

# Applicability of PCR-DGGE and 16S rDNA Sequencing for Microbiological Analysis of Otitis Media with Effusion

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## ABSTRACT

**Background:** The aim of the study was to analyze the performance of PCR-DGGE based assay and its applicability as a tool for the identification of bacteria in the middle ear of children with otitis media with effusion (OME). **Methods:** The middle ear effusions from 20 children with OME were analyzed both by bacterial culture and by 16S rDNA-gene-targeted PCR assay, DGGE fingerprinting and sequencing analysis. **Results:** In bacterial culture assay, only three middle ear effusions (15%) showed bacterial growth. None of the samples were positive for anaerobic culture. The PCR assay with 16S rDNA-gene-targeted universal primers was positive in 10 (50%) cases. The subsequent DGGE fingerprinting and 16S rDNA sequencing analysis revealed that the most commonly encountered bacteria in the middle ear effusions of children with OME are *Haemophilus influenzae*, *Alloicoccus otitidis* and *Bacteroides* spp. **Conclusions:** The present study demonstrated the applicability of PCR-DGGE based assay and 16S rDNA sequencing for analyzing of bacterial diversity in the middle ear effusion of children OME. The results of our study may contribute to a better understanding of the etiology of OME.

**Keywords:** Otitis Media with Effusion; 16S rDNA Targeted PCR; DGGE Fingerprinting

## 1. Introduction

Otitis media with effusion (OME) is defined as the presence of fluid in the middle ear cleft without clinical symptoms or signs of acute ear infection [1]. Although OME is one of the most common diseases of childhood, its etiology and pathogenesis are still under debate. OME was considered a sterile condition for a long time; however, recent studies have demonstrated bacterial growth in 21% to 70% of the middle ear effusions (MEE) [2-6]. The most frequently found pathogens are *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. Less frequently cultured aerobic bacteria have been *A. otitidis*, *S. aureus*, *S. epidermidis*, *S. pyogenes* and *Corynebacterium* spp. In some studies, different anaerobic bacteria have also been cultured from MEEs of OME patients, such as *Peptostreptococcus* spp., *Prevotella* spp., *Bacteroides* spp., *Fusobacterium* spp. and *Propionibacterium* spp. [4-6]. During the last decade, several studies have applied more sensitive molecular methods, most often PCR-based assays, for detection of middle ear pathogens in OME. The overall rate of PCR-positive effusions from patients with OME has been significantly higher than by conventional culture, varying from 46% to 100% [3,4,7,8]. The bacteria found by PCR-based assays are usually the same as listed above. The advantage of PCR-based assays over

conventional culture is their higher sensitivity and the possibility to detect fastidious and difficult to culture microorganisms, like intracellular microbes or those entrapped within the biofilm [9,10]. Compared to conventional culture, recovery rate of *A. otitidis*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, several anaerobes and various viruses is far superior by PCR [8,11,12].

However, the PCR-based assay is time consuming and requires prior knowledge of microbial composition on particular microbiota. PCR approach combined with denaturing gradient gel electrophoresis (PCR-DGGE) allows assessing the structure of complex biological systems using single step PCR. It enables to simultaneously detect broad range of different species of bacteria, as well as different strains of the single species, and is suggested for its rapidity and reliability for diseases of polymicrobial nature [13]. The general principle of DGGE is the separation of individual rRNA genes based on differences in chemical stability of genes. These methods separate multitemplate PCR products as bands on gels according to GC content, dependent on melting behaviors of the amplicons as they migrate through the gels. [14]. PCR-DGGE may be used for whole community analysis, or for the investigation of specific populations or groups within the sample. This method has been

widely used for analysing human faecal microbiota [15], monitoring dynamic changes in mixed bacterial populations over time [16], assessing the effect of antibiotic therapy, assessment of the microbial composition in mouth [17], stomach [18] and cerebrospinal fluid [19].

In the present study, the bacterial composition of MEEs of children with OME was investigated by means of PCR using 16S rRNA-targeted universal primers, followed by denaturing gradient gel electrophoresis (DGGE) and 16S rRNA sequencing analysis. The performance of PCR-DGGE based assay and its applicability as a tool for the identification of bacteria in the middle ear of children with OME was analyzed.

## 2. Material and Methods

### 2.1. Study Design and Diagnosis of OME

The study group consisted of 20 children (12 boys and 8 girls) with OME, who were referred to the Department of Otolaryngology, Tartu University Hospital, for tympanostomy tube insertion. The age of the patients ranged between 1 to 6 years, mean  $4.3 \pm 2.8$  years. The diagnosis of OME was made by finding effusion in the middle ear cleft, without symptoms and signs of acute infection. The diagnosis was supported with type B tympanogram and audiogram that indicated conductive hearing loss. The lack of response to medical treatment with oral antihistamines or topical decongestants for at least 3 months was an indication for ventilation tube insertion. OME was bilateral in all cases. Children with purulent MEE, with a systemic disease, those who received antibiotic treatment during the previous month were excluded. Informed consent from the parents of all children and approval from the local ethics committee were obtained for the use of the specimens.

