Original Article

High genetic diversity and zoonotic potential of *Staphylococcus aureus* strains recovered from bovine intramammary infections in Colombians dairy herds

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Abstract

Genotyping of *Staphylococcus aureus* isolated from mastitis has become a fundamental tool to understand its complex epidemiology and to evaluate spillover events. The aim of this study was to describe the frequency of genotypes of the *S. aureus* strains isolated from intramammary infections by *spa* typing technique, and to evaluate the association between genotypes and the ability to form biofilm under in vitro conditions. Sixty-six strains of *S. aureus* recovered from bovines intramammary infections on 56 dairy herds located in 14 municipalities of the department of Antioquia were characterized. The majority of strains (65/66) were isolated from milk samples collected from dairy cows with subclinical intramammary infections. Nineteen different *spa* types were found in this study, t521 (19.70%), t267 (15.15%), and t605 (12.12%) being the most frequent. The strains from the t605 *spa* type showed the highest biofilm production. The high frequency of *spa* types with zoonotic potential found in this study, identified cattle as an important reservoir of theses clones for people in close proximity, such as milkers and consumers of unpasteurized dairy products.

Keywords: Intramammary infection; Clonal complex; Genotyping; spa type; spa typing

1. Introduction

Staphylococcus aureus is one of the main pathogens which causes intramammary infections (IMI) in dairy cattle and represent a public health risk due to high antimicrobial resistance, food safety, and zoonotic potential (Olde Riekerink et al., 2006). Multidrug resistance of *S. aureus* and the high evasion capacity of the host immune response gives rise to persistent infections and the ability to spread fast in herds, hindering its control (Feltrin et al., 2016; Zecconi & Scali, 2013).

Genomes of *S. aureus* have demonstrated high diversity among strains, which has been associated with variability in the virulence, response to antibiotic treatment, type of host and transmission between them, geographic distribution, and infection severity (Haveri et al., 2008; Matuszewska et al., 2020). Genotyping tools provide fundamental information to understand the complex epidemiology of *S. aureus* (Boss et al., 2016; Zadoks et al., 2011). Pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and *spa* typing are some of the most widely used methods to typify strains of this pathogen (Boss et al., 2016). The *spa* typing is based on the sequencing of the spacer variable region (X region) of the *spa* gene, showing comparable resolution with the other two methods but is less expensive and laborious. This typing method has made possible the study of the clonal relatedness and genomic diversity among *S. aureus* lineages recovered from human and bovines (Asadollahi et al., 2018; Käppeli et al., 2019).

Close contact between milkers and cattle, as well as the ability of *S. aureus* clones to adapt to both hosts, show the potential of zoonotic transmission and determine cows as an important human infections source (Spoor et al., 2013). In Colombia, where *S. aureus* is one of the

main bacteria causing of IMI (Vidal et al., 2016) and hand milking continues to be frequent (43.6% - 77.7%) (Ramírez et al., 2014; Reyes et al., 2017), there is a high risk of milkers becoming infected. Furthermore, about 40% of raw milk is still commercialized under informal conditions (Business Bridge, 2015), potentially allowing that this pathogen to spread among the general population. Hence the relevance of improving knowledge by molecular typing of the *S. aureus* strains involved in IMI in order to evaluate the risk of spread into the community. Therefore, the aim of this study was to describe the frequency of genotypes of the *S. aureus* strains isolated from intramammary infections in Antioquia (Colombia) through the *spa* typing technique, and to evaluate the association between genotypes and the ability to form biofilm under in vitro conditions.

2. Materials and Methods

2.1 *Staphylococcus aureus* isolates

The selection of the 66 strains was carried out by using convenience sampling from the mastitis strains collection of the milk quality reference laboratory of the Biogenesis Research Group of the Department of Agricultural Sciences of the University of Antioquia. These strains were collected from samples as part of a milk quality control and udder health program from July-December 2015 (Table 1). The majority of these strains (65/66) were obtained from subclinical IMI. Although the selection was based on convenience, it is important to highlight that the isolates were coming from the main dairy municipalities of the department of Antioquia, considered the region with the highest milk production in the country, were included.

Isolates were initially classified as *S. aureus* using conventional microbiological techniques, based on the proposal of the National Mastitis Council (National Mastitis Council, 2017). They were stored at −80 °C in Trypticase soy agar (TSA) (Oxoid, United Kingdom) supplemented with 10% glycerol until use.

