



Frequency of *Alloicoccus otitidis*, *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae* in children with otitis media with effusion (OME) in Iranian patients

Seyed Sajjad Khoramrooz^a, Akbar Mirsalehian^{a,*}, Mohammad Emaneini^a, Fereshteh Jabalameli^a, Marzieh Aligholi^a, Babak Saedi^b, Abdollah Bazargani^c, Morovat Taherikalani^d, Pedram Borghaei^e, Ebrahim Razmpa^b

^a Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

^b Otorhinolaryngology Research Centre, Department of Otolaryngology – Head and Neck Surgery, Imam Khomeini Complex Hospital, Tehran University of Medical Sciences, Tehran, Iran

^c Department of Bacteriology and Virology, Shiraz Medical School, Shiraz University of Medical Sciences, Shiraz, Iran

^d Department of Microbiology, School of Medicine, Ilam University of Medical Sciences, Ilam, Iran

^e Department of Otolaryngology – Head and Neck Surgery, Amir-A'lam Hospital, Tehran University of Medical Sciences, Tehran, Iran

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Abstract

Objective: To determine the presence of common bacterial agents of otitis media with effusion (OME), together with investigation these agent in the adenoid tissue and antimicrobial susceptibility pattern of isolated bacteria in Iranian children with OME.

Methods: Polymerase chain reaction (PCR) and bacterial culture methods were used for detection and isolation of *Alloicoccus otitidis*, *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae* in 63 middle ear fluid samples and 48 adenoid tissues from 48 OME patients. Fifteen patients were bilaterally affected. Antimicrobial susceptibility of all bacterial isolates were determined by disk agar diffusion (DAD) method.

Results: Bacteria were isolated from 47% ($n = 30$) of the middle ear fluid samples and 79% ($n = 38$) of the adenoid tissue specimens in OME patients. *A. otitidis* was the most common bacterial isolated from the middle ear fluid 23.8% by culture and 36.5% by PCR method. *S. pneumoniae* was the most prevalent pathogen (35.5% and 31.2% by culture and PCR) in the adenoid tissues. In 10 patients the same organisms were isolated from the middle ear fluid and adenoid tissue. Antimicrobial susceptibility pattern showed that most isolates of bacteria were sensitive to ampicillin, Amoxicillin/Clavulanate and fluoroquinolones.

Conclusion: The present study, being the first report on the isolation of *A. otitidis* by culture method in Iran and Asian countries, shows that *A. otitidis* is the most frequently isolated bacterium in Iranian children having otitis media with effusion. In this study *A. otitidis*, *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* are the major bacterial pathogens in patients with OME and we found that ampicillin and Amoxicillin/Clavulanate have the excellent activity against bacterial agents in Iranian children with OME.

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Keywords: Otitis media with effusion; *Alloicoccus otitidis*; *Streptococcus pneumoniae*; *Moraxella catarrhalis*; *Haemophilus influenzae*

1. Introduction

Otitis media is one of the most common childhood diseases and it is the main cause of several otological problems [1]. Otitis media with effusion (OME) and acute otitis media (AOM) are the two major sub-classifications of otitis media

* Corresponding author at: Microbiology Department, School of Medicine, Tehran University of Medical Sciences, 100 Poursina St., Keshavarz Blvd., Tehran, Iran. Tel.: +98 21 8895 5810; fax: +98 21 8895 5810.

E-mail address: mirsaleh@sina.tums.ac.ir (A. Mirsalehian).

