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Pattern characterization of genes involved in non-specific immune response in *Staphylococcus aureus* isolates from intramammary infections

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Abstract

Staphylococcus aureus isolated from mammary gland are characterized by different genetic patterns. Ninety four isolates from 33 dairy herds were analyzed by the means of a microarray to investigate *S. aureus* virulence patterns and the distribution of genes believed to be involved in immune evasion. None of the 94 isolates considered were MRSA. However, 50% of the isolates belonged to complexes related to MRSA and to human diseases, while only about 25% of them can be considered as exclusively of bovine origin. The distribution of clonal complexes and the different gene patterns observed confirmed the presence of an influence of geographical localization.

The assessment of the influence of genes related to immune evasion on quarter milk cell count showed as four of them showed to be significantly associated to an increase quarter milk SCC. These genes could be potential target for developing new vaccines against *S.aureus*.

Keywords

Staphylococcus aureus; clonal complex; immune evasion; mastitis; enterotoxins; epidemiology

1. Introduction

Staphylococcus aureus is able to colonize several tissues in both humans and animal species (Morgan, 2008; Weese, 2010). The importance of *S.aureus* as a pathogen increased furthermore, with the increasing frequency of involvement of methicillin-resistant *S.aureus* (MRSA) in severe human disease cases. MRSA has been isolated since many years (Barber, 1961). and cows and pigs emerged as MRSA reservoirs for human infection (LA-MRSA) (van Loo et al., 2007; Vanderhaeghen et al., 2010). Among animals, dairy cows represent an important host for *S. aureus*, and particularly mammary gland provide favourable conditions for infections. Once intramammary infections are established, relevant economic losses are generally observed (Halasa et al., 2007; Zecconi, 2010; Zecconi et al., 2006a). Furthermore, *S. aureus* isolates from mammary gland are characterized by different genetic patterns, and this variability is behind the differences observed in clinic, geographical and economic aspects of infections (Fijalkowski et al., 2012; Le Marechal et al., 2011; Zecconi et al., 2006a).

Despite the large array of immune defences generally deployed by the host, *S. aureus* is able to survive in different hosts and tissues because of its impressive arsenal of virulence factors. These molecules may increase the severity of the infection, improving adhesion to the tissues, facilitating invasion, promoting immune evasion and impairing host defences (Zecconi and Scali, 2013). Indeed, *S. aureus* can impair phagocytes activity through direct cytolysis of neutrophils (PMN), delay of migration or reduction of oxidative burst (Chavakis et al., 2007;

DuMont et al., 2011; Peacock et al., 2002). Chemotaxis inhibitory protein (CHIPS) binds formyl peptide receptors and C5a receptors, hence it slow down PMN migration (Postma et al., 2004; Rooijakkers et al., 2006). Furthermore, chemotaxis may be delayed by Staphylococcal superantigen-like proteins (SSLs) 5, 7 10 and 11 (Bestebroer et al., 2007). SSL7 is able to concurrently bind Fc region of IgA and C5, therefore obstructing both recognition by phagocytes and complement activities (Langley et al., 2005; Laursen et al., 2010). Additionally, SSL7 seems to inhibit complement classical pathway via bounding Fc domain of IgG (Itoh et al., 2010). SSL10 showed to bind PMN C-X-C chemokine receptor type 4 (CXCR-4) (Walenkamp et al., 2009), and SSL11 seems to obstruct neutrophil adhesion to P-selectin-coated surfaces (Chung et al., 2007).

S. aureus have efficient defences against complement system, too. Capsular polysaccharides 1, 5 and 8 are able to interfere with C3 or C3b deposition (Chavakis et al., 2007; O'Riordan and Lee, 2004). Staphylococcal complement inhibitor (SCIN) and extracellular complement-binding protein (Ecb) inhibits convertase enzyme and *S. aureus* surface protein E (SdrE) impairs complement regulator factor H (Cunnion et al., 2011; Sharp et al., 2012). Clumping factor A (ClfA) and extracellular fibrinogen-binding protein (Efb) not only permit adherence to specific host molecules, but also provide interferences with complement system. Indeed, ClfA binds complement regulator factor I (Hair et al., 2010) and Efb inhibits both C3 and C5 convertases (Jongerius et al., 2007).

