Dynamic pattern and genotypic diversity of Staphylococcus aureus nasopharyngeal carriage in healthy pre-school children

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Objectives: It is common wisdom that persistent carriage of Staphylococcus aureus is more frequent in young children than in adults. The objectives of this study were to assess the S. aureus temporal carriage pattern among a healthy community of pre-school children, with concomitant description of genotype diversity, toxin-encoding genes and antibiotic resistance.

Methods: Among 333 children 3–6 years of age, S. aureus nasopharyngeal carriage was assessed over one school year by culture of three sequential nasopharyngeal aspirates. Identification, methicillin resistance and toxin production profile were determined by PCR. Genotyping was performed by spa sequencing and multilocus sequence typing (MLST).

Results: Out of 830 samples collected, 286 (34%) yielded S. aureus from 185 carriers (55%). Based on consecutive genotype analysis, only 40/268 (15%) children could be classified as persistent carriers, and the remaining 118 (44%) showed intermittent carriage. spa typing revealed 82 types clustered into 13 spa clonal complexes (CCs). Fourteen strains isolated from 11 (3%) children were methicillin-resistant S. aureus (MRSA), half of these strains belonged to the commonly hospital-associated spa t008-ST8-SCC_mec IV. Methicillin-susceptible S. aureus (MSSA) were genotypically more diverse. Toxic shock syndrome toxin and egc1/2 complexes were highly prevalent (24%). Contrastingly, Panton-Valentine leucocidin (PVL) was carried only by three MSSA strains (0.6% of children). Exfoliative toxins were detected in 10 (3.5%) MSSA strains, of which 5 were related to the impetigo clone CC121.

Conclusions: Although S. aureus nasopharyngeal carriage was high among healthy pre-school children, persistent carriage seems to be less frequent than previously reported. The prevalence of MRSA carriage was 3%, but was not associated with PVL.

Keywords: MRSA, kindergarten, carriers

Introduction

Staphylococcus aureus is an important human commensal bacterium colonizing various epithelial surfaces, mainly the upper respiratory tract (nose). In some circumstances, this bacterium can also be a significant pathogen, causing a wide range of mild to life-threatening infections.1 Scientific interest in S. aureus has been sustained for several years. Indeed, this bacterium has not only become increasingly antibiotic resistant, but also amazingly successful in disseminating highly virulent and/or resistant clones, both in hospitals and in the community, creating important therapeutic issues.2–4 Little is known about how some S. aureus clones have successfully evolved towards acquiring virulence/resistance determinants.5–9 How S. aureus with diverse virulence and resistance patterns are distributed among communities of healthy children remains to be determined.

It is well established that carriage of S. aureus constitutes a risk factor for developing invasive disease.7–9 The prevalence of carriage and the impact of various epidemiological factors...
have been extensively described, but results vary from one study to another, in parallel with differences in the populations included or definitions used.\textsuperscript{9,10} Three patterns of carriage are classically described: persistent carriage of one unique strain over time, intermittent carriage of various strains, and non-carriage.\textsuperscript{10–12} This distinction is of utmost interest, especially since persistent carriers have been described to have a higher rate of nosocomial invasive infections but a lower related mortality rate compared with the other groups.\textsuperscript{13,14}

Children are classically considered to be persistent carriers more frequently than adults, with the highest rate of carriage reached in the first year of life and transition from persistent to intermittent or non-carriage occurring during adolescence.\textsuperscript{9,15} However, this concept has never been confirmed on a genotypic level, and beyond the first year of life,\textsuperscript{16,17} dynamic descriptions of \textit{S. aureus} carriage in a healthy paediatric population are clearly lacking. Why a child becomes a persistent or intermittent carrier and how this carriage evolves over time with exposure to intrafamilial/community settings and multiple potentially influencing factors, including various \textit{S. aureus} genotypes, remain to be answered.

A better understanding of the epidemiology and determinants of \textit{S. aureus} nasal carriage could certainly be helpful in improving infection control and prevention strategies.

