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RESEARCH ARTICLE

Molecular epidemiology, virulence and antimicrobial resistance of Bulgarian methicillin resistant *Staphylococcus aureus* isolates

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ABSTRACT

Background: Severe infections of virulent methicillin-resistant *Staphylococcus aureus* (MRSA) are a serious health problem. The present study aimed to investigate clonal spread, virulence and antimicrobial resistance rates of Bulgarian MRSA isolates in 2016–2020. *Methods:* Molecular identification and *mecA* gene detection were performed with PCR. Clonal relatedness was evaluated by RAPD PCR and MLST. MRSA epidemiology, virulence and resistance patterns were investigated by PCR. *Results:* All 27 isolates were identified as *S. aureus* and were *mecA* positive, and all were susceptible to linezolid, tigecycline and vancomycin. The toxin genes *hlg* (in 92.6% of isolates), *seb* (77.8%), *sei* (77.8%), *seh* (59.3%), *sej* (55.6%), and *seg* (48.1%), were frequently found among the isolates. Epidemiological typing by RAPD identified 4 clones (16 isolates) and 11 were with a unique profile. MLST analysis of the same MRSA isolates showed five MLST clonal complexes and 11 ST types, including CC5 (33.3%) (ST5, ST221, ST4776), CC8 (22.2%) (ST8, ST239, ST72), CC15 (ST582), CC22 (14.8%) (ST217, ST5417), CC30 (ST30) CC398 (ST398), and CC59 (ST59). The isolates from CC5 showed higher virulence potential and almost all were macrolide resistant (*erm*B or *erm*C positive). CC8 isolates showed higher level of resistance. *Conclusion:* To the best of our knowledge, this study is the first describing the clonal spreading of Bulgarian MRSA and the association with their virulence and resistance determinants. Monitoring of MRSA epidemiology, resistance and virulence profile can lead to better prevention and faster therapeutic choice in cases of severe infections.

KEYWORDS

MRSA, RAPD, MLST, virulence, clonal complexes

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INTRODUCTION

Staphylococcus aureus is a major human pathogen causing a wide spectrum of infections with different clinical manifestations, ranging from skin and soft-tissue lesions to severe systemic infections, often associated with bacteriemia or other serious community-acquired (CA) and nosocomial, hospital-acquired (HA) diseases [1–4]. In 2020, *S. aureus* has been reported by European Centre for Disease Prevention and Control to be 17.3% of the prevalent invasive isolates in most European countries [5]. This is a pathogen that possesses a huge variety of

virulence factors, including adhesins, hemolysins, Panton Valentine leukocidin, catalase, plasmacoagulase, DNase, proteases, various staphylococcal enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-1), epidermolytic (exfoliative) toxin, surface protein A, and can fix IgG–mediated opsono-phagocytosis. Many factors which are involved in complex *S. aureus* pathogenicity are superantigens (SAgs) that abnormally activate T cells for non-effective or autoimmune answer [1, 6]. Our previous results demonstrated high virulence potential in Bulgarian invasive *S. aureus* isolates, including a wide array of genes encoding immunomodulatory molecules such as *cna, tst-1, seb, seh, sec, sed, see, seg, seh* and *sei* detected in different combinations [7]. Spread of various staphylococcal clones carrying multiple virulence factor and antibiotic resistance determinants has been a public health concern for the last two decades [1, 3, 6, 8, 9]. Methicillin-resistant *S. aureus* (MRSA) has long been recognized as a pathogen associated with nosocomial infections and also as an important causative agent of CA infections [3, 4, 7, 9]. The MRSA belongs to the ESKAPE group of pathogens (*Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacter* spp.) that have the ability to "escape" from common antimicrobial treatment through acquisition or development of a variety of resistance mechanisms [4]. Some hospital staphylococcal isolates are known as epidemic clones such as the best-known EMRSA-15, which appeared in the United Kingdom 3 decades ago and has spread to various countries around the world [10]. Evaluation of molecular epidemiology of regional circulating *S.aureus* clones, especially MRSA might provide further insights into the dissemination of emerging hyper-virulent and/or multidrug resistant (MDR) causative agents of severe infections.

The present study aimed to determine the clonal spread MRSA isolates from inpatients and outpatients in Sofia, Bulgaria in the period of 2016–2020 and to evaluate the relationship of their molecular epidemiology with the virulence profiles and antimicrobial resistance patterns.

