ARTÍCULO ORIGINAL

Design of two molecular methodologies for the rapid identification of Colombian community-acquired methicillin-resistant *Staphylococcus aureus* isolates

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Introduction. Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections are found with increasing the frequency, both in healthy individuals in the community and in hospitalized patients. In Colombia and the Andean region, CA-MRSA isolates have a genetic background that is related to the pandemic USA300 clone.

Objective. Two molecular methods are designed and standardized for the rapid differentiation of Colombian community-acquired and hospital-acquired methicillin-resistant *Staphylococcus aureus* (HA-MRSA) isolates.

Materials and methods. Two molecular methods were standardized for the identification of CA-MRSA isolates. The first method was based on the differential digestion of the carbamate kinase (*arcC*) and guanylate kinase (*gmk*) genes in the sequences type 5 (ST5) in the HA-MRSA isolates and 8 (ST8) in the CA-MRSA isolates. The second method was based on the PCR amplification of 5 specific virulence factors found in CA-MRSA and HA-MRSA isolates. The specificity and precision of each method were evaluated using 237 clinical MRSA isolates.

Results. The first method identified 100% and 93.2% of the CA-MRSA and HA-MRSA isolates, respectively. The second method also correctly identified the two isolates types (CA-MRSA and HA-MRSA).

Conclusions. These two methods are a convenient alternative for the rapid identification of the CA-MRSA isolates, compared with other techniques such as pulsed field gel electrophoresis and multilocus sequence typing, which are time-consuming and more expensive.

Key words: Methicillin-resistant *Staphylococcus aureus*, community-acquired infections, bacterial typing techniques, multilocus sequence typing, enterotoxins.

Diseño de dos metodologías moleculares para la rápida identificación de aislamientos de *Staphylococcus aureus* resistente a meticilina asociados a la comunidad en Colombia

Introducción. Los aislamientos de *Staphylococcus aureus* resistente a la meticilina asociado a la comunidad (SARM-AC), están aumentando la frecuencia de infecciones en personas sanas de la comunidad y en pacientes hospitalizados. En Colombia y en la región andina estos aislamientos tienen un componente genético relacionado con el clon pandémico USA300.

Objetivo. Diseñar y estandarizar dos metodologías para la diferenciación rápida de aislamientos colombianos de *S. aureus* resistente a la meticilina asociado a la comunidad de los asociados al hospital (SARM-AH).

Materiales y métodos. Se estandarizaron dos metodologías moleculares para la identificación de aislamientos de *S. aureus* resistente a la meticilina asociado a la comunidad. La primera se basa en la digestión diferencial con tres enzimas de restricción de los genes cinasa de carbamato (*arcC*) y cinasa de guanilato (*gmk*) para los tipos de secuencia 5 (ST5) y 8 (ST8), correspondientes a aislamientos de *S. aureus* resistente a la meticilina asociado al hospital y asociado a la comunidad, respectivamente. La segunda se basa en la amplificación por reacción en cadena de la polimerasa de cinco factores de virulencia que se encuentran de manera diferencial en estos aislamientos. Las dos metodologías fueron validadas en 237 aislamientos clínicos de *S. aureus* resistente a la meticilina.

Resultados. Con la primera metodología se identificaron el 100 % y 93,2 % de los aislamientos de *S. aureus* resistente a la meticilina asociado a la comunidad y asociado al hospital, respectivamente. Con la segunda metodología se identificaron correctamente los dos tipos de aislamientos.
Conclusiones. Estas dos metodologías son una buena alternativa en términos de ahorro en tiempo y dinero comparadas con otras técnicas, como la electroforesis en campo pulsado y la tipificación de secuencias multilocus para la rápida identificación de aislamientos de *S. aureus* resistente a la meticilina asociado a la comunidad en Colombia.

Palabras clave: *Staphylococcus aureus* resistente a meticilina, infecciones comunitarias adquiridas, técnicas de tipificación bacteriana, tipificación de secuencias multilocus, *sequence typing*, enterotoxinas.

The first infections due to community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) were reported in Australia in 1990 (1). Since then the number of reported cases has increased around the world, demonstrating a high capacity for infection and dissemination (2). In addition, the CA-MRSA isolates have begun to cause infections in hospitalized patients and to replace the clones of hospital-acquired methicillin-resistant *Staphylococcus aureus* (HA-MRSA) (3,4). These isolates differ from the HA-MRSA forms in that they are more virulent and possess genetic components that cause infections even in healthy persons without apparent risk factors or prior contact in a hospital environment (2,5,6). Even though HA-MRSA has been a clinical concern for a long time, the infections caused by CA-MRSA have become the focal point for clinical and researchconcern.

Methicillin-resistant *Staphylococcus aureus* (MRSA) presents a conserved clonal structure with a reduced number of clones that have the capacity for global dissemination. Five CA-MRSA pandemic clones that have been described based on their sequence type (ST), as determined by multilocus sequence typing (MLST): European clone (ST80); Oceanic clone (ST30); North-West Pacific clone (ST59); USA300 (ST8) and USA400 (ST1) (2). In South America, the appearance and dissemination of at least three of the CA-MRSA clones have been reported, but with different genetic characteristics. In Brazil and Uruguay the most prevalent CA-MRSA clone possesses a type of staphylococcal cassette chromosome *mec* (SCC*mec*) IVc, the Panton-Valentine leukocidin (PVL) toxin and ST30. These are genetically related to the CA-MRSA clone in Australia and Oceania. In Argentina, a CA-MRSA clone has been reported with a SCC*mec* IVa, positive PVL, ST5 and with no apparent genetic relation to one of the CA-MRSA pandemic clones (7,8). In contrast, the CA-MRSA clone that circulates in the Andean region (Colombia, Ecuador, Peru and Venezuela) is related to the USA300 clone, possesses an ST8, a *spa* type t008, has PVL and a SCC*mec* IVc (9,10).

