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Original Article

Polymerase Chain Reaction, Bacteriologic Detection and Antibiogram of Bacteria Isolated from Otitis Media with Effusion in Children, Shiraz, Iran

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Abstract

Background: Otitis media with effusion is one of the leading causes of hearing loss in children. Effective treatment of effusion in the middle ear requires appropriate empirical treatment and characterization of responsible pathogens. Objective of the present study was to detect pathogens in clinical samples from patients with otitis media with effusion in our area and to determine the sensitivity profile of isolated organisms to commonly used antibiotics.

Methods: Sixty three samples of middle ear effusion were aseptically obtained from 36 children, who had been treated up to at least two weeks before sampling. They were analyzed using standard bacteriological and multiplex polymerase chain reaction (PCR) assays. Antibiotic susceptibility tests were also performed.

Results: PCR analysis showed that DNA of *Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis* were present in 60 (95.2%) of the samples. The culture-positive effusion for *Streptococcus Pneumoniae, HaemophilusInfluenzae and Moraxella catarrhalis* was 34.9%. Almost all isolates of *Streptococcus pneumoniae*e were sensitive to ciprofloxacin and erythromycin, and none of them was sensitive to co-trimoxazole. None of *H. Influenzae* isolates was sensitive to erythromycin, cefixim, co-trimoxazole, ampicillin and amoxicillin. None of *M. Catarrhalis* isolates was sensitive to ceftriaxone, co-trimoxazole, ampicillin and amoxicillin.

Conclusion: Compared with other studies using PCR method, the number of *H. influenza* isolates was in higher in the present study (95.2%). Antibiotic sensitivity profiles of pathogens isolated in this study were different from others. Thus, we can determine empirical antibiotic therapy based on sensitivity profile in our geographic area.
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Keywords ● Otitis media with effusion ● polymerase chain reaction ● antibacterial resistance ● Iran ● antibiogram

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Introduction

Otitis media (OM) is a generic term for any inflammatory

process in the middle ear cleft behind an intact tympanic membrane (TM). Otitis media with effusion (OME) indicates collection of fluid into middle ear without any sign of acute inflammation. Several factors such as eustachian tube dysfunction, insufficiencies in the aeration of mastoid air cell, allergies, immunity, gastroesophageal reflux (GER) and previous attack of acute otitis media play an important role in the etiology of the disease.[1-3]

Otitis media with effusion is one of the most common causes of hearing loss in children. Middle ear effusion (MEE), which completely fills the middle ear cleft, usually results in moderate conductive hearing loss (CHL) that adversely affects speech, language and cognitive development in children.[1]

Antimicrobial therapy, the efficacy of which has been determined, is the mainstay of treatment of OM, and may allow cancellation or at least postponement of a surgical procedure. Moreover, antimicrobial treatment provides at least short-term relief for children with hearing loss or developmental decay for whom surgery is contraindicated.[4-6] If after treatment with a course of appropriate antibiotic sign of improvement is detected by otoscopy or tympanometry, additional observation may be warranted. However, if effusion persists and is associated with hearing loss, surgical treatment may be considered.[1] In spite of the efficacy of surgical treatment, a wide range of opinions exist about its indications. Surgical treatment does not cure OME, but substantially reduces morbidity in OME patients, when medical therapy fails. The recommended surgical procedures include myringotomy, adenoidectomy, tympanostomy tube insertion and even tonsillectomy. Although the high rate of complications of Modern tympanostomy tube outweighs its benefits, modern tympanostomy tube is the therapeutic gold standard and the most widely-used treatment option for OME.[1,7]

Treatment of effusion in the middle ear should be started empirically based on knowledge of common responsible pathogens and epidemiological information. The treatment plan should change based on the susceptibility profile of bacteria, even in those areas that appropriate empirical antibiotic therapy has been previously determined.

