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Moraxella catarrhalis Might Be More Common than Expected in Acute Otitis Media in Young Finnish Children

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According to studies based on bacterial cultures of middle ear fluids, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* have been the most common pathogens in acute otitis media. However, bacterial culture can be affected by reduced viability or suboptimal growth of bacteria. PCR detects bacterial DNA from samples with greater sensitivity than culture. In the present study, we analyzed the middle ear pathogens with both conventional culture and semiquantitative real-time PCR in 90 middle ear fluid samples obtained from children aged 5 to 42 months during acute otitis media episodes. Samples were tested for the presence of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *Alloiococcus otitidis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. One or more bacterial pathogens were detected in 42 (47%) samples with culture and in 69 (77%) samples with PCR. According to PCR analysis, *M. catarrhalis* results were positive in 42 (47%) samples, *H. influenzae* in 30 (33%), *S. pneumoniae* in 27 (30%), *A. otitidis* in 6 (6.7%), *S. aureus* in 5 (5.6%), and *P. aeruginosa* in 1 (1.1%). Multibacterial etiology was seen in 34 (38%) samples, and *M. catarrhalis* was detected in most (85%) of those cases. Fifteen signals for *M. catarrhalis* were strong, suggesting a highly probable etiological role of the pathogen. In conclusion, even though *M. catarrhalis* is often a part of mixed flora in acute otitis media, a considerable proportion of cases may be primarily attributable to this pathogen.

Bacterial culture of middle ear fluid (MEF) has been the standard for etiologic diagnosis of acute otitis media (AOM). *Streptococcus pneumoniae* is considered the most common bacterial pathogen, followed by *Haemophilus influenzae* and *Moraxella catarrhalis* (1–3). About 44% to 75% of MEF samples are positive for bacterial pathogens in conventional culture (2, 4). However, the proportions of culture-positive MEF samples decrease in patients with recurrent AOM (5) and in patients with treatment failure (6). Host defense mechanisms and previous antibiotic treatments may directly contribute to diminished survival of the pathogens in clinical samples and diminish the likelihood of detection of bacteria by culture. In addition, the presence of bacterial biofilms has been correlated with negative bacterial cultures (7) and some of the bacterial pathogens, e.g., *Alloiococcus otitidis*, grow poorly in conventional cultures (8).

Virus infections have also been considered causative agents in AOM (2, 9). Preceding or concurrent viral upper respiratory tract infection is typical in AOM (10), and interactions of viruses and bacteria in the nasopharynx may play an important role in the pathogenesis of AOM (10–12). In the era of increasing antimicrobial resistance, precise information concerning MEF pathogens may be beneficial for empirical antibiotic therapy, and therefore PCR technique applied to samples from this primarily sterile site may help to achieve more-accurate bacterial etiologic diagnosis of AOM.

Previous studies of AOM in Finnish populations have indicated that the prevalence of *M. catarrhalis* may be relatively high and may be higher than in other populations according to culture results (1–3, 13). It is of interest to determine whether this would also remain true when PCR was used to complement culture results.

This study was designed to assess the bacterial pathogens in AOM with semiquantitative real-time PCR. MEF samples were studied with conventional culture and PCR for *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *A. otitidis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Patients and their samples. We analyzed 90 MEF samples obtained during AOM episodes from 79 children aged 5 to 39 months (median, 19 months) who participated in a previously published otitis media study (14). Eleven children participated in the study during two different AOM episodes. The children were enrolled from the outpatient clinics in the city of Tampere between September 2010 and December 2011. The children were examined by an otolaryngologist in the Department of Otorhinolaryngology at Tampere University Hospital. The definition of AOM was based on the presence of MEF with signs of inflammation of the tympanic membrane or, alternatively, otorrhea through a tympanostomy tube or a spontaneous perforation of the tympanic membrane and symptoms of acute respiratory infection. In cases in which AOM was diagnosed, a myringotomy was performed using local anesthesia (topical phenol solution)

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TABLE 1 Primers and probes utilized for specific PCR detection of pathogens