MATERIAL AND METHODS

Bacterial strains

Twenty-seven non-duplicate meticillin (cefoxitin)-resistant *S. aureus* isolates from various clinical samples were collected during the period 2016–2020. They included invasive isolates (18 isolates, 66.7%) from normally sterile sites such as blood cultures - (5,18.5%), punctures of soft tissue abscesses (9,33.3%), sinus aspirates (2,7.4%), tracheal aspirate (1,3.7%), joint puncture (1,3.7%), as well as non-invasive isolates from skin and mucosal sites such as nasal secretions (4,14.8%), nasopharyngeal secretions (2,7.40%) and skin lesions (3, 11.1%). Twenty-two isolates were from inpatients and were collected from three large University Hospitals in Sofia, Bulgaria and five isolates were from outpatients, treated ambulatory.

S. aureus has been presumptively identified by colony and Gram stain morphology, positive catalase reaction and plasma-coagulase test (Rabbit Plasma, Himedia, India). Detailed biochemical identification was done by Crystal GP (Beckton Dickinson, Kelberg, Germany). Isolates were screened for methicillin resistance by the cefoxitin disk method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (http://www.eucast.org) [11]. The following antibiotic disks were used to determine antimicrobial susceptibility: benzylpenicillin (1unit), cefoxitin (30 μg), gentamicin (10 μg), amikacin (30 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), clindamycin (30 μg), ciprofloxacin (5 μg), trimethoprime/sulphomethoxazole (1.25/23.75 μg), tigecycline (15 μg) and linezolid (10 μg). To determine vancomycin susceptibility, we used a minimal inhibitory concentrations broth microdilution test (Microlatest MIC ErbaLachema, Brno, Czech Republic). For interpretations of the results of antibiotic testing EUCAST recommendations were used [11]. The staphylococcal isolates were stored in skim milk at −70°C until next testing. Before testing, *S. aureus* isolates were subcultured three times onto Brain Heart Infusion (BHI) agar (Becton Dickinson, Kelberg, Germany) and incubated at 35°C overnight.

Molecular characterization

Pure staphylococcal cultures were used for genomic DNA extraction using a DNA sorb-AM nucleic acid extraction kit (AmpliSens, Moscow, Russia), in accordance with the manufacturer's instructions. All DNA extractions were stored at −70 °C until testing.

All phenotypically identified *S. aureus* strains were PCR confirmed using species-specific primers targeting 23S rRNA genes [12]. The presence of twelve virulence genetic elements (*hlg, cna, tst-1, sea, seb, sec, sed, see, seh, seg, sei, sej)* and resistance markers (*blaZ, mec*A, *ermA, ermB, ermC*) were evaluated by multiplex or monoplex PCRs as previously described [13]. The following mixes for multiplex PCR were used: Mix I containing primers for *hlg, sea, sed* and *tst* genes; Mix II containing both *Sau 327* and *Sau 1645* primers for specific *S. aureus* 23S rRNA gene and *seg* primers for enterotoxin. Mix III contained primers for *seh, see* and *sej* genes; and Mix IV contained primers for *cna, seb, sec,* and *sei* genes. Detection by monoplex PCR of the *blaZ* gene which encodes beta-lactamase production and inactivates penicillins by hydrolyzing the beta-lactam ring, was carried out and *mecA* gene was assessed in all isolates as well to confirm the existence of MRSA [7, 13]. The genes *erm*A, *erm*B, *erm*C encoding methylases that cause macrolide resistance were detected by multiplex PCR according to previously described protocols [13].

Random amplified polymorphic DNA (RAPD)

RAPD-PCR reactions were performed in a volume of 20 μl in a buffer system with KCl, at final concentrations of $MgCl_2$ 2 mM,

with the four dNTPs and 0.2 mM each one (dATP, dGTP, dCTP, dTTP), and the C primer (5-AGGGAACGAG-3′, 0.2 mM) [14] with a total DNA of about 40 ng and 0.5 U Jumpstart™ Taq-polymerase (Sigma-Aldrich). The initial denaturation step for 5 min at 94°C was followed by 4 cycles of denaturation at 94°C for 45 s, hybridization at 30°C for 2 min and synthesis at 72°C for 30 s; 10 cycles of denaturation at 94°C for 5 s, hybridization at 36°C for 30 s and synthesis at 72°C for 30 s; 10 cycles of denaturation at 94°C for 5 s, hybridization at 36°C for 30 s and synthesis at 72°C for 40 s; 10 cycles of denaturation at 94°C for 5 s, hybridization at 36°C for 30 s and synthesis at 72°C for 50 s and 10 cycles of denaturation at 94°C for 5 s, hybridization at 36°C for 30 s and synthesis at 72°C for 60 s. The final step involved additional synthesis at 72°C for 10 min. Eight µl of the resulting products were applied to a 1.2% agarose gel with ethidium bromide. After electrophoresis, the gels were photographed under UV light.