Standard bacteriologic analysis of effusion from patients with OME has shown presence of various bacteriologic agents in 21 to 52% of the cases. *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis* and coagulase negative staphylococci are the most frequently encountered causative bacteria.[8]

The ability or disability to successfully culture a given bacteria may be due to one or more factors. If the levels an antibiotic, which reaches its site of action, become sub-lethal, a previous antimicrobial therapy may have bacteriostatic effect. Alternatively, it may not be possible to culture all strains of a given pathogenic species using the standard methods. The concept of difficulty in culturing has long been associated with chronic bacterial disease caused by slow-growing pathogens.[9] In *Streptococcus pneumoniae,* oxygen availability is a major determinant for competence development in exponentially growing cultures.[10] One of the reasons behind the low rates of bacterial growth in conventional culture conditions was reported to be the presence of L-forms of bacteria. Apparently, following the attack of OM, bacteria may survive in L-forms, which may not form colonies in culture conditions.[2] The sensitivity of bacterial detection in middle ear infections has been improved by PCR.[11-15] It is useful for the detection of pathogens that are slowly growing, difficult to culture, or hazardous to handle in a diagnostic lab.[10]

The percentage of patients given antibiotic for OM was found to vary from 31% in the Netherlands to 85% in Belgium, and more than 90% in other countries. In the Netherlands symptomatic therapy is given for the first 24-72 hours and antibiotic is prescribed only if symptoms persist. The prevalence of penicillin-resistance, either intermediate or complete, *S. pneumonia* strains ranged from 3% in the Netherlands to 53% in France.[16,17] One study showed a lower age as well as the presence of multiple bacteria as a significant factor for the presence of drug resistant bacteria.[18]

No single oral antibiotic prescription eradicates all the pathogens involved in the etiology of OM and no single management strategy is ideal for all patients. Treatment has to be administered empirically in most of the patients; therefore it has to be based on the available local epidemiological information on the most common pathogens and susceptibility patterns.[19,20]

At least one recent study showed that continuous amoxicillin treatment in OME patients resulted in more normal ears, fewer perforations, less pneumococcal carriage rates, and no increase in emerged resistant pneumococci.[21] While another study revealed relatively little benefit for such an antibiotic prophylaxis and emerging resistant bacteria.[22]

The bacteriology of OM has been studied in several parts of the world; however, current data from our region are sparse. In a previous study that was performed by Izadparast and

others, only standard bacteriologic analysis was done and sensitivity profile of pathogens was not obtained.[23]

In the present study, we carried out both PCR and bacteriologic analysis for the detection of bacteria in the middle ear effusion from patients with OME. The results of both methods were compared. Antibiograms were also done for all isolated bacteria, and sensitivity profiles of these pathogens were obtained. Thus, we can use this profile for empirical antibiotic therapy of OME patients in our geographic region. Continuous surveillance program is recommended in order to detect bacteriologic and/or susceptibility modifications that may occur over time as a baseline for appropriate antimicrobial guidelines.

Materials and Methods

A cross sectional study was performed on 36 children suffering from OME, who referred to Khalili Hospital, Shiraz University of Medical Sciences during cold seasons from September 2007 to March 2008.Otitis media with effusion was diagnosed by otomicroscopy and tympanometry. All patients underwent myringotomy and, if needed, insertion of tympanostomy tube.

The purpose of sample collection was explained clearly to the parents of each child before surgery, and written informed consent was obtained. A self-designed questionnaire comprising questions in regards to the age, sex, chief complaint, last time of antibiotic use, type of the antibiotic used, otomicroscopy finding, type of aspirated fluid, middle ear mucosa status, type of tympanometry, PCR, isolated pathogen in standard culture, and results of antibiogram was used data collection.

Exclusion criteria were the presence of underlying diseases such as craniofacial anomaly, cleft palate and primary or secondary immunodeficiency. Inclusion criteria were an age of <15 years, presence of MEE for more than three months, and not receiving antibiotic treatment for at least two weeks. Median time for the last antibiotic treatment was four weeks. None of the patients had signs of acute inflammation at surgery.

The external auditory canal was cleansed with 70% ethanol solution, and then myringotomy was performed with a paracentesis knife. A fraction of the effusions was collected, and simultaneously inoculated on three culture media including blood agar, chocolate agar and thioglycollate. The aerobic bacteria were then identified using standard microbiological methods.[24] Antibacterial susceptibility testing for all the isolated bacteria was done using Kirby-Bauer's disk diffusion method on Mueller- Hinton agar (Hi media, India) to determine their resistance pattern against common antibiotics according to the protocols of clinical and laboratory standards institute (CLSI).[25] The panels of antibiotics used included ciprofloxacin, erythromycin, ampicillin, amoxicillin, cefixim, cefotaxim, cotrimoxazole, oxacillin, cloxacillin and ceftriaxone (Pad-Tan Teb Co., Tehran, Iran).

