

Research Paper

Relationship between Oral Anaerobic Bacteria and Otitis Media with Effusion

Nursen Topcuoglu¹✉, Fahriye Keskin¹, Sevgi Ciftci¹, Ceki Paltura², Mehmet Kulekci², Duran Ustek³, Guven Kulekci¹

1. Oral Microbiology Laboratory, Istanbul University Faculty of Dentistry, Istanbul, Turkey;
2. Department of Otorhinolaryngology, Head and Neck Surgery, Taksim Training and Research Hospital, Istanbul, Turkey;
3. Department of Genetics, Institute for Experimental Medicine, Istanbul Faculty of Medicine, Istanbul, Turkey.

✉ Corresponding author: Tel: +902124142595; Fax: +902125312230, E-mail address: nursentopcuoglu@yahoo.com (N. Topcuoglu).

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Abstract

Objective: In this study hypothesing the translocation of oral bacteria from oropharynx into the middle ear cavity may be involved in the pathogenesis of otitis media with effusion (OME), we aimed to investigate the presence and similarity of *Fusobacterium nucleatum* and *Treponema denticola* in saliva, nasopharyngeal secretion and the middle ear effusion samples from the children with OME.

Methods: Totally 20 children with OME undergoing myringotomy and ventilation tube placement were attended. Stimulated saliva samples were collected after otorhinolaryngological and oral examinations were done. The middle ear effusion and nasopharyngeal secretions were collected during the operations. The presence of *F. nucleatum* and *T. denticola* were detected using 16SrRNA-based PCR. The clonal similarities of the bacteria were detected in the samples which the same bacteria had been detected in each samples of the same child. After DNA sequencing, clonal similarity was determined by 16SrRNA gene clone library analysis. The sequences from each clone were compared with similar sequences of reference organisms by FASTA search.

Results: *T. denticola* was detected only in four (20%) saliva and in one (5%) nasopharyngeal sample. *F. nucleatum* was detected in 11 (55%) saliva, eight (40%) nasopharyngeal and six (30%) middle ear effusion samples. Sequences from *F. nucleatum* clones derived from three different anatomic sites within patients were similar in 33% of OME patients, indicating their genetic relatedness.

Conclusions: Bacteria involved in this process most likely originate from the oropharynx since they show a close genetic relatedness with their oropharyngeal counterparts.

Key words: Middle ear effusion, saliva, *Fusobacterium nucleatum*, sequence analysis.

Introduction

Otitis media with effusion (OME) is an inflammation of the middle ear, accompanied by the accumulation of fluid in the middle ear cleft without the symptoms and signs of acute inflammation (1). It is a

common childhood disease which can lead to significant hearing loss in pediatric patients, resulting in developmental problems in speech, language, and the acquisition of social skills (2,3). Once a decision has

been taken to offer surgical intervention for OME in children, the insertion of ventilation tubes is recommended.

Streptococcus pneumoniae, *Haemophilus influenzae*, and *Moraxella catarrhalis* are isolated from approximately 25% of children with OME, but PCR-based methods have demonstrated sequence specific DNA and RNA for these pathogens in nearly 80% of cases (4-7). In our previous study, bacterial DNA were determined from 42 % of the middle ear effusion (MEE) samples, which, 37 % of them were positive for *Fusobacterium nucleatum* and one of them was positive for both *F. nucleatum* and *Treponema denticola* (8). *T. denticola* was found to be the second most common periodontal pathogen among the periodontally healthy children (9).

OME has been suggested to be a continuum of acute otitis media (AOM) (10). It was speculated that environmental/ecological changes in the nasopharynx during AOM episodes may favor the growth of anaerobes, which can be recovered frequently during infection in children under two years old, but not during health (11). The main anaerobes that are found in nasopharyngeal aspirates collected during AOM are *F. nucleatum* and *Prevotella melaninogenica* (12). These species are also among the principal anaerobic bacteria that are found in chronic OME (13,14,15).

Saliva contains a variety of bacteria from different oral sites; therefore it is representative for all oral microflora (16). It seems to be a transmission vehicle for translocation of oral bacteria between the oral cavity and nasopharynx. Clonal similarity of the *F. nucleatum* strains between nasopharynx and saliva of the children with AOM has been shown previously (17). It is well acknowledged that microorganisms from the nasopharynx can reach the middle ear cavity via the eustachian tube and cause middle ear infection (18,19).