Enterotoxins (SEs) should be included among the virulence factors that can modulate host immune response because of their well-known superantigen activities (Fraser and Proft, 2008). The number of these molecules identified in *S. aureus* genome steadily increased in recent years and actually at least 20 different SEs have been characterized (Pinchuk et al., 2010).

Several studies investigated roles of virulence factors in intramammary infections or as potential vaccine targets (Festa et al., 2013; Le Marechal et al., 2011; Li et al., 2013; Middleton,

2008; Piccinini et al., 2010; Tedeschi et al., 2009; Zecconi et al., 2005; Zecconi et al., 2006b). However, only few of them considered the factors involved in immune evasion (Piccinini et al., 2010; Scali et al.2015; Seo et al., 2007; Zecconi et al., 2006b).

There are several method to classify *S.aureus* (Tenover et al., 1994), and the one most frequently used is MLST (Enright et al., 2000) which allow to define clonal complexes and, consequently their epidemiological characteristics (Enright et al., 2000; Monecke et al., 2011). While this latter method is very useful to define cluster of isolates, it does not allow identifying the potential association of specific genes with infection characteristics. The availability of a diagnostic microarray that includes the analysis of about 170 distinct genes and their allelic variants, including the ones related to methicillin resistance (MRSA), allows to investigate *S. aureus* virulence patterns and, in addition, the role of specific virulence factors.

Therefore, in order to achieve further information on *S.aureus* epidemiology, potentially useful for vaccine development, we designed a study aimed to identify clonal complexes of *S.aureus* isolates from Italian dairy cows defined by MLST and the relationship between genes believed to be involved in immune evasion and in inducing changes in quarter milk somatic cell counts.

2. Materials & Methods

2.1 Sampling and Somatic cell counts

Quarter milk samples were collected from lactating cows from 33 herds enrolled in a contagious mastitis control programme based on routine sampling of cows to determine if they were infected, followed by segregation of positive ones (Zecconi, 2006; Zecconi et al., 2003). Quarters were sampled following the procedures described by NMC (Hogan et al., 1999) and were analysed by standardized procedures (Hogan et al., 1999). The isolates were presumptively identified as *S. aureus* according to the following scheme: Gram-positive cocci,

haemolytic on blood agar, catalase positive, and coagulase positive in 4-24 h. The presumptive identification was confirmed by API ID32 Staph (BioMerieux, Marcy L'Etoile, F). Isolates were stored at -80 °C in Microbank Bacterial Preservation System (Thermo Fisher Scientific Inc., Waltham MA, USA) until needed.

Quarter milk somatic cells (SCC) were counted using Bentley Somacount 150 (Bentley, USA), following standardized procedures (I.D.F., 1995).

2.2 Isolates

Ninety-four isolates from 33 different dairy herds were considered, and isolates from intramammary infection cases (*S.aureus* positive and absence of clinical signs such as milk alteration and quarter swelling) were considered for each herd, based on phenotype characteristics. Practically, to select isolates representative of each herd, they were recovered from each colony having a different phenotype appearance (i.e. color, hemolysis). This procedure led to select two or three isolates from each herd.

Forty-two isolates were recovered from herds located in Northern Italy (NIt), while 52 from herds in Southern Italy (SIt). After thawing, isolates were cultured on blood agar plates with 5% bovine blood, and then submitted for genotyping.

2.3 Genotyping analysis

Genotyping of staphylococcal DNA was performed both by DNA microarrays based on Alere StaphyType DNA microarray (Alere Technologies Gmbh, Jena D) and by MLST procedure. The microarray covers approximately 170 distinct genes and their allelic variants for a total of 334 target sequences including species markers, SCCmec, capsule and agr group typing markers, resistance genes, exotoxins, and MSCRAMM genes (Monecke et al., 2007, 2008). The array comprised the probes in twofold redundancy. Genomic target DNA was amplified in a linear manner and labelled as previously described (Monecke et al., 2007, 2008). The Iconoclust

software package (Alere Technologies Gmbh, Jena, Germany) were used combined with a defined script according to the procedure described by Monecke et al., (2007, 2008). Clonal complex were defined by standardized procedure based on MLST genotyping (Enright et al., 2000).