Grouping of strains and construction of phylogenetic trees was based on the calculation of the so-called similarity matrix and detection of sequence pairs in operational taxonomic units (OTUs) with similarity greater than 80%. The process was repeated until all OTUs were arranged and a dendrogram was obtained. Specialized software "GeneTools 4 4.01" (Syngene, UK) was used to build the dendrograms. One clone is defined as isolates with 80% similarity.

Multilocus sequence typing (MLST) analysis

DNA extractions were used to amplify 7 housekeeping genes (*arc, aro, glp, gmk, pta, tpi, yqi*) according to the guidelines described in the *S. aureus* MLST website [15]. PCR amplicons were subjected to Sanger sequencing. Purification of the PCR product for subsequent Sanger sequencing was performed using Rapid PCR Cleanup Enzyme Set (ExoSAP, Applied Biosystems, USA) and Sanger sequencing approach using BigDye® Terminator v3.1 Cycle Sequencing Kit and BigDye® Terminator v1.1 & v3. 1 5× Sequencing Buffer, on a sequencer model (Applied BiosyStems 3130xl Genetic Analyzer). The analysis of the obtained sequences was performed with Chromas Liteversion 2.01 (Technelysium Pty Ltd, Australia). Assignment to allelic numbers, sequence-types (STs) and clonal complexes (CC) were performed in the MLST database [16].

RESULTS

All 27 isolates were PCR confirmed as *S. aureus*. They were *blaZ* and *mecA* positive. The susceptibility of the isolates was as follow: penicillin 0%, cefoxitin (oxacillin) 0%, gentamicin 48.1%, amikacin 92.6%, chloramphenicol 85.2%, tetracycline 63.0%, erythromycin 11.1%, clindamycin 22.2%, ciprofloxacin 70.4%, trimethoprime/sulphomethoxazole 81.5%, linezolid 100%, tigecycline 100%, vancomycin 100%. Twenty-four (88.9%) isolates were macrolide nonsuceptible, 15 of them showed cMLST, 6- ind MLST and 3 were with MS phenotype.

The isolates gave positive PCR results for the following virulence factors with a decreasing rate: *hlg* (92.6%), *seb (*77.8%), *sei (*77.8%), *seh* (59.3%), *sej* (55.6%), *seg* (48.1%), *sec* (33.3%), *cna* (22.2%), *sea* (22.2%), *sed* (3.7%), *tst-1* (7.4%), and *see* (3.7%), The RAPD primers provided between 8 and 15 bands. Isolates with 80% similarity were classified as a clone. The results of RAPD analyses are shown in Fig. 1.

Epidemiological typing with RAPD of the 27 MRSA isolates identified 4 clones and 11 isolates with a unique RAPD profile (Fig. 1). The clones consisted of two to eight isolates and included 59.3% of the isolates. They were denoted as **a, b, c, d**. The most common clone was **RAPD type a** (in 29.6% of the tested isolates), 11.1% of the isolates were of **RAPD type b,** 11.1%were of **RAPD type c,** and 7.4% were of **RAPD type d**. The multi-locus sequence typing of all 27 MRSA isolates showed the presence of five MLST clonal complexes and 11 ST types, including **CC5** (ST5, ST221, ST4776), **CC8** (ST8, ST239, ST72), **CC15** (ST582), **CC22** (ST217, ST5417), **CC30** (ST30) as well as ST59 and ST398, which do not correspond to any clonal complex.

The most common complex was **CC5** in 33.3% of the isolates. **CC5** contained ST5 (25.9%) which is identical with RAPD type **a**, ST4776, identical with RAPD type **a** and the single isolate ST221 with unique RAPD profile. Most (77.8%) of the isolates belonging to ST5-CC5 were invasive and were isolated from blood cultures, abscesses or tracheal aspirates. Half of the isolates contained a large number of virulence genes such as *hlg, seb, sec, seg, seh, sei, sej* genes, most of which are strong superantigens (Table 1). In addition to their high virulence, all ST5-CC5 strains had a high macrolide-lincosamide resistance rate of cMLSB type (53.3% from all isolates) encoded by *ermB* or *ermC*. Members of this clone were isolated in two hospitals and only one isolate was from ambulatory patients.