The rest of samples were stored in an air tight container at -70°C until they were processed for PCR. Using Bioneer DNA extraction kit (South Korea), DNA of all the samples extracted according to the instruction by manufacturing company. The positive control bacterial strains were isolated from clinical specimens and identified by the microbiological methods.[26] For the multiplex PCR, they were grown in both media, sedimented by centrifuge and extracted as described above.

PCR Protocol

One µl of the extracted DNAs was used for PCR. The primers sets used in a multiplex PCR contained three specific forward primer (H.infulu: 5- CGT ATT ATC GGA AGA TGA AAG TGC-3' amplify 523 bp of 16srRNA, M.Cata: 5'- CCC ATA AGC CCT GAC GTT ACG -3' amplify 235 bp of 16srRNA, S. Pneu: 5' AAG GTG CACTTG CAT CAC TAC-3' amplify of 482 sbp of 16srRNA) (12), and a universal primer (Uni Per: 5'- GAC GCA TTT CAC CGC TAC A-3').

The PCR was performed in a total volume of 50 µl, including one µl of template DNA (50-500 ng/µl), 1.5 mM $MgCl_2$, 0.2 mMdNTPs, 0.4 mM each oligonucleotide primer sets, 1.5 U Taqpolymerase (Cinnagen, Iran) and five µl of 10x PCR buffer (Cinnagen, Iran). The tubes were placed in the Eppendorf thermocycler (Germany) with the following program: 95 °C for 10 minutes, 95°C for one minute as denaturation, 66°C for 45 seconds as annealing, and 72°C for one minute as elongation. This program was repeated for 35 cycles. In every run, both positive and negative controls were considered.

PCR Product Detection

Two percent agarose gel electrophoresis was used. Twenty five µl of the PCR products were mixed with two µl of 6x loading buffer dye and loaded into the individual wells. The electrophoresis was performed in Tris Acetate EDTA (TAE) buffer for one hour. At the end, the gel was stained in ethidium bromide solution (1 µg/ml) for 15 minutes. The results were analyzed according to the product length which

were visualized on gel documentation system and photographed.

Data were analyzed using Statistical Package for Social Sciences (SPSS, version 14). The results of the bacterial cultures and PCR assays were analyzed using Chi-Square test. Differences between the groups were considered statistically significant if P values were <0.05.

Results

A total of 36 patients including 23 boys and 13 girls with a mean±SD age of 6.72±2.95 years (range; 2-13 years) participated in the study. Twenty seven (75%) had bilateral and nine (25%) had unilateral OME, therefore, a total of 63 samples were obtained.

Two patients were identified incidentally during routine examination of ear, nose and throat, and remaining 34 patients presented with chief complaints of hearing impairment (70%), otalgia (24%), or both (6%). The mean duration of symptoms (hearing loss and/or otalgia) was 6 months (Range; 2-14). One patient had a history of previous tympanostomy tube insertion. Glue was the most common (n=50, 79.4%) type of aspirated fluid. Ten (15.9%) of ears had serous fluid. Purulent material was seen only in three (4.8%) of the ears.

The results of PCR and bacterial culture are presented in table 1 and 2. In the standard bacteriologic culture, bacterial growth was detected in 38 (60.3%) samples. The most frequent pathogens were *S. pneumoniae, H. influenzae, M .catarrhalis* and coagulase negative Staphylococci (49.2%). The percentages of culture positive effusions for *S. pneumoniae, H. influenzae* and *M. catarrhalis* were 15.9%, 9.5%, and 9.5%, respectively. PCR assay was done for three of frequently-occuring bacterial pathogen(s) in OME including *S. pneumoniae, H. influenzae* and *M. catarrhalis.* The DNAs of one or more of these bacteria were present in 60 (95.2%) samples. The DNAs of *S. pneumoniae, M. catarrhalis* and *H. influenzaee* were detected in 19%, 36.5% and 95.2% of the samples, respectively. In 32 (50.7%) samples, the DNA of only one bacterial species, and in 28 (44.5%) samples the DNAs of more than one bacterial species were detected. Three (4.8%) samples had no DNA content. Also, the number of *H. influenzae* isolate was significantly higher than those for other bacteria (P<0.05). The representative results of multiplex PCR are displayed in figure 1.

