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Relationship between some *Staphylococcus aureus* pathogenic factors and growth rates and somatic cell counts

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Staphylococcus aureus isolates produce several pathogenic factors. The combination of these products influences the pathogenic role of different isolates, but their specific effects are well known in the pathogenesis of udder infections. This study focused on the association of polymorphism of the coagulase gene, protein A gene, collagen-binding protein gene, and of fibrinogen-binding protein gene on somatic cell count (SCC) and on *Staph. aureus* growth rate. Fifty *Staph. aureus* isolates from 13 dairy cow herds, located in seven different provinces, were considered. The results showed a low frequency of *cna* gene, similar to the one observed in human isolates. Meanwhile, the high frequency of *efb* gene indirectly confirmed the role of this factor in bacterial pathogenesis, being associated with adhesion to epithelia. The association of these two single genes with SCC and growth rate showed to be not significant. The polymorphism of *spa* gene was confirmed to be significantly associated with inflammatory response and growth rate, albeit with a pattern different from the one suggested for human isolates. Sorting of isolates based on the clusters obtained by combining polymorphisms of *spa* and *coa* genes and the presence of *cna* and *efb* genes, showed that a single cluster (cluster V) was prevalent in the different herds and provinces, while the other six clusters identified were widely spread among the remaining 60% of the isolates. Results showed that clusters VI and VII had significantly higher growth rates at 3, 4, and 6 h in comparison with the other clusters. Meanwhile, quarters infected with these strains showed significantly lower SCC levels. The frequency of isolates from cluster V, suggested that they should possess pathogenic factors increasing their invasiveness, even if in the presence of a stronger inflammatory response. These results indirectly confirm previous findings on the different interactions between isolates and the udder immune system. They also suggest that isolates with higher growth rates and inducing a lower inflammatory response have better chances to spread among the herd. The relatively simple genomic method proposed in this study could be applied by an increasing number of diagnostic laboratories and could be useful in studying the epidemiology of *Staph. aureus* intramammary infections in dairy herds when collecting data from the field.

Keywords: *Staphylococcus aureus*, genes, somatic cell counts, growth rate.

Staphylococcus aureus is the most frequently isolated contagious mammary pathogen in many countries and it also causes different pathologies in many species. *Staph. aureus* isolates produce several surface-associated and secretory products, the combination of which influences the pathogenic role of the different isolates, but their specific effects are not well evaluated (Kerro Dego et al. 2002). These differences among isolates could also affect the infection pattern for *Staph. aureus* intramammary infections (IMI), which often differs from herd to herd (Raimundo et al. 1999; Zadoks et al. 2000; Joo et al. 2001). Indeed, in some herds, *Staph. aureus* IMI are characterized by high somatic cell counts (SCC) and mild clinical cases, while in others, most of *Staph. aureus* infected cows show rather low SCC and the infection is not readily diagnosed. These different patterns have been related to strain virulence, udder immune defences, or both (Piccinini et al. 1999; Piccinini & Zecconi 2000; Middleton et al. 2002b).

Most of the studies of *Staph. aureus* epidemiology in dairy herds have focused on the polymorphisms of the coagulase gene (Aarestrup et al. 1995) or on different genotyping methods (Matthews et al. 1994; Fitzgerald et al.

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1997; Rivas et al. 1997; Middleton et al. 2002a). All these methods discriminate between the different isolates and could be helpful in exploring different aspects of *Staph. aureus* epidemiology such as the source and spread of infections and the possible transmission of human strains to cattle and *vice versa* (Matthews et al. 1994; Joo et al. 2001; Middleton et al. 2002a; Schlegelova et al. 2003). However, this approach gives little information on the presence of genes coding for the different pathogenic factors involved in the development of *Staph. aureus* IMI and some methods could be applied only in specialized laboratories.

Our aim was to examine whether the application of a rather simple genomic method based on a small panel of pathogenic factor genes could be useful in classifying the different *Staph. aureus* isolates and, at the same time, to assess the relationship between strains and, respectively, growth rate and SCC. Therefore, this study focused on the polymorphism of coagulase gene (*coa*), the polymorphism of the X region of protein A gene (*spa*), the presence of collagen-binding protein gene (*cna*), and of fibrinogen-binding protein gene (*efb*), and their relationship with quarter milk SCC and the growth rate of isolates.