The second frequent complex was **CC8** (in 22.2%), which included two members of RAPD type **d** and four isolates with unique profiles. There were no isolates from outpatients, all isolates were invasive hospital-associated MRSA from blood culture and abscess sites. All MRSAs from ST8-CC8 clone contained the genetic determinants *hlg, seb, sec, seh, sei, sej*, and those of ST239-CC8 differed from each other and from the others isolates in CC8 by combinations of virulence, antibiotic pattern and resistance genes.

RAPD type b, corresponding to sequence type ST582-CC15 (in 18.5% of the isolates), was established in 2016–2020 in the three hospitals from which the isolates originated. In 2/3 of the cases, the isolates were invasive and contained similar genes encoding virulence *hlg, seb, sei, seh, sej,* and the resistance to macrolides/lincosamides was indMLSB type mediated by *ermA* (Table 1). The isolates showed great genetic similarity, preserved over the years, both in their virulence determinants and antimicrobial resistance rates.

CC22 (found in 14.81% of the isolates) consisted of three isolates with RAPD type **c** corresponding to ST217-CC22 and a single one ST5417-CC22 with a unique RAPD profile. They were found only in the University Orthopedics Hospital and in outpatients. Unlike the previous group, there

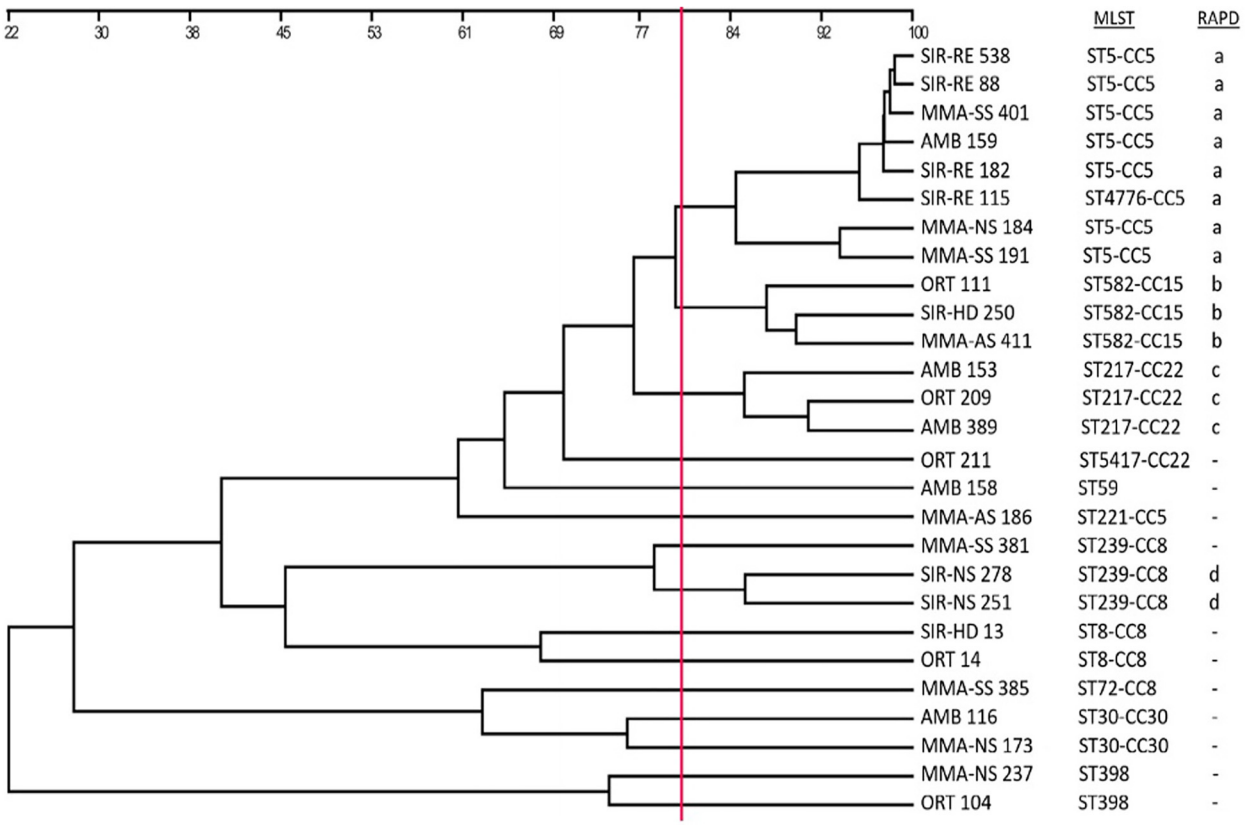

Fig. 1. Epidemiological typing with RAPD of 27 MRSA isolates from 3 University Hospitals and outpatients
Abreviations: **1**) University Hospital 'St. Ivan Rilski' (**SIR**) with its clinics: HD - Hemodialysis, NS - Neurosurgery, RE - Reumathology; **2**) University Orthopedics Hospital "Boycho Boychev" (**ORT); 3**) Military Medical Academy (**MMA**) with the clinics: AS - Abdominal surgery, NS - Neurosurgery, SS - Septic surgery.4) **Amb** - outpatients

was a significant heterogeneity in both virulence and resistance patterns (Table 1). This clone was not found in 2019–2020. The other rare ST types are shown in Table 1.

DISCUSSION

