Comparison of genomic and antimicrobial resistance features of latex agglutination test-positive and latex agglutination test-negative Staphylococcus aureus isolates causing bovine mastitis

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Abstract: The dairy industry suffers massive economic losses due to staphylococcal mastitis in cattle. The Staphaureux latex agglutination test (Oxoid, Basel, Switzerland) was reported to lead to negative results in 54% of bovine Staphylococcus aureus strains, and latex-negative strains are thought to be less virulent than Staphaurex latex-positive strains. However, comparative information on virulence and resistance profiles of these 2 groups of Staph. aureus is scarce. Our objective was to associate the latex agglutination phenotype of Staph. aureus strains isolated from bovine mastitis milk with data on clonal complexes, virulence genes, and antibiotic resistance to (1) determine the virulence profiles of the Staphaureux test positive and Staphaurex test negative groups, and (2) provide data needed to improve treatment of bovine mastitis and to identify potential vaccine targets. Seventy-eight Staph. aureus strains isolated from 78 cows on 57 Swiss farms were characterized. Latex agglutination was tested by Staphaureux kit, and resistance profiles were generated by disk diffusion. A DNA microarray was used to assign clonal complexes (CC) and to determine virulence and resistance gene profiles. By the Staphaureux test, 49% of the isolates were latex-positive and 51% were latex-negative. All latex-negative strains were assigned to CC151, whereas latex-positive strains were assigned to various clonal complexes, including CC97 (n=16), CC8 (n=10), CC479 (n=5), CC20 (n=4), CC7 (n=1), CC9 (n=1), and CC45 (n=1). Although the latex-negative isolates were susceptible to all antimicrobial agents tested, 24% of latex-positive isolates were classified as intermediate with regard to cefalexin-kanamycin and 13% were resistant to both ampicillin and penicillin. Microarray profiles of latex-negative isolates were highly similar, but differed largely from those of latex-positive isolates. Although the latex-negative group lacked several enterotoxin genes and sak, it exhibited significantly higher prevalence rates of genes encoding enterotoxin C, toxic shock syndrome toxin, and leukocidins (lukM/lukF-P83, lukD). Our findings suggest that latex-negative isolates represent a group of closely related strains with specific resistance and virulence gene patterns.

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INTERPRETIVE SUMMARY

Comparison of genomic and antimicrobial resistance features of Staphaureux latex test positive and Staphaurex latex test negative *Staphylococcus aureus* causing bovine mastitis

Moser

More than 50% of *Staphylococcus (S.) aureus* strains causing bovine mastitis are latex test negative in the Staphaureux latex agglutination test. Our study associated the latex agglutination phenotype of 78 *S. aureus* bovine mastitis isolates from 57 Swiss farms with genomic and antibiotic resistance features. We identified major differences between latex test positive and latex test negative strains with regard to antibiotic resistance, virulence gene profiles, and the assigned clonal complexes. The generated data provides new insights into genomic features of latex test positive and latex test negative strains. It also contributes to the identification of potential vaccine targets.

Comparison of genomic and antimicrobial resistance features of Staphaureux latex test positive and Staphaurex latex test negative *Staphylococcus aureus* causing bovine mastitis

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ABSTRACT

The dairy industry suffers from massive economic losses due to staphylococcal mastitis in cattle. Staphaureux latex agglutination test was reported to lead to negative results in 54% of bovine *S. aureus* strains and latex-negative strains were hypothesized to be less virulent than Staphaurex test positive strains. However, comparative information on virulence and resistance profiles of these two groups of *S. aureus* is scarce. Our objective was to associate the latex agglutination phenotype of *S. aureus* strains isolated from bovine mastitis milk with data on clonal complexes, virulence genes, and antibiotic resistance in order to 1) determine the virulence profiles of the Staphaureux test positive and Staphaurex test negative groups, and 2) provide data needed to improve treatment of bovine mastitis and to identify potential vaccine targets. A total of 78 *S. aureus* strains isolated from 78 different cows at 57 Swiss farms were characterized. Latex agglutination was tested by Staphaureux kit and resistance profiles were generated by disk diffusion. DNA microarray was used to assign clonal complexes and to determine virulence and resistance gene profiles. In the Staphaureux test, 49% of the isolates were latex-positive and 51% latex-negative. All latex-negative strains were assigned to CC151, whereas latex-positive strains were assigned to various clonal complexes including CC97 (n = 16), CC8 (n = 10), CC479 (n = 5), CC20 (n = 4), CC7 (n = 1), and CC45 (n = 1). While the latex-negative isolates were susceptible to all antimicrobial agents tested, 24% of latex-positive isolates were classified intermediate with regard to cefalexin-kanamycin and 13 % were resistant to both ampicillin and penicillin. Microarray profiles of latex-negative isolates were highly similar, but differed largely from those of latex-positive isolates. While the latex-negative group lacked several enterotoxin genes and the *sak*, it exhibited significantly higher prevalence rates of genes encoding enterotoxin C, toxic shock syndrome toxin, and leukocidins (*lukM/lukF-P83, lukD*). Our findings suggest