Target	Primers and probe	Reference(s)
Haemophilus influenzae, 16S rRNA gene	Forward primer, 5′-CGTATTATCGGAAGATGAAAGTGC-3′; reverse primer, 5′-CTACGCATTTCACCGCTACAC-3′; hydrolysis probe, 5′-FAM-CGTATTACCGCGGCTGCTGGCAC-3′[a]	Primers, 16, 24, and 32; probe, 17
Streptococcus pneumoniae, *oralis*, *mitis* and *infantis*, 16S rRNA gene	Forward primer, 5′-AAGGTGCACTTGCATCACTACC-3′; reverse primer and probe as in the assay for *H. influenzae*	
Alloiococcus otitidis, 16S rRNA gene	Forward primer, 5′-GGGGAAGAACACGGATAGGA-3′; reverse primer and probe as in the assay for *H. influenzae*	
Moraxella catarrhalis, 16S rRNA gene	Forward primer, 5′-CCCATAAGCCCTGACGTTAC-3′; reverse primer and probe as in the assay for *H. influenzae*	
Streptococcus pneumoniae, *lytA* gene	Forward primer, 5′-ACGCAATCTAGCAGATGAAGCA-3′; reverse primer, 5′-TCGTGCGTTTTAATTCCAGCT-3′; hydrolysis probe, 5′-FAM-TGCCGAAAACGCTTGATACAGGGAG-3′	21
Pseudomonas aeruginosa, *oprL* gene	Forward primer, 5′-GGGTTCATTAGGAGTTACATGA-3′; reverse primer, 5′-GGGCATAACGACTTCTTACTTC-3′; hydrolysis probe, 5′-FAM-TTCTGAGCCCAGGACTGCTCGTCGTGGCCG-3′	Primers repositioned from those reported in reference 18
Staphylococcus aureus, *nuc* gene	Forward primer, 5′-CATCCTAAAAAAGGTGTAGAGA-3′; reverse primer, 5′-TTCAATTTTMTTTGCATTTTCTACCA-3′; hydrolysis probe, 5′-FAM-TTTTCGTAAATGCACTTGCTTCAGGACCA-3′	19

[a] The reverse primer and the hydrolysis probe are common to the first four assays. FAM, 6-carboxyfluorescein.

and MEF specimens were obtained with a sterile suction tip. In children with tympanostomy tubes or spontaneous perforations of the eardrum, MEF specimens were also obtained from the middle ear by suction. One aliquot of MEF was allocated for bacterial culture and another aliquot for PCR analysis. The bacterial culture was performed after sample collection as a fresh sample. The samples for PCR analysis were frozen after collection at the study clinic and stored at −70°C until analyzed. After collection of the samples, patients were treated with antibiotics. A follow-up visit was offered to all patients to control the recovery of AOM after 3 to 4 weeks or earlier if needed.

All clinical symptoms that were present at the examination were recorded, and parents also completed a questionnaire with specific questions about the child's previous illnesses such as previous episodes of AOM and antibiotic treatments. Of the children in the study, 17 (22%) received PCV-10 vaccination. Other factors that can modulate the risk of AOM, such as attending day care, duration of breast feeding, exposure to cigarette smoke, current use of pacifier, and family history of AOMs, were also recorded.

The study was approved by the ethical committee of Tampere University Hospital (grant number R10026), and written consent was obtained from all participating families.

Bacterial culture. Bacterial culturing was performed on all MEF samples by an accredited laboratory (Department of Microbiology, Fimlab Laboratories, Tampere, Finland) using standard protocols. Samples were streaked on chocolate and 5% horse blood agar plates. The plates were incubated at 35°C for 24 h (to 48 h [chocolate agar plate for *H. influenzae*]) in a 5% CO_2 atmosphere. Species identification, including specific identification tests, was performed by conventional methods (15). Identification was primarily based on typical growth and colony morphology. *S. pneumoniae* was specifically identified by an optochin disk test, *H. influenzae* by typical growth (*H. influenzae* grows typically only in chocolate agar) and oxidase, satellite, and porphyrin tests, *M. catarrhalis* by oxidase and DNase tests, and *S. aureus* by DNase and coagulase tests. Additional identification tests were used when needed.

