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## Correlation of methicillin resistance and virulence genes of *Staphylococcus aureus* with infection types and mode of acquisition in Sofia, Bulgaria

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### Abstract:

**Background:** Infections due to methicillin resistant *Staphylococcus aureus* (MRSA) which is the most virulent species among the staphylococci have become a global health challenge. The aim of this study was to assess the correlation of genes encoding virulence and methicillin resistance in invasive and non-invasive isolates from inpatients/outpatients with staphylococcal infections in Sofia, Bulgaria.

**Materials and methods:** Non-duplicate *S. aureus* isolates were recovered from clinical samples obtained from a total of 368 in-patients with healthcare-associated infections and outpatients with community acquired infections, following overnight cultures of samples on Columbia agar with 5% sheep blood at 35°C. The isolates were presumptively identified by colony and Gram stain morphology, positive catalase reaction and plasma-coagulase test. Isolates were screened for methicillin resistance by the cefoxitin disk method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocol. The *mecA* and *mecC*, and 12 staphylococcal virulence genes were detected by a combination of monoplex and multiplex polymerase chain reaction (PCR) assays.

**Results:** The prevalence of MRSA based on carriage of *mecA* gene was 12%; 7.7% for outpatients and 16.2% for inpatients ( $p < 0.05$ ). The frequency of toxin genes detection in the staphylococcal isolates were as follows; *sei* (72.6%), *seb* (59.8%), *seh* (41.3%), *sec* (38.3%), *seg* (37.5%), *sej* (32.3%), *sea* (26.6%), *sed* (10.3%), *tst* (6.5%), and *see* (4.3%). The virulence genes, *tst*, *sea*, *seb*, *sec*, *seg*, *seh* and *sei* were more frequently associated with MRSA than methicillin sensitive (MSSA) strains ( $p < 0.05$ ). About one-third of the clinical *S. aureus* isolates harbored seven virulence genes; *sea*, *seb*, *sec*, *see*, *seg*, *seh* and *sei*, that were detected significantly more among the invasive isolates ( $p < 0.05$ ).

**Conclusions:** This study shows the occurrence of highly virulent staphylococcal isolates in our geographical region.

**Key words:** *Staphylococcus aureus*, virulence, methicillin resistance

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## Corrélation des gènes de résistance à la méthicilline et de virulence de *Staphylococcus aureus* avec les types d'infection et le mode d'acquisition à Sofia, Bulgarie

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### Abstrait:

**Contexte:** Les infections dues à *Staphylococcus aureus* résistant à la méthicilline (SARM) l'espèce la plus virulente parmi les staphylocoques, sont devenues un problème de santé mondial. Le but de cette étude était

d'évaluer la corrélation des gènes qui codent pour la virulence et la résistance à la méthicilline avec des isolats invasifs ou non invasifs de patients hospitalisés/ambulateurs infectés par le staphylocoque dans Sofia, Bulgarie.

**Matériels et méthodes:** Des isolats de *S. aureus* non dupliqués ont été récupérés à partir d'échantillons cliniques prélevés chez 368 patients atteints d'infections associées aux soins de santé et de patients ambulatoires présentant des infections acquises en communauté, après avoir effectué des cultures pendant la nuit d'échantillons sur de la gélose Columbia contenant 5% de sang de mouton à 35°C. Les isolats ont été présumés identifiés par la morphologie de la colonie et de la coloration de Gram, la réaction positive à la catalase et le test plasma-coagulase. Les isolats ont été criblés pour la résistance à la méthicilline par la méthode de la céfoxitine selon le protocole du Comité européen sur les tests de sensibilité aux antimicrobiens (EUCAST). Les gènes *mecA* et *mecC*, ainsi que 12 gènes de virulence staphylococcique ont été détectés par une combinaison de tests de réaction en chaîne de la polymérase (PCR) monoplex et multiplex.