[2]. OME is characterized by the presence of middle ear fluid without acute infection [3] and its prevalence among children is approximately 20% [4]. The peak incidence of OME occurs at the first year of age [5]. Two weeks after the beginning of otitis media, about 70% of children have fluid in the middle ear and after one month it is decreased to 40%. Within 3 months after the first signs of infection still 10% of the children have fluid in the middle ear [6]. The effect of OME on hearing loss [3] also has a negative effect on the development of language in the first 3 years of age [7]. Although the etiology of OME is still unclear, bacterial and viral infections have an important role in its pathogenesis [8]. It has been shown that bacterial agents are present in 22–52% of OME cases [9]. *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis*, isolated by culture, are the predominant bacterial pathogens of OME [10]. *Alloicoccus otitidis* (formerly *Alloicoccus otitis*) isolated for the first time by Faden and Dryja [11] and then by biochemical test and genetic analysis, was proposed as a new genus [12] and later; its name was revised to *A. otitidis* [13]. *A. otitidis* is a slow growing and fastidious organism, so it is difficult to isolate this microorganism by conventional culture [14]. However by PCR, *A. otitidis* was detected in about 18.5–60.5% of OME patients, which was more than the cases detected for the three major pathogens [15]. Some studies have suggested the association of otitis media with chronic adenoidal infection, through the transmission of bacteria from adenoidal infection via the Eustachian tubes into the middle ear [16,17]. It is demonstrated that adenoid tissue has an important role in the pathophysiology of OM [16]. Paradise et al. reported the role of adenoidectomy in the treatment of children with recurrent OME who have previously had tympanostomy tubes placement [18]. It was shown that pathogenic bacteria are more frequently isolated from the adenoid tissue of patients with recurrent or persistent otitis, compared with patients with adenoid hypertrophy alone [19]. Karlidag et al. demonstrated a similarity between isolated bacteria in the middle ear effusion and the cultured bacteria in the adenoid tissue [20]. There is no information about the etiology of bacterial agents in children with OME in Iran. The isolation of *A. otitidis*, one of the major agents of OME, has also not been reported in Asian countries. The aim of the current study was, therefore, to determine the common bacterial agents and their susceptibility pattern among patients with OME.

2. Materials and methods

2.1. Patients and sample collection

Two kinds of clinical samples were collected: Middle ear fluid ($n = 63$) and adenoid tissue ($n = 48$) obtained from 48 patients with persistent middle ear effusion without symptoms of acute otitis media. Fifteen patients were bilaterally affected whereas 33 children had unilateral disease, hence both right and left middle ear fluid were

collected. Collected samples were from patients who attended the Department of Otolaryngology of Imam Khomeini and Amir Alam, two teaching hospitals in Tehran University of Medical Sciences, during September 2009–November 2010.

Inclusion criteria for myringotomy and insertion of a ventilation tube in OME patients were the presence of middle ear effusion for more than 3 months and not being on antibiotic therapy 2 weeks before and at the time of the surgery. In addition, all of these patients had adenoid hypertrophy and they were candidate for adenoidectomy. Children with previous transtympanic ventilation tubes, tympanic membrane perforations, previous adenoidectomy, immunological defect, anatomic abnormality, respiratory tract infection and purulent middle ear fluid were excluded.

Before surgical procedure and specimen collection, the external ear canal was disinfected with povidone-Iodine for 2 min and then washed three times with sterile normal saline for eliminating the antiseptic agent. After myringotomy, middle ear fluid was aspirated into a Juhn-Tym-Tap collector (Xomed Inc., Jacksonville, USA). In bilateral cases, both middle ear fluid of the right and left ears were collected. The adenoid tissue samples obtained after adenoidectomy were placed in a sterile container. All of the clinical samples were sent to the Laboratory of Microbiology Department within two hours. Prior to sample collection, written informed consents were obtained from parents of each individual. The present study was approved by the University Ethics Committee. Past medical histories and demographic data of patients were collected from their medical records, prior to surgery.

2.2. Isolation of bacteria

Each specimen was divided into two portions; one for culture and the other for Multiplex 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 h according to Chapin, Vaneechoutte and Bosley methods [21–23]. The culture medias were as follows: Muller Hinton with 5% Sheep blood agar (for *S. pneumoniae* and *A. otitidis*), chocolate agar with vancomycin (5 µg/ml), clindamycin (1 µg/ml) and bacitracin (300 µg/ml) for *H. influenzae* [21], chocolate agar with vancomycin (5 µg/ml), clindamycin (1 µg/ml) and bacitracin (300 µg/ml) and acetazolamide (for *M. catarrhalis*) [22]. Because of the slow growth of *A. otitidis*, incubation was extended to 14 days [23]. Isolated bacteria were identified by conventional biochemical methods [24].

