Characterization of *Staphylococcus aureus* strains involved in human and bovine mastitis

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Abstract

Staphylococcus aureus is one of the main etiological agents of mastitis in different mammalian species. At present, it is unknown if strains isolated from human mastitis cases share phenotypic properties and genetic background with those obtained from animal mastitis cases. Therefore, the objective of this study was to characterize *S. aureus* strains isolated from women with lactational mastitis and to compare them with strains responsible for bovine mastitis and non-infectious strains. All the strains were genotyped by both Pulsed Field Gel Electrophoresis (PFGE) and Multilocus sequence typing (MLST) and submitted to a characterization scheme that included diverse assays related to pathogenic potential and antibiotic resistance. Apart from siderophore production, no significant association was observed between the strains from bovine and human mastitis. Statistical differences between human- and bovine-mastitis associated strains were detected for some traits and virulence determinants; such as the presence of prophages and *cna* and *hlb* genes, which were more frequently found within the bovine group. On the contrary, resistance to penicillin was significantly higher among strains isolated from human lactational mastitis, probably related to the common presence of the *blaZ* gene. A high genetic diversity was found among the strains involved in mastitis in breastfeeding women.

Introduction

Mastitis is the inflammation of the mammary gland mainly due to a bacterial infection and it is characterized by a variety of local and systemic symptoms. In Veterinary Medicine, the number of studies dealing with the distribution and characterization of the bacterial species involved in mastitis is considerable (Vasudevan *et al*., 2003; Fueyo *et al*., 2005; Smith *et al*., 2005; Kozytska *et al*., 2010). In contrast, scientific articles on the bacteria causing infectious mastitis in lactating women are scarce and most are, at least, 10 years old (Foxman *et al*., 2002). However, this condition is relatively common since its incidence oscillates between 5 and 33% of lactating mothers (WHO, 2000; Foxman *et al*., 2002). *Staphylococcus aureus* (Thomsen *et al*., 1983; Riordan & Nichols, 1990) and coagulase-negative staphylococci (Delgado *et al*., 2009) are among the most common etiological agents although, unfortunately, microbiological analysis of human milk and characterization of the strains involved in human mastitis are exceptional.

S. aureus can express a wide array of potential virulence factors, including surface proteins that promote adherence to damaged tissue, and/or exotoxins and enzymes than can cause a variety of infections in skin and soft tissues, including intramammary mastitis (Iwatsuki *et al*., 2006). Some evidence suggests that biofilm formation can be a virulence factor associated with *S. aureus* mastitis (Vasudevan *et al*., 2003). Furthermore, this microorganism can display resistance to several relevant antibiotics, making its eradication difficult (Casey *et al*., 2007).

Common genotypes of *S. aureus* causing mastitis in different ruminants have been described suggesting potential tissue specificity (Mørk *et al*., 2005; Aires de Sousa *et al*., 2007). However, different genetic clusters and lineages among strains isolated from samples of human and bovine origin have been reported (Zadoks *et al*., 2000; van

Leeuwen *et al*., 2005), which point to the concept of host specialization (Herron-Olson *et al*., 2007; Sung *et al*., 2008). In humans, most infections caused by *S. aureus* seem to derive by own carriage strains (Wertheim *et al*., 2005). The potential virulence factors possessed by the *S. aureus* strains associated with human mastitis remain still undescribed. The objective of this work was to assess and compare the presence of pathogenic traits among a number of strains of *S. aureus* isolated from both women and cows suffering lactational mastitis, alongside some non-infectious strains, by a variety of geno- and phenotypic assays.

Material and methods

S. aureus strains and growth conditions

Twenty strains of *S. aureus* isolated from cases of infectious mastitis were included in this study. Among them, 10 were isolated from breast milk of women suffering mastitis (OD111, S3B, ACLI1, S1LI12, DH3a, DI2e, BALD1, O2LI2, Z2LD1 and AXLI12; Delgado *et al*., 2008) while the remaining 10 were isolated from milk of dairy cows with clinical mastitis in Asturias, Spain (SA1, SA3, SA4, SA5, SA8, SA10, SA11, SA14, SA15 and SA16; García *et al*., 2009). In addition, 10 strains with a non-clinical origin were also included for comparative purposes: five from breast milk of healthy women (LC001, LG5081, LX153b, L1741 and L1744; Jiménez *et al*., 2008) and five from bulk tank milk (GDC3, GDC6, AC11, JFL2 and GRA16; García *et al*., 2009). Five *S. aureus* strains (CECT 435, CECT 957, CECT 976, CECT 4013, and CECT 4439) from the Spanish Culture Collection were used as controls in the different assays. The isolates were routinely grown in liquid and agarized Brain Heart Infusion (Oxoid, Basingstoke, UK) at 37 °C for 24 h and their identity was confirmed on the basis of different microbiological and biochemical tests (shape, Gram-staining, catalase and

coagulase activities) and PCR sequencing of *tuf* (Martineau *et al*., 2001), and 16S rRNA genes using primers pbl16 (5´-AGAGTTTGATCCTGGCTCA-3´) and mlb16 (5´-GGCTGCTGGCACGTAGTTAG-3´) (Kullen *et al*., 2000).