2.4 Data collection, classification and statistical analyses

Clonal complexes distributions were analyzed by FREQ procedure of SAS 9.4 software (SAS Institute, Cary NC USA), applying $\chi 2$ test on frequency tables produced. The relationship between gene patterns and quarter milk somatic cell counts was performed by General liner models (GLM) procedure of SAS 9.4 software (SAS Institute, Cary NC USA), using SCC as a response variable in a model that included all the considered genes as factors and without interactions. P value threshold for statistical significance was set at 0.05.

3. Results

3.1 Isolates characteristics

Isolates were characterized by both MLST and microarray. This latter technique showed as all the isolates carried specific *S.aureus* genes: protein A (*spa*), coagulase (*coa*), IgG-binding protein (*sbi*), and thermostable extracellular nuclease (*nuc*) as expected (Monecke et al., 2007, 2008).

None of the isolates were MRSA, being negative for methicillin-resistance regulatory protein, signal transducer protein MecR1, homologue of xylose repressor and others related genes included in the array, while 52.1% of them carried genes involved in penicillin resistance.

When isolates were grouped in clonal complexes (CC) by MLST, 12 CC including 88 isolates were identified, while 6 isolates could not be included in any group. Only 5 CC included 5 or more isolates, while the other seven had 4 or less isolates each. Therefore, for further statistical analysis, the complexes were grouped in 7 groups (Table 1). Out of them, 5 had

more than 5 isolates each (CC 1, 8, 97, 479 and 705), one including all the other clonal types (OTH) and the last one included the unclassified isolates (UNC). Within the OTH isolates, it should be emphasized that four of them were classified as CC398, which is a lineage that includes livestock-associated (LA) *S.aureus* strains able to cause diseases in humans (Monecke et al., 2011). When distribution of CC and herd location were considered, results showed significant differences at x^2 test, ($p<0.001$), (Figure 1). Indeed, CC8 was prevalent in SIt herds, while CC705 and OTH were prevalent in Nit herds. Finally, CC479 was recovered only in NIt herds, and CC1 was recovered only in SIt herds.

3.2 Virulence factors considered to be involved in immune evasion

Table 2 reports the frequency of genes involved in evasion from host non-specific immune response and included in the microarray applied in this research. Data showed as all the isolates carried genes involved in neutrophils lysis (*Hlg*, *Hld*, *PVL-F*, *PVL-S*). Only one isolate was positive for *chip* while all isolates were positive for *ClfA* and *Efb* genes. Most of the isolates carried genes codifying for factors affecting neutrophil migration (*ssl5*, *ssl 7*, *ssl 10*), while it was the opposite for *ssl 11*, and genes involved in complement inactivation (*Sak*, *SCIN*).

When capsular type was considered, 65 isolates carried capsular type 5 gene (*cap5*) and 29 carried type 8 gene (*cap8*). Among *cap8* isolates, 62.07% were from NIt herds, while among *cap5* isolates, only 36.92% were form NIt herds (statistically significant at x^2 test, $p=0.034$).

The results showed as 41.5% of isolates did not carry any enterotoxin gene, while 8.5% of isolates carried a single gene. Table 3 reports the proportion of isolates harboring the different enterotoxin genes. Enterotoxin genes never recovered were *seb*, *see*, *sek*, *seq*. The other genes were identified with a frequency between 5.3% (*seh*) and 27.7% (*sed*).

3.4 Clonal complex, gene pattern and SCC in infected quarters

To investigate if gene patterns have any relationship with mammary gland inflammatory response, quarter milk SCC measured at the time of *S. aureus* isolation was compared with CC and with the different combination of genes involved in immune evasion.

Figure 2 reports mean SCC values for the different CC. Means showed large differences, but no statistical significance was observed. Numerically, isolates in CC479 showed the highest milk SCC values when compared with the other CC, while isolates in the OTH and UNC groups showed the lowest mean SCC among the different CC.

Table 4 reports the result of GLM analysis on the influence of genes on quarter SCC. Among all the genes considered, only 4 showed a statistical significant outcome (*ssl 7, ssl 10, chip* and *cap8*). None of the enterotoxin genes showed to have significant influence, and only *sec* was close to significance level (P=0.056).

