**16S rRNA Terminal Restriction Fragment Length Polymorphism for the Characterization of the Nasopharyngeal Microbiota**

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**Abstract**

A novel non-culture based 16S rRNA Terminal Restriction Fragment Length Polymorphism (T-RFLP) method using the restriction enzymes Tsp509I and Hpy166I was developed for the characterization of the nasopharyngeal microbiota and validated using recently published 454 pyrosequencing data. 16S rRNA gene T-RFLP for 153 clinical nasopharyngeal samples from infants with acute otitis media (AOM) revealed 5 Tsp509I and 6 Hpy166I terminal fragments (TFs) with a prevalence of >10%. Cloning and sequencing identified all TFs with a prevalence >6% allowing a sufficient description of bacterial community changes for the most important bacterial taxa. The conjugated 7-valent pneumococcal polysaccharide vaccine (PCV-7) and prior antibiotic exposure had significant effects on the bacterial composition in an additive main effects and multiplicative interaction model (AMMI) in concordance with the 16S rRNA 454 pyrosequencing data. In addition, the presented T-RFLP method is able to discriminate *S. pneumoniae* from other members of the Mitis group of streptococci, which therefore allows the identification of one of the most important human respiratory tract pathogens. This is usually not achieved by current high throughput sequencing protocols. In conclusion, the presented 16S rRNA gene T-RFLP method is a highly robust, easy to handle and a cheap alternative to the computationally demanding next-generation sequencing analysis. In case a lot of nasopharyngeal samples have to be characterized, it is suggested to first perform 16S rRNA T-RFLP and only use next generation sequencing if the T-RFLP nasopharyngeal patterns differ or show unknown TFs.

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**Introduction**

Previous studies on the nasopharyngeal microbiota have mainly relied on conventional culture identification of the major pathogens *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus* and *Moraxella catharrhalis*. These bacteria colonize the human nasopharynx as a first step in the pathogenesis of acute otitis media (AOM) [1,2,3,4]. However, selective analysis focusing only on a few pathogens may lead to an underestimation of the influence of a generally disturbed or altered nasopharyngeal microbiota on disease progression.

In recent years there has been significant development of non-cultural methods to characterize microbial communities of different human body sites [5,6,7,8]. Among these, next-generation sequencing (e.g., 454 and Illumina pyrosequencing) seems to be the most appropriate technique for the future. It is based on the amplification of 16S rRNA gene which has been widely used as a bacterial classification marker [9]. Using primers specific for conserved border regions and subsequent analysis of the variable sections amplified in-between allows species identification. However, there is no standardized protocol for analyzing high throughput data and careful evaluation is needed. Among the parameters, which could have an influence on the outcome of the analysis are i) the choice of primers, ii) the DNA extraction protocols, iii) the amplification conditions, iv) the alignment of sequences and v) the Operational Taxonomic Unit (OTU) definition.

In a recent study, we investigated the influence of antibiotic consumption and vaccination with the conjugated 7-valent pneumococcal polysaccharide vaccine (PCV-7) on the nasopharyngeal microbiota in children with and without AOM using 454 16S rRNA sequencing [10]. Within this study, an alternative 16S rRNA Terminal Restriction Fragment Length Polymorphism (T-RFLP) method to characterize the microbiota of the nasopharynx was developed and validated. This novel approach offers the advantages of being quick, easy to handle and cheap.

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**Materials and Methods**

**Ethics statement.** Samples used for this study were collected within a nationwide surveillance program from outpatients with acute otitis media, which is ongoing since 1998. This surveillance program is part of the governmental public health surveillance and...
is therefore exempted from approval by Institutional Review Boards.

In silico Design of Terminal Restriction Fragment Length Polymorphism (T-RFLP)

We downloaded 38 16S rRNA sequences of bacteria reported to be characteristic for the nasopharynx [10] which were then trimmed in 4 different groups ranging from 8F-907, 8F-1391, 341–907 and 341–1391 (E. coli numbering). These groups were uploaded to the restriction Enzyme Picker website (http://rocaplab.ocean.washington.edu/tools/repk) to select enzymes which showed the highest possible discriminatory power. This website stores 16S rRNA sequences and shows a ranking and combination of enzymes for optimal bacterial 16S diversity coverage. Moreover, an emphasis was laid on the fact that the enzymes could distinguish the ‘commensal’ Mitis group members from Streptococcus pneumoniae. Based on these criteria, product sizes ranging from 8F-907 using the restriction enzymes TSP509I and Hpy166II were received.

