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ORIGINAL RESEARCH PAPER

Relationship between MLSB resistance and the prevalent virulence genotypes among Bulgarian *Staphylococcus aureus* isolates

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ABSTRACT

The aim of this study was to investigate the rate of resistance to macrolide-lincosamide-streptogramin B (MLSB) antibiotics, the mechanisms underlying this resistance and to evaluate their relationship with virulence genes profiles of 435 Bulgarian clinical isolates *Staphylococcus aureus*. The highest resistance was observed to penicillin (96.09%), followed by resistance to erythromycin and clindamycin (34.02 and 22.76%, respectively). Of the tested clinical strains of *S. aureus*, 96.09% contained the *blaZ* gene associated with penicillin resistance and 11.03%, the *mecA* gene responsible for methicillin resistance. The most prevalent were the *erm* genotypes associated with the presence mainly of *ermA* and *ermC* genes followed by *ermB*. The frequency rates of these genes, alone or in combinations were *ermA* 41.89%, *ermB* 27.70%, *ermC* 43.99%. The majority of Bulgarian macrolide resistant *S. aureus* exhibited cMLS phenotype, in 58.78% ($P = 0.0036$). The following virulence genotypes were present significantly more often in the macrolide resistant *S. aureus* isolates among the studied ones: *hlg; hlg,seb; hlg,seb,sec; hlg,seb,seh; hlg,sec; hlg,sec,sei; hlg,sec,sei; hlg,sei; hlg,sei,sej; hlg,sej*. This survey found correlation between the virulence profiles with a small number of genes and macrolide resistance among Bulgarian clinical *S. aureus* isolates, in contrast to sensitive strains, which possessed profiles predominantly with multiple genes.

KEYWORDS

macrolide resistance, virulence, *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus is a prominent human and animal pathogen causing a variety of infections: impetigo, abscess of soft tissue, mastitis, osteomyelitis, postoperative infections, pneumonia, urinary tract infections and severe infections by direct invasion and systemic dissemination such as bloodstream infections, septic arthritis, meningitis [1–5]. Staphylococcal strains with richer virulence arsenal are responsible also for toxin mediated diseases such as toxic shock and scalded-skin syndrome. *S. aureus* producing enterotoxins is capable of causing food-borne diseases [6]. Cumulative effects of many extracellular products which can play a role in pathogenesis are associated with the burden and clinical manifestations of staphylococcal infections. It is an etiological agent of both community acquired (CA) and nosocomial, healthcare-associated (HA) infections in humans [7–11]. The HA infections due to staphylococcal colonization of intravascular catheters and orthopedic devices causes significant patient morbidity and mortality worldwide [1, 2, 4].

The predominant clinically relevant type of resistance among *S. aureus* isolates, including Bulgarian ones, is that against antibiotics such as penicillin, methicillin, and macrolides–lincosamides. Macrolides and lincosamide as are the major alternative for the treatment of staphylococcal infections in patients with penicillin allergy, especially in childhood. Two recognized mechanisms of resistance to these commonly used antimicrobials are known in

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S. aureus: target modification and efflux of the antibiotic [12]. The basis of the first one is a conformational change in the ribosome, the so-called macrolide-lincosamide-streptogramin B (MLSB) phenotype. It is associated with the *erm* genes (erythromycin ribosome methylase) encoding a 23S rRNAmethylase (*ermA*, *ermB*, and *ermC*), rendering the cross-resistance to all macrolides, lincosamides and streptogramin B. The MLSB phenotype can be either constitutive (cMLSB) or inducible (iMLSB). The iMLSB variant of resistance is typical in bacteria producing inactive mRNA which is able to result ineffective methylase expression only by the help of a macrolide inducer, in contrast to the constitutive variant, which does not need an inductor for direct methylase synthesis [12]. Another mechanism of inducible resistance to erythromycin is conferred by genes *mef*(A) and *msrA*, which encode an ATP-dependent efflux pump. This is known as the M phenotype and confers resistance to the most commonly used 14- and 15-membered macrolides but with preserved susceptibility to ketolides, clindamycin and streptogramin B [12, 13]. In vivo therapy with clindamycin against sensitive etiological agents sometimes can select lincosamides resistant mutants leading to clinical therapeutic failure [12, 14]. Sometimes transmission of virulence and antimicrobial resistance elements has common regulatory and parallel transcription mechanisms [15].

