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Molecular Fingerprinting of *Staphylococcus aureus* Isolated from Patients with Osteomyelitis in Argentina and Clonal Distribution of the *cap5(8)* Genes and of Other Selected Virulence Genes

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Abstract

The molecular fingerprinting of a collection of 94 *S. aureus* isolates from patients with osteomyelitis in Argentina was performed. Twenty-three SmaI PFGE types and 37 *spa* types were identified. The isolates were assigned to 23 STs. The proportion of MRSA isolates was significantly higher among *cap5 S. aureus* (35/61) compared with *cap8 S. aureus* (8/33) isolates ($p=0.0025$). Twenty-four of the 94 isolates carried the *lukS-PV/lukF-PV* genes, which were significantly associated to *cap5* [(23/38) compared with *cap8 S. aureus* isolates (1/32) ($p=0.0001$)]. Forty of the 94 isolates carried genes of the *egc* locus (*seg/sei*). The distribution of *seg/sei* genes among isolates was related to certain clones. Isolates of the 4 *agr* types were found in the *S. aureus* collection. Whereas *agr*I isolates were evenly distributed among *cap5* and *cap8 S. aureus* isolates (32/61 and 14/33, respectively), the *agr*II group was composed of 29 *cap5 S. aureus* isolates and *agr*III was composed of 16 *cap8 S. aureus* isolates. Two clones originally associated to animals (ST 188, 7 isolates and ST 1796, 5 isolates) were associated with chronic osteomyelitis and lack of CP production. Loss of CP production remains the single factor among those investigated associated with chronic osteomyelitis.

Keywords

Staphylococcus aureus; clonal complex; *spa* type; osteomyelitis; Methicillin- resistance; capsule; *agr*

Introduction

The development of prosthetic joints has been one of the biomedical success stories of the twentieth century. Because of the increasing life expectancy in the developed nations and

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wide population segments in the developing countries the number of patients undergoing total knee arthroplasty or total hip arthroplasty has increased steadily over the past years. Despite improvements in surgical techniques, however, implantation of medical devices is associated with a definite risk of bacterial infections. Among the different etiological agents causing endoprostheses-associated infections, *Staphylococcus aureus* has emerged as one of the most prevalent and difficult-to-eradicate pathogens. There is undisputable evidence showing that certain diseases caused by *S. aureus*, such as osteomyelitis, start as acute and later develop into a protracted illness with an uncertain outcome [1]. *S. aureus*, in addition, has acquired over the years resistance against virtually all antimicrobial agents available and the worldwide emergence of multiresistant clones in hospitals and communities has been recognized [2]. More recently the emergence of non-multiresistant *S. aureus* in hospital and community settings has spurred concern [3,4].

Staphylococcus aureus displays a vast array of virulence factors, many of which exhibit broad functionality, allowing staphylococci to easily adapt to different environmental niches [5]. Among these factors, capsular polysaccharides (CP) of serotypes 5 (CP5) and 8 (CP8) are produced by a significant proportion of clinically-relevant isolates and were recognized as important virulence factors [6,7]. We have previously shown that loss of CP production, however, was associated with persistence of *S. aureus* in humans with chronic osteomyelitis [8]. This observation agrees with similar previous observations made in a mouse model of mastitis [9,10] and in bovines with subclinical mastitis [11]. A variety of mechanisms can explain the loss of CP production, including mutation in different genes [12] and deletion of *cap* cluster genes in *S. aureus* from bovine origin [12,13].

Multilocus sequence typing (MLST) and multi-strain microarray analysis have shown that most human *Staphylococcus aureus* strains belong to ten dominant clonal complexes (CCs) or lineages. Methicillin-resistant *S. aureus* (MRSA) strains currently belong to six of these lineages (CC1, CC5, CC8, CC22, CC30 and CC45) [14]. Because different *S. aureus* clones may be prevalent in different geographical regions of the world and since the potential association of loss of capsule type 5(8) production to determined clones has not yet been ascertained, the present study was conducted to investigate the population structure of *S. aureus* strains isolated from patients with osteomyelitis from seven hospitals in Argentina. The association of *cap5(8)* with *mecA* type, *agr* type and the presence of genes coding for PVL, and selected enterotoxins was also investigated.

