Multiple Colonization with *S. pneumoniae* before and after Introduction of the Seven-Valent Conjugated Pneumococcal Polysaccharide Vaccine

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Abstract

**Background:** Simultaneous carriage of more than one strain of *Streptococcus pneumoniae* promotes horizontal gene transfer events and may lead to capsule switch and acquisition of antibiotic resistance. We studied the epidemiology of cocolonization with *S. pneumoniae* before and after introduction of the seven-valent conjugated pneumococcal vaccine (PCV7).

**Methodology:** Nasopharyngeal swabs (n 1120) were collected from outpatients between 2004 and 2009 within an ongoing nationwide surveillance program. Cocolonization was detected directly from swabs by restriction fragment length polymorphism (RFLP) analysis. Serotypes were identified by agglutination, multiplex PCR and microarray.

**Principal Findings:** Rate of multiple colonization remained stable up to three years after PCV7 introduction. Cocolonization was associated with serotypes of low carriage prevalence in the prevaccine era. Pneumococcal colonization density was higher in cocolonized samples and cocolonizing strains were present in a balanced ratio (median 1.38). Other characteristics of cocolonization were a higher frequency at young age, but no association with recurrent acute otitis media, recent antibiotic exposure, day care usage and PCV7 vaccination status.

**Conclusions:** Pneumococcal cocolonization is dominated by serotypes of low carriage prevalence in the prevaccine era, which coexist in the nasopharynx. Emergence of such previously rare serotypes under vaccine selection pressure may promote cocolonization in the future.

Introduction

The epidemiology of *Streptococcus pneumoniae* is changing since the introduction of the seven-valent conjugated pneumococcal polysaccharide vaccine (PCV7) [1,2,3,4]. Non-vaccine serotypes are included in PCV7 and emergence of new serotypes may affect the pneumococcal epidemiology and different techniques employed may be responsible for such variation. Studies on cocolonization have been hampered by the lack of sensitive, economic and easy to apply methods for the detection of multiple strains in nasopharyngeal samples. Recently, we reported on a new molecular method for the detection of multiple colonization [19]. In this study we applied this technique to a large collection of nasopharyngeal swabs (n 1120) collected before and after the introduction of PCV7 in Switzerland in 2006. This report presents novel information about the epidemiology, determinants and time trends of multiple colonization with *S. pneumoniae* in the vaccine era. We hypothesized that reduction of the prevalent serotypes included in PCV7 and emergence of new serotypes may affect the rate of cocolonization and the strains or serotypes involved in cocolonization.

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Materials and Methods

Ethic statement
Nasopharyngeal swabs used for this study were collected within a nationwide surveillance program from outpatients with acute otitis media or pneumonia, which is ongoing since 1998 [20,21]. This surveillance program is part of the governmental public health surveillance and is therefore exempted from approval by Institutional Review Boards.

Collection of nasopharyngeal swabs
For this study swabs from four winter seasons between 2004 and 2009 were analyzed. Period 1 spanned from December 2004 to February 2005, period 2 from November 2006 to March 2007, period 3 from November 2007 to March 2008 and period 4 from November 2008 to March 2009. Parts of the data from period 1 have been presented earlier [19]. In Switzerland, the seven-valent conjugated pneumococcal polysaccharide vaccine (PCV7) has been recommended since May 2006 to all children under the age of 24 months in a three dose schedule given at 2, 4 and 12 months and since August 2006 the vaccine is reimbursed by health insurance.

Diagnostic work-up of nasopharyngeal swabs
Processing of swabs for culture and capsular serotyping of S. pneumoniae and for direct molecular detection of pneumococcal colonization by plyNCR PCR and subsequent restriction fragment length polymorphism (plyNCR RFLP) and terminal-RFLP (T-RFLP) has been described in detail previously [19,22]. Briefly, swabs were streaked out onto a CSBA (Columbia sheep blood agar) plates and were then eluted in 800 μl of either transport medium for chlamydia and viruses (TMCV, [19]) or in water. The swab solution was stored at −80°C until further use. All pneumococcal isolates detected by primary conventional culture were subcultured once on CSBA plates and stored at −80°C (Protect®, Technical Service, Lancashire, UK). In addition, during study period 4 the entire bacterial lawn grown on the primary CSBA culture plate was collected and stored at −80°C (Protect®, Technical Service, Lancashire, UK).

