

Molecular Epidemiology of Bulgarian Clinically Significant *Staphylococcus aureus* Isolates

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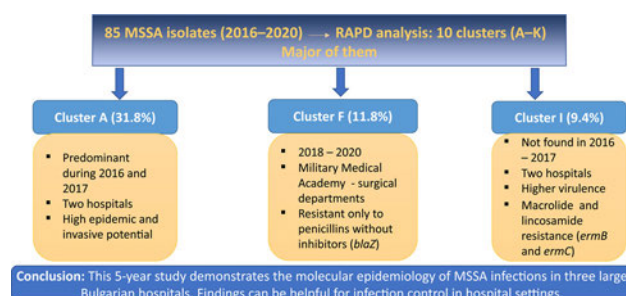
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Abstract

Severe infections due to highly virulent and resistant *Staphylococcus aureus* pose a serious health threat in Bulgaria and worldwide. The purpose of this study was to explore the clonal spread of recent clinically significant methicillin-susceptible *S. aureus* (MSSA) isolates from inpatients and outpatients treated in three university hospitals in Sofia, Bulgaria, during the period 2016–2020 and evaluate the relationship between their molecular epidemiology, virulence profiling, and antimicrobial resistance. A total of 85 isolates (invasive and noninvasive) were studied using RAPD analysis. Ten major clusters (A–K) were identified. The first major cluster A (31.8%) was found to be predominant during 2016 and 2017 and was widespread in two hospitals, unlike its case in the following years, when it was found to be replaced by newer cluster groups. All MSSA members of the second most common cluster F (11.8%) were recovered from the Military Medical Academy, mainly during 2018–2020, and were determined to be susceptible to all other groups of antimicrobials, except for penicillins without inhibitors because they harboured the *blaZ* gene. The newer cluster I, with 9.4% of the isolates absent



in 2016–2017, showed significantly higher virulence and macrolide resistance (42.9%) due to *ermB* and *ermC*. All the isolated MSSA in groups F and I were nosocomial and mostly invasive. In conclusion, this 5-year study demonstrates the molecular epidemiology of MSSA infections in three Bulgarian hospitals. Findings can be helpful for the understanding of staphylococcal infection distribution in hospital settings and their prevention.

Key words: *Staphylococcus aureus*, RAPD-PCR, virulence, antimicrobial resistance, epidemiology

Introduction

Staphylococcus aureus is responsible for a wide range of infections with various clinical manifestations, including sepsis and other severe community-acquired (CA) and hospital-acquired (HA) infections. It has been recognized as a significant cause of morbidity and mortality worldwide (Wang et al. 2018; Snygg-Martin et al. 2020; Lisowska-Łysiak et al. 2021; Potindji et al. 2022; Thabet et al. 2022). *S. aureus* has been reported by the European Centre for Disease Prevention and Control as 17.3% of the problematic human isolates in 2020

(WHO and ECDC 2020). It possesses numerous virulence factors with the role of effectively counteracting the host's immune defense, such as surface protein A, adhesins, coagulase, catalase, DNase, proteases, cytotoxins, staphylococcal enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-1), epidermolytic (exfoliative) toxin. Many factors involved in the infection pathogenesis are considered super-antigens (SAGs), which activate T-lymphocytes abnormally, resulting in cytokine storm response or non-effectively T cells proliferation and blocking of immune response (Wang et al. 2018; Cheung et al. 2021). Our previous results presented

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high virulence potential among the examined Bulgarian invasive staphylococcal isolates that have genetic determinants encoding toxins and enzymes with immunomodulatory activity, as follows: *cna*, *tst-1*, *seb*, *seh*, *sec*, *sed*, *see*, *seg*, *she*, and *sei* detected in different combinations (Gergova et al. 2019), most of which were found in nosocomial staphylococcal isolates, reported in a recent study from Northern Cyprus (Potindji et al. 2022).

