Comparison of Culture and PCR Methods in Detection of *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* in Children with Otitis Media with Effusion

Efüzyonlu Orta Kulak İltihabı Olan Çocuklarda *Haemophilus influenzae*, *Streptococcus pneumoniae* ve *Moraxella catarrhalis* Saptanmasında, Kültür ve PZR Yöntemlerinin Karşılaştırılması

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ABSTRACT Objective: The etiology and pathogenesis of otitis media with effusion (OME) is still unclear despite many studies within the last four decades. Polymerase chain reaction (PCR) based procedures are suggested for detection of the causative bacteria supposed to inflict multiple infections. In the current study, culture and PCR based approaches were used to detect the frequency of *Haemophilus influenzae, Streptococcus pneumoniae* and *Moraxella catarrhalis*, which have been known as common pathogens in middle ear effusions (MEE) of patients with otitis media. **Material and Methods:** The DNAs of these three bacteria were detected by standard and multiplex PCR techniques in MEE specimens and their diagnostic values were evaluated in comparison to the conventional culture method. **Results:** Samples from 67 OME suspected children were analysed retrospectively. Two H. influenzae and two *M. catarrhalis* isolates were recovered by conventional culture method (6.0%; 4/67). Out of the 67 samples, seven *S. pneumoniae*, nine *H. influenzae*, and eleven *M. catarrhalis* isolates were detected vith PCR. In five samples, two concurrent bacteria were detected in following combinations: two *S. pneumoniae* and *H. influenzae*, two *S. pneumoniae* and *M. catarrhalis*, and one *H. influenzae* and *M. catarrhalis*. Sensitivity, specificity, positive predictive value and negative predictive value rates of the PCR technique were 100.0%, 71.4%, 18.2% and 100.0%, respectively. The difference between culture and PCR was statistically significant ($p<0.001$). **Conclusion:** Although the specificity and positive predictive values are low, PCR, which allows rapid screening is feasible for detecting the most common fastidious bacteria that lead to OME.

Key Words: Otitis media with effusion; *streptococcus pneumoniae; haemophilus influenzae; moraxella catarrhalis;* polymerase chain reaction; culture

ÖZET Amaç: Efüzyonlu orta kulak iltihabının (EOKİ) etiyolojisi ve patogenezi, son 40 yıl boyunca yapılan çok sayıda çalışmaya rağmen hala çok açık değildir. Çoklu enfeksiyöz ajanların sorumlu olduğu düşünülen bu hastalıkların nedenini saptamak için polimeraz zincir reaksiyonu (PZR) temeline dayanan yöntemler önerilmektedir. Bu çalışmada, EOKİ şüpheli çocuklardan alınmış 67 orta kulak sıvısı (OKS) örneği incelendi. OKS'de yaygın patojenler olarak bilinen *H. influenzae, S. pneumoniae* ve *M. catarrhalis* oranlarını saptamak için kültür ve PZR teknikleri uygulandı. **Gereç ve Yöntemler:** Bu üç bakterinin DNA'ları OKS örneklerinde standart ve multipleks PZR yöntemleri ile saptandı ve bunların tanısal değerleri, altın standart olarak kabul edilen konvansiyonel kültür yöntemi ile karşılaştırmalı olarak değerlendirildi. **Bulgular:** Konvansiyonel kültür yöntemiyle iki *H. influenzae* ve iki *M. catarrhalis* kökeni izole edildi (%6,0, 4/67). PZR ile 7 *S. pneumoniae*, 9 *H. influenzae* ve 11 *M. catarrhalis* kökeni tespit edildi. Örneklerin 5'inde iki bakteri birden aşağıdaki kombinasyonlarda saptandı; 2 *S. pneumoniae* ve *H. influenzae*, 2 *S. pneumoniae* ve *M. catarrhalis*, 1 *H. influenzae* ve *M. catarrhalis*. PZR tekniklerinin duyarlılık, özgüllük, pozitif kestirici ve negatif kestirici değerleri sırasıyla, %100, %71,4, %18,2 ve %100 bulundu. PZR ve kültür arasındaki farkın istatistiksel olarak anlamlı olduğu saptandı ($p<0.001$). **Sonuç:** PZR yöntemleri, her ne kadar özgüllük ve pozitif kestirici değerleri düşükse olsa da, en azından EOKİ'ye neden olan, üretilmesi zor ve sık görülen bakterileri saptamada hızlı ve uygun tarama testleri olarak önerilebilir.