2.2 DNA extraction

The stored *S. aureus* strains were thawed and cultured in TSA medium (Oxoid, United Kingdom) and incubated at 37 °C for 24 h. DNA was extracted using the DNeasy Blood & Tissue Kit (Quiagen, Germany) according to the manufacturer's recommendation for Gram-positive bacteria. A NanoDrop kit (ThermoFisher Scientific, USA) was used to measure the purity and concentration of the extracted DNA. The DNA was then stored at −20 °C until use.

2.3 Molecular confirmation of *S. aureus* and *mecA* gene detection

The strains used in the study were confirmed as *S. aureus* using polymerase chain reaction (PCR). The PCR proposed by Graber et al. (2009) allowed the amplification of a fragment of the *nuc* gene, recognized as being species-specific (Brakstad et al., 1992). The *mecA* gene was detected according to the methodology described by Zehra et al., 2017. The PCR reactions were conducted in a PTC 200 thermocycler (Perkin-Elmer Inc., USA).

2.4 Determination of susceptibility to antibiotics

The antibiotic susceptibility profile of the *S. aureus* strains was determined with the Kirby–Bauer methodology, following the recommendations of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2020). The disc-diffusion

antibiotic used (Oxoid, United Kingdom) were oxacillin (1 µg), penicillin G (UOF), ceftiofur (30 µg), erythromycin (15 µg), doxycycline (30 µg), and tetracycline (30 µg). *S. aureus* ATCC 25922 strain was used as a control. The resistance to each of the antibiotics was recorded as a binary attribute for each isolate. Moreover, two variables were derived from the resistance profiles. The first one was the number of antibiotics to which the isolate was resistant and the second was the resistance to at least one of the tested antibiotics.

2.5 Genotyping by *spa* typing

The polymorphic region of the gene coding for protein A (*spa* gene), known as region X, was amplified by PCR for each strain. The primers used in the reaction were those described by Shopsin et al. (1999), while the thermal profile followed was that reported by Jiménez et al., 2012, with denaturation at 94 °C for 30 s, alignment at 60°C for 1 m, and 72 °C extension for 1 m, for 30 cycles. Expected band size was approximately 1.000 bp, which was later sequenced by Macrogen (USA). The *spa* types were determined using Ridom SpaServer (http://www.spaserver.ridom.de/).

2.6 Biofilm production in vitro

Biofilm production (optical densities – OD) and clusters solutions from our previous publication (Torres et al., 2019) were included as data attributes in the present study to explore further the biofilm production by *spa* types. Quantification of biofilm on microtiter plates was performed according to Stepanović et al., 2007 protocol. To facilitate the interpretation of the results, the strains were initially classified as follow: non-biofilm producer (OD ≤ ODc) and biofilm producer (OD > ODc). The cut-off value (ODc) was defined as three standard deviations above the OD mean of the negative control (Trypticase

soy broth with glucose). Biofilm quantification was performed in duplicate and repeated three times (Torres et al., 2019).

2.7 Statistical analysis

Descriptive analyses were performed for all the variables of interest. Further bivariate comparisons using Chi square and Fisher's exact tests were assessed to determine the relationship between antibiotic resistance, municipalities, and genotypes. A statistical threshold of $P < 0.05$ was considered. All the analyses were carried out on Rstudio statistical software program (version 3.6.0) (https://cran.R-project.org).

3. Results

3.1 *Staphylococcus aureus* typing

A total of 19 different *spa* types were identified. The most frequent *spa* type was t521 (19.70%), found in 64.3% (9/14) of the municipalities, followed by t267 (15.15%), t605 (12.12%), and t543 (10.61%), which were identified in seven (50.0%), three (21.4%), and five (35.7%) municipalities, respectively. The compilation of all *spa* types reported for each municipality is shown in table 2.

3.2 Biofilm production in vitro and clusters obtained

Forty-four (66.7%) strains were classified as biofilm former according to determined cut-off (OD > 0.23), 6 (9.1%) were non-biofilm former, whereas 16 (24.2%) strains were undetermined because these showed three OD that differed by at least twice ODc among them.

154 The cluster analysis excluded those strains classified as undetermined. The other 50 strains
155 were divided into three clusters: the cluster 1 included 30 (60.0%) strains with OD values
156 lower than 1.1 (30), the cluster 2 was formed by nine (18.0%) strains with higher OD values,
157 2.4 – 3.9, and 11 (22.0%) strains that showed OD between 1.3 – 2.7 were categorized into
158 cluster 3.