DNA extraction. Nucleic acid was extracted from 70 μl of MEF using a QiaAmp DNA minikit (Qiagen, Hilden, Germany) with the following modifications of the manufacturer's instructions. First, a mixture was made that was composed of 130 μl Tris-EDTA (TE) buffer (pH 8), 10,000 U of Ready-Lyse lysozyme solution (Epicentre, Madison, WI), 4 μg Carrier RNA (Qiagen), and 1 μl of a plasmid as an exogenous internal control with a fragment of a plant gene. The mixture was then added to 70 μl of MEF in a sterile tube and subjected to thorough vortex mixing. After incubation for 15 min at 25°C with mixing performed every 2 min, the tube was frozen at −70°C for at least 15 min to help disrupt cell walls and then briefly centrifuged and 20 μl of Proteinase K (Qiagen) was added. The mixture was then incubated for 30 min at 56°C with intermittent mixing and for 15 min at 95°C to inactivate lysozyme and some infectious agents. After cooling to room temperature took place, the protocol continued with steps advised by manufacturer's instructions, namely, the addition of ethanol, column binding, two washing steps, and elution. Whenever possible, a vacuum manifold was used to minimize manipulations. Nucleic acid was eluted to 100 μl EA buffer (Qiagen) and stored. The extraction was controlled by a real-time PCR assay for the exogenous internal control fragment and by quantitation of the human albumin gene. One sample was excluded from further analysis because of failure of DNA extraction. To minimize bias, we performed the analyses blind to the culture results and seven samples (7.6%) underwent the process of extraction and testing in duplicate under different identification conditions.

Specific quantitative PCR for individual pathogens. Selected individual pathogens were tested by a battery of specific PCRs in a real-time format which conferred semiquantitative information. The primers and probes used are summarized in Table 1. Four assays were targeted to the 16S rRNA genes (an *H. influenzae* assay, an assay for *S. pneumoniae*, *S. oralis*, *S. mitis*, and *S. infantis*, an *A. otitidis* assay, and an *M. catarrhalis* assay) with primers described by Holder et al. (16) complemented with a hydrolysis probe described by Nadkarni et al. (17) which allowed adaptation of the assay to the real-time PCR format. Every PCR product of the 16S-based assays was verified by bidirectional sequencing using BigDye Terminator 3.1 chemistry on an ABI 3130xl capillary sequencer (Life Technologies, Foster City, CA). The sequences were then queried against the NCBI 16S database and taxonomically classified. *P. aeruginosa* was detected using real-time PCR with a hydrolysis probe targeted to the *oprL* gene, primers were repositioned from the positions described in reference 18, and *S. aureus* was detected by testing the thermonuclease-encoding *nuc* gene (19).

The reaction described above and developed for *S. pneumoniae* by Holder et al. (16) is actually of considerably broader applicability, detecting also *S. oralis*, *S. mitis*, *S. infantis*, and a number of strains of *S. sangui-*

TABLE 2 Comparison of pathogens from 90 MEF samples detected by culture and PCR

Assay result	No. (%) of samples					
	Moraxella catarrhalis	*Haemophilus influenzae*	*Streptococcus pneumoniae*	*Alloiococcus otitidis*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
Culture +, PCR +	8 (8.9)	16 (18)	15 (17)	0	1 (1.1)	0
Culture +, PCR −	0	1 (1.1)[a]	0	0	0	0
Culture −, PCR +	34 (38)	14 (16)	12 (13)	6 (6.7)	4 (4.4)	1 (1.1)
Culture −, PCR −	48 (53)	59 (66)	63 (70)	84 (93)	85 (94)	89 (99)
Total	90	90	90	90	90	90

[a] This instance of PCR negativity of a sample containing *H. influenzae* was a consequence of the sequence of the forward primer. Some strains of *H. influenzae* are not detectable by the primer used in this study (16, 24, 32). The presence of *H. influenzae* in a high quantity was verified by sequencing the V3-to-V5 region of the bacterial 16S rRNA gene using generic primers. To reinforce the finding, and to amend the design of the detection primer, we then retrieved available sequences of the 16S region of *H. influenzae* from GenBank, aligned them, removed the ambiguous sequences, and assessed the primer annealing site in the 557 remaining sequences. The *H. influenzae*-specific forward primer designed by Holder et al. (16) and used here annealed with no mismatches to 401 of 557 retrieved sequences. Two other prevalent motifs were related to the forward primer annealing site: the first one differed by only two nucleotides in the 5′-proximal portion of the primer, so safe detection by the original primer may be reasonably expected; it was present in 53 of 557 retrieved sequences. The other motif, however, differed by eight nucleotides, including those in the 3′-distal end of the original primer; such a motif was noted in 58 of 557 retrieved sequences. Because isolates of this sequence could not be intercepted by the original primer, we amplified the sample in question using a novel forward primer, H_infl_Fv2 (5′-CGTAGTGTCGAGAGACGAAAGG-3′), and a generic reverse panbacterial 16S primer flanking the V5 region. Sequencing of the amplicon then confirmed the presence of a high quantity of *H. influenzae* in this sample. None of the remaining samples was positive for this sequence variant.