\section*{Methods}

\subsection*{Study design and population}

This study was part of a global project assessing nasopharyngeal bacterial carriage in pre-school children.\textsuperscript{18} Briefly, the study was conducted prospectively among healthy children attending 11 kindergartens from the Brussels area. Among the 11 participating schools, 7 were defined as ‘positive-discrimination’ (PD) schools because they showed the lowest socio-economic index according to a university-settled definition. The four remaining kindergartens were classified as ‘high-level’ (HL) schools. A total of 346 children aged 3–6 years were included and followed over one school year, either 2006–07 or 2007–08. \textit{S. aureus} nasopharyngeal carriage was assessed longitudinally by three sequential nasopharyngeal aspirates, performed during autumn, winter and spring. The three samples were obtained for 59% of the children. Nasopharyngeal aspirates were transported to the national reference centre for \textit{S. aureus}, where \textit{S. aureus} isolates were cultured and identified using standard methods.\textsuperscript{18} For each strain, \textit{S. aureus} identification and methicillin resistance were confirmed by triplex PCR detection of 16S rDNA, \textit{mecA} and \textit{nuc} genes.\textsuperscript{19}

\subsection*{Antimicrobial susceptibility testing}

The strains’ susceptibilities to 15 antimicrobial agents were determined by the disc diffusion method (Rosco NeoSensitabs) using CLSI criteria (2008). Inducible clindamycin resistance was detected by using the double disc diffusion method (D test).

\subsection*{Detection of toxin genes}

Exotoxin production profiles were characterized using PCR for genes encoding Panton-Valentine leucocidin (PVL) (\textit{lukS-pvK}), exfoliative toxins (eta and etb), toxic shock syndrome toxin (TSST-1) and 13 other enterotoxins, as previously described.\textsuperscript{20,21}

\subsection*{Molecular typing}

\textit{S. aureus} strains were genotyped by the spa typing method as previously described.\textsuperscript{2} The spa types were determined using Ridom StaphType software (http://www.ridom.de/staphytype/). Isolates were grouped into spa clonal complexes (CCs) using the BURP algorithm with the default parameters detailed by Hallin et al.\textsuperscript{5} Additionally, all methicillin-resistant \textit{S. aureus} (MRSA) and a subset of representative spa types from each spa CC containing at least 20 methicillin-susceptible \textit{S. aureus} (MSSA) were further analysed by multilocus sequence typing (MLST) (http://saureus.mlst.net). The most prevalent spa type in each spa CC was chosen for MLST analysis. Staphylococcal cassette chromosome mec (SCCmec) type was determined by multiplex PCR.\textsuperscript{22}

\subsection*{Pre-required definition}

Persistent carriage pattern was defined as having at least two consecutive positive cultures for \textit{S. aureus} harbouring the same spa type or closely related spa types (same spa CC).

\subsection*{Ethical considerations}

This study was approved by the Medical Ethics Committee of the Children’s Hospital Queen Fabiola. Complete information regarding the study was given to the parents and a signed informed consent was obtained for each child included before each sampling.

\subsection*{Data analysis}

Statistical analyses were performed using GraphPad Prism Software (2003; GraphPad Software, San Diego, CA, USA). The \textit{\chi}\textsuperscript{2} test or Fisher’s exact test was used to compare non-continuous variables and the Mann–Whitney test was used to compare continuous variables. A two-tailed \(P\) value <0.05 was considered statistically significant. Clone clustering within each school was assessed by calculating the epidemic index (\(E\)) using the formula of Simpson’s index diversity (\(D\)), where \(E = 1 − D\).\textsuperscript{23} It reflects the probability that two isolates from the school population will be genotypically related. An epidemic index of 1.0 means that all strains in a school belonged to the same spa CC.

\subsection*{Results}

\subsection*{Carriage rate}

Eight hundred and thirty samples were collected from 333 children (median age 4.2 years). Two hundred and eighty-six (34\%) samples yielded \textit{S. aureus}-positive cultures, corresponding to 185 children being carriers at least once over the school year (prevalence of carriage in the studied population 55\%).\textsuperscript{18} \textit{S. aureus} carriage increased significantly over the school year, from 25\% in autumn to 35\% in winter and 44\% in spring \((P<0.001)\). This seasonal pattern was observed equally during both years of sampling. Additionally, the impact of several epidemiological factors could be underlined in the first part of the study; as the \textit{S. aureus} carriage rate was shown to be significantly higher among children attending PD schools and >4 years of age.\textsuperscript{18}

\subsection*{Antimicrobial susceptibility}

Fourteen strains isolated from 11 children (3\% of the cohort) were identified as MRSA. Antimicrobial resistance profiles of MRSA and MSSA strains are listed in Table 2 and Table S1
**Table 1.** MSSA strain characteristics according to genotype (n=272)

