MAJOR ARTICLE

# Microbiology of Acute Otitis Media in Children with Tympanostomy Tubes: Prevalences of Bacteria and Viruses

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**(See the editorial commentary by Chonmaitree on pages 1423–5)**

***Background.*** Bacteria are found in 50%–90% of cases of acute otitis media (AOM) with or without otorrhea, and viruses are found in 20%–49% of cases. However, for at least 15% of patients with AOM, the microbiological etiology is never determined. Our aim was to specify the full etiology of acute middle ear infection by using modern microbiological methods concomitantly for bacterial and viral detection.

***Methods.*** The subjects were 79 young children having AOM with new onset (<48 h) of otorrhea through a tympanostomy tube. Middle ear fluid samples were suctioned from the middle ear through the tympanostomy tube. Bacteria were sought by culture and polymerase chain reaction; viruses were analyzed by culture, antigen detection, and polymerase chain reaction.

***Results.*** At least 1 respiratory tract pathogen was noted in 76 children (96%). Bacteria were found in 73 cases (92%), and viruses were found in 55 (70%). In 52 patients (66%), both bacteria and viruses were found. Bacteria typical of AOM were detected in 86% of patients. Picornaviruses accounted for 60% of all viral findings.

***Conclusions.*** In the great majority of children, AOM is a coinfection with bacteria and viruses. The patent tympanostomy tube does not change the spectrum of causative agents in AOM. A microbiological etiology can be established in practically all cases.

The bacterial etiology of acute otitis media (AOM), with or without otorrhea, is well established. However, most studies report 25%–30% of cases without any bacterial findings [1, 2]. The development of viral diagnostics over the years has elucidated the importance of viruses in the etiopathogenesis of otitis media. Studies using viral diagnostics together with bacterial culture have detected viruses in 20%–49% of cases and coinfection with bacteria and viruses in 18%–27% of patients with AOM [3, 4]. On the other hand, no etiologic agents have been shown for at least 15% of patients. One possible explanation is the small volume of middle ear fluid (MEF) obtained by tympanocentesis, which impairs the possibility of diagnostic testing for a broad spectrum of microbial pathogens. The most plausible explanation, however, may be that modern microbiological approaches have not to date been extensively used to evaluate all of the bacterial and viral causes of otitis media concomitantly.

We studied the microbiological etiology of AOM in young children with otorrhea through the tympanostomy tube. Bacteria were identified by conventional bacterial culture and also by PCR. Viruses were detected by culture, antigen detection, and PCR.

## METHODS

### Study Population and Sample Collection

***Population.*** The study population consisted of 79 children who had AOM with otorrhea through the tympanostomy tube for <48 h before study entry. In 73% of cases, the otorrhea had appeared within 24 h of study

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entry. The median age of the study population was 21 months (range, 7–71 months). Before study entry, all except 1 had experienced symptoms of a concomitant viral-type respiratory infection for 1–7 days. Presenting symptoms were rhinitis and cough (in 97% and 87% of patients, respectively); 57% of the patients had both rhinitis and cough together with fever. Of the children, 51% had smoking parents, and 60% attended day care. The median number of episodes of otitis media within 6 months before tympanostomy tube placement and during lifetime were 2 episodes (range, 1–4 episodes) and 3 episodes (range, 1–11 episodes), respectively. All children participated in a randomized, double-blind, placebo-controlled trial assessing the efficacy of amoxicillin-clavulanate on the duration of otorrhea [5]. Written informed consent was obtained from the parents. The study period covered the full respiratory season between September 1998 and June 1999.

***Collection of samples.*** All samples were collected at study entry before initiation of treatment. First, the ear canal was carefully cleaned by suction without disinfectants. Next, a sterile suction trap was used to aspirate MEF through a tympanostomy tube under otomicroscopic visual control. The MEF obtained was diluted with 1.0 mL of saline, and 10 $\mu$L of the solution was immediately inoculated on prewarmed culture plates. The solution was then frozen and kept at −70°C before thawing for analyses. All microbiological analyses were made blinded to other results. The results are presented for one ear per child. In cases of bilateral otorrhea, we chose for analysis the ear that had started to drain first or the ear that drained for a longer duration.

