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SUBTYPING OF CHILEAN METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* STRAINS CARRYING THE STAPHYLOCOCCAL CASSETTE CHROMOSOME *MEC* TYPE I

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ABSTRACT

The cassette chromosome *mec* (*SCCmec*) present in methicillin-resistant *Staphylococcus aureus* (MRSA) has two essential components, the *ccr* gene complex and the *mec* gene complex. Additionally, *SCCmec* has non-essential components called J regions which are used for MRSA subtyping. This study was performed to determine subtypes MRSA strains carrying *SCCmec* type I based on polymorphism of regions located downstream of the *mecA* gene. A total of 98 MRSA strains carrying *SCCmec* type I isolated from patients hospitalized at the County Hospital of Valdivia (Chile) between May 2007 and May 2008, were analyzed by multiplex PCR designed to amplify the *mecA* gene and 7 DNA hypervariable regions located around the *mecA* gene. MRSA strains were classified into seventeen genotypes accordingly to amplification patterns of DNA hypervariable regions. Five genotypes showed amplification patterns previously described. The remaining twelve genotypes showed new amplification patterns. Genotypes 18 and Genotype 19 were the most frequently detected. Regions HVR, Ins117 and pI258 stand out as being present in more than 60% of tested isolates. The acquisition of hypervariable regions by MRSA is a continuous horizontal transfer process through which the *SCCmec* have been preserved intact, or even may give rise to new types and subtypes of *SCCmec*. Therefore it is possible to infer that most MRSA strains isolated at the County Hospital of Valdivia (Chile) were originated from two local clones which correspond to Genotype 18 and Genotype 19.

Key words: Subtypified MRSA, polymorphism MRSA, *SCCmec* MRSA.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first isolated in England in 1961 shortly after the development of methicillin (8). Since then, MRSA has become the most prevalent pathogen causing hospital infection throughout the world, with increased incidence in many countries (2).

MRSA genome has integrated a mobile genetic

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element called staphylococcal cassette chromosome *mec* (*SCCmec*), which harbors the *mecA* gene responsible for methicillin resistance. This gene encodes PBP2a, an additional ß-lactam-resistant penicillin-binding protein (4). *SCCmec* is a unique genomic island found only in staphylococcal species that have two essential components, the *ccr* gene complex (*ccr*) and the *mec* gene complex (*mec*) (2, 5). The *ccr* gene complex is composed of *ccr* genes and surrounding open reading frames (ORFs). The *mec* gene complex is composed of the *mecA* gene, regulatory genes, and insertion sequences upstream or downstream of *mecA* gene (6, 7).

Remaining parts of *SCCmec* are called J regions (J1, J2 and J3), which constitute nonessential components of *SCCmec*. In some cases, these regions carry additional antibiotic resistance determinants (5). J1 is the region between the chromosomal left junction and the *ccr* complex; J2 is the region between the *ccr* complex and the *mec* complex and J3 is the region between the *mec* complex and the chromosomal right junction. Variations in the J regions are used for subtyping MRSA strains (9).

Currently, different genetic methods have been developed to be applied in molecular epidemiologic characterization of MRSA strains, being pulsed-field electrophoresis (PFGE) the technique of choice (14). On the other hand, through multiplex PCR technique it is possible to analyze the polymorphic downstream of *mecA* gene. This genetic polymorphism has been used as an epidemiological marker and has also been the basis of studies related to the evolutionary origin and subtyping of methicillin resistance in *S. aureus* (3).

The aim of this study was to determine subtypes of MRSA strains carrying *SCCmec* type I, through the implementation of a multiplex PCR that allows the detection of *mecA* gene and 7 DNA hypervariable regions located around the *mecA* gene.

MATERIALS AND METHODS

Clinical isolates

Ninety eight clinical isolates of MRSA previously typified as *SCCmec* type I and unrelated to nosocomial outbreaks were studied. All of them were isolated from patients hospitalized at the County Hospital of Valdivia (Chile) between May 2007 and May 2008. Strains phenotyping was performed using the semi-automated microbiological diagnosis system *Dried Gram Positive ID Type 2 panels* (Microscan®) and *SCCmec* genotyping was performed as described previously (17). The *mecA*-positive *S. aureus* ATCC 49476, which contains HVR, pT181, pI258, mecR1 and IS256 regions was used as control.

DNA hypervariable regions Subtyping: A single colony was taken from a Muller Hinton agar plate and suspended in 100 µL of sterile nuclease free water. The suspension was incubated at 100°C for 10 min for DNA extraction. After centrifugation at 20,000*g* for 2 min, 3 µL of the supernatant was taken and directly added to 25 µL of amplification mixture.