MEEs were obtained by myringotomy under the general anesthesia with the help of an operating microscope. The ear canal was first cleaned and myringotomy was performed in the anteroinferior part of the tympanic membrane. A MEE sample was aspirated under sterile conditions with an electric suction device into a Tym-Tap collector (Juhn Tym-Tap, Xomed Inc., Jacksonville, Florida, USA). MEE was collected randomly from one of the middle ear clefts of the each child. Some amount of the fluid was removed by a sterile cotton probe, placed into the Stuart transport medium and taken to the Microbiology Laboratory for aerobic and anaerobic culture.

### 2.2. Bacteriological Analysis

The samples were seeded on a Columbia agar base supplemented with 7% horse blood and chocolate agar with Vitox supplement for aerobic bacteria, and on Wilkins-Chalgren agar for anaerobic bacteria. The plates were incubated at 37°C in a microaerobic or anaerobic

atmosphere for a maximum of one week. All the media and supplements were from Oxoid Ltd., UK.

### 2.3. DNA Extraction

DNA was extracted from all MEEs by the cetyltrimethylammonium bromide (CTAB) method with slight modifications [20]. All samples were first lyophilized using freeze dryer (Christian Martin Ltd., Germany). The lyophilized powder was mixed with 1 ml of lysis buffer (200 mM Tris-HCl (pH 8.0), 25 mM EDTA, 300 mM NaCl, 1.2% sodium dodecyl sulfate) and 20 µl of proteinase K (400 µg/ml) was added. The mixture was incubated at 37°C for 24 h. Thereafter, 200 µl of 5 M NaCl was added and samples were vortexed for few seconds; 160 µl of CTAB/NaCl solution was added, followed by incubation for 10 min at 65°C. The lysate was extracted with an equal volume of phenol-chloroform and precipitated with ethanol. A DNA pellet was collected by centrifugation, washed with 70% ethanol and finally resuspended in TE buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA).

### 2.4. PCR Amplification and DGGE Analysis

The PCR was performed in a reaction volume of 50 µl containing 10 mM deoxyribonucleotide triphosphate each, 1.25 U Taq polymerase (Invitrogen, USA), 10x reaction buffer, 10 µmol of the each primer and 200 ng (1 µl) of DNA solution. The set of primers (968-GC-f, 5'-AACGCGAAGAACCTTA-3'; and 1401-r, 5'-GGTGTGTACAAGACCC-3') was used to amplify V6 to V8 regions of the 16S rRNA gene [14,21]. The PCR mixture was subjected to 35 amplification cycles (30 s at 94°C, 20 s at 55°C, and 40 s at 72°C). DGGE analysis on PCR products was performed using a Dcode™ System apparatus (Bio-Rad, Hercules, CA). Polyacrylamide gels (8% w/v) acrylamide-bisacrylamide (37.5:1) in 0.5 x Tris-acetic acid-EDTA buffers with a denaturing gradient was prepared with a gradient mixer and Econopump (Bio-Rad). Gradients from 30% to 60% were employed for the separation of the products amplified.

### 2.5. Cloning of PCR Products

The PCR was performed with primers 8f (5'-CACGGC-GGATCCAGAGTTTGAT(C/T)(A/C)TGGCTCACAG-3') and 1501r (5'-GTGAAGCTTACGG(C/T)TACCTTGTT-ACGACTT-3') to amplify the bacterial 16S rRNA [15,21]. The PCR amplicons were purified and concentrated with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and cloned in *Escherichia coli* JM109 using the pGEM-T vector system (Promega, Madison, WI). Colonies for sequencing were selected according to the migration position of the PCR fragment of the clone in

DGGE in comparison with the fragments in the original DGGE profile. The plasmid DNA of the selected transformants was isolated using the QIAprep spin miniprep kit (Qiagen).

## 2.6. Sequence Analysis

Sequencing of the cloned PCR fragments was carried out using purified plasmid DNA and the sequencing primers SP6 and T7 (Promega). Sequencing reactions were performed with Sequenase sequencing kit (Amersham, Slough, UK) according to the manufacturer's instructions. The sequences were analyzed with automatic LI-COR DNA Sequencer 4000 L (Lincoln, USA) and corrected manually. Sequence alignment of the complementary strands was carried out using the DNASTAR SEQMAN program (Madison, USA). Similarity searches for the 16S rRNA gene sequences were performed in the GenBank database using BLAST algorithm.