In Colombia, two predominant HA-MRSA clones have been found--the Chilean/Cordobés and the Pediatric clones. The first has SCC*mec* type I and the second has a SCC*mec* type IV. These clones do not possess the *LukF-PV/LukS-PV* genes, and both have an ST5 (allele combination 1, 4, 1, 4, 12, 1, 10) and belong to the clonal complex 5. In contrast, the Colombian CA-MRSA isolates are related to the USA300 clone and have an ST8 (allele combination 3, 3, 1, 1, 4, 4, 3).

In microbiological terms, CA-MRSA and HA-MRSA isolates are very difficult to differentiate. Techniques such as pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) must be used. PFGE is based on the digestion of the chromosomal bacterial DNA with a restriction enzyme (*Sma*I in the case of *S. aureus*). The DNA fragments obtained are separated by PFGE, generating a banding pattern (pulsotype) (11). The second method, MLST, identifies the allelic variation, at the sequence level, of 7 standard housekeeping genes chosen for *S. aureus*--carbamate kinase (*arcC*), shikimate kinase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*). Each combination of the 7 alleles provides a specific sequence type in accordance with the MLST database (available at www.mlst.net). Isolates with related STs determine a clonal complex (CC). The use of these techniques has allowed the differentiation of the pandemic MRSA clones (12-15).

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PFGE and MLST are expensive techniques that are time-consuming and require a staff trained in the use of specialized facilities and equipment. Additionally, analysis of results and the comparison of the pulsotypes are not straightforward tasks. For these reasons, the development of methods has become important to allow the rapid identification of CA-MRSA isolates and provide improved treatment of the infections caused by these microorganisms.

Therefore, the objective was to design and standardize two methods for the rapid identification of Colombian CA-MRSA isolates related to the USA300 clone.

Materials and methods

Staphylococcus aureus isolates

The MRSA clinical isolates were derived from strains banked at the Bacterial Molecular Genetic Laboratory of the Universidad El Bosque in Bogotá, Colombia. The reference strains used were as follows: USA300-0114 (ST8-MRSA-IVa), CHL93, corresponding to the Chilean/Cordóbes clone (ST5-MRSA-I), and HDE3, corresponding to the Pediatric clone (ST5-MRSA-IV). Two hundred and thirty seven MRSA clinical isolates were analyzed--197 isolates (153 CA-MRSA and 44 HA-MRSA) were previously classified as CA-MRSA and HA-MRSA. Genetic relationships established in circulating clones by means of PFGE and MLST and microbiological factors such as minimal inhibitory concentration (MIC) for oxacillin, gentamicin, clindamycin, erythromycin, ciprofloxacin, tetracycline, chloramphenicol, rifampicin, trimethoprim-sulfamethoxazole, vancomycin and linezolid. In addition, 40 MRSA isolates were first classified as CA-MRSA and HA-MRSA using the two methodologies standardized, and then the isolates were confirmed by molecular and genetic characterization.

DNA extraction

The isolates were recovered in brain-heart infusion broth (BHI) after incubation for 24 hours at 37°C under aerobic conditions. The DNA was extracted by resuspension of the colony in 30 µL of distilled and deionized water and later boiled at 94°C for 10 min. Finally, it was centrifuged at 5,000 rpm for five min and 5 µL of supernatant solution was used as the DNA template for each reaction.

Determination of the allelic variations and the search for specific restriction enzymes

The sequences for the alleles of the genes *arcC, aroE, glpF, gmk, pta, tpi* and *yqiL* for ST5 and ST8

were taken from the MLST website at www.mlst.net. The nucleotide variations for each of the genes and the search for differentiating restriction enzymes were determined by means of the Restriction of DNA program (16). These results were confirmed by means of multiple alignment of each allelic pair using the Multalin program, available at www.expasy.org. The cutting sites for the chosen restriction enzymes were confirmed through the programs NEBcutter (17) and Webcutter, available at http://rna.lundberg.gu.se/cutter2/.

Amplification of the genes arcC (carbamate kinase) and gmk (guanylate kinase) and cutting with restriction enzymes

The design of the primers was made using the total sequence of each gene, downloaded as part of the genome of the USA300 strain that was available in GenBank (access number gi87159884) and using the programs PRIMER and PRIMER3 (18,19). The parameters used for the design of the primers were as follows: (1) the size of the product to amplify, (2) size of the products generated after the restriction (easy identification of the alleles), (3) number of additional cuts of each restriction enzyme inside the amplified product, (4) a GC percentage between 40-60%, (5) a maximum variation of 5°C in their annealing temperatures, and (6) a lack of formation of secondary structures. The optimal concentrations of $MgCl_2$, dNTPs, primers and DNA were determined, as were the best thermal conditions, by a trial and error approach. The amplified products of each gene were digested with the selected restriction enzymes at 37°C for 2 hours. Finally, the restriction patterns were visualized in 1.5% agarose gels dyed with ethidium bromide.

