

Evaluation of antimicrobial susceptibilities and virulence factors of *Staphylococcus aureus* strains isolated from community-acquired and health-care associated pediatric infections

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SUMMARY: Karbuz A, Karahan ZC, Aldemir-Kocabaş B, Tekeli A, Özdemir H, Güriz H, Gökdemir R, İnce E, Çiftçi E. Evaluation of antimicrobial susceptibilities and virulence factors of *Staphylococcus aureus* strains isolated from community-acquired and health-care associated pediatric infections. Turk J Pediatr 2017; 59: 395-403.

The aim of this study was to investigate the enterotoxins and Panton-Valentine leukocidin (PVL) gene as virulence factor, identification of antimicrobial sensitivity patterns, *agr* (accessory gene regulator) types and sequence types and in resistant cases to obtain SCCmec (staphylococcal cassette chromosome mec) gene types which will be helpful to decide empirical therapy and future health politics for *S. aureus* species. Total of 150 isolates of *S. aureus* were isolated from the cultures of the child patients in January 2011 and December 2012. In this study, the penicillin resistance was observed as 93.8%. PVL and *mecA* was detected positive in 8.7% and in 6% of all *S. aureus* strains, respectively. Two MRSA (methicillin resistant *S.aureus*) strains were detected as SCCmec type III and SCCmec type V and five MRSA strains were detected as SCCmec type IV. SET-I and SET-G were the most common detected enterotoxins. In both community-associated and healthcare-associated MRSA strains, *agr* type 1 was detected most commonly. The most common sequence types were ST737 in 13 patients than ST22 in eight patients and ST121 in six patients. This study highlights a necessity to review the cause of small changes in the structural genes in order to determine whether it is a cause or outcome; community-acquired and healthcare associated strains overlap.

Key words: pediatric infections, pulsed field gel electrophoresis profile, *Staphylococcus aureus*, virulence factors.

S. aureus is an important pathogen causing both health-care associated (HCA) and community-acquired (CA) infections. Its prevalence is increasing particularly in pediatric intensive care units. Molecular typing data have revealed that there are a few methicillin resistant *Staphylococcus aureus* (MRSA) clones responsible for spreading MRSA infections in different regions of the world.¹

Methicillin resistance in staphylococci depends on the production of penicillin-binding protein (PBP)-2a, which has a low affinity to all beta-lactam antibiotics and can maintain cell wall synthesis in the presence of these antibiotics.

PBP-2a is encoded by a gene named *mecA* which is found on a mobile genetic element named the “staphylococcal cassette chromosome mec (SCCmec)”.^{1,2} Up to date, 11 different SCCmec types have been identified. Although HCA-MRSA strains more frequently carry the type-I, -II or -III and CA-MRSA isolates carry type-IV, -V or -VI SCCmec genes, this is not a rule.

Various virulence factors such as staphylococcal enterotoxins (ET), toxic shock syndrome toxin (TSST)-1, exfoliative toxins (ETA), and leukocidins including hemolysins and Panton-Valentine leukocidin (PVL) are responsible for the different clinical syndromes associated

with *S. aureus* infections. The expression of many virulence factors of *S. aureus* is controlled by accessory gene regulator (*agr*) locus encoding a two-component signaling pathway.³

It is quite difficult to determine the role of each staphylococcal virulence factor in the pathogenesis of invasive infections although it has been shown that PVL production is particularly associated with the development of furuncles, skin abscesses, and severe necrotic tissue infections.^{1,4}

In this study, we aimed to evaluate the antimicrobial susceptibilities, *agr* and *SCCmec* types and virulence factors of HCA- and CA-*S. aureus* strains isolated as etiological infectious agents in children, and to establish the multilocus sequence types (MLST) of representative strains showing different pulsed field gel electrophoresis (PFGE) profiles. With the data obtained, we also aimed to contribute to the molecular epidemiology of *S. aureus* strains of younger age group in our country, and guide the clinicians in the empirical treatment of coexisting infections.

Material and Methods

The study was conducted on 150 *S. aureus* strains isolated from pediatric in- or out-patients in the Department of Pediatrics between January 2011 and December 2012.

