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

Ph.D. thesis

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LIST OF ABBREVIATIONS

AB, antibiotic

agr, accessory gene regulator

ATCC, American Type Culture Collection

bp, base pair

CA-MRSA, community-acquired methicillin-resistant *Staphylococcus aureus*

CFNSA, clumping factor-negative *Staphylococcus aureus*

CFU, colony-forming unit

CIP, ciprofloxacin

CLI, clindamycin

$cMLS_B$, constitutive macrolides-lincosamides-streptogramin B

CNSA, catalase-negative *Staphylococcus aureus*

DCC, day-care centre

DNA, deoxyribonucleic acid

D-test, double disc diffusion test

erm, erythromycin ribosomal methylase gene

ERY, erythromycin

ETA / *eta*, exfoliative toxin A / exfoliative toxin A gene

ETB / *etb*, exfoliative toxin B / exfoliative toxin B gene

EUCAST, The European Committee on Antimicrobial Susceptibility Testing

for, forward primer

FOX, cefoxitin

GEN, gentamicin

HA-MRSA, hospital-acquired methicillin-resistant *Staphylococcus aureus*

HIV, human immunodeficiency virus

hVISA, heterogeneous vancomycin intermediate *Staphylococcus aureus*

I, intermediate-resistant

IgG, immunoglobulin G

$iMLS_B$, inducible macrolides-lincosamides-streptogramin B

katA, catalase-enzyme gene

MALDI-TOF MS, matrix-assisted laser desorption ionization – time-of-flight mass spectrometry

MIC, minimum inhibitory concentration

MLS_B, macrolides-lincosamides-streptogramin B

MLST, multilocus sequence typing

MRSA, methicillin-resistant *Staphylococcus aureus*

MSSA, methicillin-sensitive *Staphylococcus aureus*

MUP, mupirocin

NCE, National Center for Epidemiology

NS, not significant

nucA, thermonuclease gene

ON, overnight

OS-MRSA, oxacillin-sensitive methicillin-resistant *Staphylococcus aureus*

OXA, oxacillin

PBP, penicillin-binding protein

PCR, polymerase chain reaction

PCV, pneumococcal conjugate vaccine

PEN, penicillin

PFGE, pulsed-field gel electrophoresis

P-V leukocidin, Panton-Valentine leukocidin

R, resistant

rev, reverse primer

rRNA, ribosomal ribonucleic acid

S, sensitive

SCC*mec*, staphylococcal cassette chromosome *mec*

sea, staphylococcal enterotoxin A gene

seb, staphylococcal enterotoxin B gene

sec, staphylococcal enterotoxin C gene

spa, staphylococcal protein A gene

SSSS, staphylococcal scalded skin syndrome

TET, tetracycline

TMP/SMX, trimethoprim-sulfamethoxazole

TSS, toxic shock syndrome

TSST / *tsst*, toxic shock syndrome toxin / toxic shock syndrome toxin gene

VAN, vancomycin

VISA, vancomycin intermediate *Staphylococcus aureus*

VRE, vancomycin-resistant *Enterococcus*

VRSA, vancomycin-resistant *Staphylococcus aureus*

1 INTRODUCTION

1.1 *Staphylococcus* genus in general

Staphylococci are **Gram-positive**, nonmotile, non-spore-forming cocci, sized 0.5-1.5 µm in diameter. The genus name refers to the fact that the cells form grape-like clusters microscopically (**Figure 1**). However, in clinical samples bacteria may appear as single cells, pairs or short chains. The presence or absence of catalase enzyme production is used to subdivide different genera; staphylococci are **catalase-positive**, while streptococci are catalase-negative organisms. Catalase catabolises toxic hydrogen peroxide into water and oxygen and its production is examined by catalase-test (**Figure 2**) [1].

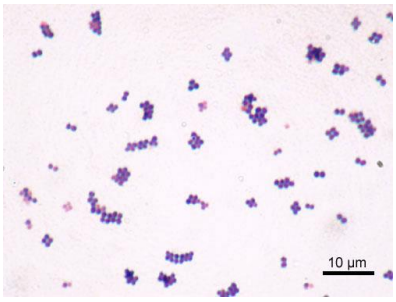

Figure 1. Gram stain of staphylococcal cells

Figure 2. A representative positive and negative catalase-test, photo by *K. Laub*

Staphylococci are ubiquitous bacteria, found on the skin and mucous membranes of humans and animals as well. They are important human pathogens causing a wide spectrum of life-threatening diseases and opportunistic infections. The species most commonly associated with human infections are *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis (S. epidermidis)*, *Staphylococcus haemolyticus*, *Staphylococcus lugdunensis (S. lugdunensis)*, and *Staphylococcus saprophyticus (S. saprophyticus)*. *S. aureus* is the only species out of them producing **coagulase** enzyme, therefore other members of the genus are collectively called as coagulase-negative staphylococci [1].

1.2 Description of *Staphylococcus aureus* in general

1.2.1 Cultivation

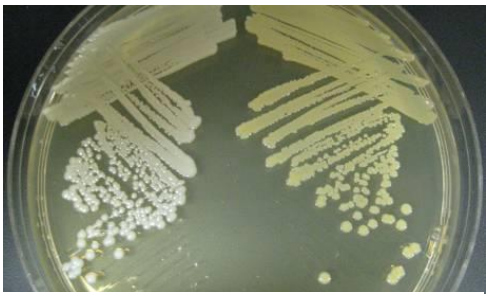

Figure 3. Appearance of cultured *Staphylococcus aureus* [2]

S. aureus is a facultatively anaerobic bacterium, growing rapidly on nonselective media with large, smooth colonies within 24 hours incubation. The species name refers to the fact that its colonies are gradually golden due to the production of carotenoid pigments, particularly when the cultures are incubated at room temperature (**Figure 3**). Nonetheless, it is able to grow in media containing a high concentration of salt and at a relatively wide temperature range (18-40°C). From clinical specimens, *S. aureus* can be isolated on selective media, like **mannitol-salt agar**. This special culture medium is supplemented with mannitol, that is fermented by *S. aureus* but not by most other staphylococci, and also contains 7.5% sodium chloride, which inhibits the growth of most other organisms. Almost all isolates of *S. aureus* produce **β-haemolysis** on sheep blood agar plates, caused by cytotoxins [1, 2].

1.2.2 Pathogenesis and major virulence factors

1.2.2.1 Structural components

The cell wall of many staphylococci is covered with a **polysaccharide capsule**, which inhibits phagocytosis of the bacteria by polymorphonuclear leukocytes [1]. Eleven capsular serotypes of *S. aureus* have been identified. Strains of serotypes 1 and 2 have very thick capsules, thus produce mucoid colonies on solid medium but are rarely encountered among clinical isolates. In contrast, isolates belonging to the remaining serotypes form nonmucoid colonies as well as strains lacking a capsule. **Serotype 5** and **8** isolates are most prevalent among human specimens, accounting about 25% and 50%, respectively. Moreover, these two serotypes are prevalent not only among clinical isolates but among commensal sources and animals as well [3].

Most staphylococci produce a loose-bound, water-soluble slime layer and can form **biofilms** on the host surfaces and prosthetic devices, protecting them from host defences and antimicrobials [1, 4].

S. aureus cell wall is usually coated with **protein A**, in contrast to coagulase-negative staphylococci. It is an immunoglobulin-binding protein with a unique affinity for the Fc receptors, inhibiting antibody-mediated clearance [5].

1.2.2.2 Enzymes

One of the most important virulence factors of *S. aureus* is coagulase enzyme. One type of it is the bound coagulase or so called **clumping factor**, binding fibrinogen and converting it to insoluble fibrin, which finally causes the aggregation of bacteria. Another type, **free coagulase** is also possessed by the bacterium, reacting with a globulin plasma factor (coagulase-reacting factor) to form staphylothrombin. This factor catalyses the same fibrinogen to fibrin conversion like clumping factor. As a result, a fibrin clot is formed around the bacteria avoiding phagocytosis [1, 4].

Numerous other enzymes are produced by *S. aureus* that function in the hydrolysis of host tissue components and help spreading the bacteria. For instance, **hyaluronidase** degrades hyaluronic acids of connective tissue; **fibrinolysin** (also called staphylokinase) dissolves fibrin clots; **lipases** hydrolyse lipids supporting the survival of bacteria in the sebaceous areas of the body. A thermostable **nuclease** is also produced being able to hydrolyse viscous DNA [1].

1.2.2.3 Toxins

S. aureus produces many toxins, including five cytolytic toxins (alpha, beta, gamma, delta and Panton-Valentine [P-V] leukocidin), two exfoliative toxins (A and B), eight enterotoxins (A to E, G to I), and toxic shock syndrome toxin-1 (TSST-1) [1].

Most strains causing human disease produce **alpha toxin**, which disrupts the smooth muscle in blood vessels and is toxic to many other types of cells, like erythrocytes or hepatocytes. **Beta toxin** (also called sphingomyelinase C) has a specificity for sphingomyelin and lysophosphatidylcholine and is toxic to variety of cells, catalysing the

hydrolysis of membrane phospholipids. **Delta toxin** has a wide spectrum of cytolytic activity as well. **Gamma toxin** and **P-V leukocidin** can lyse neutrophils and macrophages. Opposed to the other mentioned cytotoxins, P-V leukocidin has no haemolytic activity [1].

Exfoliative toxins are responsible for staphylococcal scalded skin syndrome (SSSS). Two forms of exfoliative toxin have been identified, a heat-stable (exfoliative toxin A [ETA]) and a heat-labile (exfoliative toxin B [ETB]) one. The gene of ETA (*eta*) is coded in the bacterial chromosome, whereas *etb* is plasmid mediated. Both of them are serine proteases splitting desmogelin 1 located in the stratum granulosum epidermis. ETA belongs to the class of **superantigens**, capable of inducing nonspecific activation of T-cells and massive cytokine release [6].

TSST-1 is a heat- and proteolysis-resistant toxin, furthermore another superantigen. Approximately 90% of *S. aureus* strains causing menstruation-associated toxic shock syndrome (TSS) produce this toxin. It is able to penetrate mucosal barriers, responsible for the systemic effects of TSS, while the infection itself remains localised [7].

Staphylococcal enterotoxins are stable to heating at 100°C for 30 min and resistant to gastric and jejunal enzymes. **Enterotoxin A** is most commonly associated with food poisoning, **enterotoxin C** and **D** are found in contaminated milk products, while **enterotoxin B** is responsible for staphylococcal pseudomembranous colitis. Enterotoxins are also superantigens, produced by 30% to 50% of all *S. aureus* strains [7].

1.2.3 Clinical diseases

1.2.3.1 Suppurative infections

Localised cutaneous pyogenic staphylococcal infections are folliculitis, furuncles, carbuncles and impetigo. **Folliculitis** is the pyogenic infection of the hair follicles. If the eyelid is affected, it is called a **stye**. **Furuncles** are the extension of folliculitis, more painful and occasionally surgical incision is needed to treat. If furuncles extend to the

deeper subcutaneous tissue, it is called **carbuncles**. From this type of skin lesions bacteria can spread and cause bacteraemia [1].

Impetigo is a superficial infection of the skin, mostly affecting young children and occurring primarily on the face and limbs. In 20% of the cases, *Streptococcus pyogenes* is the causative agent alone or with *S. aureus* [1].

Primarily colonising bacteria can cause **wound infections** due to a surgical procedure or after a trauma. If foreign bodies are presented in the wound, staphylococci can more likely cause infection [1].

S. aureus is one of the most common causative agents of **bacteraemia**. Approximately in one third of the cases, the initial focus of the infection remains unknown. In these cases, bacteria invade to the blood supposedly from an innocuous-appearing skin infection. If bacteraemia persists, other body sites can be infected during bacterial dissemination, including the heart. **Endocarditis** caused by *S. aureus* is a serious disease with a high mortality rate. Initial symptoms are usually non-specific, however the condition of the patient can deteriorate rapidly [5].

Respiratory diseases caused by *S. aureus* can develop either after the aspiration of oral secretions or from haematogenous spread. Aspiration **pneumonia** affects primarily the very young, the elderly, and patients suffering from other (mainly chronic) pulmonary disease. A severe form of necrotizing pneumonia is commonly caused by community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains [8].

Another severe clinical disease caused by the haematogenous dissemination of *S. aureus* is **osteomyelitis**. In children, generally a cutaneous infection is the source and the metaphyseal area of long bones are involved. In contrast, in adults the form of vertebral osteomyelitis is seen usually. Osteomyelitis can occur also as a secondary infection resulting from trauma or from the extension of a staphylococcal infection from an adjacent area of bones [8].

Septic arthritis is primarily caused by *S. aureus* in young children with a good prognosis. In adults, it can occur after applying intraarticular injections or again as a result of haematogenous spread [9].

1.2.3.2 Toxin-mediated diseases

SSSS or **Ritter disease** caused by exfoliative toxin occurs primarily in neonates and young children. The formation of large bullae and blisters is characteristic for it, followed by the desquamation of the epithelium. Within 7 to 10 days the skin becomes intact again due to the production of antibodies by the patient. Its mortality is low, unless it is complicated with a bacterial superinfection of the damaged skin. In adults, the prognosis is worse as they are often immunocompromised. **Bullous impetigo** is a localised form of SSSS caused by specific toxin-producing *S. aureus* strains affecting children mostly [6].

TSS caused by TSST-1 producing *S. aureus* strains was previously associated with menstruating women. After the recall of hyperabsorbent tampons – in which the bacterium could multiply rapidly – the incidence of the disease decreased dramatically. Clinical signs are fever, hypotension, a diffuse erythematous rash followed by the desquamation of the entire skin, including the palms and soles together with multiple organ failure. Without antibiotic therapy and elimination of the focus of infection, it can be fatal. **Purpura fulminans** is a particularly virulent form of the syndrome characterised by purpuric skin lesions and disseminated intravascular coagulation [7].

Staphylococcal **food poisoning** is an intoxication caused by toxins present is food. Commonly contaminated foods are processed meats, pastries, potato salad and ice cream. The source of the toxin-producing bacteria is a human carrier. Approximately in half of the cases, bacteria originate from the nasopharyngeal colonisation of the carrier by sneezing or contaminated hand. Heat-stable toxins cannot be inactivated by subsequent heating of the food, however, bacteria will be killed [1].

1.2.4 Identification

The successful detection of *S. aureus* from clinical specimens much depends on the quality of the sample and the type of infection as well. For example, from an abscess scraping the base with a swab will contain more bacteria than aspiration of pus, which is full of necrotic material [1].

The classical diagnostic identification of *S. aureus* is based on microscopic appearance, colony morphology, catalase- and clump-test positivity and further biochemical tests. The species-specific thermonuclease gene (*nucA*) can be detected rapidly by PCR. For epidemiologic purposes antibiotic susceptibility patterns, phage typing and several molecular typing techniques can be used, such as PFGE (pulsed-field gel electrophoresis), MLST (multilocus sequence typing), accessory gene regulator (*agr*) typing, staphylococcal protein A gene (*spa*) typing, toxin gene profiling or amplified fragment length polymorphism analysis [1, 10].

1.2.5 Antibiotic treatment and resistance mechanisms

1.2.5.1 β-lactam resistance

A common feature of Gram-positive bacteria is the thick and rigid peptidoglycan layer of the cell wall. **Penicillin-binding proteins** (PBP) are the enzymes catalysing the construction of this layer. PBPs are also the target of penicillin and other β-lactam antibiotics [1]. Within 10 years after benzylpenicillin was introduced for use in people, it was no longer effective against *S. aureus* infections due to **penicillinase** production of the bacterium [11, 12]. These enzymes are the members of β-lactamases, which hydrolyse the β-lactam ring of penicillin. Its production is plasmid-encoded, that is responsible for the rapid dissemination of resistance among staphylococci [13]. Penicillin-resistant *S. aureus* strains became pandemic in the late 1950s [11].

Then **semisynthetic penicillins** (e.g. oxacillin, methicillin, flucloxacillin, nafcillin) resistant to β-lactamases were developed, but staphylococci became resistant against them soon. The mechanism of resistance against methicillin (and related antibiotics) is the acquisition of the ***mecA*** gene coding PBP2A, a novel penicillin-binding protein that reduces the binding affinities to β-lactam antibiotics. As a result, MRSA (methicillin-resistant *Staphylococcus aureus*) strains are resistant to all β-lactam antibiotics [13]. The *mecA* gene is located on SCC*mec* (staphylococcal cassette chromosome *mec*), a distinct mobile genetic element of MRSA's chromosome while absent in MSSA's (methicillin-sensitive *Staphylococcus aureus*). SCC*mec* elements are diverse, 11 types (I-XI) have been described so far [14, 15]. The first **MRSA** strain was

reported in 1961 [16], just two years after the introduction of methicillin to treatment. During the next decades, MRSAs spread worldwide and are now endemic in many hospitals. Epidemiologically two types of MRSA infections are distinguished, health-care-associated or hospital-acquired (**HA-MRSA**), for which a predisposing risk factor or illness exists and community-acquired (**CA-MRSA**), when an otherwise healthy individual is infected [11].

A new *mecA* gene homologue, $mecA_{LGA251}$ was described in *S. aureus* isolates originating from both humans and animals in 2011. Later it was renamed ***mecC*** by the International Working Group on the Classification of Staphylococcal Cassette Chromosome (SCC) Elements. It has 69% nucleotide homology with *mecA* and encoded on a novel SCC element, SCC*mec* type XI [14, 17]. Diaz et al. calculated **0.009%** overall estimated prevalence of the *mecC* gene in a meta-analysis. Although, *mecC* positive *S. aureus* isolates are extremely rare and only reported in Europe, they suggested to take them into consideration in the future diagnostic protocols [14].

1.2.5.2 Vancomycin resistance

Vancomycin is a glycopeptide antibiotic inhibiting cell wall biosynthesis. It is still the first choice for treatment of severe MRSA infections. Unfortunately, resistant *S. aureus* isolates have been found to it causing a major concern worldwide [18]. Three levels of resistance is distinguished according to the EUCAST (The European Committee on Antimicrobial Susceptibility Testing) definition: heterogeneous vancomycin intermediate *S. aureus* (**hVISA**), *S. aureus* isolates susceptible to vancomycin (MICs [minimum inhibitory concentration] ≤2 mg/L) but with minority populations (1 in 10^6 cells) with vancomycin MIC >2 mg/L; vancomycin intermediate-resistant *S. aureus* (**VISA**) with MIC 4 - 8 mg/L and vancomycin-resistant *S. aureus* (**VRSA**) with MIC >8 mg/L [19].

The first **VISA** isolate was reported in 1997 from a patient in Japan [20]. The resistance mechanism of these strains is polygenic, involving mutations in genes responsible for cell envelope synthesis. These strains are associated with the failure of vancomycin treatment causing persistent infections with poor clinical outcomes [21].

The first **VRSA** strain was reported in the USA in 2002 [22]. The resistance of VRSA strains is mediated by the plasmid-encoded ***vanA*** gene operon, originating from vancomycin-resistant enterococci (VRE) [23]. Fortunately, the prevalence of VRSA is still very low, only 14 cases were detected in the USA from 2002 to date [21].

1.2.5.3 Macrolide resistance

As *S. aureus* strains became more resistant to β-lactam antibiotics, the use of macrolides-lincosamides-streptogramin B (MLS_B) antibiotics increased. These drugs are structurally different, but the mode of action is common, they inhibit protein synthesis by reversibly binding to 23S rRNA receptor of the 50S ribosomal subunit [24]. In staphylococci erythromycin ribosomal methylase (*erm*) genes are responsible for MLS_B-resistance. Methylases encoded by them change the bacterial ribosome resulting in reduced binding of MLS_B antibiotics to the target site [25]. Three different *erm* genes have been found in staphylococci, *ermA*, *ermB* and *ermC* [26].

Two phenotypes of MLS_B resistance is differentiated, constitutive (constitutive resistance to clindamycin [$cMLS_B$]) and inducible (inducible resistance to clindamycin [$iMLS_B$]). Bacteria expressing *erm* genes can be resistant to erythromycin, clindamycin and other MLS_B drugs *in vitro*. This type of resistance belongs to the $cMLS_B$ phenotype. On the other hand, those bacteria also possessing *erm* genes, but requiring an inducing agent to express clindamycin resistance, are members of the $iMLS_B$ phenotype. These isolates exhibit *in vitro* erythromycin-resistance and falsely clindamycin-sensitivity. To detect inducible clindamycin-resistance, the disk diffusion induction test (D-test) is used (see later) [24].

Other MLS_B resistance mechanisms are the active efflux encoded by *msr* gene and drug inactivation encoded by *lun* gene [27].

1.3 Nasal carriage of *Staphylococcus aureus*

1.3.1 Determinants of nasal carriage

The anterior nares of the nose are the most frequent carriage sites for *S. aureus*, however, extra-nasal sites can harbour this bacterium, for example skin, perineum, pharynx, gastrointestinal tract, vagina and axillae [28, 29]. As it can be seen on **Figure 4**, *S. aureus* nasal carriers more frequently carry the bacterium at extra-nasal sites as well [28].

Based on longitudinal studies, carriers can be classified into persistent, intermittent and non-carriers. Approximately 20% of humans are persistent *S. aureus* nasal carriers, while about 30% are intermittent carriers and about 50% non-carriers. Children are more commonly persistent carriers than adults. For instance, more than 70% of newborn babies' nasal samples are positive for *S. aureus* at least once. During adolescence, a transition from persistent to intermittent carriage or non-carriage states is observed (**Figure 5**) [28].