The severe infections owing to virulent and resistant *S. aureus* in Bulgaria have been a serious health problem like in many other geographical regions around the world [1, 2, 8, 9, 17]. The observed susceptibility showed high resistance rates to macrolides, lincosamides (in 88.89% of the isolates) due to production of *erm*A, B and C. We did not detect vancomycin, linesolid and tigecycline resistant isolates. Similar resistance rates to macrolides, lincosamides, tetracycline, chloramphenicol and gentamicin, and 100% susceptibility to vancomycin and linezolid have been reported for MRSA isolated from patients with bloodstream infections in Shanghai in 2013–2018 [18].

Epidemiological typing by RAPD and MLST among Bulgarian *S. aureus* isolates demonstrated wide spreading of 4 HA-MRSA clones between 2016 and 2020.

The analysis and comparison of MLST and RAPD results of the MRSA isolates showed that the most prevalent ST type in Bulgaria was **ST5-CC5** (in 25.9% of the isolates) circulating in 2016–2020 in two of the major University Hospitals University Hospital 'St. Ivan Rilski' and Military Medical Academy. **ST5-CC5** is a part of a successful international epidemic lineage. It includes USA100 (formerly designed as causing severe HA infections, called EMRSA New York/Japan clone) and USA 800 (Pediatric clone). Strains associated with this lineage have been reported worldwide-USA, Canada, Brazil, Colombia, Argentina, Australia, Asia and Europe and mainly were associated with hospital-associated infections [8, 19–23]. **ST5-CC5** has also been named as the Marseille clone and has been associated with cystic fibrosis [24]. A new China study reported that ST5-CC5 as the dominant clone (in 69.8% of MRSA isolates) from China patients with staphylococcal bloodstream infections at Shanghai in 2013–2018 [18]. These data have been consistent with immunosuppressive activity of the staphylococci and their tissue invasion and survival in biological fluids [6]. Our results confirmed these data about the clone. Six from seven isolates, belonging to **ST5-CC5,** were invasive. This could be explained by the high pathogenic potential of the isolates of the clone. Four of seven ST5 isolates contained genes encoding 6 enterotoxins *seb, sec, seg, seh, sei, sej* (also acting as superantigens) and a strong γ-hemolysin (*hlg).* The extracellular cytolytic γ–hemolysin has activity against a wide range of human and rabbit erythrocytes, as well as against neutrophils, monocytes, granulocytes and macrophages. There has been evidence

Table 1. Distribution of 27 MRSA isolates according to MLST ST-CC, RAPD profile, virulence and antibiotic resistance patterns