Multiple amplification of the genes *sek, bsaB, lukF-PV/lukS-PV, sem* and *seo*

A multiple PCR was standardized for the simultaneous amplification of the genes *sek, bsaB, lukF-PV/lukS-PV, sem* and *seo* that codify for the enterotoxin K, bacteriocin B, PVL, enterotoxin M and enterotoxin O, respectively. The primers used for the amplification of these genes are shown in table 1.

Results

First Method: Differential digestion of the carbamate kinase (arcC) and guanylate kinase (gmk) genes in the sequences type 5 (ST5) and 8 (ST8)

The sequence types ST5 and ST8 have different alleles in 6 of the 7 genes used for the MLST (*arcC,*

aroE, gmk, pta, tpi and *yqiL*)*. The allele 1 of the gene *glpF* is the same for these two types of ST. The nucleotide variations of each allelic pair were determined for each of the 6 genes in the two ST types by means of multiple alignments. In total, 17 variations were found--3 in the *arcC* gene, 2 in the *aroE* gene, 5 in the *gmk* gene, 1 in the *pta* gene, 2 in the *tpi* gene and 4 in the *yqiL* gene (table 2)*. For each allelic pair, sequences of 23 nucleotides were taken that included each variation (10 nucleotides on each side of the variation); then the differential cutting restriction enzymes were selected by means of the "Restriction of DNA", Webcutter 2.0 and NEBcutter V2.0 programs. Appropriate restriction enzymes found were for only some variations of the *arcC* and *gmk* genes*. For the *arcC* gene, the *Hinf*I enzyme (GANTC cutting and recognition sequence) cut allele 1 of ST5 in the position 198, but it did not cut allele 3 of ST8 for the nucleotide variation C198T. For the *gmk* gene, two restriction enzymes cut differently in three of the five variants. The *Hha*I enzyme (GCGC cutting and recognition sequence) cut only allele 1 for ST8 in the position 286 but it did not cut allele 4 of ST5 for the nucleotide variation T286C. The *Cai*I enzyme (with CAGNNNCTG recognition sequence) cut allele 1 of ST8 but not allele 4 of ST5 for the consecutive variations C357T and A358G (table 2).

The *arcC* gene has a total size of 942 bp; the designed primers amplify an expected product of 332 bp and include the variation C198T. The *gmk* gene has total size of 624 bp. For the primer design, the amplified product was expected to include all of the 3 variants, T286C, C357T and A358G. The expected size was 557 bp (table 1).

After a standardization process, the optimal concentrations of the reagents for the amplification of the *arcC* and *gmk* genes were as follows: 2.5 mM of MgCl$_2$, 200 µM of dNTPs, 200 nM of each primer (forward and reverse) and 2 units of *Taq* Polymerase. The two genes were amplified using the same temperature profile, which consisted of: an initial cycle of 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 1 min; and a final extension cycle of 72°C for 7 min in a final volume of 20 µL. Later, 10 µL of the PCR product, without previous purification, was incubated at 37°C with each of the respective restriction enzymes for 2 hours. For the restriction of the *arcC* gene, 7 units of the *Hinf*I enzyme were used, and the fragments expected in accordance with the ST were as follows: 332 bp for an isolate with allele 3 (ST8), the amplified product size (uncut

by the restriction enzyme), and two products of 245 bp and 87 bp for allele 1 (ST5) (figure 1). For the restriction of the *gmk* gene, 5 units of the *Cai*I or *Hha*I enzymes were used. The expected fragments with the *Cai*I enzyme were 557 bp for the allele 4 (ST5), uncut PCR product, and two products of 390 bp and 167 bp for allele 1 (ST8). In the case of the *Hha*I enzyme, this cuts the two alleles of the *gmk* gene, but produces a different number of fragments--for allele 4 of ST5 it cuts once and produces two fragments of 429 bp and 124 bp;, for allele 1 of ST8 it cuts twice and produces three fragments of 323 bp, 124 bp and 105 bp (figure 1).

The restriction of the *arcC* gene with the *Hinf*I enzyme in the Chilean clone (ST5) produced two fragments of 245 bp and 87 bp. In the case of the USA300 clone (ST8), a fragment of 332 bp was obtained that corresponded to the size of the amplified product, i.e., no cutting occurred with this enzyme. The restriction of the *gmk* gene with the *Cai*I enzyme in the USA300 clone (ST8) produced two fragments of 340 bp and 112 bp. For the Chilean clone (ST5) a fragment of 557 bp was obtained, indicating no activity of the restriction enzyme. The restriction of the *gmk* gene with the *Hha*I enzyme in the Chilean clone (ST5) produced two fragments of 428 bp and 129 bp, and for the USA300 clone (ST8) three fragments of 323 bp, 129 bp and 105 bp (figure 1). The results obtained for the Pediatric clone HDE3 (ST5) were the same as those found for the Chilean clone.