### Bacterial Analyses

***Bacterial culture.*** Bacterial culture was performed on all MEF samples. Blood agar and chocolate agar plates were incubated in a $CO_2$ atmosphere at 35°C and examined after 1 and 2 days of incubation. Pathogenic bacteria were identified using standard methods.

***Multiplex PCR.*** Multiplex bacterial PCR was performed for all 16 MEF samples that did not yield bacteria by culture and for 17 samples with positive culture results. MEF samples were lysed according to the modified protocol by Hendolin et al. [6] and purified with the Qiagen DNA Mini Kit columns and reagents. One universal reverse-strand and 4 specific forward-strand oligonucleotide primers were used for PCR detection of 4 bacterial species in 2 separate multiplex reactions: *Haemophilus influenzae* together with *Moraxella catarrhalis,* and *Streptococcus pneumoniae* with *Alloiococcus otitidis* [6]. The presence of amplifiable DNA and absence of significant PCR inhibitors was confirmed by successful amplification of the human $\beta$-globin gene with primers GH20 and PCO4. Standard PCR amplification was performed using the GeneAmp PCR system 2700 (Applied Biosystems), and DNA products of appropriate length were visualized as UV fluorescence after staining with ethidium bromide. The multiplex PCR and confirmatory reactions were performed at Mobidiag (Helsinki, Finland).

***Broad-range PCR.*** For the 7 samples that tested negative for bacteria by both culture and multiplex PCR, a broad-range PCR was performed. The 16S-rDNA genes were amplified, and amplified DNA was further sequenced using PCR primers and sequencing primers, as described elsewhere [7].

## VIRAL ANALYSES

***Viral culture.*** Viruses were cultured from all samples using standard methods, with LLC and Hela cell lines and MRC-5 fibroblast cells.

***Viral antigen detection.*** Viral antigens (respiratory syncytial virus, adenovirus, influenza A and B viruses, and parainfluenza virus types 1–3) were sought in all samples by a time-resolved fluoroimmunoassay, as described elsewhere [8].

***Virus detection by PCR.*** Nucleic acid was extracted from a 200-$\mu$L aliquot of the MEF sample using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics), eluted in a 60-$\mu$L volume, and stored at −70°C. Extracted nucleic acid was tested for virus-specific sequences by PCR assays in accordance with standard protocols in the viral diagnostic laboratory of the Department of Virology, University of Turku (Turku, Finland) [9–13]. Picornaviruses were detected by RT-PCR with subsequent agarose gel electrophoresis and microwell hybridization [9]. Picornavirus amplicons were determined as rhinoviruses, enteroviruses, or nontypeable picornaviruses as resolved in the hybridization assay with specific probes. Rhinovirus, enterovirus, and coronavirus (229E and OC-40) RNA was analyzed from all 79 samples; respiratory syncytial virus and influenza A and B virus RNA was analyzed from 63 samples. The following viruses were sought only in samples negative for the aforementioned agents: human metapneumovirus (30 samples), adenovirus (28 samples), and parainfluenza virus types 1–4 (27 samples). Furthermore, 28 samples were analyzed for coronavirus NL63 at the Department of Virology, Erasmus Medical Center (Rotterdam, The Netherlands) [14], and 21 samples were analyzed for human bocavirus at the Department of Clinical Microbiology, Karolinska University Hospital (Stockholm, Sweden) [15].

## RESULTS

At least 1 respiratory tract pathogen was noted in 76 (96%) of 79 children having AOM with otorrhea through the tympanostomy tube. The complete initial microbial findings are listed in table 1.

