

Concurrent Assay for Four Bacterial Species Including *Alloiococcus Otitidis* in Middle Ear, Nasopharynx and Tonsils of Children with Otitis Media with Effusion: A Preliminary Report

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Objectives. To detect the prevalences of *Alloiococcus otitidis*, as well as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* in children with chronic otitis media with effusion (OME) and to simultaneously investigate the colonization of these bacteria in the nasopharynx and palatine tonsils of these patients.

Methods. The study included 34 pediatric patients with OME, and 15 controls without OME. In the study group, *A. otitidis*, *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* were investigated in the samples obtained from middle ear effusions (MEE), nasopharyngeal swabs (NPS) and tonsillar swabs (TS), using multiplex polymerase chain reaction (PCR) and conventional culture methods. Only the samples obtained from NPS and TS were studied with the same techniques in the control group.

Results. *A. otitidis* was isolated only in MEE and only with multiplex PCR method. *A. otitidis*, *S. pneumoniae*, *M. catarrhalis*, *H. influenzae* were identified in 35%, 8.8%, 8.8%, and 2.9%, respectively, in 34 MEE. *A. otitidis* was not isolated in NPS or TS of the study and the control groups.

Conclusion. The prevalence of *A. otitidis* is high in children with OME and *A. otitidis* doesn't colonize in the nasopharynx or tonsil.

Key Words. *Alloiococcus otitidis*, Otitis media with effusion, Polymerase chain reaction, Culture, Nasopharynx, Tonsil

INTRODUCTION

Chronic otitis media with effusion (OME) is one of the most common diseases of the childhood and it causes acquired hearing loss in children (1). OME is a chronic inflammatory condition and the etiology of inflammation is multifactorial, including vi-

ruses, allergy, bacteria and their products, as well as the dysfunction of the Eustachian tube (2). Inflammatory stimuli of infectious agents appear as the most important factor in the development of OME (3). Bacteria can be isolated from the middle ear effusion (MEE) in about 40% of OME patients with conventional culture methods. However, the isolation rate reaches 80% when a molecular method, such as multiplex polymerase chain reaction (PCR) is used (4). Up to date, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* have been reported as the most common pathogenic microorganisms in OME. Recently, a gram-positive bacterium, *Alloiococcus otitidis* has been described as an infectious agent that plays a role in OME and acute otitis media. Although identification of this bacterium is difficult in the middle ear effusion by conventional culture methods, *A. otitidis* was detected in a high proportion of

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children with OME and acute otitis media by the multiplex PCR method, and its isolation rate was even higher than the three major pathogenic agents *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* (5-10). *A. otitidis* and the other three pathogenic agents may colonize in the nasopharynx and tonsils of the OME patients. *A. otitidis* that colonizes in the tonsils and nasopharynx may cause otitis media with effusion.

In this study, we investigated the presence of *A. otitidis*, *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* in the nasopharynx, palatine tonsils and the middle ear effusion, simultaneously, using PCR and cultures in patients with OME. To our knowledge, no studies up to date have investigated, simultaneously, the presence of *A. otitidis* and other three pathogens in these regions of patients with OME and the colonization of *A. otitidis* in the palatine tonsils.

MATERIALS AND METHODS

Patients

The study included 34 pediatric patients with OME (19 females and 15 males aged between 3-16 years with a mean age of 8 ± 3.5 years) and 15 controls subjects. The controls consisted of 9 females and 6 males, without OME, and they were aged between 6-11 years, with a mean age of 8 ± 1.9 years. In addition, they were hospitalized for either septoplasty or frenuloplasty. The children were examined and had surgery in the Department of Otorhinolaryngology in Ankara Training and Research Hospital, in 2007 and 2008. The study was approved by the Ethic Committee of Ankara Training and Research Hospital (No: 0271). Informed consents were obtained from parents of all participating children.