Genotyping of the isolates by Pulsed field gel electrophoresis (PFGE) and Multilocus sequence typing (MLST)

All isolates were submitted to PFGE genotyping according to the protocol of Murchan *et al*. (2003). Chromosomal DNA was digested with the endonuclease *Sma*I (New England Biolabs, Ipswich, USA) at 37 ºC for 16 h. Then, electrophoresis was carried out in a CHEF DR-III apparatus (Bio-Rad Laboratories, Hercules, USA) for 23 h at 14 °C at 6 V cm^{-1} with pulses from 5 to 50 s. A standard pattern (Lambda Ladder PFG Marker, New England Biolabs) was included in the gels to allow comparison of the digitally normalized PFGE profiles. Gels were analyzed with Phoretix 5.0 software (Nonlinear Dynamics Ltd., United Kingdom) using Dice's coefficient to perform the cluster analysis of the PFGE patterns.

MLST was carried out as previously described by Enright *et al*. (2000). The method involves the amplification by PCR of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*) following sequencing, and each unique sequence defines an allele. Bacterial genomic DNA used for the PCR reactions was obtained from pure cultures with the DNeasy tissue kit (QIAgen, Hilden, Germany). The resulting sequences were trimmed, aligned and compared to the allele sequences in the *S. aureus* MLST database (http://saureus.mlst.net/), and each allelic profile was assigned to a sequence type (ST). All of them were submitted to the database and registered under identity numbers 3193 to 3220. The STs were clustered into clonal complexes (CCs) by using the eBURST algorithm (www.eburst.mlst.net).

Detection of genes encoding toxins, adhesins and other virulence determinants

The presence of different toxins, adhesins and other virulence factors, including some antibiotic resistance genes, was screened by PCR using the primers described in Table 1. For the detection of the four hemolysin genes (*hla, hlb, hlg, hld*) a multiplex PCR method was developed with the following conditions: 1 cycle of 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min, and a final extension of 72 °C for 10 min. The genes encoding four adhesins (*fnbA, clfa, sdrE, ebpS*) were detected by multiplex PCR. The PCR program consisted of 1 cycle of 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min, and a final extension of 72 °C for 10 min. Each multiplex PCR reaction mixture consisted of 3 mM $MgCl_2$, 1.5X *Taq* buffer, 4 mM each dNTP, 2.5 U Eco*Taq* polymerase (Ecogen, Barcelona, Spain), 10 μM of each primer and 1.5 μl of purified DNA. Single PCR reactions were used for screening the rest of the genes with standard concentrations of the components in the mixtures and the conditions originally described (Table 1). The use of different annealing temperatures in these reactions is specified in Table 1.

Determination of minimal inhibitory concentrations (MICs) to antibiotics

The determination of the MICs to several antibiotics commonly used against staphylococcal infections was performed by a microdilution method using the Sensititre plates Staenc1F (Trek Diagnostic Systems, Cleveland, USA) following the manufacturer's instructions. The antibiotics analyzed were: penicillin, ampicillin, amoxycillin-clavulanic acid, teicoplanin, chloramphenicol, erythromycin, mupirocin, streptomycin, gentamicin, clindamycin, oxacillin, ciprofloxacin, fosfomycin, imipenem,

nitrofurantoin, trimethoprim-sufamethoxazole, tetracycline, vancomycin, linezolid, quinupristin-dalfopriscin, and rifampin.

Phenotypic assays

a) **Determination of hemolytic activity.** Hemolytic activity was assayed on Columbia agar plates supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France) following the procedure of Freney *et al*. (1999). After an incubation of 72 h at 37 °C, plates were analyzed and the strains were considered hemolytic if complete lysis of the red blood cells surrounding the colony was observed (halo ~1.5 mm).

b) **Slime production**. The ability of the isolates to form slime was assessed using the Congo Red Agar assay (Freeman *et al*., 1989). The plates were incubated at 37 °C for 24 h followed by additional storage at room temperature 24 h. Isolates displaying rough black colonies were considered as slime producers while those showing red colonies were negative.

c) **Biofilm formation ability**. Biofilm formation was assayed using a microtiter plate assay as described Vasudevan *et al*. (2003) with some modifications. Strains were grown in Brain Heart Infusion and the cultures were diluted 1:200 in Tryptic Soy Broth (Oxoid) supplemented with 0.25% glucose. Aliquots of 200 µl of each dilution were placed into 96-well "U" bottom polystyrene microtiter plates and incubated for 18 h at 37 °C. The wells were washed three times with 300 µl of sterile phosphate-buffered saline (pH 7.2) and let dried at room temperature. Afterwards, 25 µl of a 1%crystal violet solution was added to the wells for biofilm staining and the plates were incubated for 15 min at room temperature. Microtiter plates were washed by fully immersing in water and allowed to dry in inverted position. Bounded crystal violet was solubilized in 200 µl of ethanol (95% v/v), and 125 µl were transferred to another plate to measure the

optical density (OD) of each well at 570 nm using a MicroELISA Auto Reader (Labsystems, Helsinki, Finland). Each strain was tested in triplicate and a known biofilm-producer strain (*S. aureus* CECT 4013) was included as a positive control in all assays. The biofilm assays were performed on duplicate.