2.3. Multiplex-PCR

Bacterial DNA was extracted using a RTP® Bacteria DNA Mini Kit (Invitek GmbH, Berlin, and Germany) according to the manufacturer's instructions. DNA was extracted from adenoid tissue according to Bartlett and Stirling method [25]. Multiplex PCR amplification of 16S

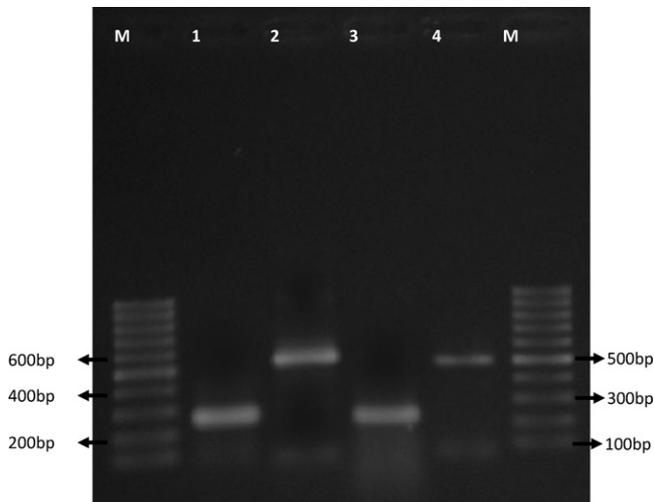


Fig. 1. Detection of *A. otitidis*, *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* by multiplex PCR. The sizes of PCR products are: 1- *A. otitidis* (264 bp), 2- *H. influenzae* (525 bp) 3- *M. catarrhalis* (237 bp) and 4- *S. pneumoniae* (484 bp). M stands for molecular size marker.

rRNA genes was done with specific primers according to Hendolin et al. method [26]. Polymerase chain reaction was carried out to a total volume of 50 μ l containing 5 μ l of DNA extracted from bacterial suspensions or adenoid tissues, 5 pmol of each primer, 1 \times PCR buffer, 5 mM MgCl₂, 100 μ l each of the deoxy-nucleotide triphosphate (Fermentas) and 1.5 U of Taq polymerase (Fermentas). PCR conditions were as follows: an initial denaturing at 94 °C for 5 min, followed by 35 cycles denaturing at 94 °C for 30 s, annealing at 60 °C for 45 s and extension at 72 °C for 40 s. The final extension was continued at 72 °C for 5 min. The multiplex PCR products were detected by electrophoresis of 20 μ l of each amplification mixture in 1.5% agarose gels. Gels were stained with ethidium bromide and then visualized by UV light illumination Fig. 1. For confirmation of *A. otitidis*, sequences of 16S rRNA of all isolates were aligned in NCBI.

2.4. Antimicrobial sensitivity

The disk agar diffusion (DAD) method was used to determine the susceptibility patterns among the strains, according to the CLSI (Clinical and Laboratory Standards

Table 1
Clinical characteristics of study subject $n = 48$.

Age ≤ 6	23
Age > 6	25
Age (mean)	7.01
Male	34
Female	14
Persistence of OME	
<3months	0
>3months	48
Previous adenotomy	0
Previous tympanotomy	0
Effusion type	
Mucous	24
Mucoserous	10
Serous	14
Antibiotic use	
At the time of Sampling	0
2 weeks ago	0

Institute) guidelines [27]. Mueller Hinton Agar with 5% Sheep Blood was used for *S. pneumoniae* and *A. otitidis*. The sensitivity of *H. influenzae* and *M. catarrhalis* were tested on Haemophilus Test Medium (HTM). Antibiotics used in this study are shown in Table 4.

3. Results

3.1. Clinical characteristics of the study groups

The clinical characteristics of patients from 1.7 to 12 years included in this study are shown in Table 1.

3.2. Determination of bacterial frequencies by culture and multiplex-PCR

The results of the culture and multiplex PCR are shown in Table 2. Bacteria were isolated by culture from 47% ($n = 30$) of the middle ear fluid samples ($n = 63$) and 79% ($n = 38$) of the adenoid specimens ($n = 48$). The most common bacteria isolated from the middle ear fluid were *A. otitidis* being 23.8% ($n = 15$), *S. pneumoniae* 9% ($n = 6$), *M. catarrhalis* 9% ($n = 6$) and *H. influenzae* being 4% ($n = 3$) respectively. However by PCR method, *A. otitidis* with 39.7% ($n = 25$), *H. influenzae* with 11%

Table 2
Isolation and detection of bacterial pathogen from clinical samples.