The spread of various clusters of *S. aureus* carrying multiple genes for virulence factors and resistance to antimicrobials poses a severe public health concern, especially in the last two decades (Tang et al. 2018; Wang et al. 2018; Cheung et al. 2021; Syed et al. 2021; Gergova et al. 2022; Silva et al. 2022). There are no clear reasons for the emergence and spread of some hyper-virulent and resistant clusters in the last few years. Methicillin-resistant *S. aureus* (MRSA) has long been recognized as a problematic pathogen associated with HA and CA infections. Unfortunately, the widespread use of macrolides in many places leads to another global problem such as growing resistance to this group of antimicrobials (Tang et al. 2018; Syed et al. 2021; Gergova et al. 2022; Silva et al. 2022). Due to recent clonal dissemination, the molecular epidemiology of staphylococcal nosocomial infections was also dynamic. Periodic examination of the regionally circulating *S. aureus* clusters might provide insights into the most widespread emerging hyper-virulent and/or multidrug-resistant etiological agents of severe infections.

The purpose of this study was to explore the clonal spread of recent clinically significant methicillin-susceptible *S. aureus* isolates from inpatients and outpatients treated in Sofia, Bulgaria, from 2016 to 2020 and evaluate the relationship between their molecular epidemiology, virulence profiling, and antimicrobial resistance.

Experimental

Materials and Methods

Bacterial strains. A total of 85 non-duplicate *S. aureus* isolates obtained from both inpatients ($n=75$) and outpatients ($n=10$) aged 1–89 were studied. Only outpatients and inpatients in University Orthopedics Hospital “Prof. Boycho Boychev” – ORT included children aged 1–17 years. The investigated strains were isolated during the period 2016–2020 from patients with diagnoses with distribution presented in Fig. 1. The first group isolates, named invasive, were from normally sterile places ($n=47$; 55.3%) as follows: blood cultures ($n=12$; 14.1%), punctures of soft tissue abscesses ($n=20$; 23.5%), bronchoalveolar lavage (BAL) ($n=6$; 7.1%), sinus punctures ($n=3$; 3.5%), middle ear fluid ($n=2$; 2.4%), and articular punctures ($n=4$; 4.7%). The non-

invasive ones ($n=38$; 44.7%) were recovered from the skin and mucosal membranes such as nasopharyngeal secretions ($n=3$; 3.5%), ocular secretions ($n=2$; 2.4%), superficial wounds / suppurative skin lesions ($n=26$; 30.6%), and urine ($n=7$; 8.2%). Only sinus punctures, ear fluid, and nasopharyngeal and ocular secretions were obtained from outpatients (with CA infections). The inpatients were treated in three university hospitals in Sofia, namely: 1) University Hospital “St. Ivan Rilski” (SIR) with its clinics: HD – Hemodialysis, ID – Therapy of Internal diseases, NS – Neurosurgery, PD – Occupational diseases, and RE – Rheumatology; 2) University Orthopedics Hospital “Prof. Boycho Boychev” (ORT); 3) Military Medical Academy (MMA) with the following clinics: AS – Abdominal surgery, CS – Chest surgery, NS – Neurosurgery, SS – Septic surgery, VS – Vascular surgery, DE – Dermatology, and ICU – Intensive Care Unit.

S. aureus was presumptively identified as a bacterial colony showing typical characteristics, including golden yellow color and hemolysis; microscopic morphology of Gram-positive cocci forming grape-like clusters; catalase- and coagulase-positive (Rabbit Plasma; Himedia, India) tests. Detailed biochemical identification was made using the BD BBL™ Crystal™ GP ID kit (Beckton Dickinson, Germany).

Antimicrobial susceptibility testing. Isolates were screened for methicillin resistance by the cefoxitin disk method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines *S. aureus* ATCC® 29213™ (MSSA) and *S. aureus* ATCC® 43300™ (MRSA) were used as control strains for species identification and antimicrobial susceptibility testing (EUCAST 2021). MRSA isolates were excluded from the present study because their epidemiology has previously been described (Gergova et al. 2022). The following antibiotic disks were used to determine antimicrobial susceptibility: benzylpenicillin (1 unit), cefoxitin (30 µg), gentamicin (10 µg), amikacin (30 µg), tetracycline (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), clindamycin (30 µg), ciprofloxacin (5 µg), trimethoprim-sulfamethoxazole (1.25 µg/23.75 µg), tigecycline (15 µg), and linezolid (10 µg). To determine vancomycin susceptibility, we implemented the minimal inhibitory concentration broth microdilution method (Microlatest MIC ErbaLachema, Czech Republic). EUCAST recommendations were applied for result interpretation (EUCAST 2021). Identified staphylococcal isolates were stored in skimmed milk at -70°C until use. Before experimental work, *S. aureus* isolates were sub-cultured three times from frozen stocks onto BD™ Brain Heart Infusion (BHI) agar (Becton Dickinson, Germany) at 35°C overnight.

