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Molecular analysis of *Staphylococcus aureus* resistance patterns encountered in a Romanian hospital from Bucharest, Romania

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ABSTRACT

Staphylococcus aureus is one of the major causes of community-acquired and hospital-acquired infections. The study objective was the analysis of antibiotic resistance genes in *S. aureus* strains isolated from hospitalized patients. The study was conducted on 20 *S. aureus* strains isolated from hospitalized patients with various cardiovascular diseases in the Microbiology Laboratory at the Emergency Institute for Cardiovascular Diseases "Prof. Dr. C.C. Iliescu" from different clinical sources, between 2014 and 2015. The results obtained from the PCR arrays showed that 8 strains possessed the *ccrB2* gene, 7 isolates the *ccrC* gene and only 1 strain expressed the *mecI* gene. The presence of the *SCC cassette* type IV which is normally associated with hospital infections in nearly 50% of the strains analyzed suggests the transfer of the staphylococcal cassettes together with resistance genes from community to nosocomial infections.

Keywords: *SCCmec cassette, nosocomial infections.*

1. INTRODUCTION

Staphylococcus aureus is one of the major causes of community-acquired and hospital-acquired infections [1, 2, 3, 4]. It produces numerous toxins including superantigens that cause a wide variety of afflictions including toxic-shock syndrome and staphylococcal scarlet fever and has acquired resistance to most commonly used antibiotics [5, 6, 7, 8, 9]. Its genetic diversity has facilitated the evolution of many virulent and drug-resistant strains [10, 11, 12, 13]. Comparative-genomics studies explored the mechanisms implicated in the evolution of the *S. aureus* genome and identify regions that influence virulence and drug resistance [14, 15, 16, 17].

First reported in a British hospital, MRSA clones are rapidly spreading across international borders.

The MRSA clones often account for an increasing percentage of nosocomial infections [18, 19, 20, 21, 22].

The impact on human health of *S. aureus* infections in community and hospital settings has lead to intensive investigation of this organism over recent years [23, 24, 25, 26].

The study objective was the analysis of antibiotic resistance genes in *S. aureus* strains isolated from patients diagnosed in the Emergency Institute for Cardiovascular Diseases "Prof. Dr. C.C. Iliescu".

2. EXPERIMENTAL SECTION

The study was conducted on 20 *S. aureus* strains isolated from hospitalized patients with various cardiovascular diseases in the Microbiology Laboratory at the Emergency Institute for Cardiovascular Diseases "Prof. Dr. C.C. Iliescu" from different clinical sources, between 2014 and 2015.

The isolation sources were represented by: nasal exudates, respiratory secretions, blood cultures, peritoneal fluids, pleural fluids, tracheal aspirates, pharyngeal exudates and venous catheter in the case of one strain. The strains identification was performed at the Prof. "C.C. Iliescu" Institute with the use of the coagulase test, the Mannitol enriched medium and the VITEK 2 system.

The susceptibility analysis was performed by Kirby -Bauer diffusion method, following the recommendations of CLSI editions 2013 and 2014.

The bacterial DNA was extracted using the alkaline extration method. Between one and five colonies of bacterial cultures were suspended in 1.5 ml tubes containing 20 µL solution of NaOH (sodium hydroxide) and SDS (sodium dodecyl sulphate).

For the permeabilization of the cell membranes the tubes were heated on a thermoblock at 95°C for 5 minutes. The following step was the addition of 180 µL TE buffer (Tris + EDTA)1X in the tubes and centrifugation at 13000 rpm for 3 minutes. The DNA in the supernatant was stored at -4°C before analysis. All PCR reactions were performed using the Thermal Cycler machine Corbet. The amplification products were visualized by electrophoresis on a 2% agarose gel, stained with ethidium bromide (10 µg / ml) and identified by comparison with the specific molecular weight marker (100pb, I Lader Bench Top, Promega, USA).

The analysis of *SCCmec* cassette types was performed using PCR methods (simplex and multiplex) in order to elucidate the presence of these constituent genetic elements. Two PCR multiplex reactions were performed using the five and four pairs

of specific primers for different components of the *SCCmec* cassette. The parameters used to conduct the reactions followed the protocol developed by Miheirico et al. [27]. The sequences of the primers used, their specificity and amplification programs used are listed in tables 1 and 2 and the components used in these reactions are shown in table 3. The PCR multiplex reactions based on four pairs of specific primers helped to distinguish between I-V *SCCmec* cassettes types and subtypes. The sequences of the primers used and the reactions parameters followed the protocol developed by Zhang et al. [28]. However, a simplex PCR reaction was used for the detection of the *ccr* genes, the *SCCmec* cassette's recombinase complex. The sequences of primers used, their specificity and the amplification programs used are listed in the 4, 5 and 6 tables and the components used in these reactions are shown in table 3.

