Original paper

Molecular Analysis of Community-Acquired Staphylococcus aureus strains isolated from Skin and Soft-Tissue Infections in Romania

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Abstract

The aim of this study was to characterise S. aureus strains from community onset Skin and Soft Tissues Infections (SSTIs) in two locations: Cantacuzino Institute (A strains) and Elias University Emergency Hospital (B strains), in the January 2014 – August 2015 interval. All strains from the A location, and three strains from the B location have been isolated from recurrent staphylococcal infections.

Materials. Seventy-one S. aureus strains (A - 42; B - 29) have been collected from different types of SSTIs. Methods: PCR was used to identify virulence factors and AMR genes, disc diffusion and broth microdilution for AST, SCCmec and spa typing.

Results and Discussions. MRSA rate of 59.52% and 17.24% among A and B strains, respectively. Twenty of A strains and one of B strains were positive for lukS/F-PV genes; two A strains and four B strains were positive for tst1 gene. Panton-Valentine Leukocidin was present in all t008 and t044 strains. Four strains from A location and seven strains from B location were positive for Staphylococcal Enterotoxins. Three new spa-types were discovered. Conclusions. The most prevalent S. aureus clone in community onset SSTIs was spa type t127, followed by spa types t044, t008. Molecular characterisation of S. aureus strains may predict the tendency to recurrence of staphylococcal SSTIs.

Keywords

CA-Staphylococcus aureus, methicillin-resistant, Panton-Valentine Leukocidin, tst1 gene, SEs genes.

Introduction

S. aureus can cause a wide spectrum of diseases attributable to the range of virulence determinants it is able to express: the production of specialized binding proteins, immune evasion molecules and immune cell targeting toxins (FOSTER [1]). The human skin, nose, throat or infected blood and the environment (surfaces, foods) can be reservoirs of susceptible or resistant to antibiotics and high virulent S. aureus strains.

Skin and soft tissue infections (SSTIs) are the most frequent forms of the disease. S. aureus is the quasi universal cause of furuncles, carbuncles and skin abscesses. Through gene acquisition and alterations in regulatory networks, S. aureus is able to counter the physical and immunological barriers of the skin. S. aureus SSTIs frequently begin as minor infectious diseases, such as boils or abscesses and may progress to severe infections involving muscle or bone and may disseminate via bloodstream to the lungs or heart valves.

S. aureus strains resistant to penicillinase-resistant β-lactam antimicrobial drugs, termed methicillin-resistant S. aureus (MRSA), were recognized from the 1960s until the 1990s as healthcare associated pathogens. Community associated strains of MRSA appeared late, in the early 1990s (GORDON and LOWY [2]). Currently, MRSA is considered the most common bacterial pathogen isolated in many parts of the world and according to the sources and ways of transmission it is divided into several categories: healthcare-associated MRSA (HCA-MRSA) (GRUNDMANN & al [3]), but also any other type of infection associated with medical care giving, while community-associated MRSA or community-acquired MRSA (CA-MRSA) is considered when there is not known evidence of host contact with a health-care institution. On the other hand, livestock-associated MRSA (LA-MRSA) may colonize people coming into close contact with animal farms (SMITH [4]). MRSA have become a significant public health problem with economic consequences worldwide (GOETGHEBEUR & al [5]).

Epidemiologically, the definition of CA-MRSA is problematic. Most studies have used a timebased definition (e.g., infections recognized within 24-72 h after hospital admission) (SALGADO & al [6]). CA-MRSA may occur when an individual with a skin lesion is in contact with others who are carrying the bacteria. This can occur with direct skin contact with infected individuals (MORAN & al [7]), colonized subjects (ELLIS & al [8]), sharing infected devices (such as razors) and public places that are not cleaned properly (BEGIER & al [9]), such as the gyms.

HA-MRSA can be transmitted during surgery, by medical devices (such as catheters) and direct skin contact without proper hand hygiene from hospital staff or visitors. However, S. aureus can persist as a colonizer for months or years (SCANVIC & al [10]), leading to misclassification of the source. Some community-acquired infections may in fact be caused by hospital-acquired strains and vice versa (SALGADO & al [6]; SCANVIC & al [10]). CA-MRSA are often isolated from hospital environments (GONZALEZ & al [11]). Thus, the distinction between CA-MRSA, HCA-MRSA and HA-MRSA is still unclear (WEBER [12]; CAMPBELL & al [13]).

Many of the community-acquired strains produce exotoxins and are epidemiologically distinct from healthcare-acquired strains (DRYDEN [14]). The presence of type IV SCCmec and Panton-Valentine Leukocidin (PVL) have been useful molecular markers of CA-MRSA strains (CARLETON & al [15]).

In the United States, a single clone of CA-MRSA (ST8/USA300) has become the most prevalent cause of staphylococcal SSTIs acquired in the community (TENOVER & al [16]), which then proved to be able to disseminate on other continents (LARSEN & al [17]) and has moved into other settings, causing not only SSTIs, but also invasive diseases (GONZALEZ & al [11]; KLEVENS & al [18]).

Among multiple virulence determinants, some S. aureus strains secrete several exotoxins directly associated with particular disease symptoms. These include Panton-Valentine Leukocidin (PVL), Toxic Shock Syndrome Toxin 1 (TSST1), Staphylococcal Enterotoxins (SEs), Exfoliative Toxins (ETs). Antibiotics resistance, particularly resistance to methicillin, can be also a predictive factor for the outcome of the disease (CAMERON & al [19]).