latex-negative isolates to represent a group of closely related strains with specific resistance and virulence gene patterns.

Key words: *Staphylococcus aureus*, bovine mastitis, latex agglutination, virulence

INTRODUCTION

The dairy industry suffers from massive economic losses due to staphylococcal mastitis in cattle (Wells et al., 1998). Stutz et al. (Stutz et al., 2011) reported that 54% of *S. aureus* isolates obtained from cases of bovine mastitis are negative in the Staphaureux latex agglutination test, a diagnostic instrument widely used to confirm putative *S. aureus* isolates. These false-negative results are due to sequence polymorphisms leading to impaired functionality of one or several of the targeted virulence factors Spa (staphylococcal protein A), ClfA/B (clumping factor A/B), and FnbA/B (fibronectin binding proteins A/B). Therefore, Staphaurex latex agglutination test negative (SLAT(-)) strains have been hypothesized to be less virulent than Staphaurex latex agglutination test positive (SLAT(+)) strains (Stutz et al., 2011). Although the assessment of the virulence potential of SLAT(-) strains is of crucial importance to the dairy industry, data on the genomic background and antimicrobial resistance of bovine SLAT(-) isolates is scarce.

Though antibiotic treatment is widely used to fight bovine mastitis, its merits are controversially discussed. Use of antimicrobial agents is not only economically questionable and favors the development of antibiotic resistance, but it is also unsuitable to address the issue of intracellular persistance of the organism (Fluit, 2012; Saini et al., 2012; Steeneveld et al., 2011). Therefore, increased efforts are now focused on the development of vaccines. Recent studies postulate extended characterization of the genetic background of bovine mastitis isolates to enable identification of proteins crucial for colonization and infection that could serve as biomarkers in the identification of vaccine targets (Fluit, 2012; Klein et al., 2012).

The objective of this study is to link the latex agglutination phenotype of *S. aureus* strains isolated from bovine mastitis milk with data on clonal complexes, virulence genes, and antibiotic resistance in order to 1) determine the virulence profiles of the SLAT(+) and SLAT(-) groups,

and 2) provide data needed to improve treatment of bovine mastitis and to identify potential vaccine targets.

MATERIALS AND METHODS

Bacterial Isolates, DNA Extraction and Species Identification

A total of 78 *S. aureus* strains was isolated from bovine mastitis milk samples collected from different cows at 57 Swiss farms between March 2011 and Feb 2012. Putative *S. aureus* isolates were identified by streaking samples onto rabbit plasma fibrinogen plates (Oxoid, Basel, Switzerland), which were subsequently incubated at 37°C and examined for coagulase activity after 48 h. A single *S. aureus* typical colony of each plate was transferred to blood agar and incubated over night at 37°C. DNA isolation kits were supplied by QIAGEN (Hilden, Germany) and handled according to the manufacturer's instructions. The concentration of nucleic acids was measured using Nanodrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Staphaureux Latex Agglutination Test

Staphaurex test kit (Oxoid, Basel, Switzerland) was used according to the manufacturer's instructions to determine latex agglutination.

Genotyping using DNA Microarray

Presence of 284 genes and allelic variants was assessed using StaphyType ArrayStrips (Clondiag chip technologies, Jena, Germany) following the manufacturer's instructions. Multiplex linear DNA amplification and microarray hybridization allowed for identification of species markers, genes conferring resistance to antimicrobial agents, and virulence determinants

such as genes encoding enterotoxins, toxic shock syndrome toxin, leukocidins, hemolysins, and MSCRAMMs. The microarray also enables assignment of strains to clonal complexes and *agr* types. DNA microarray profiles were converted to sequence-like strings as described elsewhere to allow for visualization by SplitsTree4, a software package designed to compute unrooted phylogenetic networks from molecular sequence data (Huson et al., 2006; Wattinger et al., 2012).