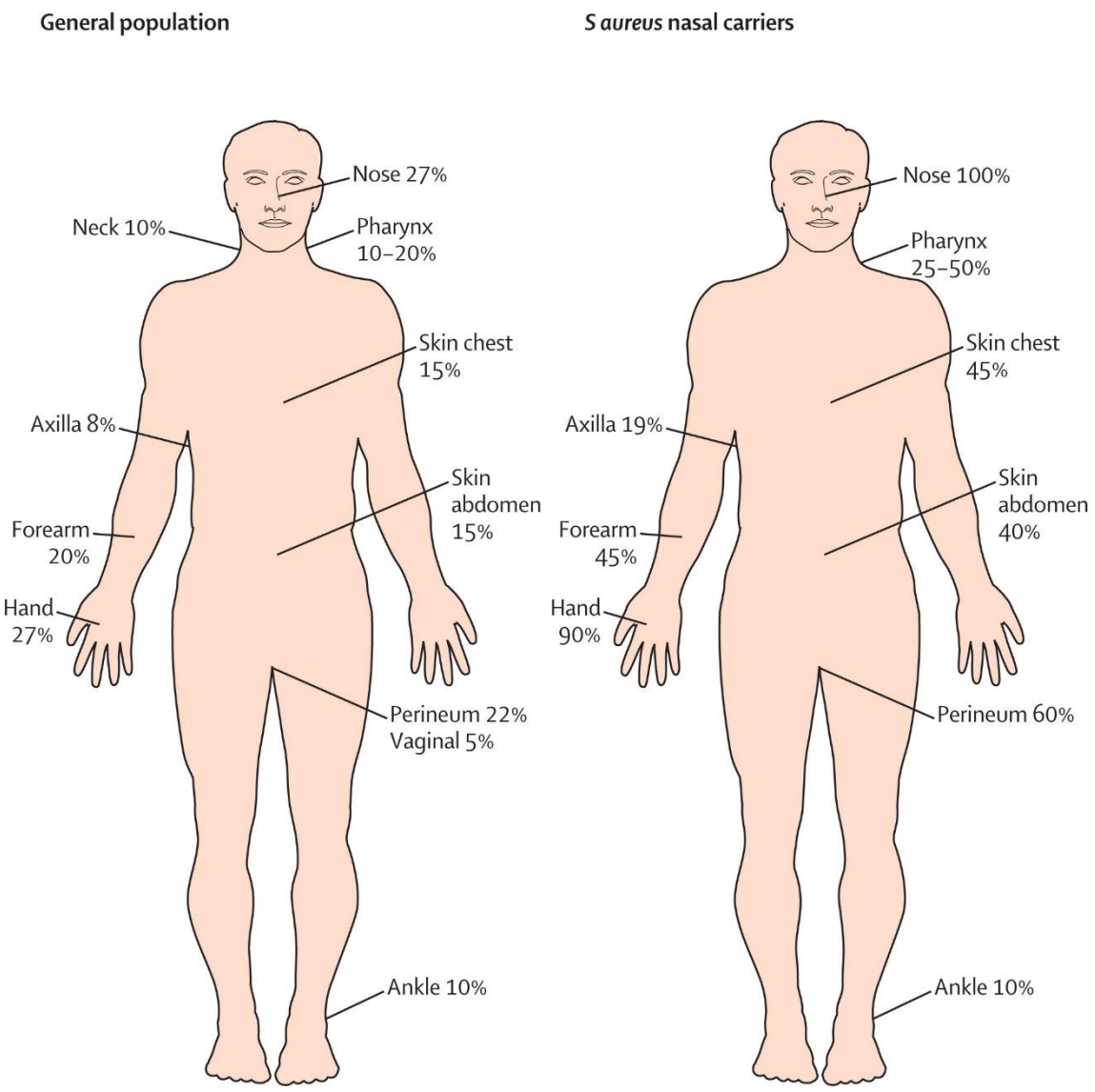

Figure 4. *S. aureus* carriage rates per body sites in adults [28]

Persistent carriers are frequently colonised by a single strain over a long time, opposed to intermittent carriers, who might carry different strains of *S. aureus* [28]. Ghasemzadeh-Moghaddam et al. demonstrated in the case of adults that *S. aureus* nasal carriage is a determinant for acquiring new *S. aureus* strains [30].

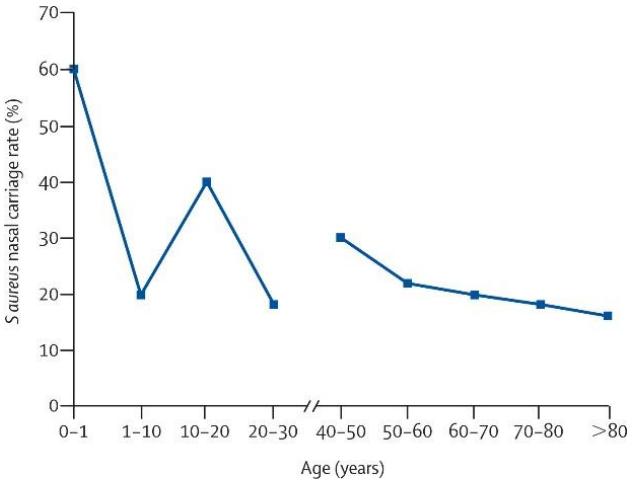

Figure 5. Rates of *S. aureus* nasal carriage according to age [28]

1.3.2 Risk factors for nasal carriage

Wertheim and co-workers have defined several risk factors for *S. aureus* nasal carriage. *S. aureus* carriage is higher among white people, in male gender and depends on age as demonstrated on **Figure 5**. Patients with diabetes mellitus (both type 1 and 2), haemodialysed or continuous peritoneal dialysed patients, patients having end stage liver disease, patients with HIV infection, obesity, previous cerebrovascular accident, *S. aureus* skin infections and chronic skin disease (like eczema, atopic dermatitis or psoriasis) have higher rates of nasal *S. aureus* carriage. Doing contact sport or other activities leading to skin lesions, as well as animal farming are further risk factors. Active cigarette smoking have been showed to be in negative association with *S. aureus* nasal carriage rate, unlike passive smoking, which is a possible risk factor for carriage [28].

Environmental factors, like seasonality, temperature or relative humidity do not correlate with nasal carriage. In contrast, hospitalisation has been proved to be an important risk factor. Furthermore, MRSA can spread among household members from and to health-care workers [28]. It has been also shown that nasal carriage predisposes to nosocomial acquisition of new *S. aureus* strains during hospitalization [30].

1.3.3 From colonisation to infection

The association between staphylococcal disease and its nasal carriage was first declared in 1931 by Danbolt studying furunculosis. [28]. *S. aureus* can be transmitted from asymptomatic carriers to other, susceptible persons, or might cause infection in the carrier itself under certain circumstances. For example, carriers were shown to have a significantly higher risk to acquire a nosocomial *S. aureus* bacteraemia [31, 32]. It is known, that persistent carriers have higher load of *S. aureus*, resulting in higher risk of getting staphylococcal infection [28]. The airborne transmission of *S. aureus* is affected by many factors, for example the number of bacteria colonising the nares. There are several papers about outbreaks due to airborne dispersal of *S. aureus* in association with skin colonisation or viral upper respiratory tract infection of either health-care workers or patients, which is called the "cloud" phenomenon [33]. This phenomenon was established in the case of newborns and adults as well. As much as 75% of newborns carrying *S. aureus* dispersed this bacterium from their noses into the air once they acquired an upper respiratory tract infection [34].

Children attending communities are well known carriers of several pathogenic bacteria such as *S. aureus* and *Streptococcus pneumoniae (S. pneumoniae* or *Pneumococcus)* [35]. Young children might play a special role in horizontal spreading of bacteria as their nasopharynx shows some special features, such as immature immunological response and high susceptibility to viral respiratory infections [36].

1.3.4 Association between *Staphylococcus aureus* and *Streptococcus pneumoniae* carriage

Carriage of *S. pneumoniae* typically occurs in childhood; colonisation is low at birth, but within a few weeks, it reaches high levels. Carriage rate peaks at 6 months to 3 years of age, and afterwards decreases steadily to the low level observed in adulthood (~3% in average), by the age of 19 years [37, 38]. In the contrary, colonisation by *S. aureus* occurs almost immediately in high levels (50-70%) after birth, but soon starts declining. However, it stays much higher also in adulthood compared to *Pneumococcus* as it was mentioned in detail above (chapter 1.3.1.). Of course, carriage rates vary widely in different geographical regions. In summary, pneumococcal colonisation peaks when *S.*

aureus colonisation is lowest. Very probably due to this fact, 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, while it was not observed by some authors in older children and adolescents [37, 39].

1.4 CA-MRSA and its importance

The first cases of community-acquired methicillin-resistant *S. aureus* infection were described in the early 1990s in Australia [11]. Since then, it spread worldwide, some of the countries reported these strains were New Zealand, USA, UK, France, Finland and Canada. Initially, outbreaks occurred amongst children and adults living in close communities. Other high-risk groups were, for example sport participants, the military, prisoners, intravenous drug users or the homeless. Typical diseases caused by CA-MRSA strains are severe skin and soft tissue infections, necrotizing pneumonia or bone and joint infections [10].

CA-MRSA strains can be distinguishable from HA-MRSA strains not only by epidemiological features, but by genetic patterns and antibiotic resistance also. CA-MRSA strains mainly harbour SCC*mec* type IV, V or VII, while most HA-MRSA harbours SCC*mec* types II and III. CA-MRSA is usually more susceptible to non-β-lactam antibiotics, especially ciprofloxacin compared to HA-MRSA strains. P-V leukocidin toxin production is strongly associated with highly transmissible, virulent CA-MRSA strains (and other *S. aureus*), particularly causing community-acquired necrotizing pneumonia [10].

In the recent years, the emergence of CA-MRSA strains became an alarming problem, possessing increased virulence and spreading abilities compared to HA-MRSA isolates [40]. Nowadays, CA-MRSA clones are more frequent cause of hospital-acquired infections as well. This has increased the importance of screening and monitoring *S. aureus* circulation in the community and its antibiotic susceptibility as well [41]. As the epidemiology of CA-MRSA is changing, the traditional epidemiological definitions become further limited highlighting the advantages of genotypic classification of the

strains. There is a growing consensus to define MRSAs by genotyping methods like SCC*mec* typing, *spa* typing, PFGE or MLST [42].

1.5 Prevention and control

The spread of *S. aureus* from person to person is difficult to prevent, because of the colonisation of the skin and mucosal surfaces. Avoiding contamination is a crucial point during surgical procedures for instance. The risk of contamination can be minimized by proper **handwashing** practice and the covering of exposed skin surfaces. In case of skin damage, staphylococci are often introduced into the wound, however proper cleansing and the application of a **disinfectant** will prevent infection in otherwise healthy persons [1].

For the eradication of nasal *S. aureus* carriage, **mupirocin** ointment is used most commonly [43]. Ammerlaan et al. stated in a systematic review, that short term (4-7 days) nasal application of mupirocin is the most effective treatment in MRSA carriage eradication. They estimated 1% risk of acquiring a drug-resistant strain during mupirocin topical treatment [44].

2 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.

3 MATERIALS AND METHODS

3.1 Study population

3.1.1 University students (Group-1)

In the first part of the study in 2009, in the frame of the regular microbiology practical classes at the Institute of Medical Microbiology, Semmelweis University, we have asked the 3rd-year students (21-24 years) on Faculty of General Medicine – on a voluntary base and anonymously – to provide nasal specimens. Totally **300** students (205 Hungarian and 95 non-Hungarian) from 32 different university groups participated in the project and they belong to **Group-1**.

3.1.2 Preschool children from different regions of Hungary (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. They belong to **Group-2**.

3.1.3 Preschool children from Szolnok (Group-3)

In Szolnok city (my hometown by the way) all 20 existing kindergartens agreed to the survey. Between February and June 2012, **1390** children have been screened there creating **Group-3**.

Altogether, we have examined 2268 healthy children in a four-year period. The location of places where samples originated from and the number of children screened are shown later on **Figure 9** (page 49).

3.2 Sample collection and transport media

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.

3.3 Identification of *Staphylococcus aureus*

3.3.1 Phenotypical identification

The nasal samples were inoculated onto Mueller-Hinton blood agar plates. After an overnight (ON) incubation at 37°C, in 5% CO_2, the *S. aureus* suspicious β-haemolytic colonies were isolated to create pure cultures. In some cases only a few β-haemolytic colonies were seen among the normal flora, while in other cases *S. aureus* had grown in nearly pure culture.

First, we confirmed the identity of *S. aureus* isolates by **catalase-test** (**Figure 2**, page 9) and **clump-test** (Pastorex Staph-Plus Kit, Bio-Rad, Marnes-la-Coquette, France). The Pastorex Staph-Plus Kit test we used simultaneously detects three components of *S. aureus*: bound coagulase or clumping factor, protein A and capsular polysaccharides. In special cases (see later), we applied DiaMondial Staph Plus Latex Reagent (DiaMondial, Sees, France) parallel to Pastorex Staph-Plus Kit.

Figure 6. A representative positive and negative tube coagulase test, photo by *K. Laub*

Besides clump-test, also in special cases (see later) **tube coagulase test** was applied to detect the production of free coagulase. To perform this, the Plasma Coagulase EDTA Selectavial kit (Mast Group, Bootle, UK) was used. The lyophilised rabbit plasma in the provided vial was rehydrated with 3 ml distilled water and 0.5 ml aliquots were distributed into thin sterile glass tubes. The reconstituted plasma was inoculated with one loopful (3-4 colonies) of bacteria from a fresh ON culture. The inoculated tubes were incubated at 37°C for up to 6 hours, but observed every half hour. In case of positivity, the plasma

coagulated (**Figure 6**). As a positive control, *S. aureus* ATCC 25923, and as a negative control, *S. epidermidis* ATCC 12228 strains were used, respectively.

At the end, every *S. aureus* suspicious isolates were frozen and kept at –80°C on cryobeads (Cryobank, Mast Diagnostica, Bootle, UK) until further examinations.

3.3.2 Genotypical identification and MRSA screening by *nucA*, *mecA* multiplex PCR

To confirm the identity of the isolates at 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 by PCR. In order to detect the *nucA* and *mecA* genes together in a multiplex reaction, we designed new primer pairs with the Primer3 programme [45] to get well distinguishable PCR products in size (**Table 1**). Primers were manufactured by Csertex Ltd., Hungary. Each reaction included ATCC 33591 as a *nucA*, *mecA* positive external control strain.

Table 1. Primers and PCR product sizes in *nucA*, *mecA* multiplex PCR reaction

Primer name	Sequence (5' → 3')	Amplicon size (bp)	Reference
nucA for	ATGGACGTGGCTTAGCGTAT	193	This study
nucA rev	TGACCTGAATCAGCGTTGTC		
mecA for	CCCAATTTGTCTGCCAGTTT	538	This study
mecA rev	ATCTTGGGGTGGTTACAACG		

bp, base pair; for, forward; rev, reverse

3.3.2.1 DNA template preparation

First of all, we inoculated one bead of the frozen isolate onto Mueller-Hinton blood agar plates and incubated ON at 37°C, in 5% CO_2. Next day, inoculation onto a fresh plate was repeated with the same incubation conditions. Approximately one loopful from the pure culture of the bacterium was suspended into 250 µl nuclease free distilled water and boiled for 15 minutes at 99°C using a PCR machine. After short vortexing, the suspension was centrifuged for 2 min at 6000 rpm (Hermle Z 160M centrifuge) and then 2.5 µl of the supernatant was used as template in the PCR reactions.

3.3.2.2 PCR mix preparation

First, every component detailed in **Table 2** was mixed except for template, which was added to the mix after aliquoting into PCR tubes.

Table 2. PCR mix for *nucA*, *mecA* multiplex reaction

Ingredients	**Volume (µl) for 1 reaction**
DreamTaq Green PCR Master Mix	12.5
nucA for*	0.5
nucA rev	0.5
mecA for	0.5
mecA rev	0.5
Water, nuclease-free	8
DNA template	2.5
Total	**25**

* Concentration of primers was 50 pmol/µl in every case

3.3.2.3 PCR cycle

The amplification temperatures and the length of the time (so called cycling parameters) are shown in **Table 3**. We used a Corbett Research PCR machine controlled by Hewlet Packard PDA and a GeneAmp PCR System 9700 (PE Applied Biosystem).

Table 3. PCR cycling parameters for the amplification of *nucA, mecA*

Repeat	Temperature	Time
1x	94°C	3 min
30x	94°C	1 min
	54°C	1 min
	72°C	30 s
1x	72°C	10 min

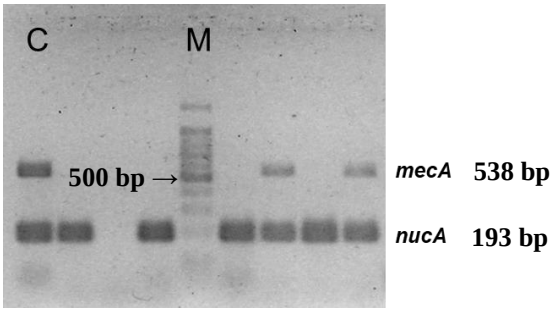

Figure 7. A representative gel image of *nucA*, *mecA* multiplex PCR, photo by *K. Laub*

C, positive control; M, marker, bp, base pair

3.3.2.4 Gel running and analysis

1.5% agarose gel was prepared with 1x TEA buffer (for solutions see **Table 18** in chapter 3.12). 10 µl of each sample was alliquoted into the gel and run together with the 100 bp DNA ladder (Promega UK Ltd, Southampton, UK) mixed with Blue/Orange Loading Dye (Promega) as molecular weight marker in every case. Running parameters were: 100 V, 36 min. For gel staining we used GelRed Nucleic Acid Gel Stain (Biotium, USA). Finally a photo image was made by a digital camera (**Figure 7**).

3.3.3 *MecC* detection by PCR

MecC is less frequently detected in MRSA strains than *mecA* [14], but for more correct screening, we checked this gene also in isolates with oxacillin MIC ≥1 mg/L by PCR in a separate reaction as described by Paterson et al. [46]. Briefly, DNA template preparation, PCR mix and gel running parameters were the same as detailed above in

chapter 3.3.2. Primers used can be seen in **Table 4**, for cycling parameters see **Table 5**. We used ATCC BAA-2312 strain as positive control.

Table 4. Primers and PCR product size in *mecC* PCR reaction

Primer name	Sequence (5' → 3')	Amplicon size (bp)	Reference
mecC for	CATTAAAATCAGAGCGAGGC	188	[46]
mecC rev	TGGCTGAACCCATTT TTGAT		

Table 5. PCR cycling parameters for the amplification of *mecC*

Repeat	Temperature	Time
1x	94°C	3 min
30x	94°C	1 min
	57°C	1 min
	72°C	30 s
1x	72°C	10 min

3.3.4 Identification by matrix-assisted laser desorption ionization – time-of-flight mass spectrometry (MALDI-TOF MS)

During the identification process we faced some diagnostic problems. There were 10 isolates, which looked like *S. aureus* by colony morphology and the *nucA* gene was detected, but the catalase- or clump-tests were negative or equivocal. To further identify these phenotypically ambiguous isolates, MALDI-TOF mass spectrometry (Bruker Daltonics, Bremen, Germany) was applied in collaboration with the Department of Laboratory Medicine, Semmelweis University. During this process the direct smear and 1 µl α-cyano-4-hydroxycinnamic acid matrix overlay method was used [47]. The parameters of the device were set applying Bruker's recommendation. Identifications were assigned using the Bruker Biotyper 2.0 software.

3.4 Antibiotic susceptibility testing

We determined the minimum inhibitory concentration (MIC) to several groups of antibiotics 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 guidelines and breakpoints were applied [48]. Breakpoints (and comments) for the tested antibiotics according to the latest guidelines (Version 7.1, valid from 2017-03-10) are shown in **Table 6**. ATCC 29213 was used as control strain.

3.4.1 Susceptibility testing by E-test and disc diffusion

For samples originated from university students, susceptibility to oxacillin, erythromycin, clindamycin, gentamicin, ciprofloxacin and vancomycin was measured by gradient test strips (Liofilchem, Roseto degli Abruzzi, Italy). In the case of trimethoprim-sulfamethoxazole (TMP/SMX), we also used this method for children's isolates. First, we set inoculum density (in physiological saline) to 0.5 McFarland by a VITEK Densichek (Biomérieux, Marcy l'Etoile, France) densitometer. Then, bacterial suspensions were inoculated onto Mueller-Hinton agar plates and 2 strips were positioned onto it in a V-shape (see later on **Figure 10**, page 51). After that plates were incubated at 37°C, in ambient air, ON.

Besides MIC measurement, penicillin susceptibility was also checked with 1 IU discs (Bio-Rad), because disc diffusion is more reliable than MIC determination for the detection of penicillinase enzyme, produced by *S. aureus* as suggested by EUCAST. If the zone diameter was <26 mm, then the isolate was reported resistant [48].

For the more accurate detection of MRSA, cefoxitin discs (30 µg, Bio-Rad) were used parallel to oxacillin MICs according to the EUCAST recommendations. Isolates with zone diameter <22 mm were reported resistant [48].

For the isolates resistant to erythromycin but sensitive to clindamycin, double disc diffusion (**D-test**) was performed to detect inducible clindamycin resistance, using 15 µg erythromycin and 2 µg clindamycin discs (Bio-Rad). The discs were positioned at a distance of 15 mm apart from one another and in case of inducible clindamycin resistance,

the original round inhibition zone flattened in the proximity of the erythromycin disc, providing a D-shape (see later on **Figure 12**, page 52).

3.4.2 Susceptibility testing by agar dilution method

In the case of isolates collected from children, MIC to penicillin, oxacillin, erythromycin, clindamycin, tetracycline, gentamicin, ciprofloxacin and mupirocin was determined by agar dilution method. First, we suspended a small amount of bacteria from pure culture in physiological saline to reach 0.5 McFarland standard (equivalent to 10^7 CFU [colony-forming unit]/mL). Then 10 µl of each bacterial suspension was inoculated on Mueller-Hinton agar plates (resulting ~10^5 CFU/spot), using an A400 Multipoint Inoculator (AQS Manufacturing Ltd., Southwater, UK) demonstrated on **Figure 8A**. After ON incubation at 37°C in ambient air, bacterial growth was detected visually and MIC values were determined (**Figure 8B**).