<table>
<thead>
<tr>
<th>spa CC</th>
<th>No. of spa types</th>
<th>MLST</th>
<th>No. of strains (%)</th>
<th>TSST-1 (%)</th>
<th>PVL (%)</th>
<th>eta/b (%)</th>
<th>egc1/2 (%)</th>
<th>sea (%)</th>
<th>seb (%)</th>
<th>sec (%)</th>
<th>sed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-166</td>
<td>11 (t240, t884, t5741)</td>
<td>ST10</td>
<td>45 (17)</td>
<td>23 (51)</td>
<td>0</td>
<td>1 (2)</td>
<td>1 (2)</td>
<td>3 (7)</td>
<td>18 (40)</td>
<td>2 (4)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>CC-364</td>
<td>3 (t1326, t364, t5739)</td>
<td>ST182</td>
<td>27 (10)</td>
<td>2 (7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CC-012</td>
<td>10 (t012, t021, t018)</td>
<td>ST30</td>
<td>26 (10)</td>
<td>21 (81)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12 (46)</td>
<td>1 (4)</td>
<td>0</td>
</tr>
<tr>
<td>CC-015</td>
<td>10 (t015, t1238, t331)</td>
<td>ST45</td>
<td>23 (8)</td>
<td>1 (4)</td>
<td>0</td>
<td>0</td>
<td>10 (43)</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>16 (70)</td>
<td>0</td>
</tr>
<tr>
<td>CC-084</td>
<td>7 (t084, t105, t5749)</td>
<td>ST15</td>
<td>22 (8)</td>
<td>0</td>
<td>1 (5)</td>
<td>3 (14)</td>
<td>0</td>
<td>1 (5)</td>
<td>0</td>
<td>2 (9)</td>
<td>0</td>
</tr>
<tr>
<td>CC-002</td>
<td>4 (t002, t003, t509)</td>
<td>ST5</td>
<td>17 (6)</td>
<td>3 (18)</td>
<td>0</td>
<td>0</td>
<td>13 (76)</td>
<td>2 (12)</td>
<td>1 (6)</td>
<td>1 (6)</td>
<td>4 (24)</td>
</tr>
<tr>
<td>CC-550</td>
<td>3 (t230, t5575, t550)</td>
<td>ST45</td>
<td>11 (4)</td>
<td>3 (27)</td>
<td>0</td>
<td>0</td>
<td>4 (36)</td>
<td>0</td>
<td>0</td>
<td>4 (36)</td>
<td>0</td>
</tr>
<tr>
<td>CC-304</td>
<td>5 (t024, t1197, t304)</td>
<td>ST8</td>
<td>10 (4)</td>
<td>1 (10)</td>
<td>0</td>
<td>0</td>
<td>3 (3)</td>
<td>0</td>
<td>1 (10)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CC-189</td>
<td>3 (t189, t224, t5224)</td>
<td>ST88</td>
<td>3 (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (33)</td>
<td>0</td>
</tr>
<tr>
<td>CCs without founder</td>
<td>8 (t065, t1159, t2778)</td>
<td>—</td>
<td>21 (8)</td>
<td>4 (19)</td>
<td>0</td>
<td>5 (24)</td>
<td>4 (19)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Singletons</td>
<td>15 (t3092, t3096, t164)</td>
<td>—</td>
<td>55 (20)</td>
<td>7 (13)</td>
<td>2 (4)</td>
<td>0</td>
<td>29 (53)</td>
<td>3 (5)</td>
<td>1 (2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Not typeable/excluded</td>
<td>—</td>
<td>—</td>
<td>12 (4)</td>
<td>1 (8)</td>
<td>0</td>
<td>1 (8)</td>
<td>6 (50)</td>
<td>1 (8)</td>
<td>1 (8)</td>
<td>3 (25)</td>
<td>4 (33)</td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
<td>272</td>
<td>66 (24)</td>
<td>3 (1)</td>
<td>10 (4)</td>
<td>67 (25)</td>
<td>26 (10)</td>
<td>23 (8)</td>
<td>30 (11)</td>
<td>9 (3)</td>
<td></td>
</tr>
</tbody>
</table>

aGenotypic analysis performed using the following default parameters: types shorter than five repeats excluded and spa types grouped into the same spa CC if cost $<6x$.

bThe three most prevalent spa types of each spa CC are given in parentheses.

cEnterotoxin gene cluster as described previously, comprising the five genes selo, selm, sei, seln and seg.

dNine strains excluded from the analysis because of conflicting PCR results.

**Genotype distribution**

spa typing of *S. aureus* strains showed 82 spa types clustered into 13 spa CCs. Each spa CC included 3–45 isolates, as detailed in Table 1 and Table 2.