Materials and methods

Bacterial isolates

S. aureus isolates were obtained from 94 patients with osteomyelitis at 7 hospitals in Argentina (4 from Buenos Aires City, 2 from Buenos Aires Province and 1 from Santa Fe) from 2005 to 2008. The isolates utilized in this study were drawn from a larger collection utilized previously to assess the prevalence of CP serotypes and the proportion of non-encapsulated isolates [8]. Isolates were obtained from patients suffering from chronic or acute osteomyelitis. The criteria for diagnosis of chronic osteomyelitis described by Cierny and Mader [15] were considered. According to these criteria, the hallmarks of chronic osteomyelitis include a nidus of infected dead bone or scar tissue, an ischemic soft tissue envelope, and a refractory clinical course. In the present study, patients with chronic osteomyelitis exhibited clinical records of infection for periods exceeding six months (range 6 months-33 years). Clinical records revealed that patients with acute infection were infected for less than 6 months. Isolation and identification of *S. aureus* was performed according to routine procedures [16]. Species was confirmed as *S. aureus* by PCR amplification of species-specific sequences [17].

Bacterial typing

The clonality of *S. aureus* isolates was assessed by SmaI-digested chromosomal DNA separated by pulsed-field gel electrophoresis (PFGE) using a CHEF-DR II apparatus (BioRad, Hercules, CA) as previously described [18]. The similarity between PFGE types was evaluated by the Dice coefficient. Bands of molecular size 30 kb to 500 kb were considered. The resultant similarity matrix was analyzed by the unweighted pair group method using arithmetic averages (UPGMA) and data were analyzed with the TREECON software for Windows. Clonality of the isolates was also investigated by multilocus sequence typing (MLST), as described previously [19]. Bacterial isolates were also analyzed using single locus sequence typing of the *Staphylococcus* protein A (*spa*) gene sequence as described elsewhere [20].

Phenotypic and genetic trait evaluation

CP5 and CP8 production was assessed by colony immunoblot and confirmed by immunodiffusion as described elsewhere [21]. Isolates yielding no positive precipitation with anti-CP5 or anti-CP8 antibodies in the immunodiffusion tests were classified as non-typeable (NT) [13]. The presence of the *cap* genes that define the CP5 and CP8 serotypes (*cap5H-J* and *cap8H-K*, respectively) were scored by PCR amplification [10]. Susceptibility to methicillin (using oxacillin disks 1 µg) was tested by the agar diffusion method and confirmed by determination of the MIC using the Etest^R strips (AB-BIODISK) as recommended by the manufacturers and the CLSI [22,23]. The heterogeneous resistance to methicillin was confirmed through the MIC to oxacillin and by the plating efficiency (EOP) method [24]. The production of the low affinity PBP2a was assessed with the SLIDEX MRSA detection kit (bioMérieux, Lyon, France). The SCC*mec* type in MRSA was determined by multiplex PCR amplification analysis as described [25]. The presence of the genes coding for expression of PVL (*lukS-PV/lukF-PV*) was evaluated by PCR amplification using the primers and conditions described elsewhere [26]. The *agr* types were determined by multiplex PCR amplification analysis as described [27]. The presence of enterotoxin genes *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei* and *sej*, as well as the *tst* (TSST-1), *eta* and *etb* (exfoliatin A and B, respectively) genes was assessed by multiplex PCR reactions [28].

Statistical analysis

The distribution of two groups of isolates into two categories (+ or –) was analyzed with 2x2 contingency tables by the two-sided Fisher exact test. When the analysis involved the distribution of more than two groups of isolates according to two categories, the Chi-square tests for independence was utilized (for instance the assessment of distribution of two categories (+/–) for four *agr* or SCC*mec* types). *P* values <0.05 were considered statistically significant. The Prism GraphPad software (version 4.0) was used for all statistical comparisons.

Results

Clonality of *S. aureus* in bone infection isolates

The *S. aureus* isolates investigated displayed high clonal diversity (Fig. 1). A total of 23 PFGE clonal groups were arbitrarily defined with a similarity threshold of 85%. Isolates were also identified by MLST and were assigned to 23 STs (Fig. 1), distributed into 9 CCs and 5 singletons. The most frequently found ST was ST5, which included 24 *cap5 agr* type II *S. aureus* isolates involving PFGE clonal groups A, D and E, and three *spa* types (12 t149, 8 t002 and 4 t311). The second most frequently found ST was composed of 13 ST97 *cap5 S. aureus* isolates that included PFGE clonal groups H and I. These 13 isolates exhibited a