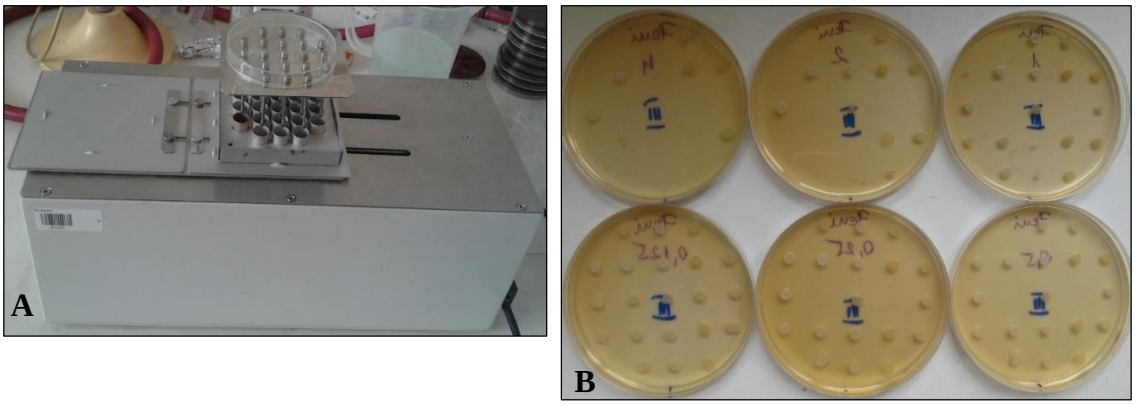

Figure 8A. Multipoint inoculator 8B. Agar dilution method

Plates contain different concentrations of penicillin (0.125 – 4 mg/L). Each spot represents a different *S. aureus* isolate. In the lowest concentration of the antibiotic all strains could grow (see lower left corner), while higher concentrations inhibited more and more strains, photo by *K. Laub.*

Table 6. Applied EUCAST breakpoints (v. 7.1.) for interpretation of MICs

Antibiotic	EUCAST breakpoint (mg/L)		
	Sensitive (S)	**Resistant (R)**	**Intermediate (I)**
Penicillin (PEN)	≤0.125	>0.125	-
Oxacillin (OXA)[1]	≤2	>2	-
Ciprofloxacin (CIP)	≤1	>1	-
Gentamicin (GEN)	≤1	>1	-
Trimethoprim-sulfamethoxazole (TMP/SMX)	≤2	>4	4
Erythromycin (ERY)[2]	≤1	>2	2
Clindamycin (CLI)[3]	≤0.25	>0.5	0.5
Tetracycline (TET)[4]	≤1	>2	2
Vancomycin (VAN)	≤2	>2	-
Mupirocin (MUP)[5]	≤1	>256	>1, ≤256

1 ”*S. aureus, S. lugdunensis* and *S. saprophyticus* with oxacillin MIC values >2 mg/L are mostly methicillin resistant due to the presence of the *mecA* or *mecC* gene.”

2 ”Erythromycin can be used to determine susceptibility to azithromycin, clarithromycin and roxithromycin.”

3 ”Inducible clindamycin resistance can be detected by antagonism of clindamycin activity by a macrolide agent. If not detected, then report as susceptible. If detected, then report as resistant and consider adding this comment to the report: "Clindamycin may still be used for short-term therapy of less serious skin and soft tissue infections as constitutive resistance is unlikely to develop during such therapy".”

4 ”Isolates susceptible to tetracycline are also susceptible to doxycycline and minocycline, but some resistant to tetracycline may be susceptible to minocycline and/or doxycycline. An MIC method should be used to test doxycycline susceptibility of tetracycline resistant isolates if required.”

5 ECOFF (epidemiological cut-off value) = 1 mg/L, “Breakpoints for nasal decontamination S≤1, R>256 mg/L. Intermediate isolates are associated with short term suppression (useful preoperatively) but, unlike susceptible isolates, long term eradication rates are low.”

3.5 Genotyping by pulsed-field gel electrophoresis (PFGE)

To determine the genetic relatedness of the isolates, pulsed-field gel electrophoresis was used on the bases of the description by Szabó et al. [49] with some modifications. We have chosen this technique as it was shown to be an excellent typing tool in similar regional surveys due to its high discriminatory power [50].

3.5.1 Chromosomal DNA preparation

A small amount of bacteria (approx. half of a 5 µl loop) from blood agar plates were suspended in 200 µl EC lysis buffer. This suspension was mixed with an equal volume of 2% chromosomal-grade low-melting agarose (Bio-Rad), prepared freshly in EC lysis buffer and dispensed into plug moulds. After solidification at 4°C, the plugs were first incubated at 37°C for 1 h in 1.5 ml of Lysis-1 buffer, and then at 54°C for 3 h in 1.5 ml of Lysis-2 buffer (**Table 7**).

Table 7. Ingredients of Lysis-1 and Lysis-2 buffers used in PFGE

Lysis-1 buffer for 1 plug		**Lysis-2** buffer for 1 plug	
Ingredient (conc.)	**Volume**	**Ingredient** (conc.)	**Volume**
EC lysis buffer	1.5 ml	ESTN buffer	1.5 ml
Lysostaphin (1 mg/ml)	16.7 µl	Proteinase K (20 mg/ml)	10 µl
Lysozyme (20 mg/ml)	20 µl		

Then the plugs were washed four times in 1.5 ml TE buffer at room temperature for 30 min each and either cut into half and processed to the digestion step, or stored at 4ºC in TE buffer until further usage.

3.5.2 Digestion

After a washing step with distilled water, one half of each plug was digested with 10 U of *Sma*I restriction enzyme (Promega, Madison, USA) in buffer SA for 3 h at 25°C (**Table 8**).

Table 8. Digestion solution for the purified PFGE plugs

Restriction mix	**Volume (μl) for 1/2 plug**
*Sma*I enzyme	1 (=10 unit)
10x cc. buffer SA	12.5
dH_2O	125
Total	**138.5**

3.5.3 Gel running

After an additional washing step with distilled water, the digested plugs were run in a 1% pulsed-field certified agarose gel (Bio-Rad, USA) prepared in 0.5x TBE buffer to separate DNA fragments in a CHEF-DR® II apparatus (Bio-Rad, USA) at 14ºC. The accurate pulse times and running parameters are detailed in **Table 9**. The N0340S Lambda Ladder PFG Marker (New England Biolabs, Hitchin, Hertfordshire, UK) was used as the molecular weight control.

Table 9. Parameters of PFGE gel running, modified after Szabó et al. [49]

Parameter	**BLOCK-1**	**BLOCK-2**
Initial time:	5 s	15 s
Final time:	15 s	60 s
Running time:	10 h	11 h
Voltage:	6 V/cm (~ 200 V)	6 V/cm (~ 200 V)

3.5.4 Gel analysis

After staining with GelRed (Biotium, USA), the gel image was captured by a digital camera. The PFGE patterns were analysed (normalisation, gel comparison) and the dendrograms were created by the Fingerprinting II software (Bio-Rad, Marnes-la-Coquette, France). A PFGE genotype was defined as isolates showing at least 90% similarity in the dendrogram created by the unweighted pair group method using arithmetic averages (UPGMA) and the different bands similarity coefficient, with a band position tolerance of 1.0%.

3.6 SCC*mec* typing

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. Parts of the respective type-specific recombinase genes (*ccr*) were amplified by using the primers designed by Milheiriço et al. [51]. The J3 region was detected in addition for type IV, with primers (dcs) published by Oliveira et al. [52]. Primers and PCR product sizes are detailed in **Table 10**, cycling parameters in **Table 11**.

Table 10. Primers used for PCR detecting SCC*mec* types IV and V

Primer name (SCC*mec* type)	**Sequence (5' → 3')**	**Amplicon size (bp)**	**Reference**
ccrB2 (IV) for	AGTTTCTCAGAATTCGAACG	311	[51]
ccrB2 (IV) rev	CCGATATAGAAWGGGTTAGC		
dcs (IV) for	CATCCTATGATAGCTTGGTC	342	[52]
dcs (IV) rev	CTAAATCATAGCCATGACCG		
ccrC (V) for	GTACTCGTTACAATGTTTGG	449	[51]
ccrC (V) rev	ATAATGGCTTCATGCTTACC		

Table 11. PCR cycling parameters for the amplification of ccr-IV, V and J3-IV

Repeat	Temperature	Time
1x	94°C	3 min
35x	94°C	45 s
	53°C	45 s
	72°C	1 min
1x	72°C	5 min

3.7 Multilocus sequence typing (MLST)

We used multilocus sequence typing for the MRSA strains and for the catalase-negative isolate (see later). During this, well-defined sections of seven housekeeping genes were amplified by PCR, using the primers provided on the MLST website [53] (**Table 12**). Cycling parameters are presented in **Table 13**.

Prior to sequencing, the PCR products were purified by the QIAquick PCR Purification Kit (Qiagen, Germany). Five volumes of buffer PB was mixed with 1 volume of the PCR reaction. To bind DNA, the mixture was transferred to a QIAquick column and centrifuged at 13000 rpm for 1 min. Flow-through was discarded and the column was washed with 750 µl buffer PE, containing ethanol. Centrifugation was repeated in order to remove any residual wash buffer and the QIAquick column was placed in a clean 1.5 ml Eppendorf tube. To elute DNA, 35 µl buffer EB was applied directly to the centre of each column, and again centrifuged at 13000 rpm for 1 minute. The DNA concentration was measured by a NanoDrop Lite machine (Bio-Rad).

The purified DNA was sent for sequencing to BIOMI Ltd., Gödöllő, Hungary. The allele sequences were compared to the MLST database and the sequence types identified (see **Appendix 2**).

Table 12. Primers and gene loci of the seven housekeeping genes of *Staphylococcus aureus* used for MLST PCR [53]

Primer name	Gene locus	Primer sequence (5' → 3')	Amplicon size (bp)
arcC for arcC rev	Carbamate kinase	TTGATTCACCAGCGCGTATTGTC AGGTATCTGCTTCAATCAGCG	570
aroE for aroE rev	Shikimate dehydrogenase	ATCGGAAATCCTATTTCACATTC GGTGTTGTATTAATAACGATATC	536
glpF for glpF rev	Glycerol kinase	CTAGGAACTGCAATCTTAATCC TGGTAAAATCGCATGTCCAATTC	576
gmk for gmk rev	Guanylate kinase	ATCGTTTTATCGGGACCATC TCATTAACTACAACGTAATCGTA	488
pta for pta rev	Phosphate acetyltransferase	GTTAAAATCGTATTACCTGAAGG GACCCTTTTGTTGAAAAGCTTAA	575
tpi for tpi rev	Triosephosphate isomerase	TCGTTCATTCTGAACGTCGTGAA TTTGCACCTTCTAACAATTGTAC	475
yqiL for yqiL rev	Acetyle coenzyme A acetyltransferase	CAGCATACAGGACACCTATTGGC CGTTGAGGAATCGATACTGGAAC	598

Table 13. PCR cycling parameters for *arcC, aroE, glpF, gmk, pta, tpi* and *yqiL* gene amplification

Repeat	Temperature	Time
1x	95°C	5 min
30x	95°C	1 min
	55°C	1 min
	72°C	1 min
1x	72°C	5 min

3.8 Determination of haemolysis type

The method described by Herbert et al. [54] was used to compare the haemolytic activity of the two *S. aureus* strains each isolated from the same child (as observed in two instances). The isolates in question were cross-streaked perpendicularly to RN4220 (kindly provided by Professor Richard Novick) on Mueller-Hinton blood agar plates and incubated at 37°C, in ambient air. Next day haemolysis type was determined based on the interaction with this test strain using the scheme shown later on **Figure 17** (page 62). RN4220 produces only beta, a hot-cold haemolysin that is synergistic with delta. Alpha is the strongest and produces a clear haemolytic zone.

3.9 Sequencing the *katA* gene

In one case, we identified a catalase-negative *S. aureus* (CNSA) isolate (L 1034). We were interested in the reason of this uncommon phenotype at the genetic level. First, we amplified its *katA* gene by PCR. DNA was prepared by the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research Corp., Irvine, CA, US). Briefly, approx. 100 mg bacterial cells (equivalent to 1-2 plateful ON culture) were resuspended in 200 µl saline buffer and transferred to a specific Lysis Tube, containing beads. After 750 µl Lysis solution was added, the suspension was rigorously vortexed for 5 minutes on full speed, in order to disrupt the bacterial cell wall by bead beating. The mixture was then centrifuged at 10000 g and the supernatant additionally filtered to remove cell debris. The filtrate – containing

the DNA – was then purified by the usual column-binding method, also provided in the kit. DNA was extracted the same way from ATCC 29213, as a control.

After that, 2 µl from the purified DNA was used as template in the PCR reaction. The complete *kat* gene was amplified by using two sets of primers yielding overlapping PCR products. Out of these four oligonucleotide primers, three derived from the paper published by Piau et al. [55], and one primer (kat-1 forward) was designed by us. The kat-1 reverse and kat-2 forward primers were also modified in one position each, as we used ATCC 25923 as a reference sequence and there were some nucleotide differences as compared to the reference strain (Mu50) used originally by the authors. The applied *kat* primers are shown in **Table 14**.

Amplification was performed using the cycling parameters shown on **Table 15**. We sent the purified PCR products for sequencing to the BIOMI Ltd., Gödöllő, Hungary.

Table 14. Nucleotide sequence of the kat primers used in this study

Primer name	**Sequence (5' → 3')**	**Amplicon size (bp)**	**Reference**
kat-1 for	AATTGTAATTCTGATGGGGTTAAA	770	This study
kat-1 rev	CAGCAGCT**W**CTTCATCAG		[55]
kat-2 for	CGTCA**Y**ATGCATGGGTTCG	1077	[55]
kat-2 rev	GCCACATTCTGTGCATGC		

W, T or A; Y, T or C

Table 15. PCR cycling parameters for *katA* gene detection

Repeat	Temperature	Time
1x	94°C	3 min
35x	94°C	45 s
	51°C	45 s
	72°C	1 min
1x	72°C	5 min

3.10 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*). Each PCR was performed in a separate 25 µl reaction, except for *eta*+*etb*. For each reaction, the appropriate positive control strain was included, provided by the National Center for Epidemiology (NCE). We used the following primers from the literature: *seb*, *eta*, *etb* published by Mehrotra et al. [56], *sec* by Becker et al. [57], and *tsst* by Monday et al. [58] as detailed below in **Table 16**. In case of *sea*, we have designed new primers using the Primer3 programme [45] also showed in **Table 16**. The PCR cycling conditions are presented in **Table 17**.

Table 16. Nucleotide sequences of sea, seb, sec, tsst, eta and etb primers used in this study

Primer name	Primer sequence (5' → 3')	Amplicon size (bp)	Control	Reference
sea for	TTATCAATGTGCGGGTGGTA	265	OEK-08-50024-TX	This study
sea rev	CCTCTGAACCTTCCCATCAA			
seb for	GTATGGTGGTGTAACTGAGC	164	OEK-08-50044-TX	[56]
seb rev	CCAAATAGTGACGAGTTAGG			
sec for	CTCAAGAACTAGACATAAAAGCTAGG	271	OEK-08-50036-TX	[57]
sec rev	TCAAAATCGGATTAACATTATCC			
tsst for	GCTTGCGACAATCGCTACAG	560	OEK-15-50026-TX	[58]
tsst rev	TGGATCCGTCATTCATTGTTAT			
eta for	AAAAACCATGCAAAAGCAGAA	372	OEK-13-50001-TX	This study
eta rev	ACCTGCACCAAATGGTTCTT			
etb for	CAGCGCAGAAGAAATCAGAA	609	OEK-13-50001-TX	This study
etb rev	CCGCCTTTACCACTGTGAAT			

OEK, Országos Epidemiológiai Központ (NCE, National Center for Epidemiology)

Table 17. PCR cycling parameters for toxin genes' detection

Repeat	Temperature	Time
1x	95°C	5 min
30x	94°C	1 min
	57°C	1 min
	72°C	1 min
1x	72°C	7 min

3.11 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.

3.12 Solutions

All ingredients were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK) and dissolved in distilled water. The pH was adjusted with solid NaOH granulate or 1 N HCl. All solutions were finally sterilised by autoclave. The composition of each solution used in this thesis is detailed in **Table 18**.

Table 18. Ingredients of different solutions used in PCR or PFGE reactions

Solution	Ingredients
1x TEA buffer	20 mM EDTA, 40 mM TRIS, 57.1 ml/L glacial acetic acid
EC lysis buffer	1 M NaCl, 100 mM EDTA, 6 mM TRIS, 0.5% Brij58, 0.2% deoxycholate, 0.5% N-lauroyl sarcosine; pH=7.6
ESTN buffer	20 mM NaCl, 100 mM EDTA, 10 mM TRIS, 1% N-lauroyl sarcosine; pH=8.0
TE buffer	1 mM EDTA, 10 mM TRIS; pH=7.5
0.5x TBE buffer	1.25 mM EDTA, 45 mM TRIS, 45 mM boric acid

3.13 Permissions

3.13.1 Parents' permission and questionnaires

Nurseries who had feedback to take part in this study were first supplied by questionnaires. It included an informed consent for the parents and several questions about the child concerning possible risk factors. In the first version of the questionnaire (used between February 2009 and February 2011), gender, pneumococcal vaccination status, siblings, history of recurrent otitis media or other severe infections were asked. From December 2011 an expanded form was adopted, including age of the child, exposure to passive smoking in the family and earlier antibiotic treatment (**Appendix 1**). Unfortunately, parents did not always answer all the questions which caused the differences in the total number of children during the analysis of risk factors.

3.13.2 Ethical permission

The number of the ethical permit for this study is TUKEB 4-3/2009, issued by the Regional and Institutional Committee of Science and Research Ethics of Semmelweis University.

4 RESULTS

4.1 Carriage rate and risk factors

4.1.1 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 (p=0.18).

4.1.2 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.

In 35 cases - in the absence of appropriate data - the gender of the child was unknown. In the remaining 843 cases, genders were represented almost equally: 50.9% males and 49.1% females. The gender of the carrier children was known in 179 cases: there were 53.6% males and 46.4% females. Another piece of information about the children was whether they had siblings or not. Among the carriers with known sibling status (n=177), slightly higher percentage had siblings (78.5%), compared to the non-carriers (73.6%). We had information about whether siblings attended any community or not in 831 cases. Out of 177 carriers, 63.8% of their siblings attended community, while 61.6% of the non-carriers' siblings attended DCC or school. As soon as we introduced the expanded questionnaire, we used this form in the last nursery in Group-2. The question about passive smoking was answered by 93 parents and the ratio in the carrier and non-carrier group was almost equal.

We found no statistically significant correlation between any of these potential risk factors and *S. aureus* carriage, as shown in **Table 19**.

Table 19. Correlation between the analysed risk factors and *S. aureus* carriage in Group-2

Risk factor	Carrier	Non-carrier	χ^2-test
Male	96 (22.4%)	333 (77.6%)	NS (p=0.41)
Female	83 (20.0%)	331 (80.0%)	
Having siblings	139 (22.4%)	482 (77.6%)	NS (p=0.18)
Not having siblings	38 (18.0%)	173 (82.0%)	
Siblings attending community	113 (21.9%)	403 (78.1%)	NS (p=0.59)
Siblings not attending community	64 (20.3%)	251 (79.7%)	
Passive smoking	9 (30.0%)	21 (70.0%)	NS (p=0.89)
No passive smoking	18 (28.6%)	45 (71.4%)	

NS, not significant

4.1.3 Preschool children from Szolnok (Group-3)

In Szolnok in 2012, we visited all nurseries that time and collected nasal samples from 1390 children. We could detect *S. aureus* in **474** cases, which equals to **34.1%** carriage rate. From two samples two different *S. aureus* were isolated (as detailed in section 4.5), hence, a total of **476** *S. aureus* were identified.

Gender of the child was unknown in only one case; considering this, out of 1389 children 52.7% were males and 47.3% were females. 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. We had information about the sibling status in the case of 470 carriers and 69.1% of them had one or more siblings. Compared to it, a quite similar proportion (70.5%) was found among the non-carriers, which is statistically not significant. Out of the 325 carriers, 88.6% of their siblings were member of some kind of community (DCC or school) in contrast to 86.4% of the non-carriers' siblings. This is also statistically non-significant. Passive smoking was taken into consideration as well, and we calculated 37.7% prevalence among the 470 carriers while 40.1% among the non-

carriers, which again was not significant. The exact numbers of children and p-values are shown in **Table 20**.

Table 20. Correlation between the analysed risk factors and *S. aureus* carriage in Group-3

Risk factor	**Carrier**	**Non-carrier**	**χ^2-test**
Male	**276 (37.7%)**	**456 (62.3%)**	**p=0.003**
Female	198 (30.1%)	459 (69.9%)	
Having siblings	325 (33.7%)	639 (66.3%)	NS (p=0.62)
Not having siblings	145 (35.1%)	268 (64.9%)	
Siblings attending community	288 (34.4%)	548 (65.6%)	NS (p=0.34)
Siblings not attending community	37 (30.1%)	86 (69.9%)	
Passive smoking	177 (32.7%)	364 (67.3%)	NS (p=0.38)
No passive smoking	293 (35.0%)	544 (65.0%)	

4.1.4 Summarised results

Summarising these three groups, **2568** individuals were screened for nasal colonisation between 2009 and 2012. 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).

Summarising just preschool children's data (Group-2 and Group-3), **29.1%** (n=661) of them (n=2268) proved to be *S. aureus* nasal carriers. Geographical distribution of the examined children as well as carriage rates are demonstrated on **Figure 9**.

We also cumulated risk factors for carriage among all examined children. Male gender was significantly higher among carriers. Interestingly, siblings attending community also significantly correlated with *S. aureus* nasal carriage as the number of samples were augmented (**Table 21**). The other two examined risk factors (having siblings and passive smoking) did not show concordance with carriage in this study.