Pathogen	Middle ear fluid ($n = 63$)				Adenoid of OME patients ($n = 48$)			
	Culture + PCR -	PCR + Culture -	Culture + PCR +	Total	Culture + PCR -	PCR + Culture -	Culture + PCR +	Total
<i>A. otitidis</i>	6(9.5%)	16(25.4%)	9(14.3%)	31(49.2%)	0(0%)	0(0%)	0(0%)	0(0%)
<i>S. pneumoniae</i>	1(1.6%)	2(3.17%)	5(7.9%)	8(12.7%)	6(12.5%)	4(8.3%)	11(22.9%)	21(43.7%)
<i>H. influenzae</i>	2(3.17%)	6(9.5%)	1(1.6%)	9(14.3%)	9(18.7%)	8(16.6%)	6(12.5%)	23(47.9%)
<i>M. catarrhalis</i>	2(3.17%)	2(3.17%)	4(6.34%)	8(12.7%)	1(2.5%)	2(2.9%)	5(7.9%)	8(16.6%)

Table 3

Frequencies of bacterial isolated from two groups of patients (age ≤ 6 and age > 6).

Bacteria	Middle ear fluids		Adenoid of OME patients	
	age ≤ 6	age > 6	age ≤ 6	age > 6
<i>A. otitidis</i>	7	8	0	0
<i>S. pneumoniae</i>	4	2	8	9
<i>H. influenzae</i>	1	2	6	9
<i>M. catarrhalis</i>	3	3	3	3

($n = 7$), *S. pneumoniae* with 11% ($n = 7$) and *M. catarrhalis* with 9% ($n = 6$) were detected as the most prevalent strains of the middle ear fluid samples.

In the adenoid tissue of patients, isolated microorganisms by culture were as follows: *S. pneumoniae* ($n = 17$), *H. influenzae* ($n = 15$), *M. catarrhalis* ($n = 6$); multiplex-PCR detected the following microorganisms: *S. pneumoniae* ($n = 15$), *H. influenzae* ($n = 14$), and *M. catarrhalis* ($n = 7$). The frequencies of all isolated microorganisms in the two age groups (≤ 6 years and > 6 years) showed in Table 3. In cultures positive of middle ear fluid of patient's ≤ 6 years, 7, 4, 1 and 3 and also in adenoid tissue cultures 0, 8, 6 and 3 grew *A. otitidis*, *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* respectively. There were no significant differences in the prevalence of all bacteria from middle ear fluids and adenoid tissues between two groups of patients; age ≤ 6 and age > 6 years. Pure cultures were detected for 28 of the middle ear fluid samples and the growth of more than one organism was observed in only one specimen. In 10 cases of the 48 OME patients, the same organisms were isolated from the adenoid tissue and middle ear fluid. 4 out of 15 patients that bilaterally affected, the same bacteria [*M. catarrhalis* (2 cases), *S. pneumoniae* (1 case) and *H. influenzae* (1 case)] were isolated from both sides of ears and adenoid.

Table 4

Resistance pattern of bacterial isolates.

	<i>A. otitidis</i> n = 15 R	<i>S. pneumoniae</i> n = 23 R	<i>H. influenzae</i> n = 18 R	<i>M. catarrhalis</i> n = 12 R
Penicillin	2(13%)	6(26%)	–	–
Vancomycin	–	0(0%)	–	–
Linezolid	–	0(0%)	6(33%)	–
Ampicillin	0(0%)	0(0%)	0(0%)	0(0%)
Amoxicillin/Clavulanate	0(0%)	0(0%)	3(16.6%)	0(0%)
Erythromycin	13(86%)	11(48%)	3(16.6%)	0(0%)
Clarithromycin	13(86%)	13(56%)	3(16.6%)	0(0%)
Azithromycin	13(86%)	13(56%)	3(16.6%)	0(0%)
Clindamycin	–	8(35%)	–	–
Cotrimoxazole	8(53%)	23(100%)	2(11%)	12(100%)
Ciprofloxacin	0(0%)	0(0%)	1(5.5%)	0(0%)
Levofloxacin	0(0%)	0(0%)	1(5.5%)	0(0%)
Rifampicin	0(0%)	–	4(22.2%)	3(25%)
Ceftriaxone	0(0%)	3(13%)	–	–
Cefepime	5(33%)	–	–	–