**Résultats:** La prévalence de SARM basée sur le portage du gène *mecA* était de 12%; 7,7% pour les patients ambulatoires et 16,2% pour les patients hospitalisés ( $p < 0,05$ ). La fréquence de détection des gènes de toxines dans les isolats de staphylocoques était la suivante: *sei* (72,6%), *seb* (59,8%), *seh* (41,3%), *sec* (38,3%), *seg* (37,5%), *sej* (32,3%), *sea* (26,6%), *sed* (10,3%), *tst* (6,5%) et *see* (4,3%). Les gènes de virulence, *tst*, *sea*, *seb*, *sec*, *seg*, *seh* et *sei* étaient plus fréquemment associés à SARM que les souches sensibles à la méthicilline (MSSA) ( $p < 0,05$ ). Environ un tiers des isolats cliniques de *S. aureus* portaient sept gènes de virulence; *sea*, *seb*, *sec*, *see*, *seg*, *seh* et *sei*, qui ont été détectés significativement plus parmi les isolats invasifs ( $p < 0,05$ ).

**Conclusion:** Cela crée un risque de propagation d'isolats très virulents dans la région géographique

**Mots-clés:** *Staphylococcus aureus*, virulence, résistance à la méthicilline

## Introduction:

*Staphylococcus aureus* is a facultative anaerobic gram-positive coccus, with grape-like arrangement. This bacterial species is a clinically relevant human and animal pathogen. In humans, *S. aureus* is a major cause of both community acquired (CA) and nosocomial or healthcare-associated (HA) infections such as skin, soft tissue and bone infections, postoperative wound infections, upper respiratory tract infections, pneumonia, bacteraemia and endocarditis (1-5). *S. aureus* is also among the most common pathogens causing food-borne diseases (6-10) and is associated with suppurative diseases in animals such as mastitis, septic arthritis, endocarditis, osteomyelitis, urinary tract infections and sepsis (7, 11, 12).

In many cases of staphylococcal infections, the clinical manifestations are associated with production of toxins, of which the most notable ones are toxic shock syndrome toxin 1 (TSST-1), heat-stable pyrogenic enterotoxins (SEs) responsible for food poisoning with 20 serologically distinct types, and epidermolytic (exfoliative) toxins (ET) responsible for scalded skin syndrome (SSS), occurring in many forms as ETA, ATB and ATD (6, 13-16). The most severe invasive and fatal infections caused by *S. aureus* are due to the cumulative effects of various extracellular products such as enzymes (thermonuclease, hyaluronidase, lipase), haemolysins/cytolysins, and other toxins, especially those exhibiting super-antigen activity (3, 6, 13).

The super-antigens (SAGs), TSST-1 and SEs, which stimulate polyclonal proliferation of T lymphocytes and non-effective immune responses are the most important toxigenic determinants and

virulence mediators in *S. aureus* infections (6, 10, 17, 18).

Methicillin resistant *S. aureus* (MRSA) can form a strong biofilm and easily colonize mucosa, endovascular catheters and/or implants, which is one reason why it causes chronic, recurrent or invasive nosocomial infections (4, 19, 20). This aim of this study is to assess the correlation between methicillin resistance, and virulence genes encoding predominantly SAGs, with invasive or non-invasive and CA or HA types of infection due to *S. aureus*.

## Materials and methods:

### Study setting and isolation of *S. aureus*

This study was conducted in three University Hospitals in Sofia, Bulgaria during the period September 2016 and November 2018. A total of 368 non-duplicate *S. aureus* were isolated from various human clinical samples obtained from in-patients with HA infections (185 isolates) and outpatients with CA infections (183 isolates), after overnight cultures of clinical samples on Columbia agar with 5% sheep blood (Himedia, India) at 35°C in aerobic atmosphere.

*S. aureus* was presumptively identified by colony and Gram stain morphology, positive catalase reaction and plasma-coagulase test (Rabbit Plasma, Himedia, India). Detailed biochemical identification, where necessary, was done with Crystal GP (Beckton Dickinson, 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>) (21). The isolates were stored in skim milk at -70°C until use.

Prior to the commencement of genotypic characterization, the isolates were sub-cultured three times on Brain Heart Infusion (BHI) agar (Himedia, India).