Patients with an effusion lasting more than three months were included in this study. The presence of OME was verified by otomicroscopic examination and tympanometry. They had multiple episodes of infection and had been treated with beta-lactam antibiotics. Eighteen children had bilateral, while 16 children had unilateral disease. The patients did not have any history of recurrent tonsillitis. The control group consisted of 15 children, who did not have any ear disorders or upper respiratory tract infection. None of the children in either group had other known chronic conditions or immunological defects.

Samples

MEE samples were obtained by myringotomy, under general anesthesia. The ear canal was cleansed with 10% povidone-iodine before myringotomy. Myringotomy was performed in the anteroinferior part of the tympanic membrane. MEE was aspirated with a specially designed device. The wider side of a sterile 2 mL syringe was connected to the suction tube and an ear suction tube was connected to its thinner side. After the aspirate was collected in the syringe, its thinner side was occluded with an

injection pin and its wider side was occluded with a plug. MEE was recorded as mucoid or serous with regard to its physical appearance. Swabs were simultaneously obtained from the nasopharynx (nasopharyngeal swab, NPS) and the palatine tonsils (tonsillar swab, TS). The samples were transported to the microbiology laboratory, within a few hours after their collection to minimize the loss of fastidious microorganisms.

A. otitidis, *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* were investigated in MEE, NPS, and TS, using multiplex PCR method and conventional cultures in the study group. In patients with bilateral disease, the more effusion in the ear was preferred for MEE sampling. In the control group, only NPS and TS were obtained, while the patients were under general anesthesia, and they were studied with the same technique.

Multiplex PCR protocol

Samples obtained for PCR (half of each MEE sample and one of each swab specimen) were preserved in 1 mL TRIS EDTA buffer at -40°C , until the test was performed. Rest of MEE, along with the second NPS and TS samples were cultured on 5% sheep blood agar, chocolate agar and eosin methylene blue (EMB) agar plates. EMB agar plates were incubated at 37°C for 24 hours before they were evaluated. Sheep blood agar and chocolate agar plates were incubated in 5% CO_2 , at 35°C , for 48 hours before their first evaluation. Since *A. otitidis* is a slow growing bacterium, incubation was prolonged to seven days. Bacterial isolates were identified by standard laboratory procedures. All specimens stored, at -40°C for molecular tests, were processed by High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. The multiplex PCR protocol was performed, as previously described by Hendolin et al. (6). Test was set up in order to detect target DNA regions of *A. otitidis*, *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae*, simultaneously, using specific forward primers for those species, as well as a common lower primer. The multiplex-PCR mixture contained 1.6 μM *A. otitidis* primer, 1.4 μM *H. influenzae* primer, 0.2 μM *M. catarrhalis* primer, 0.04 μM *S. pneumoniae* primer, 0.4 μM common lower primer, 200 μM dNTP mix, 1X PCR buffer (10 mM Tris-HCl), 1.5 mM MgCl_2 and three U Taq Polymerase (Roche) in a reaction volume of 50 μM . The reaction profile was 3 minutes of initial denaturation and 38 cycles at 94°C for 30 seconds, 66°C for 45 seconds and 72°C for one minute, followed by an extension at 72°C for five minutes. The PCR products were separated in 2% agarose gel, which contained ethidium bromide, and visualized by UV light illumination.

Statistical analysis

Data were analyzed using SPSS ver. 11.5 (SPSS Inc., Chicago, IL, USA). Data were shown as mean \pm standard deviation for continuous variables, whereas, categorical data were expressed as the number of the cases and percent. The mean age difference

between the groups was compared using Student's *t*-test. Categorical data were evaluated with Pearson chi-square or Fisher's exact test, where appropriate. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Multiplex PCR results

The samples of 34 patients with OME (MEE, NPS, and TS) and the samples of 15 controls (NPS and TS) were analyzed.