For PCR reactions, only forward primer 8F was labeled with Carboxyfluorescein (FAM) as in silico analysis revealed that labeling of reverse primer 907 would not lead to additional discrimination. In silico fragment lengths of common nasopharyngeal species using the above criteria were received using Mica (http://mica.ibest.uidaho.edu/trflp.php), assuming that experimental results will differ by about 1%.

DNA Extraction from Bacterial Cultures and PCR Amplification

To receive the in vitro terminal fragment (TF) lengths (compared to in silico as above) of common nasopharyngeal species, chromosomal DNA was extracted from Streptococcus mitis, S. pseudopneumoniae, S. pneunmoniae, S. oralis, S. gordonii, S. pyogenes, S. sanguinis, Pseudomonas aeruginosa, Rhodococcus equi, Corynebacterium pseudodiphtheriticum, Staphylococcus epidermidis, S. aureus, Haemophilus influenzae, Moraxella catarrhalis and Klebsiella pneumoniae as previously described [11,12]. PCR reactions were performed in a total volume of 50 μl using 1× fast start Taq reaction buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 1 μM of primer 8F FAM-AGA GTT TGA TCC TGG CTC AG and 1 μM of primer 907R CCG TCA ATT CMT TTG AGT TT, 0.2 μl of Fast start Taq Polymerase (Roche, Rotkreuz, Switzerland) and 2 μl of extracted DNA (2 ng/μl). PCR cycling conditions included a heat activation step at 95°C for 6 min, 35 cycles of denaturation at 95°C (30 s), annealing at 59°C (30 s) and elongation at 71°C (2 min) and a final elongation step at 71°C for 7 min. PCR products were purified using the Promega Wizard (Promega, Madison, WI).

T-RFLP Digestion and Detection Limits

Purified PCR products were checked for correct amplification size and purity using the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA) with the DNA 7500 LabChip kit.

Table 1. Predicted and observed terminal restriction fragments (TFs) in bp after separate digestion with TSP509I and Hpy166I.

| Sample species name           | TSP509I | Hpy166I |
|------------------------------|---------|---------|
|                              | In silico | In vitro | In silico | In vitro |
| Streptococcus mitis          | 207 204 | 470 465 |
| Streptococcus pseudopneumoniae | 671 665 | 470 465 |
| Streptococcus pneumoniae      | 671 664 | 206 202 |
| Streptococcus gordonii        | 186 195 | 480 475 |
| Streptococcus pyogenes        | 195 189 | 804 795 |
| Streptococcus sanguinis       | 174 169 | 472 467 |
| Pseudomonas aeruginosa        | 547 538 | 793 784 |
| Rhodococcus equi              | 523 515 | 79 73   |
| Corynebacterium pseudodiphtheriticum | 524 516 | 415 410 |
| Staphylococcus epidermidis    | 558 549 | 805 794 |
| Staphylococcus aureus         | 558 549 | 472 465 |
| Haemophilus influenzae        | 469 463 | 798 790 |
| Moraxella catarrhalis         | 541 535 | 788 780 |
| Klebsiella pneumoniae         | 549 540 | 796 784 |

TFs were received using the labeled 8F primer.

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Figure 1. Terminal fragment (TF) prevalence in nasopharyngeal swabs from 153 infants with acute otitis media. Shown are the TF frequencies (in %) for the two different restriction enzymes (blue for Tsp509I and red for Hpy166II).

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The enzymatic digestions were performed in a total volume of 20 µl using 40 ng of the amplified and purified PCR product, 1 µl NEB Reaction Buffer (New England Biolabs, Ipswich, MA) 1 for digestion with Tsp509I or 4 (Hpy166II) and 0.5 µl of the restriction enzymes Tsp509I or Hpy166II. Digestion conditions were 3 h at 65°C for Tsp509I and 3 h at 37°C with 20 min enzyme inactivation at 65°C for Hpy166II, respectively. Digested products were purified and capillary electrophoresis was performed on an AB 3130 genetic analyzer (Applied Biosystems, Rotkreuz, Switzerland) as previously described [12]. Detection limits were tested for Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis and Corynebacterium pseudoplu Hitcunicum by amplifying serial dilutions.

