Nasopharyngeal co-colonization with *Staphylococcus aureus* and *Streptococcus pneumoniae* in children is bacterial genotype independent

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Bacterial interference between *Staphylococcus aureus* and *Streptococcus pneumoniae* in the nasopharynx has been observed during colonization, which might have important clinical implications for the widespread use of pneumococcal conjugate vaccine in young children. This study aimed to determine whether the capacity of *Staph. aureus* to compete with *Strep. pneumoniae* is dependent on bacterial genotype. Demographic and microbiological determinants of carriage of specific genotypes of *Staph. aureus* in children were also studied. Children ($n=3198$) were sampled in the nasopharynx to detect carriage of *Staph. aureus*, *Strep. pneumoniae* and *Neisseria meningitidis*. *Staph. aureus* genotypes and pneumococcal sero- and genotypes were determined. Age, gender, zip code, active smoking and co-colonization with *N. meningitidis* or *Strep. pneumoniae*, both vaccine- and non-vaccine types, were not associated with colonization by specific *Staph. aureus* genotypes. Based on the whole-genome typing data obtained, there was no obvious correlation between staphylococcal and pneumococcal genotypes during co-colonization. Passive smoking showed a significant association ($P=0.003$) with carriage of a specific *Staph. aureus* cluster. This study suggests that there are no major differences between *Staph. aureus* clones (with different disease-invoking potential) in their capacity to compete with *Strep. pneumoniae* subtypes. Further studies should demonstrate whether differences in bacterial interference are due to more subtle genetic changes.

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INTRODUCTION

Staphylococcus aureus causes a wide range of human infections and the incidence of these infections is steadily

increasing, both in the community and in hospitals (Statens Serum Institut, 2004; Steinberg *et al.*, 1996). The anterior nares are the primary ecological reservoir of *Staph. aureus* in humans (Moss *et al.*, 1948), and it has been determined that most infections result from endogenous nasal carriage (Luzar *et al.*, 1990; Nguyen *et al.*, 1999; von Eiff *et al.*, 2001; Wertheim *et al.*, 2005; Yu *et al.*, 1986). The molecular basis of nasal colonization with *Staph. aureus* is not properly

Abbreviations: AFLP, amplified fragment length polymorphism; ht-AFLP, high-throughput amplified fragment length polymorphism; PCA, principal component analysis; PCV7, 7-valent pneumococcal-conjugate vaccine; RFEL, restriction fragment end labelling.

understood, but environmental factors (Bogaert *et al.*, 2004; Peacock *et al.*, 2003) as well as host factors (Cole *et al.*, 1999, 2001) play an important role (Peacock *et al.*, 2001; Wertheim *et al.*, 2005). Age, sex, fasting glucose levels, diabetes mellitus and smoking were recently demonstrated to be independent determinants of *Staph. aureus* nasal carriage in adults (Nouwen *et al.*, 2004). Furthermore, two recent large-scale population studies revealed an inverse relationship between nasopharyngeal *Staph. aureus* colonization and vaccine-type strains of *Streptococcus pneumoniae* (Bogaert *et al.*, 2004; Regev-Yochay *et al.*, 2004), suggesting natural competition between these bacterial species in the nasopharyngeal niche. This could not be confirmed in HIV-infected children (McNally *et al.*, 2006). A recent trial with the 7-valent pneumococcal-conjugate vaccine (PCV7) in children with recurrent acute otitis media (AOM) showed a shift in pneumococcal colonization towards non-vaccine serotypes and an increase in *Staph. aureus*-related AOM after vaccination (Veenhoven *et al.*, 2003). A similar trend was recently observed for bacteraemia in young children (Herz *et al.*, 2006). Hence, bacterial co-colonization is considered to have important clinical implications for the widespread use of PCV7 in young children. It has become obvious that the nasopharyngeal microbial ecology is complex, and microbial inter-species interactions, as well as host-pathogen interactions, may define whether or not potential infectious pathogens can persist locally.