Patient groups: All strains were isolated from non-duplicate patients who were younger than 18 years. The patients were divided into community-acquired and healthcare-associated

infection groups according to the definition of CDC in 2000.⁵

Detection of antibiotic susceptibilities: Kirby-Bauer disc diffusion test was performed in line with the criteria of Clinical Laboratory Standards Institute (CLSI) to determine the antimicrobial susceptibilities of the isolates.⁶

Molecular analyses: DNA was extracted by using "GeneMATRIX Bacterial Genomic DNA Extraction Kit (EUR_x, Polland) according to the manufacturer's instructions. Presence of *mecA* and *PVL* genes were evaluated by PCR analyses as described previously.^{7, 8} *SCCmec* and *agr* types and enterotoxin genes of the isolates were determined by multiplex PCR methods as described in the literature.⁹⁻¹¹

Pulsed field gel electrophoresis (PFGE): Canadian PFGE protocol was used with minor modifications.¹² Lysostaphine concentration was doubled, and incubation period in Proteinase-K buffer was extended to two hours. The principles determined by Tenover et al.¹³ were adopted in the evaluation of genotypic relationship among isolates. DNA patterns and genetic relatedness of the isolates were analyzed by using the Gene Directory Programme (Syngene, UK). The similarity index was determined by using the Dice coefficient with 1.5% band tolerance. Cluster analysis of the isolates was performed by the unweighted pair-group method on the basis of arithmetic averages (UPGMA).

Multilocus sequence typing (MLST): Representative isolates of each pulsotype were randomly chosen for MLST analysis.¹⁴

Table I. Distribution of Isolates According to Their Sites of Infection.

Diagnosis	CA	HCA	Total
Skin and soft tissue infections, n (%)	45 (64.2)	32(40.0)	77 (51.3)
Eye and ear infections, n (%)	16 (22.8)	3 (3.7)	19 (12.6)
Bacteremia-sepsis-endocarditis, n (%)	4 (5.7)	14 (17.5)	18 (12.0)
Catheter-related infections, n (%)	-	17 (21.2)	17 (11.3)
Bone and joint infections, n (%)	2 (2.8)	3 (3.7)	5 (3.3)
Meningitis, n (%)	-	4 (5.0)	4 (2.6)
Urinary tract infections, n (%)	2 (2.8)	2 (2.5)	4 (2.6)
Ventilator-associated pneumonia, n (%)	-	2 (2.5)	2 (1.3)
Peritonitis, n (%)	-	2 (2.5)	2 (1.3)
Pneumonia and empyema, n (%)	1 (1.4)	1 (1.2)	2 (1.3)
Total, n	70	80	150

CA: community acquired, HCA: health-care associated

Table II. Antimicrobial Resistance Rates of *S. aureus* Strains in Community Acquired and Health-care Associated Infections.

Antimicrobial drug	CA (% resistance)		HCA (% resistance)		Total (% resistance)	
	I	R	I	R	I	R
Penicillin	-	91.2	-	96.2	-	93.8
Ampicillin-sulbactam	1.5	7.5		5.4	0.7	6.5
Cefoxitin	-	7.1	-	5.1	-	6
Erythromycin	10.1	7.2	1.3	6.7	5.5	6.9
Rifampicin	-	-	-	5.2	-	2.8
Clindamycin	8.8	5.8	-	5.3	4.1	5.5
Gentamycin/netilmicin	-	-	1.5	1.5	0.7	0.7
Ciprofloxacin	4.6	-	7.8	7.1	6.3	2.8
TMP-SMX	-	1.4	1.2	5	0.6	3.3
Tetracycline	1.5	1.5	-	12.3	0.7	7.2

CA: community acquired, HCA: health-care associated, I: intermediate, R: resistant, TMP-SMX: trimethoprim-sulphamethoxazole

Statistical analysis

Statistical analysis was performed at the Department of Biostatistics, by using the SPSS 17.0 statistical software. Descriptive statistics were demonstrated as mean for normally distributed variables, median for abnormally distributed variables, and the number of patients and percentage (%) for nominal values. The significance of the difference between the groups with regard to the means was investigated with Student's t-test and the significance of difference with regard to the median was analyzed with Mann-Whitney U test. A p value of <0.05 was accepted to be statistically significant.