3.3. Antimicrobial susceptibility of bacterial isolates

The susceptibility patterns of 68 bacterial isolates are summarized in Table 4. All of the *A. otitidis* isolates were susceptible to vancomycin, ampicillin, amoxicillin/clavunic acid, but more than 86% were resistance to macrolides. High rates of resistance to cotrimoxazole (100%), clarithromycin and azithromycin (59%) were observed among *S. pneumoniae*. The highest susceptibility rates among *H. influenzae* strains were against amoxicillin/clavunic acid (100%), ciprofloxacin and levofloxacin (94.5%). *M. catarrhalis* isolates were most susceptible against flouroquinolones, ampicillin, amoxicillin/clavunic acid and macrolides.

4. Discussion

The main purpose of this study was to investigate the etiology of bacterial pathogens and their susceptibility in OME patients in Tehran. In this study, *A. otitidis* was the most prevalent (23.8%) bacterial isolates among middle ear fluid samples by culture, which is lower than similar reports from Australia (40%) [28] and Spain (48.2%) (14) but higher than that reported from the United States (5% and 4.7%) [11,23]. The prevalence of *A. otitidis* differs noticeably between different countries which may reflect the role of geographic regions in *A. otitidis* incidence rates. There are no data about the isolation of this bacterium in Iran's neighboring countries for comparison. According to the PCR method, as also detected by the culture method, *A. otitidis* was the most prevalent (39.7%) bacterial isolate among the middle ear fluid samples in this study. This result is almost in agreement with a similar report from Finland [29] but is higher than the 18.5%–28.1% reported by some other studies [8,15,20,26,28,30] and lower than that reported from the United Kingdom 50% [31] and Japan 60.5% [1]. There are differences between the detection rates of *A.*

otitidis in some studies which may be related to the etiology of OME and different geographic regions.

The second most isolated bacteria, by culture, from the middle ear fluid samples in this study, was *S. pneumoniae* (9%) which is higher than that reported in a similar study from Spain 3.4% [14] and lower than that in a report from Brazil 12.5% [32]. *S. pneumoniae* was detected in 11% of the middle ear fluid samples by PCR method which was not particularly in agreement with the value reported from other studies: Finland 20.9% and 46.3% [26,29], Lebanon 6.3% [33] and Japan 7.9% [15]. *M. catarrhalis* was isolated in 9% of the middle ear fluid samples whereas Matar et al. from Lebanon isolated *M. catarrhalis* in 4% of the patients in their study [33]. *M. catarrhalis* was detected in 9% of the OME patients by PCR method. This value is lower than what was reported by studies from Finland 37.3% [29] and Lebanon 21% [33] whilst is higher than that reported in a study from Japan 6.6% [15]. In contrast to the results of this study indicating *H. influenzae* in 4% of the OME patients, Matar et al. from Lebanon isolated *H. influenzae* from 19% of the patients in their study [33]. *H. influenzae* was detected in 11% of the patients by PCR method in this study which shows a different result from that detected by PCR in studies from Finland 17.9% [29] and 52% [29], Lebanon 70% [33] and Japan 7.9% [15].

The bacterial pathogens mentioned, have important roles in the etiology of OME; however, there are differences between the prevalence rates of these pathogens in different studies. Variation in the prevalence rates of these pathogens maybe related to the different health care programs in various geographical locations.

The existence of four major bacterial pathogens in OME was evaluated by culture and PCR method in the adenoid of patients with OME. *S. pneumoniae* (35% and 31%), *H. influenzae* (31% and 29%) and *M. catarrhalis* (14.5% and 10%) were isolated by culture and detected by PCR in the adenoid respectively. These bacteria were, in total, identified in 79% of the adenoid samples by PCR or culture. Karlidag et al. in Turkey have reported that the prevalence of *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* in the adenoid samples of OME patients were 70% by culture method [20]. We could not find any reasonable explanations for this controversy and only speculate that it might be due to the difference in the distribution of these pathogens among different countries.

