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Characterization of *Alloiococcus otitidis* strains isolated from children with otitis media with effusion by Pulsed-Field Gel Electrophoresis

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ABSTRACT

Objective: *Alloiococcus otitidis* is a slow growing organism which has been isolated in a few studies on patients with otitis media with effusion (OME). According to the literature review, there is no study about the molecular typing of *A. otitidis*. In this study, the characteristics of *A. otitidis* isolates from patients with OME were investigated via Pulsed-Field Gel Electrophoresis (PFGE) typing method.
Methods: A total of 50 children with OME, who underwent myringotomy or who had an insertion of a ventilation tube, were included in this study. The isolates were identified to the species level as *A. otitidis* using standard biochemical methods, following which the amplification and sequencing of the 16S rRNA gene were carried out. The molecular characteristic of *A. otitidis* was investigated by PFGE technique.
Results: Fifteen isolates of *A. otitidis* were identified in the middle ear fluid of the patients. All the isolates were susceptible to ampicillin, amoxicillin/clavulanate and fluoroquinolones. All of the 15 isolates were typed by PFGE method and were found to include 13 different PFGE types.
Conclusion: The current study, being the first reports on the molecular typing of *A. otitidis* by PFGE method, shows that *A. otitidis* is a heterogenic organism in Iranian children who have OME.

1. Introduction

Otitis media with effusion (OME) is defined as middle-ear effusion without signs or symptoms of an acute infection [1–3]. Although the etiology of OME is still unclear, the bacterial and viral infections have an important role in its pathogenesis [1]. Various bacterial isolates have been identified in 22–52% of middle ear effusion (MEE) samples in OME cases [4]. *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* are the three major pathogens in OME [5,6]. *Alloiococcus otitidis* is a slow growing and fastidious organism [7] that was found in the MEEs of patients with OME for the first time in 1989 by Faden and Dryja [8], after which, was proposed as a new genus by biochemical test and genetic analysis [9]. It is difficult to isolate *A. otitidis* by conventional cultures and therefore this bacterium has been detected in the MEEs of OME by culture, only in a few studies [7,8,10,11].

Pulsed-Field Gel Electrophoresis (PFGE) is a common tool for molecular epidemiological studies and is a valuable method in genomic analysis and comparison [12]. The usefulness of PFGE for epidemiological typing and genetic relatedness of bacterial isolates has been well established [13]. On reviewing the literature, no data was found on the molecular analysis of *A. otitidis* by various molecular typing methods. Therefore, in this study, the characteristics of *A. otitidis* isolates from patients with OME were investigated using PFGE typing method.

2. Materials and methods

2.1. Study subject and sample collection

A total of 65 specimens of middle ear effusions were obtained from 50 children, during September 2009–November 2010, who

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were candidate for myringotomy or insertion of a ventilation tube in the department of otolaryngology of two teaching hospitals of Tehran University of Medical Sciences. In 15 patients who were bilaterally affected, both right and left middle ear fluids were collected. 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. All the patients had middle ear effusion for more than 3 months and none of them were on antibiotic therapy 2 weeks before and at the time of surgery. All of the specimens were obtained during myringotomy which was performed as the treatment for OME patients. Under general anesthesia and 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 agents. After myringotomy, middle ear fluid was aspirated into a Juhn-Tym-Tap collector (Xomed Inc., Jacksonville, USA). Within 2 h after sampling, MEEs specimens were sent to the Laboratory of Microbiology Department. Written informed consents were obtained from parents of each individual before sample collection. Past medical histories and demographic data of patients were collected from their medical records, prior to surgery. The study was approved by the Ethics Committee of Tehran University of Medical Sciences.

2.2. Isolation and identification of A. otitidis

All of the MEEs samples were inoculated on Muller–Hinton agar supplemented with 5% sheep blood by streak culture method. After 5–9 days of incubation, suspected colonies were isolated and subcultured. In culture negative cases, incubation was extended to 14 days. The isolates were identified to the species level as *A. otitidis* using Gram stain, catalase test, modified oxidase test and fermentation of glycerol. To confirm the identity of the species, the 16S rRNA gene was amplified by a PCR-based method. PCR reactions were performed using the following primers: F-5′-GGGGAAGAACACGGATAGGA-3′ and R-5′-CTACGCATTTCA CCGCTA CAC-3′ [14]. Sequences of both strands of the amplicons were determined at Macrogen (Seoul, South Korea) and the 16S rRNA gene sequences of all isolates were finally aligned in NCBI.

