



Prevalence of bacteria in children with otitis media with effusion

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Abstract

Objectives: 1) To determine the prevalence of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* in middle ear effusions of children with otitis media with effusion undergoing myringotomy; 2) to compare the results obtained by culture and PCR; and 3) to determine the susceptibility of bacterial isolates to penicillin.

Methods: We analyzed 128 middle ear effusion specimens from 75 children (age = 11 months to 10 years; mean = 34.7 months). Patients with recurrent otitis media had documented middle ear effusion for = 6 weeks, and chronic otitis media with effusion for = 3 months. The patients had no signs of acute otitis media or respiratory tract infection and were not on antibiotic therapy. Aspiration was done through tympanocentesis with an Alden-Senturia trap. Bacteriological studies were initiated less than 15 minutes after specimen collection. Part of the sample was stored at -20°C for later multiplex PCR analysis. Statistical analysis employed McNemar's χ^2 test.

Results: Bacteria were cultured in 32 (25.1%) out of 128 samples and the pathogens under investigation were found in 25 (19.6%). PCR was positive for bacteria in 73 (57.0%) specimens: 50 (39.1%) for *H. influenzae*, 16 (12.5%) for *S. pneumoniae*, and 13 (10.2%) for *M. catarrhalis*. All the culture-positive samples were PCR-positive, but 48 (65.7%) of the PCR-positive specimens were culture-negative. PCR was significantly more sensitive than culture ($p < 0.01$) to identify bacteria. Resistance to penicillin was as follows: *M. catarrhalis* = 100%; *S. pneumoniae* = 62.5% and *H. influenzae* = 23% of the isolates.

Conclusions: The prevalence of bacteria in otitis media with effusion in a group of Brazilian children was similar to that reported for other countries. *H. influenzae* was the most frequent microorganism observed. This suggests that bacteria may play a role in the pathogenesis of otitis media with effusion. In addition, PCR was more sensitive to detect bacteria in middle ear effusion as compared to conventional culture methods. Penicillin resistance was similar to that reported for other countries for pneumococci and moraxella, but beta-lactamase production by *H. influenzae* was lower than that reported for other countries.

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Introduction

Otitis media with effusion (OME) is an inflammation of the middle ear in which fluid accumulates behind the eardrum, without any signs or symptoms of acute infection, and with an intact tympanic membrane. Secretory otitis media, nonsuppurative otitis media, serous otitis media and mucoid otitis media are synonymous with otitis media with effusion, but these terms are not as accurate. The frequent opacification and edema of the tympanic membrane may hinder the identification of the type of effusion.¹

OME often is considered a direct extension of the inflammatory process that occurs during long-lasting or recurrent episodes of acute otitis media (AOM), which is confirmed by the fact that almost all cases of OME follow episodes of AOM, and also corroborated by experimental animal studies.^{2,3}

The observations above suggest that OME has an infectious etiology. On the other hand, the cultures of middle ear aspirates are positive in only 20-40% of OME cases. The most frequently detected bacteria are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*.⁴⁻⁷ Recently, polymerase chain reaction (PCR) techniques were adapted to detect bacterial DNA in middle ear effusions, and their use increased the frequency of positive results for these bacteria in the examined effusions by nearly 80%.⁸⁻¹⁰

The prevalence rates mentioned above and the necessity to define beforehand which microorganisms should be identified by PCR made us opt for the investigation of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*.

The acquired knowledge about the prevalence of microorganisms responsible for or involved in OME cases may help select the most appropriate antimicrobials and minimize complications that might require surgery.

The aim of the present study was to determine the prevalence of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* in the middle ear effusions of children with OME by way of direct culture and PCR, as well as to determine the resistance of these bacteria to penicillin

Methods

A contemporary, cross-sectional and observational study was carried out with subindividual data (ears), in order to study the effusions produced by OME.

By using an expected prevalence of 65% for positive PCR results and 25% for positive culture findings, with a margin of error of 10%, we estimated a sample size with at least 72 effusions.