The results of this study showed that the isolated bacteria from the middle ear fluid samples of OME patients were the same as those isolated from the adenoid of these patients except for *A. otitidis*. The same organism was isolated both from the middle ear fluid and the adenoid tissue samples among 20% of the OME patients. Approximately similar to our results, Karlidag et al. [20] found that in 29% of the OME patients, the same pathogens which grew in the middle ear cultures were also present in the adenoid tissue cultures. Our results synchronized with Karlidag study and strengthened the hypothesis that

adenoid acts as a reservoir for the bacterial pathogens in OME. It has been suggested that the related bacteria are transmitted from adenoidal infection into the middle ear via the Eustachian tubes [16]. In this study, it has been found that the isolated bacteria from the middle ear fluid were also cultured from the adenoid tissue samples of the patients and these findings are, generally, consistent with that of other studies [20]. However *A. otitidis* were only isolated and detected from the middle ear fluids and did not isolate and detect from Adenoid. This finding is similar to De Baere et al. study that showed none of the nasopharyngeal samples positive for *A. otitidis* [34]. Some studies showed that *A. otitidis* was found in the outer ear canals [35–37] and suggested that this bacterium is a part of the normal flora of outer ear canal [35] but some studies showed that *A. otitidis* contributing to the development of an inflammatory reaction in middle ear cavity [38–40]. De Baere et al. suggest that the high incidence of *A. otitidis* in middle ear fluid may be related to the enters of bacterium through (temporarily) opened ear drums as a consequence of middle ear infection or by contamination of middle ear fluid with commensal bacteria present in the outer ear during the sampling process [34]. This is in contrast of our study because we have approximately avoided the contamination of our samples with outer ear canal microflora and also patients with opened ear drums were excluded. Our finding may be suggesting *A. otitidis* as a commensal flora in outer ear canal and potential pathogen in middle ear and may be transmitted to middle ear by unidentified mode. However, further studies are required to assess the presence of this organism at the same time in the outer ear canal, middle ear fluid and the possible route of transmission in patients with OME.

Currently, one of the major problems imposed by the presence of these bacteria is the treatment of OME patients and, therefore, the antimicrobial sensitivity of the isolated bacteria is determined in this study to select the most effective drug to be effective used on against all the detected bacteria. According to the literature review, there are a small number of investigations on the antimicrobial sensitivity of *A. otitidis*. According to the CLSI gridline for *S. pneumoniae*, Bosley et al. had reported that this bacterium shows a high resistance to trimethoprim-sulfamethoxazole and erythromycin [23] but de Miguel Martinez and Macias et al. [14] showed that all of the isolates were susceptible to amoxicillin, tetracycline and co-trimoxazole. In another study, Ashhurst-Smith et al. [28] found that all the isolated *A. otitidis* were sensitive to penicillin but were resistant or partially resistant to erythromycin. In agreement with these studies, all of the isolated *A. otitidis* in the current study were susceptible to vancomycin, ampicillin, amoxicillin/clavunic acid and fluoroquinolones but most of the isolates were resistant to macrolides. The antimicrobial sensitivity of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* was investigated and most of the isolates were susceptible to ampicillin,

Amoxicillin/Clavulanate and fluoroquinolones. In agreement with this study, Easton et al. from New Zealand [41], Hoberman et al. from United States [42] reported that Amoxicillin/Clavulanate was effective against *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. Since most isolates of *A. otitidis*, *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were sensitive to ampicillin, Amoxicillin/Clavulanate, we propose these antibiotics as the drugs of choice for the treatment of OME patients in Iranian children.

Because of the exclusion criteria in this study, the choices for patients and sample size were limited. Further studies from various geographical regions are needed to investigate the etiology of OME and determine the prevalence of these bacteria.

In conclusion, the present study, being the first report on the isolation of *A. otitidis* by culture method in Iran and Asian countries, shows that *A. otitidis* is the most frequently isolated bacterium in Iranian children having otitis media with effusion. Furthermore, in agreement with other studies, this study showed that *A. otitidis*, *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* are the major bacterial pathogens in otitis media with effusion. In this study, the role of adenoid in the etiology of OME was investigated and adenoid maybe acts as a reservoir for OME pathogens except for *A. otitidis*. Finally we found that ampicillin and Amoxicillin/Clavulanate have the excellent activity against bacterial agents in Iranian children with OME.

Conflict of interest

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