Susceptibility Testing

Disk diffusion was used to classify isolates as susceptible, intermediate or resistant depending on respective zone diameters following CLSI standard protocols (Clinical and Laboratory Standards Institute, 2008). All antimicrobial agents were chosen with regard to their relevance in mastitis therapy. Antibiotic agents tested included ampicillin (30 μg), amoxicillin (20 μg) with clavulanic acid (10 μg), cephalothin (30 μg), ceftiofur (30 μg), erythromycin (15 μg), cefoxitin (30 μg), gentamicin 10 (μg), kanamycin (30 μg), kanamycin-cefalexin (30 μg/ 15 μg), penicillin (10 U.I.), and penicillin-novobiocin (10 U.I/ 30 μg). Mueller-Hinton agar, as well as disks containing ceftiofur and penicillin-novobiocin were provided by Oxoid, while disks containing cefalexin-kanamycin (Ubrolexin®) were provided by Boehringer Ingelheim (Basel, Switzerland). All other disks containing antibiotic agents were obtained from Becton Dickinson (Basel, Switzerland). Reference strain ATCC 25923 was used as a quality control.

Statistical Analysis

The distribution of specific genes among latex-positive and latex-negative isolates was compared based on the hybridization results of the DNA microarray. SPSS Statistics 19 (SPSS Inc., Chicago, IL) was used to run Pearson's chi-squared test, identifying significant associations

between the latex-phenotype and the presence of the examined genes. Results were considered to be statistically significant for p-values < 0.050.

RESULTS

Species Confirmation and Exclusion Criteria

All isolates were confirmed to represent *S. aureus* using the species markers of the DNA microarray. To avoid bias, the sample collection was screened for identical isolates by comparison of all features tested including microarray profiles and resistance patterns, and all isolates were found to be unique.

Latex Agglutination and Clonal Complexes

Latex agglutination was tested for a all 78 confirmed bovine *S. aureus* strains. While 38 isolates (49%) were latex-positive and would have been correctly identified as *S. aureus*, 40 isolates (51%) were latex-negative (false-negative). As depicted in the Splitstree in Figure 1, all latex-negative strains exhibit very similar resistance and virulence gene profiles. They form a single cluster of isolates assigned to CC151, while latex-positive strains were assigned to various clonal complexes including CC97 (n = 16), CC8 (n = 10), CC479 (n = 5), CC20 (n = 4), CC7 (n = 1), and CC45 (n = 1).

Resistance Phenotypes (Disk Diffusion)

Resistance phenotypes determined by disk diffusion are listed in Table 1. The SLAT(-) isolates were susceptible to all antimicrobial agents tested. Among the SLAT(+) group, 24% of the isolates were classified intermediate with regard to cefalexin-kanamycin and 13 % of isolates were classified resistant to both ampicillin and penicillin.

Resistance and Virulence Gene profiles

Selected DNA microarray results on resistance, and virulence genes are depicted in Table 2. While among SLAT(-) isolates, no resistance genes were detected, some SLAT(+) isolates exhibited genes involved in resistance to antibiotic agents including *blaI/R/Z* (26%), *ermC* (3%), *fosB* (39%), and *vanB* (3%). Several SLAT(+) strains also displayed enterotoxin genes *entA* (21%), *entD* (26%), and *entJ* (16%), as well as *sak* encoding staphylokinase (26%), virulence factors that were not found among latex-negative isolates. In contrast, the SLAT(-) group exhibited significantly higher prevalence rates of both tested allelic variants of *entC*, as well as the *egc* enterotoxin gene cluster, genes encoding toxic shock syndrome toxin, and leukocidins *lukM/lukF-P83* and *lukD*. Neither *pvl*, encoding panton-valentine leukocidin, nor *etA/B/C*, encoding exfoliative toxins, were detected in this study. Microarray data on the presence of selected adhesin genes coding for target proteins of the Staphaureux test is presented in Table 3. While almost all strains were positive for one or more allelic variants of the adhesin genes tested, latex-positive and latex-negative isolates differ largely in the allelic variants found. A comprehensive overview of the prevalence rates of all genes tested is provided as supplemental file that also comprises *p*-values used to determine significant differences in the prevalence of respective genes among SLAT(+) and SLAT(-) isolates.