nis, but not *S. anginosus*. This can be documented, e.g., by bioinformatic analysis using the Silva Test Prime webpage (20). For compatibility with studies published earlier, we tested *S. pneumoniae* using the 16S-based assay and direct sequencing, but all samples were assayed in addition by quantitative PCR detection of the autolysin-encoding *lytA* gene of *S. pneumoniae* (21).

The approximate quantity of the bacteria was reported using threshold cycles. To ensure that the sensitivities of the assays were comparable, we used calibration curves derived from DNA isolated from clinical strains of the bacteria, linked through a generic quantitative 16S reaction corresponding to the mock community as the external calibrator (courtesy of BEI Resources, NIAID, NIH; genomic DNA from microbial mock community B—even, low concentration—v5.1L, for 16S rRNA gene sequencing [HM-782D]). These calibrators then served as a control of efficacy for assays detecting *H. influenzae*, *S. pneumoniae*, *M. catarrhalis*, *S. aureus*, and *P. aeruginosa*.

Statistical methods. The chi-square test was used to analyze AOM risk factors (sex, first-born status, history of AOM in siblings, attendance of day care, recurrent AOM, exposure to cigarette smoke, current use of pacifier, duration of breast feeding) that were associated with different pathogens, and the Mann-Whitney test was used for analysis of numeric data (age, number of previous antibiotic treatments). A *P* value of less than 0.05 was considered to indicate statistical significance. Statistical analysis was carried out with SPSS (IBM SPSS Statistics for Windows, Version 22.0, 2012; IBM Corp., Armonk, NY).

RESULTS

Most (79/90) of the samples were obtained after myringotomy. Samples were obtained from the middle ear via tympanostomy tubes in 9 cases and via a perforated ear drum in 2 cases. Altogether, 42 (47%) of the 90 MEF samples were culture positive. Of these 42 culture-positive samples, *H. influenzae* was present in 17 (40%), *S. pneumoniae* in 15 (36%), *M. catarrhalis* in 8 (19%), other flora in 3 (7%), and *S. aureus* in 1 (2%). Two different pathogens were cultured in 2 MEF samples. No *A. otitidis*- or *P. aeruginosa*-susceptible growth was detected in culture, and the remaining 48 (53%) samples were culture negative.

The set of PCR tests significantly increased the detection of pathogens to an overall positivity level of 69 (77%) samples (Table 2). All culture-positive samples were also positive by PCR, with the exception of one instance of *H. influenzae* not detected by the original forward primer (see footnote a in Table 2). The proportions of positive samples detected by culture and PCR and the PCR results by quantity levels are presented in Table 2 and Table 3, respectively. PCR especially increased the rate of detection of *M. catarrhalis*, the most common pathogen among the 48 samples which grew no bacteria in culture (detected in 19 of such samples). Also, 9 instances of *S. pneumoniae*, 7 of *H. influenzae*, 2 of *S. aureus*, and 2 of *A. otitidis* and 1 instance of *P. aeruginosa* were detected in those samples. There were 20 samples negative both by culture and by PCR, and in 5 of them we found sequences other than the targets of the detection reactions (namely, streptococci and staphylococci) which share sequences of the primers and *S. pneumoniae* and *S. aureus*.

Of the 42 *M. catarrhalis*-positive samples, 15 contained intermediate or large amounts of the organism (being amplified before cycle 32) and 27 contained small amounts of the organism (being amplified at cycle 32 or later). In 13 samples, *M. catarrhalis* was detected as a single pathogen. In 7 of these, it was present in an intermediate or high quantity whereas in 6 there was a low quantity of the agent. Multibacterial etiology was found in 34 (38%) of the MEF samples using PCR. These included 26 samples with 2 bacterial species and 8 samples with 3 bacterial species. Of the samples with multiple positive agents, *M. catarrhalis* was detected in 29 (85%).