The ethical approval was obtained from the Ethics Committee of Medical Faculty of University (13.06.2011/32-265). The written consent form was taken from all the patients.

Results

The study included a total of 150 patients, 54 in 2011 and 96 in 2012. Of the patients, 70 had CA-*S. aureus* infection and 80 had HCA-*S. aureus* infection; 58.7% of patients were male (n=88) and the median age was 19.5 months (3 days-18 years). The distribution of isolates according to their sites of infection are given in Table I, with skin and soft tissue infections being the most prevalent ones in both CA- and HCA- groups.

Antimicrobial resistance rates of the isolates

are given in Table II. In 6% (n=9) of the strains, *mecA* positivity was detected. All except one of the *mecA*-positive strains were phenotypically found to be methicillin-resistant. Only one phenotypically resistant strain was *mecA*-negative. Two of the *mecA*-positive MRSA strains was SCC*mec* type-III, two were SCC*mec* type-V and five were SCC*mec* type IV. Of the *mecA*-positive strains, four (44.4%) were CA-*S. aureus*.

PVL positivity was observed in 8.7% (n=13) of all *S. aureus* strains. The most commonly determined enterotoxin genes were of enterotoxins I and G. No enterotoxin gene was found in 62 of the *S. aureus* strains. The rest of the strains carried at least one enterotoxin gene. Thirty-nine (26%) of the isolates carried more than one enterotoxin gene. Enterotoxin genes, PVL-positivity, *mecA*-positivity, and *agr* types of CA- and HCA-*S. aureus* strains are shown in Table III.

Seventy-four different pulsotypes were determined by PFGE; 61 of these strains were divided into 26 groups which included at least 2 and at most 4 strains (Fig. 1).

Sequence types were determined for 89 *S. aureus* strains (Table IV). Twenty-nine different sequence types were detected in 50 CA- and in 39 HCA-*S. aureus* strains.

Discussion

Community acquired MRSA frequency has been

increasing and there is a closer relationship between CA-MRSA and invasive skin and soft tissue infections.^{15,16} In our study, the rate of MRSA was found to be 6% in the pediatric patient group. Of these strains, 2.6% were CA infections and 3.4% were HCA infections. While skin and soft tissue infections (abscess, impetigo, and omphalitis) and conjunctivitis were detected in patients with CA-MRSA, patients with HCA-MRSA had more serious clinical conditions such as endocarditis, sepsis, post-operative meningitis, surgical wound site infection, and urinary tract infection. The mean ages of patients with CA-MRSA and HCA-MRSA were 32 months and 85.6 months, respectively. According to the results of our study, MRSA does not seem to be a major problem for Turkey, at least in the pediatric

patients. However, it should be kept in mind that MRSA has a tendency to cause more serious and invasive infections.

The studies revealed that TMP-SMX is still a preferable antibiotic for empirical treatment of skin and soft tissue infections associated with MRSA strains in most regions of the world.¹⁷ While all of the CA-MRSA strains were susceptible to ciprofloxacin, rifampicin, erythromycin, and clindamycin, 25% were resistant to TMP-SMX in our study. In HCA-MRSA strains, resistance rates were found as 40% for rifampicin, 40% for TMP-SMX, 20% for erythromycin and 20% for clindamycin. Although it is a small study population, the detection of TMP-SMX resistance in 25% of patients with CA-MRSA infections suggests that empirical use of TMP-SMX in patients

Table III. Enterotoxins, PVL, *agr* Types, Positivity of *mecA* for Community Acquired and Health-care Associated *S. aureus* Strains.

Characteristic	MRSA [<i>mecA</i> (+)] (n=9)		MSSA [<i>mecA</i> (-)] (n=141)		Total (n=150)	
	CA (n=4)	HCA (n=5)	CA (n=66)	HCA (n=75)	CA (n=70)	HCA (n=80)
<i>agr</i> type 1	3	5	27	41	30	46
<i>agr</i> type 2	-	-	10	14	10	14
<i>agr</i> type 3	1	-	19	14	20	14
<i>agr</i> type 4	-	-	8	4	8	4
<i>agr</i> locus ND	-	-	2	2	2	2
SCC <i>mec</i> type I	-	-	-	-	-	-
SCC <i>mec</i> type II	-	-	-	-	-	-
SCC <i>mec</i> type III	-	2	-	-	-	2
SCC <i>mec</i> type IV	3	2	-	-	3	2
SCC <i>mec</i> type V	1	1	-	-	1	1
PVL	-	-	9	4	9	4
ET-A	-	2	1	4	1	6
ET-C	-	-	4	2	4	2
ET-E	-	-	1	5	1	5
ET-G	-	-	20	17	20	17
ET-I	1	2	36	38	37	40
ET-G + ET-I	1	1	14	13	15	14
ET-C + ET-G + ET-I	1	-	3	2	4	2
ET-A + ET-I	-	-	-	2	-	2
ET-E + ET-J	-	-	-	1	-	1
ET-A + ET-E + ET-I	-	-	-	1	-	1