PVL is one of the four pore forming bi-component toxins that may be expressed by S. aureus strains. It can lyse human cells expressing C5a receptors, including neutrophils (polymorphonuclear cells) of the host (SPAAN & al [20]). PVL has been mainly associated with CA-MRSA strains (TRISTAN & al [21]; VANDENESCH & al [22]), but some studies suggest a more common association with community-acquired methicillin-sensitive S. aureus (CA-MSSA) strains (SHALLCROSS & al [23]). PVL-producing CA-MRSA caused suppurative SSTIs, severe necrotising community-acquired pneumonia (CAP) (LINA & al [24]; KREIENBUEHL & al [25]), bone and joint infections. The effects of antimicrobial agents on S. aureus PVL-producing strains has been studied by Dumitrescu & al (26) who demonstrated an augmentation of toxin production, especially in the presence of β-lactam antibiotics. The discovery of PVL, encoded by lukSF-PV genes, was reported in the 1930s (PANTON and VALENTINE [27]). PVL has not been identified in typical epidemic or endemic HA-MRSA strains (LINDSAY and HOLDEN [28]).
Aproximately 5–25% of *S. aureus* strains are TSST1 producing. TSST1, encoded by *tst1* gene, is a superantigen that acts on host lymph cells triggering vascular reaction with inflammation, fever and shock (SHANDS & al [29]). TSST1 is a bacterial exotoxin found in patients who have developed toxic shock syndrome (TSS). In general, the toxin is not produced by bacteria growing in the blood, rather it is produced at the local site of an infection, i.e. mucosal (vaginal, nasopharyngeal, tracheal) or sequestered (empyema, abcess) sites, and then enters the blood stream (BERGDOLL & al [30]).

Some *S. aureus* strains are able to produce Staphylococcal Enterotoxins (SEs) (BERGDOLL & al [31]) and are the causative agents of Staphylococcal Food Poisonings (SFPs). Several SEs have been identified, but the most common cause of SFPs worldwide is SEA, a highly heat-stable SE (KADARIYA & al [32]). SEs found in food and drink can trigger molecular signals at the intestinal wall level and cause local or general disease. The symptoms of SFPs are abdominal cramps, nausea, vomiting, sometimes followed by diarrhea (never diarrhea alone). The onset of the symptoms is rapid (from 30 min. to 8 h after SEs ingestion) and usually spontaneous remission is observed after 24 h. Genes encoding SEs have different genetic supports, most of which are mobile genetic elements. For example, *sea* is carried by a family of temperate phages (BETLEY and MEKALANOS [33]), *seB* is chromosomally located in some clinical isolates (SFAFER and IANDOLO [34]), whereas it has been found in a 750-kb plasmid in other *S. aureus* strains (SHALITA & al [35]). *seChoNine* is encoded by a gene located on a pathogenicity island (FITZGERALD & al [36]), *seD* is carried by a plasmid (pBl485) (BAYLES and IANDOLO [37]) and *seE* is carried by a defective phage (COUCH & al [38]).

Exfoliative toxins (ETs) are serine proteases with high substrate specificity responsible for staphylococcal scalded skin syndrome (SSSS), a disease predominantly affecting infants and characterized by the loss of superficial skin layers, dehydration and secondary infections (BUKOWSKI & al [39]).

It is important for clinicians to be familiar with the incidence of CA-MRSA in their communities, as prevalence can vary from region to region (MCDONALD & al [40]).

In this article we aimed to investigate the virulence markers and resistance profiles to antibiotics of *S. aureus* strains implicated in SSTIs, isolated from two different locations in Bucharest, during one year and a half (A strains and B strains) and to evaluate strains by comparing SCCmec and *spa* molecular types.

All strains from the A location, and only 3 strains from the B location have been isolated from recurrent staphylococcal infections.

### Materials and Methods

#### Patients and Bacterial isolates

A total of 71 patients with acute and chronic SSTIs who were referred for medical care and/or laboratory investigations to locations A and B (A – 42; B – 29) were enrolled in this study. All 71 *S. aureus* isolates were collected from these patients during one year and a half in the January 2014 – August 2015 interval, from different SSTIs: superficial pustular dermatitis, acne, folliculitis, furunculosis, abcess, cellulitis, paronychia, hidradenitis or open wound. All patients from the A location have been referred for recurrent staphylococcal infections, while from B location 8 patients were hospitalized within 1-9 months before harvest sample. All the patients were outpatients by the time of SSTIs sampling.

All isolates selected in this study have been identified by a combination of phenotypic and genotypic tests: bound and free coagulase, PCR for the *nuc* gene detection. Community-acquired *S. aureus* (CA-SA) was defined as a *S. aureus* strain isolated from an outpatient or within 2 days since patient’s hospital admission.

#### Antimicrobial susceptibility testing

Antimicrobial susceptibility profiles of *S. aureus* isolates were determined by the Kirby-Bauer disk diffusion method, according to EUCAST 2014 ([41]) and 2015 guidelines ([42]). Antibiotics tested included benzylpenicillin (P, 1 unit), cefoxitin (FOX, 30 μg), erythromycin (E, 15 μg), clindamycin (DA, 2 μg), kanamycin (K, 30 μg), tobramycin (TOB, 10 μg), gentamicin (CN, 10 μg), ciprofloxacin (CIP, 5 μg), tetracycline (TE, 30 μg), rifampicin (RD, 5 μg), sulfamethoxazole-trimetoprim (SXT, 25 μg), quinupristin-dalfopristin (QD, 15 μg), linezolid (LZD, 10 μg), mupirocin (MUP, 200 μg) and fusidic acid (FD, 10 μg). The minimum inhibitory concentration (MIC) of vancomycin was detected by microdilutions in Mueller-Hinton broth. Inducible clindamycin resistance was demonstrated by the D-test. *S. aureus* ATCC 29213 was used as quality control for both the disk diffusion test and MIC detection.