Analysing coagulase gene polymorphisms can aid epidemiological investigations on *Staph. aureus* isolates in human diseases (Goh et al. 1992) and in bovine mastitis (Aarestrup et al. 1995). Protein A is a polymorph surface protein considered as one of the most important virulence factors for *Staph. aureus* (Frenay et al. 1994; Dalla Pozza et al. 1999; Shopsin et al. 2000). The gene encoding *cna* was selected as an important virulence factor associated with bacterial adhesion in orthopaedic prosthesis infection (Montanaro et al. 1999). Fibrinogen-binding protein gene is a highly conserved gene often observed among clinical isolates (Boden Wastfelt & Flock, 1995). Recent reports show that antibodies against fibrinogen-binding protein reduce the number of recovered bacteria from the mammary glands in a mouse mastitis model (Palma et al. 1998).

To assess the role of genes for the different pathogenic factors, two different response variables were considered, namely, growth rate and SCC in infected quarters. The growth rate of *Staph. aureus* can be considered as a marker for virulence (Anderson, 1983), and it has been recently shown to be related to the expression of some adhesion proteins (Foster & Hook, 1998). SCC is a well-known measure of udder inflammation already applied in similar studies (Middleton et al. 2002b), and SCC is normally correlated to the pathogenic activity of the microorganisms and to their interaction with udder immune defences (Paape et al. 2003).

Material and Methods

Quarter milk sampling

Quarter milk samples (QMS) were collected aseptically before milking. At the laboratory, an aliquot (0·01 ml) of each QMS was spread on blood agar plates to assess the presence of *Staph. aureus* as described in the following paragraph. Somatic cells were counted on a Bentley Somacount 150 (Bentley Instruments, Chaska MN, USA).

Isolates

Isolates were cultured on blood agar plates with 5% bovine blood, and then they were presumptively identified as *Staph. aureus* according to the following scheme: catalase positive, Gram-positive cocci, haemolytic on blood agar, coagulase positive in 4–18 h. Presumptive identification was confirmed by API ID32 Staph (BioMerieux, Marcy L'Etoile, France). Isolates were stored at −20 °C in BHI broth with 15% (v/v) glycerol. Fifty *Staph. aureus* isolates collected from 13 dairy cow herds, located in seven different provinces, were considered. A minimum of two isolates and a maximum of five were selected from each herd, based on herd IMI prevalence.

Growth rate

To assess bacterial growth rate, a standardized inoculum of $1{\cdot}5\times10^{8}$ colony-forming units (cfu)/ml, was used as a starter culture. Bacterial growth in tryptose broth (Oxoid, Milan, Italy), was assessed using a spectrophotometer at 3, 4, 6 and 24 h. Numbers of cfu were estimated by a calibration curve with plate count data.

PCR amplification

Each isolate was sub-cultured overnight on blood agar plate; 10 colonies were suspended in 300 μl of Tris-HCl–EDTA (TE) buffer containing 4 mg/ml of lysostaphin and the suspension was incubated at 37 °C for 40 min. After cell lysis, the DNA was purified using Rapid Prep™ DNA isolation kit (Amersham Bioscences, Milan, Italy).

The primers applied for *coa* amplification were 5′-GAGACCAAGATTCAACAAG-3′; 5′-AAAGAAAACCACTCACATCA-3′ (Goh et al. 1992). Each sample was subjected to a PCR pre-cycle of 1 min at 95 °C, followed by 32 cycles, each consisting of 45 s at 94 °C, 45 s at 55 °C and 1 min 30 s at 72 °C, with a final extension of 5 min at 72 °C. The PCR product was subsequently digested with the restriction enzyme RsaI (10 U, MBI Fermentas, Vilnius, Lithuania) for 1 h at 37 °C (Goh et al. 1992).

The primers applied for *cna* amplification were: 5′-AAAGCGTTGCCTAGTGGAGA-3′; 5′-AGTGCCTTCCCAAACCTTTT-3′ (Montanaro et al. 1998; Montanaro et al. 1999). Each sample was subjected to a PCR pre-cycle of 1 min 30 s at 95 °C, followed by 40 cycles, each consisting of 30 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C, with a final extension of 5 min at 72 °C (Montanaro et al. 1998).