We aimed to determine whether the capacity of *Staph. aureus* to compete with *Strep. pneumoniae* is dependent on bacterial genotype, the main question being whether certain successful staphylococcal clones are better equipped to compete with pneumococci. We also studied demographic and bacteriological determinants of carriage of specific genotypes of *Staph. aureus* in children.

METHODS

Study cohort and cultures. In total, 3198 healthy children from the Rotterdam area, The Netherlands, between 12 months and 19 years of age, were enrolled during a meningococcal vaccination campaign (Bogaert *et al.*, 2004). Informed consent was obtained from the child or the accompanying parent. A single nasopharyngeal sample was obtained per child with rayon-tipped pernasal swabs (Copan, Brescia, Italy). Swabs were transported in Amies medium and plated within 6 h on gentamicin blood agar and Thayer-Martin medium, and finally submerged in phenyl mannitol broth for selective isolation of *Strep. pneumoniae*, *Neisseria meningitidis* and *Staph. aureus*, respectively. Bacterial identification was performed according to standard procedures (Bannerman, 2003). *Staph. aureus* and *Strep. pneumoniae* strains were isolated from 1117 and 598 children, respectively. In this study cohort a negative correlation for co-colonization of *Staph. aureus* and vaccine-type pneumococci (odds ratio 0.68, confidence interval 0.48–0.94) was identified (Bogaert *et al.*, 2004). All isolates were stored at -80°C in broth containing glycerol. A random sample of 400 *Staph. aureus* isolates was drawn for the present study.

Demographics. Demographic information was obtained from each child through a standardized questionnaire which was completed under supervision of an instructed interviewer. Questions addressed

gender, date of birth, zip code, and active and passive smoking habits. In our analysis we grouped zip codes into four categories (north, south, centre and suburbs of Rotterdam).

DNA isolation and bacterial genotyping. The 400 *Staph. aureus* isolates were grown on Columbia III agar (Becton Dickinson) supplemented with 5% sheep blood. Three to five colonies were suspended in 25 mM Tris, 10 mM EDTA, 50 mM glucose containing lysostaphin ($50\text{ }\mu\text{g ml}^{-1}$) and incubated at 37°C for 1 h. DNA was extracted with the MagNA Pure LC DNA Isolation Kit III using the MagNA Pure LC robot (Roche Diagnostics) and stored at -20°C . High-throughput amplified fragment length polymorphism (ht-AFLP) analysis was performed as described by Melles *et al.* (2004). AFLP is a whole-genome typing method that scans for polymorphism in actual restriction sites but also among the nucleotides bordering these sites. As such it documents nucleotide sequence variation, insertions and deletions across genomes. Briefly, using the predictive software package Recomb, the optimal enzyme and primer combinations were selected. Bacterial DNA was digested with the enzymes *MboI* and *Csp6I*, and the linker oligonucleotide pairs for *MboI* (5'-CTCGTAGACTGCGTACC-3' and 5'-GATCGGTACGCAGTC-TAC-3') and for *Csp6I* (5'-GACGATGAGTCCTGAC-3' and 5'-TAGTCAGGACTCAT-3') were ligated. Subsequently, a non-selective pre-amplification was performed using the *MboI* primer (5'-GTAGACTGCGTACCGATC-3') and *Csp6I* primer (5'-GACG-ATGAGTCCTGACTAC-3'). In the final amplification, a ^{32}P -labelled *MboI* primer containing one selective nucleotide (either +C or +G) and a *Csp6I* primer containing two selective nucleotides (+TA) were used. Amplified material was analysed using standard polyacrylamide slab gels and subsequent autoradiography. Marker fragments were scored, and a binary table, scoring marker fragment absence (0) or presence (1), was compiled.