#### DNA extraction

Total genomic DNA was obtained by use of the thermal and enzymatic lysis method (lysostaphin 1 U/μL, proteinase K 2 mg/mL, 5 min. at 100°C).

#### Detection of virulence and resistance genes by PCR

A variety of significant genes in respect of their implication in *Staphylococcus aureus* pathogenicity were identified by PCR, including *nuc* (encoding thermonuclease); *lukS/F-PV* (encoding PVL) and *mecA* (encoding...
resistance to methicillin) detected simultaneously by a triplex PCR protocol optimized in our laboratory (DRĂGULESCU & al [43]): \textit{tsl} (encoding toxic shock syndrome toxin 1); \textit{eta} and \textit{etb} (encoding exfoliative toxin A and B); \textit{seA-E} (encoding enterotoxins A-E implicated in food-borne diseases).

For toxic shock syndrome toxin 1 (\textit{tsl} gene), the exfoliative toxins (\textit{eta, etb} genes) and enterotoxins A-E (\textit{seA-E} genes), simplex PCRs were performed, with 0.4 μM of each specific primer (Invitrogen) and 1.25U of Taq DNA polymerase (Promega Corporation, Madison, WI, USA). The primers used are listed in Table 1 and the amplification programs are listed in Table 2.

### Detection of antimicrobial resistance genes by PCR

Besides \textit{mecA} (see previous chapter), antimicrobial resistance genes detected by PCR were \textit{bla\textsubscript{Z}} (encoding benzylpenicillin resistance) (MARTINEAU & al [44]); \textit{erm(A)} and \textit{erm(C)} (encoding erythromycin and clindamycin resistance); \textit{aacA-aphD} (encoding gentamycin resistance); \textit{tet(K)} and \textit{tet(M)} (encoding tetracycline resistance) (STROMMENGER & al [45]).

For each simplex PCR reactions, we used 0.4 μM of each specific primer (Invitrogen) and 1.25U of Taq DNA polymerase (Promega Corporation, Madison, WI, USA). The list of the primers is presented in Table 1 and the amplification programs are listed in Table 2.

<table>
<thead>
<tr>
<th>Table 1. PCR primers used in triplex and simplex PCRs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triplex</strong></td>
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<td>Primer</td>
</tr>
<tr>
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</tr>
<tr>
<td>nucF</td>
</tr>
<tr>
<td>nucR</td>
</tr>
<tr>
<td>luk-PV-1</td>
</tr>
<tr>
<td>luk-PV-2</td>
</tr>
<tr>
<td>mecA-1</td>
</tr>
<tr>
<td>Simplex</td>
</tr>
<tr>
<td>GETAR-1</td>
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</tr>
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<td>TSST2</td>
</tr>
<tr>
<td>SEA1</td>
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<tr>
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<tr>
<td>SEE2</td>
</tr>
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</tr>
<tr>
<td>ermA</td>
</tr>
<tr>
<td>ermC1</td>
</tr>
<tr>
<td>ermC2</td>
</tr>
<tr>
<td>aacA-</td>
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<tr>
<td>aphD1</td>
</tr>
<tr>
<td>aacA-</td>
</tr>
<tr>
<td>aphD2</td>
</tr>
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</table>

**643**
Table 2. Cycling conditions for triplex and simplex PCRs

<table>
<thead>
<tr>
<th>PCR method</th>
<th>Initial denaturation</th>
<th>Cycling conditions</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Denaturation</td>
<td>Annealing</td>
</tr>
<tr>
<td>Triplex PCR</td>
<td>95°C 4 min</td>
<td>94°C 1 min 1 min</td>
<td>55°C 1 min 1 min 30 sec</td>
</tr>
<tr>
<td>Simplex PCR</td>
<td>94°C 5 min</td>
<td>94°C 2 min 2 min</td>
<td>55°C 2 min 1 min</td>
</tr>
<tr>
<td>Simplex PCR</td>
<td>94°C 5 min</td>
<td>94°C 2 min 2 min</td>
<td>57°C 2 min 1 min</td>
</tr>
<tr>
<td>Simplex PCR</td>
<td>94°C 5 min</td>
<td>94°C 2 min 2 min</td>
<td>55°C 1 min 1 min 30 sec</td>
</tr>
</tbody>
</table>

The following reference and Laboratory collection strains were used as controls: 33/2009 strain (Nosocomial Infections Laboratory collection) for nuc, lukS/F-PV, mecA genes, IC13456 strain (for eta gene), IC13455 strain (for etb gene), IC13454 strain (for tst1 gene), S. aureus National Collection of Type Cultures, U.K. (NCTC) 10652 (seA positive), S. aureus NCTC 10654 (seB positive), S. aureus NCTC 10655 (seC positive), S. aureus NCTC 10656 (seD positive), S. aureus Food Research Institute, U.S.A. (FRI) 326 (seE positive), 5681/2010 strain (Nosocomial Infections Laboratory collection) for blaZ, erm(C), aacA-aphD, tet(K) genes, 39/2008 strain (Nosocomial Infections Laboratory collection) for erm(A), tet(M) genes.