Molecular characterization. Pure staphylococcal cultures were used for genomic DNA isolation using

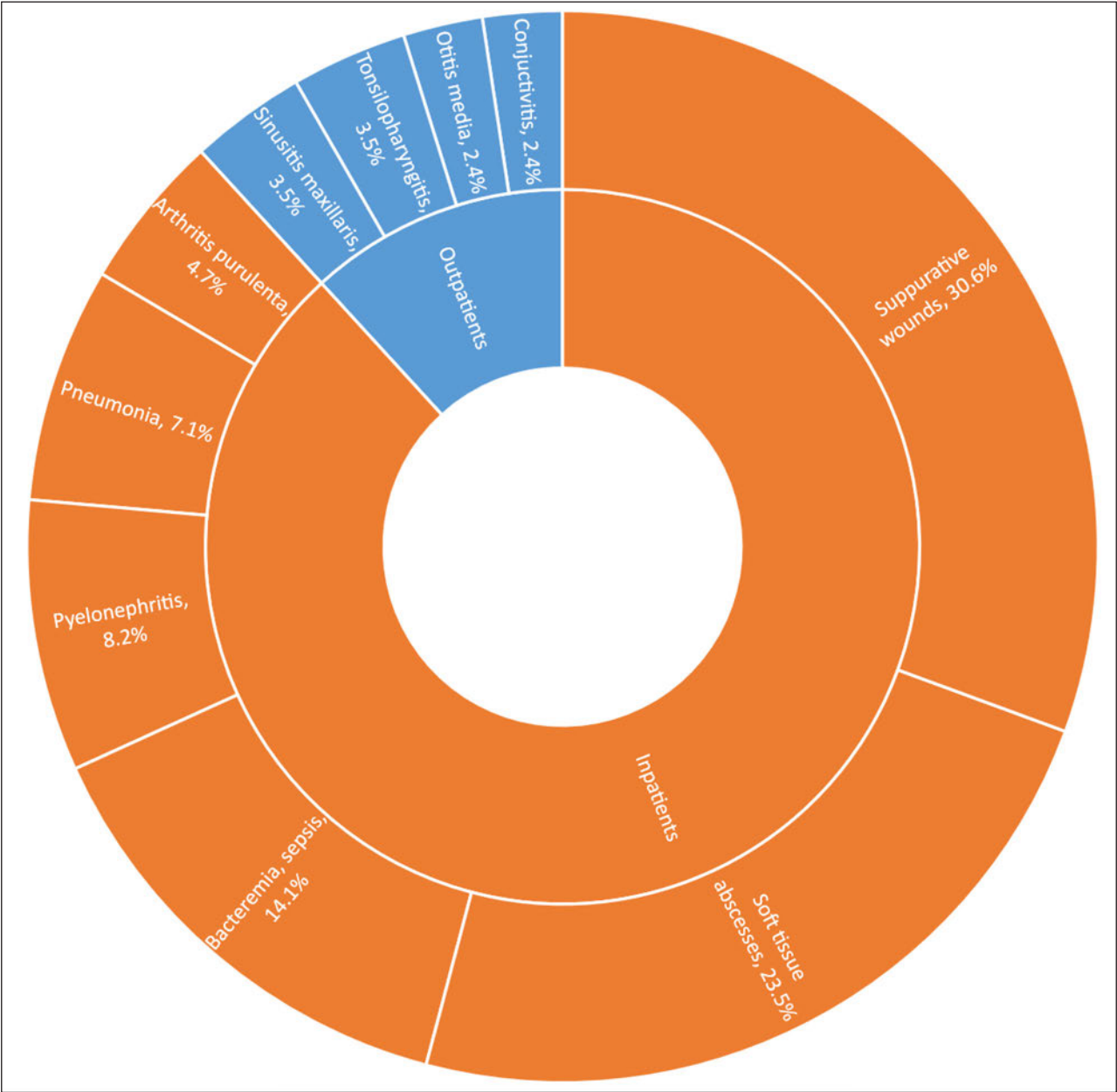


Fig. 1. Distribution of samples, according to the diagnoses of examined patients.

a DNA sorb-AM nucleic acid extraction kit (Ampli-Sens, Russia). The procedures were carried out following the manufacturer’s instructions. All DNA extractions were stored at –70°C until use.

