FREQUENCY OF ALLOIOCOCCUS OTITIDIS IN PATIENTS WITH OTITIS MEDIA USING CULTURE AND MOLECULAR METHOD

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ABSTRACT:

Background and Aims: Otitis media is one of the most common diseases of childhood that is caused by Bacteria and viruses. The most frequently isolated organisms are, \textit{Streptococcus pneumonia}, \textit{Haemophilus influenzae}, \textit{Moraxella catarrhalis}. During the last decade \textit{Alloiococcus otitidis} has been identified in specimens from patients with chronic otitis media with effusion. The aims of this study were to determine the frequency of \textit{A. otitidis} in ear discharge collected from patient with otitis media.

Materials and Methods: During a period of 10 month, 60 middle ear discharge specimens were collected from patients with otitis media with effusion. Specimens were assessed for \textit{Alloiococcus otitidis} by microscopic examination and culture. PCR technique was used for definitive identification of \textit{Alloiococcus otitidis}. The antibiotic susceptibility test for Penicillin, Ampicillin (AMP), Amoxicillin/clavulanate (30\,\mu g), erythromycin (30\,\mu g), Ciprofloxacin (CIPR 5\,\mu g), levofloxacin (LEVOF 5\,\mu g), Rifampicin (RIF 5\,\mu g) and ceftriaxone was done by Kirby-Bauer disk diffusion method according to CLSI (clinical and laboratory standard institute) criteria.

Results: Out of 60 sample of middle ear discharge specimens 3 isolates (5\%) were identified as \textit{A. otitidis} by culture methods and confirmed by PCR method. However, \textit{A. otitidis} was detected in 28(46\%) of samples by PCR method. All of three isolates of \textit{A. otitidis} were susceptible to penicillin and amoxicillin, levofloxacin, amoxicillin/clavulanate, Ciprofloxacin and ceftriaxone.
**Conclusion:** It seems that *A. otitidis* is the main factors causing middle ear infections and extremely difficult to detect with culture. Our study exhibited that the PCR was proper technique for detection of *A. otitidis*.

**Keywords:** Alloiococcus otitidis, Otitis Media with Effusion, Culture, PCR.

**INTRODUCTION**

Otitis media with effusion (OME) is a major respiratory and a common childhood respiratory disorder with most episodes occurring during early infancy[1]. In the first three years of life, at least one episode of otitis media happens in more than 80% of all young children[2]. Otitis media is the most common reason for prescribing antimicrobial medications in young Canadian and American children and there are wide discrepancies in the use of antibiotic for the treatment of otitis media worldwide[3,4]. Nontypeable *Haemophilus influenzae* and *Streptococcus pneumoniae* have historically been considered the main reason of bacterial OME. There were fewer cases of bacterial otitis media due to other microorganisms such as *Streptococcus pyogenes* and *Moraxella catarrhalis*[5].

Recently, a gram-positive cocci-shaped bacterium, *Alloiococcus otitidis* has been identified as an infectious agent that has a significant role in the development of acute otitis media (AOM) and (OME) [6]. Faden and Dryja first recover *A. otitidis* from MEE of children with OME in 1989[7]. This microorganism is a strict aerobic, gram-positive coccus and oxidase-negative by weakly catalase-reaction [8]. Probable paths of entry of *A. otitidis* to the middle ear regions might be through external ear canal or nasopharynx in persons with perforated tympanic membranes.

Isolation and accurate identification of the organism is arduous in middle ear effusions by conventional techniques, because it displays slow growth in vitro [7]. Despite this, *A. otitidis* was detected in about 50% of children with AOM and OME by molecular methods, and Identification of the bacteria rate was even higher than the three major middle ear pathogens *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis*[9,10]. Only a limited amount of investigations relating to prevalence of *A. otitidis* in patients with OME and AOM have been conducted in Asian countries [6]. In Iran, very few clinical studies are available concerning the The spread of *A. otitidis*.

In this study, we investigated the frequency of *A. otitidis* in ear discharge collected from Iranian patients with OME and AOM by culture and PCR technique. We also investigated the antibiotic susceptibility profiles of *A. otitidis* isolates.