2.3. PFGE typing

Whole genomic DNA was prepared as described by Shahsavan et al. [15] with the following modifications. A 0.5 McFarland equivalent bacterial suspension was made in 1 ml of cold TE buffer [Tris–HCL (10 mM, pH 7.6) and EDTA (5 mM)]. This aliquot of the cell suspension was then centrifuged for 2 min at 7000 × *g* at 4 °C. The supernatant was discarded and the cell pellet was resuspended in 300 μl of EC buffer [EDTA (100 mM, Ph 7.5), Tris–HCL (6 mM, pH 7.6), NaCl (1 M), Brij-58 (0.5%), N-lauroylsarcosine sodium salt (0.5%), Sodium deoxycholate (0.2%)]. Afterwards, 150 μl of cell suspension was transferred into a sterile cup and 7 μl of lysostaphin (1 mg/ml, Sigma, St Louis, USA) and 150 μl of 2% molten low melting point agarose (Sigma, St Louis, USA) were quickly added. The mixture was poured into the slots of a plastic mold and cooled for 20 min at 4 °C. Plugs were removed from the molds and placed into new sterile 50 ml falcon tube that contained 7.5 cc of EC buffer and 10 μl of lysostaphin (1 mg/ml) at 37 °C for 4 h. The EC buffer was then replaced with a new EC buffer containing 25 μl of proteinase K (50 mg/ml) and incubated overnight at 54 °C. Plugs were washed five times with TE buffer by 30 min interval and were digested by the enzyme *SmaI* (fermentase, Latvia). PFGE was performed in 0.5× TBE buffer in a CHEF DR II electrophoresis system (AP-Zoha, Iran). Electrophoresis was performed for 20 h at 14 °C at 5.0 V/cm with a ramped pulse time of 1–30 s (10 h) and 1–3 s (10 h). The gel was stained for 50 min in 1 mg/ml ethidium bromide and decolorized in distilled water for 15 min. The gel was photographed by UV transillumination. The banding patterns of isolates were analyzed with the Total Lab Quant gel analysis. Dendrograms were obtained with unweighted pair group method of arithmetic averages (UGPMA). Isolates were considered potentially genetically related if their DNA patterns differed by less than seven bands.

2.4. Antimicrobial susceptibility test

The disk agar diffusion (DAD) method was used to determine the susceptibility patterns among the *A. otitidis* isolates, according to the CLSI (Clinical and Laboratory Standards Institute) guidelines [16]. Mueller Hinton Agar with 5% Sheep Blood was used for this test. The antibiotics tested were as follows: penicillin, ampicillin, amoxicillin/clavulanate, erythromycin, clarithromycin, azithromycin, cotrimoxazole, ciprofloxacin, levofloxacin, rifampicin, ceftriaxone, and cefepime.

3. Results

The patients studied were within the range of 2–11 years old (45 males and 5 females, median age of 7.2 years). *A. otitidis* was isolated from 15 (23%) of the 65 middle ear fluid samples isolated from 13 patients (2 cases bilaterally affected). Out of the 15 isolates, 13(86.6%), 9(60%), 6(40%), 3(20%), were resistant to tested macrolides, cotrimoxazole, cefepime and cefotaxime, respectively (Table 1). All the isolates were susceptible to ampicillin, amoxicillin/clavulanate and tested fluoroquinolones. All of the 15 isolates were typed by PFGE method and were found to include 13 different PFGE types (Fig. 1).

Table 1
Clinical characteristics, resistance pattern and PFGE types of *Alloiococcus otitidis* strains.