higher diversity as ascertained by *spa* typing (6 types) but all belonged in *agr* type I. The third most frequently found ST was composed of 7 ST188 *cap8 S. aureus* isolates that clustered in PFGE clonal group O, *spa* type t189; these 7 isolates belonged in *agr* type I. The remaining 50 isolates represented 20 STs in groups of a smaller size (n=1–6), which involved 17 PFGE clonal groups and 29 *spa* types (Fig. 1). In the present study, 84% of the isolates were assigned to CC5 (27/94), CC8 (12/94), CC30 (8/94) plus two CCs: CC97 (18/94) and CC1 (14/94) not associated to any of the five major universally found CCs. The remaining 15 isolates involved one group represented by 4 isolates (ST669) and 11 isolates in other CC and ST groups of smaller size (n=1–2). It has to be noted, regarding the five major, universally occurring clusters, that no CC22 isolate and only one (2/94) CC45 isolate (included in the 15 isolates described in the previous sentence) was found in the *S. aureus* collection under scrutiny.

STs adapted to chronic bone infections

Whereas all isolates in the collection carried the *cap5* and *cap8* specific genes, 43% of them lacked CP production. Lack of CP expression was not in general associated to any major PFGE clonal group and occurred irrespective of the CP type involved. There was, however, one exception: the PFGE clonal group F was composed of 5 NT isolates assigned to the newly described ST1796 (CC97). The distribution of encapsulated and NT *S. aureus* in CC97 was not at random (ST97 CP5+: 11; ST97 NT: 2; ST1796 CP5+: 0; ST1796 NT: 5; $p=0.0025$) which suggests that the genetic changes responsible for the emergence of ST1796 may be associated with permanent loss of CP production. The distribution of isolates according to disease course in CC97 was not at random either (5 chronic and 8 acute in ST97; 5 chronic and no acute patient in ST1796; $p=0.0359$). These results confirmed the trend towards loss of CP production and chronicity. Similarly, the PFGE clone group O (CC1, ST188, *spa* type t189) *cap8 S. aureus* was composed of 7 isolates, all of them from patients with chronic osteomyelitis. Despite apparent differences in the distribution of CP8+ vs. NT isolates among chronic and acute patients in PFGE clonal group P, the trend was not significantly different. Analysis of other traits and genes revealed no statistical association between chronicity of infection and methicillin-resistance, *agr* type or presence of the *lukS-PV/lukF-PV* and enterotoxin genes.

Distribution of *cap5(8)* genes among *S. aureus* isolates

All 53 isolates in PFGE clonal groups A through K carried the *cap5* specific genes whereas most isolates in PFGE clonal groups L through W carried the *cap8* specific genes (33 of 41 isolates) (Fig. 1). The only 8 *cap5 S. aureus* within PFGE clonal groups L through W were 4 isolates assigned to ST8 (CC8), 2 isolates assigned to ST247 (CC8) and 2 isolates assigned to ST25 (CC25), clustering together within PFGE clonal groups M, N and V, respectively. The distribution of *cap5* and *cap8* among PFGE clonal groups was significantly different ($p<0.0001$) from a random distribution. *agr*I isolates were evenly distributed among *cap5* and *cap8 S. aureus* (32/61 and 14/33 isolates, respectively). The distribution of *agr* types II and III, however, was significantly different among isolates bearing the different *cap* specific genes (Table 1). All *cap5 S. aureus* isolates within PFGE clonal groups A through E (almost all CC5) belonged in the *agr* II group. All isolates within PFGE clonal groups F through O belonged in the *agr* I group. Major PFGE clonal groups P and W were composed solely of *cap8 S. aureus agr* III isolates.

Association of *cap5* with *mecA* and other selected genes

Forty-seven percent (43/94) of the isolates investigated were MRSA. The proportion of MRSA isolates was significantly higher among *cap5 S. aureus* (35 MRSA/26 MSSA) compared with *cap8 S. aureus* (8 MRSA/25 MSSA) isolates ($p=0.0025$). Only *cap5 S. aureus* carried the SCC*mec* type I and only *cap8 S. aureus* bore the SCC*mec* type III. The

SCC*mec* type IV was indistinctly found among both *cap5* and *cap8* MRSA. Twenty-four of the 94 isolates tested yielded positive PCR amplification for the *lukS-PV/lukF-PV* genes (Fig. 1). These genes were detected mostly among *cap5 S. aureus* isolates [(23 (+)/38 (-) isolates] compared with *cap8 S. aureus* isolates [1 (+)/32 (-)] ($p=0.0001$). A considerable proportion of *S. aureus* isolates (43/94) carried the genes coding for superantigens located in the *egc* locus (*seg* and *sei*) (Fig. 1). All 29 *cap5 S. aureus* isolates in PFGE clonal groups A through E (mostly ST5 and ST100) carried the *seg/sei* genes. In addition, 11 of 21 isolates in PFGE clonal groups P through W carried the *seg/sei* genes and were all *cap8 S. aureus*. The distribution of isolates bearing the *seg/sei* genes exhibited significant differences among *agr* groups ($p<0.0001$) (Table 1).