The PCR products were resolved by agarose (1.5% or 2%) gel electrophoresis, stained with ethidium bromide and visualized in an UV transilluminator.

**SCCmec typing** was performed by multiplex PCR using a scheme proposed by MILHEIRICO & al ([49]). The following reference strains were used as controls: COL (SCCmec type I), BK2464 (SCCmec type II), ANS46 (SCCmec type III), MW2 (SCCmec type IVa), WIS (SCCmec type V), HDE288 (SCCmec type VI).

**Spa-typing**

Spa-typing is a molecular typing method used to identify clones assets. Detection of polymorphism in the X region of the staphylococcal protein A (spa) gene was carried out as described by HARMSEN & al ([50]) using the forward primer spa-1113f: 5’TAAAGACGATCCTTCGGTGAGC3’ and the reverse primer spa-1514r: 5’CAGCAGTAGTGC CGTTTGCTT3’. PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA). PCR products of the spa gene were sequenced using the same PCR primers and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) as recommended by manufacturer, on an Applied BioSystems ABI PRISM 3130 Genetic Analyzer. Spa sequences were analyzed using Ridom StaphType software version 2.2.1 (Ridom GmbH, Wurzburg, Germany).

All the PCR reactions were performed either in an Applied Biosystems or in a Corbett Research Thermocyclers.


Results and Discussions

The median age of patients from the A location was 32.5 years and from the B location was 50 years (range: A: 4-76 years; B: 4 month – 88 years), while the sex distribution (male / female) was 26 / 16 (61.90% / 38.10%) for A location patients and 10 / 19 (34.48% / 65.52%) for B location patients.

Distribution of antimicrobial resistance phenotypes of A and B strains are illustrated in Figures 1 and 2.

Figure 1. Distribution of antimicrobial resistance phenotypes of A strains.

Figure 2. Distribution of antimicrobial resistance phenotypes of B strains.

59.52% (n = 25) of the A strains were found to be MRSA, while only 17.24% (n = 5) B strains were MRSA. Most of the A (95.24%) and B (93.10%) strains were resistant to P. The MLSBi phenotype was detected in 40.48% (n = 17) of A strains and 44.83% (n = 13) of B strains. Strains isolated in the A location were also resistant to: E (21-50%), DA (17-40.48%) – inducible resistance and DA (1-2.38%) – constitutive resistance, K (22-52.38%), TE (17-40.48%), QD (17-40.48%) – reported resistant according to the MLSBi phenotype, CIP (2-4.76%), FD (3-7.14%). Antimicrobial susceptibility testing of B strains revealed the following resistance rates: E (13-44.83%) and E intermediate (1-3.45%), DA (13-44.83%) – inducible resistance and DA intermediate (3-10.34%) – constitutive resistance, K (14-48.28%), TOB (5-17.24%), CN (4-13.79%), TE (13-44.83%) and TE intermediate (2 – 6.90%), CIP (2-6.90%), RD (1-3.45%) and RD intermediate (9-31.03%), QD (13-44.83%) – reported resistant according to the
MLSBi phenotype and QD intermediate (3-10.34%), FD (5-17.24%). No Vancomycin resistant isolates have been found.

The genetic analyses confirmed the presence of mecA gene in MRSA strains, included in SCCmec cassettes type IV (9 A strains) or type IVE (15 A strains and 5 B strains). A strain from the A location could not be included in any SCCmec cassette type by the using the Milheirico method. The presence of the blaZ gene was detected in all P resistant strains from the A and B locations. 40.48% (n = 17) of A strains with phenotypic resistance to TE presented the tet(K) gene, 41.38% (n = 12) of the B strains were positive for the tet(K) gene and one B strain (3.45%) was positive for the tet(M) gene. Two B strains (6.90%) with intermediate resistance to TE did not express neither the tet(K) or the tet(M) genes. The presence of the erm(C) gene in the case of certain strains was positively correlated with the E and MLSBi resistance phenotype in both A and B strains. Three A strains (7.14%) with phenotypic resistance to E and one strain (2.38%) with phenotypic resistance to E and DA were negative for erm(A)/erm(C) genes. One B strain (3.45%) with intermediate phenotypic resistance to E and three strains (10.34%) with intermediate phenotypic resistance to DA were negative for erm(A)/erm(C) genes. Two strains (6.90%) from B location, with phenotypic resistance to CN and TOB, were positive for aacA-aphD genes and 2 strains (6.90%) were PCR negative, probably due to a different resistance mechanisms.

Figures 3 and 4 show the distribution of virulence factors genes in the A and B strains. All the A and B strains were positive for the nuc gene. The lukS/F-PV genes were found in 47.62% (n = 20) isolates from the A location and only in 3.45% (n = 1) isolates from the B location; 4.76% (n = 2) strains from the A location and 13.79% (n = 4) strains from the B location were positive for tst1 gene. From the A location 2.38% of the strains (n = 1) were positive for seB gene and 7.14% (n = 1) were positive for seC gene. From the B location 10.34% (n = 3) were positive for seA gene and 13.79% (n = 4) were positive for seC gene.
**Spa-typing**

The prevalent spa type identified in strains from the A location was t127 (30.95%, n = 13), followed by t044 (11.90%, n = 5), t008 (7.14%, n = 3), t284 (7.14%, n = 3), t019 (4.76%, n = 2), t2881 (4.76%, n = 2) and other 14 spa types with 2.38% (one strain each: t005, t091, t174, t223, t280, t355, t435, t437, t948, t1211, t1889, t5841, t14512 and t14513). Spa type t127 was also the predominant type in the B location (44.83%, n = 13), along with types t005 (6.90%, n = 2), t021 (6.90%, n = 2) and other 12 spa types with 3.45% (one strain each: t002, t012, t015, t053, t084, t559, t582, t620, t701, t728, t7585, t15296). The genes responsible for the PVL production were detected in spa types t008 and t044 strains, but also in other spa types: t019, t284, t355, t435, t437, t1211, t1889, t5841, t14513.