Detection of pneumococcal colonization and multiple colonization by plyNCR PCR, RFLP and T-RFLP
Chromosomal bacterial DNA was isolated from stored swab solution and plyNCR PCR was performed as described previously [19]. In order to estimate the amount of pneumococcal DNA in swab samples as a surrogate for colonization density chromosomal DNA of three clinical isolate of S. pneumoniae (strain B109.48 of serotype 9V, strain 109.24 of serotype 19F, strain 312.57 of serotype 3) was isolated [23] and DNA concentration was determined with a Nanodrop ND-100 spectrophotometer (Nano- Technologies, Wilmington, DE). DNA was then diluted with double distilled water to amounts of 100, 10, 1, 0.1, 0.01 and 0.001 ng. After plyNCR PCR amplification DNA concentration was determined using the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). A standard curve was generated by plotting PCR-product concentration against amount of DNA. This showed a linear relationship for the range of bacterial DNA concentrations usually found in nasopharyngeal swabs (data not shown). plyNCR RFLP analysis was performed as described in [19] and samples for which the sum of the size of the bands obtained after restriction enzyme digestion was greater than the size of the undigested PCR product were further analyzed for cocolonization. In order to confirm the presence or absence of S. pneumoniae in samples with plyNCR PCR products of atypical band size or >1 band, PCR for panA was performed as described [19,24].

T-RFLP analysis was used to determine the number of strains and their relative quantity in cocolonized samples as described except that capillary electrophoresis was performed on an ABI Genetic Analyzer 3130 for samples in time periods 2 to 4 [19]. In addition, T-RFLP with the two enzymes Bsu36I and BsaI was used to detect cocolonization in samples with low pneumococcal DNA content.

Detection of capsular serotypes
Capsular serotypes were determined for cultured isolates by the “agglutination reaction” (Statens Serum Institute, Copenhagen, Denmark). Multiplex PCR and microarray were employed for molecular detection of serotypes in cocolonized samples. Both techniques are usually not performed directly on swab material, due to lower sensitivity and requirement of large amounts of swab material. Therefore, we used frozen stocks from primary culture isolates and frozen stocks from bacterial lawns from the primary culture plate for these tests. Multiplex PCR for 29 capsular serotypes/serogroups was performed according to the method described by Pai et al. [25] (except that PCR analysis was performed with the Agilent bioanalyzer). Molecular serotyping by microarray was performed using the BµG@S SP-CPSw1.3.0 microarray using standard protocols. Briefly, DNA samples were fluorescently labelled and hybridized to the Agilent 8×15K format microarray according to manufacturer’s instructions for the Agilent genomic DNA enzymatic labelling and oligo aCGH hybridisation reagent kits. Microarray data was statistically analysed using a Bayesian hierarchical model (Newton et al., unpublished data) to determine the serotype, or combination of serotypes, present in the sample and assign a relative abundance of each serotype detected.

Statistical analysis
Statistical analysis was done in StatView® version 5.0 (SAS Institute Inc., Cary, NC). Differences between means were tested by the Student’s T-test or the Mann-Whitney U test and proportions were compared with the Chi Square or Fisher’s exact test, as appropriate. Risk factors identified by univariate statistics were entered into ANOVA or a logistic regression model as appropriate. The final model contained the largest number of variables with p ≤0.05. A cut-off of p ≤0.05, two tailed, was used for all statistical analyses.