DNA No	ST type-clonal complex	RAPD type	clinical sample	hospital -clinic	year	Resistance to	virulence determinants	resistance genes	macrolide resistance phenotype
88	**ST5-CC5**	**a**	nasal swab	SIR-RE	2016	Pen, Fox, Gen, Ery, Cli	*hlg, sec, seg, sei, sej*	*blaZ; mec A; erm C*	c MLSB
159	**ST5-CC5**	**a**	sinus aspirate	Amb	2017	Pen, Fox, Gen, Tet, Ery, Cli	*hlg, sea, seb, sec, seg*	*blaZ; mec A; erm B*	c MLSB
182	**ST5-CC5**	**a**	tracheal aspirate	SIR-RE	2017	Pen, Fox, Gen, Tet, Ery, Cli	*hlg, seb, sec, seg, seh, sei, sej*	*blaZ; mec A;erm B& erm C*	c MLSB
184	**ST5-CC5**	**a**	abscess puncture	MMA-NS	2017	Pen, Fox, Gen, Ery, Cli	*hlg, seb, sed, seg, seh, sei, sej*	*blaZ; mec A; erm C*	c MLSB
191	**ST5-CC5**	**a**	blood culture	MMA-SS	2017	Pen, Fox, Gen, Ery, Cli	*hlg, seb, sec, seg, seh, sei, sej*	*blaZ; mec A; erm B*	c MLSB
401	**ST5-CC5**	**a**	wound swab	MMA-SS	2019	Pen, Fox, Gen, Tet, Ery, Cli	*hlg, seb, sec, seg, sei, sej*	*blaZ; mec A; erm B*	c MLSB
538	**ST5-CC5**	**a**	blood culture	SIR-RE	2020	Pen, Fox, Gen, Tet, Ery, Cli	*hld, seb, seh,see, seg, sei, sej*	*blaZ; mec A; erm C*	c MLSB
115	**ST4776-CC5**	**a**	nasal swab	SIR-RE	2016	Pen, Fox, Gen, Tet, Ery, Cli	*seb, seg, sei*	*blaZ; mec A; ermA*	c MLSB
186	**ST221-CC5**	*	abscess puncture	MMA-AS	2017	Pen, Fox	*hlg, cna,seb, seh, sej seg*	*blaZ; mec A;*	susceptible
111	**ST582-CC15**	**b**	nasopharyngeal swab	ORT	2016	Pen, Fox, Ery,	*seb, sei, hlg*	*blaZ; mec A; ermA*	ind MLSB
250	**ST582-CC15**	**b**	blood culture	SIR-HD	2017	Pen, Fox, Ery,	*hlg, seb, sei,seh,sej*	*blaZ; mec A; ermA*	ind MLSB
411	**ST582-CC15**	**b**	abscess puncture	MMA-AS	2020	Pen, Fox, Ery,	*hld, cna,seh,sei,sej*	*blaZ; mec A; ermA*	ind MLSB
13	**ST8-CC8**	*	blood culture	SIR-HD	2016	Pen, Fox, Gen, Ery, Cli, Cip, SxT	*hlg, sea,seb, sec, seh, sei*	*blaZ; mec A; ermB*	c MLSB
14	**ST8-CC8**	*	abscess puncture	ORT	2016	Pen, Fox, Gen,Tet, Ery, Cip, SxT	*hlg, seb, sec, seh, sei, sej*	*blaZ; mec A; ermA*	ind MLS_B
251	**ST239-CC8**	**d**	abscess puncture	SIR-NS	2017	Pen, Fox, Gen, Tet,Chl, Ery,Cip, SxT	*hlg, sea, seb, seh, sei, sej*	*blaZ; mec A; ermB*	MS phenotype
381	**ST239-CC8**	*	blood culture	MMA-SS	2018	Pen, Fox, Gen, Amk,Tet,Chl, Ery, Cli, Cip, SxT	*hlg, sej*	*blaZ; mec A; ermB*	c MLS_B
278	**ST239-CC8**	**d**	abscess puncture	SIR-NS	2018	Pen, Fox, Gen, Amk,Tet,Chl, Ery,Cip, SxT	*hlg, sei, sea,*	*blaZ; mec A; ermA*	ind MLSB
385	**ST72-CC8**	-	abscess puncture	MMA-SS	2018	Pen, Fox, Ery, Cli	*hlg, seg, sei, seb, seh*	*blaZ; mec A; erm C*	c MLSB
153	**ST217-CC22**	**c**	sinus aspirate	Amb	2016	Pen, Fox	*tst, hlg, sea, seb, sec, seg, sei*	*blaZ; mec A;*	susceptible
209	**ST217-CC22**	**c**	abscess puncture	ORT	2017	Pen, Fox	*hlg, cna,seb, sei, seh*	*blaZ; mec A; ermA*	MS phenotype
389	**ST 217-CC22**	**c**	nasal swab	Amb	2018	Pen, Fox, Ery,	*hld,seh,sei,sej*	*blaZ; mec A; ermA*	ind MLSB
211	**ST5417-CC22**	*	joint puncture	ORT	2017	Pen, Fox, Ery, Cli	*hlg, cna,seb, seh,sei*	*erm B&erm C*	c MLSB
116	**ST30-CC30**	*	nasal swab	Amb	2016	Pen, Fox, Ery, Cli	*seb,seg,sei*	*blaZ; mec A; ermA*	c MLSB
173	**ST30-CC30**	*	wound swab	MMA-NS	2017	Pen, Fox	*tst, hlg, sea,seb, seh,sei, sej*	*blaZ; mec A;*	susceptible