#### DNA extraction

Genomic DNA of pure cultures of isolates and control strains grown overnight on BHI agar (Himedia, India) at 35°C in O<sub>2</sub> containing atmosphere, were extracted using a DNAso<sub>2</sub>-AM nucleic acid extraction kit (AmpliSens) in accordance with the manufacturer's instructions.

#### Polymerase chain reaction (PCR) assay

All phenotypically identified *S. aureus* strains were confirmed by PCR assay using species-specific primers targeting *Sau* 327 and *Sau* 1645 23S rRNA genes (8, 12). All confirmed *S. aureus* strains were then screened for carriage of *mecA* and *mecC* genes which encode abnormal penicillin binding proteins of the staphylococcal cell wall of MRSA, and for 12 virulence genes using primers as shown in Table 1. The primer sequences were verified for specificity using the Basic Local Alignment Search Tool (BLAST) program available at the NCBI website, Bethesda, Maryland, USA (<http://www.ncbi.nlm.nih.gov/BLAST>).

PCR was performed in a 25 µl reaction mix. DNA was amplified after optimization of the reaction using the following protocol; initial denaturation at 95°C for 3 minutes followed by denaturation at 95°C for 35 s; annealing at 54-63°C depending on primers (Table 1), extension at

72°C for 1 minute 30 s; and final extension at 72°C for 7 minutes. Prior to multiplex PCR assay, each gene was individually amplified (monoplex PCR). After successful reaction and visualization of amplicons in the monoplex PCR, mixes with two or more pairs of primers were prepared and amplifications were repeated by multiplex PCR.

Four sets of multiplex PCR were used to detect the genes of interest with the following mixes; Mix I contain primers for *hlg*, *sea*, *sed* and *tst* genes; Mix II contains primers, *Sau* 327 and *Sau* 1645 for specific *S. aureus* 23S rRNA gene, and primers for enterotoxin *seg*; Mix III contains primers for *seh*, *see* and *sej* genes; and Mix IV contains primers for *cna*, *seb*, *sec*, and *sei* genes. Monoplex PCR was used for Mix V and Mix VI which contains primers for *mecA* and *mecC* respectively, according to the criteria and protocols (4, 22) presented in Table 1.

*S. aureus* ATCC 29213 was used as a negative control strain for methicillin resistance (MSSA), and *S. aureus* ATCC 43300 and ATCC BAA2312 were used as positive control strains (MRSA) for *mecA* and *mecC* respectively. The amplified genes were separated on 1.5% agarose gel for 25-35 minutes at 140V, stained with ethidium bromide (0.5µg/mL) and visualized by UV trans-illumination (wavelength 312 nm). The PCR products were identified on the basis of their expected fragment size as presented in Table 1.

Table 1: The primers sequences and amplification conditions for detection of virulence genes of Bulgarian human isolates of *Staphylococcus aureus*