Discussion

S. aureus of different lineages are prevalent in different regions of the world [29]. Recently, it has been shown that CC8 (19%), CC45 (18%) and CC30 (13%) were the most prevalent *S. aureus* clonal complexes found among patients with bone and joint infection in the Dresden University Hospital in Germany [30]. In the same study, capsule serotype 8 specific genes (*cap8*) were detected in 60% of the isolates whereas the remainder carried the capsule serotype 5 (*cap5*) specific genes; only 6% of the isolates were MRSA. In another study, the three more frequently found CCs in a collection of isolates from three hospitals in the Bronx, New York, USA [31] were CC8 (28%), CC5 (28%) and CC30 (6%). Very few studies have addressed the *S. aureus* ST types found in Argentina and most of them were limited to a few representative MRSA isolates [32,33]. Only very recently in 2012 Sola et al. described thoroughly the prevalence of MRSA clones in Central and Northern Argentina and emphasized the paucity of data about molecular epidemiology of community acquired MRSA infections in South America [34]. This is, to our knowledge, the first report that determined the STs of significant number of both MRSA and MSSA in the region. In the present study, nearly one-third of the isolates were assigned to CC5 (*cap5*), followed by CC97 (*cap5*) (19%) and CC1 (*cap8*) (15%); only 2 CC45 isolates were found in the collection investigated. One major cluster (CC5 PFGE types A-D) included the Cordobes HA-MRSA clone. Adjacent was the CC5 PFGE type E cluster, which included the Pediatric CA-MRSA clone and shared all the genotypic features of PFGE type A-D isolates except that the Pediatric clone isolates carried the *lukS-PV/lukF-PV* genes, a feature of CA-MRSA [35]. Isolates in the PFGE type L cluster were assigned to CC8 (ST239) and included the Brazilian HA-MRSA clone representative strain. In addition to the CCs associated to the prevalent MRSA clones, a considerable number of isolates were assigned to CC97 and CC1.

It is known that there are *S. aureus* clones better adapted to animals than humans and vice-versa [36]. However, *S. aureus* clones from animals, such as porcine CC398, may colonize and infect the human host thus becoming a potential concern for Public Health [37]. *S. aureus* CC97 has been recognized as predominant in bovines with mastitis [38] with little impact on humans [4]. In the present study, we have found a large proportion of CC97 *S. aureus* causing osteomyelitis. The ST188 animal-associated lineage [37] was also considerably represented (7%) in the collection under investigation. Whether CC97 and ST188 are clones that successfully moved from animal hosts to the humans in the geographical region investigated remains unknown since the relative prevalence of *S. aureus* CCs and STs has not been evaluated in animals of Argentina.

Previous phenotypic studies addressing the prevalence of the *S. aureus* CP serotypes in human disease have described different proportions of isolates expressing CP5 or CP8 [39,40,41]. The present genotypic study showed that a higher proportion of isolates carried the *cap5* specific genes compared with the *cap8* ones. In addition, a significantly higher proportion of isolates bearing the *cap5* specific genes also carried the *mecA* gene (SCC*mec*

types I or IV), compared with those isolates bearing the *cap8* specific genes, which were mostly MSSA. These data support previous findings of an association between the presence of CP5 and methicillin resistance [42]. We have also found in this study that almost all isolates assigned to CC5 were MRSA, carried the *cap5* specific genes and also carried the genes for enterotoxin G (*seg*) and enterotoxin I (*sei*). However, another group of isolates carrying the *seg* and *sei* genes were *cap8* *S. aureus*. These isolates clustered in contiguous PFGE clonal groups Q through W and most of them were MSSA. Therefore the higher proportion of *cap5* isolates from patients with osteomyelitis from the geographical region involved seems to be more influenced by resistance to methicillin rather than the potential production of enterotoxins G and I.