Seventy-five children diagnosed with OME at a pediatric otolaryngological clinic in Porto Alegre were assessed from June 2001 to October 2002. The study included patients aged between nine months and 12 years with middle ear effusion for six weeks or more, diagnosed with recurrent otitis media (three or more episodes of AOM within six months), or with chronic otitis media with effusion (persistent effusion for three months or more) and indication for myringotomy and tympanostomy tube insertion. The diagnostic criteria for OME included opacification, changes in color, decreased mobility, and increased vascularity of the tympanic membrane. All the selected cases were followed up by the main author for at least six weeks before the surgery. Impedance audiometry was performed, if necessary, to confirm the presence of effusion in cases

of recurrent otitis media (ROM), and all patients diagnosed with chronic otitis media with effusion (COME) were evaluated using audiometry and impedance audiometry. All patients were submitted to video otoscopy 24 hours before surgery. Patients who presented with AOM and other upper respiratory infections at the time of surgery, or who were on current use of antibiotics or had been using them until less than seven days before the surgery were excluded from the study.

The surgery was aided by microscopy. The effusion was collected from the middle ear after cleansing and antiseptics of the external auditory canal with 70% alcohol and tympanocentesis in the anteroinferior portion of the tympanic membrane. The effusion was aspirated with an Alden-Senturia trap (Storz, St. Louis, USA). The material was sent for direct culture and PCR analysis no longer than 15 minutes after collection, with no need for transport media.

For direct culture, the material was seeded onto plates containing sheep blood agar and chocolate agar (Biolab-Mérieux, Rio de Janeiro, Brazil) and incubated in the presence of oxygen for 24 hours at 37 °C. Automated bacterial identification was used (Vitek®, bioMérieux, Marcy-l'Etoile, France). Penicillin resistance of *S. pneumoniae* was determined through minimum inhibitory concentrations, using the E-test® (E test®, AB Biodisk, Soehna, Sweden). Nitrocefin test (Oxoid, Basingstoke, England) was used in colonies of *H. influenzae* and *M. catarrhalis* for the determination of the capacity of beta-lactamase production.¹¹

PCR used in this study is a method for simultaneous detection (multiplex PCR) of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*.¹⁰ The 16S rRNA gene, which contains both variable and constant sequences, was chosen as the target of PCR amplification. Constant sequences are common to several bacteria, and variable sequences are specific to each species.

The previously frozen effusions were thawed and the DNA was extracted by means of the commercially available QIAamp® kit (Qiagen, Valencia, USA). For each reaction, 1.25 U of Taq polymerase was added (GeneAmp®, Applied Biosystems, Branchburg, USA) in an appropriate buffer, following the instructions on the kit. The amplified products were then separated on a 3% agarose gel and visualized under ultraviolet light. The result was recorded as positive or negative for each of the bacteria.¹⁰

Direct culture and PCR results were described using absolute and percentage frequency. The impact of PCR on the results of direct culture was assessed by way of delta percentage ($\Delta\%$), defined as:

$$\Delta\% = \frac{\text{final value} - \text{initial value}}{\text{initial value}} \times 100$$

McNemar's chi-square test (χ^2) was used to assess associations, and a significance level of 0.05 was established.

The study was approved by the Research and Ethics Committee of the Graduate Research Program of Hospital de Clínicas de Porto Alegre. A written consent was signed by all parents or guardians after they received information about the study objectives. The indication for surgery was exclusively made by a physician before the inclusion of the patient in the study. This was, indeed, one of the requirements for inclusion in the study.

Results

A total of 128 middle ear effusions (MEE) were obtained from 75 patients submitted to myringotomy and tympanostomy tube insertion. Age ranged from 11 months to 10 years (mean±standard deviation = 34.7±18.5 months); 60% were male and all of them were white.

