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Relationship between virulence factor genes in bovine *Staphylococcus aureus* subclinical mastitis isolates and binding to anti-adhesin antibodies

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Staphylococcus aureus is the most common aetiologic agent of contagious bovine mastitis. It is characterized by a wide array of virulence factors. The differences among strains jeopardize the development of effective vaccines against *Staph. aureus* mastitis. We tested the immunogenicity of a peptide subunit vaccine coding for three different adhesion factors, fibrinogen-binding protein (Efb), fibronectin-binding protein A (FnbpA) and clumping factor A (ClfA). Then we evaluated the influence of some virulence factors on the ability of specific anti-adhesin antibodies to react with sixteen *Staph. aureus* strains isolated from bovine subclinical mastitis. Immunization with the recombinant adhesins stimulated a strong humoural (IgG and IgA) and mucosal IgA immune response in all animals tested. Hyperimmune serum recognized with diverse efficiency the sixteen *Staph. aureus* strains and this circumstance correlated well with the level of expression of adhesins. Among the different virulence factors considered to classify strains, *spa* gene polymorphisms showed the strongest influence on isolate reactions to hyperimmune serum. Our results indicate the importance of a disease- and environment-specific analysis of isolates. Thus, as opposed to other pathogens to obtain an effective vaccine we should characterize multiple strains and identify the prevalent virulence factors expressed.

Keywords: *Staphylococcus aureus*, adhesin, gene polymorphism, vaccine.

Staphylococcus aureus is the most common aetiologic agent of contagious bovine mastitis, a disease that causes relevant worldwide losses in the dairy industry (Zecconi et al. 2006a). Antimicrobial therapy is by enlarge the most frequently applied method to control contagious mastitis; however, the efficacy of this approach to control *Staph. aureus* mastitis is a matter of debate (Sol et al. 1994; Sol et al. 1997). Besides resistance factors, *Staph. aureus* expresses a wide array of surface-associated virulence factors such as a polysaccharide capsule and proteins that promote adhesion to mammary tissues and contribute to tissue colonization and resistance to phagocytosis (Foster, 2005). In addition, *Staph. aureus* secretes various exotoxins that damage the membranes of leucocytes and favour pathogen survival in host tissues (Schuberth et al. 2001; Rainard et al. 2003; von Eiff et al. 2004). Taking into account the wide range and polymorphism of virulence genes present in the genome it is not surprising that *Staph. aureus* is the most frequently isolated contagious pathogen worldwide (Peacock et al. 2002; McLaughlin & Hoogewerf, 2006; Zecconi et al. 2006b; Josefsson et al. 2008).

Several reports suggest that virulence factors potentially represent excellent targets for vaccine development (Aarestrup et al. 1995; Foster & Hook, 1998; Schuberth et al. 2001) but the heterogeneous expression of these molecules in different *Staph. aureus* isolates influences not only virulence but could be involved in the variable success of immunization protocols. These issues have contributed to the poor outcome of the field vaccine trials against *Staph. aureus* mastitis in dairy cows. Therefore the importance of evaluating the combination of *Staph. aureus* virulence factors is gaining growing attention as an indispensable step towards the development of effective vaccines (Jarraud et al. 2002; Peacock et al. 2002; Shkreta et al. 2004; Haslinger-Loffler et al. 2005).

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Table 1. Origin and genetic characteristics of the sixteen *Staph. aureus* isolates used in the study as reported by Zecconi et al. (2005, 2006b)

		Gene clusters			
Isolate	Herd	*spa*	*cna*	*efb*	*coa*
1632	A	>9 rep.	absent	1	1
1641	A	>9 rep.	absent	1	1
1673	B	7–9 rep.	absent	2	1
1676	C	⩽6 rep.	present	1	2
1697	D	7–9 rep.	present	3	2
1698	D	7–9 rep.	present	3	3
1706	F	7–9 rep.	absent	3	2
1867	G	7–9 rep.	absent	3	2
1886	H	⩽6 rep.	absent	3	2
1900	I	⩽6 rep.	absent	3	2
1937	L	⩽6 rep.	absent	2	1
1944	L	7–9 rep.	present	2	1
1951	M	⩽6 rep.	present	2	1
1973	N	>9 rep.	present	2	3
2004	M	⩽6 rep.	present	2	1
2020	M	⩽6 rep.	present	2	1