From these results, the following deductions were made. (1) The *Hinf*I enzyme cut allele 1 of the *arcC* gene in the ST5 isolates, but it did not cut allele 3 of the ST8 isolates. (2) The *Cai*I enzyme cut allele 1 of the *gmk* gene present in the ST8 isolates, but it did not cut allele 4 of ST5 isolates. (3) In the case of the *Hha*I enzyme, it cut the two alleles of the *gmk* gene, but with a different frequency and generated fragments with different sizes. (4) Allele 1, present in the isolates with ST8, was cut at two sites and generated three products. (5) For allele 4, present in ST5 isolates, *Hha*I cut in only one site and generated two products, one with a greater size (428 bp) than that expected for allele 1 (323 bp).

Second method: PCR amplification of the 5 specific virulence factors

Chavarro *et al.* (2009) carried out a study on the frequency of 24 virulence factors in 270 Colombian MRSA isolates (86 CA-MRSA isolates and 184 HA-MRSA isolates) (9). The *sek*, *seq* and *lukF-PV*/*lukS-PV* genes were found only in CA-MRSA

arcC   *gmk*

Hinf\   Cai\   Hha\

M   NC1   U   CH   NC2   U   CH   U   CH   NC3   MW
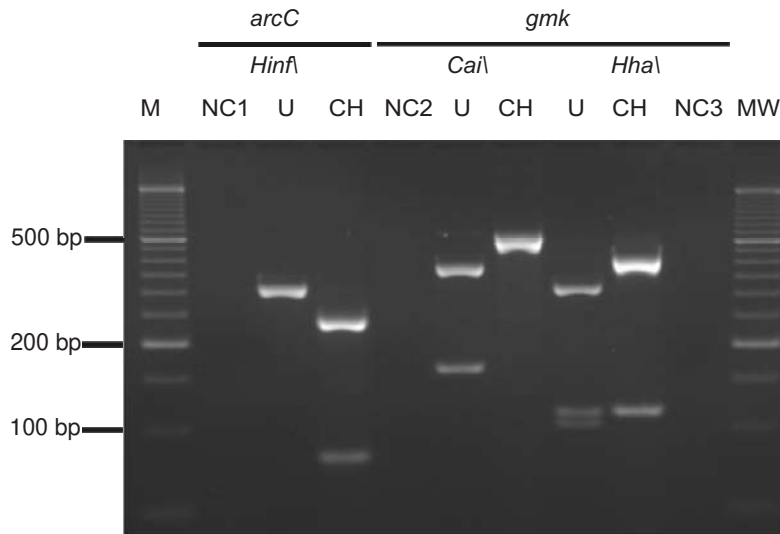
Figure 1. DNA fragments obtained after the amplification and restriction of the *arcC* and *gmk* genes for the USA300 clone (U) (ST8) and the Chilean clone (CH) (ST5). Lanes 3 and 4: restriction of the *arcC* gene with the *Hinf*I enzyme. Lanes 6 and 7: restriction of the gene *gmk* with the *Cai*I enzyme. Lanes 8 and 9: restriction of the *gmk* gene with the *Hha*I enzyme. MW: Molecular weight of 50 bp. NC1, NC2 and NC3: respective negative controls of the three restriction reactions.

isolates with a frequency of 80%, 82% and 92%, respectively. The *seg, sei, sem, sen* and *seo* genes, that make up the enterotoxin genomic cluster (egc) were found only in the HA-MRSA isolates with a frequency of 85%. Recently Álvarez *et al.* analyzed 153 MRSA isolates recovered from pediatric infections and found the *sek, seq* and *lukF-PV/lukS-PV* genes only in the CA-MRSA isolates, with frequencies of 71%, 71% and 100%, respectively. The egc genes were found in all of the HA-MRSA isolates. In addition, they found that 90% of the CA-MRSA isolates had the *bsaB* gene that codifies for a bacteriocin, whereas this gene was not found in the HA-MRSA isolates (unpublished data).

Using these results, primers were specifically designed for the simultaneous amplification of the *lukF-PV/lukS-PV, sek, bsaB, sem* and *seo* genes. The first three genes act as specific genetic markers for Colombian CA-MRSA isolates and the remaining two for Colombian HA-MRSA isolates (figure 2). The primer sequences and the size of the expected amplification products are detailed in table 1.

The optimal concentrations of the reagents found for the simultaneous amplification of the 5 genes were as follows: 2.5 mM of $MgCl_2$, 200 µM of dNTPs, 200 nM of each primer and 1 unit of *Taq* polymerase. The optimal profile of temperatures was: an initial 95°C for 5 min, followed by 30 cycles of 95°C for 45 sec, then 54°C for 45 sec and 72°C for 2 min, with a final extension of 72°C for 7 min (figure 2).