In the A strains 12 of the 13 t127 spa type strains were MRSA, while in the B strains only 4 of the 13 t127 spa type strains were MRSA. In the A location other 5 strains with spa types t019, t174, t437, t948, t2881 were MRSA and in the B location a t559 spa type strain was MRSA. In the A location t223, t1889 and in the B location t012, t021 (n = 2), t582 strains were positive for tst1 gene. In the A location one strain with spa type t437 was positive for seB gene, while 3 strains with spa types t1889 (n = 1) and t2881 (n = 2) presented the seC gene. In the B location 3 strains with spa types t053, t127, t701 were positive for seA gene and 4 strains with spa types t015, t582, t620, t728 were positive for seC gene. The distribution of spa types in the A and B locations are presented in figures 5 and 6.

![DISTRIBUTION OF SPA TYPES IN A STRAINS](image)

**Figure 5.** Distribution of spa types in A strains.

![DISTRIBUTION OF SPA TYPES IN B STRAINS](image)

**Figure 6.** Distribution of spa types in B strains.

The spa types t14512, t14513 (PVL positive strain) and t15296 were discovered for the first time during this study. The new spa-types were assigned through EU Ridom SpaServer database ([51]). Also, all the spa-types identified in this study were uploaded in the same database, except one strain’s sequence which was not accepted because of a point mutation in the 3’ signature changing the original structure 3’TAYATGTCGT into 3’TAYATATCGT.

The correlations between patterns of antibiotics resistance, spa types and PVL-positive MSSA and MRSA strains are presented in tables 3 and 4.
Table 3. The correlations between patterns of antibiotics resistance, \(spa\) types and PVL-positive MSSA and MRSA strains in the A location

<table>
<thead>
<tr>
<th>Patterns of antibiotics resistance</th>
<th>No. of strains</th>
<th>MSSA/ (spa) types/ PVL +/- (no. of strains)</th>
<th>MRSA/ (spa) types/ PVL +/- (no. of strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>2</td>
<td>t5841 +, t044 +</td>
<td>-</td>
</tr>
<tr>
<td>P</td>
<td>14</td>
<td>t005 -, t091 -, t223 -, t280 - t284 + (3), t355 +, t435 +, t1211 +, t1889 +, t2881 - t14512 +, t14513 +</td>
<td>-</td>
</tr>
<tr>
<td>P, K</td>
<td>1</td>
<td>t127 -</td>
<td>-</td>
</tr>
<tr>
<td>P, FOX</td>
<td>2</td>
<td>-</td>
<td>t019 + (2)</td>
</tr>
<tr>
<td>P, FOX, E</td>
<td>1</td>
<td>-</td>
<td>t008 +</td>
</tr>
<tr>
<td>P, FOX, E, K</td>
<td>1</td>
<td>-</td>
<td>t008 +</td>
</tr>
<tr>
<td>P, FOX, E, K, CIP</td>
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</tr>
<tr>
<td>P, FOX, K, TE, FD</td>
<td>1</td>
<td>-</td>
<td>t044 +</td>
</tr>
<tr>
<td>P, FOX, E, DA, K, CIP, TE</td>
<td>1</td>
<td>-</td>
<td>t437 +</td>
</tr>
<tr>
<td>P, FOX, E, DA (MLSBi), K, QD</td>
<td>3</td>
<td>-</td>
<td>t127 - (2), t2881 -</td>
</tr>
<tr>
<td>P, FOX, E, DA (MLSBi), K, TE, QD</td>
<td>11</td>
<td>-</td>
<td>t127 - (9), t174 -, t948 -</td>
</tr>
<tr>
<td>P, FOX, E, DA (MLSBi), TE, QD</td>
<td>1</td>
<td>-</td>
<td>t127 -</td>
</tr>
<tr>
<td>P, FOX, E, DA (MLSBi), K, TE, QD, FD</td>
<td>2</td>
<td>-</td>
<td>t044 + (2)</td>
</tr>
</tbody>
</table>

42 strains

Table 4. The correlations between patterns of antibiotics resistance, \(spa\) types and PVL-positive MSSA and MRSA strains in the B location