Primers	Oligonucleotide sequence (5'-3')	Annealing	Product (bp)	Reference
<i>Sau</i> 327	GGACGACATTAGACGAATCA-			
<i>Sau</i> 1645	CGGGCACCTATTTCTATCT-	63°C	1318	12
<i>hlg</i> F	GCCAATCCGTTATTAGAAAATGC			
<i>hlg</i> R	CCATAGACGTAGCAACGGAT	54°C	937	18
<i>cna</i> F	AGTGGTTACTAATACTG			
<i>cna</i> R	CAGGATAGATTGGTTTA	55°C	744	18
<i>tst</i> F	GTTGCTTGCGACAACCTGCTACAG			
<i>tst</i> R	TCAAGCTGATGCTGCCATCTGTG	54°C	209	18
<i>sea</i> F	ATGGTTATCAATGTGCGGGTG			
<i>sea</i> R	TGAATACTGTCTTGTGAGCACCA	54°C	344	18
<i>seb</i> F	TGGTATGACATGATGCCTGCAC			
<i>seb</i> R	AGGTAAGTCTATAAGTGCCTGCCT	55°C	196	18
<i>sec</i> F	GATGAAGTAGTTGATGTGTATGGATC			
<i>sec</i> R	AGATTGGTCAAACCTTATCGCCTGG	55°C	399	18
<i>sed</i> F	CTGAATTAAGTACCCGCGCT			
<i>sed</i> R	TCCTTTTGCAAAATAGCGCCTTG	54°C	451	18
<i>see</i> F	AGGTTTTTTCACAGGTCATCC			
<i>see</i> R	CITTTTTTCTCGGTCAATC	55°C	209	8
<i>seg</i> F	AATTATGTGAATGCTCAACCCGATC			
<i>seg</i> R	CTTATATGGAACAAAAGGTAAGTTT	63°C	642	8
<i>seh</i> F	CAATCACATCATATGCGAAAGCAG			
<i>seh</i> R	CATCTACCCAAAACATTAGCACC	55°C	376	8
<i>sei</i> F	CTCAAGGTGATATTGGGTAGG			
<i>sei</i> R	AAAAAACTTACAGGCAGTCCATCTC	55°C	577	8
<i>sej</i> F	CATCAGAAGTGTGTTCCGCTAG			
<i>sej</i> R	CTGAATTTTACCATCAAAGGTAC	55°C	142	8
<i>mecA-F</i>	TCC AGA TTA CAA CTT CAC CAG G			
<i>mecA-R</i>	CCA CTT CAT ATC TTG TAA CG	57°C	162	4
<i>mec C-F</i>	TGTTGTAGCAATGTTTCACAC			
<i>mec C-R</i>	CAAGCACCTTAATATCAACGC	59°C	138	22

### Statistical analysis

All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, United States). Results were considered statistically significant at  $p < 0.05$ .

### Results:

Of the 368 Bulgarian *S. aureus* isolates, 168 (45.7%) invasive isolates were obtained from sterile body sites; 34 from blood cultures, 88 from abscesses or soft tissue infections, 14 from middle ear fluids, 13 from sinus punctures, 12 from tracheal aspirates, 4 from joint aspirates, and 3 from bones in cases with osteomyelitis. Two hundred (54.3%) non-invasive isolates were recovered from 109 nasal/nasopharyngeal secretions, 58 from skin lesions (impetigo, furunculosis), 24 from uro-genital tract, and 9 from eye secretions (Table 2).

A total of 44 out of the 368 *S. aureus* were methicillin resistant, giving a MRSA prevalence rate of 12%. All the phenotypic MRSA strains were positive for *mecA* gene but none contained *mecC* gene. Only 7.7% (14 of 368) MRSA were isolated from outpatients, which was significantly less ( $p < 0.05$ ) compared to those from inpatients (16.2%, 30 of 368) (Table 3). The distribution of virulence genes in both MRSA and MSSA isolates as well as among inpatients with HA infection, and outpatients with CA infections, is presented in Table 3.

The most prevalent virulence gene in all the groups was *hlg*, which encodes a pore-forming gamma-toxin with a wide-spectrum cytolytic effect and found in approximately 100% of the isolates. There was no

significant difference in the frequency of *hlg* gene among patients with CA and HA infections (Table 3), and among invasive and non-invasive infections (Tables 4), but it was dominant in MSSA isolates ( $p < 0.05$ ) (Table 3). The virulence genes; *tst*, *sea*, *seb*, *sec*, *seg*, *seh* and *sei* (except *hlg*) were detected more frequently in MRSA than MSSA isolates ( $p < 0.05$ ) (Table 3). Only two genes, *seb* and *sec*, were predominantly observed in relation to staphylococcal inpatient infections ( $p < 0.05$ ).

MRSA strains were dominant (with 63.6%) among the invasive isolates compared to 36.4% among the non-invasive ones ( $p < 0.05$ ) (Table 4). The SAGs encoding genes; *sea*, *seb*, *sec*, *see*, *seg*, *seh*, and *sei* were detected more frequently among the invasive isolates ( $p < 0.05$ ) (Table 4). In addition, the *cna* gene, encoding a bacterial collagen adhesin with an inhibitory effect on complement activation, was detected in 14.2% of the *S. aureus* isolates, predominantly among in-patients and in invasive infections ( $p < 0.05$ ).