## 2.7. Statistical Analysis and Calculation of Similarity Indices

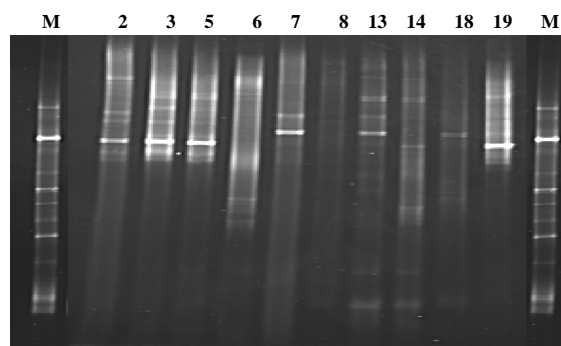
DGGE gels were scanned and analysed by using the software of Bionumerics 2.5 (Applied Maths, Belgium) [22]. The statistical analyses were performed using SigmaStat 2.0 (Jandel Scientific, USA) software programs, Chi-square test. The differences were considered statistically significant if the  $p < 0.05$ .

## 3. Results

A total of 20 MEE samples from children with OME were analyzed using bacteriological and 16S rDNA denaturing gradient gel electrophoresis (DGGE) followed by sequence analysis. The molecular analysis of MEEs was more sensitive than bacteriological (10 of 20 (50%) vs 3 of 20 (15%);  $p = 0.043$ ). *H. influenzae* was isolated in one and *M. catarrhalis* ( $\beta$ -lactamase positive) in two MEEs. All cultures for anaerobic bacteria were negative.

Following DGGE analysis of PCR amplicons revealed that each MEEs consisted of multiple 16S rDNA gene sequences, each assumed to represent a unique bacterial DNA (Figure 1). The number of clearly recognizable DNA fragments in DGGE profiles varied between 2 to 5, forming a unique DGGE profile for each MEE sample. In further analysis, the most dominating DNA fragments from DGGE profiles were isolated and sequenced. The obtained sequences were compared with the sequences in GenBank database (Table 1). The most common bacteria were *H. influenzae* and *Bacteroides* spp., each found in 4 MEE samples. *A. otitidis* was found in two samples (Table 1). Those three bacteria were predominating in all 10 PCR positive MEEs. Sequence analysis also revealed that MEEs may contain not only different species of bacteria but also different strains of single bacteria as in

case for *H. influenzae*, *A. otitidis* and *Bacteroides* spp. (patients 5, 6, 8 and 14).



**Figure 1.** PCR-DGGE profile of amplified V6-V8 regions of the 16S rDNA gene of middle ear effusions. Lane M—indicates the marker for DGGE, constructed from 16S rRNA amplicons; lanes 2, 3, 5, 6, 7, 8, 13, 14, 18, 19—DGGE profile of PCR positive patients.

**Table 1.** The results of sequence analysis of the dominant 16S rDNA gene amplicons found by PCR-DGGE fingerprinting.

No.	Age, gender	Cultivated bacteria	DGGE based sequencing/ similarity %
1	5 M	-	-
2	4 M	-	<i>H. influenzae</i> /99
3	5 M	-	<i>H. influenzae</i> /98
4	5 M	-	-
5	2 F	<i>H. influenzae</i>	<sup>1</sup> <i>H. influenzae</i> /99 <sup>2</sup> <i>H. influenzae</i> /96-99 <i>A. otitidis</i> <sup>1</sup> /96-99
6	3 M	-	<i>A. otitidis</i> <sup>2</sup> /96-99 <i>A. otitidis</i> <sup>3</sup> /95-99 <i>Alloiococcus sp.</i> /96-98
7	3 F	<i>M. catarrhalis</i>	<i>Bacteroides sp.</i> /96 <i>Acinetobacter lwoffii</i> /99
8	3 M	-	<i>Bacteroides sp.</i> <sup>1</sup> /97 <i>Bacteroides sp.</i> <sup>2</sup> /96
9	4 F	-	-
10	1 M	-	-
11	4 F	-	-
12	8 F	-	-
13	4 M	-	<i>Bacteroides sp.</i> /97
14	5 M	-	<i>A. otitidis</i> /99 <i>Lactobacillus sp.</i> /99
15	2 F	-	-
16	3 F	-	-
17	2 M	-	-
18	3 M	-	<i>Bacteroides sp.</i> /97
19	6 F	<i>M. catarrhalis</i>	<i>H. influenzae</i> /99
20	4 M	-	-

<sup>1</sup>Different strains of the species in the same patient are noted by numbers.