A. otitidis was isolated only in MEE of the patients. It was isolated in 12 out of 34 (35%) patients. The bacterium was not isolated in NPS or TS of the patients or the controls. The mean age, gender or effusion type (serous or mucous) were not different with statistical significance between *A. otitidis* positive and negative cases ($P > 0.05$). Nine out of 12 (75%) *A. otitidis* positive patients and 20 out of 22 (90.9%) *A. otitidis* negative patients had adenoid hypertrophy. There was no statistically significant difference between these groups, with regard to the presence of adenoid hypertrophy ($P > 0.05$).

A. otitidis was identified in 12 (35%), *S. pneumoniae* was identified in 3 (8.8%), *M. catarrhalis* was identified in 3 (8.8%) and *H. influenzae* was identified in 1 (2.9%) out of 34 MEE. *A. otitidis* was identified alone in 7 out of 12 (58%) cases, whereas, it was identified together with one of the other three pathogenic microorganisms in 5 out of 12 (41.7%) cases. No pathogenic agents were isolated in 20 (59%) MEE. *S. pneumoniae* was de-

tected in 17 (50%), *M. catarrhalis* was detected in 4 (11.8%) and *H. influenzae* was detected in 1 (2.9%) out of 34 NPS. *S. pneumoniae* was detected in 16 (47%), *M. catarrhalis* was detected in 1 (2.9%), and *H. influenzae* was detected in 3 (8.8%) out of 34 TS, using the same method. The multiplex PCR method results of the study groups are shown in Table 1.

In the control group, *S. pneumoniae* was detected in 3 (20%) out of the 15 NPS and in 5 (33.3%) out of the 15 TS by multiplex PCR method. *A. otitidis* or the other two pathogens could not be identified. The results of the control group are shown in Table 2.

Conventional culture method results

A. otitidis was not identified in MEE, NPS or TS samples. Although no bacteria were isolated in MEE cultures, *S. pneumoniae* was isolated in 4 (11.8%), *M. catarrhalis* was isolated in 1 (2.9%) and *H. influenzae* was isolated in 2 (5.9%) out of 34 nasopharyngeal cultures. *S. pneumoniae* was isolated in 2 (5.9%) and *H. influenzae* was isolated in 1 (2.9%) out of 34 TS cultures. The culture results of the study group are shown in Table 1.

In the control group, *S. pneumoniae* was cultured in 2 (13.3%) out of 15 NPS and in 3 (20%) out of 15 TS. Neither *A. otitidis* nor the other two pathogens could be cultured. The results of the control group are shown in Table 2.

Although *A. otitidis* was identified in the MEE by multiplex PCR method, it could not be identified by conventional cultures. Additionally, it was not identified either in the NPS or TS, by either the multiplex PCR method or the conventional cultures. Although multiplex PCR method detected *A. otitidis* and/or one of the other major three pathogens in 14 (41%) out of 34 MEE samples, no pathogenic agents were identified with conventional culture methods.

Table 1. Frequency of the pathogens in the middle ear effusion (MEE), nasopharynx and tonsil by multiplex PCR and conventional culture methods in study group

	MEE	Nasopharynx	Tonsil
Multiplex PCR method			
Negative	20 (58.8)	15 (44.1)	15 (44.1)
<i>A. otitidis</i>	7 (20.6)	0 (0)	0 (0)
<i>A. otitidis</i> + <i>M. catarrhalis</i>	2 (5.8)	0 (0)	0 (0)
<i>A. otitidis</i> + <i>S. pneumoniae</i>	2 (5.9)	0 (0)	0 (0)
<i>A. otitidis</i> + <i>H. influenzae</i>	1 (2.9)	0 (0)	0 (0)
<i>M. catarrhalis</i>	1 (2.9)	1 (2.9)	0 (0)
<i>S. pneumoniae</i> + <i>M. catarrhalis</i>	0 (0)	3 (8.8)	1 (2.9)
<i>S. pneumoniae</i>	1 (2.9)	14 (41.2)	15 (44.1)
<i>H. influenzae</i>	0 (0)	1 (2.9)	3 (8.8)
Total	34 (100)	34 (100)	34 (100)
Conventional culture method			
Negative	0 (0)	27 (79.4)	31 (91.2)
<i>A. otitidis</i>	0 (0)	0 (0)	0 (0)
<i>H. influenzae</i>	0 (0)	2 (5.9)	1 (2.9)
<i>M. catarrhalis</i>	0 (0)	1 (2.9)	0 (0)
<i>S. pneumoniae</i>	0 (0)	3 (8.8)	2 (5.9)
<i>S. pneumoniae</i> + <i>H. influenzae</i>	0 (0)	1 (2.9)	0 (0)
Total	0 (0)	34 (100)	34 (100)