d) **Lysogeny determination.** The presence of resident prophages in the *S. aureus* strains was checked after mitomycin C induction. Mid-exponential phase cultures were obtained for each strain and mitomycin C (Sigma-Aldrich, St. Louis, USA) was added at a concentration of 0.5 μg ml^{-1}. Samples were incubated for 4 h at 37 ºC and supernatants filtered and subjected to plaque assay. Each supernatant was tested against all strains. Aliquots (0.1 ml) of stationary-phase host cultures were mixed with appropriate dilutions of individual phage suspensions in 5 ml of molten 2xTY top agar (0.7% agar) and the mixture was poured on 2xTY agar plates (Sambrook *et al*., 1989).

e) **Siderophore production**. The siderophore production in liquid culture was performed following the methodology previously used by Massonet *et al*. (2006) with some modifications. For the growth of the strains, the RPMI 1640 medium (Sigma) was used. Iron was removed from the RPMI medium by overnight batch incubation with 6% (w/v) sterile Chelex 100 (Sigma) at room temperature. To avoid the reduction in the calcium and magnesium levels by the chelating effect of Chelex, the medium was afterwards enriched with filter sterilized solutions of $CaCl_2$ (0.07 mM) and $MgSO_4$ (0.7 mM). *S. aureus* strains were inoculated in the iron-depleted medium and incubated for 24 h at 37 °C in an orbital shaker. Then, the strains were subcultured (1%) in the same medium and incubated under the same conditions for 48 h. Cultures were centrifuged at 16,000 × g for 5 min and the supernatants were assayed for siderophore production. Siderophore production was measured with the Chrome Azurol S liquid assay as described by Schwyn and Neilands (1987). Briefly, 500 μl of supernatant were mixed

with 500 µl of Chrome Azurol S solution and 10 µl of shuttle solution (0.2 M 5´-sulfosalicylic acid) were added to the mixture. After 15 min of incubation at room temperature the absorbance at 630 nm was measured. The percentage of siderophore units was calculated as follows: [(Ab-As)/Ab] x 100, being Ab the absorbance of the non-inoculated medium and As the absorbance of the samples.

Statistical analysis

The distribution of putative virulence genes, antibiotic resistance, hemolysis, slime production and lysogeny among the *S. aureus* strains from different hosts (human and bovine) with mastitic infection and/or from mastitis-associated and non-infectious strains was compared using two-tailed Fisher's exact test. The non-parametric Kruskal Wallis test was used to compare the biofilm forming ability between strains isolated from bovine and human mastitis cases and the siderophore production observed in mastitis and non-infectious strains. Differences between groups were considered statistically significant if P values were ≤ 0.05. Statistical analysis was performed with the SPSS statistical package (SPSS Inc., Chicago, USA).

Results and Discussion

Genetic diversity. Twenty strains of *S. aureus* from mastitis (10 from human and 10 from bovine cases) and 10 strains that were not involved in infections (five from breast milk of healthy women and five from bulk tank milk) were included in the study. Except for GRA16 and AC11 isolates from bulk tank milk, all the strains could be individually distinguished by PFGE genotyping since each strain generated a different band pattern. Three main clusters were observed in the dendrogram obtained by computer assisted analysis (Fig. 1). Clustering of the strains was not apparently based

on the host or pathogenic origin. Strains from human mastitis were distributed along the three clusters and showed diverse genotypes. On the contrary, most of the isolates from bovine mastitis (8 out of 10) showed closely-related pulsotypes and grouped together in the same cluster. The existence of common or similar genotypes among *S. aureus* isolated from bovine mastitis has been previously reported and could reflect a long-term persistence into the bovine mammary gland (Anderson & Lyman, 2006). The strains were also typed by MLST analysis, and the results were in accordance with those obtained by PFGE (Fig. 1) even though different pulsotypes were observed within strains grouped in the same ST. Seven out the 10 isolates causing bovine mastitis grouped into ST97/ST352-CC97 and two strains were related to ST151. These lineages are rather prevalent among bovine mastitis isolates from different countries (Smith *et al*., 2005; Herron-Olson *et al*., 2007; Rabello *et al*., 2007; Sung *et al*., 2008) and seem to be adapted, not only to the mammary gland, but also to the bovine environment. This adaptation would explain their presence also among the strains obtained from bulk tank milk. Human mastitis strains presented higher diversity than the bovine ones belonging up to 7 different CCs, including the newly described ST1598-CC154 and the singleton ST1597. Some strains of human origin, including those isolated from healthy women, belong to CC5 and CC30. These clonal complexes have previously been related to *S. aureus* infections in humans but were also associated to asymptomatic human carriers (Wertheim *et al*., 2005; Rabello *et al*., 2007; Sakwinska *et al*., 2009). This could indicate that some *S. aureus* mastitis infections in breastfeeding women may derive by own carriage strains and host predisposal factors might be also important in the development of lactational mastitis.