Species identity was confirmed using a PCR-based method and primers targeting 23S rRNA genes as previously described (Cantekin et al. 2015; Gergova et al. 2019). The presence of virulence determinants (*hlg*, *cna*, *tst-1*, *sea*, *seb*, *sec*, *sed*, *see*, *seh*, *seg*, *sei*, and *sej*) and ones for predominant resistance (*blaZ*, *mecA*, *ermA*, *ermB*, and *ermC*) were determined by PCR methods and conditions as described earlier (Gergova et al. 2019). The following multiplex PCR mixes were used: Mix I containing primers for *hlg*, *sea*, *sed*, and *tst* genes; Mix II with both primers, *Sau* 327 and *Sau* 1645 for specific

S. aureus 23S rRNA genes, and primers for enterotoxin *seg*; Mix III containing primers for *seh*, *see* and *sej* genes; and Mix IV containing primers for *cna*, *seb*, *sec*, and *sei* genes. Monoplex PCR was used for *mecA* and *blaZ* separately (Gergova et al. 2019; Tsitou et al. 2021). The *ermA*, *ermB*, and *ermC* genes encoding one of the most frequent types of antimicrobial resistance were detected again by multiplex PCR, carried out with specific primers and protocols (Tsitou et al. 2021).

Phylogenetic clustering by RAPD (Random Amplification of Polymorphic DNA). RAPD-PCR reactions were performed in a 20-µl volume, in a KCl buffer system with 2 mM MgCl₂, of the four dNTP, respectively. 0.2 mM of each dNTPs (dATP, dGTP, dCTP, dTTP), and the C-primer (5'-AGGGAACGAG-3')

at a final concentration 0.2 μ M (Yoon et al. 2018) with a total DNA of about 40 ng and 0.5 U JumpStart™ Taq-polymerase (Sigma-Aldrich, USA). The initial denaturation step for 5 minutes at 94°C was followed by 4 cycles of denaturation at 94°C for 45 seconds, hybridization at 30°C for 2 minutes, and synthesis at 72°C for 30 seconds; 10 cycles of denaturation at 94°C for 5 sec, hybridization at 36°C for 30 sec. and synthesis at 72°C for 30 sec.; 10 cycles of denaturation at 94°C for 5 sec, hybridization at 36°C for 30 sec. and synthesis at 72°C for 40 sec.; 10 cycles of denaturation at 94°C for 5 sec, hybridization at 36°C for 30 sec. and synthesis at 72°C for 50 sec., and 10 cycles of denaturation at 94°C for 5 sec, hybridization at 36°C for 30 sec. and synthesis at 72°C for 60 sec. The final step involved additional synthesis at 72°C for 10 minutes. The amplification products (8 μ l of each) were applied to 1.2% agarose gel. The gels were stained with ethidium bromide and were then photographed under UV light.

UPGMA analyses. The software GeneTools v.4.1 (Syngene, UK) was used for the grouping of strains and construction of phylogenetic trees by similarity matrices calculations based on the electrophoretic gels images and the construction of the UPGMA dendrograms using the “profile” and “band position” options. One cluster was defined as isolates with 80% similarity or greater than 80%.

Statistical analysis. Differences were analyzed using unpaired descriptive statistics, chi-square test, and Z-test, SPSS for Windows, Version 19.0 (SPSS Inc., USA). A p -value < 0.05 was considered statistically significant.

Results

All studied isolates were confirmed as *S. aureus* with PCR. They were *blaZ*-positive (94.1%) and *mecA*-negative (100%). The following proportions of isolates were susceptible to penicillin – 5.9%, cefoxitin (oxacillin) – 100%, gentamicin – 97.7%, amikacin – 100%, chloramphenicol – 97.7%, tetracycline – 87.1%, erythromycin – 58.8%, clindamycin – 68.2%, ciprofloxacin – 92.9%, trimethoprim-sulfamethoxazole – 98.8%, linezolid – 100%, tigecycline – 100%, and vancomycin – 100%.