Table 21. Correlation between the analysed risk factors and *S. aureus* carriage in 2268 preschool children

Risk factor	**Carrier**	**Non-carrier**	**χ^2-test**
Male	**372 (32.0%)**	**789 (68.0%)**	**p=0.003**
Female	281 (26.2%)	790 (73.8%)	
Having siblings	464 (29.3%)	1121 (70.7%)	NS (p=0.98)
Not having siblings	183 (29.3%)	441 (70.7%)	
Siblings attending community	**401 (29.7%)**	**951 (70.3%)**	**p=0.008**
Siblings not attending community	101 (23.1%)	337 (76.9%)	
Passive smoking	186 (32.6%)	385 (67.4%)	NS (p=0.43)
No passive smoking	311 (34.6%)	589 (65.4%)	

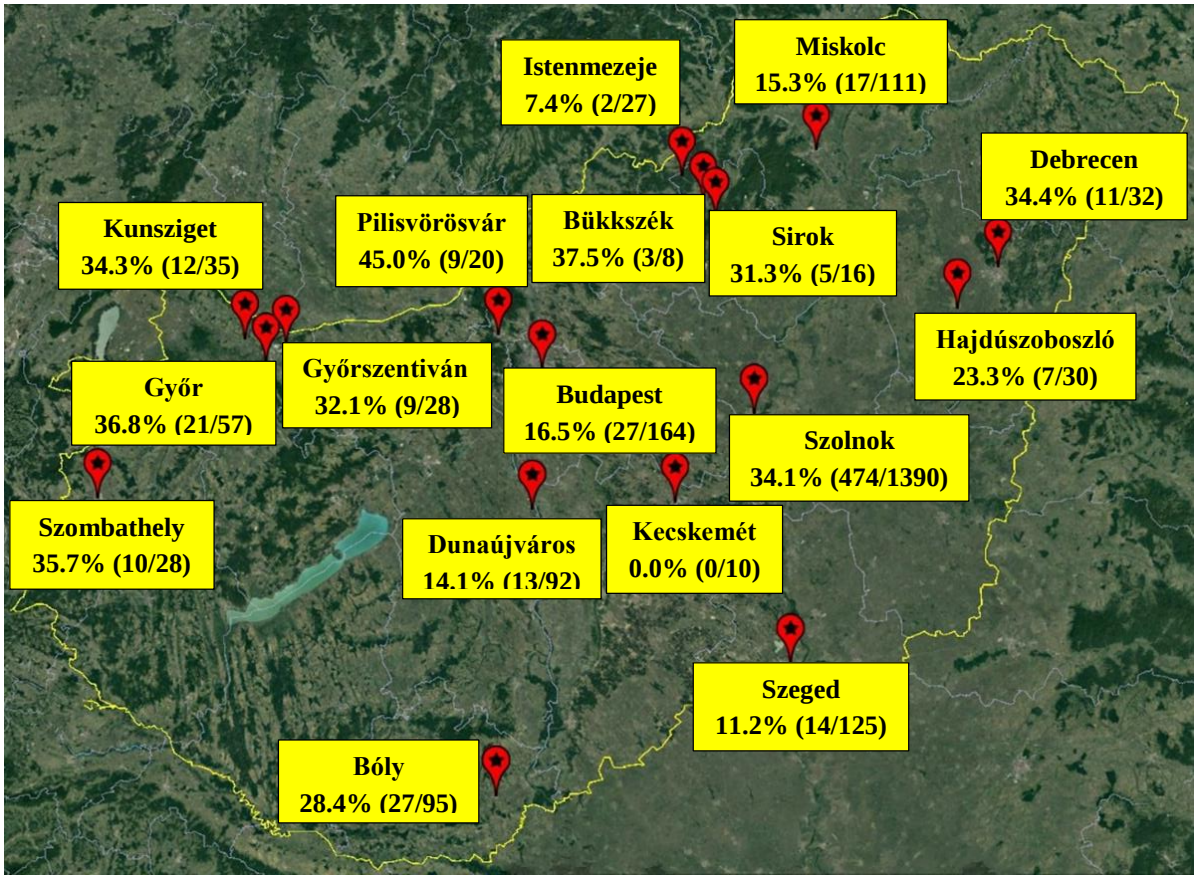

Figure 9. The location of DCCs and *S. aureus* carriage rate in each town

In brackets: number of carriers / total number of children.

4.1.5 MRSA carriage rate

In **Group-1** we could detect the *mecA* gene in two strains (**Figure 7**, page 29), so the MRSA carriage was **0.7%** (2/300) among university students, meanwhile methicillin-resistance among the *S. aureus* isolates was 2.3% (2/88).

In **Group-2** we did not find any MRSA strains, which means a **0.0%** carriage rate among the examined 878 children and 187 *S. aureus* isolates.

In **Group-3** *mecA* gene was detected in four cases. According to this, CA-MRSA carriage rate among the 1390 examined children was **0.3%**, which means a 0.8% MRSA prevalence among the *S. aureus* strains.

Among all tested children, we calculated 0.2% (4/2268) CA-MRSA nasal colonisation ratio and 0.6% prevalence of methicillin-resistance among *S. aureus* strains. 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.

4.2 Antibiotic susceptibility patterns

4.2.1 University students (Group-1)

Out of the tested six antibiotics, all isolates were fully sensitive to three: gentamicin, ciprofloxacin and vancomycin. Surprisingly, also all strains showed phenotypical sensitivity to oxacillin, including the two *mecA* positive ones. We measured OXA MIC of 0.75 and 2 mg/L in these cases, meanwhile they were both resistant to cefoxitin so then reported as MRSA (notably oxacillin-sensitive [OS]-MRSA). The only resistance was found to macrolides: 12 strains were resistant to erythromycin, three at low level (MIC 12–32 mg/L) and nine at high level (MIC >256 mg/L). Considering the ERY-R strains, one had high CLI resistance (MIC >256 mg/L) as well, while other 10 isolates showed inducible clindamycin resistance with D-test (**Table 22**).

Table 22. Antibiotic sensitivity results in Group-1

AB	S % (n)	I % (n)	R % (n)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
OXA	**100%** (88)	-	0.0% (0)	0.064 - 2	0.125	0.19
CIP	**100%** (88)	-	0.0% (0)	0.047 – 0.25	0.094	0.125
ERY*	85.2% (75)	1.1% (1)	**13.6%** (12)	0.125 - >256	0.5	32
CLI	87.5% (77)	-	**12.5%** (11)	0.064 - >256	0.125	0.125
GEN	**100%** (88)	-	0.0% (0)	0.125 – 0.75	0.25	0.5
VAN	**100%** (88)	-	0.0% (0)	0.25 – 0.75	0.38	0.5

AB, antibiotic; S, sensitive; I, intermediate; R, resistant

* Discrepancies comparing to our previously published data [59] derive from breakpoint changing in EUCAST guideline

It was very interesting to observe, that the production of the yellow pigment was markedly stronger at the border of the inhibition zones around the E-test strips (**Figure 10**).

Figure 10. E-test representing intensified pigment production at the border of the inhibition zone, photo by *K. Laub*

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

In this group, we determined MIC values for nine antibiotics. All isolates were sensitive to oxacillin, TMP/SMX and mupirocin. Most of the isolates (92.0%) were resistant to penicillin. Besides MIC measurement, we applied disc diffusion test for the better detection of penicillinase production. Thanks to it, we found one strain which was sensitive to penicillin based on its MIC (=0.064 mg/L), but it was resistant by disc

diffusion method, so it was considered resistant according the EUCAST guidelines. The isolates with the highest oxacillin MICs (0.5 or 1 mg/L) were tested also with cefoxitin discs, and all showed sensitivity (26-27 mm inhibition zones, **Figure 11**). The highest resistance (except for penicillin) was observed in the case of erythromycin (7.5%). The resistance rates for ciprofloxacin, clindamycin, gentamicin and tetracycline were 0.5%, 1.1%, 3.7% and 4.3%, respectively (**Table 23**). There were 11 isolates expressing high-level resistance to erythromycin (MIC ≥512 mg/L) but showing sensitivity to clindamycin by MIC (= 0.125 mg/L), all of these had inducible clindamycin resistance based on the double disc diffusion test and finally reported as resistant (**Figure 12**). One isolate had low level ERY-R (MIC =16 mg/L).

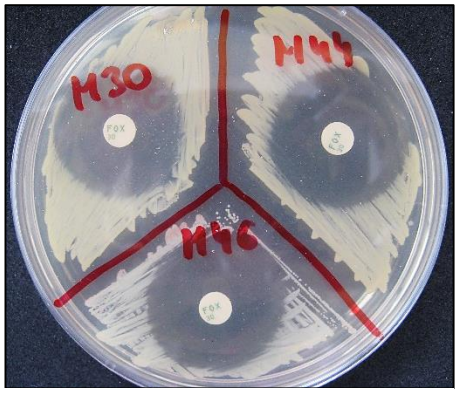

Figure 11. Cefoxitin sensitivity by disk diffusion, photo by *K. Laub*

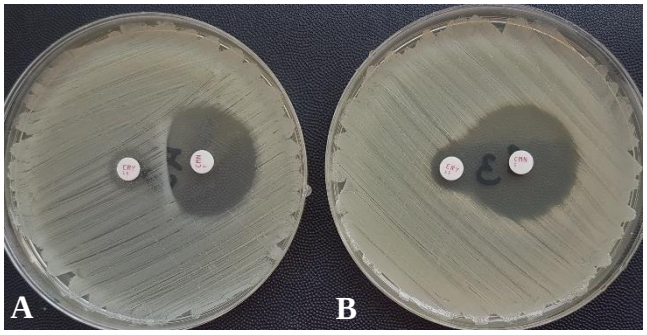

Figure 12. A representative positive (A) and negative (B) D-test, photo by *K. Laub*

Table 23. Antibiotic susceptibility pattern in Group-2

AB	S % (n)	R % (n)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
PEN	8.0% (15)	**92.0%** (172)	0.032 - 128	4	16
OXA	**100%** (187)	0.0% (0)	0.125 - 1	0.25	0.5
MUP	**100%** (187)	0.0% (0)	≤0.032 - 0.125	0.064	0.125
CIP	99.5% (186)	**0.5%** (1)	0.125 - >1	0.5	1
ERY	92.5% (173)	**7.5%** (14)	0.25 - >512	0.5	0.5
CLI	93.0% (174)	**7.0%** (13)*	0.064 - >512	0.125	0.125
GEN	96.3% (180)	**3.7%** (7)	0.125 - 2	1	1
TET	95.7% (179)	**4.3%** (8)	≤0.064 - 64	0.25	0.5
TMP/SMX	**100%** (187)	0.0% (0)	0.008 - 0.25	0.023	0.032

* 11 isolates out of these had inducible resistance

4.2.3 Preschool children from Szolnok (Group-3)

We measured MIC values for eight antibiotics in this part of the study, the same as in Group-2 with the exception of TMP/SMX. Out of the 476 isolates, three proved to be oxacillin- and cefoxitin resistant, which resulted in 0.6% resistance rate. Moreover, we found the *mecA* gene in one additional strain which showed phenotypic oxacillin-sensitivity (MIC = 1 mg/L) and cefoxitin-sensitivity (23 mm zone diameter) as well. We detected 100% sensitivity only in the case of mupirocin. For ciprofloxacin and gentamicin most of the isolates were sensitive (99.8% and 99.4%, respectively). Tetracycline-resistance appeared to be 3.4%, while 10.3% of the isolates were resistant to erythromycin. Out of the 49 ERY-R isolates, one had high level CLI-R (MIC ≥512 mg/L) and 43 were positive with D-test, interestingly, an additional isolate was CLI-R but ERY-

S. Altogether we calculated 9.5% CLI-R. The highest resistance - 91.4% - was measured for penicillin of course (**Table 24**).

Table 24. Antibiotic susceptibility pattern in Group-3

AB	**S % (n)**	**R % (n)**	**MIC range (mg/L)**	**MIC_{50} (mg/L)**	**MIC_{90} (mg/L)**
PEN	8.6% (41)	**91.4%** (435)	0.016 - ≥64	4	32
OXA	99.4% (473)	**0.6%** (3)	≤0.032 - ≥4	0.5	0.5
MUP	**100%** (476)	0.0% (0)	≤0.032 – 0.25	0.064	0.125
CIP	99.8% (475)	**0.2%** (1)	≤0.032 - ≥2	0.5	0.5
ERY*	89.5% (426)	**10.3%** (49)	0.125 - ≥512	0.5	128
CLI	90.5% (431)	**9.5%** (45)	≤0.032 - ≥512	0.125	0.125
GEN	99.4% (472)	**0.6%** (3)	≤0.064 - 2	0.5	1
TET	96.6% (460)	**3.4%** (16)	≤0.064 - 64	0.5	0.5

* One isolate (0.2%) was intermediate

4.2.4 Summarised results

Summarising antibiotic resistance data to all tested isolates, we can establish that most of the strains (>95%) were sensitive to OXA, CIP, GEN and TET. Hundred-percentage sensitivity was documented in the case of TMP/SMX, MUP and VAN. We determined >90% resistance to PEN and 9-10% resistance for CLI and ERY (**Table 25**).

Additionally, four MSSA 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.

Table 25. Combined antibiotic resistance rates of all examined *S. aureus* isolates in this thesis

AB	S % (n)	R % (n)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
PEN	8.4% (56)	**91.6%** (607)	0.016 – 128	4	16
OXA	99.6% (748)	**0.4%** (3)	≤0.032 - ≥4	0.5	0.5
MUP	**100%** (663)	0.0% (0)	≤0.032 – 0.25	0.064	0.125
CIP	99.7% (749)	**0.3%** (2)	≤0.032 - ≥2	0.5	0.5
ERY*	89.7% (674)	**10.0%** (75)	0.125 - >512	0.5	2
CLI	90,8% (682)	**9.2%** (69)	≤0.032 - >512	0.125	0.125
GEN	98.7% (740)	**1.3%** (10)	≤0.064 - 2	0.5	1
TET	96.4% (639)	**3.6%** (24)	≤0.064 - 64	0.5	0.5
TMP/SMX	**100%** (187)	0.0% (0)	0.008 - 0.25	0.023	0.032
VAN	**100%** (88)	0.0% (0)	0.25 – 0.75	0.38	0.5

* Two isolates (0.3%) were intermediate

4.2.5 CA-MRSA strains

Totally, we isolated six *mecA* gene carrier strains (see chapter 4.1.5). Two (F/2-3, G/3-2) derived from Hungarian university students and four from children living in Szolnok (L403, L745, L1108, L1112). According to their antibiotic susceptibility, three of them showed oxacillin sensitivity. Out of these isolates one was cefoxitin sensitive in parallel, two of them showed resistance to it. Referring to the EUCAST recommendations, "*S. aureus* with oxacillin MIC values >2 mg/L are mostly methicillin resistant due to the presence of the *mecA* or *mecC* gene", we considered all these isolates MRSA as they carry the *mecA* gene. Thus, 50% of the identified MRSA strains proved to

be 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-R strains had inducible CLI-R also (**Table 26**).

Table 26. Antibiotic susceptibility patterns of the isolated six CA-MRSA strains

Isolate	**OXA** (mg/L)	**FOX** (mm)	**PEN** (mg/L)	**ERY** (mg/L)	**CLI** (mg/L)	**CIP** (mg/L)	**GEN** (mg/L)	**TET** (mg/L)	**MUP** (mg/L)
F/2-3	2	**19^{R}**	**R***	**>256^{R}**	**0.125^{R,§}**	0.064	0.38	-	0.19$^{\#}$
G/3-2	0.75	**16^{R}**	**R***	0.75	0.125	0.125	0.25	-	0.19$^{\#}$
L745	1	23	**0.5^{R}**	**≥512^{R}**	**0.125^{R,§}**	0.25	1	0.5	0.064
L403	**≥4^{R}**	**12^{R}**	**32^{R}**	**≥512^{R}**	**0.125^{R,§}**	0.25	1	0.25	0.064
L1108	**≥4^{R}**	**12^{R}**	**32^{R}**	**≥512^{R}**	**0.125^{R,§}**	0.5	0.5	0.5	0.125
L1112	**≥4^{R}**	**13^{R}**	**32^{R}**	**≥512^{R}**	**0.125^{R,§}**	0.5	1	0.5	0.125

R, Resistant

* Disk diffusion method was applied

E-test was applied

§ Inducible resistance was detected

We verified the *mecA* PCR products by **sequencing** of the three oxacillin-sensitive strains (F/2-3, G/3-2, L745).

4.3 Molecular typing of the MRSA isolates

We determined the sequence types (ST) of the six MRSA isolates by MLST technique. Five out of them belonged to **ST45**, while the remaining one (G/3-2) proved to be **ST7**.

We also identified the SCC*mec* types for all six isolates. Five of them harboured the **SCC*mec* type IV** cassette and one (G/3-2) carried **type V** cassette (**Figure 13**).

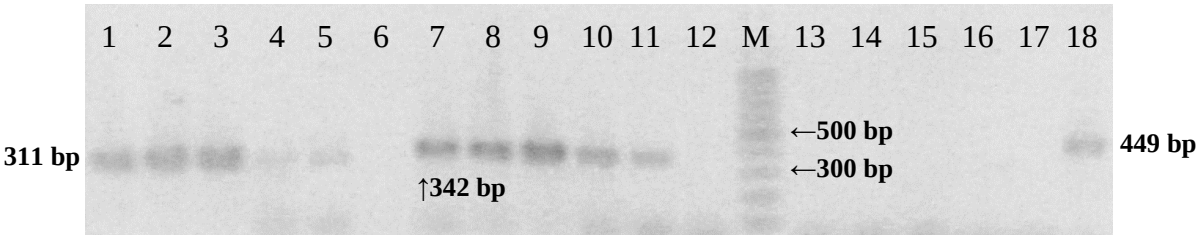

Figure 13. PCR gel image used for the detection of SCC*mec* types IV and V in the case of the six *mecA* positive isolates, photo by *K. Laub*

Lane 1-6, ccrB2 (IV); lane 7-12, dcs (IV); lane 13-18, ccrC (V); M, marker (100 bp DNA ladder). Primers are detailed above in **Table 10** (page 36).

4.4 Genetic relatedness

4.4.1 University students (Group-1)

Genetic relatedness of the isolates in Group-1 was observed by PFGE method. As it can be seen on **Figure 14**, PFGE patterns demonstrated high-grade diversity. Four bigger clusters could be assigned, however, in some cases considerable variations could be detected within the same cluster. We defined ST types of the two MRSA strains originated from this group (F/2-3 as ST45, G/3-2 as ST7) as mentioned above and two additional MSSA strains, C/2-2 as ST7 and G/3-4 as ST45 (**Figure 14**). It is interesting that in both occasions, the same MLST type could be specified within the same PFGE cluster for an MRSA and an MSSA, respectively, however, with somewhat dissimilar PFGE patterns.

In seven cases, hundred percent identity was present, although these isolates derived from five different university practice groups (**Figure 15A**). In several cases, isolates originated from the same group had definitely unrelated PFGE patterns, an example is shown in **Figure 15B**. In some occasions isolates with highly similar patterns were detected in both Hungarian and non-Hungarian students. The two MRSA strains were also genetically different (**Figure 15C**).

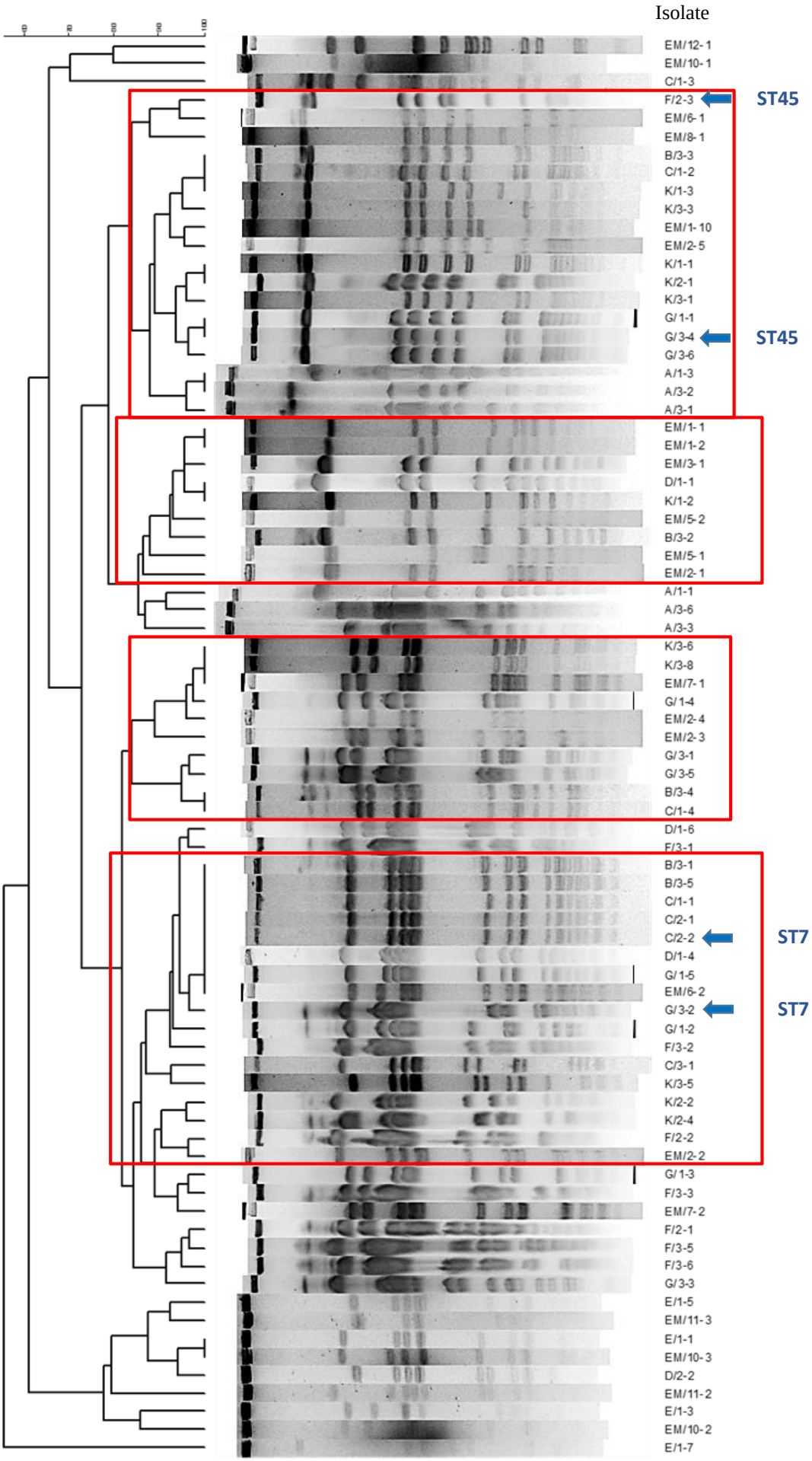

Figure 14. PFGE dendrogram of isolates from Group-1

Blue arrows, strains with known MLST type.

