

Identification of *Alloiococcus otitidis*, *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae* in Children With Otitis Media With Effusion

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Background: Based on many studies, otitis media with effusion (OME) is one of the major causes of childhood hearing loss, social malformation and medical costs. The pathogenesis still remains unclear, though it is known that this complication is closely related to bacterial infections. *Alloiococcus otitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* are the most common bacterial pathogens isolated from middle ear effusions (MEEs).

Objectives: Due to the prevalence of OME in children, we decided to investigate bacterial agents that cause diseases such as *A. otitidis*, *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* in these subjects.

Patients and Methods: Forty-five children between one and 15 years of age were selected for this study. Seventy specimens were collected from MEE by myringotomy and inoculated in PBS buffer. Conventional culture and PCR methods were used for identification of bacterial agents.

Results: The bacterial cultures in 8.6% of samples were positive by conventional culture, with *A. otitidis*, *M. catarrhalis* and *S. pneumoniae* present in 1.4%, 2.9% and 4.3% of samples, respectively. No *H. influenzae* was isolated. By the PCR method, *A. otitidis* was the most frequently isolated bacterium, found in 25.7% of samples, followed by *S. pneumoniae*, *M. catarrhalis* and *H. influenzae*, which were identified in 20%, 12% and 20% of samples, respectively. Overall, 55 out of 70 samples were positive by both the PCR and culture method.

Conclusions: It can be concluded that *A. otitidis* was the major causative agent of MEE in children with OME. Therefore clinicians should be aware that bacterial infection plays an important role in the progression of acute otitis media to OME in children of our region.

Keywords: Otitis Media with Effusion; PCR; *Streptococcus pneumoniae*; *Haemophilus influenzae*; *Moraxella catarrhalis*

1. Background

The presence of fluid in the middle ear without symptoms or signs of infection is defined as otitis media with effusion (OME). Otitis media with effusion is different from acute otitis media because it is characterized by the presence of fluid in the middle ear accompanied by signs of acute inflammation of the middle ear (1). It is a disease caused by Gram-negative and Gram-positive bacterial pathogens in the middle ear of infants or children (2). In children, it is the most common cause of hearing loss and requires antibiotic therapy and surgical management. It can lead to significant side effects on hearing, and if these effects continue for a long period of time, they can cause lack of language development and speech in children (3). The mechanism of the pathogenesis of OME is not clearly understood. However, bacterial infection, Eustachian

tube dysfunction, allergy and immunological factors are known as major causes for this disease.

Many studies have reported detection of various species of bacterial agents in middle ear effusion; *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* have commonly been identified. The bacterial etiology of acute OME varies in different individuals, where *S. pneumoniae* is the most commonly found bacteria, present in 29-32% of cultures. *Moraxella catarrhalis* as a pathogen in otitis media has increased during the last two decades. In studies based on PCR, especially on patients with a negative culture, *Alloiococcus otitidis* also was detected in a considerable proportion (4). This has resulted in the consideration of bacterial infections as an important factor in the pathogenesis of OME. The intro-

duction of polymerase chain reactions (PCR) has resulted in a highly increased sensitivity and specificity of bacterial detection in middle ear effusion (5).

2. Objectives

Due to the prevalence of OME in children, we decided to investigate bacterial agents that cause diseases such as *A. otitidis*, *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* in these subjects.

3. Patients and Methods

Forty-five children between one and 15 years of age were selected for this study. The main reason for this selection was that the prevalence of this disease (OME) is highest in children. Bacteriological analysis was performed on samples taken by swabbing the middle ear cavity during a cochlear implant operation. From the patients' medical history, particular attention was paid to the following variables: gender, age, date of onset of OME, history of conditions such as allergies, respiratory tract infection, otitis media (acute, with effusion and recurrent acute), hearing level, and unilateral or bilateral hearing impairment. Next, the external auditory canal was cleansed by 70% alcohol for one minute and a myringotomy was conducted. Middle ear fluid has high viscosity, thus an amount of 0.5 mL of normal saline was inserted into the middle ear canal and the fluid was extracted by swabbing.