DISCUSSION

While Staphaureux test kit exhibits high specificity (99.5%) and sensitivity (99.8%) when applied to *S. aureus* strains obtained from humans, it frequently leads to false-negative results when applied to *S. aureus* isolates obtained from bovine mastitis milk. We detected 51% SLAT(-) isolates, consistent with the rate of 54% reported by Stutz et al. (Stutz et al., 2011). In both

studies SLAT(+) isolates were associated with a wide range of clonal complexes, whereas the SLAT(-) isolates were assigned to CC151 only. CC151 was reported to represent the most prevalent clonal complex among *S. aureus* isolated from bovine milk obtained from both seemingly healthy, as well as clinically infected udders (Sakwinska et al., 2011; Schlotter et al., 2012; Stutz et al., 2011). The most frequent clonal complexes among SLAT(+) strains in our study included CC97, CC8, CC497, and CC20, while CC7, CC9, and CC45 were assigned to only one isolate each. Interestingly, no isolates were assigned to CC133, a dominant clonal complex among *S. aureus* isolated from the milk of seemingly healthy cows in Germany (Schlotter et al., 2012).

When we tested for antimicrobial susceptibility, the resistance proportions among clonal complexes differed, with CC151, CC479, and CC20 representing the only clonal complexes that comprised no resistant isolates. These findings are consistent with an extensive study on antimicrobial resistance among Swiss and French bovine *S. aureus* isolates, in which penicillin resistance rates among CC151 and CC20 were found to be far lower than those among CC97 (Sakwinska et al., 2011). Overall, a total of 6% of bovine *S. aureus* isolates tested in our study were resistant to both ampicillin and penicillin, and 12% exhibited intermediate susceptibility to kanamycin/cefalexin.

DNA microarray results indicate that the resistance and virulence gene profiles of the SLAT(-) strains in our study are highly similar, but differ largely from those of SLAT(+) strains (see Splitstree, Figure 1). Epidemiological studies suggest that a subset of *S. aureus* strains exhibits a distinctive genetic background that renders them highly successful in causing bovine mastitis (Herron-Olson et al., 2007). The SLAT(-) isolates characterized in our study lack several virulence genes frequently found among SLAT(+) isolates, including genes coding for resistance factors, staphylokinase, and enterotoxins A, D, and J. This particular combination of enterotoxin

genes *entA, entD,* and *entJ* was recently described to represent one of the main criteria in the identification of *S. aureus* strains classified as genotype B (Boss et al., 2011). Strains of this genotype were reported to be exclusively associated with very high (up to 65%) within-herd prevalence of mastitis (Graber et al., 2009). In our study, strains exhibiting the combination of *entA, entC,* and *entJ* were assigned to CC8 only.

Among SLAT(-) strains, we found significantly higher numbers of several other virulence genes, including genes coding for toxic shock syndrome toxins (*tst-1, tst-RF122*), enterotoxins (*entC, egc-cluster*), and leukocidins (*lukM/lukF-P83*, *lukD*). Recent studies suggest *lukM/lukF-P83* to play a role in the pathogenesis of bovine mastitis (Barrio et al., 2006; Schlotter et al., 2012).

As false-negative results in the Staphaurex test are due to impaired functionality of one or several of the targeted virulence factors Spa, ClfA/B, and FnbA/B, SLAT(-) strains have been hypothesized to be less virulent than SLAT(+) strains (Stutz et al., 2011). The DNA microarray results revealed considerable heterogeneity regarding *clfA/B* and *FnbA/B*, and we found SLAT(-) and SLAT(+) isolates to exhibit different alleles of the respective genes. ClfA/B and FnbA/B are representatives of the group of bacterial surface proteins designated MSCRAMMSs (microbial surface components recognizing adhesive matrix molecules). These proteins are of particular interest in the development of vaccines, as they mediate adherence of *S. aureus* to components of the host's extracellular matrix.

