The epidemiology of *Staphylococcus aureus* nasal carriage in preschool children and university students in Hungary

Ph.D. thesis

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is one of the most important pyogenic bacteria causing infections in the community and hospitals as well. It can colonise the skin and mucosal surfaces of humans and several animal species also. The anterior nares of the nose are the most frequent carriage sites for this bacterium, however, extra-nasal sites can harbour *S. aureus.* Approximately 20% of humans are persistent *S. aureus* nasal carriers, while 30% are intermittent carriers. Children are more commonly persistent carriers than adults. It is shown, that persistent carriers have a higher risk of getting staphylococcal infection.

Several risk factors for *S. aureus* nasal carriage was defined previously, like male gender, obesity, suffering from chronic diseases, doing contact sport, etc. *S. aureus* can be transmitted from asymptomatic carriers to other, susceptible persons, or might cause infection in the carrier itself under certain circumstances.

Children attending communities are well known carriers of several pathogenic bacteria such as *S. aureus* and *Streptococcus pneumoniae (S. pneumoniae* or *Pneumococcus).* Many authors have reported an inverse correlation between *S. pneumoniae* and *S. aureus* carriage. This negative association is strongest in young children and in the case of vaccine-serotype pneumococci.

In the mid-1990s community-acquired methicillin-resistant *S. aureus* (CA-MRSA) was described and since that it spread worldwide. In the recent years, the emergence of CA-MRSA strains became an alarming problem, possessing increased virulence and spreading abilities compared to hospital-acquired MRSA (HA-MRSA) isolates. This has increased the importance of screening and monitoring *S. aureus* circulation in the community and its antibiotic susceptibility as well. CA-MRSA strains can be distinguishable from HA-MRSA strains not only by epidemiological features, but by genetic patterns and antibiotic resistance also. There is a growing consensus to define MRSAs by genotyping methods like SCC*mec* (staphylococcal cassette chromosome *mec*) typing or MLST (multilocus sequence typing).

The spread of *S. aureus* from person to person is difficult to prevent, because of the colonisation of the skin and mucosal surfaces. For the eradication of nasal *S. aureus* as well as MRSA carriage, mupirocin ointment is used most commonly.

OBJECTIVES

The objective of the present study was to perform a surveillance on nasal *S. aureus* carriage, by screening healthy young adults (university students from the Semmelweis University) and healthy children attending day-care centres all over Hungary, as these data were missing from Hungary so far.

We aimed to specify possible risk factors for nasal carriage, the antibiotic resistance patterns of the bacteria as well as their genetic relatedness.

We especially focused on MRSA screening and their molecular typing.

As a result of the great number of collected specimens, we found some isolates showing unusual features. We aimed to specify them and find the molecular bases of the difference from typical *S. aureus* strains.

From nasal samples, *S. aureus* and *S. pneumoniae* were simultaneously identified. Thus, we also targeted to examine the co-carriage of these two pathogens.

As the 7-valent pneumococcal conjugate vaccine was introduced in the National Immunization Programme as an optional vaccine in April 2009 (became mandatory in July 2014) in Hungary, we aimed to investigate the possible effect of the vaccine on *S. aureus* carriage.

MATERIALS AND METHODS

Study population and sample collection

Group-1. In the first part of the study in 2009, **300** (205 Hungarian and 95 non-Hungarian) 3rd-year **students** (21-24 years) on Faculty of General Medicine, Semmelweis University were screened**.**

Group-2. In the second part, we have screened 3-7 years old healthy children attending day-care centres (DCC) in Hungary. Between February 2009 and December 2011, nasal

samples were taken from **878 children**, who derived from 21 DCCs in 16 different villages and cities around the country.

Group-3. In **Szolnok** city, all 20 existing kindergartens agreed to the survey. Between February and June 2012, **1390 children** have been screened.

Nasal samples were taken from both nostrils with **soft cotton swabs** and inserted into active charcoal containing Amies transport media (Transwab, Medical Wire & Equipment, Corsham, UK). The swabs were transported to the microbiology laboratory within 24 hours.

Identification of *Staphylococcus aureus*

The nasal samples were inoculated onto Mueller-Hinton **blood agar plates**. *S. aureus* suspicious **β-haemolytic** colonies were isolated to create pure cultures. Then **catalase-test** and **clump-test** (Pastorex Staph-Plus Kit, Bio-Rad, Marnes-la-Coquette, France) were applied. In special cases, tube coagulase test was applied too. Finally, every *S. aureus* suspicious isolates were frozen and kept at –80°C on cryobeads (Cryobank, Mast Diagnostica, Bootle, UK) until further examinations.