The prevalence of symptoms in episodes with *M. catarrhalis* as a single pathogen did not differ from the prevalence in other episodes (Table 4). Also, the symptoms associated with PCR-positive and PCR-negative AOM episodes were not statistically significantly different. The recovery from AOM was controlled in 82 (91%) episodes. The mean time between the study visit and the control visit was 25 days (range, 7 to 59). During the control visit, 61 (74%) patients were healed whereas 11 (13%) patients had otitis media with effusion and 10 (12%) had signs and symptoms of AOM. The antibiotic treatment was as follows: amoxicillin was given in 26 episodes, trimethoprim-sulfamethoxazole in 26 episodes, amoxicillin-clavulanate acid in 35 episodes, and ceftriaxone (Rocephalin) or cefuroxime in 3 episodes. In these treatment groups, *M. catarrhalis* was detected in 14, 11, 15, and 2 episodes,

TABLE 3 Comparison of pathogens detected by PCR results by quantity levels from 90 MEF samples

Assay result[a]	No. (%) of samples					
	Moraxella catarrhalis	*Haemophilus influenzae*	*Streptococcus pneumoniae*	*Alloiococcus otitidis*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
PCR +, any level	42 (47)	30 (33)	27 (30)	6 (6.6)	5 (5.6)	1 (1.1)
PCR +	27 (30)	11 (12)	10 (11)	1 (1.1)	4 (4.4)	0
PCR ++	15 (16)	14 (16)	13 (14)	4 (4.4)	1 (1.1)	1 (1.1)
PCR +++	0	5 (5.5)	4 (4.4)	1 (1.1)	0	0
PCR −	48 (53)	60 (66)[b]	63 (70)	84 (93)	85 (94)	89 (99)
Total	90	90	90	90	90	90

[a] The levels of positivity in PCR were derived from the result of quantitative PCR and are expressed as follows: +++, amplification before cycle 25; ++, amplification in cycle 25 to 31.9; +, any amplification before the end of PCR (45 cycles). All amplification signals were confirmed by direct sequencing of the PCR product.
[b] The data correspond to the sample later detected by the new H_infl_Fv2 primer.

respectively, and full recovery in controlled *M. catarrhalis* episodes was seen in 75%, 82%, 82%, and 100% of episodes, respectively. Of the analyzed predictors, *M. catarrhalis* results approached statistical significance with respect to history of several AOMs ($P = 0.091$), previous antibiotic treatments ($P = 0.064$), and older age ($P = 0.086$) (Table 5). None of the 11 children who participated in the study during 2 different AOM episodes had *M. catarrhalis* detected in both episodes. The presence of *H. influenzae* or *S. pneumoniae* was not associated with any analyzed potential risk factor.

DISCUSSION

This study showed that bacterial pathogens in children with AOM were detected in 47% of MEF samples using conventional culture and that adding PCR to the diagnostics resulted in an increase in the detection rate to 77%. In contrast to previous studies from other populations (22–24), *M. catarrhalis* was clearly the most common pathogen detected by PCR—in total, 47% of all MEF samples were positive for *M. catarrhalis*. Its detection was associated with the presence of other bacteria in MEF, and it was slightly more common in children with a history of several AOM episodes and antibiotic treatments.

M. catarrhalis is a Gram-negative diplococcus and has established its role as an important human pathogen. It was first recognized as an AOM pathogen in the 1980s (25, 26) and since then has been linked with AOM, particularly in young children and in first AOM episodes (3, 27). The fact that *M. catarrhalis* is associated with lower numbers of spontaneous tympanic membrane perforations and no mastoiditis (27) indicates that it might be less virulent than many other pathogens. *M. catarrhalis* has also been the predominant pathogen in children with middle ear effusion (28).