Anahtar Kelimeler: Efüzyonlu otitis media; *streptococous pneumonia; haemophilus influenza*; *moraxella catarrhalis*; polimeraz zincir reaksiyonu; kültür

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The pathogenesis of otitis media with effusion (OME) seems to be multifactorial. One of these factors is infectious agents and the most common microorganisms are bacteria, especially those with retrograde movement from the oropharynx into the middle ear cavity.[1] *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* are considered the major pathogens of OME.[2-4] Evidence on the presence of bacteria in a significant percentage of culture-sterile middle ear effusions suggests that current bacterial culture techniques cannot identify organisms in a considerable number of specimens.[5] Recently, polymerase chain reaction (PCR) was used to identify pathogenic steps in many diseases.[6] Currently PCR is the most common method in the diagnosis of bacteria that take part in the pathogenesis of OME.[7]

In this study, we aimed to detect the frequency of bacteria known as the most common pathogens in middle ear effusions (MEE) in patients with otitis media who underwent therapeutic myringotomy.

MATERIAL AND METHODS

The MEE specimens are routinely directed to the bacteriology laboratory from the Department of Otorhinolaryngology. This study included 67 samples that were cultured immediately upon arrival to the laboratory and were kept frozen at -80 °C for further analysis. Therefore, the results presented here reflect a retrospective investigation. The Ethics Committee of the Gülhane Military Medical Academy and Medical Faculty approved the research protocol and methods to be used throughout the study.

After disinfecting the external ear canal with povidone-iodine (betadine), paracentesis was performed. The effusion was immediately aspirated from the middle ear into a sterile tube before insertion of a ventilation tube. Specimens were delivered immediately to the bacteriology laboratory. An aliquot of the effusion was inoculated on sheep blood agar and chocolate agar media for conventional bacterial culture and the remaining was used for PCR testing as mentioned previously.

Standard microbiological culture methods were used for isolation of the pathogens.[8] Identification of the isolates was performed by using API NH and API 20 Strep panels according to the manufacturer's recommendations (Biomerioux, France). *H. influenzae* ATCC 49247, *S. pneumoniae* ATCC 49619, and *M. catarrhalis* ATCC 25238 were used as control strains. Molecular detection of the strains was performed by using multiplex PCR assay defined previously by Hendolin et al.[9] For the PCR analysis, 120 µl of each middle ear effusion specimen was incubated 1 h at 55°C in buffer K with 2% Sodium dodecyl sulfade (SDS) containing 20 µg/ml proteinaz K. DNA was extractred by phenol-chloroform method. The common lower primer used for this study was; 5′-CTA CGC ATT TCA CCG CTA CAC-3′. The specific upper primers were for *H. influenzae*: 5′-CGT ATT ATC GGA AGA TGA AAG TGC-3′ located at positions 177 to 200; for *M. catarrhalis*: 5′-CCC ATA AGC CCT GAC GTT AC-3′ located at positions 416 to 435; and for *S. pneumoniae*: 5′-AAG GTG CAC TTG CAT CAC TAC C-3′ located at positions 106 to 127. The amplification conditions were 3 minutes of initial denaturation prior to the addition of enzyme and 38 cycles of 94°C for 30 seconds, 66°C for 45 seconds, and 72°C for 1 minute, followed by a 5-minute final extension at 72°C. Then, 5 µl of the amplification products were subjected to 2% agarose gel electrophoresis to estimate their size by comparison with a 100-bp molecular size marker (Fermentas, USA). The gel was stained with ethidium bromide and amplicons were visualized using an ultraviolet light box (Gel Doc 2000, BioRad, USA).

STATISTICAL ANALYSIS

Mc Nemar test was used to compare conventional culture and PCR methods. $P<0.05$ was considered statistically significant.

RESULTS

Among 67 MEE samples, 2 *H. influenzae* and 2 *M. catarrhalis* were isolated by conventional culture method (6.0%; 4/67). On the other hand, PCR detected seven *S. pneumoniae*, nine *H. influenzae*, and eleven *M. catarrhalis* isolates; of these 3 *S.*

pneumoniae, 6 *H. influenzae*, and 8 *M. catarrhalis* isolates were monopathogens. In five samples, two concurrent bacteria were detected in following combinations: two *S. pneumoniae* and *H. influenzae,* two *S. pneumoniae* and *M. catarrhalis*, and one *H. influenzae* and *M. catarrhalis*. Results of conventional culture and PCR for 67 MEE samples from patients with OME were presented in Table 1 and the statistical analysis of the data was given in Table 2.

Sensitivity, specificity, positive predictive value and negative predictive value rates for PCR were 100.0%, 71.4%, 18.2% and 100.0%, respectively. The difference between culture and PCR was statistically significant (p<0.001).