Pneumococcal DNA was extracted (from 578 of the 598 pneumococcal isolates) and analysed by restriction fragment end labelling (RFEL) as described before (van Steenberghe *et al.*, 1995; Bogaert *et al.*, 2006). Briefly, purified pneumococcal DNA was digested by the restriction enzyme *EcoRI*. The DNA restriction fragments were end-labelled at 72°C with [α - ^{32}P]dATP using DNA polymerase (Goldstar; Eurogentec). After the radiolabelled fragments had been denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, NY, USA), and exposed for variable times at room temperature to ECL hyperfilm (Amersham Laboratories).

Multilocus sequence typing (MLST). MLST was carried out for a selection of 45 of the *Staph. aureus* strains using DNA arrays (van Leeuwen *et al.*, 2003). The selected strains were equally distributed across an AFLP dendrogram by selecting approximately 1 out of 10 carriage strains isolated from healthy children, going from top to bottom through the AFLP dendrogram (Melles *et al.*, 2004).

Data analysis. Analysis of the AFLP data was performed as described by Melles *et al.* (2004). The method used for 2D clustering of the AFLP data was agglomerative (successive) hierarchical. This was performed using the unweighted pair group method with arithmetic means (UPGMA). The similarity metric used was Tanimoto, which defines similarity for binary data (0 and 1) based on the number of positive attributes that two records have in common. The resulting dendrogram was ordered by mean value.

Principal component analysis (PCA) is a standard multivariate method used to reduce the dimensional space of the data to its principal components (PCs). PCA aims to reduce a large number of variables to a smaller set that explain most of the variation in the data. It is, basically, a rotation of axes after centering data to the means of the variables, the rotated axes being the PCs, which are linear combinations of the original variables. The PC computation is displayed as a 3D scatter plot

in which the position along the axes shows the PCA score of the strain. The distribution of the strains in the four phylogenetic branches was defined on the basis of PCA. Hierarchical and PCA cluster analysis was performed using Spotfire DecisionSite 7.2 software (<http://www.spotfire.com>).

To compare the distribution of strain categories in different genetic clusters, chi-squared analysis was used. Logistic regression analysis was used to adjust for possible confounding factors. A two-sided *P*-value of 0.05 or less was considered significant.

RESULTS AND DISCUSSION

Previously, we determined the overall population structure of *Staph. aureus* isolated from healthy nasal carriers in the Rotterdam area using ht-AFLP. This revealed four major genetic lineages (I, II, III and IV) of *Staph. aureus* (Foster, 2004; Melles *et al.*, 2004). This earlier study also revealed that the AFLP-clustering matched strongly with the major clonal complexes as defined by MLST (Melles *et al.*, 2004). Moreover, computer algorithms used to solve the *Staph. aureus* population structure based on MLST data (eBURST) generated clustering identical to the AFLP Spotfire analyses. In the present study, using the subset of 400 *Staph. aureus*

carriage strains obtained from children, a total of 147 different genetic markers per strain were generated, covering 58 800 AFLP fragments (Fig. 1). The AFLP data revealed two distinct, homogeneous clusters (II and III) and several other smaller subclusters within the two main ht-AFLP clusters I and IV (Fig. 1, Fig. 2a). MLST analysis of the *Staph. aureus* isolates identified different clonal complexes in AFLP group I (including CC5, CC7, CC8, CC15, CC20 and CC25), indicating its heterogeneity. In contrast, AFLP-clusters II and III harbour single clonal complexes, CC30 and CC45, respectively. The fact that these two clonal complexes account for 47.3 % of all carriage isolates in our study population suggests that they have evolved to be very successful in colonizing humans (Melles *et al.*, 2004). Clusters IVa and IVb are associated with CC22 and CC121, respectively (see Fig. 1).