agr: accessory gene regulator, CA: community acquired, ET : enterotoxin, HCA: health-care associated, ND: not detected, PVL: Panton-Valentine leucocidin, SCC: staphylococcal cassette chromosome, MRSA: methicillin resistant *Staphylococcus aureus*, MSSA: methicillin susceptible *Staphylococcus aureus*

with a suspect of MRSA infection will be inappropriate. Our study shows that the use of penicillinase-resistant beta-lactam antibiotics and clindamycin will be a more appropriate choice for empirical treatment of CA-methicillin sensitive *S. aureus* infections.

In studies conducted in the USA, PVL positivity was reported at a rate of 74-100% in MRSA strains and 9-46% in MSSA strains depending on the regions where data were collected.¹⁸

Furthermore, a relationship was found between SCCmec types of MRSA strains and PVL positivity. While PVL positivity was below 5% in MRSA strains having SCCmec type- I-III, it was between 40% and 90% in MRSA strains having SCCmec type-IV.¹⁹ The most common sequence types determined in PVL positive CA-MRSA strains were ST8 in the USA, ST80 in Europe, ST30 and ST59 in the Asian-Pacific area.²⁰⁻²³

Table IV. The Detailed Distribution of the Types of Sequences of *S. aureus* Strains According to Groups.

Sequence types (ST)	CA		HCA		Total	PVL	mecA
	MSSA	MRSA	MSSA	MRSA			
ST5	3	-	2	-	5	-	-
ST6	1	-	1	-	2	-	-
ST7	1	-	1	-	2	-	-
ST8	3	-	1	-	4	-	-
ST9	1	-	2	-	3	-	-
ST10	1	-	-	-	1	-	-
ST12	1	-	-	-	1	-	-
ST15	3	-	4	-	7	2	-
ST22	5	1	2	-	8	2	1
ST25	2	-	-	-	2	-	-
ST27	1	-	-	-	1	-	-
ST30	5	-	1	-	6	2	-
ST34	-	-	3	-	3	1	-
ST45	-	-	2	-	2	-	-
ST46	2	-	-	-	2	-	-
ST88	-	-	1	-	1	1	-
ST97	-	-	4	-	4	-	-
ST121	4	-	2	-	6	3	-
ST188	1	-	2	-	3	-	-
ST199	-	-	1	-	1	-	-
ST239	-	-	1	2	3	-	2
ST291	2	-	-	-	2	-	-
ST398	1	-	-	-	1	-	-
ST489	1	-	-	-	1	-	-
ST509	-	1	1	-	2	-	1
ST737	5	2	5	1	13	-	3
ST837	1	-	-	-	1	-	-
ST1021	1	-	-	-	1	-	-
ST1708	1	-	-	-	1	1	-
Total	46	4	36	3	89	12	7

Data is presented in numbers. CA: community acquired, HCA: health-care associated, PVL:panton-valentine leucocidin, MRSA: methicillin resistant Staphylococcus aureus, MSSA: methicillin susceptible Staphylococcus aureus