Based on the published data, we hypothesize that the translocation of oral bacteria from oropharynx into the middle ear cavity may be involved in the pathogenesis of OME. In this study, we aimed to investigate the presence and clonal similarity of *F. nucleatum* and *T. denticola* in saliva, nasopharyngeal and middle ear effusion samples from the children with OME.

Materials and Methods

Study population

A total of 20 children with OME aged 4-11 years (mean age 7.8±1.9 years) undergoing myringotomy and tube placement at the Department of Otorhinolaryngology, Head and Neck Surgery at Taksim

Training and Research Hospital, Istanbul, Turkey. At the time of study enrollment, parents provided their informed consent for all infants which was approved by the Local Ethics Committee of the Istanbul University Faculty of Medicine (2008/930).

After otorhinolaryngological examination, oral hygiene and gingival status were assessed using the Simplified Oral hygiene index (20) and gingival index (21), respectively. Paraffin stimulated saliva samples were collected immediately after clinical examination. Middle ear effusion and nasopharyngeal secretions were obtained during anesthesia for insertion of ventilation tubes (8). All the samples were stored at -80°C until use.

PCR assay

Saliva samples were diluted 1:2 and washed four times. MEE samples were lysed at 56°C for 3 h after 20µl proteinase K addition. DNA was isolated from *F. nucleatum* ATCC 25586, *T.denticola* 35405 and the clinical samples, by using a MagNA Pure automated DNA extraction platform (Roche Diagnostics) as recommended by the manufacturer.

16SrRNA primers (*F. nucleatum* Forward 5'-CGC AGA AGG TGA AAG TCC TGT AT-3', Reverse 5'-TGG TCC TCA CTG ATT CAC ACA GA-3' and *T. denticola* Forward 5'- TAA TAC CGA ATG TGC TCA TTT ACA T-3', Reverse 5'-CTG CCA TAT CTC TAT GTC ATT GCT CTT-3') were used to amplify the 264-bp-long region of *F. nucleatum* and 862-bp-long region of *T. denticola*. 50 ng DNA was added into the PCR mixture containing 10 pmol of each primer, 1.5mM MgCl₂, 50 µM of deoxyribonucleotide triphosphate (Pharmacia LKV), 0.15 U of *Taq* polymerase and 5 µl of buffer 10x.

The PCR profile for *F. nucleatum* included an initial denaturation at 95°C for 3 min followed by 30 cycles consisting of 94°C for 30 s, 66°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min. The PCR profile for *T. denticola* included an initial denaturation at 92°C for 2 min followed by 36 cycles consisting of 95°C for 30 s, 58°C for 120 s, 72°C for 60 s and a final extension at 72°C for 5 min.

Following amplification, 10 µl of PCR products were analyzed by electrophoresis on agarose gels. Gels were stained with 0.5 µg ethidium bromide ml⁻¹ and visualized by ultraviolet light illumination.

Sequence analysis

Sequences were assembled after sample purification using a purification kit (Roche Diagnostics). DNA were directly sequenced in Beckman Coulter CEQ 8000 Genetic Analysis System using Beckman Coulter DTCS Quick Start kit.

Sequence comparisons were determined by 16SrRNA gene clone library analysis. The sequences from each sample were compared with similar sequences of reference organisms by FASTA search. Clinical sequences were required to be >98% similar to reference organisms in the database for 16S rRNA gene sequence similarity (22).

Statistical analysis

The results of the conventional PCR were described using absolute and percentage frequency and the clinical conditions were compared with Fischer's Exact test and student-t test with a significance level of 0.05.

Results

A total of 20 children (4-11 years old, mean age 8 years; 11 male and 9 female) were clinically examined and sampled for microbiological analysis. Sixteen subjects (80%) were in mixed dentition and 12 subjects (60%) had been used antibiotic within last 3 months. Mean oral hygiene and gingival index scores were 1.36 ± 0.29 and 0.29 ± 0.36 , respectively.

Table 1 shows the prevalence and distribution of target bacterial species in clinical samples from 20 children. *F. nucleatum* was detected from 11 (55%), eight (40%) and six (30%) salivary, nasopharyngeal and middle ear effusion samples of the 20 subjects, respectively. *F. nucleatum* was detected from six (30%) of the children's each three samples (Patient no: 1, 4, 7, 11, 12 and 13) and two (10%) of the children's saliva and nasopharyngeal samples (Patient no: 3 and 9). The clinical conditions of the children who revealed or not revealed *F. nucleatum* in their MEE samples are shown in Table 2. The clinical conditions were not statistically different ($P>0.05$).