#### 4. Discussion

During the last decade, both conventional culture and PCR-based methods have widely been applied to assess the role of different microorganisms in the etiology of OME. The advantage of PCR-based methods over bacterial culture is the higher sensitivity and the possibility to detect fastidious and difficult to culture microorganisms. On the other hand, the positive culture is the only definite proof of the presence of viable bacteria, but PCR may indicate bacterial DNA rather than the bacteria itself. In the present study, the recovery rate of bacteria in MEEs of children with OME by culture was 15%, yielding *M. catarrhalis* and *H. influenzae* in one and two samples, respectively. These two microorganisms, together with *S. pneumoniae*, are among the most common infectious agents implicated in both AOM and OME [2,6]. The incidence of bacterial growth in the MEE of our patients was lower than in many previous studies, where the rate varies from 21% to 70% [2-6]. However, such lower rate of positive culture for OME has similarly been reported previously [8,23].

In accordance with other studies, the PCR with 16S rDNA targeted universal primers proved to be far more sensitive than conventional culture [2,3,7,8]. The presence of bacterial DNA was demonstrated 50% of MEEs of children with OME. However, the limitation of PCR-based assays is that they target only one or in some studies up to four pathogens simultaneously, and depend on prior knowledge of the microbial composition of the particular microbiota [7]. Moreover, the role of detected microorganisms in the etiology of OME is impossible to assess based solely on the colonization. Therefore, PCR amplicons were further analyzed by DGGE fingerprinting. DGGE is a technique which separates DNA fragments of the same length but with different base pair sequences, according to the point at which they denature. It also enables to assess the predominating microbes as the intensity of the fragments in DGGE corresponds semi-quantitatively with the abundance of the particular microorganism in the sample. We suggest that the predominating bacteria could be among those which are critical or even involved in the etiology of OME. The PCR-DGGE assay for detection of bacteria in MEE has not been used in previous studies. DGGE is the method of choice when the desired information does not have to be as phylogenetically exhaustive as that provided by cloning, but still relatively precise to determine the dominant members of a microbial community with medium phylogenetic resolution. The advantage of PCR-DGGE approach is that bands of interest can be excised and sequenced to obtain information about the species that they represent. The limitation of DGGE is that heterologous sequences may migrate similarly, and thus bands at the same position in the gel are not necessarily

phylogenetically related (13,15). However, application of sequencing of 16S rRNA quite solves this problem.

In the present study, PCR-DGGE fingerprinting demonstrated that each MEE from OME patients contained at least 2 to 5 predominating 16S rDNA gene sequences, each assumed to represent a unique bacterial DNA. Subsequent cloning and sequence analysis of DGGE fragments revealed that the most predominating bacteria in our patients with OME were *H. influenzae*, *A. otitidis* and *Bacteroides* spp. Those three bacteria were predominating in all 10 PCR positive MEEs. Moreover, sequence analysis also revealed that particular MEEs may contain not only different species of bacteria, but also different *H. influenzae*, *A. otitidis* and *Bacteroides* strains. The role of *H. influenzae*, *A. otitidis* and *Bacteroides* spp. in the etiology of OME is not completely understood. *H. influenzae* is a part of normal bacterial flora of the upper respiratory tract. Most *H. influenzae* strains are opportunistic pathogens, causing a disease only when host-dependent factors create an opportunity, such as in the case of AOM. The incidence of *H. influenzae* in OME has been from 4% to 22% by culture, and from 18% to 56% by PCR [2,6,7]. *A. otitidis* is another fastidious, slowly growing aerobic gram-positive diplococcus frequently encountered in the upper respiratory tract. The recovery rate of *A. otitidis* from MEEs of children with OME has been up to 5% by culture and up to 50% by PCR [7,8]. It was considered as an important middle ear pathogen after its first recovery from OME, but its etiological role in OME has been doubted in more recent studies [11,24]. *Bacteroides* spp. is anaerobic bacteria representing one of the most important groups of human commensals in the gastrointestinal, genitourinary and respiratory tracts [25]. *Bacteroides* spp. and other anaerobes could be recovered up to one-third of MEEs of OME patients [4,6]. Anaerobic bacteria have been associated with chronic and recurrent forms of otitis media [4]. All those three groups of bacteria are generally considered as the commensals in OME, which have possibly been translocated from the nasopharynx through the Eustachian tube to the middle ear. Although their exact role in the etiology of OME is unclear, their presence in the MME of OEM patients may have an impact on the treatment of once the acute recurrence of middle ear infection occurs. For example, *Haemophilus influenzae* and *Bacteroides* spp. strains are well-known beta-lactamase producers, which may help to survive pathogens in the same microbiota despite the presence of beta-lactams, the most commonly used antimicrobial agents in respiratory tract infections in childhood.

#### 5. Conclusion

The present study demonstrated the applicability of PCR-DGGE based assay and 16S rDNA sequencing for ana-

lyzing of bacterial diversity in the middle ear effusion of children OME. This method may have general usefulness in characterizing bacterial populations at the site of infection and may indicate microorganisms that are candidates for further investigation to gain a better understanding of the etiology of OME.

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