Values are presented as number (%).

Table 2. Frequency of the pathogens in the nasopharynx and tonsil by multiplex PCR and conventional culture methods in control group

	Nasopharynx	Tonsil
Multiplex PCR method		
Negative	12 (80)	10 (66.7)
<i>A. otitidis</i>	0 (0)	0 (0)
<i>S. pneumoniae</i>	3 (20)	5 (33.3)
<i>H. influenzae</i>	0 (0)	0 (0)
<i>M. catarrhalis</i>	0 (0)	0 (0)
Total	15 (100)	15 (100)
Conventional culture method		
Negative	13 (86.7)	12 (80)
<i>A. otitidis</i>	0 (0)	0 (0)
<i>S. pneumoniae</i>	2 (13.3)	3 (20)
<i>H. influenzae</i>	0 (0)	0 (0)
<i>M. catarrhalis</i>	0 (0)	0 (0)
Total	15 (100)	15 (100)

Values are presented as number (%).

DISCUSSION

OME is defined as the presence of fluid in the middle ear, without symptoms or signs of infection, and it is the most common cause of acquired hearing loss in childhood. Thus, it can result in developmental impairment of linguistic, behavioral, motor and social skills (1, 11, 12).

A. otitidis is first recovered from MEE of children with OME by Faden and Dryja (13) in 1989. This bacterium is a weakly catalase-positive, oxidase-negative and strictly aerobic coccus (4). Possible routes of entry of *A. otitidis* to the middle ear may be through nasopharynx or external ear in patients with perforated tympanic membranes. As well as nasopharynx and external ear, *A. otitidis* may originate from the upper respiratory tract, such as sinus, nasal cavity, tonsil, pharynx and oral cavity (9). It is difficult to detect *A. otitidis* in MEE with conventional culture methods, since it grows slowly and requires a special medium to grow (5). *A. otitidis* has been detected more frequently than the other middle ear pathogens by multiplex PCR (3, 6-8). Previous studies reported that *A. otitidis* was isolated in 18.5-64% of OME patients (3, 6-10) and in 25-50% of acute otitis media patients (5, 10, 14) with multiplex PCR methods. In the present study, the rate of its isolation was high in children with OME, with a rate of 35%, when studied with multiplex PCR. Further, it was identified more frequently than the other three major pathogenic agents. Our results are in accordance with the literature.

It has been hypothesized that *A. otitidis* was included in the normal flora of the middle ear cavity. However, previous *in vitro* and *in vivo* studies demonstrated that this bacterium had an immunostimulatory capacity, and this indicated that it was not included in the normal flora of the middle ear cavity (15-21). This suggestion was also supported by de Miguel Martinez et al. (12). In their study, *A. otitidis* was investigated in children with OME and acute otitis media, where the control group had a cochlear implant and did not have otitis for the previous 6 months. The bacterial analysis was performed by means of microbiological culture-specific techniques and the cultures presented negative results in all cases of the control group (12). In our study, *A. otitidis* was detected alone in 7 out of 12 (58%) cases and it was identified together with one of the other three pathogens in 5 out of 12 (41.7%) cases. We can speculate that *A. otitidis* is a pathogenic bacterium in the middle ear.