F. nucleatum was assessed in eight cases in at least two samples. The same clonal frames were detected in each sample of two (10%) children (Patient no: 4 and 13), while different frames were detected from middle ear effusion samples of the two children (Patient no: 1 and 11) and from all samples of one

child (Patient no: 7). A mixed population was determined from one child's nasopharyngeal sample, whose other two samples showed different clonal frames (Patient no: 12). Thus, the rate of genetic relatedness between *F. nucleatum* clones per patient which *F. nucleatum* were detected from three locations (middle ear effusion, nasopharyngeal secretion and saliva) indicated a clonal origin is 33% (Table 3).

The children with double *F. nucleatum* positive samples (Patient no: 3 and 9) revealed the same clonal frames in their each sample. Thus, a total of six (30%) children (Patient no: 1,3,4,9,11 and13) was found to carry the same *F. nucleatum* clonal frames in their nasopharynx and oral microflora. The rate of genetic relatedness between *F. nucleatum* clones per patient which *F. nucleatum* were detected from two locations (nasopharyngeal secretion and saliva) indicated a clonal origin is 75% (Table 3).

T. denticola was detected from four (20%) saliva samples and one (5%) nasopharyngeal secretion sample, while it was not detected in any middle ear effusion sample (Table 1). Sequence similarity could not be performed because of the fact that, none of the children harbored *T. denticola* in their each samples.

Table 1. Number and percentage of OME patient with positive samples distributed over respective locations.

Location	<i>F.nucleatum</i>		<i>T.denticola</i>	
	<i>f</i>	%	<i>f</i>	%
Saliva+NS+MEE	6	30	0	0
Only Saliva+NS	2	10	0	0
Only Saliva	3	15	4	20
Only NS	0	0	1	5
Only MEE	0	0	0	0
TOTAL	11	55	5	25

f, Frequency; NS, nasopharyngeal secretion; MEE, middle ear effusion.

Table 2. Clinical conditions of the children according to presence of *F.nucleatum* in their MEE samples.

<i>F.nucleatum</i> positive in MEE samples	n	Mixed dentition	Age	Plaque index	Gingival index	Antibiotic usage
Yes	6	5 (83%)	7.7 ± 1.9	1,36 ± 0,75	0,11 ± 0,20	5 (83%)
No	14	11 (79%)	7.9 ± 1.9	1,37 ± 0,57	0,37 ± 0,39	7 (50%)
Total	20	16 (80%)	7.8 ± 2	1,36 ± 0,29	0,29 ± 0,36	12 (60%)

Table 3. The clonal similarities of the *Fusobacterium nucleatum* obtained from the samples with reference organisms.

Patient No	Sample	Sequence No	<i>F. nucleatum</i> reference strains		
			ATCC 49256	ATCC 25586	ATCC 33693
<i>Triple samples</i>					
1	MEE	801		100	
	Saliva	807			100
	NS	813			100
4	MEE	802		100	
	Saliva	808		100	
	NS	814		100	
7	MEE	803	100		
	Saliva	809			100
	NS	815		100	
11	MEE	804		100	
	Saliva	810			100
	NS	816			100
12	MEE	805		100	
	Saliva	811			100
	NS	817		Mixed	
13	MEE	806		100	
	Saliva	812		100	
	NS	818		100	
<i>Double samples</i>					
3	Saliva	819		100	
	NS	821		100	
9	Saliva	820			100
	NS	822			100

NS: nasopharyngeal secretion; MEE: middle ear effusion.

Discussions

The pathogenesis of OME appears to be multifactorial, including immature anatomy, initiating viral infections that induce eustachian tube dysfunction, retrograde movement of bacteria from the oropharynx into the middle ear cavity, and host physiological factors (5). Eustachian tube dysfunction may create reduced oxygen pressure in the middle ear cavity and therefore this environmental change may favor the growth of anaerobes.

Knowledge about anaerobic bacteria in the pathogenesis of OME is very limited and mostly depends on cultural studies (8,23,24). The anaerobes involved in middle ear infections originate from the oropharyngeal cavity, where they constitute normal flora, outnumbering the aerobic and facultative bacteria in a 10–100 to 1 ratio (13).

