Molecular epidemiology and characterization of virulence genes of community-acquired and hospital-acquired methicillin-resistant \textit{Staphylococcus aureus} isolates in Colombia

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SUMMARY

Objective: To determine the molecular epidemiology and presence of virulence genes in community-acquired (CA) and hospital-acquired (HA) methicillin-resistant \textit{Staphylococcus aureus} (MRSA) isolates and their relationship to clinical outcomes.

Methods: An observational and prospective study of infections caused by MRSA was conducted between June 2006 and December 2007 across seven hospitals in three Colombian cities. MRSA isolates were analyzed for SCCmec. Also, pulsed-field gel electrophoresis and multilocus sequence typing were performed and 25 virulence genes were identified.

Results: Two hundred and seventy isolates were collected from 262 adult hospital patients with MRSA infections. Overall, 68% of the isolates were classified as HA-MRSA and 32% as CA-MRSA. We identified differences in the patterns of virulence genes: 85% of HA-MRSA isolates possessed the enterotoxin gene cluster (egc), whereas 92% of CA-MRSA isolates possessed the lukF-PV/lukS-PV genes. Multivariate analysis showed an increased risk of mortality for seg (p = 0.001, odds ratio 4.73) and a protective effect for etA (p = 0.018, odds ratio 0.33).

Conclusions: Our study confirms that three clones of MRSA predominantly circulate in Colombia: a Chilean clone, a pediatric clone that causes HA-MRSA infections, and a USA300-related clone (SCCmec IVc) in CA-MRSA infections, which differ in the content of clinically important virulence genes. This study confirms that PVL is not a determinant of severity or mortality in CA-MRSA infections.

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1. Introduction

Methicillin-resistant \textit{Staphylococcus aureus} (MRSA) is a pathogen mainly associated with hospitals (HA-MRSA). In South America, the two predominant HA-MRSA clones are the Brazilian clone (sequence type ST239), which bears the staphylococcal cassette chromosome (SCC) meC III (MRSA-ST239-III),\textsuperscript{1} and the Chilean/Cordobes clone (MRSA-ST5-1).\textsuperscript{2} However, in recent years, MRSA has become an important cause of community-acquired infections (CA-MRSA). USA300 is one of the main clones representing CA-MRSA and has disseminated throughout Latin America as well as some European countries.\textsuperscript{1,3} In Uruguay and Brazil, CA-MRSA isolates have been reported that display distinct genetic characteristics (MRSA-ST30-IVc).\textsuperscript{4} The dissemination of CA-MRSA isolates in hospital environments was reported in Colombia in 2009. In the Andean countries (Colombia, Venezuela,
Peru, and Ecuador), the CA-MRSA isolates present similar characteristics to the USA300 clone, including MRSA-STB-JVC-E, Panton–Valentine leukocidin (PVL) positivity, and multi-susceptibility.\textsuperscript{5,6} Staphylococcus aureus is a pathogen with the ability to produce a series of virulence factors that contribute to the severity of infections. These factors include microbial surface components that recognize adhesive matrix molecules (MSCRAMM), cytolytic toxins, exoenzymes, exotoxins, hemolysins, leukocidins (such as PVL), and superantigens. The group of superantigens includes staphylococcal enterotoxins (SE), toxic shock syndrome toxin (TSST), and exfoliative toxins. These exoenzymes and exotoxins demonstrate proteolytic activity and toxic or lytic effects in the cells, facilitating local invasion and dissemination.\textsuperscript{7}

The increase in the prevalence of infections caused by CA-MRSA in hospitals and the difficulty in differentiating them using the clinical and epidemiological criteria of HA-MRSA, has shown that it is necessary to use genetic markers for their correct classification.\textsuperscript{8} The objective of this study was to determine the presence of virulence genes in CA-MRSA and HA-MRSA isolates and to establish their possible relationship to clinical outcomes. This knowledge will contribute to our understanding of how the MRSA epidemic has evolved.