All the patients included in the study provided effusion for the analysis. Fifty-three (70.7%) of 75 patients provided effusion from both ears and in 29 (54.7%) the PCR analysis detected different bacteria in each ear. A total of 69.3% patients had ROM and 30.7% were included because they had COME. Patients with ROM had an average of 5.3±1.4 episodes of otitis every semester and those with COME showed an average duration of middle ear effusion of 4.8±1.1 months. The first episode of AOM in the patients included in the study occurred on average at 12.9±9.2 months.

The direct culture revealed pathogens in 32 (25.1%) of 128 MEE, and the major bacteria (*S. pneumoniae*, *H. influenzae* and *M. catarrhalis*) were isolated in 25 (19.6%). Bacteria of little clinical importance or nonpathogenic to

the middle ear were found in seven (5.5%) effusions (Table 1).

PCR was positive for one or more bacteria in 73 (57.0%) of 128 effusions. In 69 MEE, only one pathogen was identified per effusion, and mixed bacterial DNA was found in six positive effusions. Combining the results of individual and collective identification of bacteria, *H. influenzae* was found in 50 (39.1%), *S. pneumoniae* in 16 (12.5%) and *M. catarrhalis* in 13 (10.2%) of 128 MEE (Table 2).

PCR had a better performance than direct culture in detecting bacteria in the analyzed effusions. The difference between the proportion of positive effusions in direct culture and in PCR was statistically significant for all the studied bacteria, either individually or collectively (McNemar's test, $p < 0.01$) (Table 3). The increase in the proportion of effusions with positive culture findings, resulting from PCR ($\Delta\%$) is shown in Table 4. The inclusion of PCR results corresponds to a 192% increase in the total number of effusions that tested positive for the investigated pathogens.

The pathogens isolated in direct culture in 25 (19.6%) of 128 MEE were submitted to tests so that their level of penicillin resistance could be determined. The results consider beta-lactamase producing capacity as synonymous with resistance to penicillin and to other beta-lactamic agents (Table 5).

Discussion

In the past, OME was regarded as a strictly inflammatory process, and its effusion was considered sterile. However, in 1958, Senturia *et al.* found bacteria in OM effusions, redefining the heretofore-accepted concepts.¹²

Table 1 - Analysis of samples of 128 effusions of the middle ear

Result/strains	f	%
<i>H. influenzae</i>	12	9.4
<i>S. pneumoniae</i>	8	6.3
<i>M. catarrhalis</i>	4	3.1
<i>H. influenzae</i> + <i>M. catarrhalis</i>	1	0.8
Other*	7	5.5
Negative	96	75.0
Total	128	100.0

f: frequency.

* *Staphylococcus epidermidis* (1), *Streptococcus oralis* (2), *Brevibacterium sp* (2) and *Corynebacterium auris* (2).

Table 2 - PCR in samples of 128 effusions of the middle ear

Result/strain	f	%
<i>H. influenzae</i>	44	34.4
<i>S. pneumoniae</i>	14	10.9
<i>M. catarrhalis</i>	9	7.0
<i>H. influenzae</i> + <i>S. pneumoniae</i>	2	1.6
<i>H. influenzae</i> + <i>M. catarrhalis</i>	4	3.1
Negative	55	43.0
Total	128	100

f: frequency.

PCR: polymerase chain reaction.

Table 3 - Comparison between culture and PCR in 128 samples of effusions

	Total (%) culture effusions (+)	Total (%) PCR effusions (+)	p *
<i>H. influenzae</i>	13 (10.2)	50 (39.1)	< 0.01
<i>S. pneumoniae</i>	8 (6.3)	16 (12.5)	< 0.01
<i>M. catarrhalis</i>	5 (3.9)	13 (10.2)	< 0.01
One or more target-strains	25 (19.6)	73 (57.0)	< 0.01

Data are presented as absolute numbers and percentage.

* McNemar χ^2 test.

PCR: polymerase chain reaction.