**MATERIALS AND METHODS**

**Study population.** During a period of 10 month from March 2014 to February 2015, 60 specimens of middle ear effusions (MEE) were obtained from patients who undergoing routine tympanostomy tube placement (TTP) surgery in the department of otolaryngology of Amir Alam hospitals of Tehran University. The external ear canal was disinfected with povidone–iodine and then washed with sterile normal saline then, middle ear fluid was aspirated into a Juhn-Tym-Tap collector. All the middle ear effusions were immediately transported to the laboratory and were inoculated to Muller Hinton supplemented with 5% Sheep blood agar.

**Phenotypic identification.** Culture for *A. otitidis* was carried out using chocolate agar and Muller Hinton supplemented with 5% Sheep blood agar as recommended [11]. The plates incubated in a humidified atmosphere of 5% CO2 incubatorand were monitored daily up to 14 days Suspected alpha-hemolytic colonies are further examined by phenotypic and biochemical tests (catalase, oxidase, and gram stain).
Antibiotic susceptibility testing. Antibiotic susceptibility testing was done according to Clinical and Laboratory Standards Institute (CLSI 2013) guidelines for *S. pneumoniae* by Kirby-Bauer disk diffusion method (10, 11). Concisely, *A. otitidis* isolates were suspended in sterile normal saline to no. 3 MacFarland standard and afterwards inoculated onto sheep blood agar plates. Antibiotic discs were applied and the plates incubated at 37 degrees C for 48 hr. (12). In the study to determine the susceptibility patterns of isolated, used antibiotic included, Penicillin, Ampicillin (AMP), Amoxicillin/clavulanate (30μg), erythromycin (30μg), Ciprofloxacin (CIPR 5μg), levofloxacin (LEVOF 5μg), Rifampicin (RIF. 5μg ) and ceftriaxone which is routinely prescribed to treat otitis media All the antibiotic discs were purchased from Mast Co., UK.

Molecular detection. Extraction and purification of total DNA were performed using High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) in accordance with the manufacturer’s instructions[11,12]. PCR examination was set up in order to detect of the 16S rRNA gene of *A. otitidis* using specific primers for the species, Forward primer 5’- CTACGCATTTCACCGCTACAC -3’, Reverse primer 5’-GGGGAAGAACACGGATAGGA -3’. Polymerase chain reaction was performed to a total volume of 25 ml containing 1.6 μM *A. otitidis* primer, 1X PCR buffer (10 mM Tris-HCl), 1.5 mM MgCl2, 0.2 mM dNTP mix and three U Taq Polymerase (Roche, Mannheim, Germany). PCR program consisted of 5 minutes of initial denaturation and 30 cycles at 94°C for 30 seconds, 66°C for 45 seconds and 72°C for 60 seconds, followed by an extension for five minutes at 72°C. The PCR products were separated in 1.5% agarose gel. DNA bands were visualized by staining with ethidium bromide and observed under UV light illumination.

Statistical analysis. Data were analyzed using SPSS ver. 22.0 (SPSS Inc., Chicago, IL, USA). A P value of ≤0.05 was considered statistically significant.

RESULTS
Collection of specimens. A total of 60 samples were collected from patients with otitis media that referred to Amir Alam hospital. Forty (91.6%) of patients were children under 15 years and overall mean of age was 10± years. 33 (55%) cases were females and 27 (45%) cases were males.

Culture and antibiotic susceptibility testing. Of 60 samples of middle ear infection 3 cases (5%) were positive for culture method and all of them collected from children below 5 years of age. *A. otitidis* isolates were characterized by small pale cream colonies, slow-growing that showing alpha-haemolysis on sheep blood agar. Microscopy revealed large Gram-positive cocci arranged in pairs, tetrads and clumps. Acid was not formed from glucose or other carbohydrates. Catalase test were positive and oxidase was not produced.

Molecular assessment. Twenty-eight out of 60 samples (46%) gave positive results for the presence of 16S rRNA gene of *A. otitidis*. After sequencing, three isolates that identified as *A. otitidis* by culture were found to be *A. otitidis* with a 98.82–100% match to the GenBank library using ANG15 software.
DISCUSSION

The first and main goal of this study was to develop culture conditions for the detection of A. otitidis in samples from patients with OME and AOM and identification by PCR method and antibiotic resistance pattern of the bacteria.