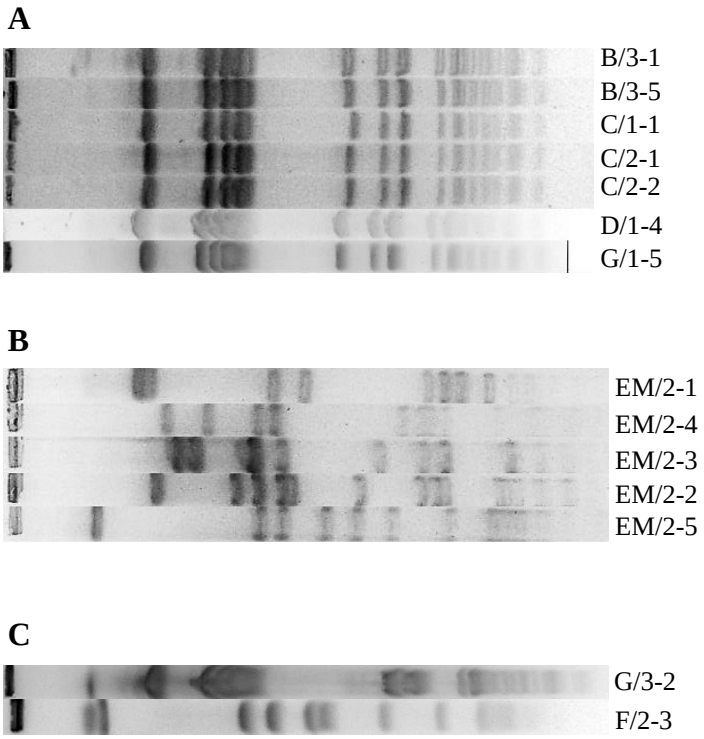

Figure 15. Representative PFGE patterns in Group-1, photo by *K. Laub*

A, Seven isolates with identical patterns, deriving from five different Hungarian university groups.

B, Isolates of five students from the same university group with different patterns.

C, Two MRSA isolates with different patterns.

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

In Group-2 the PFGE analysis was performed in 59 cases. The PFGE patterns showed high genetic diversity similarly to Group-1 (**Figure 16**). However, three major clusters could be detected. Even within the same DCC group, the PFGE patterns differed by several bands (see e.g. the upper lanes). On the other hand, sometimes the very same clone was found in two distant cities (e.g. Győr and Debrecen). MLST type of three isolates was known, KK12 belonged to ST7, D4 belonged to ST45 and Szi28 was the member of ST30. It is interesting to observe that the same MLST clones (i.e. ST45 and ST7) could be found among both the students and the small children.

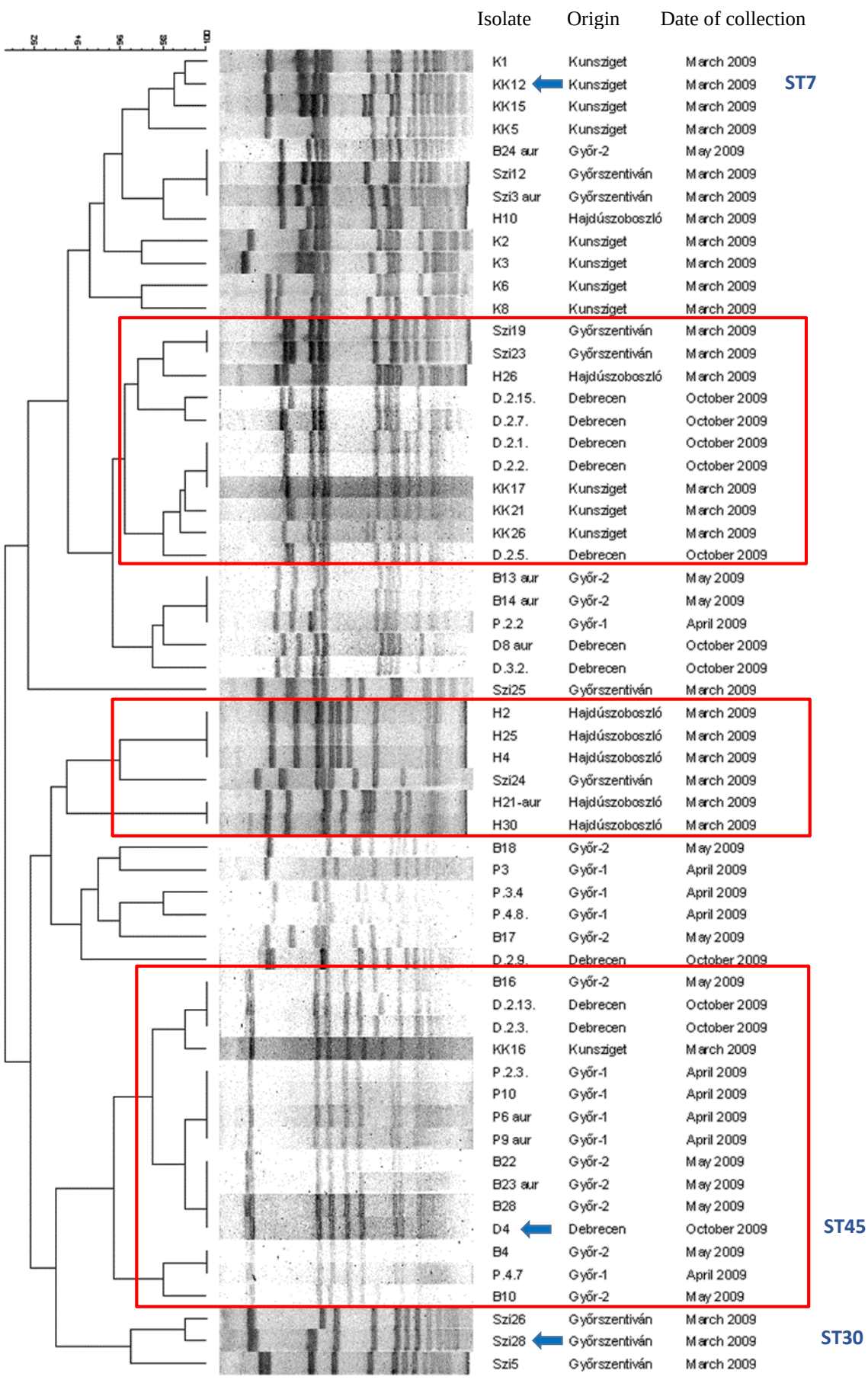

Figure 16. PFGE dendrogram of the *S. aureus* isolates from Group-2

Blue arrows, strains with known MLST type

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

As we mentioned above (see chapter 4.1.3), in two occasions, two phenotypically different *S. aureus* were isolated from the same nasal sample (L265/1-2 and L850/1-2). They seemed to be different based on colony morphology, especially the image of β-haemolysis. First, we compared their antibiotic susceptibility patterns and we found conspicuous difference in the PEN MIC values, but their MICs for the other antibiotics were almost the same (sensitive to all antibiotics tested, except for penicillin), as shown in **Table 27**.

Then, their haemolysis type was classified by the use of a scheme illustrated on **Figure 17**. According to this, we also found differences in haemolysin production of the strains (**Figure 17; Table 27**).

Table 27. Comparison of the *S. aureus* isolate pairs carried by the same child

Isolate	**Haemolysis type**	**MIC (mg/L)**							
		PEN	OXA	ERY	CLI	TET	GEN	MUP	CIP
L265/1	delta	0.25	0.25	0.5	0.125	0.25	0.5	0.064	0.25
L265/2	alpha+beta+delta	16	0.5	0.5	0.125	0.25	0.5	0.064	0.25
L850/1	delta	0.064	0.25	0.5	0.125	0.25	0.25	0.064	0.25
L850/2	alpha+beta+delta	2	0.25	0.5	0.125	0.25	0.25	0.064	0.5

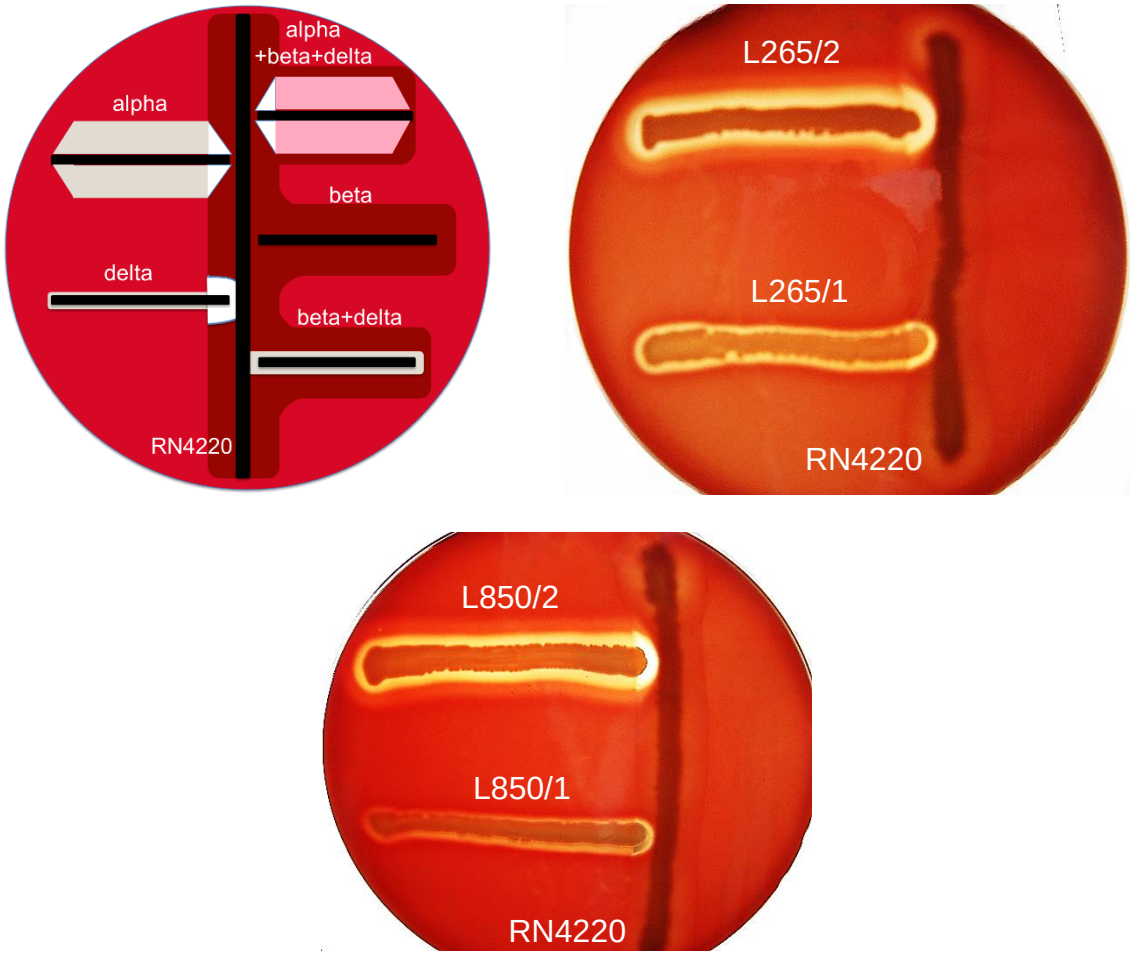

Figure 17. Determination of haemolytic phenotypes of the co-carried *S. aureus* isolates (L265/1,2 and L850/1,2), photo by *K. Laub*

RN4220, test strain

Finally, to unequivocally determine whether these isolates were identical or different on the genetic level, their PFGE patterns were compared. These were also different in both cases, however, interestingly, identical PFGE pattern was obtained in case of L265/2 and L850/2, although these strains derived from different nurseries (**Figure 18**). In conclusion, we could prove in both cases that the two *S. aureus* isolates carried by the same child were different from one another.

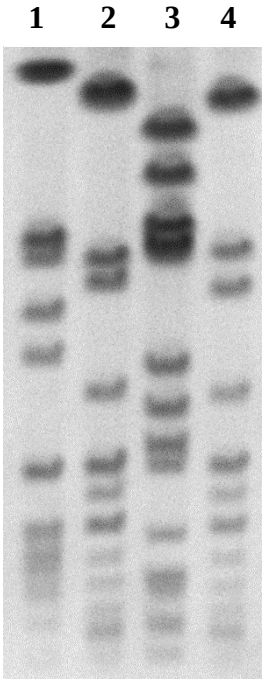

Figure 18. PFGE patterns of the two *S. aureus* isolates each deriving from the same child, photo by *K. Laub*

Lane 1, L265/1; lane 2, L265/2;
lane 3, L850/1; lane 4, L850/2.

4.6 Clumping factor-negative *S. aureus* isolates (CFNSA)

Among all examined (n=751) isolates, we found nine, which seemed to be *S. aureus* by colony morphology, but were clump-negative with both Pastorex Staph-Plus Kit and DiaMondial Staph Plus Latex Reagent tests. 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 (**Table 28**). For further identification, MALDI-TOF MS technique was applied, and its results confirmed all of them as *S. aureus* (Biotyper score value ≥1.9). Considering this, **1.2%** (9/751) of the isolates proved to be clumping factor-negative *S. aureus*. These strains derived from four different DCCs, so again we were interested whether they had a clonal origin or not. All isolates showed the same antibiotic sensitivity (sensitive to all tested drugs, except for PEN), although there were some variations in MIC values (**Table 29**).

Table 28. Results of tube coagulase test in the case of the nine clumping factor-negative isolates

Isolate	Detection at		
	2.5 h	**4 h**	**6 h**
L21	~	+	++
L30	~	++	+++
L67	+	++	+++
L82	+	++	++
L245	+++	+++	++
L248	~	+	++
L453	─	~	+
L456	~	++	++
L1251	~	+	++

+, positive; ─, negative; ~ border-line

Table 29. Antibiotic sensitivity results of the nine CFNSA isolates

Isolate	MIC (mg/L)							
	PEN	**OXA**	**ERY**	**CLI**	**TET**	**GEN**	**MUP**	**CIP**
L21	8	0.5	0.5	0.125	0.25	0.125	0.064	0.125
L30	16	0.5	0.5	0.125	0.5	0.125	0.064	0.125
L67	16	0.5	0.5	0.125	0.5	0.125	0.125	0.125
L82	8	0.25	0.5	0.064	0.25	1	0.064	0.125
L245	8	0.5	0.5	0.125	0.25	0.5	0.064	0.5
L248	16	0.5	0.5	0.125	0.25	0.5	0.125	0.5
L453	2	0.5	0.5	0.125	0.5	0.25	0.064	0.125
L456	16	0.5	0.5	0.125	0.5	0.25	0.064	0.125
L1251	16	0.5	0.5	0.125	0.5	0.25	0.064	0.125

The PFGE macrorestriction pattern was identical in eight cases (**Figure 19**). Based on these results, a clonal origin could be concluded.

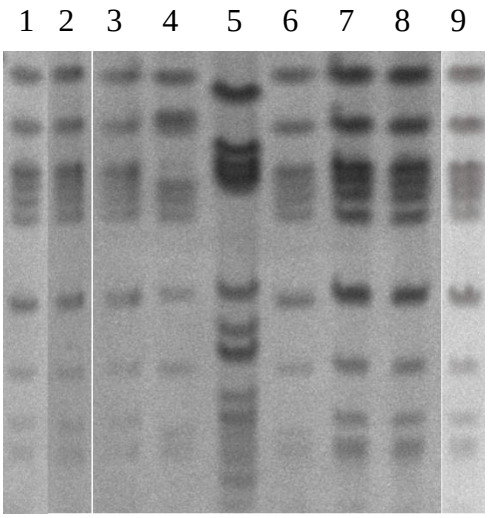

Figure 19. PFGE patterns of the clumping factor-negative *S. aureus* isolates, photo by *K. Laub*

Lane 1, L21; lane 2, L30; lane 3, L67; lane 4, L82; lane 5, L245; lane 6, L248; lane 7, L453; lane 8, L456; lane 9, L1251.

4.7 Catalase-negative *Staphylococcus aureus* (CNSA)

4.7.1 Identification of the catalase-negative strain L1034

As mentioned above (see chapter 3.3.1), during the phenotypical identification process, we frozen every *S. aureus* suspicious isolate despite of producing negative catalase- or clump-test. Thank to this kind of foresight, we could identify one *nucA* positive, but catalase-negative strain showing a typical *S. aureus* colony morphology. In the Gram-stained smear Gram-positive cocci appeared being arranged in grape-like clusters. We applied two different slide coagulase tests (Pastorex Staph-Plus Kit and DiaMondial Staph Plus Latex Reagent) on this isolate, and both gave a positive result. Finally, MALDI-TOF MS was used for further verification, and it identified this strain as

S. aureus with a high score (=2.3). Among all *S. aureus* isolates, only this one proved to be catalase-negative. This equals to a **0.1%** prevalence (1/751). The carrier of L1034 was a 6-year-old girl from Szolnok.

4.7.2 Identification of mutation in the *katA* gene

Sequencing the *katA* gene of the catalase-negative isolate, a single nucleotide substitution (G491A) was identified compared to the control strain (ATCC 29213). This modification resulted in a **nonsense mutation**, changing the TGG triplet to TAG, which encodes a stop codon instead of tryptophan. The gene sequence comparison of the region around the mutation is shown on **Figure 20**. This results the production of a truncated gene product of only 164 amino acids, i.e. 67.5% (341 amino acids) of the catalase protein is lost (**Figure 21**).

Figure 20. Sequence comparison of the *katA* gene region around the mutation of the catalase-negative isolate L1034 and the control strain ATCC 29213

Yellow highlighting stands for the place of the nonsense point mutation.

4.7.3 Nucleotide sequence accession number

The *katA* gene sequence data of the isolate L1034 has been submitted to the GenBank database under accession number KY587223. Details of data submission can be found at: GenBank (http://www.ncbi.nlm.nih.gov).

Met S Q Q D K K L T G V F G H P V S D R E N S M T A G P R G P L L M Q D I Y F L E Q M S Q F D
R E V I P E R R M H A K G S G A F G T F T V T K D I T K Y T N A K I F S E I G K Q T E M F A R F S T
V A G E R G A A D A E R D I R G F A L K F Y T E E G N W D L V G N N T P V F F F R D P K L F V S L
N R A V K R D P R T N M R D A Q N N **Stop** D F W T G L P E A L H Q V T I L M S D R G I P K D L R
H M H G F G S H T Y S M Y N D S G E R V W V K F H F R T Q Q G I E N L T D E E A A E I I A T D R
D S S Q R D L F E A I E K G D Y P K W T M Y I Q V M T E E Q A K N H K D N P F D L T K V W Y H D
E Y P L I E V G E F E L N R N P D N Y F M D V E Q A A F A P T N I I P G L D F S P D K M L Q G R L
F S Y G D A Q R Y R L G V N H W Q I P V N Q P K G V G I E N I C P F S R D G Q M R V V D N N Q
G G G T H Y Y P N N H G K F D S Q P E Y K K P P F P T D G Y G Y E Y N Q R Q D D D N Y F E Q P G
K L F R L Q S E D A K E R I F T N T A N A M E G V T D D V K R R H I R H C Y K A D P E Y G K G V A
K A L G I D I N S I D L E T E N D E T Y E N F E K Stop

Figure 21. Translated amino acid sequence of the *katA* gene of strain L1034

Yellow highlighting stands for the place of the stop codon.

4.7.4 Antibiotic susceptibility of L1034

The isolate was resistant to penicillin (MIC = 8 mg/L) and erythromycin (MIC ≥ 512 mg/L), but sensitive to all other tested antibiotics. It was a methicillin-sensitive strain, with an oxacillin MIC of 0.5 mg/L. The rest of the MICs were as follows: clindamycin 0.125 mg/L, gentamicin 1 mg/L, tetracycline 0.5 mg/L, ciprofloxacin 0.25 mg/L and mupirocin 0.064 mg/L. The isolate had inducible clindamycin resistance.

4.7.5 Detection of toxin genes and determination of sequence type

Out of the six toxin genes tested, isolate L1034 possessed the **enterotoxin A** (*sea*) gene and was negative for *seb*, *sec*, *tsst*, *eta* and *etb* (**Figure 22**). The MLST revealed that the isolate belongs to **ST5**.

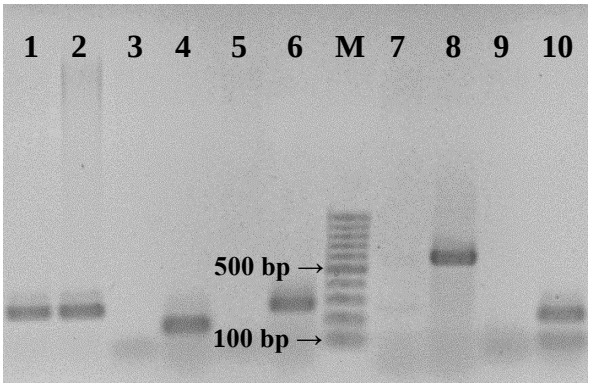

Figure 22. Detection of toxin genes for isolate L1034 by PCR,
photo by *K. Laub*

Lane 1, L1034 *sea*; lane 2, *sea* + control (265 bp); lane 3, L1034 *seb*;
lane 4, *seb* + control (164 bp); lane 5, L1034 *sec*; lane 6, *sec* + control (271 bp);
M, 100 bp size marker; lane 7, L1034 *tsst*; lane 8, *tsst* + control (560 bp);
lane 9, L1034 *eta* and *etb*; lane 10, *eta* and *etb* + controls (93 bp and 226 bp).

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

4.8.1 Rate of dual 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, which means that *S. pneumoniae* carriage is in negative correlation with *S. aureus* nasal carriage (**Table 30**, page 71).

In one case, two different *S. pneumoniae* (according to serotype and antibiotic resistance pattern) were isolated from the same specimen, so totally three pathogenic bacteria were carried by a single child at the same time.

4.8.2 Serotype distribution of the co-colonising pneumococci

Pneumococcus isolates originated from Group-2 and Group-3 were deeply analysed by A. Tóthpál [60]. In the present study, 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 as demonstrated on **Figure 23**.