### Table 2. DNA sequencing and subsequent taxa assignments of the most prevalent Tsp509I terminal fragments (TFs) derived from 15 samples with different T-RFLP patterns.

| In silico TF in bp | In vitro TF in bp | Taxa assignment | Number of samples with TF (%) | Number of DNA Sequences |
|-------------------|------------------|----------------|-------------------------------|-------------------------|
| 671               | 665              | Streptococcus spp. 1* | 105 (68.6)                   | 84                      |
| 541               | 535              | Moraxella spp. 1     | 48 (31.4)                    | 7                       |
| 524               | 518              | Corynebacterium spp. | 45 (29.4)                    | 32                      |
| 207               | 204              | Streptococcus spp. 2* | 32 (20.9)                    | 14                      |
| 469               | 464              | Haemophilus influenzae | 21 (13.7)                    | 3                       |
| 358               | 352              | Neisseria spp.       | 13 (8.5)                     | 8                       |
| 153 & 156         | 148              | Moraxella spp. 2 & Sphingobacterium spp. | 9 (5.9)                   | 3 & 14**                |
| 558               | 550              | Staphylococcus spp.  | 7 (4.6)                      | 8                       |
| 501               | 496              | Prevotella spp.      | 5 (3.3)                      | 5                       |
| 195               | 190              | Streptococcus pyogenes | 5 (3.3)                    | 4                       |
| 561               | 552              | Gemella spp.         | 4 (2.6)                      | 1                       |
| 591               | 583              | Granulicatella spp.  | 4 (2.6)                      | 3                       |
| 551               | 544              | Stenotrophomonas maltophilia | 3 (2.0)                   | 8                       |
| 190               | 185              | Chrysebacterium spp. 1 | 3 (2.0)                  | 6                       |
| 702               | 697              | Chrysebacterium spp. 2 | 2 (1.3)                   | 6                       |
| 204               | 199              | Microbacterium spp. 1 | 2 (1.3)                  | 14                      |
| 374               | 371              | Fusobacterium spp.   | 2 (1.3)                      | 1                       |
| 169               | 167              | Turicella spp.       | 1 (0.7)                      | 6                       |
| 549               | 540              | Klebsiella spp.      | 1 (0.7)                      | 1                       |
| 645               | 640              | Propionibacterium spp. | 1 (0.7)                   | 3                       |
| 209               | 206              | Porphyromonas spp.   | 1 (0.7)                      | 1                       |
| 529               | 524              | Microbacterium spp. 2 | 1 (0.7)                   | 1                       |
| TOTAL             |                  |                   | 153 (100)                    | 233                     |

*Streptococcus pneumoniae and pseudopneumoniae are included in Streptococcus spp. 1 while other members of the Mitis group are included in Streptococcus spp. 2. Using the second restriction enzyme (Hpy166II) Streptococcus spp. 1 can be further divided into S. pneumoniae and S. pseudopneumoniae (see text).

**Two different bacterial assignments were retrieved for TF with 148 base pairs (bp).

The enzymatic digestions were performed in a total volume of 20 µl using 40 ng of the amplified and purified PCR product, 1 µl NEB Reaction Buffer (New England Biolabs, Ipswich, MA) 1 (for digestion with Tsp509I) or 4 (Hpy166II) and 0.5 µl of the restriction enzymes Tsp509I or Hpy166II. Digestion conditions were 3 h at 65°C for Tsp509I and 3 h at 37°C with 20 min enzyme inactivation at 65°C for Hpy166II, respectively. Digested products were purified and capillary electrophoresis was performed on an AB 3130 genetic analyzer (Applied Biosystems, Rotkreuz, Switzerland) as previously described [12]. Detection limits were tested for Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis and Corynebacterium pseudopneumoniae.(see text). Detection limits were tested for Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis and Corynebacterium pseudopneumoniae by amplifying serial dilutions.