3.1. Isolation of Bacteria

Seventy middle-ear samples were collected from 45 children between one and 15 years old, who had been afflicted to OME. Each specimen was divided into two portions; one portion of the extracted middle ear fluid was transmitted into micro tubes with PBS buffer for ordinary bacterial culture and the other was stored at -70°C for the PCR assay. For primary isolation of bacteria, specimens were inoculated to several culture media under aerobic conditions with 5% CO₂ at 35°C for 24-72 hours according to Chapin. The following culture media were used: Muller Hinton with 5% sheep blood agar (*A. otitidis* and *S. pneumoniae*), chocolate agar with vancomycin (5 mg/mL), clindamycin (1 µg/mL) and bacitracin (300 µg/mL) for *H. influenzae*, chocolate agar with vancomycin (5 µg/mL), clindamycin (1 µg/mL) and bacitracin (300 µg/mL) and acetazolamide (for *M. catarrhalis*). Since *A. otitidis* has slow growth incubation was done for two weeks. Isolated bacteria were recognized by a conventional biochemical test (6-8). Thus Gram staining was performed. All media were incubated at 37°C, with the blood agar and chocolate agar plates in a microaerophilic atmosphere and Brucella agar in aerobiosis.

3.2. PCR Assay

The genomic DNA was extracted by High Pure PCR Tem-

plate preparation kit (Roche, Germany). Furthermore, PCR was done with DNA extracts using universal primers (Table 1). The specific primers that were used for this study included; RDRI25: ACTTTTG-GCGGTTACTCTAT and DG74: TGTGCCTAATTTACCAGDAT for *H. influenzae* (5, 9, 10), STR1: GATCCTCTAAATGATTCTCAGGTGG and DG74: ACTATAGAAGAAAGGG-AAGTTTCCA for *S. pneumoniae* (5, 9, 10), MCA: TTGGCTGTGCTAAAATATC and MCAT2: GTCATC-GCTATCATTACCT for *M. catarrhalis* (5, 9, 10), and mer21: CTACGCA-TTTCACCGCTACAC and mer20: GGGGAAGAA-CACGGATAGGA for *A. otitidis* (11-14). Initial denaturation at 95°C for five minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at (66°C for *S. pneumoniae* and *A. otitidis*, 55°C for *M. catarrhalis* and 52°C for *H. influenzae*) for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for seven minutes were carried out using a DNA thermal cycler eppendorf. Electrophoresis was used for 60 minutes in 2% agarose gel for the detection of amplified products. Specimens of this study with consistent PCR results were sequenced by the Bioneer Company (Korea) and used as positive controls, while distilled water was used as a negative control.

Table 1. Microorganisms Isolated in Otitis Media with Effusion by Culture and Polymerase Chain Reaction^a

Bacteria	PCR +, Culture +	PCR -, Culture -
<i>Alloiooccus otitidis</i>	1 (1.4)	17 (24.3)
<i>Haemophilus influenzae</i>	0	14 (20)
<i>Streptococcus pneumoniae</i>	2 (2.8)	12 (17.15)
<i>Moraxella catarrhalis</i>	3 (4.2)	6 (8.4)
Total positive (n = 55)	6 (8.4)	49 (70)

^a Data are presented as No. (%).

4. Results

All middle-ear samples were taken by aspiration during the surgery and were then analyzed. Based on the conventional method (culture and staining), polymorphonuclear leukocytes were not seen in any of the patients.

4.1. Results of Culture

Of the 70 samples that were obtained from 45 children with OME, the average age of onset was 4.5 years. Of those affected, 56% were boys and 44% girls. About 20 out of 45 patients (45%) with unilateral involvement and 25 out of 45 patients (55%) with bilateral involvement had samples positive for bacterial DNA. During surgery, the quality of the effusion was analyzed visually and was mucous in 32 (71.2%) children and serous in 13 (28.8%) patients. A total of 70 samples were taken from the 45 children, 25 cases were positive for bilateral presentation; it was possible to take a sample from both ears. When analyzing bilateral samples, the samples that were obtained from both sides were 100% similar. Among the 70 samples, bacterial cultures

were positive in six (13.4%) samples. The microorganisms that were isolated from cultures, included: *A. otitidis*, *M. catarrhalis* and *S. pneumoniae*. *Haemophilus influenzae* was not isolated from any of the subjects (Table 1).