2. Materials and methods

2.1. Study population

A prospective cohort study was carried out between June 2006 and December 2007. The study was based on an active surveillance of all MRSA infections in patients older than 18 years who attended seven third-level hospitals in Colombia. MRSA isolates included in the present study were collected prospectively and the first strain isolated from each patient was analyzed. The quality of the sample was monitored by the study coordinator at each hospital, in accordance with the local protocol. Clinical specimens included blood, secretions from complicated skin and soft tissue infections and postoperative wound infections, bronchoalveolar lavage, and endotracheal aspirate culture and sterile liquid samples. Isolates recovered from catheters and spum and those recovered from skin without clinical justification were excluded. All MRSA isolates identified at each hospital were sent to a reference laboratory where identification was confirmed by phenotypic and genotypic methods. Clinical data related to the type of infection and outcome (death, improvement, and relapse), as well as other data, were collected and stored in a database designed for this purpose for up to a period of 30 days after the diagnosis of infection by MRSA.

2.2. Bacterial isolates and reference strains

Two hundred and seventy isolates were collected from 262 adult hospital patients diagnosed with an infection caused by MRSA. The reference strains used were Mu50, JCSS4744, JCSS2172, 81/108, JCSS4469 (donated by Teruyo Ito), NPS123, USA500, TC146, FR11151 (kindly provided by Dr Jerome Etienne), and USA300-0114.

2.3. SCCmec typing and virulence factor gene detection

Confirmation of the species and methicillin resistance in the isolates was achieved through the amplification of the nuc and mecA genes, respectively.\textsuperscript{9} The types and subtypes of SCCmec were also evaluated.\textsuperscript{10} The presence of the genes that code for PVL (lukF-PV and lukS-PV), 15 staphylococcal enterotoxins (see–see, seg–seo, and seq), two exfoliative toxins (eta and etb), gamma hemolysin (hlg), and the toxic shock syndrome toxin ( tst) was determined using previously reported methodologies, with some modifications.\textsuperscript{11,12} Additionally, isolates were examined for the presence of clfA, clfB, fib, fnbA, and fnbB genes for adhesive proteins and the ica operon, which is related to biofilm formation.\textsuperscript{13} The localization of seq and sek genes into pathogenicity island 5 was confirmed by PCR.

2.4. Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)

The genetic relationship of the isolates was determined by PFGE.\textsuperscript{2} The electrophoretic patterns were analyzed with the Gelcompare II program (Bio-Rad Laboratories, Hercules, CA, USA) using a Dice similarity coefficient. The isolates with \textgreater75\% similarity were clustered in patterns. The isolates were classified according to the criteria described by Tenover et al.\textsuperscript{14} MLST was carried out in accordance with the protocol described by Enright et al.\textsuperscript{15}

2.5. Antibiotic susceptibility profile

The minimal inhibitory concentration (MIC) was determined for 11 antibiotics (oxacillin, gentamicin, rifampin, erythromycin, ciprofloxacin, vancomycin, linezolid, tetracycline, clindamycin, trimethoprim, and sulfamethoxazole) by the agar dilution method.\textsuperscript{16} The inducible clindamycin resistance was determined by D-test.\textsuperscript{16}

2.6. Statistical analysis

Relative risk or virulence factor protection was evaluated using the diagnosis of the patient’s infection, and clinical outcome (mortality or improvement) was determined by applying the asymptotic or exact Pearson’s Chi-square test (expected values \textless5) to two tails or one tail when significant clinical differences were expected. The force of the association was evaluated using the odds ratio (OR) with reliability intervals of 95\%. The multilinear regression between the virulence factors and the coefficient of force was previously evaluated by the contingency coefficient, Phi, and Cramer’s V. A model for the explanation and control of confounding variables was constructed for infections where the results were either improvement or mortality, using a multivariate analysis with the model of unconditional logistic regression. The statistical tests were evaluated as having a level of significance at 5\% (p \textless0.05).