Typing of MSSA revealed a wide genotypic diversity, with 54% distributed into five spa CCs (Table 1). After MLST analysis, a distinct sequence type was found for each spa CC. Each spa type was found to be associated with different spa CCs, as detailed in Table 1 and Table 2. Overall, the TSST-1 gene was the most prevalent, found in 70 *S. aureus* isolates (24%) and equally distributed among MSSA and MRSA. In contrast, the PVL gene was carried by only three methicillin-susceptible strains. The prevalence of PVL carriage in this healthy community of children was 0.6% (2/333). Exfoliative toxin genes were detected in 10 (3.5%) MSSA strains. Half of these harboured a spa type related to the clone CC121:ST123 (spa type t159 or t171), but only one was resistant to fusidic acid (MIC=2 mg/L). Finally, 24% of strains carried the egc1/2 complexes, as defined previously.

**Dynamic pattern**

Among 268 children who underwent at least two consecutive samplings, 88 (33%) had a single positive culture and 68 (26%) had two or more consecutively (two additional children had two non-consecutive positive samplings). However, genotype analysis revealed that only 41/268 (15%) could be classified as persistent carriers and the remaining 118 (44%) as intermittent carriers, according to the aforementioned definition. The proportion of MRSA isolates did not significantly differ between both groups (5% and 8% for persistent and intermittent carriers, respectively; *P*=0.7). Further analysis revealed that persistent carriage status was independent from gender, previous antibiotic prescription and co-colonization with *S. pneumoniae* (see Table S2, available as Supplementary data at JAC Online). The temporal carriage distribution was also unaffected by socio-economic status (*P*=0.27). Finally, no particular spa CC could be associated with the persistent carriage pattern (*P*=0.12).
Discussion

Thanks to its longitudinal design over a whole school year, our dynamic study showed for the first time that the persistent carriage pattern of *S. aureus* is not as prevalent during childhood as initially believed. Moreover, this series offers an extensive description of *S. aureus* carriage in a healthy paediatric community.
Children were classically considered to be persistent carriers of \( \text{S. aureus} \) more frequently than adults, with the highest rate of carriage reached in the first year of life.\(^{9,15} \) Nevertheless, this concept was only supported by one old longitudinal study, assessing \( \text{S. aureus} \) carriage status by culture without genotype confirmation.\(^{15} \) Further paediatric studies were mainly conducted on a descriptive cross-sectional design, using a single nasopharyngeal sample to classify individuals;\(^{9,25–27} \) except for two recent publications reporting that the prevalence of carriage progressively decreased over the first year of life and that ‘persistent carriers’ of the same genotype were rare among infants.\(^{16,17} \)

Since persistent carriers have been described to have more invasive infections but a lower fatality rate compared with the other groups,\(^ {12,13} \) it becomes of utmost interest to further assess the dynamics and determinants of \( \text{S. aureus} \) carriage, especially considering \( \text{S. aureus} \) vaccination development.\(^ {26} \) In this context, we notably wonder whether, among intermittent carriers, those people carrying highly recurrent \( \text{S. aureus} \) isolates but of various genotypes could constitute a novel group at risk for staphylococcal invasive diseases as they face many strain exchanges over time but without any immune protection.

One limitation regarding the interpretation of our data was the lack of a standardized definition of ‘persistent carriage’ status in the medical literature. This definition varies from one study to another, rendering any comparison between them quite unreliable.\(^ {12,17,25,30} \) To address this issue, Nouwen et al.\(^ {12} \) recently proposed a ‘culture rule’ that determines persistent carriage status following the culture results of two serial nasal samples using both qualitative and quantitative methods. However, the quantitative cultures are not always available and this rule doesn’t consider the results from genotype analysis, still recommended by others.\(^ {7,25} \) Considering all the above, we decided to define persistent carriage as ‘having at least two consecutive positive cultures (obtained at 3 month intervals) with \( \text{S. aureus} \) strains harbouring the same spa type or two closely related spa types’ (same spa CC). Nevertheless, this definition was arbitrarily chosen. Moreover, it cannot be excluded that some individuals could carry simultaneously more than one single genotype of \( \text{S. aureus} \) inside the nasopharyngeal niche.\(^ {31} \)