<table>
<thead>
<tr>
<th>Patterns of antibiotics resistance</th>
<th>No. of strains</th>
<th>MSSA/ (spa) types/ PVL +/- (no. of strains)</th>
<th>MRSA/ (spa) types/ PVL +/- (no. of strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1</td>
<td>t002 -</td>
<td>-</td>
</tr>
<tr>
<td>P</td>
<td>3</td>
<td>t701 -, t728 -, t7585 -</td>
<td>-</td>
</tr>
<tr>
<td>P, DA(I)*</td>
<td>2</td>
<td>t005 -, t127 -</td>
<td>-</td>
</tr>
<tr>
<td>P, TE</td>
<td>1</td>
<td>t084 -</td>
<td>-</td>
</tr>
<tr>
<td>P, RD(I)</td>
<td>2</td>
<td>t005 -, t021 +</td>
<td>-</td>
</tr>
<tr>
<td>P, TE, RD(I)</td>
<td>1</td>
<td>t021 -</td>
<td>-</td>
</tr>
<tr>
<td>P, E(I), TE, QD(I)</td>
<td>1</td>
<td>t582 -</td>
<td>-</td>
</tr>
<tr>
<td>P, CIP</td>
<td>1</td>
<td>t012 -</td>
<td>-</td>
</tr>
<tr>
<td>P, DA(I), K, TE, RD(I), QD(I)</td>
<td>1</td>
<td>t127 -</td>
<td>-</td>
</tr>
<tr>
<td>P, FD</td>
<td>2</td>
<td>t015 -, t620 -</td>
<td>-</td>
</tr>
<tr>
<td>CIP, RD, QD(I), FD</td>
<td>1</td>
<td>t053 -</td>
<td>-</td>
</tr>
<tr>
<td>P, FOX, E, DA (MLSBi), K, TOB, CN, TE, RD(I), QD</td>
<td>1</td>
<td>-</td>
<td>t127 -</td>
</tr>
</tbody>
</table>
Patterns of antibiotics resistance | No. of strains | MSSA/ spa types/ PVL +/− (no. of strains) | MRSA/ spa types/ PVL +/− (no. of strains)
--- | --- | --- | ---
P, FOX, E, DA (MLSbi), K, TOB, CN, RD(I), QD | 1 | - | t127 -
P, FOX, E, DA (MLSbi), K, TE(I), RD(I), QD | 1 | - | t559 -
P, FOX, E, DA (MLSbi), K, TE, RD(I), QD | 1 | - | t127 -
P, FOX, E, DA (MLSbi), K, TE, QD | 1 | - | t127 -
P, E, DA (MLSbi), K, TE, QD | 4 | t127 - (3), t15296 - | -
P, E, DA (MLSbi), K, TOB, CN, QD, FD | 1 | t127 - | -
P, E, DA (MLSbi), K, TOB, CN, TE, QD | 1 | t127 - | -
P, E, DA (MLSbi), K, TOB, TE(I), QD | 1 | t127 - | -
P, E, DA (MLSbi), K, TE, RD(I), QD, FD | 1 | t127 - | -
--- | --- | --- | ---
29 strains
(I)* - Intermediate

Discussion

Epidemiological studies have shown an increase not only in healthcare-associated *S. aureus* infections, but also in *S. aureus* infections acquired in the community (DRYDEN [14]).

SSTIs caused by *S. aureus* are a diverse group of infections that can be classified into uncomplicated and complicated infections. Host factors such as co-morbidities and microbial factors, in particular drug resistance, complicate the management of these infections. MRSA is an important pathogen involved in complicated SSTIs (cSSTIs) in Europe and its involvement can be associated with poor patient outcomes. Therapy should be initiated without delay and on an empiric base, before microbiological analysis have been accomplished, waiting for culture and antimicrobial susceptibility testing for documentation of the presence of MRSA (LEE & al [52]).

In 2008, Europe showed a strong presence of MRSA strains, accounting for approximately 44% of hospital infections (KOCK & al [53]). Other European studies showed an overall oxacillin resistance rate for *S. aureus* of 26.7%. There was a great variation of MRSA rates between countries, the resistance level ranging from over 40% in Great Britain, Belgium, Greece, Ireland and Island to only 0.6% in Sweden (SADER & al [54]). Twenty five % or more of *S. aureus* isolates have been reported in Bulgaria, Croatia, Cyprus, Greece, Israel, Italy, Malta, Portugal, Ireland, Romania, Spain, Turkey and the UK (KOCK & al [53]). According to the ECDC Antimicrobial Resistance Surveillance Network (EARS-Net formerly EARSS) report, in Romania the MRSA rate among invasive infections was 49.5% in 2011, 53.3% in 2012, 64.5% in 2013 and 56% in 2014 ([55]). Fortunately, this is improving because of surveillance programs and stringent outbreak control criteria (HUMPHREYS [56]).

HA-MRSA reach also a high prevalence in Australia (HUMPHREYS [56]), North Africa, the Middle East and East Asia (IPPOLITO & al [57]).

*Spa* type t030 has been described as the prevalent MRSA *spa* type in Turkey (ALP & al [58]) and Romania (GRUNDMANN & al [3]), being reported by other several studies as the most widespread MRSA in Romanian hospitals (OPREA & al [59]; IONESCU & al [60]), and considered as being endemic (SZEKELY & al [61]). ST239/ t030 highly associated with the SCCmec type III is now widely disseminated in many Asian countries and China, although it might have originated from European countries (CHEN & al [62]). In Romanian hospitals, the ST239 clone was reported by the Seqnet.org structured survey (GRUNDMANN & al [63]) and by other authors (CİRLAN & al [64]). *Spa* type t127 reported frequently in Romanian hospitals (OPREA & al. [59]; SZEKELY & al [61]; CODIŢĂ & al [65]), was described also by Franco & al ([66]) to be associated with animals (e.g., pigs).