Table 1. Nucleotide sequences of primers used, their specificity and the size of the amplicons obtained (after Milheirico et al. 2007).

Primers	Nucleotide sequence	Amplicon dimension	Primer specificity
CIF2 F2 CIF2 R2	5'-TTC GAG TTG CTG ATG AAG AAG G-3' 5'-ATT TAC CAC AAG GAC TAC CAG C-3'	495	**I**, J1 region
RIF5 F10 RIF5 R13	5'- TTC TTA AGT ACA CGC TGA ATC G-3' 5'- GTC ACA GTA ATT CCA TCA ATG C-3'	414	**III**, J3 region
ccrB2 F2 ccrB2 R2	5'-AGT TTC TCA GAA TTC GAA CG-3' 5'-CCG ATA TAG AAW GGG TTA GC-3'	311	**II** şi **IV**, *ccr* genes
mecI P2 mecI P3	5'-ATC AAG ACT TGC ATT CAG GC-3' 5'-GCG GTT TCA ATT CAC TTG TC-3'	209	**II** şi **III**, *mec* complex
mecA P4 mecA P7	5'-TCC AGA TTA CAA CTT CAC CAG G-3' 5'-CCA CTT CAT ATC TTG TAA CG-3'	162	**mecA** gene
SCCmecV J1 F SCCmecV J1 R	5'-TTC TCC ATT CTT GTT CAT CC-3' 5'-AGA GAC TAC TGA CTT AAG TGG-3'	377	**V**, J1 region
dcs F2 dcs R1	5'-CAT CCT ATG ATA GCT TGG TC-3' 5'-CTA AAT CAT AGC CAT GAC CG-3'	342	**I**, **II**, **IV** and **VI**, J3 region
kdp F1 kdp R1	5'-AAT CAT CTG CCA TTG GTG ATG C-3' 5'-CGA ATG AAG TGA AAG AAA GTG G-3'	284	**II**, J1 region
SCC mec III J1 F SCCmec III J1 R	5'-CAT TTG TGA AAC ACA GTA CG-3' 5'-GTT ATT GAG ACT CCT AAA GC-3'	243	**III**, J1 region

Table 2. PCR conditions used to amplify the SCCmec elements (after Milheirico et al. 2007).

The amplification program					
Temperature	94°C	94°C	53°C	72°C	72°C
Duration	4 min	30 sec	30 sec	1 min	4 min
Number of cycles	1	30			1

Table 3. Reaction components used in the PCR reactions.

Primers volume (10μM)	PCR volume Master Mix*	Ultra pure water Volume	ADN volume	The reaction volume
0,3 μl	10 μl	6,5 μl	0,5 μl	20 μl

Table 4. The nucleotide sequences of the primers used, their specificity and size of the amplicons produced [28].

Primers	The nucleotide sequence	Amplicon size	Specific primers (cassette mec type)
Type I-F Type I-R	5'-GCT TTA AAG AGT GTC GTT ACA GG-3' 5'-GTT CTC TCA TAG TAT GAC GTC C-3'	613	SCC*mec* I
Type II-F Type II-R	5'-CGT TGA AGA TGA TGA AGC G-3' 5'-CGA AAT CAA TGG TTA ATG GAC C-3'	398	SCC*mec* II
Type III-F Type III-R	5'-CCA TAT TGT GTA CGA TGC G-3' 5'-CCT TAG TTG TCG TAA CAG ATC G-3'	280	SCC*mec* III
Type IVa-F Type IVa-R	5'-GCC TTA TTC GAA GAA ACC G-3' 5'-CTA CTC TTC TGA AAA GCG TCG-3'	776	SCC*mec* Iva

Type IVb-F Type IVb-R	5'-TCT GGA ATT ACT TCA GCT GC-3' 5'-AAA CAA TAT TGC TCT CCC TC-3'	493	SCC*mec* IVb
Type IVc1-F Type IVc1-R	5'-TCT ATT CAA TCG TTC TCG TAT T-3' 5'-TCG TTG TCA TTT AAT TCT GAA CT-3'	200	SCC*mec* IVc
Type IVd1-F Type IVd1-R	5'-AAT TCA CCC GTA CCT GAG AA-3' 5'-AGA ATG TGG TTA TAA GAT AGC TA-3'	881	SCC*mec* IVd
Type V-F Type V-R	5'-GAA CAT TGT TAC TTA AAT GAG CG-3' 5'-TGA AAG TTG TAC CCT TGA CAC C-3'	325	SCC*mec* V
ccrC-F ccrC-R	5'-CGT CTA TTA CAA GAT GTT AAG GAT AAT-3' 5'-CCT TTA TAG ACT GGA TTA TTC AAA ATA T-3'	495	*ccr* Tip 5

Table 5. The PCR amplification conditions used for the genetic elements characteristic of SCCmec cassettes types [28].