On the genetic level, we detected the *S. aureus* species-specific thermonuclease gene by ***nucA* PCR** for all isolates. For MRSA screening, we applied (also for all isolates) ***mecA*** gene detection (together with *nucA* gene detection) in a multiplex PCR reaction. We checked ***mecC*** gene also in isolates with oxacillin MIC ≥1 mg/L by PCR in a separate reaction.

To further identify phenotypically ambiguous isolates, **MALDI-TOF** (matrix-assisted laser desorption ionization – time-of-flight) mass spectrometry (Bruker Daltonics, Bremen, Germany) was applied using the Bruker Biotyper 2.0 software.

Antibiotic susceptibility testing

We determined the minimum inhibitory concentration (**MIC**) to **penicillin** (PEN), **oxacillin**, **erythromycin** (ERY), **clindamycin** (CLI), **gentamicin** (GEN), **ciprofloxacin** (CIP), **trimethoprim-sulfamethoxazole** (TMP/SMX), **tetracycline** (TET), **mupirocin** (MUP) and **vancomycin** by E-test or agar dilution method. Disk diffusion method was used for penicillin, **cefoxitin** and for **D-zone test** to detect inducible clindamycin-resistance. In all cases the **EUCAST** (The European Committee on Antimicrobial Susceptibility Testing) guidelines and breakpoints were applied.

Genotyping by pulsed-field gel electrophoresis

To determine the genetic relatedness of the isolates, pulsed-field gel electrophoresis (PFGE) was used. The PFGE patterns were analysed and the dendrograms were created by the Fingerprinting II software (Bio-Rad, Marnes-la-Coquette, France).

Molecular typing of the MRSA strains

SCC*mec* typing was performed in the case of the *mecA* positive isolates by PCR. As CA-MRSA isolates mostly belong to the SCC*mec* types IV or V, we checked these two types only.

We used **MLST** for the MRSA strains, for some methicillin-sensitive strains and for the catalase-negative isolate.

Determination of haemolysis type

We used this method to compare the haemolytic activity of the two *S. aureus* strains each isolated from the same child.

Sequencing the *katA* gene

In one case, we identified a catalase-negative *S. aureus* (CNSA) isolate. First, we amplified its *katA* gene by PCR. DNA was prepared by the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research Corp., Irvine, CA, US). Then, the complete *kat* gene was amplified by PCR using two sets of primers yielding overlapping products.

Detection of toxin genes

In the case of the catalase-negative isolate (L 1034), we checked the presence of several toxin genes by PCR: enterotoxin A (*sea*), enterotoxin B (*seb*), enterotoxin C (*sec*), toxic shock syndrome toxin (*tsst*), exfoliative toxin A (*eta*) and exfoliative toxin B (*etb*).

Statistical analysis

We applied khi-square test for statistical analysis to determine risk factors. Applying a 95% confidence interval, p value <0.05 was considered significant.

RESULTS

Carriage rates and risk factors

University students (Group-1)

Out of the screened 300 third-year students, we isolated *S. aureus* in **88** cases, which equals to **29.3%** carriage rate. The colonisation ratio was otherwise higher among the Hungarian students (65/205 = 31.7%) compared to the non-Hungarian ones (23/95 = 24.2%), but this difference is not statistically significant.

Preschool children from different regions of Hungary (Group-2)

From 16 different Hungarian cities and villages, 878 DCC attending children took part in this survey. Out of them, **187** nasal samples were positive for *S. aureus* resulting in a **21.3%** carriage rate.

We found no statistically significant correlation between any of the following potential risk factors and *S. aureus* carriage: gender, having siblings, siblings attending community or passive smoking.

Preschool children from Szolnok (Group-3)

We could detect *S. aureus* in **474** cases, which equals to **34.1%** carriage rate. From two samples two different *S. aureus* were isolated, hence, a total of **476** *S. aureus* were identified.

Out of the 474 carriers 58.2% were males while among the non-carriers this ratio was 49.8%, and this difference was found to be statistically significant (p=0.003). For the other examined risk factors, differences were not statistically significant.

Summarised results

Altogether, we detected **749** *S. aureus* carriers – which equals to **29.2%** overall carriage rate – and totally **751** *S. aureus* isolates (due to the two double carriages).

We also cumulated risk factors for carriage among all examined children. Male gender was significantly higher among carriers (p=0.003). Interestingly, siblings attending community also significantly correlated with *S. aureus* nasal carriage as the number of samples were

augmented (p=0.008). The other two examined risk factors (having siblings and passive smoking) did not show concordance with carriage in this study.