In Europe, CA-MRSA was first described at the beginning of this decade. The highest rates of CA-MRSA carriage (>50%) are reported in North and South America, Asia and Malta. Intermediate rates (25-50%) are reported in China, Australia, Africa and some European countries, such as Portugal (49%), Greece (40%), Italy (37%) and Romania (34%). Other European countries, including the Netherlands and Scandinavian Countries reported generally low prevalence rates (ALAKLOBI & al [67]).

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CA-MRSA had a higher prevalence in the United States, Canada (CARROLL [68]) and Australia (KOCK & al [53]). Both in Europe and in United States there is a dramatic raise in the number of reports showing the increase of MRSA prevalence in community-associated infections which differs with area and population. Before 2000, in United States, the percent of MRSA strains isolated from SSTIs was very low (3%) and raised in some regions to 30% and then to 64% between 2001-2004, with 97% of isolates belonging to clone USA300 (MORAN & al [7]).

Other United States clones include USA400 (ST1), USA1000 (ST59), and USA1100 (ST30) (PAN & al [69]).

In China, the most common clone among CA-MRSA isolates reported in the case of children with SSTIs was ST59-MRSAIV/V-4378 (WU & al [70]; LI & al [71]). In Beijing, one recent study reported the livestock ST398 clone as having a high prevalence in S. aureus SSTIs, where 64.3% were harbouring the lukSF-PV genes (ZHAO & al [72]). In Japan, the most common clone among SSTIs-associated MRSA was MLST-CC8/spa-CC008-SCCmec-IV (MAEDA & al [73]). In New York, the United States CC8 is the most common type in S. aureus SSTIs, especially among PVL-positive S. aureus (KALTSAS & al [74]).

Vandenesch & al [22]) showed a limited number of clones with a preferential geographic distribution before 2003. Afterwards, a much higher genetic diversity was reported in Europe among the CA-MSSA and the CA-MRSA strains (ROLO & al [75]). It is clear that some strains are found geographically restricted while others are found worldwide, but it is not clear why certain MRSA strains predominate in hospitals and other MRSA strains predominate in community settings (DIEP & al [76]). Some strains showed significant variation in the ability to cause outbreaks.

The most prevalent European CA-MRSA clone detected was ST80/CC80 and is resistant to kanamycin/amikacin and fusidic acid (STEGERG & al [77]), while in the United States the most prevalent clones were ST8 (USA300) and ST1 (USA400), and in Oceania ST30. The study by Tristan & al. [21]) suggests intercontinental exchanges of several continent-specific clones: the ST8 clone from the United States towards Europe, the ST1 clone from the United States towards Europe and Asia, the ST59 clone (USA1000) from the United States towards Asia, the ST80 clone from Europe towards Asia (HSU & al [78]) and the ST30 clone from Oceania towards Europe. The epidemic character of CA-MRSA, especially the geographically widespread clone USA300 is poorly understood. USA300 isolates carry a type IV SCCmec element conferring \( \beta \)-lactam antibiotic class resistance and a putative pathogenicity island, arginine catabolic mobile element (ACME), which may be involved in increasing persistance in the skin environment and in inhibiting polymophonuclear cells function. Physical linkage between SCCmec and ACME suggests that selection for antibiotic resistance and for pathogenicity may be interconnected (DIEP & al [79]).

The main European clone, t044/ST80, was detected in Austria, Belgium, Bulgaria, Chile, Croatia, Cyprus, Czech Republic, Finland, France, Germany, Greece, Hungary, Iceland, Iran, Ireland, Italy, Jordan, Lebanon, Netherlands, Norway, Romania, Spain, Sweden, Switzerland, United Arab Emirates, United Kingdom (EU Ridom SpaServer database), but also in northern Europe (e.g., Denmark), where MRSA strains are rare in hospitals (FARIA & al [80]). ST30 is also a major clone in Asia and Oceania (HO & al [81]; VLACK & al [82]) and is referred to as the South West Pacific clone (VLACK & al [82]).

ST1 and ST30, the PVL-positive clones, can now be considered pandemic. They are detected in America, Europe and Asia. Some continent-specific clones described in 2003, such as clone ST8, have now spread all over the world and the PVL-positive CA-MRSA have spread to several countries on various continents (TRISTAN & al [21]; VANDENESCH & al [22]). In 2002, PVL has been associated with 1.6% of S. aureus strains in England and Wales (HOLMES & al [83]). Currently, the prevalence of PVL-positive CA-MRSA varies considerably from continent to continent. In contrast to the United States, where CA-MRSA now accounts for the majority of S. aureus infections in the community, in Northern Europe, the prevalence of PVL-positive CA-MRSA is lower, at ≈1–3% (DEL GIUDICE & al [84]), while Southern European countries, such as Greece and countries located at the southern boundaries of Europe, such as Algeria, have a greater prevalence.PVL-positive MSSA/MRSA have also occasionally been described in Romania (IONESCU & al [60]; NĂSTASE & al [85]; CODIŢĂ & al [86]; MONECKE & al [87]). Studies have reported a high prevalence of the PVL-positive strains in our country (31% among MRSA, 14% among MSSA). One study revealed a relatively high rate of PVL-producing strains (23.93%), mainly CA-MRSA (51.11%) in North-Eastern region of Romania (VREMERA & al [88]).