3. Results

3.1. Genetic relationship by PFGE

Nine electrophoretic patterns were obtained from the 270 MRSA isolates. Pattern F, present in 162 isolates (60\%), had a genetic similarity with the Chilean clone of \textgreater77.8\%. Pattern U, related to the USA300-0114 clone, was found in 86 (31.8\%) isolates of which only one was genetically indistinguishable, while 85 isolates presented a genetic similarity with the clone of \textgreater79.9\%. Pattern D, present in four isolates (1.5\%), showed a similarity with the pediatric clone of 79\% (Figure 1). Eighteen isolates presented pulsortypes that were not related to these three patterns (patterns G, H, K, O, and P).

3.2. Analysis by MLST

Twelve isolates were selected for typing by MLST according to their PFGE pattern. Two new alleles were obtained for the arcc gene with variations in only one nucleotide: ST1110 (938-4-1-4-12-1-10) in two isolates related to the Chilean clone and ST1111
(20–4–1–4–1–1–1–0) in one isolate related to the pediatric clone. These two ST are a single locus variant (SLV) of ST5. Three isolates were related to the Chilean clone, and one isolate was related to the pediatric clone with ST5. Five representative isolates of the predominant patterns U1, U2, and U3 were variants of ST8.

3.3. SCCmec typing, detection of the arginine catabolite mobile element (ACME), and susceptibility profiles

All isolates with patterns F and D had SCCmec types I and IV, respectively. In pattern G, nine isolates were type II and one isolate was type I. In pattern H, the isolates were type III; in patterns O and P, the isolates were type I; and in patterns R and K, the isolates were type II. Among the 86 CA-MRSA isolates, SCCmec Ivc was found in 82 isolates (95%), followed by Iva and Ivb in three (3%) and one (2%) of the isolates, respectively (Figure 1). The presence of ACME was detected in only one CA-MRSA isolate (COL207), which was genetically indistinguishable from the USA300-0114. In accordance with the PFGE patterns and SCCmec type, 86 (31.9%) isolates were classified as CA-MRSA and 184 (68.1%) as HA-MRSA.

In HA-MRSA, the presence of SCCmec I, II, and III was related to the resistance profiles reported, of which 31.5%, 60.9%, and 7.6% presented co-resistance to four, five, and six antibiotics, respectively. Among CA-MRSA isolates, 37.2% did not exhibit resistance to non-β-lactam antibiotics and 30.2% of the isolates were resistant to tetracycline. Co-resistance to three antibiotics was found in 16.2% (oxacillin–clindamycin–tetracycline and oxacillin–erythromycin–clindamycin), co-resistance to four antibiotics in 3.4%, and co-resistance to five antibiotics in 4.6% of the isolates.

3.4. Virulence factors

Among isolates classified as HA-MRSA, the sem, sen, and seo genes were detected in 184 isolates (100%); the seg gene was found in 173 isolates (94%) and 154 isolates (85%) possessed the sei gene. These five genes make up the enterotoxin gene cluster (egc). The exfoliative toxin A was found in 52 (60.5%) CA-MRSA isolates and 171 (92.9%) HA-MRSA isolates. The exfoliative toxin B was found in four (2.1%) isolates, and the sed and sei genes were found in 2 (1.0%) isolates from the pattern F (HA-MRSA). The sek, seq, and lukF-PV/lukS-PV genes were detected only in the CA-MRSA isolates (Figure 1). The fibronectin binding proteins A and B (fiba and fibb) were mainly detected in CA-MRSA isolates (Figure 1). The clfA, clfB, and fib genes were detected in all isolates. The hlg, tst, sea, seb, sec, see, seh, and sel genes were not found.
3.5. Relationships of genetic and clinical features, and mortality

During the study, the clinical data of 247 patients with 250 MRSA isolates were analyzed. One hundred and eighty-four isolates (73.6%) were classified as HA-MRSA and 66 isolates (26.4%) were classified as CA-MRSA. Our study showed a statistically significant association between mortality and HA-MRSA infection compared with CA-MRSA infections (26.1% vs. 10.6%; relative risk (RR) 2.46, 95% confidence interval (CI) 1.17–5.16, p < 0.009). Similarly, an increased mortality was found in patients with bacteremia (45.9%) and pneumonia (43.8%) compared with surgical site infection (10.8%) and other diagnoses (6.7%) (p < 0.001). Table 1 shows the association between clinical diagnosis and the presence of different virulence genes. The enterotoxin gene cluster (egc) was detected in a higher proportion in patients with bacteremia and pneumonia caused by HA-MRSA (p < 0.001). sek, seq, and lukF-PV/lukS-PV genes were significantly associated with soft tissue infection, and proved to be significant markers of CA-MRSA isolates (p < 0.001). The lukF-PV/lukS-PV genes were not found in MRSA isolates causing pneumonia.