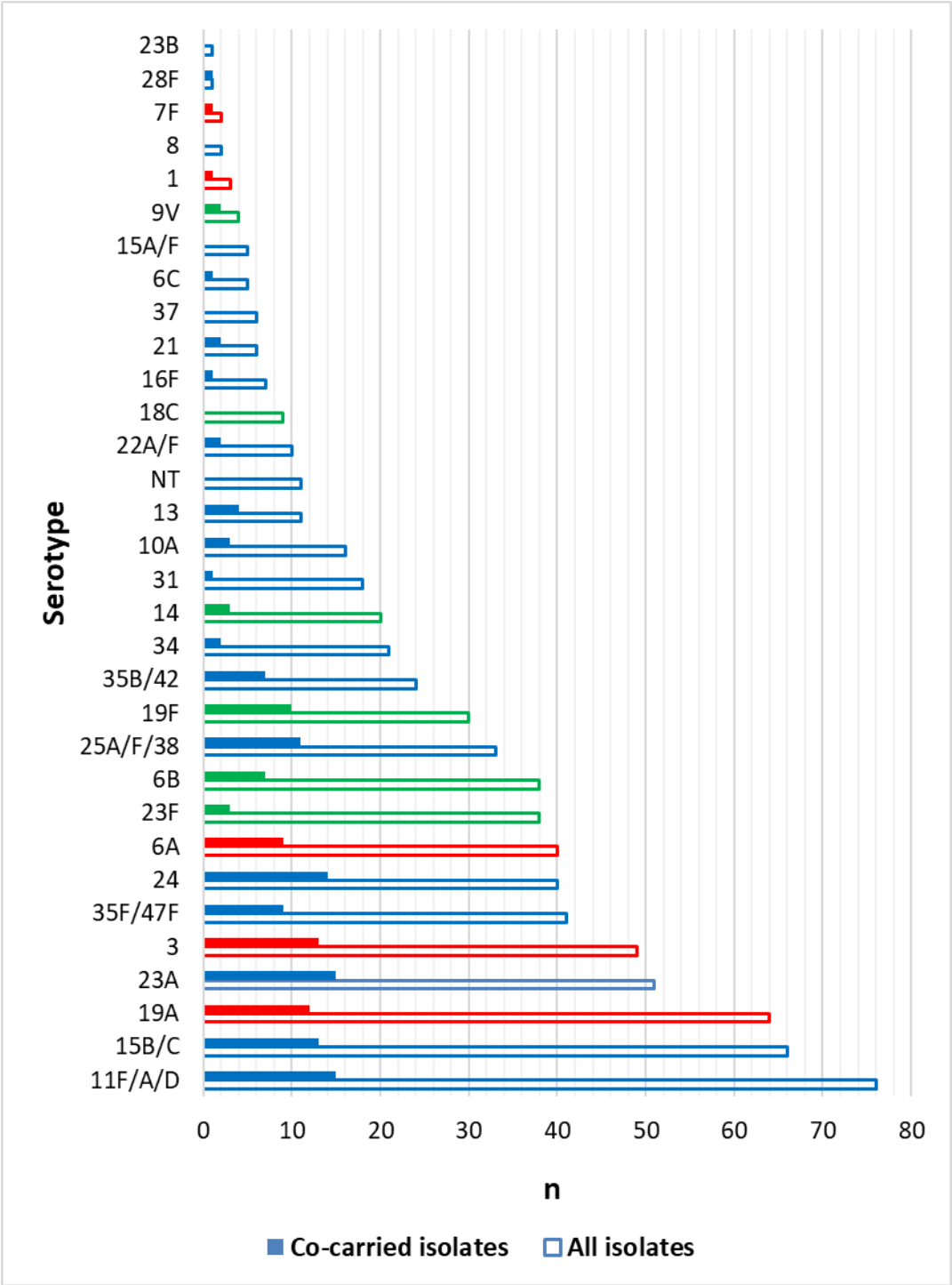

Figure 23. Serotype distribution of all (n=748) versus co-carried (n=162) *S. pneumoniae* isolates among all children (n=2268)

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

4.8.3 Correlation between PCV7 vaccination and *Staphylococcus aureus* carriage

We also analysed the possible effect of PCV7 vaccination on *S. aureus* carriage. DCCs were divided into two groups based on the level of vaccination rate. We had information about the vaccination status of the child in 74.7% of the cases. Analysing these data, 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 *S. aureus* nasal carriage is obvious (**Table 30**).

On the other hand, we were also interested in whether the vaccination status of the individual is associated with *S. aureus* carriage or not. In order to check this, the total number of vaccinated and non-vaccinated carriers was calculated. Among 1677 children (whom vaccination status was known), 897 (53.5%) had been vaccinated with PCV7 while 780 (46.5%) had not received vaccination against *Pneumococcus*. The *S. aureus* carriage rate in these two groups of children was almost equal, as it can be seen in **Table 30**. In conclusion, *S. aureus* nasal carriage in children is not associated with previously administered PCV7 vaccination of the individual.

Table 30. Correlation between *S. aureus* and *S. pneumoniae* carriage and connection with PCV7 vaccination in preschool children (Group-2 + Group-3)

Risk factor	***S. aur.* carrier**	***S. aur.* non-carrier**	**χ^2-test**
***S. pneumo.* non-carrier**	**500 (32.8%)**	**1024 (67.2%)**	**$p=3.9 \times 10^{-8}$**
S. pneumo. carrier	161 (21.6%)	583 (78.4%)	
High-level vaccination	**481 (33.4%)**	**959 (66.6%)**	**$p=5.2 \times 10^{-9}$**
Low-level vaccination	173 (21.7%)	625 (78.3%)	
PCV7 vaccinated	256 (28.5%)	641 (71.5%)	NS
PCV7 non-vaccinated	221 (28.3%)	559 (71.7%)	

5 DISCUSSION

5.1 *Staphylococcus aureus* carriage rate

5.1.1 Carriage rate in university students (Group-1)

S. aureus nasal colonisation rate observed by us (29.3%) correlates well with other countries' data. For example, in the USA Rohde et al. investigated also university students in Texas, and examined almost the same *S. aureus* carriage (29.6%) like us [61]. A European example from Ireland reported a carriage rate of 22% among a thousand healthy young adults between 18 and 35 years [62]. In an Australian paper, also similar result (28.9%) was published in the general adult population [63].

5.1.2 Carriage rate in preschool children (Group-2 and Group-3)

In the two groups of children screened (Group-2 and Group-3) we observed quite dissimilar carriage rates, 21.3% versus 34.1%. The calculated average carriage rate (29.1%) shows that approximately one third of the Hungarian healthy 3-7 years old children are nasal *S. aureus* carriers. It is in good concordance with other studies' findings and very similar to the carriage rate in Group-1. For instance, Ciftci et al. found 28.4% prevalence screening 1134, 4-6 years old healthy Turkish children [64]. In Brazil 31.1% of 1192 children attending DCCs carried *S. aureus* in their nose, as demonstrated by Lamaro-Cardoso et al. [65]. A cross-sectional study was published in 2013 by den Heijer et al., comparing the prevalence of nasal *S. aureus* carriage of healthy patients (specifically, patients visiting their family doctor's practice for a non-infectious disorder) in nine European countries including Hungary. They involved >32 thousand people in it (aged between 4 and ≥65 years) and the overall carriage rate was found to be 21.6%, lower, than the average carriage rate found by us. In the age category of 4-19 years, the lowest prevalence was found in Hungary (19.9%, out of 1133 patients tested), while the highest (36.2%) was seen in The Netherlands [66]. Van Bijnen et al. involved eight European countries (including Hungary) in their study in 2015, in which nearly 29 thousands healthy persons (aged >4 years) were screened for nasal commensal *S. aureus*.

They presented an average carriage rate of 21.2%, practically the same as published by den Heijer et al. before [67]. Nonetheless, there are even higher differences in carriage rates, for instance, in an Indian study the prevalence of *S. aureus* nasal colonisation was 52.3% in 5-15 years old children [68]. Another survey was conducted in Turkey, where 18% of healthy preschool children (aged 0-6 years) was colonised by *S. aureus* [69].

5.1.3 CA-MRSA carriage rate

MRSA prevalence varies widely in different geographical areas. We estimated 0.2% average CA-MRSA carriage rate, which is equivalent to 0.8% methicillin-resistance among carried *S. aureus* isolates. It can be ascertained that CA-MRSA carriage rate is low in Hungary according to our observation. Van Bijnen et al. found a similarly low prevalence of MRSA (1.3%) among 6093 *S. aureus* isolates in Europe in the above mentioned study [67]. In another European example, Mollaghan et al. reported 0.57% MRSA carriage rate form Ireland [62]. In a recent Serbian paper, higher (3.8%) MRSA carriage rate was examined among 1362 healthy adults in 2011-2013 [70]. In a large survey in the USA, based on nearly ten thousand participants, 0.8% were colonised with MRSA [71]. However, a much higher rate, 7.4% was found among university students in Texas [61]. In the above mentioned study conducted in Australia, Munckhof et al. published also a very similar rate to our (0.7%) [63]. In contrast, in an Indian study a really high percent of carried *S. aureus* isolates, 29% were MRSAs among 1002 preschool children [40]. In an Iranian study, 5.8% of 2-6 years old children were colonised with CA-MRSA [72].

National annual antibiotic sensitivity data for clinical *S. aureus* isolates are available from the National Center for Epidemiology (NCE), separated to inpatients and outpatients. In separate files, antibiotic resistance data originated from intensive care units are also available. We averaged NCE data (available for several thousands of people yearly) at the time period when our samples were collected (i.e. 2009-2012). According to this, average oxacillin resistance among *S. aureus* isolates was 7.2% in outpatients and 22.9% in inpatients in this period [73]. Summarising data from 2005 to 2015 represents a slow but continuous increase in MRSA rate among outpatients over the last decade,

reaching already 9.8% in 2015 (last available data). Meanwhile, MRSA prevalence in inpatients was stable in the last five years (**Figure 24**).

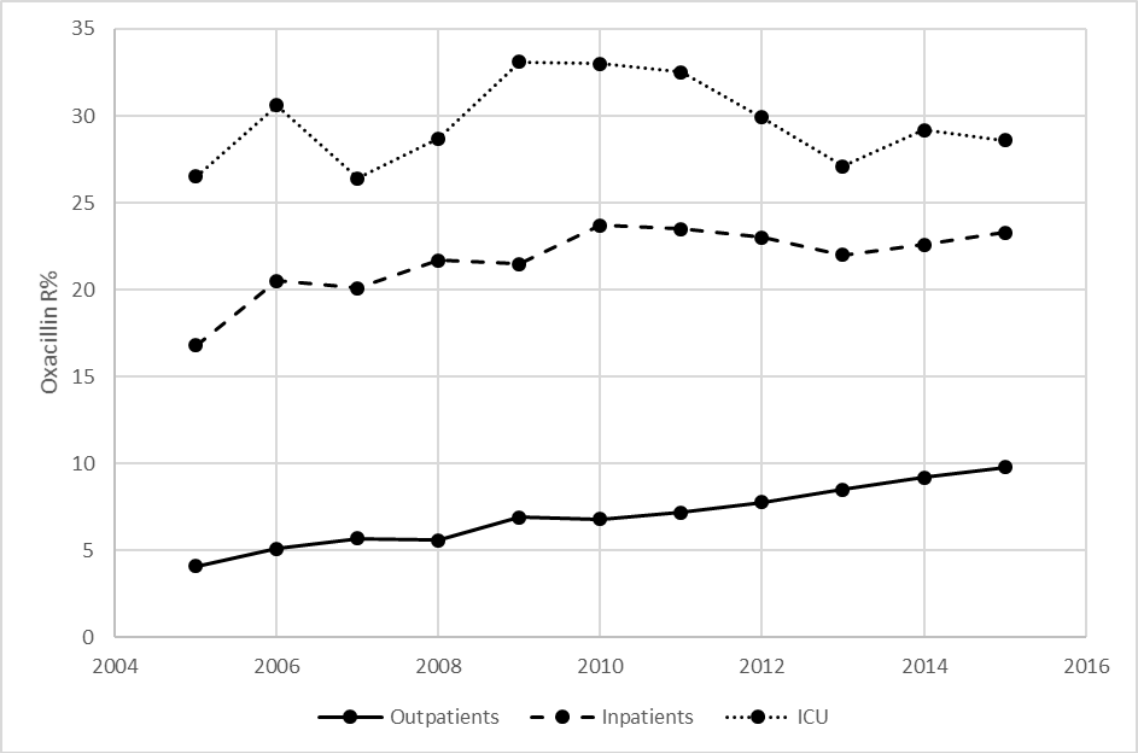

Figure 24. MRSA prevalence in Hungary among clinical isolates between 2005 - 2015 (data obtained from the National Center for Epidemiology)

ICU, intensive care unit

Based on our results, we can declare that unlike observed with the clinical isolates, CA-MRSA carriage rate in Hungary is very low yet.

5.2 Possible risk factors for *Staphylococcus aureus* carriage

We analysed possible risk factors for *S. aureus* nasal colonisation in preschool children. From the literature, there are several known risk factors for carriage – like having chronic diseases (diabetes mellitus, end stage liver or kidney disease, HIV infection, etc.) or doing contact sport [28] – being generally irrelevant in the case of small children. That is the reason we studied only the gender and some external factors, like

sibling status and explosion to passive smoking. We found two factors, male gender and siblings attending community to be in positive correlation with nasal carriage of *S. aureus*. Wertheim et al. in the previously mentioned work stated that *S. aureus* carriage rate is higher in men [28]. Den Heijer et al. established a similar conclusion [66]. On the other hand, there are several papers where there is no correlation between gender and carriage [40, 41, 74, 75].

Similar to our results, having siblings in the family was not shown to be statistically significant in an Italian study among school children [41]. Regev-Yochay et al. concluded parental *S. aureus* colonisation as an independent predictor for staphylococcal carriage in young children, but not having young siblings [76]. Surveys published in the literature examine only the number or age of siblings in association with *S. aureus* carriage. Until now, we did not find any paper dealing with siblings attending community as a possible risk factor. Based on this, we are the first proving that *S. aureus* carriage in young children can be partially dependent on whether their siblings attend any community (DCC or school) or not.

Esposito et al. did not find passive smoking a risk factor for carriage in children either [41]. On the other hand, Melles et al. revealed an association between passive smoking and carriage of a specific *S. aureus* genetic cluster in children [77].

5.3 Antibiotic susceptibility

In the current study, all isolates were sensitive to TMP/SMX, MUP and VAN. We examined <5% resistance rate to CIP, OXA, GEN and TET (0.3%, 0.4%, 1.3% and 3.6%, respectively). Higher resistance was observed in the case of CLI (9.2%) as well as ERY (10.0%), and the highest for PEN (91.6%), as expected. Antibiotic susceptibility of carried *S. aureus* isolates in other European countries is more or less comparable to our results. For example, den Heijer et al. documented ERY resistance varying between 1.6% in Sweden and 16.5% in France; TET resistance between 1.8% in Spain and 7.2% in Croatia [66]. Somewhat higher resistance rates were observed in Turkey screening 200 healthy preschool children: ERY 16.7%, CLI and TET 8.3% [69]. In the study of van Bijnen et al., PEN-R among the 539 Hungarian isolates was 76.1% and considering all the eight countries, it varied between 64.8% and 87.1%; ERY-R was 12.1% (range 1.5%

- 16.5%); CLI-R was 12.1% (range 1.4% - 14.6%); TET-R was 6.7% (range 1.8% - 7.2%); GEN-R was 0.0% (range 0.0% - 2.0%) and CIP-R was 2.0% (range 0.6% - 2.5%) [67]. Compared to our results it can be concluded, that among young children TET resistance is lower while ERY, CLI, GEN and CIP resistance is similar.

Far distant from Europe, in a recent study conducted in Malaysia similar resistance results to ours were observed for doxycycline (4.2%) and GEN (0.0%). Nonetheless, they measured much higher resistance to CIP (27.1%) and ERY (20%) [78]. However, there are countries with extremely high antibiotic resistance, such as India, where TET-R was found to be 41%, GEN-R 32%, TMP/SMX-R 28% [40] and CIP-R 23% [79].

We compared our resistance results to clinical isolates reported by NCE between 2009 and 2012 on **Figure 25**. It can be concluded, that inpatients data show higher resistance than outpatients data, especially for OXA and CIP (because of higher MRSA prevalence in hospitals), but the resistance rates of the carried isolates are even lower, except for PEN. Vancomycin resistance is not shown on **Figure 25**, because it was 0.0% in all three groups (however, there was 0.1% intermediate among inpatients in 2010).

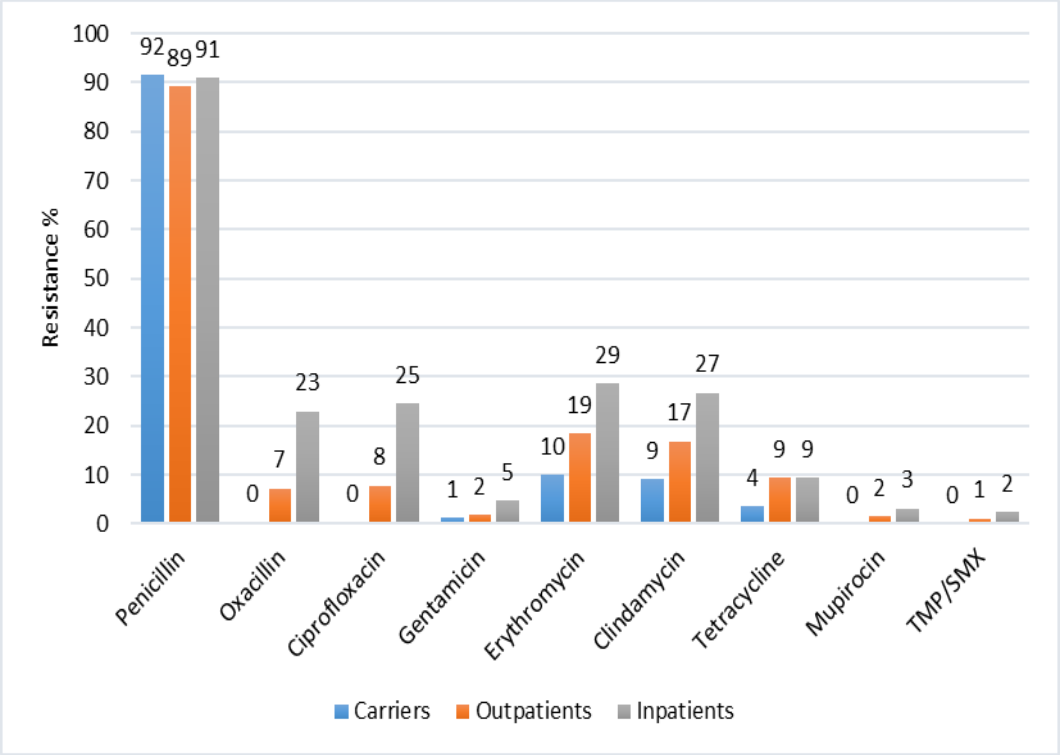

Figure 25. Antibiotic resistance rates of carried (n=751) versus clinical (data obtained from the National Center for Epidemiology, based on several thousands isolates each year) *S. aureus* isolates from Hungary, in the period of 2009-2012

TMP/SMX, trimethoprim/sulfamethoxazole

5.3.1 CA-MRSA strains

The genotypic method was very helpful in the determination of antibiotic sensitivity, especially methicillin-resistance. We identified three OS-MRSA isolates (out of six MRSAs) that harboured the *mecA* gene, although they had oxacillin MICs below the EUCAST resistance breakpoint and one of them was cefoxitin sensitive as well. A survey from India also emphasises the importance of *mecA* gene detection parallel to phenotypic test in the characterisation of *S. aureus* strains. In this paper, 19 oxacillin and cefoxitin susceptible *mecA* positive (OS-MRSA) strains were identified out of 39 *S. aureus* isolates causing bovine mastitis [80]. Another example is from Brazil, where surprisingly high, 33.7% (30/89) of clinical *S. aureus* isolates proved to be cefoxitin-sensitive but *mecA* gene positive [81]. Jannati et al. reported two out of eight nasal

colonising MRSA strains as *mecA*-positive and OXA sensitive, screening 173 nurses in an Iranian teaching hospital [82]. To conclude, without genetic identification of *S. aureus* isolates, MRSA can be easily misclassified as MSSA.

All six isolated MRSA strains proved to be generally sensitive to most antibiotics (CIP, GEN, TET, MUP), with the exception of macrolides and lincosamides. Five out of the six isolates (83.3%) represented high-level ERY resistance (MIC >256 mg/L) and inducible CLI resistance. The NCE database has antibiotic resistance data not only for *S. aureus* but for clinical MRSA isolates (HA-MRSA) as well. We again compared four years' averaged resistance data to our CA-MRSA's resistance rates to the tested antibiotics on **Figure 26**. The most striking difference is the high CIP-R and then a moderate GEN-R of clinical isolates, whereas carried strains were hundred percentage sensitive to these antibiotics. This is in line with our current knowledge that, CA-MRSA strains differ from HA-MRSA strains by possessing smaller SCC*mec* elements, encoding for fewer resistance genes [83].

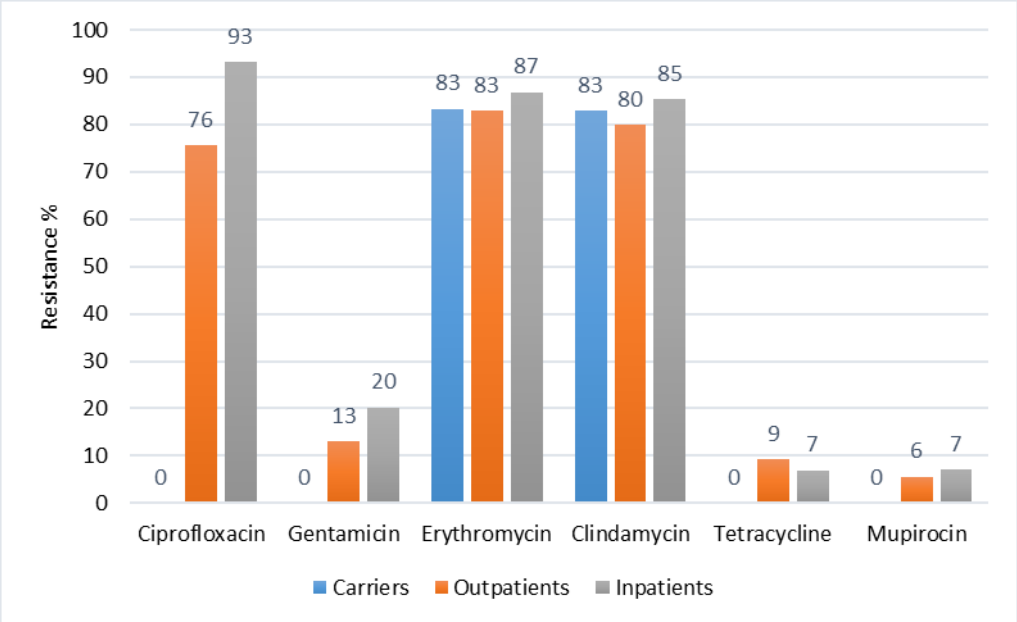

Figure 26. Antibiotic resistance rates of carried (n=6) versus clinical (data obtained from the National Center for Epidemiology, based on several thousands isolates each year) MRSA isolates from Hungary, in the period of 2009-2012

5.4 Molecular typing of MRSA strains

From the numerous molecular typing method, we chose SCC*mec* typing and MLST to better describe MRSA isolates. In most of the world, SCC*mec* types IV and V are most prevalent among CA-MRSA strains, however, majority of such data were obtained from clinical isolates. In this work, 83.3% (5/6) of the strains harboured SCC*mec* type IV and 16.7% (1/6) type V. Similar results were published in the investigation of Lepsanovic et al., where 80% (40/50) of the isolates belonged to these two types [70]. In Jordan, Alzoubi et al. collected nasal specimens from children aged 6-11 years and all 15 MRSA identified were type IV [84]. Another very similar project to ours was conducted in Argentina, where 316 healthy children were screened in a given city, and here again all 14 MRSA strains carried the SCC*mec* type IV cassette [85]. On the other hand, in India, only 7 out of the 17 MRSAs (41.2%) belonged to type IV and none were identified as type V either in a study screening 1503 school-age children [86].