TABLE 4 Symptoms of patients with *M. catarrhalis* only compared to other pathogens

Symptom or duration	No. (%) of samples	
	M. catarrhalis-only episodes ($n = 13$)	Episodes with other pathogen[a] ($n = 77$)
Cough	8 (61.5)	59 (77.6)
Rhinitis	11 (84.6)	64 (84.2)
Irritability	11 (84.6)	60 (78)
Restlessness	12 (92.3)	67 (88.2)
Earache	11 (84.6)	58 (76.3)
Lack of appetite	7 (53.8)	44 (57.9)
Fever (>38°C)	5 (38.5)	33 (43.4)
Diarrhea	1 (7.7)	6 (7.9)
Vomiting	0	10 (13.2)
Duration of symptoms		
<7 days	6 (46.2)	45 (58.4)
≥7 days	7 (53.8)	32 (41.6)

[a] Data include cases with infections by *M. catarrhalis* together with other pathogens.

PCR increases the detection of pathogens compared to conventional culture (29, 30). Many of the previous PCR-based studies were carried out by detecting pathogens from MEF samples taken during episodes of non-acute otitis media, e.g., otitis media with effusion, or during tympanostomy tube replacement (22, 31). In children with the presence of purulent MEF samples during tympanostomy tube placement, the most common pathogens were *H. influenzae* (53%), *M. catarrhalis* (26%), *A. otitidis* (23%), and *S. pneumoniae* (19%) in the study by Holder et al. (22). Ruohola et al. reported that *M. catarrhalis* was detected in 28% of AOM episodes in Finnish children with tympanostomy tubes in 2006 (13). However, the researchers used PCR only for those MEF samples that were culture negative ($n = 16$) and for 29% (17/58) of the culture-positive samples, which makes it difficult to compare their findings with our results.

To the best of our knowledge, there have been only a few other previous reports of detection of bacterial pathogens with a panel of PCR tests applied to MEF samples of patients with AOM. One of the previous studies was conducted in Finland (30). Among children in a group with a median age of 30 months and a 25% rate of being otitis prone, *M. catarrhalis* was the most common pathogen (27%), followed by *A. otitidis* (25%), *S. pneumoniae* (20%), and *H. influenzae* (11%) (30). In a Japanese study by Harimaya et al. (23), *A. otitidis* was detected in 55% of the samples and *M. catarrhalis* was the next-most-common pathogen (20%). The median age of the children was 42 months (23), while in our study the children were clearly younger (median age, 19 months). A study of children in an age group similar to that examined in our study (6 to 36 months) in a different geographical area (the United States) detected *S. pneumoniae* in 51% of MEF samples, *H. influenzae* in 35%, *A. otitidis* in 19%, and *M. catarrhalis* in 14% during AOM with PCR (24). It seems that relatively high proportions of *M. catarrhalis* in MEF samples have been reported in Finnish studies compared to studies from other parts of the world (32), and our results confirm these previous observations. Furthermore, our

TABLE 5 Analysis of risk factors for *M. catarrhalis* infection

Risk factor	*M. catarrhalis* + MEF samples	MEF samples with other pathogen	*P* value
Patient demographics [no. (%)]			
Male	24 (57.1)	25 (52.1)	0.631
First born	10 (23.8)	16 (34.0)	0.289
History of AOM in sibling(s)	16 (38.1)	14 (29.8)	0.408
Attending day care	23 (54.8)	18 (38.3)	0.120
>3 AOM episodes in history	28 (66.7)	23 (48.9)	0.091
Exposure to cigarette smoke	16 (38.1)	19 (40.4)	0.822
Use of pacifier	20 (47.6)	31 (66.0)	0.081
Breast feeding for ≤6 mo	16 (38.1)	19 (40.4)	0.822
No. of previous antibiotic treatments, median (range)	5 (0–23)	3 (0–15)	0.064
Age (mos), mean (range)	20 (6–42)	14 (5–39)	0.086

study showed that *M. catarrhalis* is particularly common among younger AOM patients. In all, it is obvious that the prevalence of AOM pathogens varies geographically and that the incidence of *M. catarrhalis* in AOM appears to be increased in younger Finnish children for some reason.

An important feature of *M. catarrhalis* is that about 95% of strains produce beta-lactamase. This may also cause treatment failures in multibacterial AOM episodes. Previous studies have shown that the beta-lactamase enzyme produced by *M. catarrhalis* protects other pathogens against beta-lactam antibiotics in mixed infections (33). Although our study was not primarily designed to research the efficacy of different antibiotics against *M. catarrhalis* and the number of patients in each treatment subgroup was too small to draw definite conclusions, it is noteworthy that more treatment failures happened in the amoxicillin group than in the others. In our study, *M. catarrhalis* was detected in 85% of the MEF samples with multibacterial etiology, most often with *H. influenzae* or *S. pneumoniae*, which are usually susceptible as sole pathogens to beta-lactam antibiotics.