159 The bivariate analysis performed between *spa* types and biofilm production cluster showed
160 significant association (P = 0.023). It is important to note that the most (6/8) of the t605
161 strains tested were included intro the cluster 2, which was mainly composed by strains that
162 showed the highest biofilm production in vitro. In contrast, most (8/10) of isolates belonging
163 to t267 and all (4/4) of the t1236 were classified into cluster 1, which had the lowest biofilm
164 formation. The distribution of the clusters for the nine more frequent *spa* types (around 85%
165 of the total isolates evaluated) is presented in table 3.

166 3.3 Antibiotic susceptibility and molecular detection of the *mecA* gene

167 The *S. aureus* strains evaluated showed greater resistance to the antimicrobials penicillin and
168 lincomycin (59.1%), followed by cefoperazone (10.6%), ampicillin/sulbactam (9.1%),
169 trimethoprim/sulfa (7.6%), amoxicillin/clavulanic acid (4.5%), and cloxacillin (3%). Out of
170 the 66 strains evaluated, only one was positive for the *mecA* gene. Moreover, 80% (n=53) of
171 the strains were resistant to at least one antimicrobial.

172 The bivariate analysis performed between resistance to at least one antibiotic and
173 municipality was not significant (P = 0.3744), and between antibiotic resistance to at least
174 one antibiotic and genotype did not show significant association (P = 0.2885).

175

4. Discussion

High diversity of *spa* types was identified (n=19) from 66 isolates. The two most common *spa* types in this study were t521 (19.70%) and t267 (15.15%), both genotypes commonly reported as causing subclinical mastitis in cattle (Schmidt et al., 2017). Theses *spa* types are part of the clonal complex 97 (CC97), which is recognized as the most reported complex in genotyping studies of strains obtained from cattle and an emerging cause of human infections (Feltrin et al., 2016; Wang et al., 2018).

Unlike our results, t521 has not been so frequent in other studies in which typing *S. aureus* strains were also recovered from bovines with IMI, agreeing with what is reported in the Ridom SpaServer (https://spa.ridom.de/frequencies.shtml). This database shows a frequency of 0.05% for t521 at a global level. The frequencies found in studies performed in Brazil, Canada, and Tunisia were 2.1%, 1.98%, and 13.95%, respectively (Ben Said et al., 2016; Bonsaglia et al., 2018; Pichette-Jolette et al., 2019). On the other hand, in USA (Albrecht et al., 2015) and Kuwait (Boswihi et al., 2020), t521 was also isolated from human samples, suggesting its zoonotic potential. Some of the strains recovered from patients in Kuwait were even Methicillin-Resistant *S. aureus* (MRSA). In our study MRSA strains belonging to t521 were not found.

The t267 *spa* type is more frequent at global level than t521, as is observed in the Ridom SpaServer (0.35%) and in most studies where it has been reported, being in general the first or second more predominant *spa* type isolated from bovines in several countries. Although t267 was the second most common genotype in our study, its frequency was lower (15.15%) than in other studies carried out in China, India, Sweden, and Canada, which ranged between

18% - 25% (Käppeli et al., 2019; Li et al., 2017; Mitra et al., 2013; Veh et al., 2015). In Canada, t267 is one of the more predominant genotypes (Demontier et al., 2021). MRSA strains belonging to t267 *spa* type were also isolated from patients in Kuwait and USA (Albrecht et al., 2015; Boswihi et al., 2020), but in our study, we did not find t267 *mecA* positive strains. These finding highlight the ability of t267 to infect also humans.

With respect to t605 *spa* type, third (12.12%) most frequent in this study, it is a genotype less common than t267 on a global level (0.09% in Ridom SpaServer). According to reports from Brazil, the t605 frequency varied between 37.5% - 92.52% (Bonsaglia et al., 2018; Santos et al., 2020; Silva et al., 2013), which is a higher frequency than those obtained in our study. Recently, we performed the whole-genome sequencing (WGS) of a t605 strain, previously isolated from bovine mastitis in Antioquia (Colombia) (Torres et al., 2020). Our results allowed the classification of this clone as Sequence Type 126 (ST126), one of the genotypes widely reported in Brazil as a cause of mastitis and related to persistent infections. In addition, we found that this strain was a carrier of *bap* gene, which has been associated with a strong biofilm-forming phenotype (Cucarella et al., 2004; Demontier et al., 2021; Torres et al., 2020). Earlier, we also demonstrated that six out of eight strains typified as t605 in this study were *bap* positive, which was consistent with the highest amount of biofilm observed, suggesting the role of the *bap* gene in the pathogenesis of *S. aureus* (Torres et al., 2019). Cucarella et al., (2004) found in *bap* positive strains caused more persistent infections compared to native strains (*bap* negative). Likewise, a study performed with six major Canadian *spa* types isolated from bovine mastitis (among theses t267 and t605), described that t605 strains *bap* positive were in vitro biofilm hyperproducers (Demontier et al., 2021). The authors suggested that this genotype would be more related to chronic mastitis, which

221 also was confirmed by Rossi et al., (2019), who demonstrated persistence of this clone for
222 up to four months.