MRSA carriage rate

Among all individuals screened in this study, MRSA carriage was found to be **0.2%** (6/2568) and **0.8%** (6/751) *mecA* gene carriage of *S. aureus* isolates.

Antibiotic susceptibility patterns

Summarising antibiotic resistance data to all tested isolates, we can establish that most of the strains (>95%) were sensitive to oxacillin, ciprofloxacin, gentamicin and tetracycline. Hundred-percentage sensitivity was documented in the case of TMP/SMX, mupirocin and vancomycin. We determined >90% resistance to penicillin and 9-10% resistance for clindamycin and erythromycin.

Additionally, four MSSA (methicillin-sensitive *S. aureus*) isolates exhibited **multi-drug resistance** (i.e. resistant to three or more different classes of antibiotics): one resistant to PEN, ERY, CIP; one resistant to PEN, ERY, GEN; one resistant to PEN, GEN, TET; and another resistant to PEN, ERY and TET.

Three of the MRSA isolates carrying the *mecA* gene showed oxacillin sensitivity. Out of these isolates one was cefoxitin sensitive in parallel, two of them showed resistance to it. Thus, 50% of the identified MRSA strains proved to be oxacillin-sensitive MRSA (OS-MRSA). Most of the isolates had high-level resistance to ERY, otherwise they were sensitive to CIP, GEN, TET and MUP at the same time. All the ERY-resistant strains had inducible CLI-resistance also. We verified the *mecA* PCR products by **sequencing** of the three oxacillin-sensitive strains.

Molecular typing of the MRSA isolates

Five out of six belonged to **ST45** (sequence type), while the remaining one proved to be **ST7**. Five of them harboured the **SCC*mec* type IV** cassette and one carried **type V** cassette.

Genetic relatedness of the isolates

PFGE patterns demonstrated high-grade diversity among the isolates originated from university students. Four bigger clusters could be assigned, however, in some cases considerable variations could be detected within the same cluster. The same MLST types (ST45 or ST7) could be specified within the same PFGE cluster for two MRSA and two MSSA isolates, however, with somewhat dissimilar PFGE patterns.

The PFGE patterns of several isolates deriving from children showed high genetic diversity similarly to university students'. However, three major clusters could be detected. MLST type of three isolates was known from this group, they belonged to ST7, ST45 or ST30.

Co-carriage of two different *Staphylococcus aureus* isolates by the same child

In two occasions, two phenotypically different *S. aureu*s were isolated from the same nasal sample. According to haemolysis type classification, we found differences in haemolysin production of the strains. Their PFGE patterns were also different in both cases. According to this, we could prove in both cases that the two *S. aureus* isolates carried by the same child were different from one another.

Clumping factor-negative *S. aureus* isolates

Among all examined (n=751) isolates, we found nine, which seemed to be *S. aureus* by colony morphology, but were clump-test negative. At the same time, all of them carried the *nucA* gene. Tube coagulase test was also carried out and gave positive result in all cases. MALDI-TOF technique confirmed all of them as *S. aureus* (Biotyper score value ≥1.9). Totally, **1.2%** of the isolates proved to be clumping factor-negative *S. aureus* (CFNSA). The PFGE macrorestriction pattern was identical in eight cases, based on this, a clonal origin could be concluded.

Catalase-negative *Staphylococcus aureus* (CNSA)

We could identify one *nucA* positive, but catalase-negative strain showing a typical *S. aureus* colony morphology. MALDI-TOF MS identified this strain as *S. aureus* with a high score (i.e. 2.3). Sequencing the *katA* gene of this isolate, a single nucleotide substitution (G491A) was identified compared to the control strain (ATCC 29213). This modification resulted in a nonsense mutation and due to it, the production of a truncated gene product. The

katA gene sequence data has been submitted to the GenBank database under accession number KY587223 (http://www.ncbi.nlm.nih.gov). Out of the six toxin genes tested, this isolate possessed the **enterotoxin A** (*sea*) gene and was negative for *seb*, *sec*, *tsst*, *eta* and *etb*. The MLST revealed that the CNSA isolate belongs to **ST5**.

Correlation between *Staphylococcus aureus* and *Streptococcus pneumoniae* carriage

In case of children (Group-2 and Group-3, n=2268) *S. aureus* and *S. pneumoniae* were screened simultaneously. *S. pneumoniae* carriage rate was 32.8% in this combined population. In **161** occasions, we detected both bacteria from the same nasal sample. Altogether, it is equal to **7.1%** dual carriage rate among all examined children. Considering just *S. aureus* carriers, 24.4% of them were co-colonised with *Pneumococcus*, while among *S. aureus* non-carriers this ratio was 36.3%. Considering just *Pneumococcus* carriers, 21.6% of them carried *S. aureus* also. By statistical analysis, this difference proved to be significant ($p=3.9 \times 10^{-8}$), which means that *S. pneumoniae* carriage is in negative correlation with *S. aureus* nasal carriage.