The rates of detection by PCR (95.2%) and bacteriological assays (34.9%) were significantly (P<0.05) different. Culture positive results in serous, glue and purulent aspirated fluids was 58.3% (7), 60% (30) and 100% (1), respectively, but there was no statistically significant association between the type of aspirated fluid and the results of standard cultures (P=0.495). PCR-positive results in serous, glue and purulent aspirated fluids was 91.7% (11), 96% (48) and 100% (1) respectively but again there was no statistically significant (P=0.665) association between the type of aspirated fluid and PCR results. Five different antibiotics were used by patients until two weeks prior to the surgery. Culture-negative results for those patients who used co-amoxiclave, amoxicillin, erythromycin, cefixim and cephalexin were 53.8% (7), 38.7% (12), 0% (0), 16.7% (2) and 66.7% (4), respectively. There was no statistically significant association between the type of pre-operative antibiotic treatment and culture-negative results (P=0.559). PCR-negative

Table 1: The results of bacteriological culture and PCR assay for single isolated bacteria

Type Bacteria	Culture positive (%)	PCR positive (%)
Streptococcus Pneumoniae,	10 (15.9%)	12 (19%)
Haemophilus Influenzae	6 (9.5%)	60 (95.2)[a]
Moraxella Catarrhalis	6 (9.5%)	23 (36.5%)
Coagulase negative Staphylococci	9(14.3%)	ND
Pseudomonas aeruginosa	2(3.2%)	ND
Staphylococcus aureus	2(3.2%)	ND
Viridans streptococci	1(1.6)	ND
Coagulase-positive Staphylococci	2(3.2)	ND
Total	38 (60.3 %)	

[a] Significant (P<0.05) difference between the rate of detection by PCR and bacteriological assays. ND; not detected

Table 2: The results of bacteriological culture and PCR assay for mixed bacteria

Mixed Bacteria	Culture positive (%)	PCR positive (%)
Streptococcus Pneumoniae+ Haemophilus Influenzae+Moraxella Catarrhalis	0 (0%)	7 (11.1%)
Streptococcus Pneumoniae+Haemophilus Influenza	0 (0%)	5 (8%)
Haemophilus Influenzae+Moraxella Catarrhalis	0 (0%)	16 (25.3%)
Streptococcus Pneumoniae+Moraxella Catarrhalis	0 (0%)	0 (0%)
Total	0 (0%)	28 (44.4%) [a]

[a] Significant (P<0.05) difference between the rate of detection by PCR and bacteriological assays. ND; not detected