We have developed a subunit peptide vaccine based on three adhesion factors, fibronectin-binding protein A (FnbpA) and clumping factor A (ClfA) that did not show genetic polymorphism, and fibrinogen-binding protein (Efb), whose limited polymorphism has been suggested to be uninfluential on immune response (Palma et al. 2001). The importance of these proteins in the pathogenesis of *Staph. aureus* infections is described by Höök & Foster (2000). To our knowledge, studies on the influence of single gene polymorphisms on vaccine response are not available. Therefore, we used readily available anti-adhesins IgG and IgA raised in mice using a model peptide vaccine to study the relationship between the ability of anti-adhesin antibodies to recognize different *Staph. aureus* isolates from bovine subclinical mastitis and *Staph. aureus* genetic polymorphism based on a combination of virulence genes shown to be simple and informative (Zecconi et al. 2005; Zecconi et al. 2006b).

Materials and Methods

Bacteria

Staphylococcus aureus was isolated from routine milk samplings performed during a control programme aimed to reduce *Staph. aureus* intramammary infections in cows (Zecconi et al. 2003). The isolates, randomly selected, were genetically characterized as described (Zecconi et al. 2005) Isolate characteristics are reported in Table 1. To explore whether polymorphism of *Staph. aureus* isolates could play a role in interaction with antibodies produced following vaccination, bacteria were classified in clusters as previously described (Zecconi et al. 2006b). As reference strain we used a *Staph. aureus* subsp *aureus* from American tissue culture collection (ATCC catalog number 29213).

Preparation of bacterial extracts

Single isolates were grown in Brain Heart Infusion (BHI), under aerobic conditions at 37 °C and under agitation. Cells were harvested by centrifugation in the late logarithmic phase of growth and then washed in sterile phosphate-buffered saline (PBS). Bacterial pellets were resuspended in Tris-buffered saline (TBS) supplemented with a mixture of proteinase inhibitors (50 mM-EDTA, aprotinin 120 μg/ml, TLCK 150 μg/ml, pepstatin A 20 μg/ml, leupeptin 10 μg/ml, antipapain 10 μg/ml and chymostatin 10 μg/ml) (Roche, Milan, IT), lysostaphin (10 μg/ml) and DNAase (20 μg/ml) (Sigma-Aldrich, Milan, Italy). The suspension was incubated at 37 °C in a rotating incubator for 30 min, then supplemented with additional 10 μg/ml of lysostaphin and incubated for further 2.5 h. Digestion was stopped by heating the mixture at 88 °C for 20 min. Insoluble bacterial debris were removed by centrifugation, the supernatant was collected, the pH was adjusted to 7·4 and stored at −20 °C.

Genomic DNA was extracted by standard procedures (Sambrook et al. 1989) and DNA sequences encoding *efb*, *fnbpA* and *ClfA* were amplified by PCR using a proof reading thermostable polymerase (Vent polymerase; New England Laboratories, Beverly MA, USA). Primers and PCR conditions are summarized in Table 2. Integrity and reliability of all recombinant constructs was verified by sequence analysis.

Preparation of recombinant proteins

Recombinant *Staph. aureus* Efb (all peptide), FnbpA (1–878 aminoacids), and ClfA (1–562 aminoacids) were expressed in *Escherichia coli* BL21 and purified as previously reported (Castagliuolo et al. 2006). The concentration of recombinant proteins was determined by micro-Bradford test using the commercial Bio-Rad protein assay, whereas integrity and purity of proteins was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Castagliuolo et al. 2006).

Immunization of mice and generation of specific anti-adhesin antibodies

Male Balb/c mice, 10 weeks old (Charles River, Oderzo, Italy) were used in all experiments. Mice were housed under controlled humidity and temperature conditions and were given free access to commercial rodent food and water. Animals studies were approved by the Institutional Animal Care and Use Committee of the University of Padua.