The primers applied for *spa* (region X) amplification were: 5′-TGTAAAACGACGG CCAGTGCTAAAAAGCTAAACGATGC-3′; 5′-CAGGAAACAGCTATGACCCC

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ACCAAATACAGTTGTACC-3′ (Dalla Pozza et al. 1999). Each sample was subjected to a PCR pre-cycle of 3 min at 95 °C, followed by 40 cycles, each consisting of 1 min at 94 °C and 3 min at 72 °C, with a final extension of 10 min at 72 °C. The PCR product was subsequently digested with the restriction enzyme RsaI (10 U, MBI Fermentas) for 1 h at 37 °C (Dalla Pozza et al. 1999).

The primers applied for *efb* amplification were: 5′-ACGGTCCAAGAGAAAGAAACC-3′; 5′-TTGTCCAG-ACTACTTGCATCTGC-3′ (Smeltzer et al. 1997). The amplification of *efb* gene was obtained by PCR pre-cycle of 1 min 30 s at 95 °C, followed by 32 cycles, each consisting of 45 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, with a final extension of 5 min at 72 °C (Smeltzer et al. 1997).

All the oligonucleotide primers were synthesized by Invitrogen (Milan, Italy). PCR products were analysed by electrophoresis in agarose gel in the presence of ethidium bromide and a DNA marker: GeneRuler DNA ladder 50 bp (MBI Fermentas) for *coa*, *cna*, *spa* genes, GeneRuler DNA ladder 100 bp (MBI Fermentas) for *efb* gene. Gels were photographed under u.v. illumination and digital images were taken and stored.

Data analysis

The electrophoresis patterns of the four genes considered were analysed using software Gel Compar II version 2.5 (Applied Maths, Sint-Martens-Latern, Belgium). The basis of the software is a relational database consisting of entries corresponding to individual organisms. Each organism was characterized by four fingerprints (*efb*, *spa*, *cna* and *coa*). Gels were normalized by choosing the molecular weight (MW) ladder as standard for each fingerprint type and all following gels added to the database were normalized against the specific MW. In the next step, the MW ladders on the incoming gels were aligned against the database standard. The bands were assigned by manual editing of the software-identified bands, and the position tolerance and optimization were set to 2% for all four fingerprints types. The similarity coefficients of DNA fingerprints were calculated based on band positions. The Dice coefficient was used for *spa* and *coa* fingerprints, and the different bands coefficient was used for *cna* and *efb*. The clustering was performed combining the four fingerprints types. The similarity matrices were taken from the individual genes and a combined matrix was calculated by averaging the values. Each value was considered equally important and no extra weight was assigned to each fingerprint. The patterns were then clustered by the unweighted pair group method using arithmetic algorithms (UPGAMA).

Data were analysed by SAS 8.2 GLM procedure for repeated measurements (SAS Institute, Cary NC, USA) considering as independent variables, respectively, the presence of *cna*, *efb*, the number of *spa* repetitions and the cluster obtained as previously described. The $\log_{10}$ of SCC value and the growth rate (ratio to starting cfu at 3, 4, 6 and 24 h) were considered as dependent variables.

Table 1. Frequency of the different genes and their polymorphism observed in the 50 *Staph. aureus* isolates considered

Gene	Number of positives, %
cna	15 (30%)
efb	44 (88%)
Repetitions of *spa* X region	11 (22%)
2	4 (8%)
3	4 (8%)
4	4 (8%)
6	3 (6%)
8	5 (10%)
9	16 (32%)
10	3 (6%)
11	
coa types	11 (22%)
1	4 (8%)
2	2 (4%)
5	4 (8%)
6	3 (6%)
7	5 (10%)
13	2 (4%)
16	7 (14%)
17	3 (6%)
18	9 (18%)
all others (9)	

Results

Data description

The frequency of the considered genes and their polymorphism are reported in Table 1. *Efb* gene showed a high frequency, while it was rather low for *cna*. *Staph. aureus* isolates showed a number of repetitions in the X region of the protein A gene in the range 2–11: eleven isolates had two repetitions, 16 isolates had 10 repetitions, representing together more than 50% of the isolates considered.

The polymorphism of *coa* gene showed similar results to previous reports (Aarestrup et al. 1995; Annemuller et al. 1999). Eighteen different polymorphisms were identified. However, nine of them were shown to be represented by a single isolate, while type 1 was the most frequent one, with 11 isolates. The polymorphism of this gene was included in the clustering procedure and therefore further analyses were not performed.