The aim of this study was to investigate the rate of resistance to macrolide-lincosamide-streptogramin B (MLSB) antibiotics, the mechanisms underlying this resistance and to evaluate their relationship with virulence genes profiles.

MATERIALS AND METHODS

Strains

A collection of 435 clinically significant bacterial isolates of *S. aureus* was prepared (1 per patient) from various human clinical specimens: blood 39 (8.97%), pus 177 (40.69%), naso-pharengeal secretions 93 (21.38%), uro-genital secretions 58 (13.33%) and body fluids – aspirates 68 (15.63%) from both in-patients 269 (61.84%) and out-patients (38.16%) during the period January 2017 – December 2019.

The isolates were identified based on routine criteria, using colony and microscopic morphology, a positive catalase reaction and positive plasmo-coagulase tests (Coagulase Plasma from Rabbit, Hi Media, India) for presumptive detection. For more detailed biochemical identification, when needed, Crystal GP (Becton Dickinson, Kelberg, Germany) was used. The verified strains were stored in skim milk at −70 °C, and before the initiation of experiments, they were sub-cultured three times on Brain Heart Infusion agar (Hi Media, India).

All procedures involving patient's participants were performed in accordance with the ethical standards of the Medical University of Sofia, Bulgaria and the 1964 Helsinki declaration and its later amendments. For this type of study, formal consent is not required.

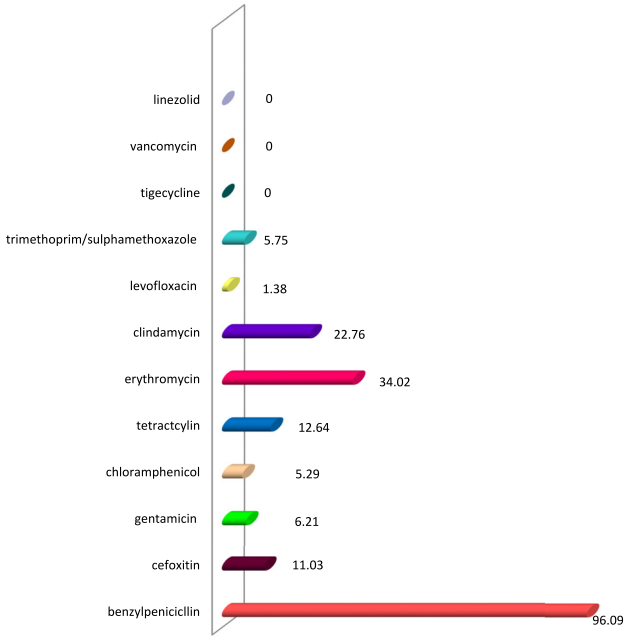

Fig. 1. Percentage resistance to various groups antimicrobials among 435 isolates *S. aureus*

Antibiotic susceptibility assay

The antimicrobial susceptibility assay was performed by the disc diffusion method for the following antibiotics: benzylpenicillin (1 unit), cefoxitin (30 μg), gentamicin (10 μg), erythromycin (15 μg), clindamycin (2 μg), tetracycline (30 μg), chloramphenicol (30 μg), trimethoprim/sulphamethoxazole (1.25/23.75 μg), levofloxacin (5 μg), linezolid (10 μg), tigecycline (15 μg), and the susceptibility to vancomycin determined by MIC (broth microdilution test). The antimicrobial susceptibility testing was done by the disk diffusion method as routine screening including detection of MRSA by the cefoxitin disk diffusion test and by determining of minimal inhibitory concentrations (MICs) according to the criteria of EUCAST 2020 (http://www.eucast.org) [16]. Blunting of the clindamycin inhibition zone from the nearby erythromycin disk on the petri dish was scored as an iMLSB; resistance to both erythromycin and clindamycin according to the disk-diffusion method was indicated as a cMLSB and resistance to erythromycin with preserved susceptibility to clindamycin with no blunting around the disks was considered as the M phenotype according to the EUCAST 2020 criteria [16]. The MIC values of erythromycin and clindamycin were determined for all isolates by Epsilometer test known as E-tests (obtained from Laboratories Pvt. Limited, Mumbai, India). The MICs of vancomycin were determined according to the EUCAST 2020 guide for broth microdilution – the other method currently accepted for susceptibility testing using Microlatest MIC (Erba Lachema, Brno, Czech Republic). After detection of MICs, we determined the MIC_{90} values for all the tested staphylococcal strains. MIC_{90} was defined as the lowest concentration of the antimicrobial agent at which 90% of the *S. aureus* isolates were inhibited. American Type Culture