4.2. Results of PCR Assay

As visualized by agarose gel electrophoresis of the PCR products, *A. otitidis* was the bacterium most frequently isolated; there were 18 positive samples amongst a total of 55 (25.7%), and bacterial DNAs related to *A. otitidis* were detected in 33% and 67% of unilaterally and bilateral affected patients, respectively. Hearing levels were normal in all patients. Amongst 12 patients with bilateral OME caused by *A. otitidis*, unilateral infection was caused by *H. influenzae* and *S. pneumoniae* in one and two cases, respectively. The distribution of bacterial agents isolated from patients with OME based clinical data; sex and age are summarized in Table 2. The PCR assay results showed that 55 samples were positive for bacterial DNA as tested by universal primers. In these samples, *A. otitidis*, *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* were detected (Figure 1).

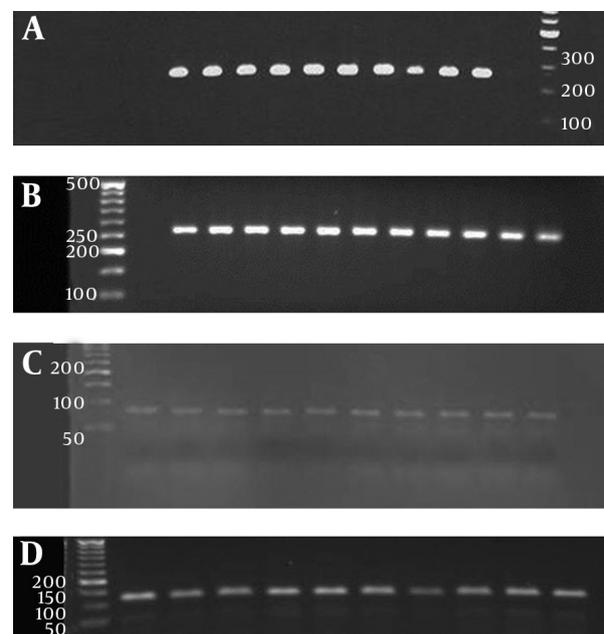
5. Discussion

In the pathogenesis of OME, bacterial infection has been known as an important factor, and many studies have demonstrated that *A. otitidis*, *M. catarrhalis*, *S. pneumoniae* and *H. influenzae* are the most common bacterial pathogens in this infection. It has been supposed that these bacteria are included in the normal flora of the middle ear canal (15). However, many previous studies have demonstrated that some of these bacteria, such as *A. otitidis* have immune-stimulatory ability (16) thus could not be part of the normal flora of this tract. Detection of these bacterial agents could be performed by PCR assay and ordinary bacterial culture. However, isolation of *A. otitidis* via conventional culture methods is difficult because it grows slowly and requires a special medium to grow in vitro (11).

The rate of isolation of these bacteria by standard culture does not yield more than 45%. Nonetheless, when the PCR method is used the isolation rate of these bacterial

agents is significantly increased and, *A. otitidis* is often the most common pathogen isolated (11, 16). Therefore, PCR is useful for the detection of pathogens that have slow growth rate, are difficult to culture in a diagnostic laboratory or require a special medium. Furthermore, PCR is the most functional technique for determination of the existence of pathogenic bacteria DNA in culture-sterile effusions of the middle ear. Thus these bacterial agents can easily be detected by the PCR assay (4, 16). We used both methods to study the bacterial etiology of OME in 70 children under 15 years old. The average age of onset was 4.5 years and the most common age of onset was less than five years.