M. catarrhalis was found only in 9% of the samples by the use of conventional culture, while PCR detected it 5 times more frequently. This is well in line with a systematic review by Ngo et al. in which *M. catarrhalis* was detected 4.5 times more frequently by PCR than by culture (32). Taking the results together, 19/48 (40%) of culture-negative MEF samples were positive for *M. catarrhalis* in PCR analysis. One possible explanation for this phenomenon might be the presence of biofilms, since several studies have shown a correlation between negative culture and the presence of bacterial biofilms (7, 34). In biofilms, the bacterial communities are covered by a protective polysaccharide matrix that protects them from antibiotics (24). *M. catarrhalis* has been identified in such biofilms of the middle ear mucosa (7). It has also been reported that previous antibiotic treatments increase biofilm formation (35). The presence of biofilms was not analyzed in this study. Nevertheless, *M. catarrhalis* was mostly detected in culture-negative and mixed infections and in patients with a history of previous AOMs and antibiotic treatments (likely due to previous AOMs), and this might reflect the presence of biofilms in these patients. Interestingly, antibiotic resistance has been reported to be particularly common in biofilms with mixed infections of *H. influenzae* and *M. catarrhalis* (36).

A debated issue is whether the DNA detected by PCR comes from viable or nonviable bacteria. As the samples had been frozen, we could not utilize propidium iodide techniques for separating DNA from live bacteria only. Studies in chinchillas have shown that bacterial DNA in MEF samples disappears within 3 days of bacterial cell death (37). Accordingly, we assume that the bacterial DNA in MEF samples originated from bacteria involved in the present AOM and did not represent remnants from a previous AOM episode. It is also possible that *M. catarrhalis* is constantly transported to the middle ear from the nasopharynx during AOM, as about 40% of otitis-prone children are colonized with this pathogen (38). In this respect, it may be important to assess the quantitative information that was categorized into three levels of positivity depending on the threshold cycle of PCR; admittedly, of the 42 samples positive for *M. catarrhalis*, 27 contained small amounts of the organism. However, in the remaining 15 samples, the quantities reflected not only passive transport but also active replication. In addition, none of the children in the study had *M. catarrhalis* detected during two different AOM episodes, which speaks against the continuous feed of this pathogen from the nasopharynx unless they had been colonized between the two episodes. In agreement to other studies (4, 13, 39), we consider specific PCR a reliable method to detect causative pathogens from MEF samples, especially when semiquantitative information is available.

A total of 20 MEF samples were negative in culture and bacterial PCR, which may reflect a viral etiology of these AOM cases, a low number of bacteria present in MEF, or a bacterial species not included in the detection panel. A strength of our study was that we were safeguarded against the loss of DNA during the extraction procedure or the presence of PCR inhibitors in the sample by the use of exogenous controls, consisting of a fragment of *Arabidopsis thaliana* detected in a separate real-time PCR. Furthermore, to maintain specificity, we sequenced all samples with positive findings, and to avoid contamination, all of the samples were collected with sterile instruments and only a few samples were collected per day. One of the samples obtained via a perforated ear drum grew *S. aureus* in culture, and it might have been a colonizing pathogen from the outer ear canal. Water controls were used in PCR tests, and they all were negative. A limitation of our study was the relatively small study population. However, it still represented a background population of children with AOM. We also could not quantify the bacterial load with absolute accuracy, because there is no quantitative reference in the ear fluid and because our quanti-

tative standards for individual assays were linked only indirectly to an internationally recognized external calibrator.

In conclusion, we found evidence suggesting that the role of *M. catarrhalis* in AOM may be more important than previously estimated on the basis of traditional bacterial culture of MEF samples. The presence of *M. catarrhalis* was common in multibacterial infections and was minimally associated with a history of previous AOMs and previous antibiotic treatments. Its role might be associated with biofilms, and its interaction with other pathogens might be notable. *M. catarrhalis* should be considered an important pathogen in AOM. If further studies in other populations support these findings, developing vaccines against *M. catarrhalis* could be one option to reduce the burden caused by AOM.

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