Table 1. Used primer sequences for resistance-associated genes detection.

Gene	Primers sequence (5′–3′)	Amplicon size (bp)	Annealing	Reference
*erm*A	F: TAT CTT ATC GTT GAG AAG GGA TT R: CTA CAC TTG GCT TAG GAT GAA A	139	54 °C	[17]
*erm*B	F: CTA TCT GAT TGT TGA AGA AGG ATT′ R: GTT TAC TCT TGG TTT AGG ATG AAA	142	54 °C	[17]
*erm*C	F: CTT GTT GAT CAC GAT AAT TTC C R: ATC TTT TAG CAA ACC CGT ATT C	190	54 °C	[17]
*msr*A	F: TCC AAT CAT TGC ACA AAA TC R: AAT TCC CTC TAT TTG GTG GT	163	50 °C	[17]
mefA	F: CAA TAT GGG CAG GGC AAG R: AAG CTG TTC CAA TGC TAC GG	317	58 °C	[18]
blaZ	F: ACT TCA ACA CCT GCT GCT TTC R: TGA CCA CTT TTA TCA GCA ACC	173	54 °C	[17]
mecA	F: TCC AGA TTA CAA CTT CAC CAG G R: CCA CTT CAT ATC TTG TAA CG	162	57 °C	[8]

Table 2. Percentage resistance to 12 groups of antimicrobials among 435 *S. aureus* isolates

Antimicrobial agent	MIC range	MIC_{90}[a]	R[b] (%)
Benzylpenicillin[c]			96.09
Cefoxitin[c]			11.03
Erythromycin	0.75 – >256 mg/L	4 mg/L	34.02
Clindamycin	0.25 – >256 mg/L	2 mg/L	22.76
Gentamicin	0.125–8 mg/L	0.5 mg/L	6.21
Levofloxacin[c]			1.38
Tetracyclin	0.125 – >256 mg/L	4 mg/L	10.11
Chloramphenicol[c]			5.29
Trimethoprim/sulphamethoxazole	0.125–4 mg/L	0.5 mg/L	5.75
Tigecycline	0.064–0.25 mg/L	0.125 mg/L	0
Vancomycin	0.5–2 mg/L	1mg/L	0
Linezolid	0.25–2 mg/L	0.5 mg/L	0

[a]MIC_{90}, the lowest concentration of the expected antimicrobial agent at which 90% of the isolates had been inhibited.
[b]R, resistant.
[c]The results for these antimicrobials are according diffusion disc method (EUCAST 2020).

Collection *S. aureus* strains ATCC29213 and ATCC25923 were used as quality controls.

DNA extraction

All staphylococcal isolates were screened for important genes of virulence and resistance mechanisms to penicillin, methicillin, macrolides and lincosamides by PCR.

Pure staphylococcal cultures were used for genomic DNA extraction using a DNAsorb-AM nucleic acid extraction kit (AmpliSens, Inter Lab Service, Moscow, Russia), in accordance with the manufacturer's instructions.

Polymerase chain reaction (PCR) assay

PCR was performed in a 25 µL reaction mix. All phenotypically and biochemically determined *S. aureus* strains were again genetically confirmed by PCR assay using species-specific genes: *Sau* 327 and *Sau* 1645 23S rRNAas described previously [8]. Twelve virulence determinants (*hlg, sea, seb, sec, sed, see, seg, seh, sei, sej, tst, can*) were detected based on the previously described protocols [8].