Tables 2 and 3 show the relationships between virulence factors and clinical outcomes. In patients with HA-MRSA infections, the presence of sem, sen, seo, sei, and seq genes and the diagnosis of bacteremia or pneumonia significantly increased the risk of mortality (RR 4.66, 95% CI 2.73–7.95, p < 0.001 for sem, sen, seo genes, RR 3.93, 95% CI 2.44–6.35, p < 0.001 for the sei gene, and RR 5.00, 95% CI 2.93–8.52, p < 0.001 for the seq gene). Paradoxically, in the bivariate analysis, the sek and seq genes showed a protective effect with regard to mortality. This effect was also related to clinical improvement for the same toxins combined with the eta factor. However, in the multivariate analysis and measurement of the multicollinearity between the detection of different virulence factors, increases in the risk of mortality for seq (p = 0.001, OR 4.73, 95% CI 1.88–11.87) and in the protective effect for eta (p = 0.018, OR 0.33, 95% CI 0.13–0.83) were observed. The multivariate model of clinical improvement showed the same results, with seg (p = 0.002, OR 0.29, 95% CI 0.13–0.63) and eta (p = 0.022, OR 2.74, 95% CI 1.15–6.52).

4. Discussion

Staphylococcus aureus possesses an extensive repertoire of virulence factors that contribute to its survival and the development of pathologies of variable complexity. It is not easy to establish a clear profile of the virulence factors that explain the differences in the pathogenic processes that cause CA-MRSA and HA-MRSA. This is due to the variations in the genetic content of the MRSA lineage that contribute to its diversification and evolution.8

The present study, through the genetic detection of the virulence factors, aimed to contribute to the understanding of the genetic basis underlying the possible association of MRSA with a particular epidemiological behavior.

In Latin America, the presence of some virulence factors in CA-MRSA isolates has been reported. However, the epidemiological and genetic correlation between the profile of the virulence genes present in the different CA-MRSA and HA-MRSA clones and their clinical results has not yet been determined.6,17 This study details the coexistence of three MRSA clones that are predominant in Colombia: the Chilean clone and the pediatric clone that cause HA-MRSA infections, and a USA300-related clone (SCCmec IVc) that causes CA-MRSA infections. This study also highlights the discovery of two new allelic combinations, ST1110 and ST1111, both SLV of ST5, in the Chilean and Pediatric clones, respectively. This finding demonstrates the existence of constant genetic change in not just CA-MRSA but also in HA-MRSA. These bacteria undergo genetic variations that can give rise to isolates with greater capacities for environmental adaption.

In terms of virulence factors, differential content was reported between CA-MRSA and HA-MRSA isolates. Eighty-five percent of the HA-MRSA isolates possessed the enterotoxin gene cluster (egc), generally contained in the genomic island B type I. In contrast, the lukF-PV/lukS-PV, sek, and seq genes were found in the majority of CA-MRSA infections (92%, 80%, and 82% of CA-MRSA isolates, respectively). The last ones are transported by the pathogenicity island 5 (SalP5).