Presence of potential virulence genes. Multiple properties of *S. aureus* contribute to its virulence. In this study, the presence of 18 genes encoding a variety of potential virulence factors was determined by PCR (Fig. 2), and the results are shown in Table 2. Statistical analysis was only applied to 11 out of the 18 genes, since *hla*, *hld*, *fnbA*, *clfA*, *icaA* were present in all the strains and *pvl-S/F*, *se-adej* were absent in all of them. Significant differences between strains isolated from human and bovine cases of infectious mastitis were observed for two genes; *cna* and *hlb* genes, encoding a collagen adhesin and the β-hemolysin, respectively, that were more common in the bovine than in the human group (10/5 strains, Fisher's exact test $P = 0.033$, for *cna* gene and 8/1 strains, Fisher's exact test $P = 0.006$, for *hlb* gene). No significant differences were observed in the rest of tested genes, even though the occurrence of *blaZ* gene (encoding resistance to β-lactams antibiotics) was higher among the human strains (9 *versus* 4 strains, $P = 0.057$). Pyrogenic toxin superantigens genes such as those encoding the toxic shock syndrome toxin (*tst*) and the exfoliative toxin (*eta*) were found in a small number of strains independently of the source, while the *pvl-M/F* gene, implicated in the damage of host defense cell membranes, was present in four bovine strains. The important role of these toxins in bovine mastitis has been previously reported and the presence of the leukocidin M/F variant has been described as characteristic of bovine *S. aureus* strains (Fueyo *et al*., 2005; Yamada *et al*., 2005; Monecke *et al*., 2007). When bacterial strains from clinical and non clinical origin were compared, presence of *sir*B gene (encoding an iron-siderophore transporter) was found to be associated to strains isolated from cases of infectious mastitis independently of their human or bovine origin (Fisher's exact test, $P = 0.008$).

Antibiotic resistance. Regardless of their origin, all the strains were sensitive to gentamicin (≤2 μg ml^{-1}), tetracycline (≤4 μg ml^{-1}), nitrofurantoin (≤32 μg ml^{-1}), mupirocin (≤4 μg ml^{-1}), streptomycin (≤1,000 μg ml^{-1}), clindamycin (≤0.5 μg ml^{-1}), imipenem (≤0.12 μg ml^{-1}), rifampin (≤1 μg ml^{-1}), trimethoprim/sulfamethoxazole (≤1/38 μg ml^{-1}), fosfomycin (≤16 μg ml^{-1}) and teicoplanin (≤0.5 μg ml^{-1}). Most of the strains from cow's bulk tank milk and healthy women were susceptible to the lowest concentration of most antibiotics (data not shown). All strains recovered from mastitis infections were susceptible to oxacillin, ciprofloxacin, vancomycin and quinupristin/dalfopristin according to the breakpoints established by the Clinical and Laboratory Standards Institute (CLSI, 2010). In general, strains involved in human mastitis showed higher MICs than isolates from bovine mastitis, with intermediate resistances observed for some antibiotics (such as chloramphenicol and erythromycin) among the strains involved in human mastitis (Table 3). A significant difference was detected for penicillin resistance between isolates from human and bovine cases of infectious mastitis (Fisher's exact test, $P = 0.020$). Three out of the 10 strains recovered from bovine mastitis were ampicillin resistant, in contrast to seven out of 10 obtained from human mastitic milk, even though the difference was not statistically significant (Fisher's exact test, $P = 0.101$). In concordance with these results, the *mecA* gene was not detected among the mastitis-related strains and the presence of the *blaZ* gene was higher among the human isolates from mastitic milk (Fisher's exact test, $P = 0.057$). This enhanced antibiotic resistance of human *S. aureus* has been previously described (Reinoso *et al*., 2008) and might be a consequence of higher antibiotic pressure in human hosts.

Hemolysis. All the non-infectious strains displayed a non hemolytic behaviour on sheep blood agar plates. In contrast, most of the isolates from bovine mastitis cases (8/10) showed a complete lysis of the red blood cells surrounding the colony causing a clearing of the blood from the medium, while only four of the strains from human mastitis presented this type of hemolysis (Table 4). However, this difference was not statistically significant (Fisher's exact test, $P = 0.170$). Strains of *S. aureus* from bovine milk typically produce β-hemolysin (Larsen *et al*., 2002; Monecke *et al*., 2007), while many human isolates have β-hemolysin-converting bacteriophages inserted in the *hlb* gene (van Wamel *et al*., 2006). Although α-, β-, and δ-hemolysins are relevant in several infections caused by *S. aureus*, the pathogenic significance of such toxins in human disease has not yet been fully established (Iwatsuki *et al*., 2006). Some authors have reported the ubiquitous nature of some of these hemolysins (like the α-hemolysin encoded by *hla* gene) regardless of the strain source (Peacock *et al*., 2002).

Slime and biofilm production. Slime production was a relatively frequent phenotype among the mastitis-associated strains, with 12 out of the 20 strains positive in the Congo Red Agar assay (Table 4) in contrast with only three out 10 non-infectious strains. However this difference was not statistically different (Fisher's exact test, $P = 0.245$). In parallel, the potential for biofilm production was determined by a quantitative assay using microtiter plates and the results are shown in Table 4. Globally, the adhesion to polystyrene plates was low when compared with a positive control (OD_{570}~1) in spite of the fact that all of them contained the gene *icaA*. There was not a statistically significant difference in the ability to form biofilm *in vitro* between the strains associated to bovine and human mastitis (Kruskal Wallis test, $P = 0.070$). Biofilm formation has been described in many cases of mastitis caused by *S. aureus*