A total of 85 isolates representing differing origins, sites of infection, types of infections (invasive or non-

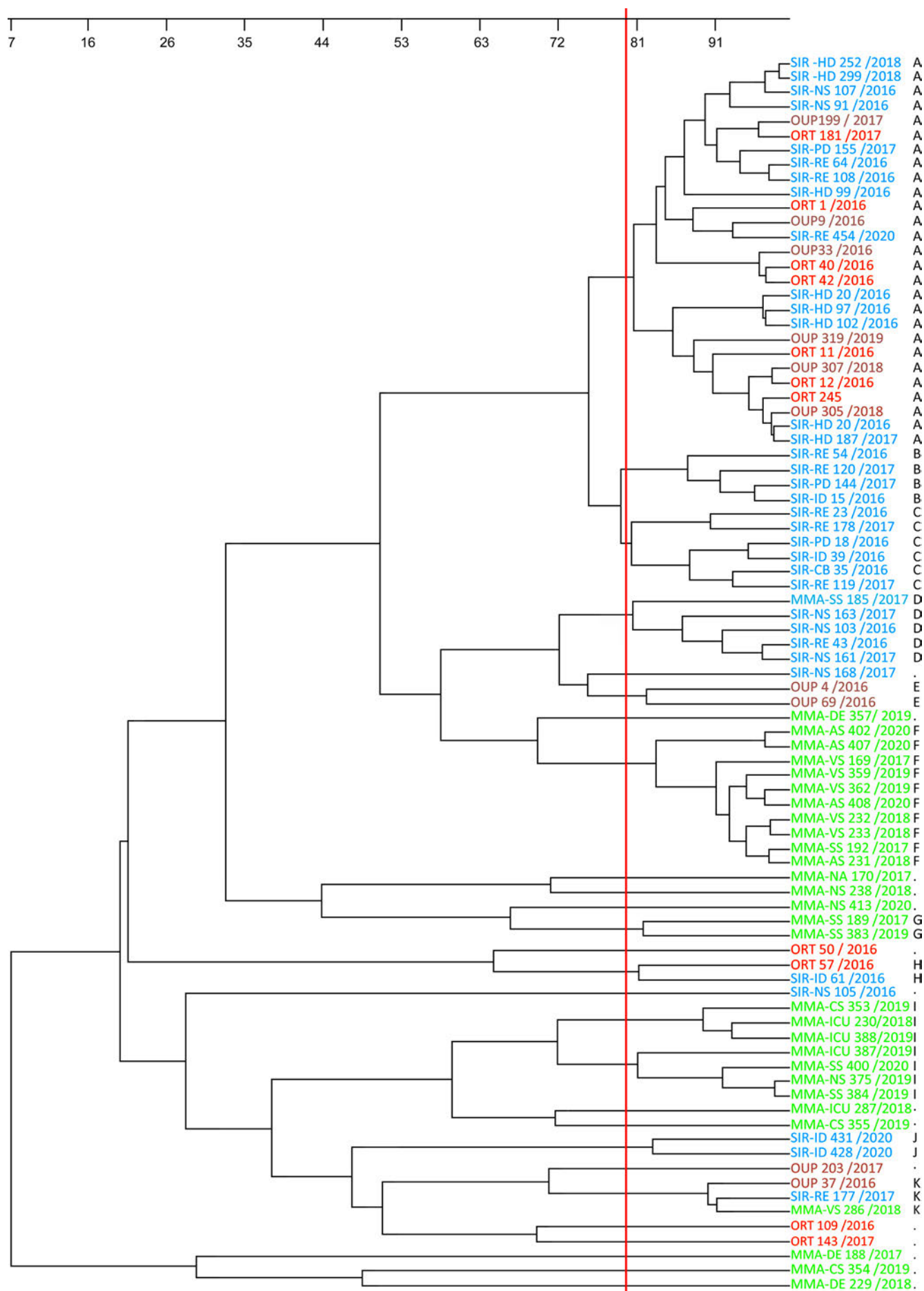
invasive), and isolation from inpatients (hospitals) or outpatients were studied by RAPD. The distribution of samples by diagnosis is presented in Fig. 1.