CONCLUSIONS

The genomic background of SLAT(-) and SLAT(+) strains differs significantly, including a vast range of genes encoding crucial virulence and resistance factors. In consideration of the high heterogenity among MSCRAMM genes detected by DNA microarray, we conclude that a

232 combination of diverse antigens is crucial to the development of highly functional adhesin-based
233 diagnostic tools and vaccines.
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238
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282

283 **TABLES**

284

285 Table 1: Antimicrobial resistance phenotypes determined by disk diffusion among Staphaurex
286 latex agglutination test positive (SLAT(+)) and Staphaurex latex agglutination test negative
287 (SLAT(-)) isolates

	Antimicrobial agents[1]										
	AM	AMC	CF	EFT	ERY	FOX	GM	K	K/CFX	P	P/NB
SLAT(-)											
CC151 (n = 40)	0	0	0	0	0	0	0	0	0	0	0
SLAT(+)											
CC7 (n = 1)	1	0	0	0	0	0	0	0	0	1	0
CC8 (n = 10)	1	0	0	0	0	0	0	0	(1)[2]	1	0
CC9 (n = 1)	1	0	0	0	0	0	0	0	(1)[2]	1	0
CC20 (n = 4)	0	0	0	0	0	0	0	0	(3)[2]	0	0
CC45 (n = 1)	1	0	0	0	0	0	0	0	0	1	0
CC97 (n = 16)	1	0	0	0	0	0	0	0	(4)[2]	1	0
CC479 (n= 5)	0	0	0	0	0	0	0	0	0	0	0
total (n = 78)	5	0	0	0	0	0	0	0	(9)[2]	5	0

288 [1)] Abbreviations: AM = ampicillin, AMC = amoxicillin with clavulanic acid, CF = cephalothin,
289 EFT = ceftiofur, ERY = erythromycin, FOX = cefoxitin, GM = gentamicin, K = kanamycin,
290 K/CFX = kanamycin-cefalexin, P = penicillin, P/NB = penicillin-novobiocin

291 [2)] The number of isolates that exhibited intermediate sensitivity to the respective antimicrobial
292 agent is presented in brackets.
293
294 Table 2: Prevalence of selected virulence and resistance genes detected by DNA microarray
295 among Staphaurex latex agglutination test (SLAT(+)) and Staphaurex latex agglutination test
296 negative (SLAT(-)) bovine mastitis isolates

Group	Gene/Probe	Function	SLAT(+) (n = 38)	SLAT(-) (n = 40)
agr typing	agrI	accessory gene regulator, type I	84%	0%*
	agrII	accessory gene regulator, type II	16%	100%*
Capsule	capsule-5	capsule type 5	79%	0%*
	capsule-8	capsule type 8	21%	100%*
Resistance	blaI, blaR, blaZ	beta lactamase resistance	26%	0%*
	fosB	putative marker for fosfomycin, bleomycin	39%	0%*
Enterotoxins	entA	enterotoxin A	21%	0%*
	entC	enterotoxin C	3%	28%*
	entCM14	enterotoxin-like protein	3%	100%*
	entD	enterotoxin D	26%	0%*
	entJ	enterotoxin J	16%	0%*
	egc-cluster	enterotoxin gene cluster	29%	100%*
Toxic shock	tst-1	toxic shock syndrome toxin	0%	13%*
	tst-RF122	allelic variant of toxic shock syndrome toxin from RF122	0%	15%*

Leukocidins	lukM/lukF-P83	bovine leukocidin	42%	100%*
	lukD	leukocidin D component	87%	100%*
Staphylokinase	sak	staphylokinase	26%	0%*

*The prevalence of the respective gene differs significantly between bovine SLAT(+) and SLAT(-) *S. aureus* isolates tested in this study (p <0.050).

Table 3: DNA microarray data on the prevalence of allelic variants of *spa, clfA/B, fnbA/B*, coding for proteins targeted by the Staphaurex test.