Resistance of *S. aureus* to beta-lactam antibiotics is one of the major contemporary challenges to public health. Methicillin-resistant *S. aureus* (MRSA) causes not only hospital-acquired (HA) but also community-acquired infections [29]. Moreover, the emergence of livestock-associated (LA) MRSA and its dissemination into hospitals has to be noted [37]. The Pediatric and the Cordobes MRSA clones prevalent in Argentina share a common lineage [43]. The MRSA Pediatric clone that carried the SCC*mec* type IV has been associated to CA-MRSA isolates whereas the MRSA Cordobes clone that carried the SCC*mec* type I has been associated to HA-MRSA isolates [32]. Therefore, it is not a surprising finding that all isolates in PFGE clonal group E (ST5), which includes the Pediatric clone representative, carried the *lukS*-PV/*lukF*-PV genes (PVL). Isolates of the Pediatric CA-MRSA clone bore the *cap5* genes and the SCC*mec* type IV.

The distribution of the *cap5* or *cap8* specific genes in isolates of the collection investigated was highly clonal. All isolates of PFGE types A through N carried the *cap5* specific genes, except those assigned to PFGE type L (ST239), whereas all PFGE types from O through W carried the *cap8* specific genes, except PFGE type V (ST25). It is known that the ST239 chromosome is a hybrid of distinct ST8-like and ST30-like fragments, inherited in a recombination event from ST8-like and ST30-like parents, respectively, plus additional elements that have been subsequently acquired [44,45]. The *cap* locus of ST239 is located in the ST30 region of the chromosome and, therefore, ST239 isolates bear the *cap8* like ST30 isolates instead of the *cap5* like the ST8 isolates. In contrast, the *agr* locus is located in the ST8 region of the chromosome. Therefore, ST239 isolates are *agr*I like the ST8 isolates, instead of *agr*III like the ST30 isolates. The results obtained from the collection under investigation are consistent with the described mechanism of ST239 emergence. With regard to the origin of methicillin resistance in the Brazilian clone, the SCC*mec* type III element is a composite element of SCC*mec* and SCC*Hg* that together encode resistance to multiple antibiotics, antiseptics, and heavy metals [46]. It is not clear where SCC*mec* III originated because neither ST8 nor ST30 are known that carry this element. The mechanism responsible for the association of the *cap5* specific genes to the two isolates assigned to ST25 (PFGE type V) remains undisclosed.

No other study has assessed before, to our knowledge, the prevalence of *S. aureus* STs in MSSA and MRSA in a sizeable collection of clinical isolates from Argentina. From the present study, it can be concluded that many *S. aureus* clones cause osteomyelitis in patients from the geographical region under scrutiny. There was no major specific clone associated with chronicity of the infection, except two clones where loss of CP production may be a fixed trait (ST1796 and ST188). The clonal distribution of genes coding for selected pathogenesis factors confirms the multifactorial basis of *S. aureus* osteomyelitis. We have shown in the *S. aureus* collection utilized in this study an association between the *cap5* genes and methicillin resistance, irrespective of CP5(8) production. Finally, we showed that loss of CP production remains the single factor among those investigated associated with chronic osteomyelitis.

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Figure 1. Dendrogram obtained by analysis of SmaI PFGE band patterns from *S. aureus* isolates. PFGE clonal groups were defined considering 85% similitude (dotted line). Each column depicts the reported feature for each isolate. The “PFGE” column represents the 23 PFGE clonal groups, from A to W. The column labeled “Course” indicates whether the isolate was obtained from a patient with acute or chronic osteomyelitis. The column labeled ST indicates the multilocus sequence type and the column labeled CC indicates the clonal complex. The column labeled *spa* indicates the *spa* type. The remaining columns were labeled to indicate the gene, phenotypic trait or group investigated. The presence of the *eta*

and *etb* genes was investigated but none of the isolates carried the genes coding for exfoliatin toxins A and B.

Table 1

The association between *agr* types and CP5 and CP8, and between *agr* types and enterotoxins G and I genes in *S. aureus* isolates.

	Number of isolates			
	<i>agr</i> I	<i>agr</i> II	<i>agr</i> III	<i>agr</i> IV
<i>cap5 S. aureus</i> ^a	32	29	0	0
<i>cap8 S. aureus</i>	14	0	16	3
<i>seg/sei</i> (+) ^b	6	29	8	3
<i>seg/sei</i> (-)	40	0	8	0

^aThe distribution exhibited significant differences (p<0.0001, Chi-Square test) from *cap5* and *cap 8 S. aureus* even distribution among *agr* types.

^bThe distribution exhibited significant differences (p<0.0001, Chi-Square test) from *seg+/sei+* and *seg-/sei-* *S. aureus* even distribution among *agr* types.