Epidemiological characterization of 85 MSSA strains was performed. Isolates with 80% coincidence in the phylogenetic tree were included in each cluster. Upon analysis of the results, ten major clusters were designated: A, B, C, D, E, F, G, H, I, J, and K (Fig. 2). Of these, cluster A was predominant among the investigated MSSA strains and comprised 27 strains (31.8%). The next most common was cluster F, grouping 11.8% of the tested isolates. In third place was cluster I, with a frequency of 9.4% of the examined strains. The remaining cluster groups contained significantly fewer or single isolates from staphylococcal infections found on an outpatient basis or only in some clinics. Cluster A was dominated by isolates from the University Hospital SIR (14 total), mainly from the HD Clinic – eight isolates (seven from blood cultures and one from puncture). Three isolates from this cluster were found in the RE Clinic, two isolates from patients of the NS Clinic, and one from a patient at the PD Clinic. This cluster included seven more strains isolated from patients operated on in the ORT and six outpatient isolates (Fig. 1). Every MSSA originated from a different type of sample, but most of them were from blood culture (14.1%) with a significant difference ($p < 0.0001$) between patients with bacteremia and sepsis (29.6). Over 77% of isolates in this cluster were invasive. They were predominantly isolated from patients undergoing hemodialysis at the SIR-HD or with severe fractures, less frequently after joint prosthetics operated at the ORT, which raises suspicion of a higher virulence of this cluster. The virulence genes in the cluster A isolates were *hlg*, *can*, *tst-1*, *sea*, *seb*, *sec*, *sed*, *seg*, *sei*, *sej*, *she*, *hlg*, *seg*, *sei*, and *sej* with different combinations. They were present in all blood culture isolates. In superficial skin wounds, eye, and nasopharyngeal secretion isolates, the combination of *hlg*, *seb*, *sec*, *sei*, and *sej* prevailed.

The second largest group, designated as cluster F, contained ten *S. aureus* isolates, and all circulated in the MMA in recent years. There were no outpatient isolates, indicating a regional distribution, so far demonstrated solely in this hospital. Most of these staphylococcal strains were isolated from the VS clinic ($n = 5$), followed by the AS clinic ($n = 3$), and in the SS clinic ($n = 2$). Unlike the first group, all isolates in

Fig. 2. Epidemiological typing with RAPD of MSSA isolates from three University Hospitals and outpatients.

SIR – University Hospital ‘St. Ivan Rilski’: HD – Hemodialysis, NS – Neurosurgery, RE – Reumatology,
ID – Therapy of Internal Diseases, OD – Occupational Diseases
ORT – University Orthopedics Hospital ‘Boycho Boychev’
MMA – Military Medical Academy: AS – Abdominal surgery, CS – Chest surgery, NS – Neurosurgery,
SS – Septic surgery, VS – Vascular surgery, DE – Dermatology, ICU – Intensive Care Unit
OUP – outpatients



cluster F were HA from wound infections, half of which were aspirated from abscesses, while others were from superficial wound secretions. Another difference with the first group was that the strains in cluster F were isolated mainly from 2018–2020. There were only two isolates from 2017 and none from 2016. There was no significant difference in the virulence factors studied. Combinations of the genes *hlg*, *sea*, *seb*, *sec*, *sed*, *sei*, *sej*, and *seh* were found again, only the *seg* gene was missing in the Cluster F isolates. The genetic elements *hlg*, *sei*, and *sej* were found predominantly in isolates belonging to this cluster.

In the third RAPD group, cluster I, all staphylococci were isolated from the MMA. They were not recovered from outpatients or isolated from the other two hospitals. This cluster consisted of strains isolated in the period 2018–2020 in the MMA clinics as follows: ICU (n = 3), SS (n = 2), NS (n = 2), and TS (n = 1). The isolates belonging to this cluster were invasive, mainly obtained from abscess infections (punctures and aspirates) from intensive care clinic patients. This cluster was new, since its isolates were not found in samples from 2016–2017. Macrolide resistance established in representatives of this cluster was significantly higher than 42.9%. Genetic determinants encoding resistance to macrolides and lincosamides were found to be *ermB* and *ermC*.