Unsuccessful isolation of A. otitidis in MEE with customary culture methods have been confirmed in many References, because it requires a complex medium to grow and grows slowly [9]. Nevertheless, A. otitidis has been identified more frequently than the other middle ear pathogens by molecular methods such as multiplex PCR [13]. Some researchers reported that the bacterium was identified in approximately 20-70% of patients with OME and in 25-50% of patients with AOM by multiplex PCR technique [10,14]. It has been theorized that A. otitidis is part of the normal bacterial flora in the middle ear cleft of the human. However, earlier in vitro and in vivo studies established that this microorganism had an immunostimulatory ability, and this showed that it was not included in the normal flora of the middle ear cavity [15,16]. A. otitidis was evaluated in the upper respiratory tract, such as nasopharynx, tonsil, sinus, pharynx, oral cavity and nasal cavity in some researches. Tano et al. conducted a study among 129 patients with upper respiratory infections without otitis media and recognized A. otitidis in the nasopharynges out of nine children, by multiplex PCR [17]. In the study that conducted by Aydin E et al., prevalence of A. otitidis in children with OME and simultaneously the colonization of the bacterium in the palatine tonsils and nasopharynx of the patients were investigated [18].

PCR is a method that permits for the specific and sensitive identification of pathogens[19]. It is valuable for the detection of middle ear pathogens, which are problematic to culture, slowly growing or dangerous to handle in laboratories [20]. Many authors indicated that PCR approaches especially multiplex PCR methods are more advantageous than conventional culture methods for detecting A. otitidis [18, 21]. In agreement with these studies, we found in our study that PCR was much better method than of the conventional cultures for identifying A. otitidis.
Standard guidelines for antibiotic susceptibility analysis do not comprise \textit{A. otitidis}. Based on studies conducted before reporting antibiotic susceptibility of \textit{A. otitidis}, \textit{Streptococcus pneumoniae} was used to guide antibiotic sensitivity[22].

All of three isolates of \textit{A. otitidis} were susceptible to penicillin and amoxicillin, levofloxacin amoxicillin/clavulanate, Ciprofloxacin,ceftriaxone.

According to what was said above. Our antibiotic susceptibility results were in agreement with Emaneini et al. in Iran in 2012 for penicillin and amoxicillin, levofloxacin amoxicillin/clavulanate, Ciprofloxacin and ceftriaxone [23]. Smith et al in Australian reported that all isolates of \textit{A. otitidis} had large zones of inhibition indicating sensitivity to penicillin[22]. Resistance to erythromycin and other macrolides may result in treatment failure and complicate the antimicrobial therapy of persons with allergies to penicillins. In the present study, one of the three isolated \textit{A. otitidis} was resistant to erythromycin. \textit{A. otitidis} has been reported to be resistant to some macrolides such as erythromycin and azithromycin [22, 23]. Miguel Martinez and Macias in 2008 described that all \textit{A. otitidis} strains tested were susceptible to cefotaxime and ampicillin, but they were resistant to macrolides and cotrimoxazol.

The studied samples included 110 patients aged between 1 and 12 years. Bacteria were present in the culture of 72.5% (29) of the patients with OME. \textit{Alloioococcus otitidis} was the highest frequent bacterium in OME (48.27%) as well as \textit{Haemophilus influenzae} (17.24%). \textit{Streptococcus pneumoniae} was the most The most common pathogen detected in acute otitis media (37.5%), and then \textit{H. influenzae} (25%). For most of the OME cases, only \textit{A. otitidis} bacteria were isolated [24]. Harimaya et al. indicated that this bacterium was commonly identified in patients who received erythromycin or beta-lactam antibiotics [6]. Considering of these results, it is proposed that \textit{A. otitidis} can be resistant to beta-lactams in the near future. Therefore, as this bacterium seems to be existing in patients with otitis media in various parts of the world, guidelines for susceptibility testing of this species need to be developed.

In conclusion, in the present study, we indicated that molecular assays such as PCR was more effective and proper than culture in detecting \textit{A. otitidis}. In addition, our data revealed that the pathogen was frequently detected in children with otitis media in Iran.

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REFERENCES