DISCUSSION

Otitis media is an important worldwide health problem during childhood and brings a significant economic burden for the society.[10] Otitis media is generally considered a bacterial infection but bacterial pathogens cannot be easily isolated from MEE. In this study, we aimed to compare the PCR method with conventional culture in the detection of the most common bacterial pathogens of otitis media. The conventional culture method yielded two *H. influenzae* and two *M. catarrhalis* isolates (6.0%; 4/67). Some possible causes for low detection rates might be prolonged use of antibiotics before ventilation tube insertion, inhibition of bacteria by secretory immunoglobulin and lysozyme in the middle ear, presence of biofilm in the MEE,[7] or survival of bacteria in L-forms.[11] The effect of antibiotic use is controversial; Harimaya et al. reported that there was no difference in the frequency of bacterial isolation between the groups with and without antibiotic treatment. However, the number of specimens in that study was too small for statistical evaluation.[3] Hendolin et al. stated that only 20-30% of MEE specimens would yield positive results in ordinary cultures.[4]

TABLE 1: Frequencies bacteria in conventional culture and PCR assays.

Bacterial Species	Culture-positive (%)	PCR-positive (%)
H.influenzae	2/67 (3.0)	6/67 (9.0)
S.pneumoniae	0/67 (0)	3/67 (4.5)
M.catarrhalis	2/67 (3.0)	8/67 (12.0)
S.pneumoniae+M.catarrhalis	0/67 (0)	2/67 (3.0)
H.influenzae+M.catarrhalis	0/67 (0)	1/67 (1.5)
H.influenzae+S.pneumoniae	0/67 (0)	2/67 (3.0)
Total	4/67 (6.0)	22/67 (32.8)

TABLE 2: The statistical analysis of the data.

			Culture		
	PCR	Positive	Negative	Total	Statistics
OME	Positive	4	18	22	p< 0.0001
	Negative	0	45	45	
	Total	4	63		

OME, otitis media with effusion; PCR, polymerase chain reaction.

Since conventional culture methods fail to make clear the pathogenic cascade underlying OME, PCR has been used to detect bacterial DNA.[3] In addition, some studies reported that PCR technique was more specific and sensitive in detecting bacteria in MEEs.[6,7] In our study we detected 7 *S. pneumoniae*, 9 *H. influenzae*, 11 *M. catarrhalis* isolates with multiplex PCR. In other words, we detected 22 (32.8%) samples as positive in terms of these three pathogens by using PCR, whereas only 4 (6%) samples were positive by convensional culture methods. The difference between culture and PCR was statistically significant (p<0.001).

If we compare our results with other studies, up to 75% of the MEEs yield positive results for pathogenic bacteria by PCR.[9] In a study from Turkey conventional culture and bacterial DNA positivity rates for *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were 24.3% and 94.5%, respectively in 37 MEE samples.[11] The results of culture and PCR positivities for *S. pneumoniae* and *M. catarrhalis* in other similar studies were as follows; 21.3% and 70.2%,[12] 5.3% and 78.9%,[5] 32.0% and 84.0%,[3] 28.9% and 77.3%,[1] 13.6% and 36.4%,[13] 7.9% and 26.3%,[2] and 24.0% and 92.0%, respectively.[14] The prevalence rates for pathogens vary significantly in various studies. Geographic variations could effect the incidence of organisms.[15] Only the results of Jbara[13] and Harimaya's[2] studies were close to our results (6.0% and 32.8%, respectively). Sen-

sitivity, specificity, positive predictive value and negative predictive value rates for PCR were 100.0%, 71.4%, 18.2% and 100.0%, respectively. Jbara[13] reported similar rates-100.0%, 73.7%, 37.5% and 100.0%, respectively. Culture-positive and PCR-negative specimens were not reported in any study except for the report of Harimaya et al. where two MEE specimens were culture-positive and PCR-negative.[2] The findings of the present study and other studies suggest a high ability for PCR-based assays on detecting bacterial DNAs in culture-negative MEE specimens. This may be attributed to the ability of PCR to detect DNA from dead bacteria as well viable ones. This was shown in a study where antibiotic-sensitive bacteria instantly became culture negative after the initation of treatment, while the DNA remained amplifiable for up to three weeks postinfection.[16] However, the detection of bacterial genomic DNA by PCR-based assays is not proof of an active bacterial infection, therefore its clinical significance is currently unknown.[1] It will not be possible to understand the etiology of otitis media completely until the microbiology of bacterial pathogens is clearly characterized.[5]

The most common bacteria identified in MEE specimens are *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. Other potential pathogens found in the MEEs are coagulase-negative staphylococci,[15] *Streptococcus pyogenes,*[7] viruses[10] and *Alloiococcus otitidis,* which is detected solely in patients with OME.[9] In a study the detection rate of *Alloiococcus otitidis* was 60%, while the same rate was 26.3% for all three major pathogens.[2] We did not investigate such agents in this present study. This may be another reason of the low rate of positive results.

Although the specificity and positive predictive values are low, PCR, which allows rapid screening is feasible for detecting the most common fastidious bacteria that lead to OME. Further studies investigating all pathogenic bacteria in sufficient numbers of MEE specimens are needed to reveal their clinical role and the rates of bacteria. However, the first issue to be clarified is whether or not the bacterial DNA detected by PCR shows the presence of metabolically active bacteria.

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