Clonal similarity of salivary and nasopharyngeal *Fusobacterium nucleatum* in infants with acute otitis media experience

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The environment of an infant’s nasopharynx during acute otitis media (AOM) favours the growth of anaerobic bacteria, which can be recovered frequently during infection, but hardly at all if the infant is healthy. The aim of this investigation was to identify the potential source and inoculation route of anaerobes that were present in the nasopharynx. Eleven *Fusobacterium nucleatum* isolates that were collected through the nasal cavity from the nasopharynx of eight infants with a history of AOM, and 161 *F. nucleatum* isolates from the saliva of the same infants, were typed to the clonal level by using arbitrarily primed PCR (AP-PCR). In five of the eight infants examined, identical AP-PCR types were found among nasopharyngeal and salivary isolates. As anaerobes seem to be present only transiently in the nasopharynx and salivary contamination of the nasopharyngeal samples can be excluded, this observation indicates that the source of nasopharyngeal anaerobes is the oral cavity and that saliva is their transmission vehicle.

**INTRODUCTION**

The most common bacterial infection in young children, acute otitis media (AOM), generally follows the colonization of the nasopharynx with common respiratory pathogens such as *Streptococcus pneumoniae* and *Haemophilus influenzae* (Bluestone, 1982; Faden et al., 1998). In addition, the environment of the infant’s nasopharynx during AOM seems to favour the growth of anaerobic bacteria, which can be recovered frequently during infection, but not during health (Könönen et al., 2003). The main anaerobes that are found in nasopharyngeal aspirates collected during AOM are *Fusobacterium* species and saccharolytic *Prevotella* species, especially *Fusobacterium nucleatum* and *Prevotella melaninogenica* (Könönen et al., 1999c). These species are also among the principal anaerobic bacteria that are found in chronic otitis media with effusion (Brook & Frazier, 1996; Brook et al., 2000).

Translocation of bacteria from one anatomical body site to another within the same individual has not received much attention. In children, the main focus of research has been the transmission of oral (Asikainen & Chen, 1999) and respiratory (Yano et al., 2000) pathogens between individuals, especially with respect to the transmission of antibiotic-resistant *S. pneumoniae* (Sá-Leão et al., 2000). Recent observations of the absence of anaerobes in the nasopharynx during health (Könönen et al., 2003) raised the question of the source of anaerobes that are present in the nasopharynx during infection. *F. nucleatum* was selected as a representative to test our working hypothesis, according to which, the most plausible origin for nasopharyngeal anaerobes is the oral cavity and, conceivably, saliva is the most likely transmission vehicle.

*F. nucleatum* is considered to be a key species in building the community structure in dental plaque and on various oral mucosal surfaces. Although strictly anaerobic, these bacteria can be isolated from edentulous infants (Könönen et al., 1999b). It is assumed that *F. nucleatum* is capable of surviving in aerobic environments because of its coaggregation with oxygen-consuming bacteria (Kolenbrander, 2000). The frequency of isolation rises with age, probably due to the better living environment that is created with the eruption of teeth (Könönen et al., 1999b). Bacteria are being shed constantly from different oral surfaces into the saliva, which therefore gives insight into a variety of ecological niches and reflects the overall oral microflora. Furthermore, saliva acts as a potential vehicle for transmission of various oral anaerobes between individuals (Könönen et al., 1994, 2000). Similarly, it appears...
to be the most plausible vehicle for translocation of oral bacteria between close anatomical sites, such as the oral cavity and nasopharynx, within an individual.

In the present investigation, we used arbitrarily primed PCR (AP-PCR) to assess the clonality of *F. nucleatum* isolates that were collected from the nasopharynx and saliva of the same infants. This species was chosen as a representative of the oral anaerobic bacteria because of its crucial role in the formation of biofilms. The aim was to demonstrate the oral origin of nasopharyngeal *F. nucleatum* and a plausible inoculation route via saliva in infants with experience of AOM during their first 2 years of life.