Table 4 - Comparison between the results of the culture analysis and PCR according to strain

Bacteria	PCR	Culture (%)		n	$\Delta\%$
		(+)	(-)		
<i>H. influenzae</i>	(+)	13 (100.0)	37 (32.2)	50	285
	(-)	-	78 (67.8)	78	
<i>S. pneumoniae</i>	(+)	8 (100.0)	8 (6.7)	16	100
	(-)	-	112 (93.3)	112	
<i>M. catarrhalis</i>	(+)	5 (100.0)	8 (6.5)	13	160
	(-)	-	115 (93.5)	115	
Total	(+)	25 (100.0)	48 (46.6)	73	192
	(-)	-	55 (53.4)	55	

Data are presented as absolute values, as percentage obtained taking into consideration the analysis of the culture.

n: number of effusions.

 $\Delta\%$: percentage increase in the positivity of the culture provided by the informative addition of PCR.

PCR: polymerase chain reaction.

Table 5 - Profile of resistance to penicillin (*) in 26 bacterial isolates of effusion samples and middle otitis with effusion

Culture (+)	Sensitive		Intermediate		Resistant	
	f	(%)	f	(%)	f	(%)
<i>S. pneumoniae</i> (n=8)	3	(37.5)	3	(37.5)	2	(25)
<i>H. influenzae</i> (n=13)	10	(77)	-		3	(23)
<i>M. catarrhalis</i> (n=5)	0	(0)	-		5	(100)

Data are presented as absolute numbers and percentage.

(*) Assessment of *S. pneumoniae* by E-test.

Production of β -lactamase by *H. influenzae* and *M. catarrhalis*: (-) = sensitive; (+) = resistant.

f: frequency.

In the literature, the lack of a uniform definition of OME, the duration of MEE, and the absence of criteria for the use of antibiotics by the patients who provided the effusions are a hindrance to the proper relevance of information and for a comparison with the results obtained in the present study. The difficulty in interpreting MEE bacteriological data results, on many occasions, from the virtual impossibility to determine the stage of otitis media of the patients from whom the effusions were obtained. It is common knowledge that the studies that investigate the characteristics of prolonged middle ear effusion use the material aspirated by myringotomy. The criteria for myringotomy are relatively uniform, but few publications provide clear information on the duration of effusion.

The long-term follow-up of a child by the same physician or researcher, with the aim of determining the type of otitis media and the duration of effusion, is not at all an easy task in outpatient clinics. In this regard, our study offers the advantage of including only patients from the pediatric otolaryngological clinic owned by the main author, who was exclusively in charge of the initial evaluations, as well as of the follow-up, which included video otoscopy in every appointment.

The culture findings regarding the type and frequency of bacteria in effusions from OME patients are similar to those reported in the world literature (20 – 40%).⁴⁻⁷ The higher prevalence of *H. influenzae*, followed by *S. pneumoniae* and *M. catarrhalis* reproduces, in our sample, the findings of other studies, which also show an inverse order between pneumococcus and *haemophilus*, when comparing the data with those obtained from AOM patients.⁶ The individual percentage rates for *H. influenzae* (10.2%), *S. pneumoniae* (6.3%) and *M. catarrhalis* (3.9%) are very similar to the ones recently observed by Sutton *et al.* when they assessed ROM and COME samples – 12.1%, 9.6% and 5.6%, respectively.⁷

On the other hand, the comparison of the results of the present study with those obtained from Brazilian studies shows some discrepancy. The results varied from negative culture findings in all samples to 33.4% of positive findings. Among the predominant pathogens, in addition to *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* (not necessarily in this order), *S. aureus*, *S. epidermidis* and *P. aeruginosa* also were highly prevalent.¹³⁻¹⁵ Very likely, the different inclusion criteria, microbiological methodology, geographical variations, and variability influenced by the small sample size are the cause for the discrepancies observed in the comparison of the results of these studies between themselves and with the present study.