The distribution of the staphylococcal toxin genes detected in this study compared with data from other countries is shown in Table 5. The frequency of virulence genes detected in this study are; *sei* (72.6%), *seg* (67.5%), *seb* (60.3%), *seh* (41.3%), *sec* (38.3%), *sej* (32.3%), *sea* (26.6%), *sed* (10.3%), *tst* (6.5%), and *see* (4.3%). Fig 1 shows the gel electrophoresis picture of the amplified products of *S. aureus mec* and virulence genes in the 4 multiplex PCR reactions (Mixes I-IV) and the two monoplex PCR reactions (Mixes V and VI).

Table 2: Distribution of *Staphylococcus aureus* isolates by clinical specimen types

Specimen types/isolates	No of isolates (%)
Invasive isolates	168 (45.7)
Blood culture	34 (9.2)
Abscess/Soft tissue	88 (23.9)
Middle ear fluid	14 (3.8)
Sinus punctures	13 (3.5)
Trachea aspirates	12 (3.3)
Joint aspirates	4 (1.1)
Bone samples	3 (0.8)
Non-invasive isolates	200 (54.3)
Nasopharyngeal secretion	109 (29.6)
Skin lesion	58 (15.8)
Urogenital tract	24 (6.5)
Eye secretion	9 (2.4)
Total	368 (100)

Table 3: The frequency of virulence genes among MRSA and MSSA isolates from inpatients and outpatients

Genes	MRSA (n=44)			MSSA (n=324)			p value (between MRSA and MSSA)	Total number of <i>S. aureus</i> isolates (n=368)			
	Inpatient % (n=30)	Outpatient % (n=14)	Total (%)	Inpatient (%) (n=155)	Outpatient % (n=169)	Total (%)		Inpatient % (n=183)	Out-patient (%) (n=185)	p value (between Inpatient and Out-patient)	Total (%)
<i>hlg</i>	93.3	100	88.6	98.1	98.8	98.5	<b>0.0031*</b>	95.7	98.9	0.1046	97.3
<i>cna</i>	33.3	0	22.7	23.2	3.6	13.0	0.1035	24.8	3.2	<b>0.0001</b>	14.2
<i>tst</i>	20.0	28.6	22.7	4.5	4.1	4.3	<b>0.0001</b>	7.0	6.0	0.8334	6.5
<i>sea</i>	53.3	85.7	63.6	23.9	19.5	21.6	<b>0.0001</b>	28.6	24.6	0.1257	26.6
<i>seb</i>	86.7	100	90.9	66.5	46.7	56.2	<b>0.0001</b>	69.7	50.8	<b>0.0003</b>	60.3
<i>sec</i>	66.6	57.1	63.6	49.7	21.3	34.9	<b>0.0004</b>	52.4	29.5	<b>0.0004</b>	38.3
<i>sed</i>	10.0	7.1	9.1	12.3	8.3	10.5	1.0000	12.4	8.2	0.2304	10.3
<i>see</i>	0	0	0	7.7	2.4	4.9	0.2350	6.5	2.2	0.0706	4.3
<i>seg</i>	86.7	100	90.9	66.5	69.8	64.2	<b>0.0002</b>	69.7	65.0	0.3741	67.4
<i>she</i>	53.3	85.7	63.6	43.2	33.7	38.3	<b>0.0018</b>	44.9	37.7	0.1703	41.3
<i>sei</i>	90.0	100	93.2	69.7	69.8	69.8	<b>0.0005</b>	73.0	72.1	0.9072	72.6
<i>sej</i>	33.3	50.0	38.6	34.2	29.0	31.5	0.3908	34.0	30.6	0.5049	32.3

\*Results were considered statistically significant at  $p < 0.05$  - in dark field