All confirmed *S. aureus* strains were screened for the following resistance-associated genes: *ermA, ermB, ermC, msrA, mefA, blaZ, mecA,* using the primer sequences [8, 17, 18] presented in Table 1. Their specificity was verified by the Basic Local Alignment Search Tool (BLAST) program available at the NCBI website (Bethesda, MD) (http//:www.nbi.nlm.nih.gov/BLAST).

Statistical analysis

Differences were analyzed using unpaired descriptive statistics, Fisher exact test Quick cal. The differences were considered statistically significant at $P < 0.05$.

RESULTS

The antimicrobial susceptibility of the studied isolates and their resistance to 12 antimicrobials (Fig. 1) was determined by the disc-diffusion method and MIC technique (Table 2). Almost all of the tested 435 *S. aureus* strains were resistant

Table 3. Distribution of resistance genes in 102 strains *S. aureus* according MLSB phenotype and epidemiology of infection

Resistance genes	cMLS phenotype Number positive	iMLS phenotype Number positive	*P* value* between cMLS and iMLS	Total Number positive
ermA	22	3	**0.0001**	25
ermB	16	2	**0.0008**	18
ermC	33	4	**0.0001**	37
msrA	0	4	0.1213	4
mefA	0	8	**0.0068**	8
ermA + *ermB*	17	0	**0.0001**	17
ermA + *ermC*	19	0	**0.0001**	19
ermB + *ermC*	4	1	0.3688	5
ermA + *ermB* + *ermC*	3	0	0.2463	3

Resistance genes	CA	HA	*P* value* between CA and HA	Total
ermA	15	10	0.3935	25
ermB	8	10	0.8058	18
ermC	11	26	**0.0065**	37
msrA	3	1	0.6213	4
mefA	5	3	0.7211	8
ermA + *ermB*	2	15	**0.0015**	17
ermA + *ermC*	8	11	0.6310	19
ermB + *ermC*	0	5	0.0594	5
ermA + *ermB* + *ermC*	0	3	0.2463	3

*Bold numbers are statistically significant ($P < 0.05$).

to penicillin (96.09%) and 11.03% of them were cefoxitin-, respectively methicillin-resistant (MRSA). The level of resistance to gentamicin, levofloxacin, tetracycline, chloramphenicol, and to the combination trimethoprim/sulphamethoxazole was as follows: 6.21, 1.38, 12.64, 5.29, and 5.75% respectively and was evaluated as no relevant types of resistance for medical practice. No strain was found to be resistant to vancomycin, tigecycline or linezolid. The resistance to the group of macrolides-lincosamides (MLSB) antibiotics was significantly more frequent than resistance to antibiotics of other groups. Among the studied staphylococcal isolates collected in 2017–2019, those not susceptible to erythromycin and clindamycin were 34.02 and 22.76%, respectively. The MIC values of these antimicrobials were in the range of 0.75 – ≥256 mg/L for erythromycin and from 0.25 to ≥256 mg/L for clindamycin (Table 2).

Based on amplification of seven genes (Table 1), we determined the potential mechanisms of clinically relevant resistance to beta-lactams and MLSB antibiotics. Of the tested clinical *S. aureus* strains, 96.09% carried the *blaZ* gene associated with penicillin resistance and 11.03%, the *mecA* gene, respectively, responsible for methicillin resistance. The most prevalent genotypes were the *erm* genotype associated with the presence mainly of *ermA* and *ermC* genes and more rarely with *ermB*. The frequency rates of these genes, alone or in combinations were: *ermA* 62 (41.89%), *ermB* 41 (27.70%), *ermC* 65 (43.99%). The distribution of resistance genes according to the phenotype of MLS and the type of infection (CA or HA) is shown in Table 3 The studied isolates were most commonly carriers of gene *ermC* only, followed by *ermA* only, both of these genes being significantly predominant in the cMLS phenotype ($P < 0.05$). The most common gene combinations *ermA*+*ermC* and *ermA*+*ermB,* were detected only in cMLS strains ($P < 0.05$), but *mefA* was predominant in iMLS ($P = 0.0068$). Only two resistant genotypes (*ermC* and *ermA*+*ermC*) were demonstrated to be significantly associated with HA infections ($P < 0.05$).