Bacteria were found altogether in 73 cases (92%). Cultures for bacteria showed bacteria in samples from 63 (80%) of 79 children. However, in the 16 negative samples, the presence of

**Table 1. Initial microbial findings for 79 young children who have acute otitis media with new onset (<48 h) of otorrhea.**

| Bacterial result, viral result | No. of cases |
|---|---|
| *Streptococcus pneumoniae* | |
| Rhinovirus | 7 |
| NT picornavirus | 3 |
| Respiratory syncytial virus | 7 |
| Human metapneumovirus | 2 |
| Respiratory syncytial virus and NT picornavirus | 1 |
| Negative for viruses | 7 |
| *Haemophilus influenzae* | |
| Rhinovirus | 4 |
| NT picornavirus | 1 |
| Parainfluenza virus 3 | 1 |
| Human bocavirus | 2 |
| Negative for viruses | 6 |
| *Moraxella catarrhalis* | |
| Rhinovirus | 1 |
| Enterovirus | 1 |
| NT picornavirus | 2 |
| Respiratory syncytial virus | 1 |
| Parainfluenza virus 3 | 2 |
| Influenza A virus | 1 |
| Rhinovirus and coronavirus | 1 |
| Human bocavirus | 1 |
| Negative for viruses | 3 |
| *Staphylococcus aureus* | |
| Enterovirus and parainfluenza virus 3 | 1 |
| Negative for viruses | 1 |
| *Pseudomonas aeruginosa* | |
| Rhinovirus | 1 |
| Enterovirus | 1 |
| *Corynebacterium pseudodiphtheriticum:* negative for viruses | 1 |
| *S. pneumoniae* and *H. influenza* | |
| Rhinovirus | 1 |
| Enterovirus | 2 |
| Coronavirus | 1 |
| *S. pneumoniae* and *M. catarrhalis* | |
| Enterovirus | 1 |
| NT picornavirus | 1 |
| Respiratory syncytial virus | 1 |
| Negative for viruses | 1 |
| *H. influenzae* and *M. catarrhalis* | |
| Enterovirus | 1 |
| Negative for viruses | 1 |
| *S. pneumoniae, H. influenzae,* and *M. catarrhalis* | |
| Parainfluenza virus 3 | 1 |
| Influenza A virus | 1 |
| *S. pneumoniae* and *S. pyogenes:* rhinovirus | 1 |
| *S. pneumoniae, H. influenzae, M. catarrhalis,* and *S. aureus:* negative for viruses | 1 |
| Negative for bacteria | |
| Enterovirus | 1 |
| NT picornavirus | 1 |
| Respiratory syncytial virus | 1 |
| Negative for viruses | 3 |

**NOTE.** NT, nontypeable.

bacteria could be shown in 8 cases by multiplex PCR and in 2 cases by broad-range PCR. Furthermore, in 4 samples that tested positive by culture, additional bacterial species were found by multiplex PCR. Typical bacterial pathogens of AOM *(S. pneumoniae, H. influenzae,* and/or *M. catarrhalis)* were cultured from 58 of children (73%) and additionally detected by PCR in samples from 10 (13%). The most common bacterial finding was *S. pneumoniae,* found in 39 cases (49%), followed by *H. influenzae* in 23 (29%) and *M. catarrhalis* in 22 (28%). *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Streptococcus pyogenes* were found in 3 children, 2 children, and 1 child, respectively. One child had *Corynebacterium pseudodiphteriticum* as the only microbiological finding. *A. otitidis* was not detected in any of the 33 samples tested. Multiple bacterial species were found in samples from 14 children (18%).

Viruses were noted in 55 cases (70%). Picornaviruses were most abundant, detected in 41% of 79 samples. Rhinovirus was found in 16 cases (20%), enterovirus in 8 (10%), and a nontypeable picornavirus in 9 (11%). Respiratory syncytial virus was found in 11 children (14%), parainfluenza virus in 5 (6%), and human bocavirus in 3 (4%). Influenza A virus, human metapneumovirus, and coronavirus were each found in 2 children (3%). Adenovirus and human coronavirus NL63 were not found in any samples analyzed. Dual detections of virus were observed in 3 children (4%). Picornaviruses occurred randomly over the study period, whereas respiratory syncytial virus had a typical seasonal occurrence. During the season when the 11 cases of respiratory syncytial virus were detected, 15 picornaviruses were detected.