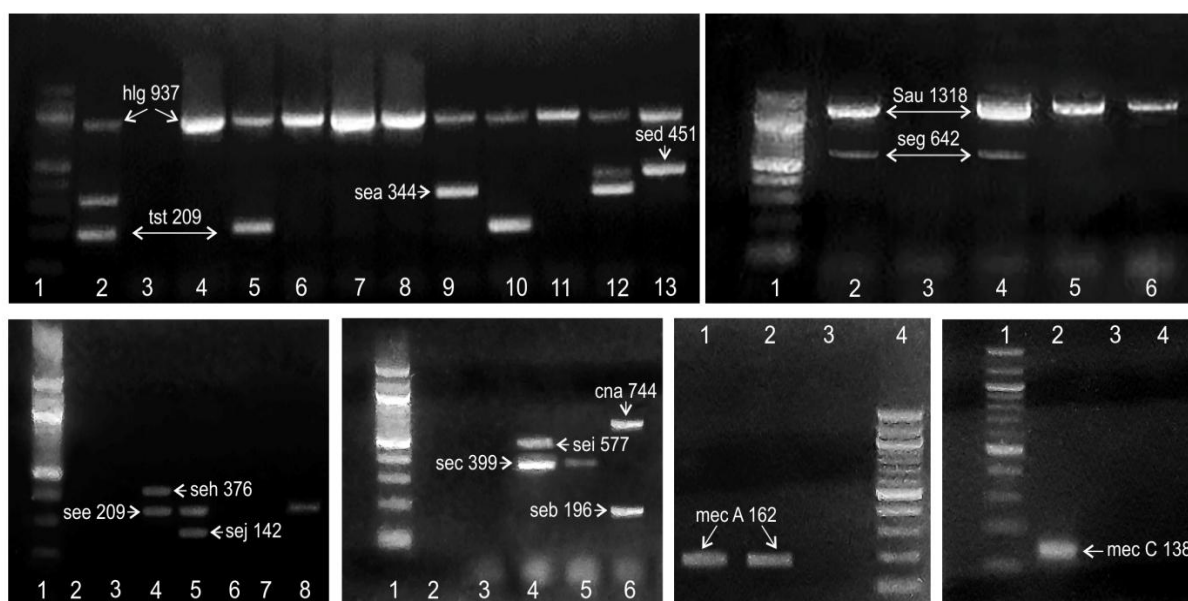
Table 4: The frequency of virulence genes among MRSA and MSSA invasive and noninvasive isolates

Genes	MRSA (n=44)			MSSA (n=324)			p value (between invasive and non-invasive)	Total number of <i>S. aureus</i> isolates (n=368)		
	Invasive isolates % (n=28)	Non-invasive isolates % (n=16)	Total (%)	Invasive isolates (%) (n=140)	Non-invasive isolates % (n=184)	Total (%)		Invasive isolates % (n=168)	Non-invasive isolates % (n=200)	Total (%)
<i>hlg</i>	92.9	81.25	88.6	100.0	78.3	98.5	98.8	96.0	0.1179	97.3
<i>cna</i>	35.7	0	22.7	25.7	3.3	13.0	24.8	3.0	<b>0.0001*</b>	14.1
<i>tst</i>	28.6	12.5	22.7	5.7	3.3	4.3	9.5	4.0	<b>0.0358</b>	6.5
<i>sea</i>	60.7	68.75	63.6	24.3	19.6	21.6	30.4	23.5	0.1159	26.6
<i>seb</i>	96.4	81.25	90.9	72.1	44.0	56.2	76.2	47.0	<b>0.0001</b>	60.3
<i>sec</i>	71.4	50.0	63.6	48.6	24.5	34.9	52.4	26.5	<b>0.0001</b>	38.3
<i>sed</i>	7.1	12.5	9.1	16.4	6.0	10.5	14.9	6.5	<b>0.0098</b>	10.3
<i>see</i>	0	0	0	8.6	2.2	4.9	7.1	2.0	<b>0.0201</b>	4.3
<i>seg</i>	85.7	100.0	90.9	77.9	53.8	64.2	79.2	57.5	<b>0.0001</b>	67.4
<i>seh</i>	57.1	85.0	63.6	43.2	31.0	38.3	49.4	34.5	<b>0.0042</b>	41.3
<i>sei</i>	100.0	81.25	93.2	47.9	64.1	69.8	81.0	65.5	<b>0.0010</b>	72.6
<i>sej</i>	34.1	50.0	38.6	37.9	26.6	31.5	36.9	59.5	0.0940	32.3