Results
Pneumococcal carriage rate by culture and PCR
The overall pneumococcal carriage rate was 45.8% (514 of 1120 swabs) by culture or PCR, the rate was 39.7% by bacterial culture, and 45.6% by PCR. Sixty nine samples (6.1%) were positive by PCR only and three samples were positive by culture only. A total of 389 of 511 (74.3%) PCR positive samples were screened for cocolonization by plyNCR RFLP and T-RFLP. The remaining 122 samples could not be processed, because quantity of DNA recovered from swabs was insufficient for enzyme digestion (n = 83), or because plyNCR PCR amplified more than one band (n = 40), which inhibited interpretation of restriction enzyme patterns [19].

Pneumococcal vaccination rates
Patients were mostly children and about half of patients were below the age of 5 years (49.0%, n 549). PCV7 vaccination rates increased during the study period. Among children less than 5 years of age
Vaccination rates were 3.2% for study period 1, 33.3% for study period 2, 50% for study period 3, and 77.8% for study period 4. The corresponding rates for children 2 years of age for time periods 1, 2, 3, and 4 were 1.4%, 47.3%, 76.3%, and 88.4%, respectively. Therefore, study period 1 was considered the “prevaccine era” and the later seasons combined were defined as the “vaccine era”.

Figure 1. Ratio of strains present in cocolonized nasopharyngeal swabs. Strain ratios were determined from the peak heights in the chromatograms obtained by terminal-restriction fragment length polymorphism analysis (T-RFLP, as described in the methods section). The X-axis indicates the fold-higher presence of the more abundant strain. The three samples with three strains were excluded.

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Table 1. Univariate analysis for factors associated with carriage of single or multiple strains of *Streptococcus pneumoniae*.

| Factor                                | Cocolonization | Single colonization | Not colonized | p     |
|---------------------------------------|----------------|---------------------|---------------|-------|
|                                       | N %            | N %                 | N %           |       |
| Total                                 | 41 100         | 473 100             | 606 100       |       |
| Introduction of PCV7<sup>a</sup>     |                |                     |               |       |
| prevaccine era                         | 10 25.6        | 139 29.4            | 138 22.8      |       |
| vaccine era                           | 31 74.4        | 334 70.6            | 468 77.2      |       |
| Age (years)<sup>b</sup>              |                |                     |               |       |
| <2                                    | 14 34.1        | 138 29.2            | 119 19.8      | <0.001|
| 2–4                                   | 14 34.1        | 150 32.0            | 113 18.8      |       |
| 5–16                                  | 11 26.8        | 117 24.8            | 144 24.0      |       |
| 17–64                                 | 2 4.9          | 55 11.7             | 170 28.3      |       |
| >64                                   | 0 0            | 11 2.3              | 54 9.0        |       |
| Female gender<sup>c</sup>            | 17 41.5        | 207 44.0            | 286 47.6      | 0.43  |
| Antibiotic treatment<sup>d</sup>     | 8 20.5         | 55 12.9             | 84 15.4       | 0.29  |
| Day care<sup>e</sup> in children <5 years old | 7 26.9 | 106 39.5 | 72 33.6 | 0.24 |
| Recurrent otitis<sup>f</sup> media in children <5 years old | 4 15.4 | 55 21.2 | 47 22.3 | 0.71 |
| PCV7 vaccinated<sup>g</sup> in children <5 years old | 9 34.6 | 102 36.3 | 98 45.4 | 0.03 |

<sup>a</sup>PCV7 = 7-valent conjugated pneumococcal polysaccharide vaccine.
<sup>b</sup>No information was available about age for 7 patients.
<sup>c</sup>No information was available about gender for 4 patients.
<sup>d</sup>Antibiotic treatment during the 8 weeks before swab collection, no information was available for 108 patients (9.6%).
<sup>e</sup>No information about current day care use was available for 152 patients (13.5%).
<sup>f</sup>More than one otitis media episode during the past 12 months. No information was available about recurrent otitis media for 183 patients (16.3%).
<sup>g</sup>Vaccination with the seven-valent conjugated pneumococcal vaccine.