The majority of the Bulgarian macrolide resistant *S. aureus* isolates tested here exhibited cMLS phenotype, 58.78% ($P = 0.0036$). The rate of macrolide resistance was significantly higher in MRSA (60.42%) than in MSSA (30.75%) ($P = 0.0001$).

The association between virulence profiles and macrolide resistances shown in Table 4. The PCR assay revealed many virulence genetic elements present both in the macrolide sensitive and in the macrolide resistant staphylococcal isolates without statistical difference, but some genotypes, such as *hlg;hlg,seb; hlg,seb,sec; hlg,seb,seh; hlg,sec; hlg,sec,sei; hlg,sec,sei; hlg,sei; hlg,sei,sej; hlg,sej,* were significantly more frequently detected in the resistant ones.

It seems that the isolates were carriers of only one, two or three genes. The genotypes with multiple genes were not detected as often in the resistant *S. aureus* isolates as in the sensitive strains.

DISCUSSION

Penicillin resistance has been known for a long time and has progressed in Bulgarian comparison to earlier data from 2014, when 90% of Bulgarian staphylococcal isolates were

Table 4. Distribution of virulence determinants in tested isolates 435 *S. aureus*

Prevalent genotypes of virulence	Virulence genes in macrolide sensitive strains ($N = 333$) Number positive	Virulence genes in macrolide resistant strains ($N = 102$) Number positive	P value*
hlg	30	17	**0.0432**
hlg,sea seb	2	2	0.2353
hlg,sea,seb,sec,seg	0	2	0.0546
hlg,sea,seb,sec,seg,sei	0	1	0.2345
hlg,sea,seb,sec,seg,seh,sei	2	2	0.2353
hlg,sea,seb,sec,tst	1	2	0.1385
hlg,sea,seb,sed,seg,seh,sei	2	1	0.5523
hlg,sea,seb,sec,sei, eh,tst	1	1	0.4144
hlg,sea,sed,sei	2	0	1.0000
hlg,seb	11	17	**<0.0001**
hlg,seb,sec	18	15	**0.0044**
hlg,seb,sec, seg,sei,tst	0	2	0.0546
hlg,seb,sec,sei	4	3	0.3625
hlg,seb,sed,sej,can	3	0	1.0000
hlg,seb,seh	2	4	**0.0290**
hlg,seb,seg,sei	3	2	0.3343
hlg,seb,seh,sei,can	6	1	1.0000
hlg,seb,seh,sei,sej	2	3	0.0866
hlg,seb,seh,sei	2	0	1.0000
hlg, seb, sei	4	3	0.3625
hlg,seb,sei,tst	0	2	0.0546
hlg,seb,sei,sej	0	2	0.0546
hlg,sec	6	9	**0.0022**
hlg,sec,seg,sei	2	2	0.2353
hlg,sec,sei,sej	2	0	1.0000
hlg,sec,sei	2	5	**0.0091**
hlg,seh	5	3	0.3978
hlg,seh,sei	2	1	0.5523
hlg,seh,sei,sej	2	2	0.2353
hlg,seh,sei,tst	0	2	0.0546
hlg,sei	9	23	**0.0001**
hlg,sei,sej	2	4	**0.0290**
hlg,sej	11	12	**0.0020**
seb,seg,sei	2	3	0.0866

*Bold numbers are statistically significant ($P < 0.05$).