311 (Melchior *et al*., 2006; Oliveira *et al*., 2006) but the phenotypic expression of this
312 property can be influenced by numerous factors such as the method, or the growth
313 media and conditions used in the assay. Discrepancies between slime production,
314 biofilm formation *in vitro*, and the presence of the *ica* gene have been previously
315 reported in bovine *S. aureus* (Vasudevan *et al*., 2003).
316
317 **Presence of prophages.** The incidence of lysogenic strains was investigated since
318 many genes associated with virulence factors in *S. aureus* can be encoded by mobile
319 genetic elements such as bacteriophages. Lysogeny was observed only in two of the 10
320 strains isolated from human milk supplied by women with mastitis and in one strain
321 from milk of a healthy woman (Table 4). In contrast, phages were detected in most
322 strains from bovine origin, with the exception of strain SA1 isolated from bovine
323 mastitis and strain GDC3 from bulk tank milk. Globally, lysogeny was associated more
324 frequently to bovine than to human mastitis-causing strains (Fisher's exact test, P =
325 0.006). Some authors have proposed that lysogenization of a strain with phages carrying
326 *pvl* genes may transform a non-virulent strain into a pathogenic one (Melles *et al*., 2004;
327 Yamada *et al*., 2005). In this study, positive isolates for the long-known Panton-
328 Valentine leukocidin gene (*pvl*-S/F) were not detected but presence of the more recently
329 described leukocidin M/F-PV like gene (*pvl-M/F*) was revealed in three of the lysogenic
330 strains isolated from bovine mastitis infections. This gene was originally described on a
331 prophage (φPV-83) and has been associated frequently with mastitis-causing strains in
332 different ruminants (Rainard *et al*., 2003; Yamada *et al*., 2005; Monecke *et al*., 2007),.
333
334 **Siderophore production**. Another potential pathogenic trait investigated was the
335 production of siderophores because the ability to acquire iron from the host by bacterial

siderophores may facilitate the establishment of infection. Different siderophores have been characterized in *S. aureus* including iron-uptake ABC transporters (Dale *et al*., 2004). The production of siderophores was highly variable among the strains tested (Table 4), but it was significantly higher in the strains involved in mastitis than in the non-infectious group (Kruskal Wallis test, $P = 0.039$). In concordance with this result, the presence of *sir*B gene (encoding an iron-siderophore transporter) was also found to be associated to mastitis-causing strains (Fisher's exact test, $P= 0.008$).

No clear association could be observed between the strains from human and bovine mastitis studied, except for siderophore production. In fact, some properties of the strains seemed to depend on their source. The human mastitis strains were characterized by a higher resistance to antibiotics, specifically to β-lactams. Although the small sample size is a clear limitation of this work, and further studies involving a larger number of strains will be required to confirm these results, this is the first time, to our knowledge, that *S. aureus* involved in human lactational mastitis have been examined in detail. High genetic diversity was found among these strains and two novel sequence types (ST1597 and ST1598) were registered.

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Figure legends

Fig. 1. Dendrogram obtained after clustering analysis of pulsed field gel electrophoresis profiles of the *Staphylococcus aureus* strains included in this study. Multilocus sequence typing results, including sequence types (STs) and clonal complexes (CCs), are also shown.

Fig. 2. Multiplex PCR assays for the simultaneous detection of the genes encoding different hemolysins (a) and adhesins (b). (a) M_1, molecular weight marker (DNA HyperLadder II, Bioline, London, UK); Lanes: 1, *Staphylococcus aureus* ACLI1; 2, *S. aureus* BALD1; 3, *S. aureus* SA2; 4, *S. aureus* SA15; 5, *S. aureus* LX153b. *hlg* gene: 535 bp; *hlb* gene*:* 309 bp; *hla* gene: 209 bp; *hld* gene: 111 bp. (b) M_2, molecular weight marker (DNA HyperLadder I, Bioline); Lanes: 1, *S. aureus* SA10; 2, *S. aureus* O2LI2; 3, *S. aureus* BALD1. *clfA* gene: 1,548 bp; *fnbA* gene*:* 1,226 bp; *sdrE* gene: 749 bp; *ebpS* gene: 506 bp.