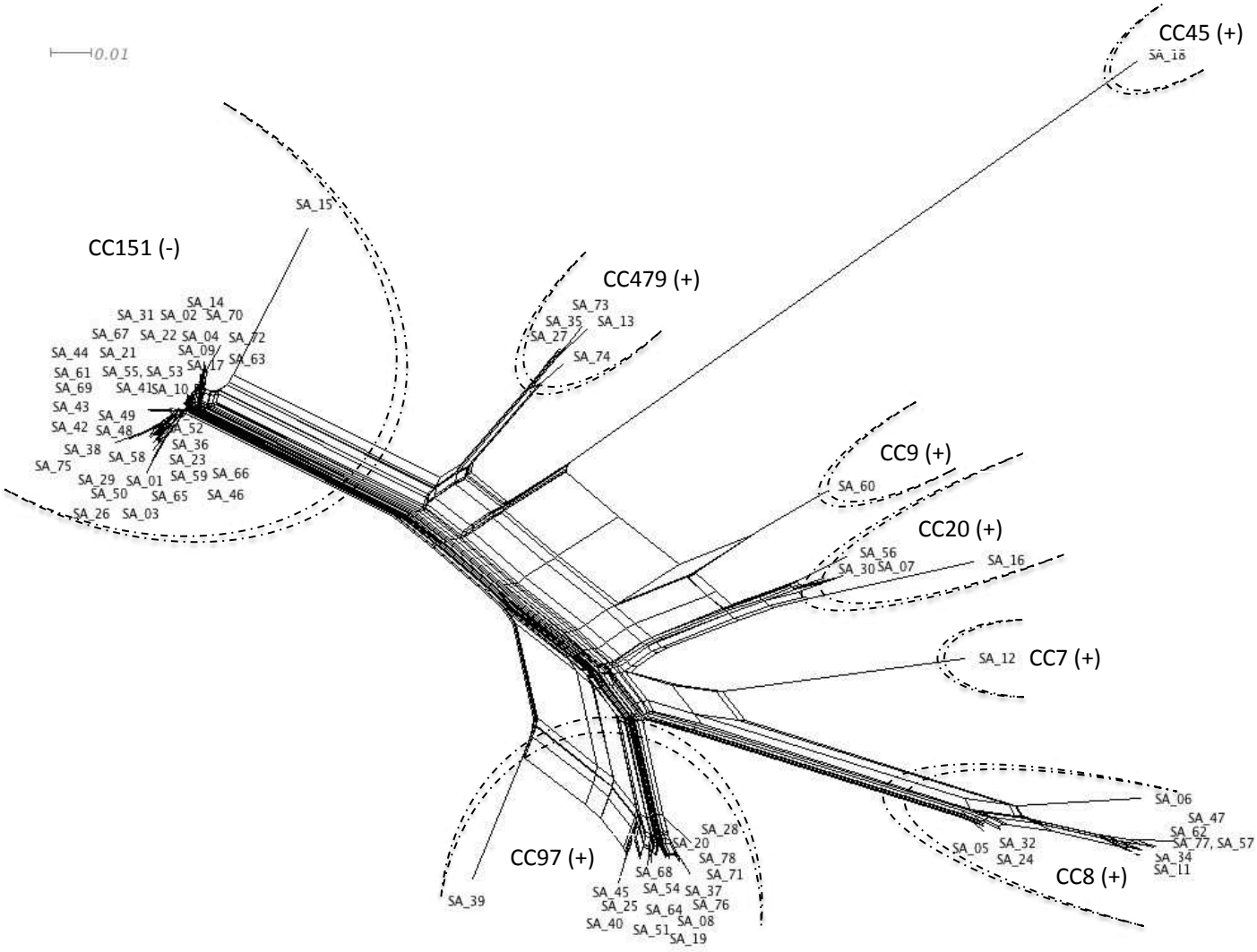
Target	Gene/Probe	Function	SLAT(+) (n = 38)	SLAT(-) (n = 40)
Spa	spa	staphylococcal protein A	100%	100%
ClfA/B	clfA-all	clumping factor A (ClfA)	97.4%	100%
	clfA-COL+RF122	ClfA, allele from COL/ RF122	71%	100%*
	clfB-COL+Mu50	ClfB, allele from COL/ Mu50	26%	0%*
	clfB-MW2	ClfB, allele from MW2	5%	100%*
	clfB-RF122	ClfB, allele from RF122	18%	100%*
FnbA/B	fnbA	fibronectin-binding protein A (FnbA)	89%	100%*
	fnbA-COL	FnbA, allele from COL	29%	0%*
	fnbA-MRSA252	FnbA, allele from MRSA252	16%	0%*
	fnbA-RF122	FnbA, allele from RF122	0%	100%*
	fnbB-COL+Mu50+MW2	fibronectin-binding protein B (FnbB)	100%	0%*

*The prevalence of the respective gene differs significantly between the SLAT(+) and SLAT(-) bovine *S. aureus* isolates tested in this study (p <0.050).

305 **FIGURES**

306

307 Figure 1: Splitstree depicting similarity of resistance and virulence gene profiles among SLAT(+)
308 and SLAT(-) bovine mastitis isolates. CC151 represents the only cluster comprised of SLAT(-)
309 isolates. The latex phenotype of all isolates clustered in one clonal complex is indicated in
310 brackets.



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