We studied potential determinants of carriage of different *Staph. aureus* genotypes. Table 1 summarizes the main characteristics of the study population. Age, gender, zip code, active smoking and co-colonization with *N. meningitidis* or *Strep. pneumoniae* were not significantly associated with carriage of a particular *Staph. aureus* genotype as

Fig. 1. Two-dimensional hierarchical clustering of the 400 *Staph. aureus* strains. The green/red figure represents 58 800 binary outcomes, generated by ht-AFLP with 147 marker fragments. Green represents marker absence and red represents marker presence. The dendrogram on the y-axis represents the phylogenetic clustering of the 400 strains. The dendrogram on the x-axis shows the clustering of the 147 AFLP markers, many of which segregate in specific groups. In conjunction with PCA, four major clusters (I, II, III and IV) could be identified, represented by the black-and-white bar on the right of the figure. Several subclusters could be identified, which are indicated by dotted lines. MLST data are plotted next to this bar. The numbers indicate clonal complexes determined by MLST analysis.

Fig. 2. (a) PCA of the AFLP data of 400 *Staph. aureus* isolates. Each cube in the figure represents one strain. The *Staph. aureus* strains that are marked purple were isolated from children with co-colonization of *Strep. pneumoniae*, and the strains marked grey were isolated from children without *Strep. pneumoniae* co-colonization. (b) PCA of the RFEL data of 578 *Strep. pneumoniae* isolates. Each cube in the figure represents one strain. The *Strep. pneumoniae* strains that are marked blue were isolated from children with co-colonization of *Staph. aureus*, and the strains marked red were isolated from children without *Staph. aureus* co-colonization.

Table 1. Demographic and microbiological characteristics of the study population

	Study-population (<i>n</i> = 400)
Age (years)	
Mean	9.93
Range	1–18
Age: no. (%)	
1–6 years	113 (28.3)
7–12 years	175 (43.8)
13–18 years	112 (28.0)
Gender (male): no. (%)	234 (58.5)
Zip code (area): no. (%)	
North	69 (17.3)
Centre	257 (64.3)
South	54 (13.5)
Suburbs	20 (5.0)
Co-colonization: no. (%)	
<i>N. meningitidis</i>	7 (1.8)
<i>Strep. pneumoniae</i>	74 (18.5)
Vaccine-type	24 (32.4)
Non-vaccine-type	42 (56.8)
Non-typable	8 (10.8)
Active smoking: no. (%)	22 (5.5)
Passive smoking: no. (%)	186 (46.5)

defined by AFLP. Although age is an important determinant of *Staph. aureus* colonization in children, with highest incidences at the age of 11 years (Bogaert *et al.*, 2004), we found no association between age distribution and the various genotypes of *Staph. aureus*. This suggests that unknown host or environmental factors are more likely to be prime determinants of the parabolic age-versus-carriage distribution in children (Bogaert *et al.*, 2004). Seventy-four (18.5 %) of the 400 children with *Staph. aureus* carriage were co-colonized with *Strep. pneumoniae*. Twenty-four (32.4 %) of the 74 pneumococcal isolates expressed capsular serotypes included in PCV7 (vaccine-type pneumococci) (Table 1). We found no association between co-colonization with vaccine- or non-vaccine-type pneumococci and the different genotypes of *Staph. aureus* (Table 2, Fig. 2a). In addition, when the RFEL types obtained for the pneumococci were included in the comparison, no additional overlaps in co-colonizing types were observed (Fig. 2b). Notably, genetic heterogeneity among the pneumococci was much higher (showing no genetic clusters; Fig. 2b) (see also Bogaert *et al.*, 2006), than in *Staph. aureus* (showing four major genetic clusters; Fig. 2a).