**METHODS**

**Study subjects and specimens.** The present data are from eight infants who were positive for carriage of nasopharyngeal *F. nucleatum* by 2 years of age. They belonged to a satellite subpopulation of the Finnish Otitis Media (FinOM) cohort study, where 50 healthy, 2-month-old (at baseline) Caucasian infants were recruited to a prospective longitudinal study on the development of microflora in the upper respiratory tract (Könoenen et al., 1999b, 2003). The infants were followed in a study clinic at scheduled healthy visits up to 24 months of age and, in addition, between visits if an infant became sick. Infections were diagnosed and treated in the same clinic, as described in detail by Syrjanen et al. (2001). All eight infants included in the present study had at least one AOM episode by 2 years of age. Salivary samples and nasopharyngeal swab (NP) samples were collected at scheduled healthy visits at 2 (± 2 weeks), 6 (± 2 weeks), 12 (± 2 weeks), 18 (± 4 weeks) and 24 (± 4 weeks) months of age and nasopharyngeal aspirate (NPA) samples were collected at every visit that was related to AOM. Unstimulated saliva was collected in the buccal sulcus area of the mouth, as described previously (Könoenen et al., 1999b). NP and NPA samples were taken through the nasal cavity by inserting a flexible metal-shaft electric suction device, respectively, as described by Syrjanen et al. (2001). Samples were placed in VMGA III transport medium (Dahlen et al., 1994) and transported to the laboratory via express mail. Written informed consent was obtained from parent(s); the Ethical Issues Committees of the National Public Health Institute, Tampere University Hospital, and the Department of Social and Health Care of Tampere City, Finland, approved the study protocol.

**F. nucleatum isolates.** Specimens were cultured within 24 h of collection on several media, including neomycin/vancomycin agar (which is selective for fusobacteria), and were identified by using established biochemical methods as described previously (Könoenen et al., 1999b, c). One specific target of the FinOM satellite study was to collect multiple *F. nucleatum* isolates per infant and store them at −70 °C until further testing. Eight *F. nucleatum* isolates from NPA samples (infants A, C, H, I, M, N and P) and three isolates from NP samples (infants C and O) were available for AP-PCR typing. These 11 nasopharyngeal isolates were compared with 161 *F. nucleatum* isolates from saliva of the same eight infants, which was collected at scheduled healthy visits that preceded (n = 39) and/or followed (n = 113) AOM episodes, and/or were collected simultaneously with an NP sample (n = 9).

Type strains of the four human *F. nucleatum* subspecies, *F. nucleatum* subsp. *nucleatum* ATCC 25586T, *F. nucleatum* subsp. *polymorphum* ATCC 10953T, *F. nucleatum* subsp. *fusiforme* NCTC 11326T and *F. nucleatum* subsp. *vincentii* ATCC 49256T, were used to select potential primers for amplifying DNA from human *F. nucleatum* subspecies.

**DNA isolation.** *F. nucleatum* isolates were revived from frozen stocks and grown on Brucella blood agar (5 % horse blood) plates in an anaerobic environment (10 % CO₂, 10 % H₂, 80 % N₂) at 37 °C for 3–7 days. Bacterial growth (a few colonies) was harvested from the agar plates, suspended in 600 μl 5 % Chelex 100 (Bio-Rad) and boiled for 10 min. The suspension was then mixed briefly on a vortex mixer and centrifuged for 10 min; a 5 μl aliquot of supernatant was used for AP-PCR.

**Oligonucleotide primers.** For separation of *F. nucleatum* clones, 12 primers (Amersham Biosciences) were tested by using the four reference strains, Four primers, C1 (5’-GATGAGTTCGTGCCTGACAATCGG-3’), C2 (5’-GGTTATCGAATCGCCAGCAGGC-3’), D8635 (5’-GACGGGCGCAAGGGAGCAGCAG-3’) and D11344 (5′-AGTGAATTCCGGTGGAATGCTCACA-3’), were chosen for AP-PCR typing of the 172 clinical *F. nucleatum* isolates.