No information about vaccination status was available for 100 patients (8.9%). P-value is for the comparison between carriage of single or multiple strains of *S. pneumoniae* versus no carriage.

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Detection of multiple colonization

Cocolonization was detected in 41 nasopharyngeal swabs by RFLP or T-RFLP. Only seven of these 41 cocolonization events were also detected by conventional serotyping of multiple (n = 20) colonies from primary swab culture plates. Two different strains were present in 38 of the 41 samples and three samples contained three strains. The relative ratio of strains present in the same sample ranged from 1:1 to a maximum of 1:45, with a median ratio of 1:3.8 (Figure 1).

Frequency of multiple colonization

The rate of cocolonization was 3.6% among all samples (n 1120) and 7.9% among samples with detection of S. pneumoniae (n 514). The rate of cocolonization was similar before and after introduction of PCV7 with a prevaccine rate of 3.4% (10 of 287 samples) or 6.7% (10 of 149 samples with detection of S. pneumoniae) as compared to 3.7% (31 of 833 samples) or 8.4% (31 of 365 samples with detection of S. pneumoniae) after introduction of PCV7. Cocolonization rates were also stable over the four time periods analysed (data not shown).

Factors associated with multiple colonization

Cocolonization was more frequent at younger age (Table 1). The probability of cocolonization was not affected by recent antibiotic treatment, day care use, a history of recurrent acute otitis media or PCV7 vaccination status. However, pneumococcal colonization density (the concentration of the plyNCR PCR product) was significantly higher in samples with multiple colonization (p<0.001) (Table 2).

Multiple colonization and plyNCR RFLP-types

We have previously shown that plyNCR RFLP can be used for molecular typing of pneumococcal strains [22]. We were interested to analyze whether strains involved in cocolonization belonged to distinct RFLP types. A RFLP-type could be determined for 81 of the 85 pneumococcal strains present in samples with multiple colonization. The 81 strains grouped into 30 different RFLP types, of which 13 occurred at a relative frequency of ≥2% relative to all RFLP types detected in this study. The remaining RFLP types with a prevalence of <2% were designated “rare” and were grouped together into the category “others” for analysis. RFLP type 15 (p 0.01), type 17 (p 0.02), and type 19 (p 0.03) were significantly more frequent among cocolonized samples, and samples with only one strain had a higher proportion of type 2 (p 0.02) and type 11 (p 0.01). There were no time trends of RFLP type distribution between the prevaccine and vaccine era (data not shown). Pairs of RFLP types in cocolonized samples showed no predominance of a certain combination of RFLP-types (data not shown).

Multiple colonization and capsular serotypes

A serotype could be determined for 64 of 85 strains detected in the 41 cocolonized samples. The 64 strains grouped into 30 different serotypes and non-typeable pneumococci. Serotypes with

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**Table 2.** Univariate analysis of DNA quantity of plyNCR PCR products as a surrogate for pneumococcal colonization density in the nasopharynx.

| DNA concentration of PCR product | Proportion of samples with <10 ng/µl |
|---------------------------------|-------------------------------------|
|                                | mean  | SD  | p    | N | % | p |
| All                             | 26.6  | 20.2| 0.07 | 149| 31.7
| Age (years)*                    |       |     |      |   |   |   |
| <2                              | 29.1  | 20.5| 0.07 | 37 | 24.5
| 2–4                             | 27.2  | 19.4|      | 44 | 26.9
| ≥5                              | 24.3  | 20.5|      | 68 | 34.8
| Gender                          |       |     |      |   |   |   |
| Female                          | 29.0  | 20.3| 0.02 | 54 | 24.2
| Male                            | 25.0  | 19.9|      | 92 | 32.2
| PCV7 vaccinated*, <2 years olds |       |     | 0.06 | 22 | 30.9
| yes                             | 26.1  | 20.0|      | 22 | 30.9
| no                              | 32.4  | 20.8|      | 14 | 18.6
| Cocolonization                  |       |     | 0.003| <0.001
| yes                             | 35.3  | 16.5|      | 0  | 0
| no                              | 25.9  | 20.3|      | 149| 31.7
| PCV7 serotype†, <2 years olds   |       |     | 0.20 | 5  | 12.1
| yes                             | 38.0  | 19.0|      | 5  | 12.1
| no                              | 32.6  | 19.9|      | 7  | 16.2