Analysis of the zip code categories versus genotypes of *Staph. aureus* again revealed no significant association ($P=0.268$; Table 2). This suggested that AFLP clusters were evenly distributed geographically; this contrasts with *Staph. aureus*

Table 2. Determinants of nasal carriage for each genetic lineage of *Staph. aureus*

	AFLP cluster				Chi-squared <i>P</i> -value (2-sided)
	I (<i>n</i> = 162)	II (<i>n</i> = 103)	III (<i>n</i> = 86)	IV (<i>n</i> = 49)	
Age (%)					0.722
1–6 years	32.1	26.2	25.6	24.5	
7–12 years	39.5	48.5	43.0	49.0	
13–18 years	28.4	25.2	31.4	26.5	
Gender (male) (%)	63.0	49.5	57.0	65.3	0.278
Zip code (%)					0.268
Centre	66.0	64.1	62.8	61.2	
North	14.8	19.4	18.6	18.4	
South	13.6	15.5	14.0	8.2	
Suburbs	5.6	1.0	4.7	12.2	
Co-colonization (%)					
<i>Strep. pneumoniae</i>	19.8	18.4	17.4	16.3	0.943*
Vaccine-type	6.8	4.9	7.0	4.1	0.839*
Non-vaccine-type	9.9	12.6	9.3	10.2	0.907*
Non-typable	3.1	1.0	1.2	2.0	0.606*
<i>N. meningitidis</i>	1.2	1.9	2.3	1.8	0.927
Active-smoking (%)	6.2	3.9	5.8	6.1	0.870
Passive-smoking (%)	40.7	44.7	47.7	67.3	0.012†

*Compared to number (percentage) of children with cultures negative for pneumococci.

†Chi-squared (2-sided): AFLP cluster IV vs I + II + III; $P=0.003$.

infection in specific disease categories, where local spread of certain clones is well established (Koning *et al.*, 2003).

However, passive smoking was significantly associated with carriage of *Staph. aureus* AFLP-cluster IV ($P=0.003$; see also footnote to Table 2). An earlier study had already revealed that passive smoking is associated with an increased risk of *Staph. aureus* colonization, with active smoking being protective against colonization (Bogaert *et al.*, 2004). This suggests that passive smoking excites colonization opportunities for specific genotypes of *Staph. aureus* that have been shown to be possibly hypervirulent before (AFLP cluster IV) (Melles *et al.*, 2004). We additionally performed logistic regression analysis with AFLP cluster IV as outcome, and active smoking, passive smoking, age, gender, co-colonization and zip code as possible confounders in the model. This analysis revealed that passive smoking was independently associated with carriage of AFLP cluster IV (odds ratio 2.8; confidence interval 1.5–5.5; $P=0.002$). Additional statistical analysis of the different subclusters from major AFLP clusters I and IV (dotted lines in Fig. 1) did not reveal novel associations with carriage of specific *Staph. aureus* genotypes (data not shown).

In our search for factors co-determining success of colonization with certain *Staph. aureus* genotypes, we found no evidence for involvement of age, gender, zip code, active smoking and co-colonization with *N. meningitidis* and vaccine or non-vaccine serotypes of *Strep. pneumoniae*. This suggests that with respect to *Staph. aureus*, bacterial inter-species competition in the nasopharynx probably depends on host characteristics or currently unspecified microbial features (e.g. receptor and ligands), rather than AFLP-defined overall *Staph. aureus* genotypes. Further studies involving molecular typing or gene expression testing at a more detailed level may still generate data that could help identify strain- rather than clone-specific factors involved in bacterial interference. Maturation of the nasopharyngeal niche, including its diverse innate immunity factors during ageing, might be an important driving force, but this hypothesis should be substantiated by further investigations. Recently, it was suggested that hydrogen peroxide production by *Strep. pneumoniae* is important in the bacterial interference process, a lead that warrants further investigation (Regev-Yochay *et al.*, 2006). In conclusion, neither of the staphylococcal clones identified by AFLP has a better competitive edge over the pneumococcus. Thus, success of *Staph. aureus* clones is not explained on the basis of improved competition with co-colonizing pneumococci. Furthermore, we found an association between passive smoking and carriage of a specific *Staph. aureus* cluster in children.

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