Up to date, apart from the middle ear, *A. otitidis* was investigated in the outer ear canal (4, 22-24), nasopharynx (4, 9, 22, 24) and maxillary sinus (25). It was most frequently identified in the ear canals of healthy adults (24). Some authors stated that *A. otitidis* was a commensal inhabitant and is a part of the normal bacterial flora of the human outer ear canal (4, 23, 24).

A. otitidis was investigated in the nasopharynx in a few studies. Durmaz et al. (22) studied 50 healthy children and identified the bacterium in the nasopharynxes of 4 children with multiplex PCR. Tano et al. (4) studied 129 patients with upper respi-

ratory tract infection without OME and identified *A. otitidis* in the nasopharynxes out of 9 children, using multiplex PCR. Harimaya et al. (9) showed that nasopharyngeal colonization of *A. otitidis* increased in otitis-prone children, when compared to those in non-otitis-prone children. Contrary to aforementioned studies, De Baere et al. (24) showed the absence of the bacterium in nasopharynxes of 70 healthy adult volunteers. Our study showed that *A. otitidis* was not present in the nasopharynxes of children with OME. However, we detected the bacterium in MEE in 35% of them with multiplex PCR method. Additionally, we did not detect the bacterium in nasopharynxes of the control group. These findings suggest that *A. otitidis* does not colonize in the nasopharynx, and is not included in the normal flora of this region. The discrepancies between the results of this study and the above mentioned studies may depend on different geographic locations and variations in the study protocols, and inclusion criteria among the studies.

To the best of our knowledge, no studies up to date investigated the colonization of *A. otitidis* in the palatine tonsils of children with OME. We did not identify *A. otitidis* in the tonsils of study and the control groups.

PCR is a technique that allows for the sensitive and specific detection of bacteria. It is useful for the detection of pathogens that are slowly growing, difficult to culture, or hazardous to handle in a diagnostic laboratory. PCR has been used to improve the sensitivity of bacterial detection (26) and to demonstrate the existence of pathogenic bacterial DNA in culture-sterile effusions in the middle ear from pediatric patients (27). There were some clinical and experimental studies, which suggest that viable, intact bacteria were required for the detection of bacterial DNA by PCR-based assays (10, 28).

Some authors showed that multiplex PCR methods were more useful than that of the cultures for identifying *A. Otitidis* (6-8). In accordance with these studies, we observed in our study that multiplex PCR was more useful than that of the conventional cultures for detecting *A. otitidis* and other three major pathogens.

In Leskinen et al. (7)'s study, *A. otitidis* was detected more frequently with greater significance in mucous effusions, which represent a more chronic stage of otitis media, when compared to mucoserous effusions. However, we did not find any relationship between the presence of *A. otitidis* and the effusion type, in our study.

de Miguel Martinez and Macias (12) reported that *A. otitidis* was sensitive to ampicillin and cefotaxime, but it was resistant to macrolides and cotrimoxazol. Harimaya et al. (5) reported that *A. otitidis* was frequently detected in patients who received a beta-lactam antibiotics or erythromycin. In our study, although all cases had received beta-lactam antibiotics for more than three months, *A. otitidis* was identified in 35% of the patients with OME. In light of these findings, we supposed that *A. otitidis* might be resistant to beta-lactams.

In conclusion, we showed that *A. otitidis* was frequently iso-

lated in MEE of children with OME, when studied with multiplex PCR. However, it could not be identified in the nasopharynx or tonsils of the study and either with the control groups with multiplex PCR or conventional cultures. This study results showed that the prevalence of *A. otitidis* was high in children with OME. Our results also revealed that *A. otitidis* did not colonize in the nasopharynx or in the tonsil, and it was not included in the normal flora of these regions. New, larger studies are needed to further clarify these issues.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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