Cluster C (7.1%) was also interesting, including only a few invasive isolates (n = 6), but circulating only in the SIR clinics. These strains were isolated in 2016–2017 only. Again, all isolates (except one from urine) were invasive – from blood cultures and BALs. Resistance to the macrolide-lincosamide group was predominant in 30% of all samples and was genetically encoded by the *ermA* and *ermC* genes. As early as 2016, a combination of the *ermA* and *ermC* genes was proven in a BAL strain isolate (n = 1).

Cluster D, circulating only in the SIR clinics and representing 5.9% of all RAPD types, was similar to the previously mentioned one. Its representatives (n = 5) were mostly invasive – from blood cultures, a BAL, and only one noninvasive was obtained from a urine sample. Resistance to the MLS group antibiotics was established in 40% of the isolates. Genes encoding this resistance were *ermA* and *ermC*, which proved to be dominant in 2016–2017 and in isolates circulating mainly in the SIR during this period, as established in the aforementioned A and C clusters. Moreover, cluster D had not been found after 2017.

Discussion

Severe infections due to highly virulent and resistant *S. aureus* in Bulgaria are a serious health problem like in many other geographical regions worldwide (Enright

et al. 2000; Lisowska-Łysiak et al. 2021; Syed et al. 2021; Thabet et al. 2022). RAPD analysis regarding recent MSSA isolates showed that various strains circulate in the studied hospitals and outpatients from Sofia, which was noted as a specific feature for the MSSA group in other studies from various countries (Wang et al. 2018; Aung et al. 2021). Furthermore, there is increased recognition of the considerable clinical importance of these strains – some MSSA lineages are highly virulent and cause fatal infections. A recent study showed that 10% to 30% of patients with bacteremia due to MSSA die due to this infection (Cheung et al. 2021). MSSA infections have not been monitored as strictly as MRSA infections, and the implemented restrictions do not lead to a similar decrease in MSSA infections, as reported in some countries, such as the USA and the UK (Kavanagh 2019).

RAPD typing of Bulgarian staphylococcal isolates revealed nosocomial dissemination of 10 MSSA clusters between 2016 and 2020. The first primary MSSA RAPD cluster A circulated extensively between 2016 and 2017 and was widespread in two hospitals, unlike the following years, when other, newer strains replaced it. The strains of dominant cluster A demonstrated high epidemic and invasive potentials. The strains of dominant cluster A demonstrated high epidemic and invasive potentials. The strains from cluster A were isolated predominantly from 12 males and 15 females aged 41–89 years from the SIR clinics. Twelve patients were immunocompromised after hemodialysis, rheumatoid or occupational diseases and the other seven isolates were from patients aged 11–67 years after surgery in the ORT hospital and two in SIR NS. All MSSA members of the second most common cluster F were isolated mainly in the period 2018–2020 and were susceptible to macrolides and lincosamides, as well as to other groups of antimicrobials, except for penicillin and aminopenicillins without inhibitors, because all of them harboured *blaZ*. All isolates from this cluster were from MMA surgical departments and were mainly associated with invasive procedures or operations in 10 patients aged 18–78 years. They were predominantly males (8). The newer cluster I (including isolates from the ICU clinic and from some surgical departments of the MMA) was only from males aged 23–69 years. These strains were missing in 2016–2017 and had significantly higher virulence and macrolide resistance (42.9% of resistant strains), encoded by *ermB* and *ermC*. Many virulence determinants were found in the isolates from blood cultures enclosed in cluster A and cluster F (*hlg*, *cna*, *tst-1*, *sea*, *seb*, *sec*, *sed*, *seg*, *sei*, *sej*, *seh*, *hlg*, *seg*, *sei*, and *sej*), which are the signs of the high pathogenic potential of these clusters to disseminate in other hospitals in the future. *S. aureus* produces a vast array of virulence factors in contrast to many other pathogens (Gergova