*No information was available about age for 7 patients.
†PCV7 = seven-valent conjugated pneumococcal vaccine.
‡This associations became statistically significant after adjustment for carriage of a serotype contained in PCV7 in a logistic regression model (p = 0.02).
§Carriage of a serotype contained in the seven-valent conjugated pneumococcal vaccine; i.e. one of the following serotypes: 4, 6B, 9V, 14, 18C, 19F.

No information about vaccination status was available for 100 patients (8.9%). P-values compare the carriage of single or multiple strains of S. pneumoniae to no carriage.

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a relative frequency of less than 2% were designated “rare” and grouped together as “other serotypes” for analysis. These rare serotypes/-groups included 1, 8, 9 (except 9V), 10, 16, 17, 17F, 18 (except 18C), 20, 21, 28, 33, 34, 35, 38 and non-typeable isolates. Interestingly, in cocolonized samples rare serotypes were more frequently represented than the prevalent serotypes (23.4% versus 11.4%, p = 0.01), but difference in the frequency of individual serotypes (for example for the serotypes 11, 15, and 19F) did not reach statistical significance. B, Distribution of rare serotypes (group “other” in Figure 2A) in samples with or without cocolonization. The difference between the proportion of non-typeable strains in cocolonized samples (4.7%) and in those with a single strain (0.5%) was statistically significant (Fisher’s exact test p = 0.02).

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Discussion

Simultaneous colonization of the human nasopharynx with more than one strain or serotype of *S. pneumoniae* is an intriguing event. It sets the stage for pneumococcal evolution through horizontal exchange of genes, which may result in the acquisition of antibiotic resistance or capsule switch [9]. In addition, the study of cocolonization offers insight into the interaction between different strains or serotypes of *S. pneumoniae*. This study shows that PCV7 selection pressure did not affect the cocolonization rate up to three years after the introduction of the vaccine. Therefore, opportunities for horizontal gene transfer between pneumococci in the nasopharynx will continue to occur at the same frequency in the vaccine era as in the prevaccine era and new strains may emerge under vaccine selection pressure as has been described [5].

One might have expected that cocolonization would involve more frequently the prevalent serotypes with high incidence of acquisition and long duration of carriage [21,26,27]. However, in this study, rare serotypes predominated among cocolonizing...
pneumococcal strains. For example, serotype 19F, one of the most carried serotypes, was relatively rare among cocolonizing strains already in the prevaccine era and serotype 19A did not emerge among cocolonizing strains in the vaccine era as it did among single colonizing strains. The observed predominance of rare serotypes during cocolonization suggests that simultaneous colonization is not simply a consequence of cumulative exposure to transmission opportunities. This hypothesis is also supported by the observed lack of an association of cocolonization with day care usage, a history of recurrent otitis media and recent antibiotic exposure.

It has been shown that pneumococcal serotypes differ for their competitiveness in colonizing the nasopharynx [28]; some serotypes, such as 6B can outcompete others, such as 23F. In our study, colonization density was significantly higher in nasopharyngeal swabs containing multiple pneumococcal strains as compared to those swabs with a single strain. Also, the median ratio of two pneumococcal strains in a given sample was balanced (median 1.3). Therefore, cocolonizing strains seem to co-exist rather than to compete with each other for example for resources (i.e. nutrients, space) or by production of bacteriocins. Margolis and colleagues recently showed in a rat colonization study that sequential cocolonization with two pneumococcal strains can lead to stable co-existence and a higher density for cocolonization compared to single colonization [29]. Whether the tendency for co-existence between some pneumococcal serotypes, as described here, may also apply to co-existence with other pathogens such as *H. influenzae* and *S. aureus* needs to be studied [30,31,32,33].