Bacteria and viruses were concomitantly found in 52 patients (66%) (figure 1). Viruses were detected in 77%, 65%, and 73% of samples that yielded positive results for *S. pneumoniae, H. influenzae,* and *M. catarrhalis,* respectively. No association of any bacteria with any specific virus was observed.

## DISCUSSION

Our key finding is that acute middle ear infection is a coinfection with bacteria and viruses in the majority of young patients. Previously, 10 studies have reported concomitant detection of bacteria and viruses from MEF samples of patients with AOM [3, 4, 16–23]. The rates of coinfections have varied between 5% and 27%, compared with 66% in this study. On the other hand, we were able to specify etiology in 96% of cases, compared with the 59%–85% of the previous studies. This discrepancy is explained by the microbiological methods used rather than by the characteristics of the study populations. The traditional method of viral diagnostics is culture, which has low sensitivity, especially for picornaviruses. Yoshie [24], in the early 1950s, was the first to show viruses in the middle ear by culturing influenza A viruses from MEF specimens obtained from 4 patients. This was followed by the studies of Berglund

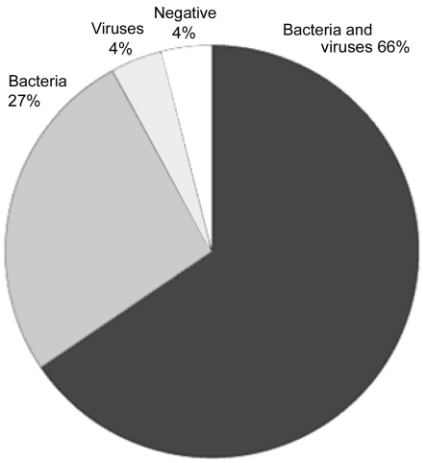


**Figure 1.** Proportions of microbial findings in children with acute middle ear infection (because of rounding, the total is 101%).

et al. [25, 26] in Turku, who cultured respiratory syncytial virus from MEF and showed that viruses and bacteria may coexist in an acute middle ear infection. Since then, the etiology of AOM has had 3 possibilities: bacterial infection, viral infection, or coinfection [27]. The landmark study of Henderson et al. [28], showing a clear association of viral upper respiratory tract infection with otitis media, as well as the availability of antigen detection, boosted investigations into viral etiology of AOM. Klein et al. [16] reported viral antigens in 23% of AOM cases and coinfection with bacteria and viruses in 8% of patients. Comparable rates were presented by the successors of Berglund from our center [17]. Chonmaitree and colleagues [3, 19, 20] detected coinfection in almost 20% of patients with AOM, but this was due to a better bacterial yield, not to a higher rate of findings of viruses. The advent of PCR enabled Pitkäranta et al. [4] to double the rate of detection of viruses, which increased the proportion of coinfections up to 27%, although they sought only 3 common viruses in MEF. The chain of these studies confirms that viral detection techniques are of fundamental importance, as was pointed out in early reviews of the role of viruses as etiologic agents of otitis media [27, 29]. Our study is the first to use a variety of sensitive methods to identify a broad spectrum of bacteria and viruses. As a consequence, this study strongly suggests that acute middle ear infections in young children are mainly coinfections with bacteria and viruses.