et al. 2019; Cheung et al. 2021). Some virulence determinants, such as *cna*, promote adhesion and host colonization, while others encode immune evasion factors with a strong toxic effect (*hlg*, *tst-1*, *sea*, *seb*, *sec*, *sed*, *seg*, *sei*, *sejseh*, *hlg*, *seg*, *sei*, and *sej*). Different combinations of the three enterotoxin-coding genes *seb*, *seg*, and *sei*, which are strong SAGs, were detected in 48.15% of strains within the prevalent cluster A. In this study, they were found in the invasive isolates ($p < 0.05$) of more than 55% of the examined staphylococcal isolates. Strains of these characteristics have not been identified in the MMA. However, the significant incidence of this RAPD type in outpatients means that it is only a matter of time before they can be admitted to other hospitals with CA infections. All MSSA in clusters F and I were HA and mostly invasive. The major mechanism behind an HA infection route is the capacity of staphylococci to adhere to plastic devices, implants, and vascular catheters. Soon after insertion and initial attachment, *S. aureus* forms a biofilm on the device at the insertion site. This process is aided by many extracellular substances (DNA, proteins, enzymes), and cell proliferation (Schilcher et al. 2020; Snygg-Martin 2020). The specific behavior of bacteria within the biofilm is due to the so-called “quorum sensing” mechanism. Each bacterial cell secretes signaling molecules into the environment and simultaneously receives signals from other bacteria present nearby. Reaching a particular concentration of bacteria is perceived as a signal to reach critical microbial mass; therefore, bacterial cells switch to protein expression – characteristic of biofilms, with considerable increases in metabolism and expression of virulence factors. It is generally thought that biofilm formation prevents the antimicrobial effects of antibiotics and the immune system (Kavanagh 2019; Schilcher et al. 2020). The spread of significant clusters was typical for their local distribution in MMA’s intensive clinics and showed their increased virulence potential. Two other small circulating clusters were associated with invasive nosocomial infections. The other clusters included single outpatient isolates, collected simultaneously from different hospitals through the years, without a clear epidemiological relationship. This finding indicates a divergence of MSSA over the years and settlement in different hospital units by incoming patients, probably staff, similar to the previously reported results (Kavanagh 2019; Schilcher et al. 2020). Clusters C and D circulated in SIR predominantly. Cluster D was older than cluster C; the latter was found to be on a steady rise since 2018. They encompass the strains isolated from males (6) and females (5), aged 18–74 years.

Resistance to antimicrobials, especially penicillins and macrolides-lincosamides, is also widespread in *S. aureus* (Tang et al. 2018; Silva et al. 2022). MLSB resistance (*ermA* and *ermC*) detected in cluster C and

cluster I was found in more than 30% of isolates. The spread of specific and nonspecific antibiotic resistance within biofilms plays a major role in staphylococcal biofilm-associated infections (Schilcher et al. 2020; Cheung et al. 2021).

Very little data on the RAPD typing of *S. aureus* exists in the literature (Reinoso et al. 2004; Hakimi et al. 2017). Based on the constructed dendrogram, authors from Argentina reported that all tested *S. aureus* strains were divided into nine major clusters (A–I) with an 80% similarity, which was validated by our results. Some clusters consisted of staphylococcal strains isolated from one source, while others were comprised strains isolated from more than one source. The results demonstrated that most *S. aureus* strains from the same source were placed in the same clusters. Many strains of different sources shared the same RAPD profiles. It indicates a possible transmission of MSSA and MRSA strains among human and animal hosts (Reinoso et al. 2004). A research group from Iran conducted a similar study using isolates from bovine and human hosts. The resulting eleven clusters (A–K) are nearing the cluster count in our study. Group A included 95% of the human staphylococcal isolates; the other groups (B–K) consisted of bovine isolates. The genetic diversity among *S. aureus* isolates from bovine hosts was relatively low compared to those from humans (Hakimi et al. 2017).

In conclusion, this study gives a novel view of the spread of pathogenic MSSA clusters among large hospitals in Sofia, Bulgaria. Investigating the molecular epidemiology of infections is a prerequisite for understanding the dynamics of staphylococcal populations and cases of replacement with more resistant clusters carrying new genetic determinants for antimicrobial resistance. Findings can be helpful for the understanding of staphylococcal infection distribution in hospital settings and their prevention.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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