Serotypes, which predominated in multiple colonization in this study, did not include serotypes covered by the seven-, ten- or thirteen-valent conjugated polysaccharide vaccines, and the prevalence of these rare serotypes increased in the vaccine era.
of pneumococcal serotypes has also been used for the detection of coccolization [11]. Whilst multiplex PCR for serotype cannot detect coccolization by different pneumococcal strains of the same serotype the method used in this study, plyNCR RFLP and T-RFLP, will miss coccolization by two serotypes with an identical RFLP type. We compared the performance of multiplex PCR and T-RFLP for the detection of coccolization in 141 consecutive nasopharyngeal swabs (from time period 4) and found a slightly higher sensitivity for the T-RFLP assay than multiplex PCR: coccolization was detected by both methods in six samples, by T-RFLP only in 8 samples, and by multiplex PCR only in one sample (Table 3). Therefore, we believe that this study did not underestimate the coccolization rate in comparison to studies using multiplex PCR.

In summary, this study showed that pneumococcal coccolization rates have not been affected by the selection pressure of PCV up to 3 years after the introduction of universal vaccination. Multiple colonization involves more often serotypes that have been of low prevalence in the prevaccine era, but may now emerge under vaccine selection pressure. This may lead to an increase in the coccolization rate in the future, with an increasing opportunity for pneumococcal evolution through horizontal gene exchange.

Author Contributions
Conceived and designed the experiments: SDB KM. Performed the experiments: SDB PF SA JH. Analyzed the data: SDB PF SA JH KM. Contributed reagents/materials/analysis tools: JH KM. Wrote the paper: SDB KM.