Validation of the two methods

The methods were validated evaluating 197 and 40 MRSA clinical isolates, with and without micro-biological, genetic and molecular characterization, respectively. Among the 197 MRSA isolates previously characterized, 153 (77.6%) were classified as CA-MRSA and 44 (22.3%) were classified as HA-MRSA. With the first method, the 153 CA-MRSA isolates evaluated showed a restriction profile of the genes *arcC* and *gmk* expected of CA-MRSA isolates, while of the 44 HA-MRSA isolates analyzed, 41 showed restriction profiles that were characteristic of HA-MRSA isolates and 3 (6.8%) isolates presented profiles with restriction characteristics of CA-MRSA isolates for the *Hinf*I and *Cai*I enzymes. However, the amplified fragment of the *gmk* gene was not cut by the *Hha*I enzyme in those three isolates, an unexpected result for the ST5 and ST8 isolates. This suggests the possibility that these 3 isolates possess a different allele of the gene *gmk*. To confirm this hypothesis, the allele of this gene will be sequenced for these samples. These results show that the first standardized method had a high specificity (98.5%) and correctly identified all of the CA-MRSA isolates. The second method correctly identified both the CA-MRSA and HA-MRSA isolates (table 3). The 153 CA-MRSA isolates amplified at least one of the three genes (*sek, bsaB* or *lukF-PV/lukS-PV*). One hundred thirteen (73.8%) isolates amplified the three genes, 29 (18.9%) only amplified the *lukF-PV/lukS-PV* gene, 8 (5.2%) amplified the *sek* and *bsaB* genes and 3 (2.0%) isolates amplified the *lukF-PV/lukS-PV* and *bsaB* genes. All of the HA-MRSA isolates amplified the *sem* and *seo* genes.

Among the 40 MRSA clinical isolates without previous molecular characterization, 29 (72.5%) were classified as CA-MRSA and 11 (27.5%)
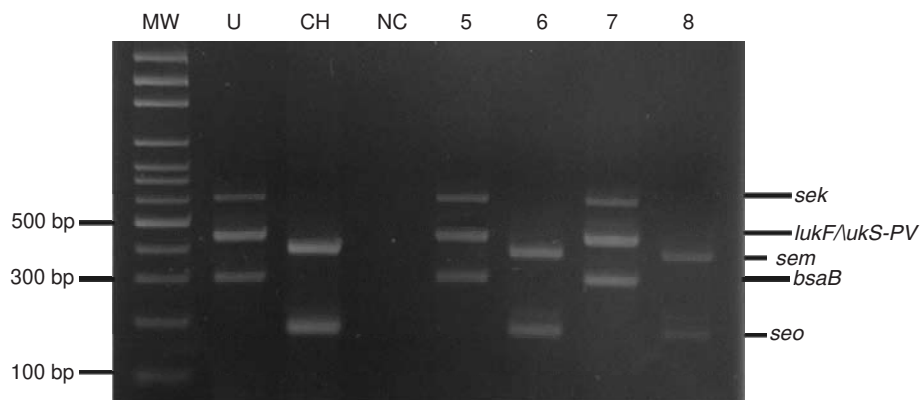
Figure 2. Multiple PCR of the *lukF-PV/lukS-PV, sek*, *bsaB*, *sem* and *seo,* genes amplified in the USA300 clone (U), Chilean clone CH93 (CH) and the clinical isolates 5sau23, 5sau46, 5sau78 and 5sau80 (Lanes 5 and 8). MW: Molecular weight of 100 bp. NC: negative control.

Table 1. Primers used in this study

Primer	Sequence (5' to 3')	product (bp)	Target gene	Reference
GP287	ATCATTAGGTAAAATGTCTGGACATGAT	433	*lukF-PV /lukS-PV*	(20)
GP286	GCATCAACTGTATTGGATAGCAAAAGC			
GP265	GTGTCTCTAATAGTGCCAGC	596	*seK*	(9)
GP264	ACTTTTTTGGTAACCCATCATC			
GP336	TTGGATTTGGCGAGGAATTA	296	*bsaB*	This study
GP337	AATTGTTGTCCCGAAACCTG			
GP259	CGGTGGAGTTACATTAGCAGGT	376	*sem*	(9,20)
GP258	TTCAGTTTCGACAGTTTTGTTGT			
GP255	AGTTTGTGTAAGAAGTCAAGTGTA	180	*seo*	(20)
GP254	ATCTTTAAATTCAGCAGATATTCCATC			
GP223	TCACCAGCGCGTATTGTCAT	332	*arcC*	This study
GP222	AAGACTGAGTCTGGCTGTTC			
GP225	CAGGACCATCTGGAGTAGGT	557	*gmk*	This study
GP224	CTTAGCTTCTACGCGCTCTC			

Table 2. Nucleotide variations found in the six alleles of the six housekeeping genes used for MLST in *S. aureus*. The ST5 alleles are used as a reference to indicate where the nucleotide changes position.

Gene	Protein	Allele (ST)	Nucleotide variations*
arcC	Carbamate kinase	1 (ST5)	-------
		2 (ST8)	C198T,A199G,C210T
aroC	Shikimate dehydrogenase	4 (ST5)	-------
		3 (ST8)	T102A, G435A
gmk	Guanylate Kinase	4 (ST5)	-------
		1 (ST8)	T129C, T286C,T318A, C357T,A358G
Pta	Phosphate acetyltransferase	12 (ST5)	-------
		4 (ST8)	A294C
tpi	Triosephosphate isomerase	1 (ST5)	-------
		4 (ST8)	T69A,G195A
yqil	Acetyl coenzyme A acetyltransferase	10 (ST5)	-------
		3 (ST8)	A192G,G303A,C333T,T495A

*The ST5 alleles are used as a reference to indicate where the nucleotide changes position.

were classified as HA-MRSA using the two methods. With the first method, the 29 CA-MRSA isolates evaluated showed a restriction profile of the genes *arcC* and *gmk* expected of CA-MRSA isolates, the 11 HA-MRSA isolates analyzed showed restriction profiles that were characteristic of HA-MRSA isolates (100% exactitude). With the second method, the 29 CA-MRSA isolates amplified at least one of the *sek*, *bsaB or lukF-PV/lukS-PV* genes and all HA-MRSA isolates amplified the *sem* and *seo* genes. The subsequent molecular and genetic characterization showed that all isolates classified CA-MRSA were related to USA300 clone and all isolates classified HA-MRSA were related to Chilean clone. These results confirm that the two methods correctly identified both the CA-MRSA and HA-MRSA isolates (100% exactitude) (table 3).