This study confirms that HA-MRSA clones predominantly carry the egc cluster, as reported in Europe, the USA, and Japan.18 The egc cluster is predominant in the HA-MRSA isolates related to the Chilean clone. From a clinical point of view, 40% of isolates came from patients with bacteremia and 35% from patients with surgical wound infections. It is therefore probable that one or more of the enterotoxins codified from the egc is not found in severe infections, but this conclusion seems contradictory if we consider that the enterotoxins, due to their nature as superantigens, induce an increase in capillary permeability, hypertension, and other symptoms related to toxic shock syndrome.7 Ferry et al.19 described a greater prevalence of the egc-positive isolates recovered from surface infections compared to invasive infections. The prevalence of the egc cluster significantly declined as the severity of the infection increased. van Belkum et al.20 did not find any evidence of an association of the egc cluster with mortality in patients with MRSA-related bacteremia. The bivariate analysis does not demonstrate a global association with mortality. After analyzing all of the models and measuring their multicollinearity, a significant increase in the risk of mortality was evident among

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### Table 2

<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Presence</th>
<th>Mortality, n %</th>
<th>p-Value</th>
<th>RR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>sem, sen, seo</td>
<td>Pos</td>
<td>48 (26.1)</td>
<td>0.006</td>
<td>2.46</td>
<td>1.17–5.16</td>
</tr>
<tr>
<td></td>
<td>Neg</td>
<td>7 (10.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sei</td>
<td>Pos</td>
<td>41 (26.1)</td>
<td>0.028</td>
<td>1.74</td>
<td>1.00–3.01</td>
</tr>
<tr>
<td></td>
<td>Neg</td>
<td>14 (15.1)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>seq</td>
<td>Pos</td>
<td>48 (27.1)</td>
<td>0.001</td>
<td>2.83</td>
<td>1.34–5.96</td>
</tr>
<tr>
<td></td>
<td>Neg</td>
<td>7 (9.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sek</td>
<td>Pos</td>
<td>5 (9.6)</td>
<td>0.009</td>
<td>0.38</td>
<td>0.16–0.91</td>
</tr>
<tr>
<td></td>
<td>Neg</td>
<td>50 (25.3)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>seq</td>
<td>Pos</td>
<td>6 (11.1)</td>
<td>0.019</td>
<td>0.44</td>
<td>0.20–0.98</td>
</tr>
<tr>
<td></td>
<td>Neg</td>
<td>49 (25.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVL</td>
<td>Pos</td>
<td>6 (10.3)</td>
<td>0.009</td>
<td>0.41</td>
<td>0.18–0.90</td>
</tr>
<tr>
<td></td>
<td>Neg</td>
<td>49 (25.5)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

MRSA, methicillin-resistant *Staphylococcus aureus*; RR, relative risk; CI, confidence interval.

---

### Table 3

<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Presence</th>
<th>Improvement, n (%)</th>
<th>p-Value</th>
<th>RR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>sem, sen, seo</td>
<td>Pos</td>
<td>127 (60.0)</td>
<td>0.017</td>
<td>0.83</td>
<td>0.72–0.96</td>
</tr>
<tr>
<td></td>
<td>Neg</td>
<td>55 (83.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sei</td>
<td>Pos</td>
<td>108 (68.8)</td>
<td>0.043</td>
<td>0.87</td>
<td>0.75–1.00</td>
</tr>
<tr>
<td></td>
<td>Neg</td>
<td>74 (79.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>seq</td>
<td>Pos</td>
<td>120 (67.8)</td>
<td>0.004</td>
<td>0.80</td>
<td>0.69–0.92</td>
</tr>
<tr>
<td></td>
<td>Neg</td>
<td>62 (84.9)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>sek</td>
<td>Pos</td>
<td>44 (84.6)</td>
<td>0.021</td>
<td>1.21</td>
<td>1.05–1.41</td>
</tr>
<tr>
<td></td>
<td>Neg</td>
<td>138 (69.7)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>seq</td>
<td>Pos</td>
<td>45 (83.3)</td>
<td>0.033</td>
<td>1.19</td>
<td>1.03–1.39</td>
</tr>
<tr>
<td></td>
<td>Neg</td>
<td>137 (69.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eta</td>
<td>Pos</td>
<td>164 (89.4)</td>
<td>0.048</td>
<td>1.31</td>
<td>0.95–1.80</td>
</tr>
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<td></td>
<td>Neg</td>
<td>18 (68.4)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>PVL</td>
<td>Pos</td>
<td>48 (82.8)</td>
<td>0.035</td>
<td>1.19</td>
<td>1.02–1.38</td>
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<td></td>
<td>Neg</td>
<td>134 (65.8)</td>
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<td></td>
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</tbody>
</table>

MRSA, methicillin-resistant *Staphylococcus aureus*; RR, relative risk; CI, confidence interval.
patients with infections where the seg gene was present ($p = 0.001$).