The amplification program								
Temperature	94°C	94°C	65°C	72°C	94°C	55°C	72°C	72°C
Duration	5 min	45 sec	45 sec	1,5 min	45 sec	45 sec	1,5 min	10 min
Number of cycle	1	10			25			1

Table 6. PCR conditions used for *ccr* gene amplification [28].

The amplification of program					
Temperature	94°C	94°C	50°C	72°C	72°C
Duration	5 min	1 min	1 min	2 min	10 min
Number of cycles	1	30			1

3. RESULTS SECTION

The results obtained from the PCR arrays showed that 8 strains possessed the *ccrB2* gene, 7 isolates the *ccrC* gene and only 1 strain expressed the *mecI* gene (tabel 7, figure 1). In regard to the types of *SCCmec* cassettes our findings pointed out that 7 strains expressed the Type Iva cassette (tabel 7). Our results in relation to the presence of the *SCC cassette* type IV in 35% of cases are confirmed by the findings of Cuevas et. al. which found that 70% of the *S. aureus* isolates studied were positive for the *SCCmec*IV cassette (Cuevas et. al., 2002).

Tabel 7. Expression of resistance genes associated with the *SCCmec* cassettes.

Bacterial strains	*SCCmec* cassettes										
	ccrB2	*mecI*	*mecA*	*CIF 2*	*ccrC*	*SCCmecV J1*	*TypeIVa*	*TypeIVb*	*TypeIVc*	*TypeII*	*TypeIVd*
1											
2	+	+			+						
3	+										
4	+				+						
5	+				+		+				
6	+				+						
7	+						+				
8	+				+		+				
9	+				+		+				
10							+				
11							+				
12											
13							+				

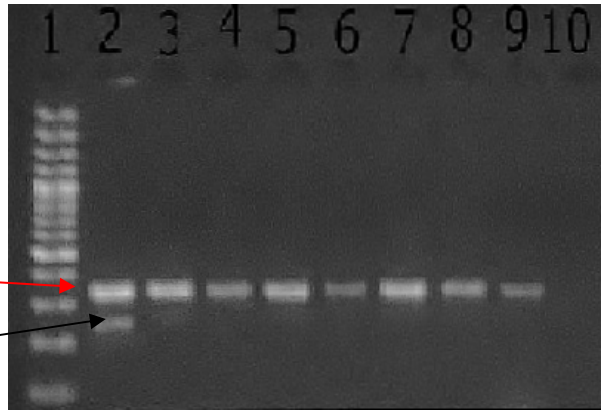

Figure 1. Electrophoresis gel with corresponding primers for elements of the *SCCmec* cassettes: *ccrB2, mecI, mecA*. The figure shows that MRSA isolates 2, 3, 4, 5, 6, 7, 8, 9 express the *ccrB* gene and only strain the second isolate presented the *mecI* gene. Well 1(top and bottom) marker gm: 100pb.

4. CONCLUSIONS

In the context of the emergence of a significant number of multi-resistant staphylococcal strains to antibiotics, the MRSA high prevalence Eastern Europe, in Romania, in 2010, between 25 and 50% of *S. aureus* strains isolated from blood cultures were methicilin resistant, understanding the mechanism responsible for this resistance is essential. The incidence of MRSA strains associated with community infection makes it even more important to analyze the horizontal transmission mechanisms of intra- and inter-species mobile genetic elements responsible and the resistance genes responsible for this resistance patterns.

The presence of the *SCC cassette* type IV which is normally associated with hospital infections in nearly 50% of the strains analyzed coupled with the high incidence of the *ccr* genes responsible the mobility of this genetic elements suggest the transfer of the staphylococcal cassettes together with resistance genes from community infections to nosocomial infections making more urgent the need to better understand the mechanism of gene transmission and the development of methods efficient in combating the emergent health problem represented by multidrug resistant MRSA strains.

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