**AP-PCR.** AP-PCR was performed in a volume of 25 μl in a 500 μl Ready-To-Go-PCR tube (Amersham Biosciences), which contained 5 μl DNA suspension and 80 nM one primer, in an Eppendorf thermal cycler. A negative control (without DNA) was included in each AP-PCR run. Amplification was performed by using a slightly modified version of the method of George et al. (1997). Briefly, 5 min initial denaturation at 94 °C and annealing at 35 °C for 5 min were followed by five cycles of denaturation at 94 °C for 3 min, annealing at 37 °C for 3 min and elongation at 72 °C for 3 min. This was followed by 30 cycles of 94°C for 1 min, 55 °C for 1 min and 72 °C for 3 min, and a final elongation phase of 72 °C for 10 min. Amplified products were kept at 4 °C until they were separated by 1.5 % agarose/TBE electrophoresis, stained with ethidium bromide and photographed digitally (Alpha Innotech) under UV light. A 100 bp ladder (Amersham Biosciences) served as a molecular size marker.

**RESULTS AND DISCUSSION**

*F. nucleatum*, a bacterium that is found commonly in infants’ mouths (Könoenen et al., 1999b) and nasopharynges during AOM (Könoenen et al., 1999c), was selected as a representative to demonstrate the possible translocation of anaerobic bacteria via saliva to the nasopharynx. In the present study, AP-PCR was used for the clonal typing of 172 clinical *F. nucleatum* isolates from the saliva and nasopharynx of eight infants who were positive for nasopharyngeal *F. nucleatum*.

Although numerous investigators have used AP-PCR to demonstrate similarity and/or dissimilarity between bacterial strains, only a few have used this method for the differentiation of *F. nucleatum* (George et al., 1997; Avila-Campos et al., 1999). Of the 12 primers tested for AP-PCR typing, eight resulted in poor amplification when tested on the type strains of the four human *F. nucleatum* subspecies, whereas four primers, C1, C2, D8635 and D11344, revealed unique and reproducible fingerprints (Fig. 1a–d). This is in line with previous studies that reported discriminating AP-PCR patterns with the four selected primers for different *Fusobacterium* strains (George et al., 1997; Narongwanichgarn et al., 2001). Amplification patterns of the 172 clinical isolates generally consisted of two to five major amplicons, but ranged up to 13 amplicons (Fig. 1a–d). Major amplicons and consistent minor bands of each isolate were inspected visually and compared with the amplification patterns of other isolates from the same infant. Isolates that shared an amplification pattern derived from one primer usually...
shared the patterns constructed with the other three primers. In a few cases, the other primers separated isolates that shared an identical AP-PCR pattern with one primer. Generally, two or three AP-PCR types were detected among salivary isolates from each infant on one sampling occasion and, in some cases, the same AP-PCR type could be seen on a subsequent sampling occasion, 6 months later (data not shown). Fig. 1(a–d) presents the AP-PCR patterns, revealed by using the four selected primers, for the \textit{F. nucleatum} isolates from five infants (C, I, M, O and P) with matching nasopharyngeal and salivary isolates. Two nasopharyngeal \textit{F. nucleatum} isolates were available from each of three infants; the NP isolate collected during health and the NPA isolate collected during AOM from infant C at 6 and 14 months of age, respectively, represented different clones, as did the simultaneously collected NP isolates from infant O at 18 months and the NPA isolates from infant P at 23 months of age (Table 1).

In five of the eight infants who were positive for nasopharyngeal \textit{F. nucleatum}, identical AP-PCR types were found among salivary and nasopharyngeal isolates (Table 1). Saliva was collected from the buccal area of the mouth, whereas NP and NPA samples were collected through the nasal cavity, thus excluding salivary contamination. As anaerobes seem to be present only transiently in the nasopharynx (Könnönen et al., 2003), the present observation indicates that the source of nasopharyngeal anaerobes is the oral cavity and that saliva is their transmission vehicle. In infant C, AP-PCR typing revealed an identical pattern from salivary \textit{F. nucleatum} collected at a healthy visit at 12 months of age and the subsequent NPA isolate that was collected during an AOM episode, 2 months later. In infants I, M and P, nasopharyngeal \textit{F. nucleatum} strains shared identical AP-PCR patterns with salivary \textit{F. nucleatum} that was isolated 3.5, 2.5 and 1 months after their AOM episodes, respectively (Fig. 1a–d and Table 1). In infant O, an identical AP-PCR pattern was found among the salivary and nasopharyngeal \textit{F. nucleatum} isolates that were collected simultaneously at 18 months of age (Fig. 1a–d and Table 1). Our failure to detect matching