The MLST typing revealed that the majority (5/6) of our MRSA isolates belonged to ST45, a clone which has been very frequently reported from numerous countries from all over the world, based on the MLST database [53]. One isolate was a member of ST7. Very interestingly, the same MLST clones could be found also among MSSA isolates from both university students and children from DCCs, indicating that probably some world-wide prevalent clones are circulating in Hungary. However, the PFGE patterns of isolates with the same ST type are somewhat dissimilar. This suggests that the *S. aureus* strains are evolving continuously over time – the more sensitive PFGE method can show these small differences. Furthermore, apparently these strains can acquire resistance genes and mechanisms, hence the same ST type was found among both MSSA and MRSA isolates. One of these MLST types was ST45. This was for instance found among Slovenian clinical MRSA isolates, being the second most frequent clone in 2010 [87]. The same ST45 clone was shown to be associated with high-level mortality and vancomycin insensitivity in the US [88]. In our study, five of the six carried MRSA isolates belonged to this type, but luckily were not associated with high resistance levels. The other ST type found in our study, ST7, on the other hand rarely causes human infections, meanwhile it is often associated with asymptomatic colonisation [89], or could be detected from animal originated food [90].

In the Indian description of OS-MRSAs from cows, the authors observed four different SCC*mec* types (III, IVc, V, III+IVc) and six different ST types among the 19 strains [80]. Andrade-Figueiredo and Leal-Balbino also examined high diversity by MLST and SCC*mec* typing from clinical isolates [81].

5.5 Clumping factor-negative *Staphylococcus aureus* (CFNSA)

During the identification process, we faced diagnostic problems not only in MRSA detection but in *S. aureus* identification too. We found 1.2% of all strains to be clump-test negative but *nucA* gene positive. Again, without applying a genetic method parallel to commonly used diagnostic techniques for species-specific identification, these strains would have been lost. On the other side, it is an interesting investigation, because the primarily used Pastorex Staph-Plus Kit detects three components of the bacterium simultaneously. Therefore test negativity means, these isolates have no endocoagulase, no protein A and no capsule either, or at least they are masked somehow. We also checked the isolates with another diagnostical latex test (DiaMondial Staph Plus Latex Reagent) to exclude false negativity. This test detects clumping factor, protein A and capsular 5 and 8 antigens and gives positive result if the tested bacterium possesses at least one of them. According to the manufacturer's description, its sensitivity and specificity are 100% [http://www.diamondial.com/index.php?id=48]. On the other hand, all these isolates produced free coagulase by tube coagulase test. Based on these three different tests' results, we identified them as clumping factor-negative *S. aureus*. Although, the isolates originated from four different DCCs in the same city, their clonality was obvious by their PFGE patterns.

In the literature, coagulase-negative and clumping factor-negative *S. aureus* strains are distinguished. In a Polish study by Krasuski et al., 6.5% of 248 clinical *S. aureus* isolates (245 human, 3 animal origin) were found to be clumping factor-negative by sodium citrate rabbit plasma coagulation test. They also identified 58 (23.4%) coagulase-negative strains by tube coagulase test, but all of them gave positive reactions with the Pastorex Staph-plus latex agglutination test used. One of the 16 CFNSA isolates (0.4% of all strains) gave negative, and two of them nonspecific reaction with this kit

[91]. Piechowicz et al. isolated 18 CFNSA and 59 coagulase-negative *S. aureus* strains between 1997 and 2003 from clinical specimens and determined the protein A production and the presence of *spa* gene. They established, that the disability of protein A production occurred more frequently in CFNSA strains compared to coagulase-negative isolates. Moreover, almost all of protein A-negative CFNSA strains were methicillin-resistant. Protein A production deficiency was not found to be in connection with the loss of *spa* gene [92]. German authors analysed 50 clumping factor-negative and protein A-negative clinical *S. aureus* strains. All strains were not only oxacillin-resistant, but also multi-resistant. They amplified that part of the gene, which is responsible for IgG-binding and detected about 170 bp loss in the PCR product. Similarly to us, 49 isolates possessed identical PFGE patterns [93].

Coagulase-, clumping factor-, or protein A-negative *S. aureus* isolates described in the literature are mostly MRSAs deriving from clinical samples. Based on this, the CFNSA isolates identified by us can be considered novelty, as they are carried MSSA isolates and susceptible to many antibiotics. In the era of current new generation microbiological identification systems such as MALDI-TOF, when a clinical specimen can directly be examined, clumping factor negativity or other discrepancies in the phenotype might not influence the diagnosis, nevertheless these could result in misleading identification during conventional laboratory workflow.

5.6 Catalase-negative *Staphylococcus aureus* (CNSA)

One of the most important virulence factor of *S. aureus* is catalase, that protects the bacterium from oxidative damage. Toxic hydrogen peroxide is produced for example during infection by host phagocytes [94, 95], but also by co-colonising bacteria like *Pneumococcus* in the upper respiratory tract. This latter is a very likely reason for the frequently observed antagonistic relationship between these two species by the way [96].

There are relatively few reports about CNSA isolates in the literature, probably because such bacteria are often thought not to be *S. aureus*. The first mention of a CNSA derives from 1955 [97], and the next report is from more than 20 years later [98]. Subsequently about 20-25 cases have been reported until this date in total, all deriving from clinical cases. The first catalase-negative MRSA was found in 2002, causing

pneumonia. It was the tenth case described of this type of strain since 1955 [99]. The first outbreak caused by a CNSA clone was reported from a Brazilian hospital, involving four patients [100]. The first veterinary case was detected in a dog in 2013 in Italy. The animal was suffering from severe pododermatitis (the inflammation and/or infection of the skin and connective tissue of the foot), where a catalase-negative MRSA strain was isolated together with a methicillin-resistant *Staphylococcus pseudintermedius* from the skin lesions [101]. In 2013, the first catalase-negative non-aureus species (*S. lugdunensis*) was also described from a patient in China, diagnosed with chronic suppurative otitis media [102]. Finally, the strain L1034 identified by us is the first report of a CNSA ever isolated from asymptomatic carriage in humans.

Del'Alamo et al. have reviewed all 19 reports between 1955 and 2004 [100]. Until that date, no molecular characterisation was done to any of the isolates. The first molecular description was provided in 2007 by Grüner et al., who have identified a 5-base deletion towards the end of the gene (nucleotide positions 1388-1392), resulting in frameshift which lead to an early truncation of the protein product [103]. Subsequently four more frameshift mutations were reported: both Horiuchi et al. and Corrente et al. found a single-base deletion (637G and 487A) [101, 104]; while Ellis et al. and Teo et al. have identified insertions (a single T-insertion after position 31 and a CA-insertion after position 1157, respectively) [105, 106]. Four authors have detected one or more missense point mutations, resulting in changes in essential parts of the protein playing key role in catalase activity [55, 104, 107, 108]. However, the most interesting cases are the nonsense mutations, when a single nucleotide change results in conversion to a stop codon, casing the prematurely termination of the protein product. The first nonsense mutation was found in 2011 by To et al. [109], the second in 2016 by Lagos et al. [110], and finally we reported the third nonsense mutation in a CNSA. Notably, these three cases derive from three different continents that we summarised in **Table 31**.

Table 31. Summary of the nonsense mutations found to date in the *katA* gene of CNSA isolates

Country	**Specimen (origin)**	**Nonsense mutation**	**Truncation to**	**Reference**
Hong Kong	blood (endocarditis/ pericarditis)	G802T GAA → TAA Glu → Stop	267 amino acids	[109]
Chile	blood + synovial tissue (infectious arthritis)	G852A TGG → TGA Try → Stop	283 amino acids	[110]
Hungary	nasal swab (carriage)	G491A TGG → TAG Try → Stop	164 amino acids	this study

Additionally, a nonsense mutation was also found in the above mentioned *S. lugdunensis* strain found by Lu et al. in 2013 [102]. All missense, nonsense and frameshift mutations reported to date are summarised on **Figure 27**. These can be found all across the *katA* gene and we could not identify any specific "hot spots".

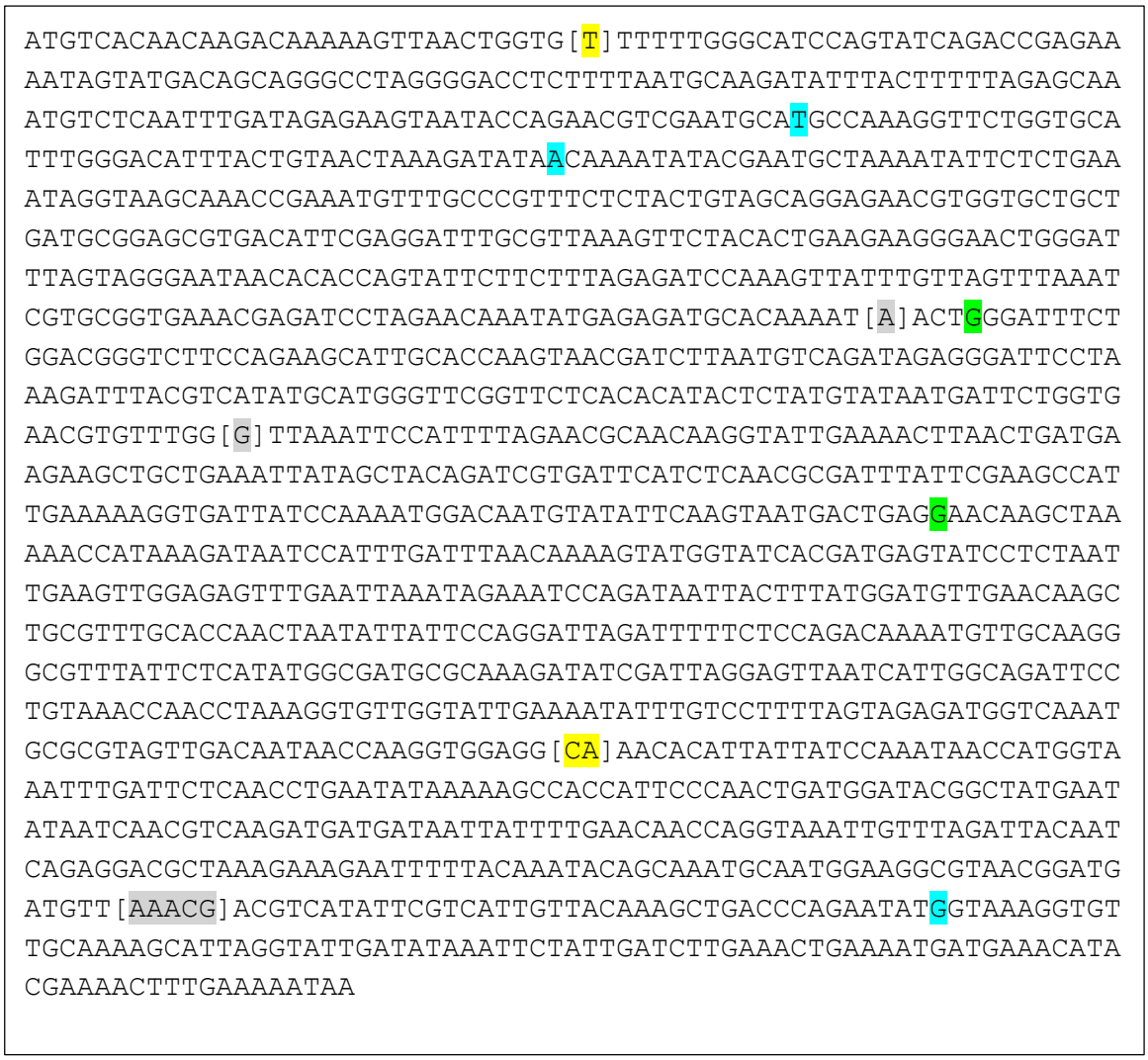

Figure 27. Mutations, deletions and insertions in the *katA* gene reported in the literature

Turquoise shade, missense point mutations (i.e. leading to an amino acid change);
green shade, nonsense point mutations (i.e. coding for a stop codon);
[] brackets and grey shade, deletions; [] brackets and yellow shade, insertions.

The multilocus sequence type was also determined of the isolate, and it was shown to be ST5. This sequence type is a typical MRSA clone belonging to clonal complex 5 (CC5), which has been associated with haematogenous complications before [111]. In the literature, the sequence types of the isolates was determined in four cases, additional to sequencing the *katA* gene. Interestingly, the MRSA isolate from the Italian dog was also ST5, while the strain characterised by Berenger et al. was ST25. Both ST5 and ST25 have

been frequently found in human infections from all continents. In the other two cases (Teo et al. from Singapore and Lagos et al. from Brazil) very rare or novel STs were identified (ST2250 and ST3145). Nonetheless, ST3145 was found to be highly similar to other common STs such as ST10 or ST145.

Before us, toxin genes of catalase-negative isolates was checked only in one occasion: Lee at al. found that all 17 catalase-negative MRSAs involved in the outbreak possessed enterotoxin A, B and TSST genes [107]. Our isolate was positive only for enterotoxin A.

Catalase negativity does not seem to be related to antibiotic sensitivity. Among all reported CNSA strains, both methicillin-resistant and sensitive ones were presented.

Catalase has been thought for a long time to play a key role in the virulence of *S. aureus*. For example, Mandell found a good correlation between staphylococcal catalase activity and mouse lethality in his experiments in 1975 [95]. Although, later it was shown in a mouse model of chronic granulomatous disease, that catalase-negative strains retained equivalent virulence compared to those possessing catalase [112]. This lack of correlation between catalase and virulence is further supported by the fact that all CNSA isolates reported in the literature – except for our strain – derived from clinical cases, representing a wide spectrum of staphylococcal infections such as pneumonia, post-operative wound infection, chronic leg ulcer, infectious arthritis, cutaneous abscess, endocarditis, etc. Furthermore, hospital outbreaks occurred with high fatality rate as well. Out of the four Brazilian patients involved in the outbreak in 2002, three died due to bacteraemia [100]. Similarly, among the 17 patients with respiratory syndromes in Korea, three died during the outbreak [107].

Similarly to its correlation with virulence, catalase has been found necessary for nasal colonisation previously. Cosgrove et al. stated that both AhpC and KatA (two antioxidant proteins) are required for environmental persistence and nasal colonisation, as neutralising H_2O_2 is essential for aerobic survival. They concluded that the major component involved in resistance to externally applied H_2O_2 is KatA. They have also shown in a cotton rat model (an animal often used to study human respiratory pathogens based on several similarities) that nasal colonisation by either *ahpC* or *katA* or double

mutants was significantly less successful compared to the wild type bacteria [113]. Park et al. have used a mouse model as well to investigate the role of catalase in the successful competition against *S. pneumoniae* in the nasopharyngeal niche. By generating a catalase knockout mutant strain, they could confirm that catalase significantly contributes to the survival of *S. aureus* in the presence of *S. pneumoniae* [114].

During our study, we isolated a CNSA from a symptomless healthy child's nasal mucosa, showing for the first time that the presence of this type of bacterium in the nasopharynx is indeed possible and catalase is apparently not required for nasal colonisation. No *Pneumococcus* was however present in this child. Nota bene, Grüner et al. have found a catalase-negative MRSA strain in the tracheal secretion of a 65-year old patient during routine microbiological investigation upon admission to the intensive care unit. Although the patient had no signs of any acute infection being apparent at that time, he was suffering from multiple diseases, such as alcohol-toxic liver cirrhosis, arterial hypertension, coronary artery disease, heart failure, diabetes mellitus, and chronic obstructive pulmonary disease [103]. We think that probably the latter disease on its own could be related to the presence of *S. aureus* and generally speaking, such a multimorbid patient cannot be considered as a symptomless carrier in contrast to the healthy young child in our case.

Our case further underlines the importance of CNSA in both causing infections as well as colonising the mucosal surfaces of the nasopharynx. The presence of such a bacterium in the normal flora finally can also lead to the development of infection. Similarly to CFNSA cases, we would also emphasise the importance of further investigation, if a bacterium shows typical *S. aureus* morphology but has unusual phenotypical behaviour (such as catalase or clump-test negativity). Otherwise, a serious and virulent pathogenic isolate could be missed, potentially leading to therapeutic failure due to the lack of early initiation of adequate treatment.

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

Most studies in the literature report a definite inverse relationship between *S. pneumoniae* and *S. aureus* carriage, especially in the case of PCV serotypes; however, other results can also be found. In a study conducted in Israel during 2002, Regev-Yochay et al. screened 790 children aged 40 months or younger who did not receive pneumococcal vaccine. They measured lower *S. aureus* carriage rate (6.5%) among *S. pneumoniae* carriers than us (21.6%), while a similar proportion (27.5%) of *S. aureus* carriers was co-colonised with *S. pneumoniae* compared to our results (24.4%). They found dual carriage in 2.8% of all examined children, which is also much lower than we found in this current study (7.1%). They observed the highest *S. aureus* carriage rate (30%) among children aged 3 month or younger, in whom *Pneumococcus* prevalence was lowest (9%). In contrast, the highest *Pneumococcus* prevalence (approx. 50%) was detected in older children (aged 7-40 months) with the lowest *S. aureus* prevalence (5-9%). Finally, they established the same conclusion like us, that *S. pneumoniae* carriage is inversely associated with *S. aureus* nasal carriage and vice versa. It is important to note, that this negative association persisted when data were controlled for age as well as restricted to vaccine-type pneumococci. The authors did not find any association between *S. aureus* and non-vaccine-type *S. pneumoniae* carriage. As pneumococcal conjugate vaccines reduce vaccine-type *Pneumococcus* nasopharyngeal carriage, they predicted for the future the increase of *S. aureus* carriage rates in children [115].

Another study by Lee at el. could not establish statistically significant association between *S. aureus* and *S. pneumoniae* nasal carriage among 1968 American healthy children in the post-PCV7 era. Meanwhile, they observed higher dual carriage rates compared to us, 11.4% of children colonised with PCV7 *Pneumococcus* strains carried *S. aureus* and a very similar rate (12.7%) of children carrying non-PCV7 strains were concomitantly colonised with *S. aureus* [35]. Van Gils et al. investigated the effect of PCV7 on *S. aureus* colonisation among healthy Dutch newborns in a randomised controlled trial. They observed a negative correlation in co-colonisation of *S. aureus* and *S. pneumoniae* for both vaccine and non-vaccine serotypes [116]. Esposito *et al.* investigated older children, aged 6-17 years, and found no significant association between

carriage of pneumococci and *S. aureus*, regardless of age, PCV7 vaccination status and pneumococcal serotype. Dual carriage was detected in 27.3% of the children [39]. A very interesting comparative study was performed in the Netherlands, a country with high vaccine coverage. Bosch et al. sampled children several times between 2005 (before PCV7) and 2013 (5.5 years post-PCV7). Pneumococcal carriage dropped from 67% in 2005 to 47% in 2009 (three years post-PCV7), but later it started increasing again, up to 59% in 2013 (with, naturally, a strong serotype re-arrangement). On the other hand, carriage of *S. aureus* showed an inverse pattern: it first increased from 5% in 2005 to 9% in 2009, later further to 14%, but it went back to 7% by 2013 [117].

6 CONCLUSIONS

The 29.3% *S. 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. On the other hand, 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.

There is much higher variety in CA-MRSA carriage rate worldwide. 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.

From the possible risk factors analysed, we found positive correlation between *S. aureus* carriage and male gender, as well as children's siblings attending community. Nonetheless, 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. The only exception was PEN-R, which is similarly about 90% in both carried and clinical isolates. We found low resistance to CIP, OXA, GEN and TET, and higher to CLI and ERY. Fortunately, all isolates were sensitive to TMP/SMX, MUP and VAN, which is important as VAN is the first choice antibiotic for the treatment of MRSA infections and MUP is used for nasal *S. aureus* as well as MRSA decolonisation. According to international data, our antibiotic sensitivity results are similar or lower than detected in other European countries.

Half of the MRSA strains (3/6) totally observed were phenotypically OXA-S. 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 in 5 out of 6 cases and type V in one case. 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 – which resulted again 5 out of 6 strains belonging to ST45 – it can be concluded, that our strains are not unique.

We could observe a noticeably great genetic diversity by PFGE patterns, even among strains deriving from students of the same university group. On the other hand, sometimes the same macrorestriction pattern could be identified in different university groups, or in distantly located cities in Hungary. This 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.

Not only *mecA* gene detection but *nucA* gene detection was helpful during the identification of *S. aureus*. 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. We defined them as CFNSA isolates, which are known from the literature, but was observed in clinical isolates so far. We found high clonality of these strains by PFGE method, as 8 out of 9 had identical patterns.

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.

7 SUMMARY

S. aureus is one of the most common human pathogens causing suppurative skin and soft tissue infections as well as life-threatening diseases, like bacteraemia or endocarditis. It can colonise the skin and mucosal surfaces and from carriers can easily spread to the community. Since the discovery of penicillins, several resistance mechanisms were developed against different antibiotics. One of the most important is methicillin-resistance.