4. Discussion

4.1 Distribution of CC and MRSA

Characterization of isolates with microarrays resulted in useful information both from epidemiological and practical point of view. The first important information was the absence of any MRSA isolates. Even if this is, apparently, a positive outcome, it should be considered together with the genomic characterization. Indeed, nearly 50% of isolates was classified in CC8, which is a lineage that includes pandemic MRSA strains (Monecke et al., 2011). Frequency of CC8 isolates is higher than usually observed in other Countries (Hata et al., 2010; Ikawaty et al., 2009; Monecke et al., 2011). However, a recent study in Switzerland reported results similar to ours (Sakwinska et al., 2011). Similarly four isolates were classified in CC398, another potential LA-MRSA lineage (Vanderhaeghen et al., 2010), with a frequency, once more, similar to the one observed by Sakwinska et al., (2011). Next to CC8, the highest frequencies were observed for other two complexes CC97 (12.8%) and CC705 (11.7%) two

lineages usually isolated in cattle (Hata et al., 2010; Monecke et al., 2011; Sakwinska et al., 2011), but rarely associated to MRSA. The number of isolates belonging to complexes related to MRSA and to human diseases (CC1, CC5, CC8, CC20, CC398) represent more than 50% of the isolates considered, while only about 25% of them can be considered as exclusively of cow origin. These data support the importance of monitoring dairy cows as a potential reservoir of LA-MRSA and to apply a prudent use of antibiotics to reduce the development of antibiotic resistance (Fluit, 2012; Graveland et al., 2011; Trevisi et al., 2014).

Significant differences were also observed within CC distribution among Italian herds, when northern herds were compared to southern ones. These two areas are characterized by very intensive dairy farming with high production levels (northern Italy) compared with southern area where dairy herd are less efficient and smaller, due to the adverse environmental conditions (water scarceness, poor soil fertility). Even if there is a trade of animals between these two areas, the results of this study suggest that CC spread more frequently within their area of origin. The differences in CC frequencies observed in comparison to other investigations and the significant variations in frquencies when herd location was considered confirm that *S. aureus* genetic characteristics may be related to geographical location (Costa et al., 2012; Piccinini et al., 2010; Sakwinska et al., 2011; Zecconi et al., 2006a). This latter observation has important implication for vaccine development, because effective vaccines should be able to be provide protection even in presence of strains with different genetic characteristics (Middleton et al., 2009).

4.2 Virulence factors considered to be involved in immune evasion

When genes related to immune evasion were considered, data confirmed the presence of several of them within the isolates considered. All the isolates harbor genes involved in neutrophil lysis and in most cases genes impairing neutrophil migration were also present. To the opposite, genes involved in complement inactivation showed variable frequencies. Within these latter genes, the frequency of capsular types (*cap5*, *cap8*) showed some peculiarity.

Gene frequencies were different from the ones previously reported in a large study involving isolates from Europe and North America (Tollersrud et al., 2000). In this paper, only 63% of European isolates was cap5 or cap8 typeable, while in our case all isolates were positive for either one or the other gene. Moreover, in our study *cap5* was the prevalent gene, while *cap8* was the most prevalent in Tollersrud et al. (2000) study. The differences in gene frequency could be explained by the different diagnostic methods applied, microarray in our case and serological in the other study. However, large geographical differences were observed in both studies, supporting previous observations on the presence of a geographical effect on *S. aureus* characteristics. This effect could be related to a genetic drift driven by control measures against *S. aureus*, which are different from one country to another, and by the adaptation of the bacteria to different hosts.

Staphylococcal enterotoxins genes were identified in 41.5% of our isolates. Enterotoxins, being superantigens, have been considered as an important virulence factor (Pinchuk et al., 2010). However, the frequency of isolates carrying SE showed to be largely variable and the evidence of a role in subclinical mastitis is not consistent (Larsen et al., 2002; Larsen et al., 2000; Oliveira et al., 2011; Younis et al., 2003; Zecconi et al., 2006b). The relative low frequency of SE genes in our study and the presence of only a weak relationship with quarter milk SCC for *sec* gene (Table 4), would be consistent with the contention that SE did not play a major role in bovine mastitis pathogenesis.

3.4 Clonal complex, gene pattern and SCC in infected quarters.

The analysis of a potential association between CC and quarter SCC (Figure 2) showed large variability among CC, but the difference among CC was not statistically significant. These result confirm that MLST is useful in clustering *S.aureus* isolates, but this approach is unable to discriminate the influence of isolate characteristics on host immune response.