Regarding the significant association with mortality, Dauwalder et al. showed in septic shock models that seg does not induce a proinflammatory response related to increasing the severity of the infection. The prevalence of egc-harboring strains was higher among strains causing suppurative infections than in those causing invasive diseases.21 Ferry et al. also observed a negative relationship between the egc cluster and clinical severity.19 A similar analysis can infer that the presence of the seg gene is related to a worsening clinical result. Bae et al. described similar deterioration in clinical results through the presence of some seg genes, but they also found a paradoxically higher rate of clinical healing through the presence of some adhesive genes.22

The fnbA and fnbB genes were mainly detected in CA-MRSA isolates compared to HA-MRSA isolates (77% vs. 3%). These genes have been related to the increase in the adhesive and formation of biofilm capacity of S. aureus.23 From the physiopathological point of view, this is related to the clinical diagnosis, as it was found in 64% of patients with skin and soft tissue infections (SSTI) and in 20% of patients with bacteremia. The adhesion of S. aureus to the endothelium depends on the fnbA and fnbB genes. These also significantly improve the movement of bacteria to the bloodstream, the colonization of organs such as the kidneys,24 and the development of sepsis.25 In this study, 92% of the CA-MRSA isolates carried the luikF-PV/lukS-PV genes, which is a higher percentage than that reported in the USA26 and some European countries.27

The isolates related to the USA300 clone were characterized by the presence of the PVL gene, which was found in 85% of patients with SSTIs, a rate similar to that reported in the USA.28 There was no statistically significant association between the presence of PVL and mortality, as it was only detected in 10% of patients with CA-MRSA infections who died compared to 82% of patients who improved ($p = 0.009$), similar to the findings reported by Wang et al.29 In a multicenter study by Bae et al. conducted with 522 patients with complicated SSTIs in the USA, 85% of isolates were associated with the luikF-PV/lukS-PV genes and these patients exhibited a significantly higher probability of recovery compared to those infected by PVL-negative MRSA (91.6% vs. 80.7%, $p = 0.015$).22 These results are consistent with previous studies of patients with bacteremia and SSTIs and suggest that the presence of PVL is related to favorable and improved clinical results.30 Additionally, this study did not find a relationship between the presence of PVL and pneumonia. This finding is in contrast to the relationship previously described in Spanish patients with pulmonary diseases.31

An important finding in this study was the high detection rate of the eta gene in both CA-MRSA and HA-MRSA isolates, as well as its absence in all cases that were related to the clinical presence of bupleus petigito (data not shown). Surprisingly, the eta gene was related to favorable results compared with those described in other publications, in which its detection was minimal or absent.32 This finding is consistent with previously reported studies of these genes, but there is still a lack of correlation with clinical results.21 Furthermore, it has to be taken into account that the clinical outcome of an infection is not dependent only on the virulence factors of the bacteria; in fact, the outcome is due to multiple factors, such as immunological factors, co-morbidity of the host, anatomic site of the infection, proper antibiotic treatment, and prompt diagnosis and treatment, among others. Such clinical variables were not included in the multivariate analysis, this being a limiting factor of this study in the interpretation of our findings related to mortality and clinical recovery.

In conclusion, isolates of CA-MRSA and HA-MRSA in our study expressed differential patterns of virulence genes, predominantly the enterotoxin gene cluster (egc) in HA-MRSA isolates and sek, seq, and lukF-PV/lukS-PV genes detected only in the CA-MRSA. In patients with HA-MRSA infections, the presence of sem, sen, and seo genes and the diagnosis of bacteremia or pneumonia significantly increased the risk of mortality (RR 4.66, 95% CI 2.73–7.95, $p < 0.001$). PVL was not found to be a determinant of severity or mortality in CA-MRSA infections. It is necessary to conduct additional studies to clarify the interactive processes of the virulence, pathogenicity, and clinical results.

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