The distribution of clusters obtained by the combination of polymorphisms of the four different genes considered is reported in Table 2. Seven different ones were identified and among them, cluster V represented 42% of the isolates considered, whereas all the other showed nearly equally distributed frequencies. Clusters I, VI and VII had a frequency of 6%, while clusters II, III and IV showed frequencies of 14, 12 and 14%, respectively.

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Table 2. Distribution of *Staph. aureus* isolates sorted by the 7 clusters considered and, respectively the 13 dairy cow herds and the 7 provinces where herds were located

Cluster	Herd A	B	C	D	E	F	G	H	I	L	M	N	O
I				1		1					1		
II				1	3	1							2
III					1	1		4					
IV	5		1						1				
V		4	1	2	1	1	1		3	4		4	
VI							1						2
VII											3		

Cluster	Provinces A	B	C	D	E	F	G
I		1	2				
II		3	1				3
III			1			4	1
IV		1			1	5	
V	1	9	1	4	5		1
VI	1	2					
VII			3				

The distribution of isolates among the 13 herds (Table 2) showed that only one cluster was identified in 5 out of 13 herds; in all the other herds, two or more clusters were identified. In herd F, the four isolates considered came from four different clusters. The distribution of clusters among the seven provinces (Table 2) showed that cluster V was the only one isolated in province D (1 herd), and in 5 out of 6 isolates in province E (2 herds). In all the other provinces different clusters were isolated, although in province B, cluster V was the prevalent one.

Influence of cna *and* efb *genes*

The ratio of bacteria counts at 3, 4, 6 and 24 h to the initial counts showed that numerically *cna*- and *efb*-negative isolates had slower growth rates up to 6 h in comparison with *cna*- and *efb*- positive ones, but the differences were not statistically significant (results not shown). Comparison of SCC in *Staph. aureus* infected quarters between *cna*- and *efb*- negative and positive isolates also showed non-significant differences (results not shown).

Influence of spa *gene*

Evaluation of the influence of *spa* gene polymorphism on SCC variance (Table 3) showed that isolates with six repetitions were associated with a significantly lower response, when compared with isolates with two and ten repetitions. Among the other isolates, no significant differences were observed.

Table 3. Somatic cell counts and growth rate at 6 h by the different number of repetitions of protein A region X

Values are means with SD for $n=50$

Protein A Region X repetitions	SCC, $\log_{10}$/ml Mean	SD	Growth rate, cfu Mean	SD
2	$5{\cdot}56^{a}$†	1·44	$29{\cdot}29^{a,b}$	8·34
3	$4{\cdot}85^{a,b}$	1·26	$30{\cdot}12^{a,b,c}$	14·21
4	$4{\cdot}58^{a,b}$	0·59	$33{\cdot}07^{a,c}$	5·88
6	$4{\cdot}33^{b}$	1·59	$19{\cdot}42^{b}$	0·64
8	$5{\cdot}05^{a,b}$	0·74	$39{\cdot}63^{a,c}$	11·34
9	$5{\cdot}37^{a,b}$	2·23	$39{\cdot}72^{a,c}$	12·70
10	$5{\cdot}77^{a}$	0·70	$36{\cdot}69^{c}$	13·37
11	$4{\cdot}71^{a,b}$	0·09	$36{\cdot}21^{a,c}$	6·54

† Values without a common superscript letter are statistically different ($P<0{\cdot}05$)

Table 4. Somatic cell counts and growth rate at 6 h by strain cluster

Values are means with SD for $n=50$

Strain cluster	SCC, $\log_{10}$/ml Mean	SD	Growth rate, cfu Mean	SD
I	$3{\cdot}91^{a}$†	2·03	$33{\cdot}07^{a,b}$	9·43
II	$6{\cdot}17^{b}$	0·46	$29{\cdot}85^{a,b}$	8·30
III	$5{\cdot}26^{a,b}$	1·17	$27{\cdot}85^{a,b}$	11·56
IV	$5{\cdot}09^{a}$	1·68	$21{\cdot}65^{b}$	5·66
V	$5{\cdot}44^{b}$	1·02	$39{\cdot}16^{a,c}$	11·97
VI	$5{\cdot}05^{a}$	0·73	$39{\cdot}64^{a,c}$	11·34
VII	$4{\cdot}51^{a}$	0·70	$35{\cdot}81^{a,c}$	2·57

† Values without a common superscript letter are statistically different ($P<0{\cdot}05$)

Growth rates at 3, 4 and 6 h showed the same trend and the respective statistical analysis gave similar results, while no statistically significant differences were observed for growth rate at 24 h. Therefore we focused on the 6-h growth rate pattern. *Staph. aureus* isolates with six repetitions of *spa* gene had a significantly lower mean growth rate than all the other isolates, out of isolates with two and three repetitions. Growth rates in the other strain groups were not statistically different.

