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Genetic similarity between adenoid tissue and middle ear fluid isolates of *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* from Iranian children with otitis media with effusion

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ABSTRACT

Background: Otitis media with effusion (OME) is a common disease among children, in the pathogenesis of which bacterial infections play a critical role. It was suggested that adenoid tissue could serve as a reservoir for bacterial infection, the eustachian tubes being the migration routes of bacteria into the middle ear cavity. The aim of this study was to investigate the genetic similarity between isolates of *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis*, obtained from adenoid tissue and middle ear fluid.

Methods: A total of 60 specimens of middle ear fluids (MEFs) and 45 specimens of adenoid tissue were obtained from 45 children with OME. All the samples were inoculated on culture media for bacterial isolation and identification. The genetic similarity between bacterial isolates was determined by pulsed-field gel electrophoresis (PFGE).

Results: The same bacterial species were simultaneously isolated from adenoid tissue and MEFs of 14 patients, among which, 6 pairs of *M. catarrhalis*, 5 pairs of *S. pneumoniae* and 3 pairs of *H. influenzae* were identified.

Conclusions: Based on the genetic similarities between isolate pairs, found by PFGE analysis, this study suggested that *M. catarrhalis*, *S. pneumoniae* and *H. influenzae* colonize the adenoid tissue, then migrate to the middle ear cavity and, hence, contribute to the total pathogenesis of OME.

1. Introduction

Otitis media is one of the most common diseases during childhood for which children visit doctors and receive antibiotics before ten years old [1]. Otitis media with effusion (OME) is one of the sub-classifications of otitis media, defined as the presence of fluid in the middle ear without symptoms or signs of infection [2,3]. Bacterial and viral infections are known as the agents contributing to the pathogenesis of OME, but the etiology of OME is still unclear [4], Although Kubba et al. [5] stated that the presence of bacteria in the middle ear mucosa have role in primary event of inflammation and associated with releasing of inflammatory mediators, then cause secretion of a mucin-rich effusion and finally by prolonged stimulation of the this response, lead to the clinical presentation of OME.

Bacterial culture of OME showed positive result for only 20–30% of patients [6]. *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* are the three most common bacterial species, isolated by culture, in the middle ear effusion [7]. *Alloiococcus otitidis* is a fastidious slow growing organism isolated from middle ear fluid of OME patients. Different prevalence rates of this organism have been reported in different studies [3,8–12].

Brook et al. [13] suggested that adenoid tissue may serve as a reservoir for infection, providing a bacterial source for OME. Some studies proposed the association of otitis media with chronic adenoidal infection, through the transmission of bacteria from

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adenoidal infection via the Eustachian tubes into the middle ear [14,15]. Many studies describe that colonization of nasopharynx or adenoid is an essential step in development of otitis media [16,17].

The role of adenoid tissue in the pathophysiology of OME was demonstrated by Nguyen et al. [14]. Adenoidectomy has an important role in the treatment of children with recurrent OME who have previously had tympanostomy tubes placement [18]. McClay et al. [19] reported that the isolation rate of *S. pneumoniae, H. influenzae* or *M. catarrhalis* from the adenoid tissue samples of OME patients was higher than that in patients with adenoid hypertrophy alone. In another study by Karlidağ et al. [20], the similarities between the three mentioned bacteria in the middle ear fluid and adenoid tissues were shown and the possible role of these bacteria in the development of OME was then suggested. Therefore, the pathogenesis of otitis media due to *S. pneumoniae*, *H. influenzae* or *M. catarrhalis* appears to involve spread of the bacteria from the adenoid tissue to the middle ear fluid. Conventional epidemiological analysis based on serotypes, biotypes and antimicrobial susceptibilities have been performed to show that paired isolates, recovered simultaneously from the adenoid (nasopharynx) and middle ear fluids, are identical [21]. Pulsed-field gel electrophoresis (PFGE) is a common tool for molecular epidemiological studies and is a valuable method in genomic analysis and comparison [22]. The usefulness of PFGE for epidemiological typing and genetic relatedness of bacterial agents has been well established [23]. PFGE is ideal for the rapid clustering of genetically related isolates. After digesting of the chromosomal DNA with restriction endonucleases the DNA banding patterns are then compared between isolates and clonal relationships are inferred [24]. PFGE clones were defined as isolates sharing 80% or higher similarity on the dendrogram according to their banding pattern [25]. If the genetic similarity was shown by PFGE method, the hypothesis of migration of bacteria from adenoid to middle ear cavity is strengthen and by treatment of children with bacterial colonization of adenoid or adenoid hypertrophy, the risk of OME will be decreased.