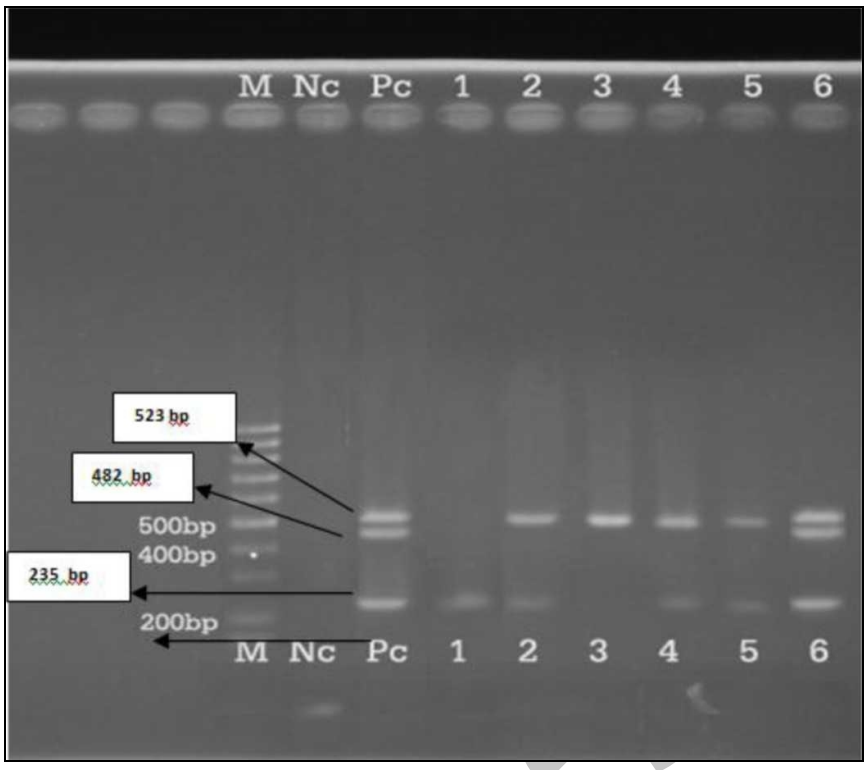

Figure 1: A representative analytical agarose gel for OME specimens. The specimen number is given below and above each lane. Lane M; molecular size marker, Nc; Negative control, Pc; Positive control, (235 bp; *M Catarrhalis,* 482bp; *S. Pneumoniae,* 523bp; *H. Influenzae),* Lane 1; *(M. Catarhalis+*), Lane 2; *(M. Catarrhalis, H. Influenzae+),* Lane 3; (H. .*Influenzae+*), Lane 4; *(M Catarrhalis+, H. Influenzae+*), Lane 5; *(M. Catarrhalis+, H. Influenzae+)*, Lane 6; *(M. Catarrhalis+, S. Pneumoniae +, H. Influenzae+*).

results for the patients treated with co- amoxiclave, amoxicillin, erythromycin, cefixim and cephalexin were 0% (0), 6.5% (2), 0% (0), 8.3% (1) and 0% (0), respectively. There was no statistically significant association between type of pre-operative antibiotic therapy and PCR- negative results (P=0.792).

There was no significant association between the duration of the last pre-operative antibiotic therapy and culture positivity. Also, no significant association was found between the duration of the last pre-operative antibiotic treatment and PCR positivity.

Antibiotic susceptibility tests were done for the all of the isolated bacteria. Among the *S. pneumonia* isolates, the numbers of strains with susceptible, and intermediate and complete resistance were as follows: ampicillin; 40%, 10% and 50%, respectively, amoxicillin; 40%, 20% and 40%, respectively, cefixim; 40%, 0%, and 60%, respectively, cefotaxim and ceftriaxone; 70%, 10% and 20%, respectively, and erythromycin; 90%, 0% and 10%, respectively. For ciprofloxacin 100% of the strains were susceptible. None of the strains was sensitive to co-trimoxazole. Among the *H. Influenzae* isolates, the numbers of strains with susceptible, intermediate or complete resistance were as follows: ciprofloxacin; 33%, 0% and 77%, respectively. For ceftriaxone and cefotaxim 100% of the strains were susceptible. None of the strains was sensitive to ampicillin, amoxicillin, cefixim, erythromycin, or co-trimoxazole. The sensitivity profile for *M. catarrhalis* isolates were as follows: 100% of the strains were susceptible to cefotaxim, cefixim and erythromycin. None of the strains was sensitive to ceftriaxone, ampicillin, amoxicillin, ciprofloxacin or co-trimoxazole.

Pseudomonas aeruginosa isolates were resistant to all of the above-mentioned antibiotics, except for ciprofloxacin. None of the *coagulase- negative staphilococcus* isolates was sensitive to co-trimoxazole, cloxacillin, oxacillin, erythromycin, ampicillin, amoxicillin or cefixim. Eighty six percent and 71% of the isolates were susceptible to ciprofloxacin and ceftriaxon, respectively. None of the *coagulase-positive staphilococcus* isolates (table 1) was sensitive to co-trimoxazole, oxacillin, ampicillin, amoxicillin or cefixim. The susceptibility of this pathogen to ciprofloxacin, ceftriaxon and cefotaxim was 100%, 60 % and 40%, respectively.

Discussion

The results of this study demonstrate that effusions from OME in children from the city of Shiraz were largely infected with bacteria. In the present study, DNAs of S. *pneumoniae, H. influenzae* and M. *catarrhalis* were detected in 60 (95.2%) of samples that were obtained under aseptic conditions. It probably represented those bacterial species in the effusions. This shows the extraordinary sensitivity of PCR. On the other hand in the standard bacterial culture method, only 22 (34.9%) of samples were positive for *S. pneumoniae, H. influenzae*and or *M. catarrhalis.*

In this study, DNA of *H. Influenza* was found in 95.2% of samples. This rate is well above the rate reported in other studies, which found a rate of 32-70% for *H. influenzae* DNA in effusions.[11-13,26] Since in all experiments, negative and positive controls were included in the assay system, the likelihood of false positivity is disregarded. The high percentage *of H. influenzae* in the present study may be due to the lack of *H. influenza* vaccination in our country. In a report by Post et al, it was shown that genetic material degraded two days following the death of bacteria.[9] Thus, it is likely that DNA detected in our study did also originate from live bacterial species.