Table 1. Primers and PCR conditions used in this study

Gene	Product	Primers sequence (5'–3')	Amplicon size	Annealing temperature	Control strain	Reference
eta	Exfoliative toxin A	Fw: GCAGGTGTTGATTTAGCATT Rv: AGATGTCCCTATTTTTGCTG	100 bp	58 °C	Isolate Z2LD1	van Leeuwen *et al*. 2005
hla	α-hemolysin	Fw: CTGATTACTATCCAAGAAATTCGATTG Rv: CTTTCCAGCCTACTTTTTTATCAGT	209 bp	58 °C[a]	CETC 976	Jarraud *et al*. 2002
hlb	β-hemolysin	Fw: GTGCACTTACTGACAATAGTGC Rv: GTTGATGAGTAGCTACCTTCAG	309 bp	58 °C[a]	CETC 976	Jarraud *et al*. 2002
hlg	γ-hemolysin	Fw: GTCAYAGAGTCCATAATGCATTTAA Rv: CACCAAATGTATAGCCTAAAGTG	535 bp	58 °C[a]	CECT 435	Jarraud *et al*. 2002
hld	δ-hemolysin	Fw: AAGAATTTTTATCTTAATTAAGGAAGGAGTG Rv: TTAGTGAATTTGTTCACTGTGTCGA	111 bp	58 °C[a]	CETC 976	Jarraud *et al*. 2002
tst	Toxic shock syndrome toxin	Fw: AAGCCCTTTGTTGCTTGCG Rv: ATCGAACTTTGGCCCATACTTT	404 bp	62 °C	CETC 957	van Leeuwen *et al*. 2005
pvl-S/F	Panton-Valentin leukocidin (component S and F)	Fw: ATCATTAGGTAAAATGTCTGGACATGATCCA Rv: GCATCAASTGTATTGGATAGCAAAAGC	433 bp	65 °C	CECT 435	Jarraud *et al*. 2002
pvl-M/F	Panton-Valentin leukocidin (component M and F)	Fw: AACTTTCAATGATGTTAAACAAAATAGAG Rv: AAAATAGTCTCTAGCATTAGGTCC	405 bp	57 °C	CETC 957	Yamada *et al*. 2005
se-adej	Enterotoxins SEs	Fw: AAAGATTTGCGAAAAAAGTGTGAATT Rv: TTKYRTATAAATABAWRTCAATATG	669 bp	50 °C	CECT 4439	Martín *et al*. 2003
fnbA	Adhesin for fibronectin	Fw: CACAACCAGCAAATATAG Rv: CTGTGTGGTAATCAATGTC	1,226 bp	50 °C[b]	CETC 976	van Leeuwen *et al*. 2005
cna	Adhesin for collagen	Fw: AGTGGTTACTAATCATG Rv: CAGGATAGATTGGTTTA	1,722 bp	45 °C	CETC 976	van Leeuwen *et al*., 2005
clfA	Adhesin for fibrinogen	Fw: GTAGGTACGTTAATCGGTT Rv: CTCATCAGGTTGTTCAGG	1,548 bp	50 °C[b]	CETC 976	van Leeuwen *et al*. 2005
sdrE	Putative adhesin	Fw: CAGTAAATGTGTCAAAAGA Rv: TTGACTACCAGCTATATC	749 bp	50 °C[b]	CETC 976	Peacock *et al*. 2002
ebpS	Adhesin for elastin	Fw: CAATCGATAGACACAAATTC Rv: CAGTTACATCATCATGTTTA	506 bp	50 °C[b]	CETC 976	Peacock *et al*. 2002
icaA	Polysaccharide intercellular adhesin	Fw: GATTATGTAATGTGCTTGGA Rv: ACTACTGCTGCGTTAATAAT	770 bp	50 °C	CETC 976	Peacock *et al*. 2002
mecA	Methicillin resistance	Fw: GGTCCCATTAACTCTGAAG Rv: AGTTCTGCAGTACCGGATTTTGC	163 bp	57 °C	CECT 4439	Mehrotra *et al*. 2000
blaZ	β-lactamase	Fw: ACTTCAACACCTGCTGCTTTC Rv: TGACCACTTTTATCAGCAACC	173 bp	61 °C	CETC 976	Martineau *et al*. 2000
sirB	Iron-siderophore transporter	Fw: CAGCTACGGCTACCGAAATA Rv: CATTTTTGGGGGCTATTGTTGT	399 bp	61 °C	CECT 976	Dale *et al*. 2004

[a, b] primers used in the same multiplex PCR reaction

Table 2. Presence of putative virulence genes in the *S. aureus* isolates

Virulence determinant	Mastitis			Non-infectious		
	Human (n=10)	Bovine (n=10)	*P*-value [a]	Human (n=5)	Bulk tank (n=5)	*P*-value [b]
Adhesins						
fnbA	10	10	-	5	5	-
cna	5	10	0.033	3	2	0.231
clfA	10	10	-	5	5	-
sdrE	6	7	>0.99	2	3	0.694
ebpS	9	9	>0.99	5	5	0.540
Toxins						
eta	1	0	>0.99	0	0	>0.99
hla	10	10	-	5	5	-
hlb	1	8	0.006	2	4	0.700
hlg	3	0	0.211	3	1	0.181
hld	10	10	-	5	5	-
tst	1	1	>0.99	1	1	0.584
pvl-S/F	0	0	-	0	0	-
pvl-M/F	0	3	0.211	0	1	>0.99
se-adej	0	0	-	0	0	-
Others						
icaA	10	10	-	5	5	-
mecA	0	0	-	2	0	0.103
blaZ	9	4	0.057	4	2	>0.99
sirB	10	10	-	2	4	0.008

[a] Fisher's exact test to compare the presence of the virulence determinant in strains from human and bovine mastitis infections

[b] Fisher's exact test to compare the presence of the virulence determinant in mastitis isolates and non-infectious strains

Table 3. Antibiotic susceptibility of *S. aureus* isolated from human and bovine mastitis cases according to the microbiological breakpoints (MICs) defined by the Clinical and Laboratory Standards Institute (CLSI)