In this study we screened a total of 2568 healthy individuals – 300 university students and 2268 young children – for *S. aureus* nasal colonisation between 2009 and 2012 in Hungary. The overall carriage rate was calculated 29.2% while the MRSA prevalence was 0.2%. Male gender and having community attending siblings among children were determined to be possible risk factors for carriage. All of the isolates were sensitive to TMP/SMX, MUP and VAN, while low resistance was observed in the case of OXA, CIP, GEN and TET. Resistance to ERY and CLI was about 10%. Half of the MRSA strains proved to be OS-MRSA, otherwise they belonged to well known SCC*mec* types and ST types. In general, the examined isolates were genetically diverse, even within the same university group. Special isolates could be identified, like clumping-factor negative or catalase-negative *S. aureus*. *S. aureus* carriage was inversely associated with *S. pneumoniae* carriage in children and high-level of PCV7 vaccination rate in the given community, but not at the individual level, seems to be in positive correlation with *S. aureus* carriage.

To conclude, it is important to examine the epidemiology of *S. aureus* carriage over time, as the antibiotic resistance is growing worldwide and MRSA strains are emerging even in the community causing antibiotic treatment challenges.

8 ÖSSZEFOGLALÁS

A *S. aureus* a gennyes bőr és lágyrész fertőzések egyik leggyakoribb kórokozója, illetve életet veszélyeztető kórképeket is okozhat, mint a véráramfertőzés vagy a szívbelhártyagyulladás. Gyakran kolonizálja a bőrt és a különböző nyálkahártya felszíneket, így a hordozók révén könnyen terjed a közösségekben. A penicillin felfedezése óta számos antibiotikum-rezisztencia mechanizmust kifejlesztett. Ezek közül az egyik legfontosabb a methicillin-rezisztencia.

Jelen tanulmányban 2009 és 2012 között 2568 személynél vizsgáltuk a *S. aureus* orrban történő tünetmentes hordozását Magyarországon, közülük 300 egyetemi hallgató és 2268 óvodás kisgyermek volt. Az átlagos hordozási arány 29,2%-nak bizonyult, míg az MRSA-k aránya 0,2% volt. A gyermek populációban a fiúk között szignifikánsan magasabb volt a hordozási arány, illetve még az bizonyult lehetséges rizikó tényezőnek, hogy a testvérük közösségbe jár. Az összes törzs TMP/SMX, MUP és VAN érzékeny volt, ezen kívül alacsony rezisztenciát mértünk OXA, CIP, GEN és TET esetében. Az ERY- és CLI-rezisztencia 10% körül mozgott. Az MRSA izolátumok fele OS-MRSA-nak bizonyult, egyébként pedig közismert SCC*mec* és ST típusúak voltak. A törzsek általában genetikailag különböztek egymástól, még az egyes hallgatói csoportokon belül is. Sikeresen azonosítottunk különleges törzseket is, mint clumping-faktor negatív vagy kataláz-negatív *S. aureus*. A gyerekek között az *S. aureus* hordozás negatív összefüggést mutatott a *S. pneumoniae* kolonizációval, viszont pozitív korrelációban volt a magas szintű PCV7 átoltottsági aránnyal.

Következésképpen fontosnak tartjuk a *S. aureus* hordozás időről-időre történő ismételt epidemiológiai vizsgálatát, mivel az antibiotikum rezisztencia világszerte folyamatosan növekszik és az MRSA-k is egyre nagyobb fenyegetést, illetve terápiás kihívást jelentenek, már a közösségben szerzett típusuk is.

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Internet source was used for Figure 1:

https://en.wikipedia.org/wiki/Staphylococcus_aureus#/media/File:Staphylococcus_aureus_Gram.jpg

10 LIST OF PUBLICATIONS

10.1 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.

Laub K, Tóthpál A, Kovács E, Sahin-Tóth J, Horváth A, Kardos S, Dobay O. (2017) High prevalence of *Staphylococcus aureus* nasal carriage among children in Szolnok, Hungary. Acta Microbiol Immunol Hung, In press, DOI: 10.1556/030.65.2018.001.

Laub K, Tóthpál A., Kardos S, Dobay O. (2017) Epidemiology and antibiotic sensitivity of *Staphylococcus aureus* nasal carriage in children in Hungary. Acta Microbiol Immunol Hung, 64: 51-62.

Tóthpál A, Laub K, Kardos S, Tirczka T, Kocsis A, van der Linden M, Dobay O. (2016) Epidemiological analysis of pneumococcal serotype 19A in healthy children following PCV7 vaccination. Epidemiol Infect, 144: 1563-1573.

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.

10.2 Not related to the thesis

Tóthpál A, Kardos S, Laub K, Nagy K, Tirczka T, van der Linden M, Dobay O. (2015) Radical serotype rearrangement of carried pneumococci in the first 3 years after intensive vaccination started in Hungary. Eur J Pediatr, 174: 373-381.

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12 APPENDIX

Appendix 1. Parent's permission

Szülői beleegyezés a mintavételhez

Kérem a Tisztelt szülőt, hogy nyilatkozzon a megfelelő szöveg aláhúzásával!

GYERMEKEM ORRÁNAK SZŰRŐVIZSGÁLATÁHOZ

HOZZÁJÁRULOK NEM JÁRULOK HOZZÁ

Gyermekem neve[1]:……..

Gyermekem neme: fiú lány

Gyermek életkora :……..

Testvére(i): van nincs

Ha van, hány testvére van:……

Ha testvére van, ő közösségbe jár nem jár

ha igen, az: (kérjük aláhúzni) bölcsőde óvoda iskola felsőfokú int.

Gyermekem ***Prevenar*** oltást[2] kapott-e igen nem nem tudom

Gyermekem ***Pneumovax*** oltást[3] kapott-e igen nem nem tudom

Szokott-e középfülgyulladása lenni igen nem

Volt-e már súlyos tüdőgyulladása, agyhártyagyulladása vagy középfülgyulladása? (A megfelelő aláhúzandó)

Kapott-e antibiotikumos kezelést a megelőző 2 hónapban, ha igen: mit, mikor és miért?

…….

Volt-e a gyermek kórházban az elmúlt 3 hónapban? igen nem

Dohányoznak-e a családban? igen nem

Dátum:

Szülő aláírása:

Szülői tájékoztató

Értesítjük a tisztelt szülőket, hogy egy egészségfelmérő program keretén belül szeretnénk óvodás és bölcsődés korú gyermekek orrában megbújó néhány baktériumot (pneumococcus, *Staphylococcus aureus*) kiszűrni. Ebben az életkorban a kisgyermekek kb. fele hordozza az orrában ezeket a baktériumokat, **ami teljesen természetes állapot**. A felmérés célja, hogy adatokat kapjunk az óvodás / bölcsődés korú gyermekek tünetmentes baktérium hordozásának mértékéről és a hordozott baktériumok tulajdonságairól. Ezen adatok segítséget nyújtanak például annak a kérdésnek az eldöntésében, hogy a rendelkezésre álló pneumococcus ellenes védőoltások (*Pneumovax* és *Prevenar*) mennyire alkalmasak a visszatérő középfülgyulladás, orrmelléküreg gyulladás, illetve a súlyos gyermekkori tüdőgyulladás és agyhártyagyulladás megelőzésére.

A mintavétel fájdalmatlan beavatkozás során vékony vattapálcával történik, az a gyerekekre nézve semmiféle hátránnyal, károsodással nem jár. A mintalevételt és az eredmények kiértékelését egyaránt a Semmelweis Egyetem Orvosi Mikrobiológiai Intézetének munkatársai végzik el, **a felmérés kizárólag tudományos célokat szolgál**. Az eredmény munkatársunk diplomamunkájának alapjául is szolgál.

Kérem, hogy néhány adat közlésével tegye lehetővé a kapott eredmények feldolgozását. A mintákat a továbbiakban név nélkül kezeljük, minden baktérium izolátum egy számot fog kapni, a minta beazonosítására a felmérésben szereplő adatok miatt van csak szükség. Ezeket az adatokat senkinek tovább nem adjuk.

Köszönettel:

Dr. Dobay Orsolya	Tóthpál Adrienn	Laub Krisztina
egyetemi adjunktus	PhD hallgató	PhD hallgató

Semmelweis Egyetem, Orvosi Mikrobiológiai Intézet

Budapest, 2011. október 24.

Appendix 2. Nucleotide sequences of the seven housekeeping genes of *S. aureus* examined during MLST

Purple highlighting, location of the PCR primers; yellow highlighting, location of the internal MLST allele fragments.

A, Sample gene for *arcC*

Source: *S. aureus* subsp. *aureus* USA300_TCH959 contig00030, whole genome shotgun sequence; GenBank database accession number: AASB02000025.1

MLST fragment length: 456 bp

ATGTTTTTTAAAAGGAGCGACAAAAATATGAAAGAGAAAATTGTCATTGCATTAGGCGGTAATGCGATAC
AGACAAAAGAAGCAACAGCTGAAGCACAACAAACAGCTATTAGATGTGCGATGCAAAACCTTAAACCTTT
ATTTGATTCACCAGCGCGTATTGTCATTTCACATGGTAATGGTCCACAAATTGGAAGTTTATTAATCCAA
CAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGCAATGTCACAGGGTA
TGATAGGCTATTGGTTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGG
CACAATCGTTACACGTGTGGAAGTAGATAAAGATGATCCACGATTTGATAACCCAACTAAACCAATTGGT
CCTTTTTATACGAAAGAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTAAAGAAGATG
CAGGACGTGGTTATAGAAAAGTAGTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCG
AACTTTAGCAGACGGTAAAAATATTGTCATTGCATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAA
AATACCTATGAAGGTGTTGAAGCGGTTATAGATAAAGATTTTGCTAGTGAGAAATTAGCAACGCTGATTG
AAGCAGATACCTTAATGATTCTTACGAATGTAGAAAATGTATTTATTAACTTTAATGAACCTAATCAACA
ACAAATCGATGATATTGATGTAGCAACACTGAAAAAATACGCGGCACAAGGTAAGTTTGCGGAAGGATCG
ATGTTGCCAAAAATAGAAGCTGCGATACGATTTGTTGAAAGTGGGGAAAACAAAAAAGTTATCATTACCA
ATTTAGAGCAGGCATACGAAGCTTTGATTGGTAATAAAGGTACACACATTCACATGTAG

B, Sample gene for *aroE*

Source: *S. aureus* D30 contig00395, whole genome shotgun sequence; NCBI reference sequence: NZ_ABFB01000005.1

MLST fragment length: 456 bp

ATGAAATTTGCAGTTATCGGAAATCCTATTTCACATTCCTTGTCGCCCGTTATGCATAGAGCAAATTTTA
ATTCTTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAAGA
AATTATTTCGAAAAAAGAATTAGAAGGCTTTAATATCACAATTCCTCATAAAGAACGTATCATACCGTAT
TTAGATTATGTTGATGAACAAGCGATTAATGCAGGTGCAGTTAACACTGTTTTGATAAAAGATGGCAAGT
GGATAGGGTATAATACAGATGGTATTGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAA
TGCATACATTTTAATTTTGGGCGCAGGTGGTGCAAGTAAAGGTATTGCTTATGAATTAGCAAAATTTGTA
AAGCCCAAATTAACTGTTGCGAATAGAACGATGGCTCGTTTTGAATCTTGGAATTTAAATATAAACCAAA
TTTCATTAGCAGATGCTGAAAAGTATTTAGCTGAATTCGATATCGTTATTAATACAACACCAGCGGGTAT
GGCTGGAAATAACGAAAGTATTATTAATTTAAAGCATCTTTCTCCCAATACTTTAATGAGTGATATTGTT
TATATACCGTATAAAACACCTATTTTAGAGGAAGCAGAGCGCAAGGGAAACCATATTTATAATGGCTTAG
ATATGTTTGTTTACCAGGGTGCGGAAAGCTTTAAAATTTGGACTAATAAAGATGCTGATATTAATTCTAT
GAAAACAGCAGTTTTACAACAATTAAAAGGAGAATAA

C, Sample gene for *glpF*

ATGAATGTATATTTAGCAGAATTCCTAGGAACTGCAATCTTAATCCTTTTTGGTGGTGGCGTTTGTGCCA
ATGTCAATTTAAAGAGAAGTGCTGCGAATGGTGCTGATTGGATTGTCATCACAGCTGGATGGGGATTAGC
GGTTACAATGGGTGTGTTTGCTGTCGGTCAATTCTCAGGTGCACATTTAAACCCAGCGGTGTCTTTAGCT
CTTGCATTAGACGGAAGTTTTGATTGGTCATTAGTTCCTGGTTATATTGTTGCTCAAATGTTAGGTGCAA
TTGTCGGAGCAACAATTGTATGGTTAATGTACTTGCCACATTGGAAAGCGACAGAAGAAGCTGGCGCGAA
ATTAGGTGTTTTCTCTACAGCACCGGCTATTAAGAATTACTTTGCCAACTTTTTAAGTGAGATTATCGGA
ACAATGGCATTAACTTTAGGTATTTTATTTATCGGTGTAAACAAAATTGCCGATGGTTTAAATCCTTTAA
TTGTCGGAGCATTAATTGTTGCAATCGGATTAAGTTTAGGCGGTGCTACTGGTTATGCAATCAACCCAGC
ACGTGATTTAGGTCCGAGAATTGCACATGCGATTTTACCAATAGCTGGTAAAGGTGGTTCAAATTGGTCA
TATGCAATCGTTCCTATCTTAGGACCAATTGCCGGTGGTTTATTAGGTGCAGTGGTATACGCTGTATTTT
ATAAACATACATTTAATATTGGTTGTGCAATTGCAATTGTTGTAGTTATTATTACTTTGATTTTAGGTTA
CATTTTAAATAAATCATCAAAAAAAGGTGATATCGAATCAATTTACTAA

D, Sample gene for *gmk*

Source: *S. aureus* subsp. *aureus* strain MRSA252, complete genome; GenBank database accession number: BX571856.1

MLST fragment length: 417 bp

ATGGATAATGAAAAAGGATTGTTAATCGTTTTATCGGGACCATCTGGAGTAGGTAAAGGTACTGTTAGAA
AACGAATATTTGAAGATCCAAGTACATCATATAAGTATTCTATTTCAATGACAACACGTCAAATGCGTGA
AGGTGAAGTTGATGGCGTAGATTACTTTTTTAAAACTAGGGATGCGTTTGAAGCTTTAATTAAAGATGAC
CAATTTATAGAATATGCTGAATATGTAGGCAACTATTATGGTACACCAGTTCAATATGTTAAAGATACAA
TGGACGAAGGTCATGATGTATTTTTAGAAATTGAAGTAGAAGGTGCAAAGCAAGTTAGAAAGAAATTTCC
AGATGCGTTATTTATTTTCTTAGCACCTCCAAGTTTAGATCACTTGAGAGAGCGATTAGTAGGTAGAGGA
ACAGAATCTGATGAGAAAATACAAAGTCGTATTAACGAAGCACGTAAAGAAGTCGAAATGATGAATTTAT
ACGATTACGTTGTAGTTAATGATGAAGTAGAACTTGCGAAGAATAGAATTCAATGTATTGTAGAAGCTGA
GCACTTAAAAAGAGAGCGCGTAGAAGCTAAGTATAGAAAAATGATTTTGGAGGCTAAAAAATAA

E, Sample gene for *pta*

Source: *S. aureus* subsp. *aureus* CO-85 contig00088, whole genome shotgun sequence; GenBank database accession number: JHQG01000054.1

MLST fragment length: 474 bp

ATGGCTGATTTATTAAATGTATTAAAAGACAAACTTTCTGGTAAAAACGTTAAAATCGTATTACCTGAAG
GAGAGGACGAACGTGTTCTAACAGCTGCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTT
AGGTGATGAGACTAAGGTTCAATCTTTAGCGCAAAAACTTGATCTTGATATTTCTAATATTGAATTAATT
AATCCTGCGACAAGTGAATTGAAAGCTGAATTAGTTCAATCATTTGTTGAACGACGTAAAGGTAAAGCGA
CTGAAGAACAAGCACAAGAATTATTAAACAATGTGAACTACTTCGGTACAATGCTTGTTTATGCTGGTAA
AGCAGATGGTTTAGTTAGTGGTGCAGCACATTCAACAGGCGACACTGTGCGTCCAGCTTTACAAATCATC
AAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAAGGTGATGAACAATACATCT
TTGGTGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAGTGCAAA
ATCAGCATTAAGCTTTGGCATGGATCCAAAAGTTGCAATGTTAAGCTTTTCAACAAAAGGGTCTGCTAAA
TCAGACGACGTGACAAAAGTTCAAGAAGCTGTCAAATTAGCACAACAAAAAGCTGAAGAAGAAAAATTAG
AAGCAATCATTGATGGCGAATTCCAATTTGATGCTGCGATTGTACCAGGTGTTGCTGAGAAAAAAGCGCC
AGGTGCTAAATTACAAGGTGATGCAAATGTCTTTGTATTCCCAAGTTTAGAAGCTGGTAATATTGGTTAC
AAAATTGCACAACGTTTAGGTGGATATGATGCAGTTGGTCCAGTATTACAAGGTTTAAATTCTCCAGTAA
ATGACTTATCACGTGGCTGCTCAATTGAAGATGTATACAATCTTTCAATTATTACAGCAGCGCAAGCCTT
ACAATAA

F, Sample gene for *tpi*

Source: *S. aureus* subsp. *aureus* N315 DNA, complete genome; GenBank database accession number: BA000018.3

MLST fragment length: 402 bp

ATGAGAACACCAATTATAGCTGGTAACTGGAAAATGAACAAAACAGTACAAGAAGCAAAAGACTTCGTCA
ATGCATTACCAACATTACCAGATTCAAAAGAAGTAGAATCAGTAATTTGTGCACCAGCAATTCAATTAGA
TGCATTAACTACTGCAGTTAAAGAAGGAAAAGCACAAGGTTTAGAAATCGGTGCTCAAAATACGTATTTC
GAAGATAATGGTGCGTTCACAGGTGAAACGTCTCCAGTTGCATTAGCAGATTTAGGCGTTAAATACGTTG
TTATCGTTCATTCTGAACGTCGTGAATTATTCCACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGC
TATTTTCAAACATGGAATGACTCCAATTATTTGTGTTGGTGAAACAGACGAAGAGCGTGAAAGTGGTAAA
GCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAGCTGTTGCAGGTTTATCTGAAGATCAACTTAAATCAG
TTGTAATTGCTTATGAGCCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGAAGATGCAAATGA
AATGTGTGCATTTGTACGTCAAACTATTGCTGACTTATCAAGCAAAGAAGTATCAGAAGCAACTCGTATT
CAATATGGTGGTAGTGTTAAACCTAACAACATTAAAGAATACATGGCACAAACTGATATTGATGGGGCAT
TAGTAGGTGGCGCATCACTTAAAGTTGAAGATTTCGTACAATTGTTAGAAGGTGCAAAATAA

G, Sample gene for *yqiL*

Source: *S. aureus* subsp. *aureus* MRSA252 chromosome, complete genome; NCBI reference sequence: NC_002952.2

MLST fragment length: 516 bp

AAAGTAGATGGTGAAATTGATGGTGAAACAAGCTGTGAATAGCGATGCAATGATAGATAGCATTTAATTA
GAGTCATTACGCGAAATGATTAATGATAATTTGTGGTAAATCAAAGCATAATTTTGTACTATAGATGAGG
ATGATAGAGCATATTTAAGAGTGTGAAATGTTAAAGTGAAACCGTTTACGTTTCCGATTGCCCAAACAAA
TTACATCATTGTATAATATGATTTGTTAAATGCATAACAAGAATGAAAATGTAACATACGTAGCAATTGG
TTTCATAAATTGGATGTTAGTGGCGTATTGGTTCATTAGACGTATTAGTAATAAAATTGTATATATCATA
AGGAGATGAATATGACATGACGAGAGTCGTATTAGCAGCAGCATACAGGACACCTATTGGCGTTTTTGGA
GGTGCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAACATATTATTAAAGAGACGG
GTTTGAATCCAAGTGAGATTAATGAAGTCATCATCGGTAACGTACTACAAGCAGGACAAGGACAAAATCC
AGCACGAATTGCTGCTATGAAAGGTGGCTTGCCAGAAACAGTACCTGCATTTACAGTGAATAAAGTATGT
GGTTCTGGGTTAAAGTCGATTCAATTAGCATATCAATCTATTGTGACTGGTGAAAATGACATCGTGCTAG
CTGGCGGTATGGAGAATATGTCTCAATCACCAATGCTTGTCAACAACAGTCGCTTTGGTTTTAAAATGGG
ACATCAATCAATGGTTGATAGCATGGTATATGATGGTTTAACAGATGTATTTAATCAATATCATATGGGT
ATTACTGCTGAAAATTTAGTAGAGCAATATGGTATTTCAAGAGAAGAACAAGATACATTTGCTGTAAACT
CACAACAAAAAGCAGTACGTGCACAGCAAAATGGTGAATTTGATAGTGAAATAGTTCCAGTATCGATTCC
TCAACGTAAAGGTGAACCAATCGTTGTCACTAAGGATGAAGGTGTACGTGAAAATGTATCAGTTGAAAAA
TTAAGTCGCTTAAGACCAGCTTTCAAAAAAGACGGTACAGTTACAGCAGGTAATGCATCAGGAATCAATG
ATGGTGCTGCGATGATGTTAGTCATGTCAGAAGACAAAGCTAAAGAATTAAATATCGAACCATTGGCAGT
GCTTGATGGCTTTGGAAGTCATGGTGTAGATCCTGCTATTATGGGTATTGCCCCAGTTGACGCTGTTGAA
AAGGCTTTGAAACGTAGTAAAAAAGAATTAAGTGATATTGATGTATTTGAATTAAATGAAGCATTTGCAG
CACAATCATTAGCTGTTGATCGTGAATTAAAATTACCTCCTGAAAAGGTGAATGTTAAAGGTGGCGCTAT
TGCATTAGGACATCCTATTGGTGCATCTGGTGCTAGAGTTTTAGTGACATTATTGCATCAACTGAATGAT
GAAGTTGAAACTGGTTTAACATCATTGTGTATTGGTGGCGGTCAAGCTATCGCTGCAGTTGTATCAAAGT
ATAAATAATAAGAAAACAGGTTATCACAACAGTATTAATTACATGTTGGCATAACCTGTTTTTATTTGTT
TATGGATTTATTGGGTAATATTAGTCATTTGATGGTTTAATTGCAAATGCTCTAACAGGGAACCCAGGTG
CATCTTTTGGTTTAGGGCTGATAGCGTAAATGATGGCGCCACGAGTTGGTAATTGATCTAAATTAGTTAA
TAACTCGACTTGGTATTTATCCTGACCAAGAATATAACGTTCGCCAACTAAATCACCATTTTTTACAACG