(continued)

Table 1. Continued

DNA No	ST type-clonal complex	RAPD type	clinical sample	hospital -clinic	year	Resistance to	virulence determinants	resistance genes	macrolide resistance phenotype
158	**ST59-CC59**	*	nasopharyngeal swab	Amb	2017	Pen, Fox, Tet, Chl, Ery, Cip	*hlg, sea, seb, sec, seg, seh,sei*	*blaZ; mec A; ermA*	MS phenotype
104	**ST398-CC398**	*	abscess puncture	ORT	2016	Pen, Fox, Ery, Cli, Cip	*hlg, cna, sei, sej*	*blaZ; mec A; erm B&erm C*	c MLSB
237	**ST398-CC398**	*	abscess puncture	MMA-NS	2017	Pen, Fox, Gen, Ery, Cli, Cip	*hlg, cna, seb, sei, sej*	*blaZ; mec A; ermB*	c MLSB

Abreviations: *, unique RAPD type; **SIR,** University Hospital 'St. Ivan Rilski' with its clinics: HD - Hemodialysis, NS - Neurosurgery, RE - Reumathology; **ORT,** University Orthopedics Hospital "Boycho Boychev"**; MMA,** Military Medical Academy with the clinics: AS - Abdominal surgery, NS - Neurosurgery, SS - Septic surgery; **Amb**, out patients.
Pen-benzylpenicillin, Fox-cefoxitin, Gen - gentamicin, Amk - amikacin, Tet-tetracycline, Chl-chloramphenicol, Ery - erythromycin, Cli - clindamycin, Cip-ciprofloxacin, SxT-trimethoprime/sulphomethoxazole, *hlg*- γ-hemolysin; *cna* - collagen adhesin; *sea,seb, sec, sed, see, seg, seg, seh,sei, sej*-enterotoxins; cMLSB - constituitive, indMLSB - inducible, MSphenotypes of resistance to macrolides, lincosamides, streptogramines.

that *hlg* and its extracellular toxic product can cause abnormalities in immune response, its modulation and potential staphylococcal survival in biological fluids, making isolates more invasive [25]. In the present study, the *seg* gene was found only in members of ST5, a similar observation was observed in a Shanghai hospital [18]. In addition to their inherent increased virulence, ST5-CC5 isolates demonstrated a high level of cMLSB-type macrolide-lincosamide resistance (Table 1), predominantly encoded by *erm B* or *ermC*. **ST5** have been previously detected in Bulgaria (in 33.7% of MRSA isolates) during in 2005–2011, although it did not harbor resistance to macrolides and lincosamides and other antimicrobials, it showed resistance only to oxacillin and some aminoglycosides such as gentamicin and kanamicin [26]. The present study showed an additional acquisition of resistance determinants among the isolates of this clone.