Antibiotic[a]	Sample origin	Number of resistant (R), intermediate (I) and susceptible (S) strains according to MICs breakpoints (μg ml^{-1}) established by the CLSI (2010)		
PEN		R (≥ 0.25)[b]		S (≤ 0.12)
	Human	**9**		1
	Bovine	**3**		7
AMP		R (≥ 0.5)		S (≤ 0.25)
	Human	7		3
	Bovine	3		7
OXA		R (≥ 4)		S (≤ 2)
	Human			10
	Bovine			10
CIP		R (≥ 4)	I (2)	S (≤ 1)
	Human			10
	Bovine			10
CHL		R (≥ 32)	I (16)	S (≤ 8)
	Human	1	6	3
	Bovine	1		9
ERY		R (≥ 8)	I (1-4)	S (≤ 0.5)
	Human		4	6
	Bovine			10
VAN		R (≥ 16)	I (4-8)	S (≤ 2)
	Human			10
	Bovine			10
LNZ		R (≥ 8)		S (≤ 4)
	Human	1		9
	Bovine			10
Q/D		R (≥ 4)	I (2)	S (≤ 1)
	Human			10
	Bovine			10

[a] PEN: penicillin; AMP: ampicillin; OXA: oxacillin; CIP: ciprofloxacin; CHL: chloramphenicol; ERY: erythromycin; VAN: vancomycin; LNZ: linezolid; Q/D: quinupristin/dalfopristin

[b] Bold indicates significant differences in the number of resistant strains from human and bovine mastitis infections (Fisher's exact test, $P = 0.020$)

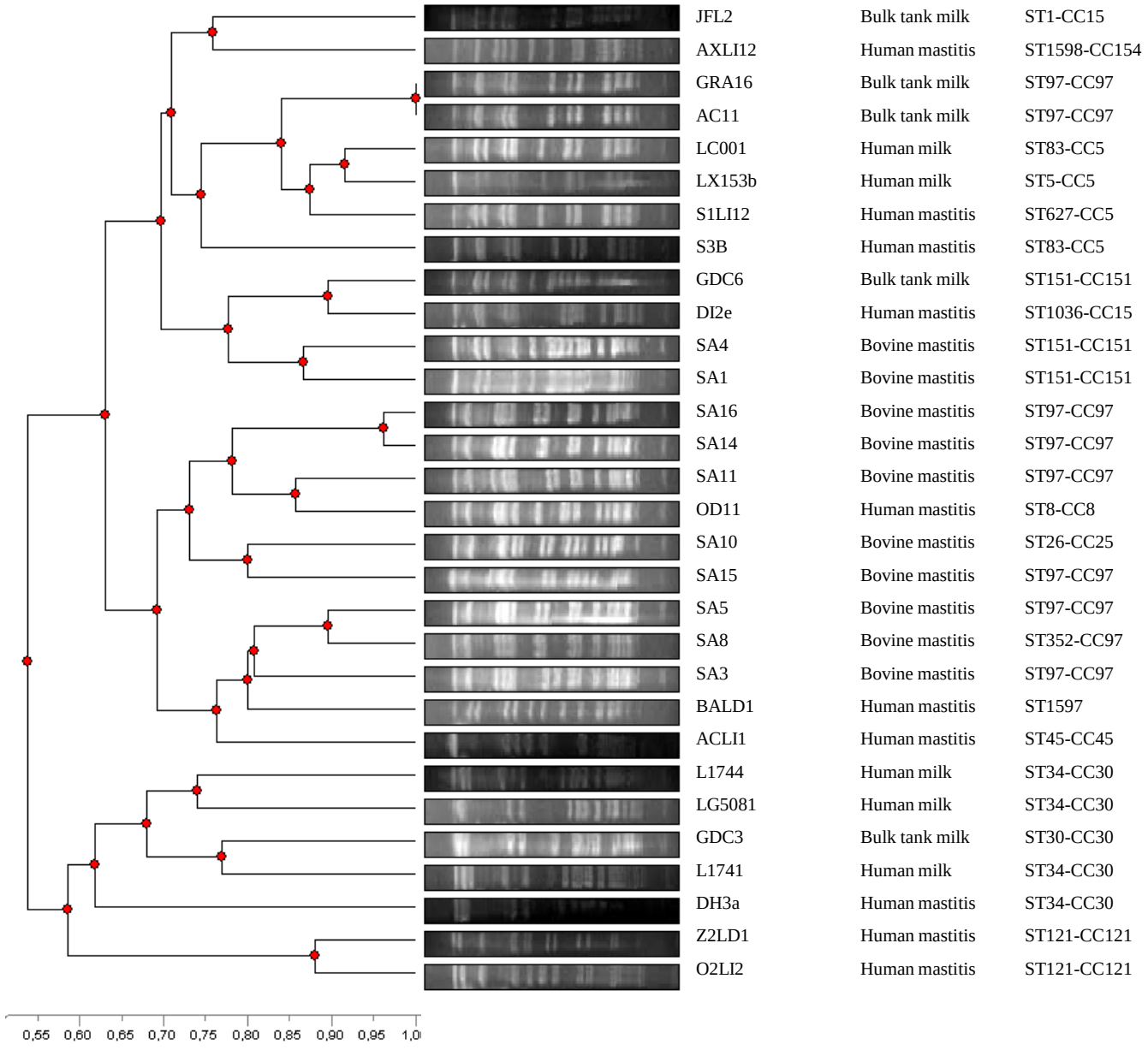
Table 4. Phenotypic traits of the *S. aureus* isolates