Discussion

Several international studies have demonstrated that the CA-MRSA isolates are more virulent than the HA-MRSA isolates, due to greater production of virulence factors such as the Panton-Valentine leukocidin (PVL) and the phenol-soluble modulins (PSM), capable of lysing macrophages and neutrophils (5,6). Additionally, the CA-MRSA isolates contains genetic mobile elements that give them greater environmental adaptability-- as is the case with the arginine catabolic mobile element (ACME), specifically identified in the USA300 clone (21). Clinically, the CA-MRSA isolates have acquired the capacity not only to cause minor infections in healthy persons, but also to cause severe infections such as pneumonia and

necrotizing fasciitis (16). The entry of CA-MRSA to hospitals and their greater virulence indicates an expected increase in patient mortality and morbidity in affected institutions. This underlines the importance of developing methods that allow rapid identification of CA-MRSAs, and hence, more effective treatment, control and eradication of these infections.

Currently, the CA-MRSA and HA-MRSA isolates are differentiated with greater accuracy using robust and expensive techniques like PFGE and MLST. In developing countries such as Colombia and others in the Andean region, faster and cheaper alternative methods are necessary to differentiate between these types of microorganisms in hospitals. Herein,methods have been standardized for the rapid identification of Colombian CA-MRSA isolates; the methods were designed based on isolates from several multi-centre studies (with epidemiological data as well as molecular and genetic characterizations), as well as new isolates without molecular characteristics and later confirmed by PFGE and MLST (4,9,10,22,23).

The first method was based on the detection of polymorphism, by means of restriction enzymes, found in two constitutive genes of *S. aureus*, which are used in MLST. This method correctly identified the 153 CA-MRSA isolates (100%) and 41 of the 44 HA-MRSA isolates (93.2%). A more detailed analysis of the molecular characteristics of the three HA-MRSA isolates classified as CA-MRSA was conducted. This revealed that these have a SCC*mec* III and a PFGE pulsotype (named as H,

Table 3. Classification of the 237 MRSA clinical isolates by means of the two standardized methods.

| Type of Isolate* | PFGE (n) pulsotype** | SCC*mec* type | PVL n (%) | Possible ST according to the first method | | | Type of isolate - first method | Type of isolate - second method |
				*Hin*fI	*Hha*I	*Cai*I		
Isolates with previous molecular and genetics characterization (n=197)								
CA-MRSA	U (153)	Type IVc	149 (97%)	8	8	8	CA-MRSA	CA-MRSA
HA-MRSA	F (32)	Type I	0 (0%)	5	5	5	HA-MRSA	HA-MRSA
	D (4)	Type IV	0 (0%)	5	5	5	HA-MRSA	HA-MRSA
	G (4)	Type II	0 (0%)	5	5	5	HA-MRSA	HA-MRSA
	H (3)	Type III	0 (0%)	8	NC***	8	CA-MRSA	HA-MRSA
	R (1)	Type II	0 (0%)	5	5	5	HA-MRSA	HA-MRSA
Isolates without previous molecular and genetics characterization (n=40)								
CA-MRSA	U (29)	Type IVc	24 (100%)	8	8	8	CA-MRSA	CA-MRSA
		Type IVa	4 (100%)	8	8	8	CA-MRSA	CA-MRSA
		Type IVb	1 (100%)	8	8	8	CA-MRSA	CA-MRSA
HA-MRSA	F (11)	Type I	0 (0%)	5	5	5	HA-MRSA	HA-MRSA

*Classified by molecular techniques such as PFGE, MLST and / or *spa* typing.
**Two isolates belong to different pulsotypes when they present >6 bands of difference in their restriction profile.
***NC: No restriction enzyme cuts.

table 3) possibly related to the Brazilian clone. This clone possesses a SCC*mec* IIIa and an ST239. Its allelic combination is 2-3-1-1-4-4-3, a single locus variant (SLV) from ST8 (3-3-1-1-4-4-3); allele 2 of the *arcC* gene also possesses the mutation C198T, as found in ST8. For this reason,the restriction enzyme *Hinf*I did not cut the *arcC* gene in the three isolates, and consequently classified as CA-MRSA. The epidemiological data indicated that the circulation of isolates related to the Brazilian clone is very low, with a frequency of less than 1% (unpublished data). The first method correctly identified all of the "new" 29 CA-MRSA isolates and 11 HA-MRSA isolates (100%).

Various methods have been developed to try to identify CA-MRSA isolates at an international level (24,25). For example, Diep *et al.* (2003) reported a method that established the genetic relationship between two or more isolates from *S. aureus* by analyzing the polymorphism of amplified fragments of the 7 genes used for MLST, utilizing restriction enzymes (26). Unfortunately, this method does not allow the easy differentiation between ST5 and ST8 isolates--it generates DNA fragments with very similar size because it uses the primers employed by the MLST.