We summarised the serotypes of the co-carried *Pneumococcus* isolates with a special focus on those covered by PCV7 (7-valent pneumococcal conjugate vaccine) and PCV13 (13-valent pneumococcal conjugate vaccine). The vaccine coverage of PCV7 was calculated 15.4% (25/162), while PCV13 coverage would have been 37.7% (61/162). Considering all pneumococci in the examined population, similar vaccine coverage was determined (PCV7: 18.6%, PCV13: 39.7%). Comparing the serotype distribution of the co-carried isolates to all pneumococci, the most frequent serotypes are also quite similar (**Figure 1**).

We also analysed the possible effect of PCV7 vaccination on *S. aureus* carriage. The vaccination rate of the DCCs was considered as low-level under 50% (average: 24.6%, ranging from 0.0-42.1%), or high-level above 50% (average: 67.9%, ranging from 54.2-85.3%). According to statistical analysis, the positive correlation between high-level of PCV7 vaccination among the children community and *Staphylococcus aureus* nasal carriage is obvious ($p=5.2 \times 10^{-9}$). In contrast, *S. aureus* nasal carriage in children is not associated with previously administered PCV7 vaccination of the individual.

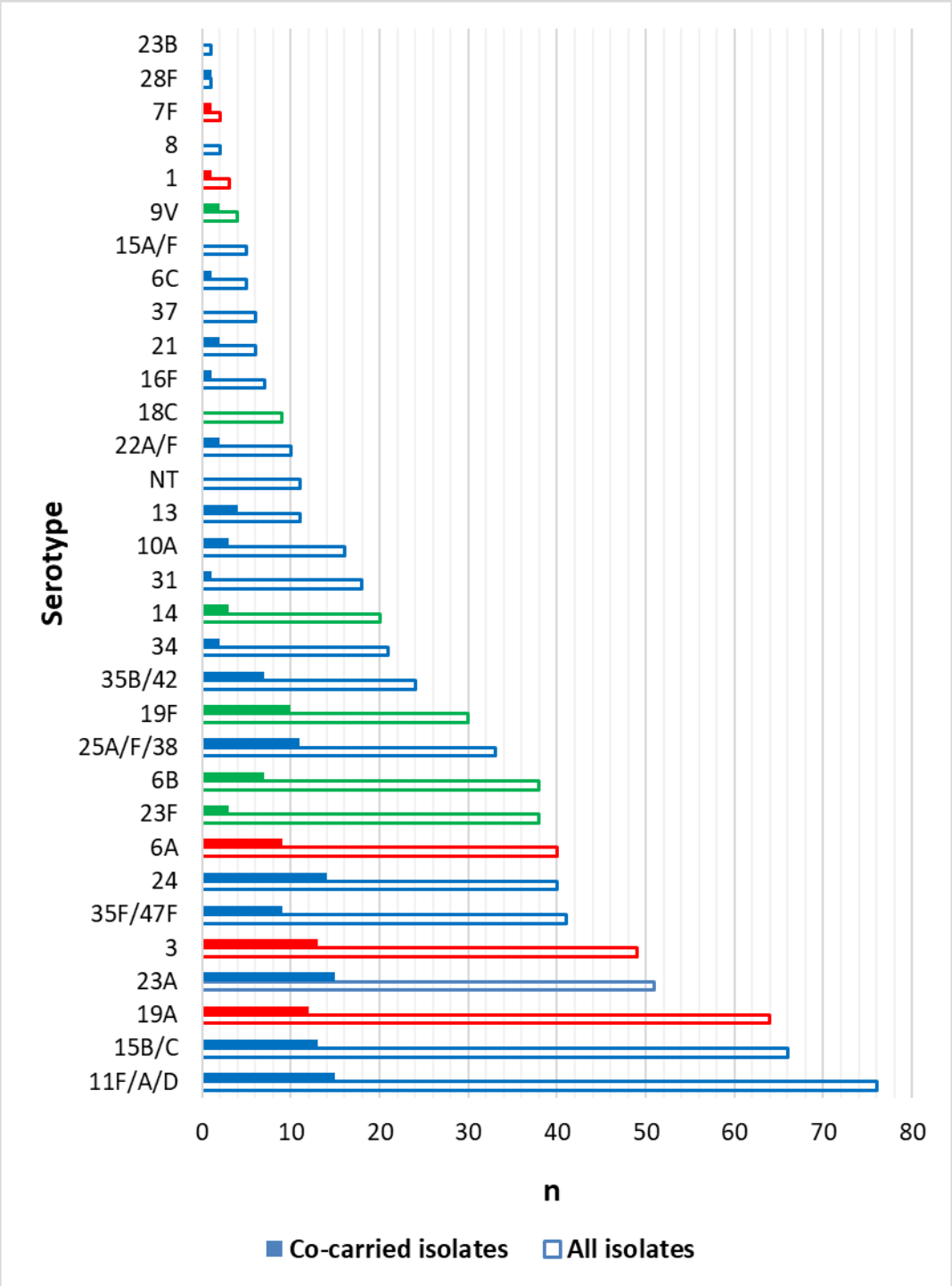

Figure 1. Serotype distribution of all (n=748) versus co-carried (n=162) *S. pneumoniae* isolates among all children (n=2268)

Green column, PCV7 serotypes; red column, additional serotypes in PCV13, blue column, non-PCV serotypes; NT, non-typable; n, number of isolates.

CONCLUSIONS

The 29.3% *Staphylococcus aureus* nasal carriage rate we observed among university students correlates well with international data and is similar to the prevalence usually detected in the general adult population.

The carriage rate determined in the two groups (Group-2 and Group-3) of screened preschool children were quite dissimilar, 21.3% versus 34.1%. However, the average nasal colonisation prevalence of children (29.1%) is comparable to other European countries' data.

Our observation calculating 0.2% average nasal MRSA colonisation prevalence is still low. Mostly in the Eastern part of the world, much higher data are examined causing an alarming problem.

We found positive correlation between *S. aureus* carriage and male gender, as well as children's siblings attending community. The latter correlation was observed only when we analysed the combined children population. This emphasises the great significance of the number of samples during statistical analysis.

Carried isolates are generally more sensitive to antibiotics than clinical ones. Our data supports well this fact, as we compared the resistance rates to clinical isolates' data from Hungary observed in the same time period. According to international data, our antibiotic sensitivity results are similar or lower than detected in other European countries.