Strains	Phenotypic traits				
	Hemolysis	Slime	Biofilm (OD_{570})	Siderophores[a] (%)	Prophages[c]
	Mastitis				
Human					
OD111	+	+	0.962	45.4	+
S3B	+	+	0.953	30.9	-
ACLI1	-	+	0.151	46.0	-
S1LI12	-	+	0.138	45.8	-
DH3a	-	+	0.155	33.5	-
DI2e	+	-	0.068	47.7	+
BALD1	-	-	0.115	33.8	-
O2LI2	+	+	0.797	27.5	-
Z2LD1	-	+	0.387	29.8	-
AXLI12	-	+	0.140	50.6	-
Bovine					
SA1	+	+	0.175	59.3	-
SA3	+	+	0.421	25.4	+
SA4	+	-	0.086	26.3	+
SA5	-	-	0.077	49.8	+
SA8	-	-	0.081	36.8	+
SA10	+	+	0.095	32.0	+
SA11	+	+	0.067	37.7	+
SA14	+	-	0.099	55.5	+
SA15	+	-	0.240	58.9	+
SA16	+	-	0.136	48.3	+
	Non-infectious				
Human					
LC001	-	-	0.143	34.7	-
LG5081	-	+	0.168	24.4[b]	+
LX153b	-	-	0.087	31.3	-
L1741	-	-	0.169	29.2[b]	-
L1744	-	-	0.046	31.5[b]	-
Bulk tank					
GDC3	-	-	0.116	24.1[b]	-
GDC6	-	-	0.418	40.5	+
AC11	-	+	0.904	32.7	+
JFL2	-	+	0.517	28.6	+
GRA16	-	-	0.319	47.4	+

[a] Siderophore production in mastitis strains differed significantly from non-infectious strains (Kruskal-Wallis test, P =0.039)

[b] No *sirB* gen was detected.

[c] Presence of prophages in bovine mastitis strains differed significantly from human mastitis strains (Fisher's exact test, P =0.006)


Strain
Origin
MLST
JFL2 Bulk tank milk ST1-CC15
AXLI12 Human mastitis ST1598-CC154
GRA16 Bulk tank milk ST97-CC97
AC11 Bulk tank milk ST97-CC97
LC001 Human milk ST83-CC5
LX153b Human milk ST5-CC5
S1LI12 Human mastitis ST627-CC5
S3B Human mastitis ST83-CC5
GDC6 Bulk tank milk ST151-CC151
DI2e Human mastitis ST1036-CC15
SA4 Bovine mastitis ST151-CC151
SA1 Bovine mastitis ST151-CC151
SA16 Bovine mastitis ST97-CC97
SA14 Bovine mastitis ST97-CC97
SA11 Bovine mastitis ST97-CC97
OD11 Human mastitis ST8-CC8
SA10 Bovine mastitis ST26-CC25
SA15 Bovine mastitis ST97-CC97
SA5 Bovine mastitis ST97-CC97
SA8 Bovine mastitis ST352-CC97
SA3 Bovine mastitis ST97-CC97
BALD1 Human mastitis ST1597
ACLI1 Human mastitis ST45-CC45
L1744 Human milk ST34-CC30
LG5081 Human milk ST34-CC30
GDC3 Bulk tank milk ST30-CC30
L1741 Human milk ST34-CC30
DH3a Human mastitis ST34-CC30
Z2LD1 Human mastitis ST121-CC121
O2LI2 Human mastitis ST121-CC121
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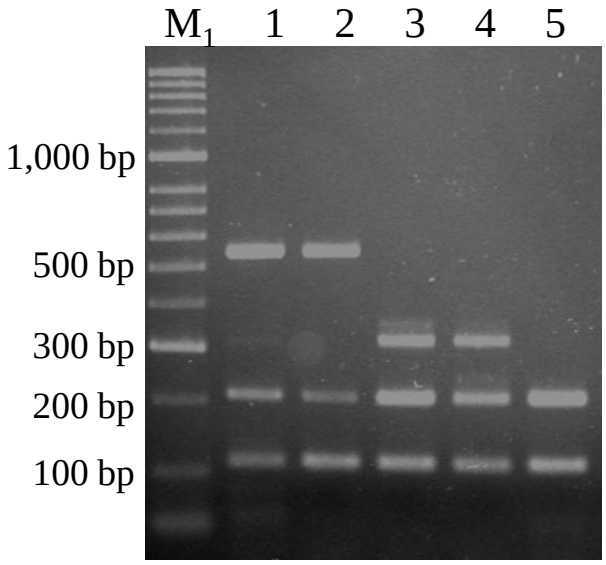
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2
3
4
5
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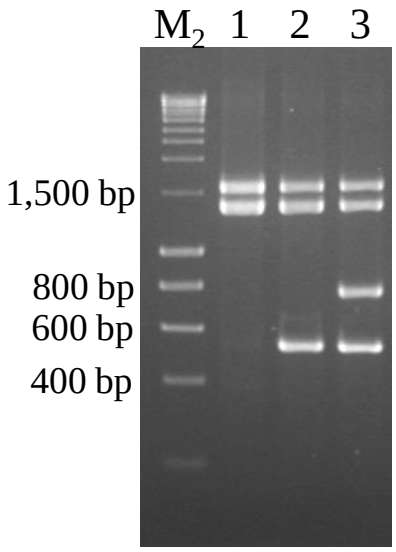
500 bp
300 bp
200 bp
100 bp

(a)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2
3
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600 bp
400 bp

(b)