References
1. Poehling KA, Talbot TR, Griffin MR, Craig AS, Whitney CG, et al. (2006) Invasive pneumococcal disease among infants before and after introduction of pneumococcal conjugate vaccine. JAMA 295: 1668–1674.
2. Kyaw MH, Lynfield R, Schaffner W, Craig AS, Hadler J, et al. (2006) Effect of introduction of the pneumococcal conjugate vaccine on drug-resistant Streptococcus pneumoniae. N Engl J Med 354: 1455–1463.
3. Whitney CG, Farley MM, Hadler J, Harrison LH, Bennett NM, et al. (2003) Decline in invasive pneumococcal disease after the introduction of protein-poly saccharide conjugate vaccine. N Engl J Med 348: 1737–1746.
4. Singleton RJ, Hennessy TW, Bullock LR, Hammitt LI, Zula T, et al. (2007) Invasive pneumococcal disease caused by nonvaccine serotypes among alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. JAMA 297: 1794–1792.
5. Brueggemann AB, Pai R, Crook DW, Beall B (2007) Vaccine escape recombinants emerge after pneumococcal vaccination in the United States. PLoS Pathog 3: e168.
6. Moore MR, Gertz RE, Jr., Woodbury RL, Barkocy-Gallagher GA, Schaffner W, et al. (2008) Population snapshot of emergent Streptococcus pneumoniae serotype 19A in the United States, 2003. J Infect Dis 197: 1016–1027.
7. Pai R, Moore MR, Pilishvili T, Gertz RE, Whitney CG, et al. (2003) Postvaccine genetic structure of Streptococcus pneumoniae serotype 19A from children in the United States. J Infect Dis 192: 1988–1995.
8. Temime I, Boile PY, Opatsowski I, Guillenot D (2008) Impact of capsular switch on invasive pneumococcal disease incidence in a vaccinated population. PLoS One 3: e3244.
9. Coffey TJ, Dowson CG, Daniel M, Zhou J, Martin C, et al. (1991) Horizontal transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of Streptococcus pneumoniae. Mol Microbiol 5: 2255–2260.
10. Johnsburg O, Havarstein LS (2009) Regulation of natural genetic transformation and acquisition of transforming DNA in Streptococcus pneumoniae. FEMS Microbiol Rev 33: 627–642.
11. Rivera-Olivero EA, Blommaart M, Bogert D, Hermans PW, de Waard JH (2009) Multiplex PCR reveals a high rate of nasopharyngeal pneumococcal 7-valent conjugate vaccine serotypes co-colonizing indigenous Warao children in Venezuela. J Med Microbiol 58: 564–567.
12. Haenner RE, Dagan R, Porath N, Waas AD, Klugman KP (2000) Lack of utility of serotyping multiple colonies for detection of simultaneous nasopharyngeal carriage of different pneumococcal serotypes. Pediatr Infect Dis J 19: 1017–1020.
13. O’Brien KL, Millar EV, Zell ER, Bronsdon M, Weatherholtz R, et al. (2007) Effect of pneumococcal conjugate vaccine on nasopharyngeal colonization among immunized and unimmunized children in a community-randomized trial. J Infect Dis 196: 1211–1220.
14. Auranen K, Mehta J, Tanskanen A, S Kaltoft M. Between-strain competition in acquisition and clearance of pneumococcal carriage—epidemiologic evidence from a longitudinal study of day-care children. Am J Epidemiol 171: 169–176.
15. Bronsdon MA, O’Brien KL, Facklam RR, Whitney CG, Schwartz B, et al. (2004) Immunoblot method to detect Streptococcus pneumoniae and identify multiple serotypes from nasopharyngeal secretions. J Clin Microbiol 42: 1596–1600.
16. Billal DS, Hotomi M, Suzumoto M, Yamauchi K, Arai J, et al. (2008) Determination of pneumococcal serotypes/genotypes in nasopharyngeal secretions of otitis media children by multiplex PCR. Eur J Pediatr 167: 401–407.
17. Have KM, Morris P, Smith-Vaughan H, Leach AJ (2008) Random colony selection versus colony morphology for detection of multiple pneumococcal serotypes in nasopharyngeal swabs. Pediatr Infect Dis J 27: 178–180.
18. Abdullahi O, Wanjurul G, Muysyni R, Glass N, Scott JA (2007) Validation of nasopharyngeal sampling and culture techniques for detection of Streptococcus pneumoniae in children in Kenya. J Clin Microbiol 45: 3408–3410.
19. Bradbur BR, Hadaway LJ, Muhlemann K (2009) Detection of Streptococcus pneumoniae strain coccolization in the nasopharynx. J Clin Microbiol 47: 1750–1756.
20. Muhlemann K, Matter HC, Tauber MG, Bodmer T (2005) Nationwide surveillance of nasopharyngeal Streptococcus pneumoniae isolates from children with respiratory infection, Switzerland, 1998-1999. J Infect Dis 178: 399–406.
21. Kronenberg A, Zaas P, Dov S, Muhlemann K (2006) Distribution and invasiveness of Streptococcus pneumoniae serotypes in Switzerland, a country with low antibiotic selection pressure, from 2001 to 2004. J Clin Microbiol 44: 2032–2038.
22. Hadaway LJ, Brugger S, Martynova A, Aebi S, Muhlemann K (2007) Use of the Agilent 2100 bioanalyzer for rapid and reproducible molecular typing of Streptococcus pneumoniae. J Clin Microbiol 45: 803–809.
23. Meier PS, Utz S, Aebi S, Muhlemann K (2005) Low-level resistance to rifampin with low antibiotic selection pressure, from 2001 to 2004. J Clin Microbiol 44: 2032–2038.
24. Morrison KE, Lake D, Crook J, Carlone GM, Ades E, et al. (2000) Confirmation of pspA in all 90 serotypes of Streptococcus pneumoniae by PCR and potential of this assay for identification and diagnosis. J Clin Microbiol 38: 454–457.