Oligonucleotides sequences used for the amplification of *mecA* gene and 7 DNA hypervariable regions are listed in Table 1 (3, 17).

The amplification protocols originally described by Huygens *et al*., and Wilson *et al*., were modified due to the similar size of PCR amplicon (3, 15). The analysis of each strain was performed in four individual reactions. i) The first reaction included primers to amplify *mecA* gene, pI258 (I) and mecR1 regions. ii) The second reaction included primers to amplify pI258 (II) and IS256 regions. iii) The third reaction included primers to amplify pUB110 and pT181 regions. iv) The fourth reaction included primers to amplify HVR and Ins117 regions.

The PCR mixture consisted of 3 µL of cell lysate, 0.2 mM concentrations of each deoxynucleoside triphosphate (dNTPs), 0.5 µM concentrations of each primer, 1 Uof DFS *Taq* DNA polymerase (Invitrogen®), 10X PCR buffer and 1.5 mM $MgCl_2$ contained in a total volume reaction of 25 µL. The program DNA amplification consisted of an initial cycle of 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s, with a final extension step of 72°C for 10 min. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide.

Table 1. Primers used for subtyping MRSA. The analysis of each strain was performed in four individual reactions.

Primers	Oligonucleotides sequences	PCR amplicon size (pb)	Target	GenBank accession nº.
HVRPF	TGCAACATCTAACTCCAACC	300	HVR	AF181950
HVRP2	TGGAGCTTGGGACATAAATG			
DF4	TAACATGCTGTTTTAACC	331	pUB110	M19465
MR1	TGAACGTGGCTCTGACCG			
MDVF1	GCTTGGGTAACTTATCATGG	215	Ins117	AF181950
IS117R1	CTAAATATAGTAAATTACGG			
DF1	CACGAGATGAAATGATTTGG	255	pT181	JO1964
DR1	GCATCTGCATTATCTTTACG			
DF2	ATAGAAAGGAAAAAACATGG	295	pI258 (I)	L29436
DR2	TTTATACGTAAACCAGTCGG			
EF1	CAAAGTGTAAGTAACCCG	270	pI258 (II	L29436
ER1	TATACGTAAACCAGTCGG			
AF1	TGATATGGGTATTTGG	406	mecR1	AF142100
AR1	TTTTTCACAGTCATTGTCC			
DF3	ACTAATGGAAAATCAACG	371	IS256	M18086
DR3	TTTTTTTCTGATAATAAACG			
*MecA*147F	GTGAAGATATACCAAGTGATT	147	*mecA*	X52593
*MecA*147R	ATGCGCTATAGATTGAAAGGAT			

First reaction included primers *MecA*147F - *MecA*147R, DF2-DR2, and AF1-AR1
Second reaction included primers EF1-ER1, DF3-DR3
Third reaction included primers DF4-MR1, DF1-DR1
Fourth reaction included primers HVRPF-HVRP2, MDVF1-IS117R1

RESULTS

The present study showed that all MRSA strains, previously typified as *SCCmec* type I, were classified into seventeen genotypes according to amplification patterns of DNA hypervariable regions (Table 2).

Genotypes 2, 6, 14, 15 and 16 showed amplification patterns previously described by Huygens *et al*. and Wilson *et al*. A serial number, starting with the genotype 18, was assigned to the remaining twelve new amplification patterns (Table 2).

The most frequent amplification patterns found were genotypes 18 and genotype 19 with 24,5% and 20,4% respectively (Figure 1 and Table 2). On the other hand, five strains were classified into genotype 29 which did not detect any of the DNA hypervariable regions (Table 2).

Finally, the detection percentage of DNA hypervariable regions was: HVR 92,9% - Ins117 and pI258 69,4% - IS256 46,9% - pT181 13,3% - pUB110 2%. In addition, we found that no strains included in the analysis amplified the mecR1 region.

Table 2. Classification of genotypes according to amplification patterns of DNA hypervariable regions and their frequency.

Genotype	DNA hypervariable regions							Frequency %
	HVR	pUB110	Ins117	pT181	pI258	*mecR1*	IS256	
2	+	-	+	-	-	-	-	10.2
6	+	-	-	-	-	-	-	4.0
14	+	-		+	+	-	-	2.0
15	+	-	-	-	+	-	-	8.2
16	+	-	-	-	+	-	+	4.0
18	+	-	+	-	+	-	+	24.5
19	+	-	+	-	+	-	-	20.4
20	+	-	+	+	+	-	+	6.1
21	+	-	-	-	-	-	+	5.1
22	+	-	+	+	-	-	+	3.0
23	+	-	+	-	-	-	+	2.0
24	-	-	-	-	+	-	+	1.0
25	-	+	+	-	-	-	-	1.0
26	+	+	+	-	+	-	-	1.0
27	+	-	-	+	+	-	+	1.0
28	+	-	+	+	+	-	-	1.0
29	-	-	-	-	-	-	-	5.0

98 MRSA strains isolated from patients hospitalized at the County Hospital of Valdivia (Chile), previously typified as *SCCmec* type I, were subtypified into seventeen genotypes according to amplification patterns of 7 DNA hypervariable regions. The genotypes 2, 6, 14, 15 and 16 showed amplification patterns previously described. A serial number, starting with the genotype 18, was assigned to the remaining twelve new amplification patterns. Amplification patterns corresponding to genotypes 18 and genotype 19 were the most frequent.