Influence of isolates classified by clustering

When isolates were classified by the presence of *efb* and *cna* genes and by polymorphisms of *spa* and *coa* genes (Table 4), significantly higher values for SCC were observed for clusters II and V, when compared with all the other clusters. Analysis of growth rate at 6 h showed that isolates in cluster IV had significantly lower values than isolates in clusters V, VI and VII, but not lower than the other clusters.

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Discussion

The frequency of the genes considered confirmed previous reports for human or bovine *Staph. aureus* strains. Indeed, the frequency of *cna* gene was similar to the one reported for human isolates (Montanaro et al. 1999). *Efb* gene is involved in adhesion to skin and epithelia (Jarp et al. 1989; Cho et al. 2001). The high frequency of this gene observed in the present study, suggests that the proteins encoded by this gene could play an important role also in the pathogenesis of mastitis. However, the association of these latter two genes with SCC and growth rate was not significant, confirming that interactions between the genes are more important than the activity of single adhesion-protein genes (Foster & Hook, 1998; Elasri et al. 2002).

The polymorphism of *spa* gene showed a significant association with the inflammatory response and strain growth rate, albeit with a pattern different from the one reported for human isolates (Frenay et al. 1994). Indeed, isolates with six repetitions were associated with lower mean values for both SCC and growth rates up to 6 h, compared with isolates with two and ten repetitions. *Coa* gene was confirmed to be highly polymorphic, and therefore significant associations could not be expected.

Sorting of isolates based on the clusters obtained by combining polymorphisms of *spa* and *coa* genes and the presence of *cna* and *efb* genes, showed that a single cluster (cluster V) was prevalent (40%) in the different herds and provinces. The other isolates were widely spread among the other six clusters. These results support previous findings, using different genomic methods, suggesting that there are predominant strains both within herds and between herds (Zadoks et al. 2000). However, the presence of several isolates in clusters other than cluster V suggests a high risk of potential outbreaks from the spread of emergent strains (Middleton et al. 2002a).

Clusters VI and VII had significantly higher growth rates at 3, 4, and 6 h in comparison with the other clusters, but they were associated with significantly lower SCC levels in infected quarters. Isolates in these clusters have obviously some common features: five out of six are *cna* negative and *efb* positive, they have between four and six repetitions of *spa* region X, and they are included in *coa* type 17. Cluster V, the most frequently isolated one, showed growth rates similar to cluster VI and VII, but SCC values were significantly higher. These isolates are mainly *cna* negative (17/22) and *efb* positive (20/22); *spa* region X repetitions are >8, and they are spread throughout the different *coa* types. Comparison of the behaviour of cluster V with clusters VI and VII suggests that *spa* could play a role in inducing an inflammatory response as suggested for human cases (Frenay et al. 1994). Moreover, the frequency of isolates from cluster V suggests that these isolates should have other pathogenic factors that could increase their invasiveness, even if in the presence of a stronger inflammatory response, when compared with clusters VI and VII, which have a similar growth rate. These results apparently do not agree with an earlier report of within-cow SCC differences (Middleton et al. 2002b). Differences of study design and the importance of cow and herd effects on the inflammatory response (Piccinini et al. 1999, 2004) cannot be ignored, and might explain the apparent difference.

This uncertainty and the observation of an increasing frequency of *Staph. aureus* IMI characterized by low SCC (Zecconi & Piccinini, 2002) support the need for further research on *Staph. aureus* epidemiology. Genomic techniques showed to be efficient tools to characterize isolates and to assess relationships between their characteristics and the spread and severity of *Staph. aureus* infections in both human and veterinary medicine. The relativly simple genomic method proposed in this study could be used by an increasing number of diagnostic laboratories and could be useful in studying the epidemiology of *Staph. aureus* IMI in dairy herds when collecting data from the field.

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