PCR significantly increased the identification of bacteria in MEE of several types of otitis media. Positive PCR findings in this study (57%) are consistent with the available literature, in which a rate between 50 and 77.3% was observed.⁸⁻¹⁰ The frequency with which the bacteria were identified revealed a higher prevalence of *H. influenzae* (39.1%), followed by *S. pneumoniae* (12.5%) and *M. catarrhalis* (10.2%). The comparison of these data with those obtained by the previously mentioned authors shows that they fit into the ranges described for *H. influenzae* (12.5–70.2%) and for *S. pneumoniae* (6.4–35%). On the other hand, the rates obtained in the present study are a bit lower than those found for *M. catarrhalis* (16–63%), but without a significant difference. We understand, and so do other authors, that the small differences observed might be easily explained by the variability of the samples¹⁶. Therefore, there are no signs that the findings of the present study are actually different from those reported in the literature. Finally, since no Brazilian study could be found that investigated MEE in OME patients by using PCR, further studies are necessary, especially because Brazil is a continental country with different populations.

By comparing the results of direct culture and PCR, we note that all samples with positive culture findings also were positive for the same pathogen in the PCR analysis, and the difference between positive effusions in direct culture (19.6%) and in PCR (57%) was statistically significant for the analyzed bacteria, both individually and collectively ($p < 0.01$). These results are similar and have the same statistical significance of those reported in the international literature.^{8-10,16} The inclusion of PCR results represented a 192% increase in the total number of positive effusions for one of the pathogens studied, comparatively to direct culture. This results is similar to the 268% increase observed by Post *et al.* and the 260% increase described by Hendolin *et al.*^{9,10}

The use of PCR in the investigation of MEE may be seen as unsatisfactory, since the detection of single genes does not necessarily indicate the presence of viable bacteria; indeed, they could be only DNA fragments or "fossilized remnants" of bacteria, as pointed out by Cantekin.¹⁷ Animal studies have shed some light on the use of PCR for the investigation of the infectious etiology of middle ear infections. By using the OM model in chinchillas, Post *et al.*¹⁸ demonstrated that purified bacterial DNA and the DNA of heat-inactivated bacteria did not remain amplifiable for more than three days after inoculation. In addition, antibiotic-sensitive bacteria could not be cultured after the third day of treatment, but the DNA remained amplifiable for three weeks. Aul *et al.*,¹⁹ using the same animal model, showed that strains of ampicillin-resistant *haemophilus* could be identified by PCR and direct culture for over one month, whereas the DNA of pneumococci inoculated in a low number of colonies were amplified for 21 days, although they could not be isolated in culture after the third day. As in the previous study, the purified DNA of *haemophilus* and the DNA of heat-inactivated moraxella did not persist for longer than two days. Additional evidence was produced by Rayner *et al.*,²⁰ who identified the mRNA of *H. influenzae*, a molecule with an average life of seconds to minutes, in human MEE that tested negative for the same pathogen. Also, they demonstrated that the DNA of the same pathogen was only identified in the samples in which the mRNA was also amplified, suggesting that the method for identification of the former one, which is more common and less expensive, is sufficient for the diagnosis. These results suggest that middle ear effusions have efficient mechanisms for the removal of non-viable bacteria, in addition to a quick process of DNA degradation. On the other hand, these findings provide varied hypotheses regarding the complex relationship in the middle ear between pathogen and host, which cannot be detected by traditional culture techniques. Metabolically active bacteria may remain in the middle ear, and the lower frequency of bacterial identification by direct culture, compared with PCR, may be explained by one or more of the following hypotheses: (a) the amount of microorganisms is lower than the limits of detection by direct culture, often indicated as 10^4 CFU/ml;²¹ (b) pathogens take L shapes, bacterial

variants that lost their capacity to synthesize the peptidoglycan layer of their cell wall and that, on these grounds, change their original shape, becoming resistant to beta-lactamic antibiotics, and imposing restrictions on their culture;²² (c) bacteria are harbored in a biofilm, a community in which bacteria stick to a surface or to one another, resulting in reduced metabolic activity and higher resistance to antimicrobial agents.²⁰ The three situations may be caused by antibiotic therapy and by the host's immune response. In addition, L-shaped microorganisms or microorganisms in biofilm, with lesser metabolic and reproductive activity, when compared with planktonic bacteria, are believed to be partly responsible for chronic inflammation and for the persistence of middle ear effusion in children, without showing profuse symptoms. Certainly, further investigation is necessary to confirm these hypotheses.