16S rRNA T-RFLP of Nasopharyngeal Swabs from Infants with AOM

We processed nasopharyngeal swabs collected from children younger than 2 years within an on-going nationwide surveillance program during four winter seasons in Switzerland [13]. The presence of S. pneumoniae was verified by culture and phyNCR-PCR data generated in earlier studies [11,12,14]. PCR and 16S gene T-RFLP digestions with Tsp509I or Hpy166II were performed as described above. The resulting T-RFLP electropherograms were uploaded to the Peak Scanner Software v1.0 (Applied Biosystems, Carlsbad, CA). Background noise was eliminated by setting the cut off at 50 fluorescence units (FU). The resulting sizing table was exported and the data was prepared for uploading to the T-RFLP analysis expedited software (T-Rex), which included creating a ‘genemapper’ and label file [15,16]. For the genemapper file, a different fluorophor was assigned to the values derived from Hpy166II (Green) as compared to Tsp509I (Blue), which allowed the combined analysis of both enzymes for each sample at the same time. For processing the data with T-Rex, no additional filter for noisy peaks was set but clustering thresholds of 2.0 were used to align the TFs.

Cloning and Sequencing

Purified PCR products were cloned into the pGEM-T Easy Vector (Promega) as per manufacturer’s instructions. 20 colonies per ligation were picked at random. Plasmid DNA was purified using the Qiagen Plasmid Miniprep Kit and sequenced as described [6]. This protocol still results in longer reads as compared to pyrosequencing protocols and therefore allows precise assignment of bacterial taxa. Resulting sequences were submitted to GenBank (accession numbers JQ745789–JQ746034).

Statistical Analysis

For comparing TF mean richness (mean number of TFs within all samples) between different environments, we performed unpaired t-test using Statview software defining significance if the two tailed p-value was <0.05. For the additive main effects and multiplicative interaction model (AMMI) of T-Rex, data included was based on TF presence, but not height or peak area [15,16].
AMMI plot was derived by R as suggested by T-Rex. The function “mantel” in R was used to find the Mantel statistic as a matrix correlation between two dissimilarity matrices derived by the two enzymes.

Results

In silico Design of Terminal Restriction Fragment Length Polymorphism (T-RFLP) for the Nasopharynx

We first downloaded the complete 16S rRNA sequences of 38 bacterial strains characteristic for the nasopharynx based on previous studies from the ncbi webpage (www.ncbi.nlm.nih.gov), which we sheared in 4 different primer pair groups ranging from 8F-907, 8F-1391, 341–907 and 341–1391 (E. coli numbering). All commercially available restriction enzymes (n = 189) were then tested for optimal discrimination of these 16S rRNA gene sequences. The groups 8F-907 and 8F-1391 were found to be superior and primers for 8F-907 were subsequently designed. We then manually inspected the top 10 restriction enzymes for the additional ability to discriminate between S. pneumoniae and other members of the Mitis group streptococci but also within Haemophilus spp. and Staphylococcus spp. (Table S1). We identified 2 enzymes (Tsp509I and Hpy166II), fulfilling these criteria which we subsequently tested in the laboratory.

In vitro Evaluation of the Restriction Enzymes Tsp509I and Hpy166II

For a proof of principle, we subsequently verified in vitro 14 of the 38 species that had been examined by in silico analysis. The strains represented the following taxa: Corynebacteriaceae, Streptococcaceae, Staphylococcaceae, Haemophilus influenzae, Moraxella catarrhalis and Klebsiella pneumoniae (Table 1). Results showed that TF sizes calculated in silico differed consistently about 1% of the predicted in vitro values (Table 1). The detection limit of the in vitro T-RFLP experiment was about 30 genome copies ml⁻¹ with the exception of H. influenzae and M. catarrhalis (100 copies ml⁻¹; data not shown).

A differentiation between S. pneumoniae and other members of Mitis group streptococci could also be achieved (Table 1). However, the restriction enzyme TSP509I alone could not differentiate between S. pneumoniae and S. pseudopneumoniae. This could only be achieved when results of TSP509I and Hpy166I were analyzed in combination (Table 1).