The assessment of the potential influence of genes related to immune evasion on quarter milk SCC gave some unexpected results. Among the genes retained in the final model, four of them (*ssl7*, *ssl11*, *chip* and *cap8*) showed to significantly associated to quarter milk SCC. As all these genes are involved in the lysis of neutrophils or in the impairment of their motility, therefore a decrease in SCC should be expected in gene-positive isolates, nevertheless our results were the opposite. There are at least two possible explanations for these results. The simplest one is that genes are not expressed; therefore, there is no influence on cellular immune response. An alternative explanation is that *S. aureus* expressing these genes have an advantage in invading mammary gland by antagonizing host initial cellular response. Thus, the amount of tissue infected and the relative damages induce a higher level of inflammation (resulting in an increase of SCC) compared to less virulent isolates, probably mediated by macrophages, as recently reported (Luscinskas, 2014).

5. Conclusions

Staphylococcus aureus is still one of the most dangerous and costly pathogen in human and veterinary medicine. The findings of this study supports this contention as it was confirmed that genetic lineages common to human and dairy cows that includes life-threatening isolates such as MRSA.

This study confirms the presence of genetic differences related to geographical origin of the isolates, suggesting that may need to consider the region of interest in developing vaccines against the *S. aureus* mastitis. Moreover, genes related to host-immunity could be also considered in the development of *S. aureus* vaccines.

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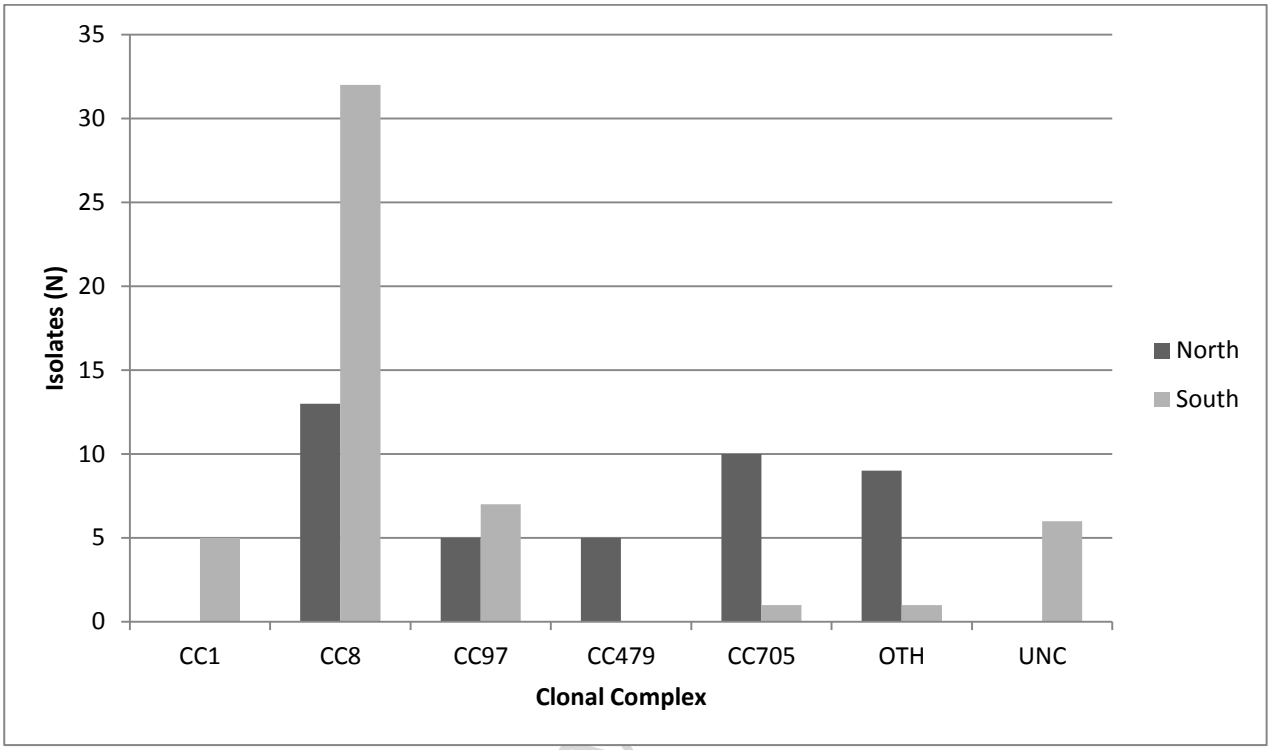

Figure 1: Distribution of Clonal Complexes by herd location. Significant differences were observed when herd location (Northern or Southern Italy) was considered (x^2 test, $p<0.05$).