The low number (26 isolates) certainly restricts the value of the findings related to the level of penicillin resistance. In the present study, *S. pneumoniae* showed to be penicillin-resistant in 62.5% of the cases, when the data on intermediate resistance (37.5%) were combined with those on total resistance (25%). Penicillin-resistant pneumococci are much more common in children, and have become a major cause for therapeutic failure in OM patients. The increase in the rates of penicillin-resistant *S. pneumoniae* has been a hindrance to empirical treatment, has caused the reassessment of antibacterial drugs of choice, and awakened the interest in microbiological monitoring. It is common knowledge that the rates of penicillin-resistant pneumococci vary considerably from one region to another, reaching, for instance, 1-5% in Sweden and 71% in Israel.²³⁻²⁴ On the other hand, specific studies of MEE obtained from OME patients revealed rates between 38 and 70%.^{7,25,26} In Brazil, monitoring studies on pneumococcal susceptibility that did not include MEE found a resistance of 3.2 to 40%, being the lowest rates reported in older studies.^{27,28}

All the risk factors for resistant pneumococcal infection described by Klein²⁹ were found in the patient population of the present study, except for past history of hospitalization. The patients were young, attended day care centers, and had a long history of past exposure to antibiotics, as they certainly presented several OM episodes treated with these medications. Jacobs *et al.*³⁰ showed that the rates of penicillin-resistant *S. pneumoniae* varied considerably depending on the site of infection, between 38% in eye infections and 60% in otitis media, which may explain the high rates observed herein. McCracken Jr.³¹ posited that most strains of penicillin-resistant pneumococci have intermediate resistance, which also occurred with the small sample analyzed here.

Twenty-three percent of penicillin-resistant *H. influenzae* was found. In the 1980s, Bluestone *et al.* identified 20-30% of *haemophilus* found in children with OM as beta-lactamase producers, and the specific analysis of OM effusions revealed 41.5 to 65% of resistant *H.*

influenzae.^{7,25,26} In Brazil, the SENTRY program detected 11.8% of penicillin-resistant *haemophilus* in specimens obtained from the respiratory tract,³² and the study conducted by Sih³³ showed that 14% of pathogens detected in AOM effusions produced this enzyme. These reports suggest that the rates of beta-lactamase producing *H. influenzae* may be a bit lower in Brazil than in other countries, which may explain the rate observed in the present study. Once again, the small number of *H. influenzae* isolates (13) may restrict the relevance of the findings.

The data about the capacity of *M. catarrhalis* to produce beta-lactamase are easier to interpret and are probably less amenable to restrictions resulting from the small number of isolations of this bacterium in culture. The moraxellae identified in the present study were resistant to penicillin and to other beta-lactamic agents. North-American studies with AOM and OM effusions have revealed 90-100% of beta-lactamase production since the late 1980s.^{6,7,25,26} In Brazil, a study of children with AOM also showed 100% beta-lactamase producing moraxellae, which suggests that the behavior of this pathogen in our setting is the same as that reported in the international literature.³³

Medical literature has shown an increase in the incidence of antibiotic-resistant bacterial strains. Several cofactors have been implicated, among which the frequent use of antimicrobial agents is highlighted.^{6,31} Myringotomy with aspiration and tympanostomy tube insertion is, in our opinion, excellent for collecting material for the microbiological monitoring of otitis media, which could allow us to define local trends for resistance to certain antibiotics, and to identify populations at higher risk for infections caused by resistant pathogens.

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