Initially PVL-positive CA-MRSA isolates were susceptible to most antistaphylococcal antimicrobial agents, then they have acquired new antimicrobial resistance determinants. In Asia (Singapore, People’s Republic of China) or Africa (Algeria) most PVL-positive CA-MRSA strains showed multiple antimicrobial resistance determinants (TRISTAN & al [21]). One study from 1999 showed the prevalence of resistance to aminoglycosides in S. aureus SSTIs: MSSA – gentamicin 2% (4/217), tobramycin 4% (8/217), kanamycin 5% (11/217) and MRSA – gentamicin 67% (39/57), tobramycin 91% (52/57), kanamycin 93% (53/57) (SCHMITZ & al [89]), while another study from 2001 showed the distribution of the tetracycline resistance genes of MRSA and MSSA strains

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in Europe (SCHMITZ & al [90]). In Romania, most of the PVL-positive CA-MRSA isolates were resistant to erythromycin (91.3%), and susceptible to clindamycin, fluoroquinolones, rifampicin, chloramphenicol or fusidic acid (VREMERA & al [88]). CA-MSSA/CA-MRSA, PVL-positive strains isolated from skin and soft tissue infections in A and B location proved different patterns of antimicrobial resistance covering a large spectrum, from sensitive to all classes of antimicrobials tested to resistant to one up to all of the following antibiotics: erythromycin, clindamycin, kanamycin, ciprofloxacin, tetracycline, quinupristin-dalfopristin, fusidic acid. In B location, one CA-MSSA PVL-positive strain was intermediate to rifampicin, but in this location we found also this intermediate resistance in other 8 PVL negative strains, amongst 4 were MSSA and 4 MRSA. Three CA-MRSA t044 PVL positive strains isolated in the A location and 5 MSSA PVL negative strains from B location were found resistant to fusidic acid, widely used for skin and soft tissue infections topical therapy.

In countries of high prevalence, PVL-positive CA-MRSA are responsible for an increasing number of HA infections. Among the different clones, sequence variations of PVL have been observed. The clone most frequently observed in Europe, ST80 harbours an H2 haplotype which is described as the ancestral allele that gave rise to the other variants. These trends, although largely regionalised, must be taken into account when targeting skin infections as well as other infections, including CAP. These emerging tendencies are directed towards increasing incidence of CA-MRSA and decreasing susceptibility to antimicrobial agents. It must be considered when choosing empiric treatment options (VANDENESCH [91]).

CA-MRSA strains often cause more severe diseases such as deep skin infections. A clear epidemiological association between PVL-positive S. aureus strains and necrotizing pneumonia has been shown (LINA & al [24]). In the United States, the high prevalence of PVL-positive CA-MRSA is mostly responsible for necrotizing infections, whereas in Europe the majority of cases of necrotizing pneumonia are caused by PVL-positive MSSA strains that are as virulent as the PVL-positive CA-MRSA clones (SICOT & al [92]).

In Romania, one study and one communication presented case reports on fatal sepsis due to PVL-positive CA-MRSA spa type t044 clone (SZEKELEY & al [93]; ALEXANDRESCU & al [94]). Our study allowed us to identify PVL-positive spa types t008 and t044 strains, as being implicated in community acquired SSTIs. Over the time, PVL-positive t044 strains have been involved in necrotizing pneumonia, fatal sepsis with community-onset, associated with influenza but also with skin infections evolving in teenagers or preschoolers in Romania (SZEKELEY & al [93]; ALEXANDRESCU & al [94]). In a previous study we found PVL-positive spa type t008 strains involved in newborns or infants infections with hospital-onset (personal communication). We presume that t008 strain has the capacity to multiply and to disseminate in hospital outbreaks.

However, little is known on the precise Romanian situation. In recent Romanian studies the MRSA rates in hospitals are very high, ranging from approximately 30% up to 85%, whereas CA-MRSA has been identified inside hospitals (COMAN & al [95]; GRIGORE & al [96]; DORNEANU & al [97]; SZEKELY & al [98]; DOROBĂȚ & al [99]; IONESCU & al [60]; KOCK & al [53]; NÂSTASE & al [85]; NICA & al [100]; VREMERA & al [88]; IONESCU & al [101], MEREZEANU & al [102], COTAR & al [103], CRISTEA & al [104]). From our knowledge, there are no national reports on MRSA prevalence among community onset SSTIs. Our study revealed a prevalence of 59.52% in location A and 17.24% in location B.

In our study the prevalent community onset SSTI spa type was t127 in strains isolated from outpatients both at the Cantacuzino Institute and at the Elias University Emergency Hospital.

Conclusions

The most prevalent S. aureus clone in community onset SSTIs was spa type t127, followed by spa types t044 and t008. Previous reports on possible link between human and animal t127 strains support future development by using an integrated One health approach. On the other hand, we proved that t127 MRSA was involved in several hospital outbreaks in the last years.

The genes responsible for PVL production were detected in strains of t008 and t044 spa types, but also in other spa types: t019, t284, t355, t435, t437, t1211, t1889, t5841, t14513.

The detection of PVL, TSST and SEs positive S. aureus strains by using molecular methods and antimicrobial resistance testing revealed differences between mostly recurrent infections (A strains) and mainly not recurrent infections (B strains).

Detection of virulence and AMR genes may support enhanced surveillance and public health interventions aiming to prevent the dissemination of clones implicated in necrotizing pneumonia, complicated suppurative skin infections, toxic-septic shock and food poisonings both in community and hospitals settings. Systematic surveillance of both hospital and community isolates is required and should be done regularly for proper treatment, together with measures designed to limit their spread, including an integrated One health approach.

Our study results confirmed that containment of S. aureus MRSA strains remains a first emergency public health constraint.
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