PVL positivity is not so common in our country as in most European countries. In a study performed in Turkey, 242 *S. aureus* strains isolated from skin and soft tissue infections were evaluated for the presence of PVL genes, and PVL positivity was detected in 9.1% of the strains, all of which were MSSA; 63.6% of the PVL-positive strains were isolated from CA- and 36.4% were from HCA infections. PVL positivity was not detected in any of the MRSA strains.²⁴ In another study conducted in Turkey, PVL-positivity was reported to be <10% for MSSA and <3% for MRSA.²⁵ In our study, PVL positivity was found in 8.7% of the strains, all of which were MSSA. PVL-positive *S. aureus* was found in 69.2% of CA-MSSA strains and in 30.8% of HCA-MSSA strains. Of the PVL producing strains, 77% were isolated from patients with skin and soft tissue infections, 7.7% from bone and joint infections, 7.7% from patients diagnosed with pneumonia-empyema, and 7.7% from patients with catheter-related infection. Of the MRSA strains, seven were SCCmec types-IV and -V. None of them carried the PVL gene. PVL negativity particularly in the CA-MRSA strains can be explained with their sequence types (ST737, ST509 and ST22). As in other studies, a stronger relationship was found between PVL positivity and skin and soft tissue infections in our study.²⁶

When *mecA* positivity is regarded as the gold-standard, the sensitivity and specificity of oxacillin/cefoxitin disc diffusion method for determining methicillin resistance have been reported to be 100% and 89%, respectively.²⁷ The rates of sensitivity and specificity were found to be 88.8% and 99.2% for oxacillin/cefoxitin disc diffusion method in our study. One, *mecA*-negative strain was phenotypically methicillin-resistant, and one *mecA*-positive strain was phenotypically MSSA. Misidentification of one *mecA*-positive strains as MSSA might have resulted from heterogeneous appearance of methicillin resistance, or technical problems such as the use of inappropriately stored beta-lactam antibiotic disks, incubation conditions, and amount of inoculum. The determination of *mecA*-negative strain as MRSA may be due to point mutations in penicillin binding proteins, excess production of penicillinase, or a decrease in the binding affinity of methicillin.²⁸

In a study carried out in Taiwan, 91.9% of the

isolates were *agr* type-1, 7.8% type-2, 0.2% type-3, and 0.1% type 4.²⁹ In another study conducted in England, 26% of HCA-MSSA isolates represented *agr* group 2.³⁰ In another study, while *agr* type 3 was observed to be associated with non-invasive diseases, *agr* type 1 was associated with invasive diseases such as bacteremia.³¹ In our study, 50.7% were found to be *agr* type 1, 16% type 2, 22.7% type 3, and 8% type 4. The most common *agr* type in CA and HCA infections was type 1 (42.9% and 57.5%, respectively). While *agr* types 1 and 2 were most frequently detected in HCA-infections, *agr* types 3 and 4 were more prevalent in community acquired infections ($p>0.05$). In MRSA strains, *agr* types 1 and 3 were detected in 88.8% and 12.2% of the strains. *agr* type 1 was more prominent in strains isolated from invasive infections such as meningitis and bacterial endocarditis and in bone-joint infections, compared to other types. On the other hand, *agr* type 4 was more frequently found in skin and soft tissue infections. Considering infection types and antibiotic susceptibility profiles, it is seen that these data are generally similar to the literature all over the world.

In a study that was performed in Germany, 25.6% of the *S. aureus* strains were found to be negative for enterotoxin genes. Enterotoxins with at least two and more combinations were observed in 63.9% of strains and single enterotoxin gene was found at in 10.5% of the strains.³² In another study, enterotoxins G and I were more frequently detected and a statistically significant difference was found between MRSA and MSSA strains (77% for MRSA and 49% for MSSA). The coexistence of enterotoxins G and I, as well as D and J, was also remarkable (17% for MRSA, 8% for MSSA) in another study.¹¹ In our study, enterotoxins B, D, H and TSST-1 were not detected in any of the strains. No enterotoxin was found in 62 isolates (41.3%). At least one, at most three coexistences of enterotoxin genes were observed. Enterotoxin I presence was most frequent (51.3%), followed by G (24.6%), A (4.6%), C (4%), E (4%), and J (0.66%).

The results of PFGE typing in our study, grouped the strains in 74 different pulsotypes. Detection of so many different pulsotypes may be due to the long study time of two years,

no occurrence of an outbreak in our hospital during this period, collection of the samples from different clinics, and the higher number of patients with community acquired infections.