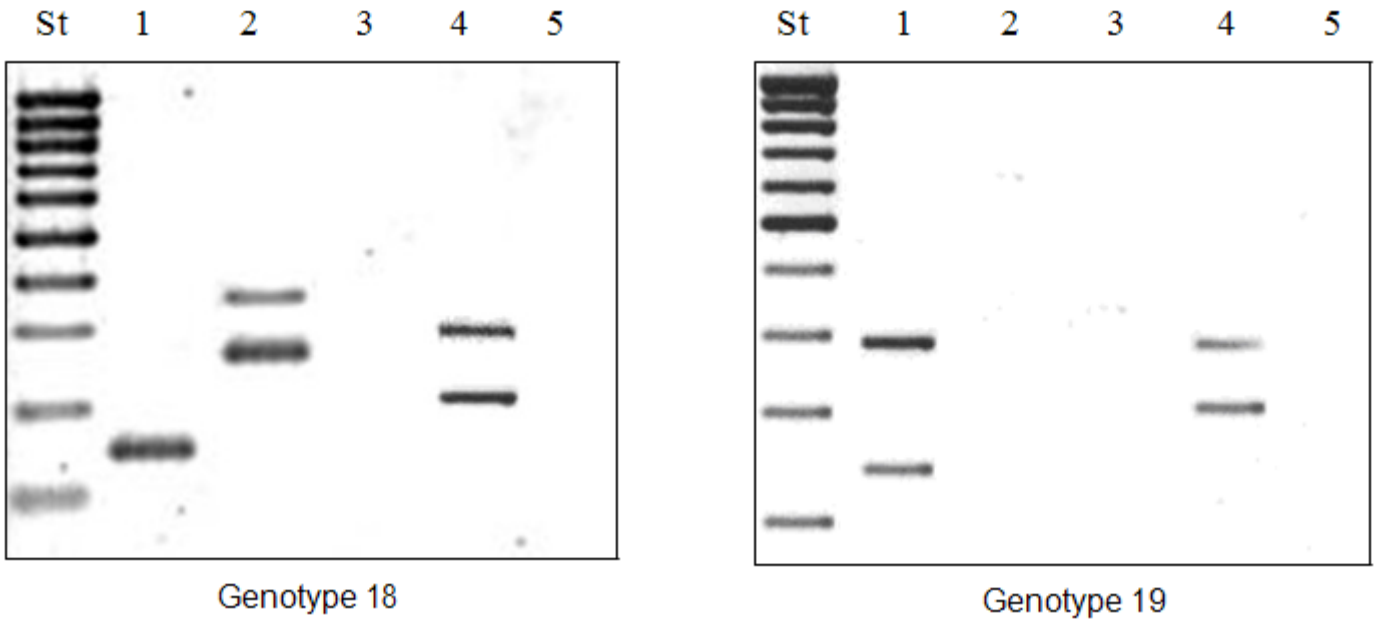

Figure 1. Amplification patterns of genotype 18 and genotype 19. The most frequent amplification patterns. Genotype 18= St: standard molecular size, 1: *mecA*, 2: pI258 – IS256, 4: Ins117 – HVR, 5: negative control. Genotype 19= St: standard molecular size, 1: *mecA* - pI258, 4: Ins117 – HVR, 5: negative control.

DISCUSSION

SCCmec typing is one of the most important molecular tools available for understanding the epidemiology and clonal strain relatedness of MRSA (14). However, due to the very complex and diverse structure of the *SCCmec* element, *SCCmec* subtyping is a powerful tool applicable to clinical and epidemiological surveillance purposes (10). Based on the horizontal transfer of *SCCmec* and the polymorphism of regions located "downstream" of the *mecA* gene, we suggest that genotypes identified through the presence of hypervariable regions can be classified as subtypes of MRSA strains previously typified as *SCCmec* type I.

In the present study ninety eight MRSA strains isolated from patients hospitalized at the County Hospital of Valdivia (Chile), were subtypified into seventeen genotypes according to amplification patterns of 7 DNA hypervariable regions located around the *mecA* gene.