If \( \text{S. aureus} \) persistent carriage is supposed to result from an optimal fit between host and bacteria,\(^ {10,32} \) the determinants of temporal carriage status still remain to be elucidated. Like other reports,\(^ {17,29} \) our study failed to find any determinant for the persistent carriage pattern concerning either host socio-economics or bacterial genotype. So far, no bacterial genetic polymorphism has been demonstrated to correlate with the pattern of carriage.\(^ {10} \) Currently several other factors believed to be involved in \( \text{S. aureus} \) carriage dynamics are under extensive investigation, such as competition with other pathogens,\(^ {17,18,33} \) regulatory adaptation of the organism itself\(^ {34} \) and human DNA polymorphism.\(^ {29,32} \)

As expected,\(^ {5,27} \) the distribution of MSSA showed wide genotypic diversity without any clonal clustering, independent of socio-economic level or season of sampling. This suggests a relatively low pressure by previous antibiotic therapies and/or a high exchange rate inside and outside schools. Only the subgroup of strains carrying exfoliative toxin genes was less diversified, with half of the isolates belonging to the highly transmissible European impetigo clone CC121:ST123, as determined by MLST.\(^ {35} \) Although this clone has been described as increasingly resistant to fusidic acid in Scandinavia and the UK,\(^ {35} \) our strains remained mainly susceptible, probably because they were isolated from healthy children without skin infections or recurrent antibiotic therapies. Finally, from our strain collection, 22% of MSSA isolates could be assigned to the same three successful clones as the predominant MRSA circulating in Belgium during the same period: ST45, ST8 and ST5 by MLST.\(^ {36} \)

Compared with other European\(^ {25,27} \) and American\(^ {2,37} \) paediatric surveys, our series reported a relatively higher prevalence of MRSA carriage among healthy children in the community (0.1%–1% versus 3%, respectively). However, none of them carried the gene encoding PVL. Half of our MRSA isolates belonged to the international hospital-associated ST8–SCCmec IV clone, a major clone circulating inside Belgian healthcare institutions at this time\(^ {38} \) and related to the Lyon clone, largely disseminated in France.\(^ {38} \) This observation suggests that the main source of MRSA carriage in this population is the spread of resistant strains from healthcare settings through the paediatric community rather than the emergence \textit{de novo} of new successful clones by acquisition of SCCmec. However, a minority of isolates in our collection still harboured the characteristics of classic community-associated MRSA, with two strains of spa \( t437-ST59-SCCmec \text{V} \) related to the ‘Taiwan clone’\(^ {39} \) and two other TSST-1-positive spa \( t586-ST5-SCCmec \text{I} \) belonging to the French Geraldone clone.\(^ {38} \) Furthermore, a recent series conducted in Brussels reported a low rate of ‘community-onset’ MRSA infections in children.\(^ {40} \) Those infections were remarkably limited and mainly due to hospital-associated/PVL-negative strains. Taken together with our results, all these findings provide a very different picture than the one traditionally observed in the USA\(^ {37} \) and some other European countries,\(^ {41,42} \) where MRSA circulating in the community are usually carrying the PVL gene (i.e. USA 300 ST8–SCCmec IV) and frequently involved in deep infections instead of being carried by healthy people.

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**Figure 2.** Assessment of temporal carriage pattern based on consecutive genotype analysis. **Including two patients with two non-consecutive \( \text{S. aureus} \) (SA)-positive cultures.**
In contrast with the low rate of PVL, the TSST-1-encoding gene was extremely prevalent in our series, isolated in one-quarter of the strains collected. As this toxin is commonly carried by S. aureus recovered from healthy people but has been associated with the development of toxic shock syndrome or severe cutaneous infections, several other factors involved in the triggering of disease certainly have to be identified. While the relationship between genotype and virulence remains a controversial issue, some authors suggest that the enterotoxin genes could significantly influence the strain’s invasiveness. However, as shown here, those toxins are also commonly recovered in simple carriage. Unquestionably, understanding the gap between carriage and disease will require further long-term investigation.

Conclusions

In contrast, S. aureus nasopharyngeal carriage is common among pre-school children, with wide genotype diversity and significant prevalence of strains producing enterotoxins and exfoliative toxins. In the healthy paediatric community, we still reported a relatively high rate of MRSA carriage (3%), but MRSA isolates were not associated with PVL and mainly belonged to commonly hospital-associated clones. Basically, our dynamic study revealed for the first time that, unlike common wisdom, S. aureus persistent carriage was not more prevalent among pre-school children than adults. Despite increasing knowledge, the dynamics and determinants of S. aureus carriage remain unclear but arouse great interest in this era of S. aureus vaccination development.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).
S. aureus nasopharyngeal carriage in healthy pre-school children