223 Currently, the clonal complex classification of the t605 strains is unclear. Most studies have
224 included it in CC126 (Alves et al., 2018; Bonsaglia et al., 2018; Rossi et al., 2019; Silva et al.,
225 2013). However, we previously found this clone to be more closely related to ST126 (Torres
226 et al., 2020), which, according to PubMLST (https://pubmlst.org/organisms/staphylococcus-
227 aureus), belongs to CC97. This clonal complex has become one of the major MRSA clones,
228 as observed in Italy, where it is recognized as one of the most prevalent MRSA lineages in
229 pig and dairy cattle (Feltrin et al., 2016). These finding are consistent with our results, since
230 the only positive *mecA* strain identified belonged to t605 *spa* type. Furthermore, some CC97
231 lineages, among these t605, are an emerging cause of human infections. For example, in
232 Denmark humans infections caused by clones belonging CC97 increased 11-fold between
233 2007 and 2011 (Spoor et al., 2013). More recently in Iran, t605 clones carrying of *mecA* gene
234 were isolated from human and it was recognized as an emerging genotype (Goudarzi et al.,
235 2020).

236 Regarding the antibiotic susceptibility profiles obtained, the penicillin and lincomycin
237 showed the highest resistance profile. These results are consistent with those reported by
238 other studies carried out in the same region (Ramírez et al., 2018; Ramírez et al., 2011) and
239 in studies performed in China, as well as in Sweden and Tunisia, which also reported high
240 penicillin resistance (Ben Said et al., 2016; Käppeli et al., 2019; Liu et al., 2020).

241 **Limitations**

There are several limitations than need to be considered to interpret the results of this research. First, the authors acknowledge that the selection of isolates where not part of a systematic sampling process by geographical region, since these isolates were obtained from the mastitis strain collection of the Milk quality reference laboratory of the Biogenesis Research Group of the Department of Agricultural Sciences of the University of Antioquia. However, as a reference laboratory of milk quality in the region, a large amount of milk samples was processed as part of milk quality control and udder health programs. The second limitation is the lack of epidemiological information about the hosts and sites, from which the strains were collected.

5. Conclusions

Despite of the limitations, the study showed the high frequency of *spa* types belonging to CC97 with zoonotic potential found in this study, identify cattle as an important reservoir of theses clones for people in close proximity, such as milkers and consumers of unpasteurized dairy products. Hence, our results highlight the importance of the molecular surveillance of *S. aureus* strains that cause IMI with potential risk of occupational exposure in the dairy industry and spreading to the community.

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Author contributions

G. T. Conceptualization, methodology, investigation, and writing – review and editing. **K. V.** Conceptualization, methodology, investigation, and writing – review and editing. **J.R.V.** Formal analysis and writing – review and editing. **N. J.** Conceptualization, methodology, data curation, and formal analysis. **A. B.** Data curation and formal analysis. **M. O. A.** Resources, supervision, and funding.

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468 **TABLES**

469 **Table 1.** Distribution of strains by municipality

Municipality	Dairy herds number	Strains number (%)
Abejorral	2	2 (3.03%)
La Ceja	1	1 (1.52%)
Rionegro	1	1 (1.52%)
La Unión	1	1 (1.52%)
Urrao	4	5 (7.58%)
Bello	7	7 (10.61%)
Donmatías	4	6 (9.09%)
Belmira	7	8 (12.12%)
Entrerrios	6	7 (10.61%)
San Pedro de los Milagros	11	16 (24.24%)
Santa Rosa de Osos	7	7 (10.61%)
San José de la Montaña	2	2 (3.03%)
Carolina del Príncipe	2	2 (3.03%)
San Jerónimo	1	1 (1.52%)
14 Municipalities	**56 herds**	**66 strains** (100%)

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476 **Table 2**. *spa* types found by municipality