Figure 1. Agarose Gel of Polymerase Chain Reaction Products Amplified by Species-Specific Primers of *Alloiooccus otitidis*



A, 1: 50 bp DNA Ladder 2: Negative control 3: Positive control 4-13: Samples, *Haemophilus influenzae*; B, 1: 100 bp DNA Ladder 2: Negative control 3: Positive control, 4-12: Samples, *Streptococcus pneumoniae*; C, 1: 50 bp DNA Ladder 2: Positive control 3-11: Samples 12: Negative control, *Moraxella catarrhalis*; D, 1: 50 bp DNA Ladder 2: Positive control 3-11: Samples 12: Negative control.

Table 2. The Frequency of Bacterial Agents Isolated from OME Based Sex and Age^a

Variables	<i>Alloiooccus otitidis</i>	<i>Haemophilus influenzae</i>	<i>Streptococcus pneumoniae</i>	<i>Moraxella catarrhalis</i>	Total Positive
Age, > 5 y	13 (73)	11 (78)	6 (43)	3 (37)	33 (47.1)
Age, ≤ 5 y	5 (27)	3 (22)	8 (57)	6 (63)	22 (31.4)
Male	12 (67)	6 (43)	8 (57)	7 (75)	33 (47.1)
Female	6 (33)	8 (57)	6 (43)	2 (25)	22 (31.4)
Unilateral	6 (33)	9 (64)	9 (64)	6 (67)	30 (54.6)
Bilateral	12 (67)	5 (36)	5 (36)	3 (33)	25 (43.4)
Total samples	18 (25.7)	14 (20)	14 (20)	9 (12.8)	55 (78.5)

^a Data are presented as No. (%).

In this study, using ordinary bacterial culture, bacteria were detected in only six (8.6%) cases, while using the PCR assay bacterial DNA was detected in 55 (78.6%) cases. This rate is lower than that reported by previous studies performed in Iran (17). In this study, the rate of culture positivity was 8.6% (6 out of 70 samples), while the rate of culture positivity that was reported by Khoramrooz et al. was about 47.6% (17); overall, our results were lower than other reports (4, 11, 16). *M. catarrhalis* was the most prevalent (4.3%) bacterial isolate among middle ear fluid samples that was detected by culture, however, our detection rate of this isolate was lower than that previously reported by Khoramrooz et al. (9.5%) (17) yet similar to reports from Lebanon (4%) (10). Furthermore, *A. otitidis* was isolated from 1.4% of samples while other studies from various regions have reported different isolation rates; Iran 23.8% (17), Spain 48.2% (11), Turkey 58% (18), and the United States 5% and 4.7% (7).

The third most isolated bacteria, by culture, from the middle ear fluid samples in this study, was *S. pneumoniae* (2.9%), which was isolated at lower rates than that reported by a similar study from Iran 9% (17), yet similar to a study from Spain 3.4% (19) and Brazil 12.5% (20). However, no *H. influenzae* was isolated using ordinary culture in this study. Furthermore, in this study we compared PCR with culture as a possible means of obtaining evidence of bacterial involvement in this study. According to the PCR method, which confirmed the results of the culture method, *A. otitidis* was the most prevalent (25.7%) bacterial isolate among the middle ear fluid samples. This isolation rate was lower than that reported by a similar study from Iran (17), but higher than that reported by some other studies (18.5%) (11) and lower than that reported from turkey 35% (18) and Japan 60.5% (13).

Using the PCR assay *M. catarrhalis*, *S. pneumoniae* and *H. influenzae* were detected in 12.8%, 20% and 20% of samples, respectively, and these results were somewhat similar to that reported by a study from Iran (17). The specificity and sensitivity of the PCR assay was higher than the culture method in detection of bacterial infection that caused OME. There is no reasonable description for this disagreement due to the difference in the distribution of these pathogens. In conclusion, based on this study, we accepted that *A. otitidis*, *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* are major bacterial pathogens in otitis media with effusion, where *A. otitidis* was the most often isolated bacterium in Iranian children with middle ear effusion. Many studies have suggested that the remaining bacteria due to an inadequate use of antibiotics may be the cause of OME. Thus bacterial infection plays an important role in the progression of acute otitis media to OME, which in turn causes hearing loss, social malformation and high medical costs. This is an important factor that must be considered while treating OME in children.

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Authors' Contributions

We hereby confirm that all authors participated in the research design and contributed to the research.

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