Several other international epidemic clones of MRSA (EMRSA) found to circulate in different countries and continents were ST22-CC22 (EMRSA-15), ST239-CC8 (Aus-2 and Aus-3 EMRSA), ST8-CC8 Pediatric (Irish-2 EMRSA; USA300), ST36-CC30 (EMRSA-16), ST8-CC8 variant (Irish-1 EMRSA) and the classic MRSA clone ST250-CC8. [20–23, 26]. In the present study, we found only members of **ST239-CC8** and **ST8-CC8** isolates in Bulgaria and they were showed extremely resistant phenotype. ST239-CC8 was the older predominant clone in some China hospitals and one of the most extensively distributed MRSA lineages throughout the world [18]. In the neighboring countries such as Serbia, Greece and Turkey the predominance of ST239 in HA-MRSA infections remains constant more than ten years, although new clones were emerging [27–30]. ST239 was predominant in Serbian human MRSA isolates, while ST5 was prevalent in canine ones. Human-associated clones belonging to both of two MRSA clones were discovered in companion animals, which suggests anthropozoonotic transmission [28]. A single predominant clone of ST239 was circulated in all hospitals in different regions of Turkey, also and only few new types of MRSA were introduced over the past years there [29, 30]. The Turkish strains from this lineage were susceptible to vancomycin, teicoplanin, tigecycline, trimethoprim-sulfamethoxazole, quinupristin-dalfopristin, and linezolid [30]. ST239 isolates showed resistance to 10 antimicrobials, including and were more resistant than the other clones. Interestingly, a previous Bulgarian study in 2005–2011 identified **ST239** as the major clone (in 43.2% of isolates) with very similar resistant patterns [25]. Our results (Table 1) were also similar to those of Aung et al. [17] who found that ST239 isolates were MDR to 12 antimicrobials, including macrolides and quinolones with resistance rates of 82–100%. Other authors also have reported high resistance rates in ST239 [8]. ST239-MRSA was described as the oldest pandemic MRSA strain, being prevalent worldwide [18, 23]. ST239-MRSA clones have been phylogenetically divided into several clades [17]. Our ST239-CC8 isolates were from inpatients and were invasive isolates, confirming the global spread of healthcare-associated strains [23]. Interestingly, the pathogenicity of these isolates was not high as two of the three ST239 isolates had 2 to 3 virulence factors. This confirmed finding of other authors that ST239/241 isolates should be discussed separately as they exhibit distinct hybridization patterns [20]. The other member of CC8 in the present study was ST8 and contained 6 virulence (*hlg, seb, sec, seh, sei,* and *sej*) determinants, more than many other MRSA strains and were hospital acquired invasive isolates as well. The China MRSA ST8 isolates were highly virulent, with similar virulence potential as the epidemic USA300 strain [9]. Only one isolate of our collection was ST72, this was a distinct lineage that differs from other CC8 strains and has been already found in West Africa, Australia and in Latin America [20]. Only this isolate among the members of CC8 possessed *ermC*. Similarly to **CC5, CC8** has been a pandemic MRSA lineage reported in many European, African and Asian

countries [20–23]. Both of **CC5** and **CC8** were predominant in the Afghanistan/Pakistan border region [22]. Only 3 EMRSA clones in the world have been considered as MDR: ST239-CC8, ST8-CC8 pediatric and ST39 [19], he last one was missing in our collection. ST39 and similar ST398 (which we detected among our MRSA isolates) were observed in community-acquired infections in Iran, India in humans and in pigs [31].

In 2016-2020, **ST582-CC15**, was found in all three hospitals from which the isolates originated, predominantly from invasive samples, although with smaller number of genes encoding virulence factors (*hlg, seb, sei, seh, sej*) and resistance to macrolides/lincosamides was of MLSB type, mediated by *ermA* that distinguishes the clone from other MRSAs of the clone **ST5-CC5**. However, it also included strains with high epidemic and invasive potential. This was the next sequence type that can be associated with infections in cystic fibrosis patients [24]. Other authors showed that CC15-MSSA was abundant among healthy carriers, but MRSA isolates from this lineage are very rare, only among Italian MRSA strains [24]. Exotoxin gene *sea*, that have been detected in Italian MRSA strains was missing in Bulgarian ST582-CC15 isolates [24].

ST217-CC22 was represent among our strains with 4 isolates. It was found in only from one hospital strain and in outpatients. Unlike previous groups, in this clone, a significant heterogeneity was observed. The variable virulence and resistance markers in Bulgarian isolates corresponded to those from other surveys [21, 22, 24]. This clone, ST217, was predominant among colonized Greek health care workers at the last years [32]. Some authors have reported a high prevalence of TSST-1 genes among MRSA - CC22 sequence type, which has been prevalent in Gaza, Palestine and this fact correlated with the common finding of *tst-1* there [33]. Our results were in concordance with this finding since Bulgarian MRSA harbored *tst-1* gene in only 7.4%, however, its rate in **ST217-CC22** isolates was 25%. Geographical differences in the frequency of *tst-1* have been found in many studies, and the prevalence of specific clones may be related.

In conclusion, to the best of our knowledge, this study is the first describing the clonal spread detected by MLST combined with RAPD among Bulgarian MRSA isolates and their virulence and resistance determinants. Monitoring of MRSA epidemiology, resistance and pathogenic profiles, especially the causes of hospital-associated infections, can improve prevention and control of the associated infections in the hospitals as well as the prompt therapeutic choice in cases of severe infections.

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