolysin gene, and only 2 isolates have shown a clear positive result. Some isolates have shown aberrant amplification curves or small quantities resulting in ambiguous interpretation (T. Kaijalainen, A.S., E. Herva, and M.L., unpublished data). However, α-hemolytic streptococci are not common in MEF samples obtained during AOM: in the FinOM Cohort Study, α-hemolytic streptococci were isolated in <2% of MEF samples. Thus, α-hemolytic streptococci probably do not play any significant role in this context.

One explanation for the PCR-positive, culture-negative samples would be the lower sensitivity of conventional culture, compared with PCR. In the FinOM studies, special emphasis was given to the optimal isolation of Pnc from MEF samples [4]. We have compared the sensitivities of bacterial culture and real-time pneumolysin PCR by testing different dilutions of pneumococcal broth culture by use of both methods. Depending on the dilution of the sample and the DNA-extraction method used, the number of genome equivalents detected by PCR was 2–30 times higher than the number of colonies detected by culture [23, 24], a finding that indicates the higher sensitivity of PCR, compared with culture. Keeping in mind that pneumococci are present as diplococci or as chains of diplococci in both broth cultures and clinical samples, we can assume that these chains form only 1 colony on the culture plate; however, PCR detects all the genomes separately.

Antibiotic treatment decreases the yield of conventional culture methods. Also in the present study, current antibiotic treatment was associated with an increased proportion of PCR-positive, culture-negative samples. Of interest, no association with the previous antibiotic treatment was found, suggesting that dead bacteria are efficiently removed from the middle ear cavity. In-
Table 4. Tympanic membrane (TM) findings and middle-ear fluid (MEF) characteristics, according to the pneumococcal findings in the MEF samples obtained from 2335 ears of children aged 2–24 months.

<table>
<thead>
<tr>
<th>Finding</th>
<th>MEF samples, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pnc culture positive&lt;sup&gt;a&lt;/sup&gt; (n = 506)</td>
</tr>
<tr>
<td>Opaque or cloudy</td>
<td>72</td>
</tr>
<tr>
<td>Bulging</td>
<td>44</td>
</tr>
<tr>
<td>Immobile</td>
<td>44</td>
</tr>
<tr>
<td>Tympanogram</td>
<td></td>
</tr>
<tr>
<td>Type B</td>
<td>80</td>
</tr>
<tr>
<td>Negative pressure&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4</td>
</tr>
</tbody>
</table>

**NOTE.** MEF samples were obtained after myringotomy through an intact tympanic membrane (ears with discharge were excluded) during acute otitis media. PCR, polymerase chain reaction; Pnc, *Streptococcus pneumoniae.*

<sup>a</sup> Pure culture only.

<sup>b</sup> All pathogens.

<sup>c</sup> Also includes MEF samples that were positive for pathogens other than Pnc.

<sup>d</sup> Pressure < 100 daPa.

deed, Post et al. [25] have shown, in a chinchilla model, that DNA from dead bacteria does not remain detectable in MEF. We also showed, in the present study (figure 2), that the rate of PCR positivity for MEF samples decreases with time after a culture-confirmed case of pneumococcal AOM. The MEF samples (Pnc culture negative) that were included in the analysis were not obtained systematically but, rather, for clinical reasons. It is possible that these MEF samples came from subjects with more severe cases (e.g., from subjects prone to have recurrence of AOM). Even so, the trend of decreasing DNA positivity suggests that DNA remnants are removed from the middle-ear cavity after resolution of the episode of otitis.

Although PCR is able to detect both dead and viable bacteria, PCR-positive, culture-negative otitis events, at least in some cases, have been shown to be caused by metabolically active bacteria; mRNA (with a half-life of seconds to a few minutes) of *H. influenzae* has been detected by reverse-transcriptase PCR in PCR-positive, culture-negative samples obtained from ears with chronic otitis media with effusion [26]. Furthermore, elevated endotoxin levels in MEF during otitis media with effusion have been associated with PCR positivity for *H. influenzae* and *M. catarrhalis* [27]. However, middle-ear effusion may have nuclease-inhibiting activity, and, then, both DNA and mRNA could persist longer in MEF than previously was anticipated [28].

The presence of bacterial biofilms on mucosal membranes has been proposed as an explanation for cases having positive PCR results yet negative culture results [29, 30]. In a biofilm, the bacteria may form a complex bacterial community attached to middle-ear epithelium and covered by a protective polysaccharide matrix. These biofilms are resistant to antimicrobials and immune defense mechanisms. While in the biofilm phenotype, the bacteria are less active with regard to their interactions with the environment and are more difficult to isolate in culture, but they may survive in the middle ear for long periods. The results of the present study support the biofilm theory: Pnc that are dormant after culture-positive AOM can reside in the middle ear and can be detected by PCR but not by culture. On the other hand, a high proportion of samples were PCR positive and culture negative in the absence of documented previous culture-confirmed Pnc AOM or, even, in the absence of any previous documented AOM. Therefore, other types of mechanisms may also account for the phenomenon.

Pneumococcal DNA and/or whole bacteria present in the nasopharynx could reach the middle ear as an innocent bystander or as a secondary pathogen during infection caused by some other microbe. In the present study and in the study by Virolainen et al. [8], the nasopharyngeal carriage of Pnc was associated with PCR-positive, culture-negative AOM. Although nonviable bacteria and isolated DNA do not survive in the middle ear for longer than only a few days, it is possible that their continuous feed from the nasopharynx may produce positive PCR results.

Pnc tends to cause more-severe AOM, compared with other etiologic agents [1, 31, 32]. However, the clinical features (i.e., symptoms and tympanic membrane signs) of PCR-positive AOM more closely resembled (although usually between the 2 groups) the culture-negative Pnc AOM cases than the culture-positive Pnc AOM cases. The features most typical of Pnc AOM (i.e., fever and the presence of earache and bulging tympanic membrane) were clearly different in culture-positive cases of Pnc AOM and PCR-positive cases of Pnc AOM. The less severe
clinical disease could be associated with the biofilm formation, insidious onset of disease (subacute disease), and, also, the theory of ascending nonreplicating Pnc from the nasopharynx acting as secondary invaders.

In conclusion, pneumolysin PCR is a highly sensitive method for the detection of pneumococcal DNA in MEF samples. A positive finding seems to indicate the presence of viable, although often nonculturable, Pnc within the middle-ear cleft. PCR-positive AOM clearly represents a clinically less active Pnc disease, compared with the culture-positive AOM. Additional studies using animal models and clinical samples, performed by use of quantitative, real-time PCR assay for the demonstration of pneumococcal DNA and mRNA during acute otitis media, are needed to elucidate both the pathogenetic mechanism of AOM and the clinical significance of diagnosis by PCR.

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References