Overall TF Richness and Taxonomic Assignment

16S rRNA T-RFLP was performed for 153 nasopharyngeal samples from infants with otitis media using the two different restriction enzymes. Overall only 5 (using Tsp509I) and 6 (Hpy166II) TFs achieved a prevalence of more than 10% (Figure 1). Overall, the mean TF richness was 2.3 TFs (±1.5) (Tsp509I) and 3.5 TFs (±2.0) (Hpy166II) for the 153 samples analyzed and we received sample heterogeneity values of 17.0
We then chose 15 nasopharyngeal samples covering all TFs with a prevalence >6% for subsequent cloning and sequencing for taxa assignment. A total of 245 sequences of approximately 900 bp were received of which 232 could be assigned to a TF (Table 2). All TFs with a prevalence >6% could be identified with DNA sequencing allowing a sufficient description of bacterial community changes for the most important bacterial taxa. *Streptococcus* spp., *H. influenzae*, *Moraxella* spp., *Corynebacterium* spp. and *Staphylococcus* spp. were identified most frequently (Table 2).

**Correlation of Distance Matrices and Comparison with 454 Pyrosequencing and Pneumococcal Culture Data**

The performance of the 2 restriction enzymes was evaluated by creating pairwise distance matrices for all 153 samples and a subsequent mantel test. This revealed a high correlation between the two restriction enzymes ($r = 0.65$; Figure 2).

Comparing T-RFLP with recently published 454 pyrosequencing data revealed that *Moraxellaceae*, *Streptococcaceae*, *Pasturellaceae*, *Staphylococcaceae* and *Corynebacteriaceae* were the most frequent bacterial families within the 153 samples with both methods [10]. However, the absolute frequencies of *Moraxellaceae and Pasturellaceae*, were increased in the 454 compared to the T-RFLP data set. This therefore means that the two methods showed different absolute sensitivity values.

Pneumococcal culture detected *S. pneumoniae* in 80 samples (52.3%). In comparison, 79 *S. pneumoniae* could be identified with the T-RFLP method if results of TSP509I and Hpy166II were analysed in combination (100% specificity and 98.8% sensitivity).

**Demographic Factors Influencing the Mean Richness of TFs and Whole Microbial Community Comparison**

Measuring mean richness differences within sex, day care and recent treatment with antibiotics did not reveal significant outcomes (Table 3). In contrast, PCV-7 vaccination status, year of sampling (prevaccine versus vaccine era) and age were identified as factors significantly influencing TF mean richness. Using an Additive Main Effects and Multiplicative Interaction Model (AMMI) revealed higher percentages of signal compared to noise interaction effects for prior antibiotic exposure (4.8 versus 1.0), samples from the vaccine era (3.4 versus 0.8) and age (1.5 versus 1.2). AMMI findings therefore conclude that antibiotic exposure and sampling in vaccine era mostly influenced the nasopharyngeal microbiota which was also illustrated in a Principal Component Analysis (PCA) plot (Figure 3).

These findings are in line with the pyrosequencing data published recently and therefore show the validity of the proposed

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Figure 3. Additive Main Effects and Multiplicative Interaction Model (AMMI) analysis of bacterial T-RFLP data sets. Using the software T-Rex (T-RFLP analysis Expedited) [15], T-RFLP profiles were first analyzed individually and then grouped according to antibiotic exposure, vaccine season and vaccine status in the software R. The quadrates illustrate the centroids of the individual data points and represent the following: A) prevaccine era, not vaccinated, no antibiotic exposure. B) vaccine era, not vaccinated, no antibiotic exposure. C) vaccine era, vaccinated, no antibiotic exposure. A_ab) prevaccine era, not vaccinated, antibiotic exposure. B_ab) vaccine era, not vaccinated, antibiotic exposure. C_ab) vaccine era, vaccinated, antibiotic exposure. According to AMMI, antibiotic exposure (grey versus black) and vaccine season (A and A_ab versus others) significantly influences the microbiota (see text and Table 3). doi:10.1371/journal.pone.0052241.g003
Sequencing additionally revealed that the prevalence of community members within study subjects was small and fell beyond detection limit of the 16S rRNA gene T-RFLP. Their importance as persistent or transient colonizers has to be revealed in the future.