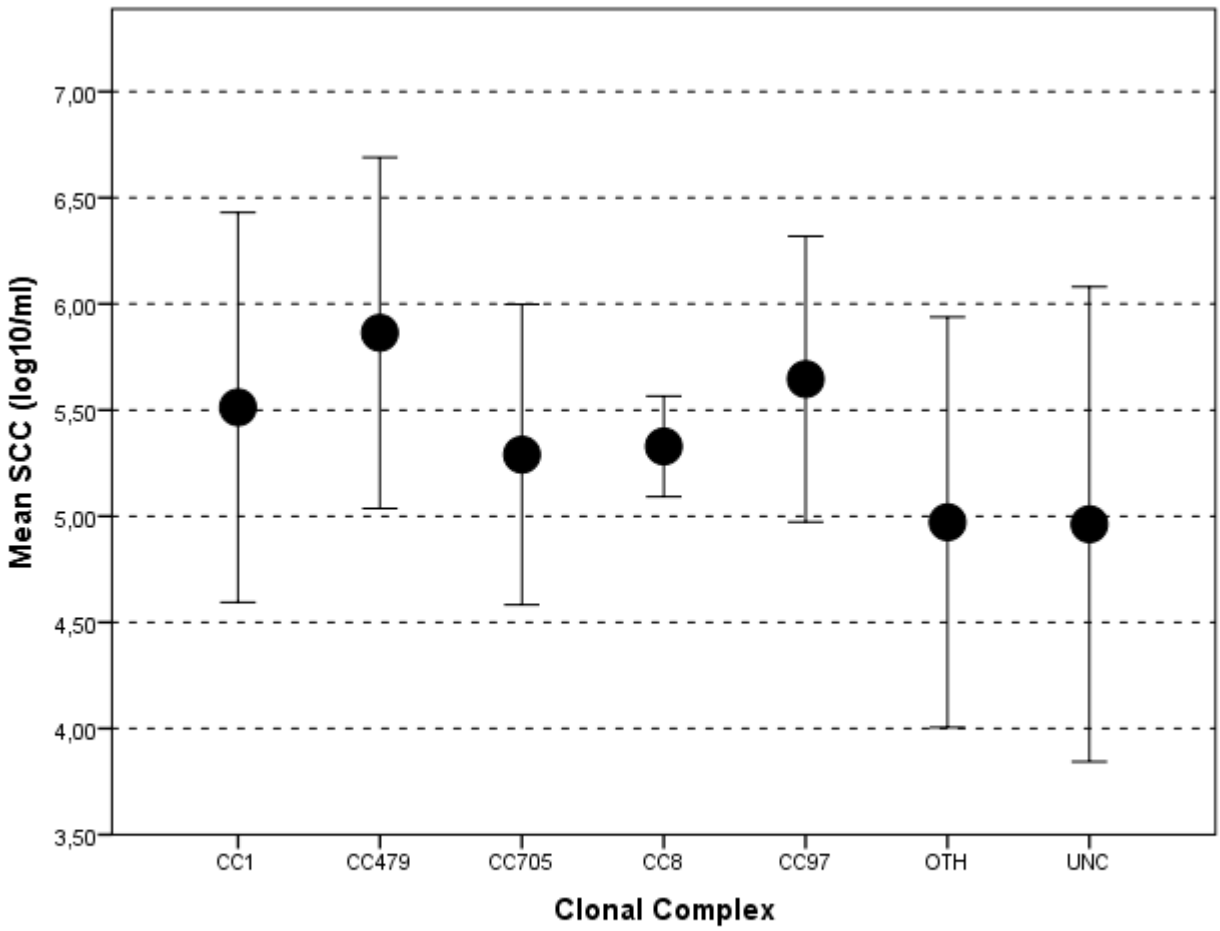

Figure 2: Mean quarter milk SCC observed by *S. aureus* clonal complex (differences among groups are not statistically significant)

Table 1: Distribution of clonal complex frequencies among 94 *S. aureus* isolates considered

Clonal Complex	Frequency (%)	Group
CC1	5 (5.3%)	CC1
CC8	45 (47.9)	CC8
CC97	12 (12.8%)	CC97
CC479	5 (5.3%)	CC479
CC705	11 (11.7%)	CC705
Other types (5, 20, 101, 398)	10 (10.6%)	OTH
Unclassified	6 (6.45)	UNC

Table 2: Frequency of virulence genes enabling *S. aureus* to evade innate immune defenses among 94 *S. aureus* isolates considered.