The clinical impact of coinfections of the middle ear is based on observations that bacterial eradication and clinical outcomes are poorer in coinfections than in solely bacterial infections [19, 20]. Viruses are recovered more frequently from MEF specimens from patients with infections unresponsive to antimicrobials than from MEF specimens from unselected patients with AOM [30]. A plausible explanation could be that concentrations of antimicrobials in MEF may be lower in coinfections than in infections due solely to bacteria [31]. Furthermore, viruses strengthen the bacteria-induced inflammation in the middle ear, which is evident by the higher concentrations of inflammatory mediators in coinfections than in cases of AOM due solely to bacteria [21]. Thus, viruses do not seem to be "innocent bystanders" in the middle ear. Of interest, in other respiratory tract infections as well, concomitant detection of bacteria and viruses is related to more severe symptoms and/or poorer therapeutic responses than are seen in infections due either solely to bacteria or solely to viruses [32, 33]. Our findings suggest that coinfection with bacteria and viruses in the middle ear may be a more common cause for a poor treatment response in AOM than previously thought [19].

Typical AOM bacteria could be cultured in 73% of our cases, comparable to rates in populations with AOM with an intact tympanic membrane [1]. The use of PCR revealed that more than one-half of our patients with initially negative culture results actually harbored bacteria in their middle ears. Thus, AOM bacteria were found in 86% of children. Del Beccaro et al. [34] have achieved comparable results by using broth subcultures in addition to conventional culture. In young children with acute tube otorrhea, AOM bacteria have been previously found in 45%–62% of cases [2, 35, 36]. In our population, both *S. aureus* and *P. aeruginosa* were rare findings, as expected, considering the young age group and the exclusion of summer months [2, 35]. An equally important explanation, however, may be the true acute nature of the infection, lasting in most of our cases for <24 h before sampling. The previous surveys reporting high prevalences of *S. aureus* and *P. aeruginosa* have included patients with prolonged otorrhea lasting as long as 3 weeks [36]. Furthermore, in all previous studies, specimens were collected by swabbing the ear canal, in contrast to the direct visually controlled MEF suctioning through the tube in the present study. Brook et al. [37] have convincingly shown that the sampling technique affects the rate and spectrum of bacteria; thus, specimen collection from the ear canal may give misleading results. This gives further support to our finding that, although the presence of the tympanostomy tube alters the physiological conditions of the middle ear, the patent tube does not change the etiology of AOM in young children.

Respiratory viruses were demonstrated in 70% of our patients. Earlier studies have emphasized respiratory syncytial virus in the etiology of AOM. However, these studies have used only viral culture and/or antigen detection, and overall positivity rates for viruses have been modest (10%–24%) [16, 17, 20, 21, 38]. Use of PCR techniques has revealed the significance of picornaviruses in AOM [4, 22, 39]. We found rhinoviruses and enteroviruses in 42% of patients, and 60% of all findings of viruses were picornaviruses. Picornaviruses were the most frequent findings even during respiratory syncytial virus season, as also shown by Nokso-Koivisto et al. [39]. This suggests that the overall high prevalence of picornaviruses could raise rhi-

noviruses and enteroviruses to be the leading viral etiologic agents of AOM, although the relative rate of respiratory syncytial virus as a predisposer to AOM is important [38]. However, differences in the sensitivity of the viral detection methods may hamper the situation. In our study, the children were an average of 6 months older than in the studies described above that included children with intact tympanic membranes. This may have decreased detection rates of respiratory syncytial virus, although our sample collection period covered the whole respiratory season. To draw a complete picture of viral infections in AOM, future studies should use viral detection methods of equal sensitivity for each virus and collect the samples at least during 1 fall-winter-spring period.

In recent years, new respiratory viruses have been discovered. This is the first study to show that the newly discovered human bocavirus [15] is also related to the etiopathogenesis of middle ear infections. We also showed, in agreement with others, that human metapneumovirus is an otitis media pathogen [40]. Unfortunately, these viruses were sought from only a limited number of samples in our study. Future studies exploring the importance of each virus in the etiology of AOM should combine optimal diagnostics with sample collection during the whole season of respiratory infections.

This study provides evidence that the majority of acute middle ear infections in children are due to coinfection with bacteria and viruses. Furthermore, our results confirm that AOM with an intact tympanic membrane and AOM with otorrhea through the tympanostomy tube have similar spectrums of pathogens in young children who have a truly acute onset of the disease and a concomitant respiratory infection.

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