reported resistant [19]. A similar rate to the present resistance in Bulgaria (96.09%) has been found in recent years in Turkish pediatric patients with HA infections [2]. The basic mechanism underlying the resistance to penicillins is associated with *blaZ* and the production of extracellular beta-lactamases that hydrolyze the beta-lactam ring, which is the active center of the antimicrobial agent, and can interfere with the successful treatment of other respiratory pathogens [19]. The geographical distribution of MRSA attributed to *mecA* at present varies in a very wide ranged depending on the origin of the infection, CA or HA. There are reports of 2.9% in Turkish nasal carriers and 36.2% in Turkish hospital isolates [2, 5], 25% in Nepal [14], up to 40.9% from Iranian HA infected patients [20], about 46% MRSA in U.S. hospitals [11], 47% MRSA in Ghana Hospitals [6]. MRSA were 52% in HIV positive persons in contrast to 20.80% detected in HIV negative ones in Ethiopia [21] and 55% in Hospitals in China [22]. The methicillin resistance rate among the Bulgarian *S. aureus* isolates both of CA and HA infections during 2017–2019 observed in this study was 11.03% and the EARS-Net data about 2017–2018 for MRSA of invasive isolates in Bulgaria were between 13.7 and 17.6% [23]. Some decreasing trend during the last 5 years can be noted when compared with data of about 20% MRSA in 2014 from a previous study in Bulgaria [19] and EARS-Net data for 20.8% MRSA of invasive isolates in Bulgaria during 2014 [24].

The macrolide resistance in the Bulgarian staphylococcal isolates was more problematic in this period. The present results were in agreement with other data about significant prevalence of macrolide resistance in MRSA, as it is known that resistance to multiple antibiotics arises more often in MRSA strains than in MSSA [3, 13, 14, 25]. Wide differences in the geographical distribution of this resistance was exhibited among clinical isolates *S. aureus* during this period. The present rate of 34.02% in the tested Bulgarian isolates (both of CA and HA) is similar to 40.2% reported in Central Greece [13], up to 48.4% in HIV positive patients in

Ethiopia [21], 64.1% in Hospitals in China [22], 79% in a tertiary hospital in Ismir during 2011–2012 [26] and 91% in Tokyo, Japan [27]. The resistance to MLSB antibiotics (per EUCAST) in Bulgarian clinical *S. aureus* isolates has not increased as rapidly in recent years but varied from of 30% in 2014 [19] to a higher incidence of 34% in 2018–2019, with an increasing trend during the last 5 years of the investigation. Another problem that was found was that the most prevalent mechanisms of resistance to this group of antimicrobials in the tested strains were *erm* genotypes, which confer a higher level of resistance, including clindamycin, which is important for severe soft tissue infections (Tables 2 and 3). These results are in contrast to data from Japan, where iMLS prevailed [27], but are in concordance with other reports from Korea [28], Uruguay [29] and some countries in Europe [30]. Our results agree with data from various studies that the most prevalent macrolide resistance determinants are *ermC* followed by *ermA,* and more rarely by *ermB* alone or in combinations; and that *msrA* and *mefA* were either missing or present in an insignificant number of macrolide resistant isolates [13, 20, 23, 26, 28]. Some authors reported that none of the MRSA isolates had the *ermB* gene [25] but the present study did not confirm that. The results from our work (Table 3) confirm some data from a recent report from a public hospital in Uruguay [29] and a previous European multicenter study [30] about significant predominance of *ermA* alone in cMLS isolates and *mefA* in iMLS respectively. However, in contrast to other previous data, we observed a similar association of cMLS with carriage of *ermB* only or combinations of *ermA*+*ermC* and *ermA*+*ermB* ($P < 0.05$).

Two theoretical models about the interface of virulence and resistance to antimicrobials have been reported [15]. One mechanism is the horizontal transfer of genetic elements for virulence and antimicrobial resistance, often facilitated by biofilm formation, especially in patients with chronic infections. Enhancing virulence and the emergence of new antibiotic resistance can arise almost simultaneously. This can lead to major changes in gene expression, increasing virulence, and fast acquisition of antimicrobial agent resistance [15]. In the second mechanism, in concordance with the model of negative association between high resistance to antimicrobials and low virulence [15], some experiments demonstrated activation in MRSA due the presence of *mecA* and subsequent changes in the virulence of strains, which become with low-level toxicity [31]. The present results of predominance of profiles with a smaller number of gene combinations in macrolide resistant *S. aureus* isolates can be attributed to the second type of virulence – resistance interface.

CONCLUSION

Accurate detection of the resistance mechanisms to antimicrobials and regional information about the virulence traits of staphylococcal etiological agents, especially of life-threatening infections is very important and useful for the right choice of complex treatment, which is extremely important for saving patients' lives.

DISCLOSURE

The authors declare no conflict of interest.

ETHICAL APPROVAL

Not acquired.

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