***S. aureus* gene**	**Effector mechanism**	**Abbre viation**	**Positive (%)**
Staphylococcal superantigen-like 5	Neutrophil migration	SSL5	68 (72.3%)
Staphylococcal superantigen-like 7		SSL7	90 (95.7%)
Staphylococcal superantigen-like 10		SSL10	77 (81.2%)
Staphylococcal superantigen-like 11		SSL11	45 (47.9%)
Chemotaxis inhibitory protein		CHIPS	1 (1.1%)
γ toxin	Neutrophils lysis	Hlg	94 (100%)
δ toxin		Hld	94 (100%)
Panton-Valentine leukocidin-F		PVL-F	94 (100%)
Panton-Valentine leukocidin-S		PVL-S	94 (100%)
Capsular polysaccharides - 5	Complement inactivation	cap5	65 (69.15)
Capsular polysaccharides - 8		cap8	29 (30.95)
Staphylokinase		Sak	16 (17.0%)
Staphylococcal complement inhibitor		SCIN	16 (17.0%)
Clamp factor A		ClfA	94 (100%)
Staphylococcus aureus surface protein E		SdrE	72 (76.6%)
Extracellular fibrinogen-binding protein		Efb	94 (100%)

Table 3: Frequency of enterotoxin genes enabling *S. aureus* to evade innate immune defenses among 94 *S. aureus* isolates considered.

S. aureus gene	Abbreviation	Positive (%)
Enterotoxin A	sea	14 (14.9%)
Enterotoxin B	seb	0 (0.0%)
Enterotoxin C	sec	6 (6.4%)
Enterotoxin D	sed	26 (27.7%)
Enterotoxin E	see	0 (0.0%)
Enterotoxin G	seg	21 (22.3%)
Enterotoxin H	seh	5 (5.3%)
Enterotoxin I	sei	21 (22.3%)
Enterotoxin J	sej	25 (26.6%)
Enterotoxin K	sek	0 (0.0%)
Enterotoxin L	sel	6 (6.4%)
Enterotoxin M	sem	21 (22.3%)
Enterotoxin N	sen	21 (22.3%)
Enterotoxin O	seo	21 (22.3%)
Enterotoxin Q	seq	0 (0.0%)
Enterotoxin R	ser	25 (26.6%)
Enterotoxin U	seu	21 (22.3%)

Table 4: Results of general linear model analysis of the influence of the genes retained in the final model on quarter milk SCC and relative means observed in presence or absence of these genes.

Gene	F value	P=	SCC means. (Log_{10}/ml)	
			Negative	Positive
Staphylococcal superantigen-like 5	1.11	0.294	5.28	5.37
Staphylococcal superantigen-like 7	4.79	0.031	5.32	5.58
Staphylococcal superantigen-like 10	4.67	0.033	5.16	5.41
Staphylococcal superantigen-like 11	0.07	0.789	5.37	5.33
Chemotaxis inhibitory protein	5.46	0.021	5.34	6.58
Capsular polysaccharides - 8	6.10	0.015	5.25	5.56
Enterotoxin A	0.45	0.503	5.36	5.32
Enterotoxin C	3.75	0.056	5.33	5.64
Enterotoxin D	2.37	0.127	5.25	5.60
Enterotoxin G	0.70	0.406	5.25	5.51
Enterotoxin J	0.77	0.382	5.27	5.58

Highlights

- Ninety-four isolates from 33 different dairy herds were analyzed by microarray
- Most of the isolates carried genes affecting neutrophil migration
- *S. aureus* clonal complexes are related to geographical location
- Clonal complexes are not related to quarter milk SCC
- *ssl7*, *ssl11*, *chip* and *cap8* genes were associated to an increase of quarter milk SCC