The second method correctly identified the 197 CA-MRSA and HA-MRSA isolates, including the 3 HA-MRSA isolates possibly related to the Brazilian clone. This method allowed the differentiation between the Colombian CA-MRSA and HA-MRSA isolates by the detection of specific virulence factors for each type of isolate. The fundamental characteristic of these virulence factors are their location on mobile genetic elements, which are acquired differently in the two types of isolates. The *sek*, *bsaB, lukF-PV/lukS-PV* genes were selected using results previously obtained by the current study and as reported in other literature (9,10), and the selection supported by a bioinformatics analysis of their location on the genome. In accordance with the genome sequence of the USA300 clone (21), the *sek*, *bsaB, lukF-PV/lukS-PV* genes are found on different mobile genetic elements. The first is transported on the pathogenicity island 5 (SaPI5), the second on the genomic island β (vSaβ) type II and the third on the prophage Sa2usa. The selection of specific virulence factors for the HA-MRSA isolates was made using information from studies previously carried out in our laboratory (the genomes of the Chilean/Cordóbes and Pediatric clones have not yet been sequenced).

These studies verified that approximately 90% of the HA-MRSA isolates contained the egc cluster (9). This cluster has been found on the genomic island β (vSaβ) type I of the strains N315, Mu50 and Mu3 (27). Fossum *et al.* (2009) analyzed 821 MRSA isolates and found that each genetic lineage possessed a specific repertoire of enterotoxins. This supports the hypothesis that mobile genetic elements are not inserted in all isolates and are not randomly distributed (28). Miranda *et al.* (2007) reported the emergence of isolates that were genetically related to the USA800 clone (ST5-SCC*mec* IV) in Brazil. These were related to the Pediatric clone, with all of the isolates carrying the *egc* enterotoxin cluster. These observations confirmed that the cluster is widely disseminated in isolates related to the Pediatric clone, not only in Colombia, but also in neighboring countries (29). The second method identified correctly both the CA-MRSA and HA-MRSA isolates (100%), as well as CA-MRSA and HA-MRSA isolates without previous molecular characterization (100%). These results indicate that the two methods may be used for a correct determination a MRSA isolate may be either a CA-MRSA or HA-MRSA isolate.

The two methods standardized in this study allow the identification of the CA-MRSA isolates in a short period of time (approximately 5 hours) and with a lower cost compared to techniques such as PFGE and MLST. Furthermore, even if these methods were based on Colombian CA-MRSA isolates, they have the potential to identify CA-MRSA isolates in the for the entire Andean region (Colombia, Venezuela, Ecuador and Peru), given that the epidemiology of these countries is similar to that of Colombia. The pulsotypes obtained from PFGE show that the majority of the HA-MRSA isolates have a close genetic relation to the Chilean clone; and the CA-MRSA isolates, as in Colombia, are genetically related to the USA300 clone (10,30). These determinations will be of value even if additional studies are required to confirm the STs and whether the STs possess these same specific virulence factors.

The development of these diagnostic tools is an important advance that allows faster and more accurate identifications of the MRSA isolate. Rapid identifications will in turn quickly orientate the medical staff toward a more appropriate empirical antibiotic treatment and assist an adequate therapeutic management of CA-MRSA infections. Finally, without the of knowledge of the genetic

and molecular characteristics of the isolates, inappropriate treatments can result in increased resistance of bacterial agents to antibiotics, and, once established, reduces the therapeutic possibilities for treatment.

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Conflicts of Interest

The authors declare that during the conduct of the present study, no conflicts of interest occurred that will have affected the experimental results or the opinions expressed herein.

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References

1. **Udo EE, Pearman JW, Grubb WB.** Genetic analysis of community isolates of methicillin-resistant *Staphylococcus aureus* in Western Australia. J Hosp Infect. 1993;25:97-108.

2. **Deleo FR, Otto M, Kreiswirth BN, Chambers HF.** Community-associated meticillin-resistant *Staphylococcus aureus*. Lancet. 2010:375:1557-68.

3. **Popovich KJ, Weinstein RA, Hota B.** Are community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) strains replacing traditional nosocomial MRSA strains? Clin Infect Dis. 2008;46:787-94.

4. **Álvarez CA, Yomayusa N, Leal AL, Moreno J, Méndez-Álvarez S, Ibáñez M, *et al.*** Nosocomial infections caused by community-associated methicillin-resistant *Staphylococcus aureus* in Colombia. Am J Infect Control. **¿Año?**;38:315-8.

5. **Diep BA, Palazzolo-Ballance AM, Tattevin P, Basuino L, Braughton KR, Whitney AR, *et al.*** Contribution of Panton-Valentine leukocidin in community-associated methicillin-resistant *Staphylococcus aureus* pathogenesis. PLoS One. 2008;3:e3198.

6. **Wang R, Braughton KR, Kretschmer D, Bach TH, Queck SY, Li M, *et al.*** Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat Med. 2007;13:1510-4.