There is not much data about molecular characterization of CA-MRSA strains in our country, especially in the pediatric population. This is the first study covering the detection of enterotoxin and PVL genes, as well as SCC_{mec} types, *agr* types, and PFGE profiles with MLST analysis in the pediatric patient group in Turkey. In a study which evaluated nine possibly HA-MRSA strains in Turkey between 2003 and 2004, MLST analysis was performed and their sequence types were found to be ST239.³³ In our study, MLST analysis was performed in 100 strains. Based on the allelic profiles, sequence types could be determined in 89 strains. 29 different sequence types were found and the most common ones were ST737 (14,6 %), ST22 (8,9 %), ST15 (7,8 %), ST30 (6,7 %), ST121 (6,7 %), and ST5 (5,6 %). While ST737, ST22, ST30, and ST121 were the most common types in CA-strains, ST737, ST97, ST15, ST239, and ST34 were most prevalent in HCA-strains. There are not many reports on ST737 sequence type, which has one-allele difference with ST22. The frequency of ST737 strains was found to be 4% in *S. aureus* strains collected from different regions of our country between 2005 and 2007³⁴, it was 14.6% in our study. Rapid spread of this strain both in the community and hospital environment is noteworthy. To investigate whether the pathogenicity of ST737 strain is different from others or not will reveal significant results in terms of preventive public health measures and empirical treatment. It was interesting to observe the presence of various sequence types within the same pulsotype in our study, which may reflect that even in the same PFGE group, different clones may exist (Fig. 1).

In conclusion, our study has provided important epidemiological data on *S. aureus* strains isolated from pediatric patients in Turkey. Although CA-*S. aureus* infections were not a major problem in our study population; considering the process followed in different regions of the world, we assume that CA-infections would increase over time. PVL-positivity is still more prevalent among MSSA strains, although gaining the *mecA* gene among these populations may lead

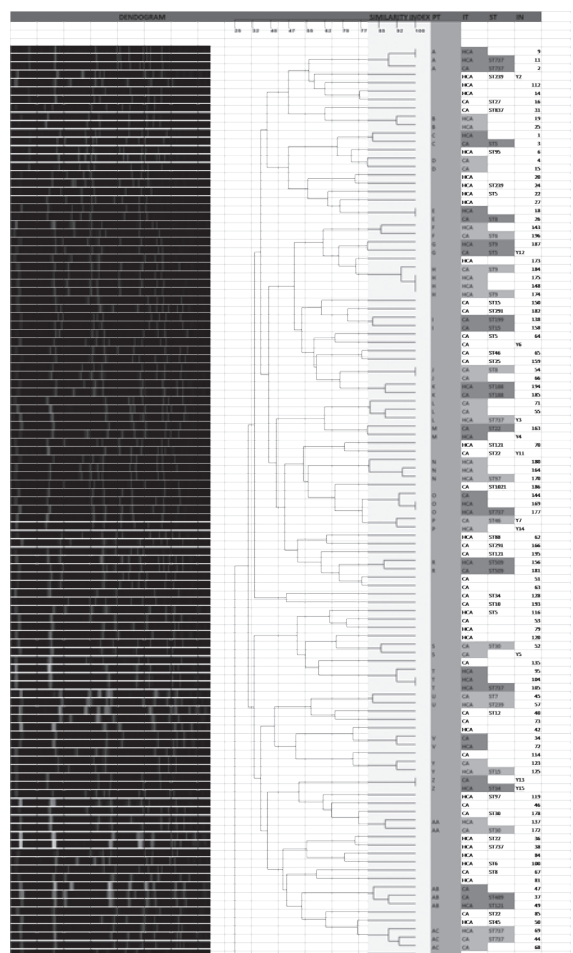


Fig. 1. Pulsed field gel electrophoresis (PFGE) pulsotypes and corresponding sequence types in *S. aureus* strains CA: community acquired, HCA: health-care associated, IN: isolate numbers, IT: infection types, PT: pulsotypes, ST: sequence types

to an increase in PVL-positive MRSA infections with higher morbidity and even mortality. Penicillinase-resistant beta-lactam antibiotics could still be used as the first choice in empirical treatment of pediatric patients with *S. aureus* infections, if they are not methicillin-resistant. The rate of PVL positivity in Turkey was not found as high as other countries. When sequence types of both community and hospital acquired *S. aureus* strains were evaluated, the presence of interwoven strains was found to be consistent with the tendency recently reported across the world.

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