\*Results were considered statistically significant at  $p < 0.05$  - in dark field

Table 5: Distribution of staphylococcal virulence genes (SAGs) among *S. aureus* isolates from Bulgaria and other countries

Virulence genes (SAGs)	Bulgaria (this study) %	Turkey <sup>3,23</sup> %	Czech Republic <sup>21</sup> %	France <sup>32</sup> %	Colombia <sup>16</sup> %	Canada <sup>35</sup> %	China <sup>30</sup> %	India <sup>20</sup> %	Korea <sup>33</sup> %	Iran <sup>25,26</sup> %
<i>tst</i>	6.5	27.9	2	0-38	6.2	24.3	48.1	24.3	52.6	25.0
<i>sea</i>	26.6	4.6-13.5	12	6-34	11.1	19.6	44.4	38.6	47.4	40.6
<i>seb</i>	59.8	8.7	3	0-31	9.9	5.6	6.48	26.8	0	25.0
<i>sec</i>	38.3	4.0-52.9	2	0-19	16.0	7.5	9.26	0		0
<i>sed</i>	10.3	4.8	17	0-14	3.7	1.9	5.56	0	0	0
<i>see</i>	4.3	0	0	0	3.2	0	0	0	0	0
<i>seg</i>	67.4	24.6-89.4	77	75	51.9		40.7		61.1	27.8
<i>seh</i>	41.3	35.6	0	0-20	35.8		1.85			
<i>sei</i>	72.6	51.3-65.4	77	75	44.4		14.8		70.5	
<i>sej</i>	32.3	0.66-22.1	17		3.7		13.9			

Fig 1: Gel electrophoresis of the PCR products of *Staphylococcus aureus* virulence and *mec* genes (Amplicons from mixes I, II, III, IV, V, VI)

## Discussion:

There are wide differences in the reported geographical distribution of MRSA, from 2.9% to 8.7 % in Turkish nasal carriers and respiratory tracts of ill persons (3, 23), up to 38.7% in Colombia (16), 39.6% in India (24), 41-48% in Iran (25, 26) and 44% in Nigeria from animal samples (27). The methicillin resistance rate observed in this

study was 12%, with 7.7% from outpatients and 16.2% from inpatients. The prevalence of MRSA is known to vary in relation to where the infection was acquired, whether in the community (CA) or in healthcare (HA) setting (3, 4, 5, 19, 20).

In almost all the *S. aureus* isolates in our study, we found the *hlg* gene in the genome of the organism in both MSSA and MRSA (though more frequent in MSSA),

invasive and non-invasive, and CA and HA isolates. This gene is responsible for the synthesis of extracellular toxic polypeptide (gamma-haemolysin) with strong cytolytic activity against a broad spectrum of eukaryotic cells (erythrocytes, neutrophils, monocytes, lymphocytes, macrophages) and non-specific activity against intracellular membrane structures in various mammalian cells with detergent-like action (28). Available data suggest that *hlg* extracellular product contributes to immune evasion and potential staphylococcal survival in biological fluids. In high concentration, this bi-component pore-forming toxin has dermo-necrotic and lethal activity in experimental animal model and is being studied as a potential component of a multivalent vaccine (28). Our results are similar to data from the USA and Iran reporting high prevalence of *hlg* among MRSA and MSSA isolates (25, 29).

In contrast, the *cna* gene was detected in only about 14% of the staphylococcal isolates. *S. aureus* can produce collagen adhesin (CNA) to interact with collagen in humans and animals. This adhesin plays a leading role in colonization, prolongation and persistence of *S. aureus* infection in human host. Another mechanism of CNA action is inhibition of complement activation. In experimental animal models, progression of bacterial infections as a result of staphylococcal CNA or other adhesins such as YadA of *Yersinia enterocolitica*, *Enterococcus faecalis* adhesin ACE, *E. faecium* adhesin ACM, and *Streptococcus mutans* adhesin CNM, have been established (30). The frequent correlation of *cna* with invasive isolates predominantly in inpatients suggests *cna* encodes a product with high and specific virulent potential.