Half of the MRSA strains totally observed were phenotypically oxacillin-sensitive. Without a PCR screening of the *mecA* gene, these isolates would have been lost. It proves the significance of molecular techniques during diagnostic process.

During SCC*mec* typing, we found type IV and V. Both types are traditionally observed in CA-MRSA strains all over the world, which further supports the community-acquired origin of our isolates.

Based on MLST typing it can be concluded, that our strains are not unique.

Based on the high diversity of PFGE patterns and the presence of the same MLST types suggests that although certain successful clones might circulate in the country, these strains are in a dynamic change over time, which is reflected by the more sensitive PFGE method.

The commonly used clump-test in routine laboratories was negative in 1.2% of all our *S. aureus* cases, however they carried the *nucA* gene. This finding also proves the importance of specific gene detection in diagnostics.

One further special isolate was examined by us, showing negative catalase-test. Applying *nucA* gene detection and MALDI-TOF method, we could identify it as *S. aureus*. Without these modern techniques, this isolate would have been also lost during the identification process. We were able to prove by *katA* gene sequencing, that the molecular base of the absence of catalase production is a novel nonsense mutation in the gene. This is the first case in the literature, when a catalase-negative *S. aureus* was isolated from a healthy carrier.

The rate of *S. aureus* and *S. pneumoniae* co-colonisation observed (7.1%) more or less fits with international data. We found negative association between these two pathogens, as many others before.

Previously, association between the level of PCV7 vaccination and *S. aureus* carriage was not analysed in the literature. Our novel finding is the statistically significant positive correlation between high-level of vaccination rate in the children community – but not at the individual level – and *S. aureus* nasal carriage.

At the end we can state, that understanding the epidemiology of nasal carriage can lead us closer to find solutions for the management and control of infections caused by *S. aureus*, especially MRSA.

LIST OF PUBLICATIONS

Papers related to the thesis

Laub K, Kristóf K, Tirczka T, Tóthpál A, Kardos S, Kovács E, Sahin-Tóth J, Horváth A, Dobay O. (2017) First description of a catalase-negative *Staphylococcus aureus* from a healthy carrier, with a novel nonsense mutation in the *katA* gene. Int J Med Microbiol, In press, DOI:10.1016/j.ijmm.2017.10.011.

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Laub K, Kardos S, Nagy K, Dobay O. (2011) Detection of *Staphylococcus aureus* nasal carriage in healthy young adults from a Hungarian University. Acta Microbiol Immunol Hung, 58: 75-84.