Municipality	Strains number evaluated (%)	*spa* type found (strains number)
Abejorral	2 (3.03%)	t18436 (1), t267 (1)
La Ceja	1 (1.52%)	t543 (1)
Rionegro	1 (1.52%)	t13485 (1)
La Unión	1 (1.52%)	**t521** (1)
Urrao	5 (7.58%)	t527 (2), t267 (1), t605 (1), t1190 (1)
Bello	7 (10.61%)	t267 (3), **t521** (1), t543 (1), t605 (1), t1885 (1)
Donmatías	6 (9.09%)	**t521** (3), t2207 (2), t527 (1)
Belmira	8 (12.12%)	t543 (2), t1236 (2), **t521** (1), t4911 (1), t2207 (1), t2112 (1)
Entrerrios	7 (10.61%)	**t521** (3), t267 (1), t2207 (1), t2112 (1), t18438 (1)
San Pedro de los Milagros	16 (24.24%)	t605 (6), t527 (2), t543 (2), t267 (1), **t521** (1), t571 (1), t2207 (1), t3626 (1), t18437 (1)
Santa Rosa de Osos	7 (10.61%)	t267 (2), t 1236 (2), **t521** (1), t4911 (1), t6664 (1)
San José de la Montaña	2 (3.03%)	**t521** (1), t693 (1)

Carolina del Príncipe	2 (3.03%)	t267 (1), t543 (1)
San Jerónimo	1 (1.52%)	**t521** (1)

The most frequent *spa* type is highlighted in bold

507 **Table 3**. Distribution of the most prevalent *spa* types by cluster

***spa* type**	**Cluster 1**	**Cluster 2**	**Cluster 3**	**Undetermined**	**Strains total**
t521	6	0	1	6	13
t267	8	0	1	1	10
t605	0	6	2	0	8
t543	4	1	1	1	7
t527	2	1	1	1	5
t2207	1	2	1	1	5
t1236	4	0	0	0	4
t2112	1	0	0	1	2
t4911	1	0	0	1	2

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TABLES

Table 1. Distribution of strains by municipality

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Abejorral	2	2 (3.03%)
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San Pedro de los Milagros	11	16 (24.24%)
Santa Rosa de Osos	7	7 (10.61%)
San José de la Montaña	2	2 (3.03%)
Carolina del Príncipe	2	2 (3.03%)
San Jerónimo	1	1 (1.52%)
14 Municipalities	**56 herds**	**66 strains** (100%)

Table 2. *spa* types found by municipality

Municipality	Strains number evaluated (%)	*spa* type found (strains number)
Abejorral	2 (3.03%)	t18436 (1), t267 (1)
La Ceja	1 (1.52%)	t543 (1)
Rionegro	1 (1.52%)	t13485 (1)
La Unión	1 (1.52%)	**t521** (1)
Urrao	5 (7.58%)	t527 (2), t267 (1), t605 (1), t1190 (1)
Bello	7 (10.61%)	t267 (3), **t521** (1), t543 (1), t605 (1), t1885 (1)
Donmatías	6 (9.09%)	**t521** (3), t2207 (2), t527 (1)
Belmira	8 (12.12%)	t543 (2), t1236 (2), **t521** (1), t4911 (1), t2207 (1), t2112 (1)
Entrerrios	7 (10.61%)	**t521** (3), t267 (1), t2207 (1), t2112 (1), t18438 (1)
San Pedro de los Milagros	16 (24.24%)	t605 (6), t527 (2), t543 (2), t267 (1), **t521** (1), t571 (1), t2207 (1), t3626 (1), t18437 (1)
Santa Rosa de Osos	7 (10.61%)	t267 (2), t 1236 (2), **t521** (1), t4911 (1), t6664 (1)
San José de la Montaña	2 (3.03%)	**t521** (1), t693 (1)
Carolina del Príncipe	2 (3.03%)	t267 (1), t543 (1)
San Jerónimo	1 (1.52%)	**t521** (1)

The most frequent *spa* type is highlighted in bold

Table 3. Distribution of the most prevalent *spa* types by cluster

***spa* type**	**Cluster 1**	**Cluster 2**	**Cluster 3**	**Undetermined**	**Strains total**
t521	6	0	1	6	13
t267	8	0	1	1	10
t605	0	6	2	0	8
t543	4	1	1	1	7
t527	2	1	1	1	5
t2207	1	2	1	1	5
t1236	4	0	0	0	4
t2112	1	0	0	1	2
t4911	1	0	0	1	2

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

High genetic diversity and zoonotic potential of *Staphylococcus aureus* strains recovered from bovine intramammary infections in Colombians dairy herds

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