The combinations of three genes, *sei*, *seg* and *seb*, which codes for strong super-antigens (SAGs) were detected in more than 60% of the Bulgarian staphylococcal isolates tested in this study, predominantly among invasive isolates. About one-third of the clinical *S. aureus* isolates harbored seven genes, *sei*, *seb*, *seh*, *sec*, *seg*, *sej*, and *sea*, encoding super-antigenic toxins, and as many as 25% of the isolates carried at least nine genes while nearly 40% carried at least six virulence genes. This high carriage rate put the populace at risk of infections from highly virulent *S. aureus* strains in our geographical region.

The frequency of SAGs gene detection reported varies from countries to countries as shown in Table 5. The high prevalence of *sei* and *seg* genes in our study is similar to what has been reported in Turkish paediatric patients and healthy students (3, 23), and in Czech Republic, France, Colombia, Mexico and Korea (16, 20, 31, 32, 33) but data from

India, China, Iran and Canada present *sea* as the most frequent SAGs gene (24, 25, 34, 35).

Among the MRSA isolates in our study, the genes encoding SEs that were dominant with high frequency (63.6% to 93.2%) and significantly associated with methicillin resistance ( $p < 0.05$ ) were *sea*, *seb*, *sec*, *seg*, *seh* and *sei*. This is similar to the study of Ortega et al., (5) who reported *sea*, *seb*, *sed*, *seg*, *sei* and *sej* genes as the most prevalent in their MRSA isolates. However, they detected *tst-1* gene more frequently among their MSSA isolates, whereas in our study, *tst-1* gene was more frequently detected in the MRSA isolates. The prevalence of *tst-1* gene in the *S. aureus* isolates in the present study and from other European countries as shown in Table 5 is low compared to the prevalence in China and Korea (about 50% of isolates). The *tst-1* gene was detected equally in isolates from inpatients and outpatients ( $p > 0.05$ ) but was more frequently detected in the invasive staphylococcal isolates ( $p < 0.05$ ). The prevalence of both *seb* and *sec* genes, which are the most frequently detected genetic elements in patients with HA *S. aureus* infections and in invasive disease, was significantly higher ( $p < 0.05$ ) in our study than from other countries.

The genetic profile of the invasive *S. aureus* isolates was different in the present study with more virulence genes detected compared to non-invasive isolates ( $p < 0.05$ ). The genes mainly detected in the invasive staphylococcal isolates were *cna*, *tst-1*, *seb*, *seh*, *sec*, *sed*, *see*, *seg*, *seh* and *sei* in various combinations. Some of these genes have been reported to correlate with nosocomial and invasive infections (16, 20, 24). In a Colombia study (16), five SAG genes out of 22 tested genetic elements were found in invasive isolates. Some of the genes, *sei*, *sea* and *seg*, most frequently detected in our study were reported to be harbored by more than 33% of *S. aureus* isolates in patients who died from staphylococcal bacteraemia (24).

## Conclusion:

The prevalence of MRSA in the Bulgarian human staphylococcal isolates in our study was 12%, and the strains were recovered mainly from patients with HA infections. More than 60% of the staphylococcal isolates carried *sei*, *seg* and *seb* genes which encode strong Sags predominantly among the invasive isolates, in addition to the *hlg* gene which encode a pore-forming toxin. Furthermore, approximately one-third of the clinical *S. aureus* isolates

harbored seven (*sei*, *seb*, *seh*, *sec*, *seg*, *sej* and *sea*) out of the twelve virulence genes encoding super-antigen toxins, in addition to the *hlg* gene. The results from the present study showed that the genome of clinical *S. aureus* isolates in Bulgaria contain wide array of genes encoding immuno-modulatory molecules. This may put the populace at risk of acquisition and spread of highly virulent *S. aureus* strains in our geographical region.

### Conflict of interest:

No conflict of interest is declared

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