Isolate	Age (year)	Gender	Secretion type	Resistance pattern	PFGE type
1, 2	4	F	Seroumucus	CLA, ATM, E	1
3	10	M	Mucus	CLA, ATM, E, CPM, TS	4
4	9	M	Mucus	CLA, ATM, E, TS	5
5	7	M	Serous	–	3
6	6	M	Mucus	CLA, ATM, E, CPM, CTX, TS	6
7	2	M	Seroumucus	TS	9
8	11	M	Mucus	CLA, ATM, E	8
9	5	M	Serous	CLA, ATM, E, CPM, CTX	11
10	7	M	Seroumucus	CLA, ATM, E	7
11	6	M	Serous	CLA, ATM, E, CPM, CTX, TS	10
12	11	M	Mucus	CLA, ATM, E, TS	2
13	9	M	Mucus	CLA, ATM, E, TS	12
14,15	7	F	Mucus	CLA, ATM, E, CPM, PG, TS	13

TS, cotrimoxazole; CTX, cefotaxime; F, Female; M, male; CLA, clarithromycin; ATM, azithromycin; E, erythromycin; CPM, cefepime; PG, penicillin.

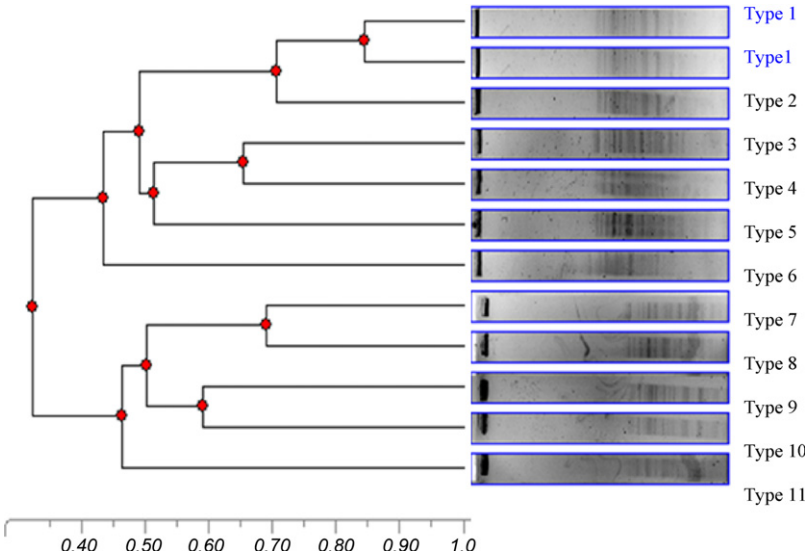

Fig. 1. Dendrogram showing estimates of the percent similarities among some of *Alloiococcus otitidis* strains typed by PFGE.

4. Discussion

Although *A. otitidis* has been frequently detected in OME, it is still unclear whether this organism has enough pathogenic potential to induce otitis media [17]. *A. otitidis* is a bacterium with special growth requirements which is detected only by difficulty in culture media [6,8,17]. So far, only a few clinical strains of *A. otitidis* have been isolated [7,10–12].

Molecular methods have been able to elucidate the role of this bacterium in OME. Very little is still known, however, about *A. otitidis* and its possible role in the pathogenesis of otitis media. PFGE typing method was used as a means of characterization and discrimination of the isolates based on their genotypic characteristics and may be used to establish genetic relationships between strains and to trace the geographic dissemination of the bacterial isolates [14,15]. In the present study, the considerable genomic variability in *A. otitidis* strains is reflected by the high number of PFGE patterns obtained for the 15 strains studied. These data revealed *A. otitidis* as a heterogenic organism with a genetic diversity. This genetic heterogeneity may demonstrates that there are several distinct *A. otitidis* strains present in children with OME in Tehran and shows that *A. otitidis* infection is sporadic in this area and there is no common source for infections. However in this study, in two patients, who were bilaterally affected, *A. otitidis* showed the same PFGE type and antibiotic susceptibility pattern.

Hence there is no data about the genetic analysis of *A. otitidis* from divergent geographic origins for comparisons between strains. Further studies from various geographical regions are needed to investigate the molecular characteristics of *A. otitidis* by various typing methods and to determine the genetic nature of this bacterium.

In conclusion, to our knowledge, this is the first study reports the molecular characteristics of *A. otitidis* by PFGE method, reveals that *A. otitidis* is a heterogenic organism in Iranian children who have OME.

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