7. **Sola C, Saka HA, Vindel A, Bocco JL.** Emergence and dissemination of a community-associated methicillin-resistant Panton-Valentine leucocidin-positive *Staphylococcus aureus* clone sharing the sequence type 5 lineage with the most prevalent nosocomial clone in the same region of Argentina. J Clin Microbiol. 2008;46:1826-31.

8. **Gardella N, von Specht M, Cuirolo A, Rosato A, Gutkind G, Mollerach M.** Community-associated methicillin-resistant *Staphylococcus aureus*, eastern Argentina. Diagn Microbiol Infect Dis. 2008;62:343-7.

9. **Chavarro B.** Determinación de la presencia y transcripción de factores de virulencia en aislamientos colombianos de *Staphylococcus aureus* resistente a meticilina adquirido en la comunidad. Bogotá: Facultad de Medicina, Universidad el Bosque; 2009.

10. **Reyes J, Rincon S, Díaz L, Panesso D, Contreras GA, Zurita J, *et al.*** Dissemination of methicillin-resistant *Staphylococcus aureus* USA300 sequence type 8 lineage in Latin America. Clin Infect Dis. 2009;49:1861-7.

11. **Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, *et al.*** Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. J Clin Microbiol. 1995;33:2233-9.

12. **Tristan A, Ferry T, Durand G, Dauwalder O, Bes M, Lina G, *et al.*** Virulence determinants in community and hospital meticillin-resistant *Staphylococcus aureus*. J Hosp Infect. 2007;65(Suppl.2):105-9.

13. **Tenover FC, McDougal LK, Goering RV, Killgore G, Projan SJ, Patel JB, *et al.*** Characterization of a strain of community-associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. J Clin Microbiol. 2006;44:108-18.

14. **Deurenberg RH, Stobberingh EE.** The evolution of *Staphylococcus aureus*. Infect Genet Evol. 2008;8:747-63.

15. **Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG.** Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J Clin Microbiol. 2000;38:1008-15.

16. **Bikandi J, San Millan R, Rementeria A, Garaizar J.** *In silico* analysis of complete bacterial genomes: PCR, AFLP-PCR and endonuclease restriction. Bioinformatics. 2004;20:798-9.

17. **Vincze T, Posfai J, Roberts RJ.** NEBcutter: A program to cleave DNA with restriction enzymes. Nucleic Acids Res. 2003;31:3688-91.

18. **Rozen S, Skaletsky H.** Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol. 2000;132:365-86.

19. **Krawetz SA, Womble DD.** Design and implementation of an introductory course for computer applications in molecular genetics. A case study. Mol Biotechnol. 2001;17:27-41.

20. **Jarraud S, Mougel C, Thioulouse J, Lina G, Meugnier H, Forey F, *et al.*** Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. Infect Immun. 2002;70:631-41.

21. **Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, *et al.*** Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. Lancet. 2006;367:731-9.

22. **Miller LG, Perdreau-Remington F, Rieg G, Mehdi S, Perlroth J, Bayer AS, *et al*.** Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. N Engl J Med. 2005;352:1445-53.

23. **Álvarez-Olmos MI, Enríquez SP, Pérez-Roth E, Méndez-Álvarez S, Escobar J, Vanegas N, *et al*.** Pediatric cases from Colombia caused by a panton-valentine leukocidin-positive community-acquired methicillin-resistant *Staphylococcus aureus* ST8-SCCmecIVc clone. Pediatr Infect Dis J. 2009;28:935.

24. **Bonnstetter KK, Wolter DJ, Tenover FC, McDougal LK, Goering RV.** Rapid multiplex PCR assay for identification of USA300 community-associated methicillin-resistant *Staphylococcus aureus* isolates. J Clin Microbiol. 2007;45:141-6.

25. **Strommenger B, Braulke C, Pasemann B, Schmidt C, Witte W.** Multiplex PCR for rapid detection of *Staphylococcus aureus* isolates suspected to represent community-acquired strains. J Clin Microbiol. 2008;46:582-7.

26. **Diep BA, Perdreau-Remington F, Sensabaugh GF.** Clonal characterization of *Staphylococcus aureus* by multilocus restriction fragment typing, a rapid screening approach for molecular epidemiology. J Clin Microbiol. 2003;41:4559-64.

27. **Baba T, Bae T, Schneewind O, Takeuchi F, Hiramatsu K.** Genome sequence of *Staphylococcus aureus* strain Newman and comparative analysis of staphylococcal genomes: Polymorphism and evolution of two major pathogenicity islands. J Bacteriol. 2008;190:300-10.

28. **Fossum AE, Saltyte J, Alm-Kristiansen K, Bukholm G.** Exotoxin-encoding gene content in community-acquired and hospital-acquired methicillin-resistant *Staphylococcus aureus*. Clin Microbiol Infect. 2009;15:1139-45.

29. **de Miranda OP, Silva-Carvalho MC, Ribeiro A, Portela F, Cordeiro RP, Caetano N, *et al*.** Emergence in Brazil of methicillin-resistant *Staphylococcus aureus* isolates carrying SCCmecIV that are related genetically to the USA800 clone. Clin Microbiol Infect. 2007;13:1165-72.

30. **Rodríguez-Noriega E, Seas C, Guzmán-Blanco M, Mejía C, Álvarez C, Bavestrello L, *et al*.** Evolution of methicillin-resistant *Staphylococcus aureus* clones in Latin America. Int J Infect Dis. 2010;14:e560-6.