Standard bacteriologic analysis of effusion from patients with OME has shown that in 21 to 52% of the cases various bacteria were present, and that the most frequently encountered causative bacteria were *S. pneumoniae, H. influenzae, M. catarrhalis* and coagulase-negative *Staphylococci*.[2,8] In this study the overall culture-positive samples were 60.3% that is higher than that of previous studies (21-52%). Of the culture-positive samples, 22 (34.9%) samples were positive for *S. pneumoniae, H. influenzae* or *M .catarrhalis.* The other bacterial isolates included coagulase-negative Staphylococci, coagulase-positive Staphylococci, *P. aeruginosa, S. aureus, viridans streptococci and a- hemolytic streptococci.*

In Izadparast et al. study in Shiraz (1998), the rate of samples positive for bacteria was only 19%, which is lower than that of the present study (60.3%). This may be due to the difference in time intervals between last antibiotic use and the operation (one week in their study *vs* two weeks in the present study). In our study antibiotic was discontinued in all patients at least two weeks prior to the surgery. Similar to our findings *S. pneumoniae* was the most common isolated pathogen, but antibiogram was not performed in their study.[23]

Antibiogram of the isolated bacteria was performed in our study. None of *S. pneumonia* isolates was sensitive to co-trimoxasole. Moreover, none of *H. influenza* isolates was sensitive to erythromycin, cefixim, ampicillin or amoxicillin. In addition, none of *M .catarrhalis* isolates was sensitive to ceftriaxone ciprofloxacin, ampicillin or amoxicillin.

Fahimzad and others investigated antibiotic susceptibility in *H. influenza type b* isolates in day care units in Tehran. Ampicillin resistance was detected in 32.3% of the isolates. Also 58.8% of the isolates were resistant to cefixim. Isolates resistant to azithromycin and clarithromycin were 19.6% and 35.3%, respectively.[27] In this study all isolates of *H. influenzae* were resistant to ampicillin, amoxicillin and cefixim. Also, none of the isolates was sensitive to erythromycin.

Previous studies,[7,19,20] did recommend amoxicillin as the first-line drug for the treatment of OM in the era of antibiotic-resistant organisms. Continuing treatment with amoxicillin or switching to an alternative antibiotic was based on clinical responses after 48 hours of treatment.[7,19,20] None of *H. influenzae* and *M. catarrhalis* isolates in the present study was sensitive to ampicillin or amoxicillin; however, only 40% of *S. pneumonia* isolates were sensitive. It is seems that these antibiotics are not a good choice for the initial treatment of OM in our area.

Slinger study showed that rifampin and ciprofloxacin combination were most effective against *H. influenza* biofilm. The biofilm of *H. influenza,* which may explain why OME did not respond well to antibiotic therapy, was demonstrated in OME.[28] Rifampin was not included in sensitivity profile of our study. Moreover, only 33% of *H. influenza* isolates were sensitive to ciprofloxacin.

There are different ideas about antibiotic prophylaxis in the literature.[21,22] Somehow, we found an association between the mean duration of the last antibiotic therapy and PCR or culture-negative results in the present study. However, this association did not reach statistical significance, which might be due to small size of the sample employed. Thus, a similar study with a larger sample size, which provides a better evaluation of antibiotic prophylaxis for the OME patients, especially in the cold seasons, is recommended.

Conclusion

The findings of bacteriological testing on samples from children with OME at our center are

different from those reported in the literature. H. i*nfluenzae* was found in 95.2% of the effusions, which is higher than the results of previous studies (32-70%). This difference may be due to lack of *H. influenzae* vaccination in our region. In this study, bacteriologic analysis of our effusion showed the presence of various bacteria in 60.3 % of all samples that is higher than results reported in the literatures (21-52%). Antibiotic sensitivity profiles of pathogens in this study were also different from those of others. Thus, we can recommend empirical antibiotic therapy based on the sensitivity profile in our geographic area. In our PCR assays, more than 40% of all specimen had mixed bacterial DNA; therefore, it is seems that amoxicillin, ampicillin and even cefixim alone are not good choices in our area. We recommend combination therapy comprising of macrolide plus cephalosporin in patients, who don't respond well to single initial antibiotic therapy. We also recommend further studies involving larger population to better evaluate antibiotic prophylaxis in cold seasons.

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Conflict of Interest: None declared

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