Seventeen genotypes detected in our environment contrasts with the five genotypes previously identified by Wilson *et al*., who detected only five genotypes of MRSA strains isolated from patients hospitalized at the County Hospital of Valdivia (Chile) between March 2004 and December 2005 (15). This situation is because in our study we included a greater number of strains and we identified hypervariable regions not detected previously.

The new amplification patterns detected in this study were ranked between genotype 18 and genotype 29. There was a predominance of genotype 18 and genotype 19 with 24.5% and 20,4% respectively (Figure 1 and Table 2). From these data we could infer that most of MRSA strains were originated from two local clones. In fact, we suggest that strains belonging to genotype 18 are different from those belonging to genotype 19, does not possess the IS256 region. Therefore, we infer that the strains belonging to genotype 18 come from a strain belonging to genotype 19, in which the IS256 region was integrated.

IS256 region, located downstream of a fragment 2 Kb called *dcs* (downstream constant segment), is an insertion sequence that can be independent or as part of the transposon Tn*4001*. This transposon carries the *aacA-aphD* gene, which encodes resistance to aminoglycoside (1, 11). IS256 region was detected in 46.9% of MRSA strains. These results are different from those obtained by Wilson *et al*., who detected this region in 9.4% of MRSA strains (15).

The increase in the prevalence of IS256 region is probably due to a clonal expansion of some MRSA strains that possess this region in their *SCCmec*.

Moreover, this situation reflects the constant genomics evolution of MRSA strains in our environment. In two years (2005 - 2007), almost half of strains incorporated the IS256 region in their *SCCmec*. This is worrying because IS256 region allows the insertion of Tn*4001* encoding resistance to aminoglycoside (11).

Ins117 region is a short sequence of 117 bp, flanked by two 15 bp direct repeats, contained within *orfX* region (11). This region was detected in 69.4% of MRSA strains. These results are different from those obtained by Wilson *et al*., who did not detect this region in MRSA strains (15). The increase in the prevalence of Ins117 region is probably due to a clonal expansion of some MRSA strain that possess this region in their *SCCmec* as happened with IS256 region. This is also worrying because Ins117 region, along with IS431, allows the insertion of plasmid pUB110 which encodes resistance to tetracycline and aminoglycoside (11, 12).

pUB110 region is flanked by IS431 and was integrated during the period when *mec* DNA was being formed and prior to the emergence of the first outbreaks of MRSA infections in European hospitals in the early 1960s (11, 12). This region was detected in 2% of MRSA strains. In the previous study of Wilson *et al*. MRSA strains carrying pUB110 region were not detected (15). Spread of strains possessing pUB110 region would be a problem due to the resistance that this region encodes. Moreover, pUB110 region is present in subtypes *SCCmec* IA, II-A, II-b, II-A, II-B and II-C. MRSA strains showed this region can be classified as *SCCmec* subtype IA (16).

HVR region is a DNA sequence composed by direct

repeat unit elements (DRUs) located between IS*431mec* and *mecA* (13). This region was detected in 92.9% of MRSA strains. A similar situation is reported by Wilson *et al* (15). This fact reflects the high degree of conservation of the HVR region at the strains isolated in our hospital environment.

pI258 and pT181 regions are plasmid flanked by IS431 that encodes resistance to mercury and tetracycline respectively (11). pI258 region was detected in 69.4% of our strains. Previously Wilson *et al*. detected the pI258 region in 81% of their MRSA strains (15). pT181 region was detected in 13.3% of our strains. These results are different from those obtained by Wilson *et al.* who detected this region in 41.5% of their MRSA strains (15).

Located upstream of *mecA* gene lies the mecR1 gene, that encodes the protein MecR1, which activates the *mecA* gene transduction generating the synthesis of PBP2a (2, 16). This region was not detected in any of our MRSA strains, as it was previously reported by Wilson *et al* (15). This situation is because mecR1 gene is characterized by suffering deletions. This characteristic is highly conserved among strains isolated in our environment (2).

Finally, based on the results obtained in this study and the results obtained previously by Wilson *et al* (15) we suggest that acquisition of hypervariable regions by MRSA is a continuous horizontal transfer process through which the *SCCmec* has been preserved intact, or even may give rise to new types and subtypes of *SCCmec*. This means that MRSA strains could maintain or increase their resistance, but in no case it would decrease.

Continue surveillance studies are needed to make annual checkups to determine the prevalence of MRSA subtypes in our environment, as well as controlling the emergence of new subtypes. On the other hand, it would allow retrospective studies to detect evolutionary changes and would establish an accurate antimicrobial therapy, which would shorten the hospitalization stay, resulting in a significant decrease in health costs caused by MRSA infections.

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