**Discussion**

In this study, a novel 16S rRNA T-RFLP method was developed using 2 restriction enzymes (TSP509I and Hpy166II) able to discriminate colonizers of the nasopharynx most efficiently. Despite known high 16S rRNA gene sequence homology for members of the Mitis group, the enzymes were also able to discriminate among streptococcal species. So far, differentiation of *S. pneumoniae* from other Mitis group members was hampered by the close genetic homogeneity and horizontal gene transfer [17]. Analyzing 634 strains, Scholz et al. recently demonstrated a mutation in the 16S rRNA gene which reliably discriminated pneumococci from other members of the Mitis group [18]. This SNP is the corresponding cutting site for the two restriction enzymes TSP509I and Hpy166II and therefore enables this specific discrimination.

Within our study, we used the *T-REX* (T-RFLP analysis EXPedited) for data analysis [15]. This software has originally been designed for the analysis of T-RFLP data from soil communities but its applicability for studies of the human microbiome has recently been demonstrated [19]. This software offers two approaches for an automated alignment (binning) of peaks. We used the option which models the approach taken by the software program T-Align [20] and set the clustering threshold at 2.0. Setting an appropriate value for the clustering threshold is crucial for the evaluation of the TF richness. Setting it too low or too high may lead to an over- and underestimation of TF richness, respectively. This is a drawback of the methodology but newly developed, high throughput sequencing technologies face the same challenge as analyzing sequencing data also involves defining of Operational Taxonomic Units (OTU) based on an incidental cut-off (most often 97% sequence identity) [21].

Another important factor for the optimal detection sensitivity is the choice of primers for the experiment. A recent study using the same forward but a different reverse primer revealed that T-RFLP was able to detect *S. aureus* when there were more than 30 genome

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**Table 3. Terminal fragment (TF) richness and interaction effects resulting from two restriction enzymes (Tsp509I and Hpy166II).**

|                                | Sex          | Day care**    | Antibiotic treatment | Seven valent pneumococcal polysaccharide vaccine (PCV-7) | Season                     |
|--------------------------------|--------------|---------------|----------------------|----------------------------------------------------------|-----------------------------|
|                                | Female       | Male          | Yes                  | Yes                                                      | Vaccination era             |
| TF richness Tsp509I             | n            | Mean SD       | p                    | Mean SD p                                               | p                           |
|                                |              |               |                      |                                                          |                             |
|                                | 74           | 2.3 1.6 0.68  | 0.05                 | 3.45 2.03 0.76                                          | -0.1 1.4                    |
|                                | 79           | 2.2 1.4       | 0.05                 | 3.48 2.10                                               |                             |
| TF richness Hpy166II            |              |               |                      |                                                          |                             |
|                                | 3.52 1.96 0.78 | 0.05         |                      | 4.08 2.25 <0.01                                         | 1.5 1.2                     |
|                                | 3.38 2.08     |               |                      |                                                          |                             |
| Interaction effects (%)*        |              |               |                      |                                                          |                             |
|                                |              |               |                      |                                                          |                             |
|                                | -0.1 1.4     |               |                      |                                                          |                             |
|                                | 1.5 1.2      |               |                      |                                                          |                             |

*Received by Additive Main Effects and Multiplicative Interaction Model (AMMI). Variation due to interaction effects can have a considerable range and reflect how similar or dissimilar the microbial communities are [15]. Therefore the more positive the difference between signal and noise, the more likely the parameters are influencing the communities [15,16].

**No data concerning day care for 10 children.**
copies µl⁻¹ in 50 µl DNA extract from a nasal swab [22]. While we discovered a comparable detection limit for Streptococcus spp. and Corynebacterium spp., this was not the case for Haemophilus influenzae and Moraxella spp., probably due to a SNP present in the conserved region of the forward primer and therefore suboptimal PCR efficiency. Indeed, when comparing T-RFLP with 454 sequencing approach, but, in contrast, is able to discriminate individual bacterial members of the nasopharynx in concordance with previously described results [10].