\textbf{Fig. 1.} Amplification patterns of DNA from matching nasopharyngeal and salivary \textit{F. nucleatum} isolates from five infants and from four \textit{F. nucleatum} reference strains, which were amplified with primers C1 (a), D8635 (b), C2 (c) and D11344 (d). Lanes 2–3, infant C; lanes 4–5, infant I; lanes 6–7, infant M; lanes 8–9, infant O; lanes 10–11, infant P; lane 12, \textit{F. nucleatum} subsp. \textit{nucleatum} ATCC 25586\textsuperscript{T}; lane 13, \textit{F. nucleatum} subsp. \textit{polymorphum} ATCC 10953\textsuperscript{T}; lane 14, \textit{F. nucleatum} subsp. \textit{fusiforme} NCTC 11326\textsuperscript{T}; lane 15, \textit{F. nucleatum} subsp. \textit{vincentii} ATCC 49256\textsuperscript{T}; lane 16, negative control; lanes 1 and 17, 100 bp ladder.
nasopharyngeal and salivary isolates in three infants may be explained, on one hand, by limited numbers of salivary isolates typed and, on the other hand, by strain turnover among oral F. nucleatum populations in early childhood (Haraldsson et al., 2003). Whether anaerobic bacteria colonize the nasopharynx purely because of ecological changes that favour their growth or whether they could play an active role in the pathogenesis of AOM is not known. Respiratory pathogens present in the nasopharynx can be translocated through the Eustachian tube to the middle ear (Bluestone, 1996). A similar event may occur with anaerobes that are present in the nasopharynx during AOM (Könönen et al., 2003). Variation between F. nucleatum strains exists, for example, in terms of properties associated with virulence, such as bacterial attachment to and invasion of host epithelial cells (Han et al., 2000). It can be speculated that F. nucleatum clones with a high affinity for epithelial cells may have an advantage to persist on mucosa and may, conceivably, be involved in polymicrobial infections.

**ACKNOWLEDGEMENTS**

This work was presented in part at the 80th General Session of the International Association for Dental Research (IADR), San Diego, California, in March 2002. We thank Dr Ritva Syrjänen for clinical screening of the infants and Susan Nyfors and Anne Bryk for their assistance with handling of the strain collection. We also acknowledge the contribution of the late Professor Hannele Jousimies-Somer for her assistance with handling of the strain collection. We also acknowledge the contribution of the late Professor Hannele Jousimies-Somer for her contribution to this work. This work was supported financially by the Icelandic Research Fund for Graduate Students and Nordisk Forskerrudanningsakademi (NorFA).

**REFERENCES**


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**Table 1.** Match (+) or mismatch (−) between nasopharyngeal F. nucleatum (‘test strain’) and salivary F. nucleatum available from preceding, simultaneous or following samples collected from eight infants by 24 months of age

<table>
<thead>
<tr>
<th>Infant</th>
<th>Nasopharyngeal sample</th>
<th>Nasopharyngeal F. nucleatum isolated at age (months)</th>
<th>Salivary F. nucleatum at age (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A NPA</td>
<td>8.5</td>
<td>−</td>
<td>6</td>
</tr>
<tr>
<td>C NP</td>
<td>6</td>
<td>−</td>
<td>12</td>
</tr>
<tr>
<td>NPA</td>
<td>14.5</td>
<td>−</td>
<td>18</td>
</tr>
<tr>
<td>H NPA</td>
<td>21.5</td>
<td>−</td>
<td>24</td>
</tr>
<tr>
<td>I NPA</td>
<td>8.5</td>
<td>−</td>
<td>18</td>
</tr>
<tr>
<td>M NPA</td>
<td>9.5</td>
<td>−</td>
<td>18</td>
</tr>
<tr>
<td>N NPA</td>
<td>13.5</td>
<td>−</td>
<td>13</td>
</tr>
<tr>
<td>O NP</td>
<td>18</td>
<td>−</td>
<td>18</td>
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<tr>
<td>P NPA</td>
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<td>−</td>
<td>23</td>
</tr>
<tr>
<td>NPA</td>
<td>23</td>
<td>−</td>
<td>23</td>
</tr>
</tbody>
</table>

NA, Not available; ND, not determined.