Anaerobes contribute to the severity and chronicity of infections that occur in and around the oral cavity. *F. nucleatum* is a commensal oral bacterium on various oral mucosal surfaces and in dental plaque. *F. nucleatum* can be isolated from oral mucosal surfaces of the edentulous infants. The frequency of isolation rises with age, probably due to the better living environment that is created with the eruption of teeth (25). In our study, five of six *F. nucleatum* positive children in MEE were in mixed dentition.

Fusobacterium nucleatum is considered to be a key species in building the community structure in dental biofilm and on various oral mucosal surfaces. Although strictly anaerobic, it is assumed that *F. nucleatum* is capable of surviving in aerobic environments because of its coaggregation with oxygen-consuming bacteria (26). In our study, while the salivary presence rate was 55%, mean oral hygiene and gingival levels were similar between the children with and without *F. nucleatum* in MEE samples.

F. nucleatum is frequently found in middle ear effusions from children with OME (8,14). Sequences from *F. nucleatum* clones derived from three different anatomic sites were similar within two of the six (33%) OME patients. This result indicates the genetic relatedness between oropharyngeal flora and middle ear effusion. The source of the bacteria is important, because many *F. nucleatum* strains present in the oral cavity of young children produce β -lactamases (27). Since, β -lactam antibiotics are commonly used for upper respiratory tract infections, *F. nucleatum* strains, which translocate to middle ear cavity, can persist to colonize even the antibiotic therapy. In our study, five of the six (83%) of the children with *F. nucleatum* in MEE samples had been used antibiotics within the last 3 months, while totally 12 (60%) of the children used antibiotics. As it is not statistically significant, in these circumstances, it may be significant if we increase the numbers of the samples.

Clonal similarities of *F. nucleatum* isolates collected from the nasopharynx and saliva of the infants with AOM has been shown using arbitrarily primed PCR (17). It was also reported that environmental/ecological changes in the nasopharynx during AOM was favor the growth of anaerobes (12). In our study, similar clonal frames were found among salivary and nasopharyngeal samples in six of the eight children whose nasopharyngeal secretion samples were *F. nucleatum* positive. The present observation indicates that the source of nasopharyngeal anaerobes may be the oral cavity and the saliva may be their transmission vehicle.

F. nucleatum is a heterogeneous species with numerous genotypic profiles and high heterogeneity

of serovars and ribotypes have been found within individuals (28-30). The high intra-individual clonal heterogeneity and frequent turnover of clones among oral *F. nucleatum* populations was claimed to allow the species to escape the host immune response, which is targeted against pathogens, and persist to colonize the oral cavity. In this study, intra-individual clonal heterogeneity was detected in each sample of two children. Thus, the translocation of oral bacteria from oropharynx into the middle ear cavity may be involved in the pathogenesis of OME. Nasopharyngeal microflora may be considered to be the source of other regions, because, in this study, different clones was observed in one child's saliva and MEE sample, while a mixed population was observed in his nasopharynx.

In our previous study, which *F. nucleatum* had not been searched, *T. denticola* (32%) was the second most prevalent periodontal bacteria following *Prevotella nigrescens* (80%) in 41 periodontally healthy Turkish children in mixed dentition (9). As our study population was similar to that study, we thought it was important to detect *T. denticola* which was also previously detected in MEE samples (8). In our current study, *T. denticola* was detected from only four (20%) saliva samples and one (5%) nasopharyngeal secretion sample, while it was not detected in any middle ear effusion sample. The detection rates could be higher by using a quantitative real-time PCR assay, which is more sensitive, but in this study, we aimed to detect a sequence-based similarity which a conventional PCR had to be used.

Conclusions

F. nucleatum in middle ear effusions of OME patients most likely originate from the oropharynx, since they show close genetic relatedness to their oropharyngeal counterparts. With the limitations of this study, it can be concluded that oral bacterial species could gain entry through retrograde movement from the oropharynx into the middle ear cavity and may also play a role in the aetiopathogenesis of OME. The similarities for other oral bacteria can be shown after further detection the microbial profiles of the MEE samples using metagenomic analysis.

Acknowledgements

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Abbreviations

OME: otitis media with effusion; AOM: acute otitis media; MEE: middle ear effusion; NS: nasopharyngeal secretion.

Competing Interests

The authors have declared that no competing interest exists.

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