In order to show the validity of the proposed T-RFLP method we additionally investigated if the same factors were influencing the microbiota of the 153 samples as received by recently published 454 pyrosequencing data [10]. We hypothesized that although the detection limit and amplification protocols of the two methods are slightly different the outcome had to be same in order to show the robustness of the T-RFLP method. The two main factors influencing the composition of the nasopharyngeal flora were prior exposure to antibiotics and PCV-7 vaccination. However, 16s rRNA gene T-RFLP only identified seasonality (prevaccine versus vaccine era) and not PCV-7 status as a driving factor for the composition of the microbiota while 454 data showed an influence of both. Age, day care and sex did not show an influence on the microbiota which was in accordance with our previous study [10].

In conclusion, this study describes the development and validation of a 16s rRNA T-RFLP method to characterize the nasopharyngeal microbiota. It is not as sensitive for Haemophilus influenzae and Moraxella spp. as a recently published 454 pyrosequencing approach, but, in contrast, is able to discriminate bacterial species within the Mitis group of streptococci as the

| Table 4. Prevalence of the most frequent bacterial taxa. |
|---|---|---|---|---|---|---|---|---|
| | Total | Haemophilus influenzae** | Corynebacterium spp.** | Moraxella spp.** | Streptococcus spp. (1)** | Streptococcus spp. (2)** |
| | n | n (%) | n (%) | n (%) | n (%) | n (%) |
| Sex | | | | | | | | |
| female | 74 | 8 (11) | 0.35 | 22 (30) | 1 | 26 (35) | 0.38 | 51 (69) | 1 | 15 (20) | 1 |
| male | 79 | 13 (16) | | 23 (29) | 1 | 22 (28) | 1 | 54 (68) | | 17 (22) |
| Day care* | | | | | | | | | | | |
| yes | 54 | 7 (13) | 1 | 14 (26) | 0.2 | 19 (35) | 0.71 | 41 (76) | 0.14 | 11 (20) | 1 |
| No | 89 | 13 (15) | | 31 (35) | 1 | 28 (31) | | 56 (63) | | 18 (20) |
| Age | | | | | | | | | | | |
| 0 years | 61 | 9 (15) | 0.81 | 22 (36) | 0.15 | 22 (36) | 0.37 | 37 (61) | 0.11 | 16 (26) | 0.22 |
| 1 years | 92 | 12 (13) | | 23 (25) | 1 | 26 (28) | | 68 (74) | | 16 (17) |
| Antibiotic treatment | | | | | | | | | | | |
| Yes | 39 | 10 (26) | 0.03 | 7 (18) | 0.1 | 10 (26) | 0.43 | 21 (54) | 0.03 | 11 (28) | 0.25 |
| No | 114 | 12 (11) | | 38 (33) | | 38 (33) | | 84 (74) | | 21 (18) |
| Seven valent pneumococcal polysaccharide vaccine (PCV-7) | | | | | | | | | | | |
| yes | 87 | 13 (15) | 0.64 | 19 (22) | 0.02 | 28 (32) | 0.86 | 57 (66) | 0.38 | 17 (20) | 0.69 |
| no | 66 | 8 (12) | | 26 (39) | 1 | 20 (30) | | 48 (73) | | 15 (23) |
| Season | | | | | | | | | | | |
| prevaccine era | 31 | 5 (16) | 0.77 | 16 (52) | 0.004 | 10 (32) | 1 | 23 (74) | 0.52 | 9 (29) | 0.22 |
| vaccine era | 122 | 16 (13) | | 29 (24) | | 38 (31) | | 82 (67) | | 23 (19) |

*No data concerning day care for 10 children.

**Data based on Tsp509I received TFs of 464 bp (Haemophilus influenzae), 518 bp (Corynebacterium spp.), 535 bp (Moraxella spp.), 665 bp (Streptococcus spp. 1) and 204 bp (Streptococcus spp. 2). Streptococcus pneumoniae and pseudopneumoniae are included in Streptococcus spp. 1 while other members of the Mitis group are included in Streptococcus spp. 2 (see Table 2).

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In conclusion, this study describes the development and validation of a 16s rRNA T-RFLP method to characterize the nasopharyngeal microbiota. It is not as sensitive for Haemophilus influenzae and Moraxella spp. as a recently published 454 pyrosequencing approach, but, in contrast, is able to discriminate bacterial species within the Mitis group of streptococci as the
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