Only one study reported the high genetic similarity between *S. pneumoniae* and *H. influenzae* isolates from paired nasopharynx and middle ear fluids following PFGE analysis.

Therefore, this study was aimed to strengthen this idea that the pathogenesis of OME involves the migration of bacteria from the adenoid into the middle ear cavity and that the paired isolates recovered simultaneously from the adenoid tissue and the middle ear fluid are identical using PFGE method. If our finding shown the genetic similarity between OME bacterial isolates and their adenoid counterparts, supported the concept that the removal of the adenoid may remove the reservoir of potential pathogens for OME. In addition, to evaluate the contributing pathogen for OME, obtaining culture from nasopharyngeal or adenoid is less invasive and easier than obtaining cultures of middle ear fluid by invasive surgical procedure such as tympanocentesis or myringotomy in children that suggested before [26].

2. Materials and methods

2.1. Patient population and sampling methods

This study was approved by the Ethics Committee of Tehran University of Medical Sciences.

A total of 60 specimens of middle ear fluids (MEFs) and 45 specimens of adenoid tissue were obtained from 45 children with otitis media with effusion (OME) during 2009–2010, who were candidate for myringotomy or insertion of a ventilation tube in the department of otolaryngology of two teaching hospitals in Tehran University of Medical Sciences. Out of the 45 children with otitis media, 15 were bilaterally affected, from which both right and left middle ear fluids were collected. Children with previous trans-tympanic ventilation tubes, tympanic membrane perforations, previous adenoidectomy, immunological defect, anatomic abnormality, respiratory tract infection and purulent middle ear fluid and any malformations were excluded. None of these patients had received antibiotic therapy for at least two weeks prior to surgery. The criterion for being candidate for surgery was the presence of middle ear effusion for more than 3 months. All the patients had adenoid hypertrophy and were, therefore, candidate for adenoidectomy.

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). Adenoids were removed with a curette under general anesthesia after myringotomy and placed in a sterile container. Both operations were performed as the treatment for OME patients. Within two hours after sampling, all the clinical samples 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. Bacterial culture

Adenoid tissue samples were homogenized with tissue grinder under sterile condition, then adenoid tissue and middle ear fluid samples were inoculated on the following specific culture media: Muller Hinton with 5% Sheep blood agar for *S. pneumoniae*, chocolate agar with vancomycin (5 μg/ml), clindamycin (1 μg/ml) and bacitracin (300 μg/ml) for *H. influenzae* [27], chocolate agar with vancomycin (5 μg/ml), clindamycin (1 μg/ml) and bacitracin (300 μg/ml) and acetazolamide for *M. catarrhalis* [28]. The media were incubated under aerobic conditions with 5% CO_2 at 35 °C for 24–72 h. The bacteria grown after incubation were identified with conventional methods [29]

2.3. PFGE typing

All the isolated bacteria were grown overnight on selective media. Whole genomic DNA was prepared as described by Shahsavan et al. [30] 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)]. The cells were harvested by centrifugation 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%)]. An equal volume of 2% molten low melting point agarose (Sigma, St Louis, USA) were added to 150 μl of cell suspension of *S. pneumoniae* with 7 μl of lysostaphin (1 mg/ml, Sigma, St Louis, USA) and, for *H. influenzae* and *M. catarrhalis*, with 7 μl Lysozyme (500 μg/ml, Cinnagene) into a sterile cup. The mixture was poured into the slots of a plastic mold and chilled at 4 °C for 20 min. 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 for *S. pneumoniae* or 10 μl of lysozyme for *H. influenzae* and *M. catarrhalis* 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) for