Table 3. Comparison between multiplex PCR and plyNCR RFLP/T-RFLP for the detection of multiple colonization in 141 nasopharyngeal swabs collected during study period 4 for which culture revealed growth of a pneumococcal strain.

| Multiple colonization by multiplex PCR | Multiple colonization by plyNCR RFLP and T-RFLP |
|--------------------------------------|-----------------------------------------------|
| Yes                                  | No                                            |
| 6                                    | 1                                             |
| 8                                    | 126                                           |

Multiplex PCR was performed on DNA extracted from frozen stocks of the primary culture plate. plyNCR RFLP and T-RFLP detected more episodes of multiple colonization than multiplex PCR positive and in 3 of the 8 coccolization events not detected by multiplex PCR non-typeable pneumococcal strains were involved.

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(Figure 3). Therefore, we can not exclude that rates of multiple colonization may increase in the future with the emergence of these serotypes. The follow-up period of three years after introduction of PCV7 may have been too short to observe such a trend.

A limitation of this study is that the study population was restricted to patients with acute otitis media or pneumonia. The conclusions drawn from this study may therefore not apply to healthy persons. Also, the epidemiology of cocolonization may be different in populations with overall higher carriage rates than observed in our study [11,17,34]. Multiplex PCR for the detection
25. Pai R, Gertz RE, Reall B (2006) Sequential multiplex PCR approach for determining capsular serotypes of Streptococcus pneumoniae isolates. J Clin Microbiol 44: 124–131.

26. Brueggemann AB, Peto TE, Crook DW, Butler JC, Kristinsson KG, et al. (2004) Temporal and geographic stability of the serogroup-specific invasive disease potential of Streptococcus pneumoniae in children. J Infect Dis 190: 1203–1211.

27. Sleeoman KL, Griffiths D, Shackley F, Diggle L, Gupta S, et al. (2006) Capsular serotype-specific attack rates and duration of carriage of Streptococcus pneumoniae in a population of children. J Infect Dis 194: 602–608.

28. Lipsitch M, Dykes JK, Johnson SE, Ades EW, King J, et al. (2000) Competition among Streptococcus pneumoniae for intranasal colonization in a mouse model. Vaccine 18: 2893–2901.

29. Margolis E, Yates A, Levin BR. The ecology of nasal colonization of Streptococcus pneumoniae, Haemophilus influenzae and Staphylococcus aureus: the role of competition and interactions with host’s immune response. BMC Microbiol 10: 59.

30. Pettigrew MM, Gent JF, Revai K, Patel JA, Chonmaitree T (2008) Microbial interactions during upper respiratory tract infections. Emerg Infect Dis 14: 1504–1509.

31. Bogaert D, van Belkum A, Suijker M, Luijendijk A, de Groot R, et al. (2004) Colonisation by Streptococcus pneumoniae and Staphylococcus aureus in healthy children. Lancet 363: 1871–1872.

32. Madhi SA, Adrian P, Kuwanda L, Cutland C, Albrich WC, et al. (2007) Long-term effect of pneumococcal conjugate vaccine on nasopharyngeal colonization by Streptococcus pneumoniae—and associated interactions with Staphylococcus aureus and Haemophilus influenzae colonization—in HIV-Infected and HIV-uninfected children. J Infect Dis 196: 1662–1666.

33. Regev-Yochay G, Bogaert D, Malley R, Hermans PW, Veenhoven RH, et al. (2008) Does pneumococcal conjugate vaccine influence Staphylococcus aureus carriage in children? Clin Infect Dis 47: 289–291; author reply 291–282.

34. Hill PC, Cheung YB, Akisanya A, Sankareh K, Lahai G, et al. (2008) Nasopharyngeal carriage of Streptococcus pneumoniae in Gambian infants: a longitudinal study. Clin Infect Dis 46: 807–814.