Three doses of vaccine were given at 2-week intervals. Purified recombinant proteins (10 μg/dose) were administered into the right tibialis anterior muscle (i.m.) emulsified

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Table 2. Polymerase chain reaction (PCR) primers and conditions used in the study

	Forward	Reverse	T_M, °C	Length, bp
Efb	5′tcaatggacagaatatcaaccaac3′	5′cttttccatcttcttctttgggat3′	55	511
FnbpA	5′ccggagaggagacttcacaga3′	5′tccacgatttcccagagaac3′	62	1214
ClfA	5′atgcaggactttaagggttacttg3′	5′actgccttgctcttattttcacag3′	56	1700

with an equal volume of Freund's adjuvant in a total volume of 30 µl/dose. Four groups of animals, each of three mice, were injected either with a single recombinant protein (Efb, FnbpA or ClfA) or with the mixture of the three recombinant adhesins. Blood samples were collected before immunization (to exclude pre-existing specific immunity, by puncture of the dorsal tail vein) and than 2 weeks after the last immunization (by cardiac puncture in deeply anaesthetized animals). Antibodies present on the intestinal mucosa (mucosal IgA) were collected by washing the small intestine with 2 ml of ice-cold PBS containing the proteinase-inhibitor mixture described above. After centrifugation clear supernatants were collected and stored at −20 °C.

Recognition of specific Staph aureus *isolates by anti-adhesin antibodies in enzyme-linked immunosorbant assays* (*ELISAs*)

ELISAs were developed to determine the ability of specific anti-adhesin (IgG and IgA) induced with a peptide vaccine to recognize different clinical *Staph. aureus* isolates. Immunoplates (96-wells) were coated overnight at 4 °C with 100 µl/well of bacterial extracts prepared from the different isolates of *Staph. aureus* used in this study. As previously reported, to reduce the high background signal in the ELISAs using total bacterial lysate as coating antigen, the plates were pre-incubated with 20% rabbit serum in PBS–0·05% Tween 20 (PBS–T) for 90 min at 25 °C to saturate immunoglobulins-protein A binding activity (Castagliuolo et al. 2006). Then appropriately diluted serum and intestinal washes were added to the wells and incubated for 2 h at 22 °C on an orbital mixer. Plates were washed three times with PBS–T and the immunocomplexes were detected by adding the chromogenic substrate solution (TMB, Sigma-Aldrich, Milan, Italy). The reaction was stopped after 15 min incubation with an equal volume of 1 M-H_3PO_4. The optical density (OD) at 450 nm was determined using an ELISA plate-reader (Spectra I Tecan, Gratz, Austria). To support the specificity of the antibodies generated we performed preliminary Western Blot assays that confirmed the ability of each immune serum to identify the recombinant protein used for immunization; to recognize a single protein band of the expected molecular weight in *Staph. aureus* total protein lysates; and not to recognize *Esch. coli*-derived total protein proteins at the dilutions used. To standardize the working dilutions of immune serum and intestinal washes to be used in ELISAs, we first determined the antibody titre against the single antigens in hyperimmune serum and intestinal washes. Immunoplates were coated with a solution (10 µg/ml) of the specified recombinant protein (Efb, FnbpA and ClfA) and probed with 2-fold serial dilutions of immune and non-immune serum and intestinal washes. The antibody titre was defined as the highest serum or intestinal wash (secretory IgA, sIgA) dilution able to generate an OD at least 2-folds greater than non-immune serum or intestinal wash, respectively. Therefore we used serum dilutions at 1 : 5000 for serum IgG and 1 : 1000 for serum IgA, whereas intestinal washes were used at 1 : 20 dilution.

Data analysis and statistical procedures

The electrophoretic patterns obtained from PCR for the genes considered were analysed following a three-step procedure as described elsewhere (Zecconi et al. 2005). First, gels were digitally acquired and bands were identified based on position (tolerance, 2%) by means of Gel-Pro Analyzer 3.1 software (Media Cybernetics, Silver Springs MD, USA). Then gene patterns were clustered by hierarchical clustering with percent distances on SYSTAT 11.0 statistical software (Systat software, Point Richmond CA, USA). Finally, the relationship between gene patterns and immune responses after vaccination was analysed by general linear model with statistical software SAS 9.1 GLM procedure (SAS Institute, Cary NC, USA). The general linear model included as independent variables the different gene clusters and the different replicates (number of mice), while IgG, IgA and mucosal IgA were considered as response variables. Statistical significance level was 0·05.

Results

Production of specific anti-adhesin antibodies and activity against Staph. aureus *isolates*

Following immunization with a single recombinant *Staph. aureus* adhesin or with a mixture of three recombinant proteins, significant levels of anti-Efb, anti-FnbpA, and anti-ClfA IgG, IgA and mucosal IgA were detectable in the serum and in the intestinal washes of mice. Serum and mucosal surface antibodies reacted with specific recombinant proteins as well as with native proteins obtained from lysates of a reference *Staph. aureus* isolate as determined by ELISA. Following immunization with a mixture containing the three recombinant adhesins, the antibody

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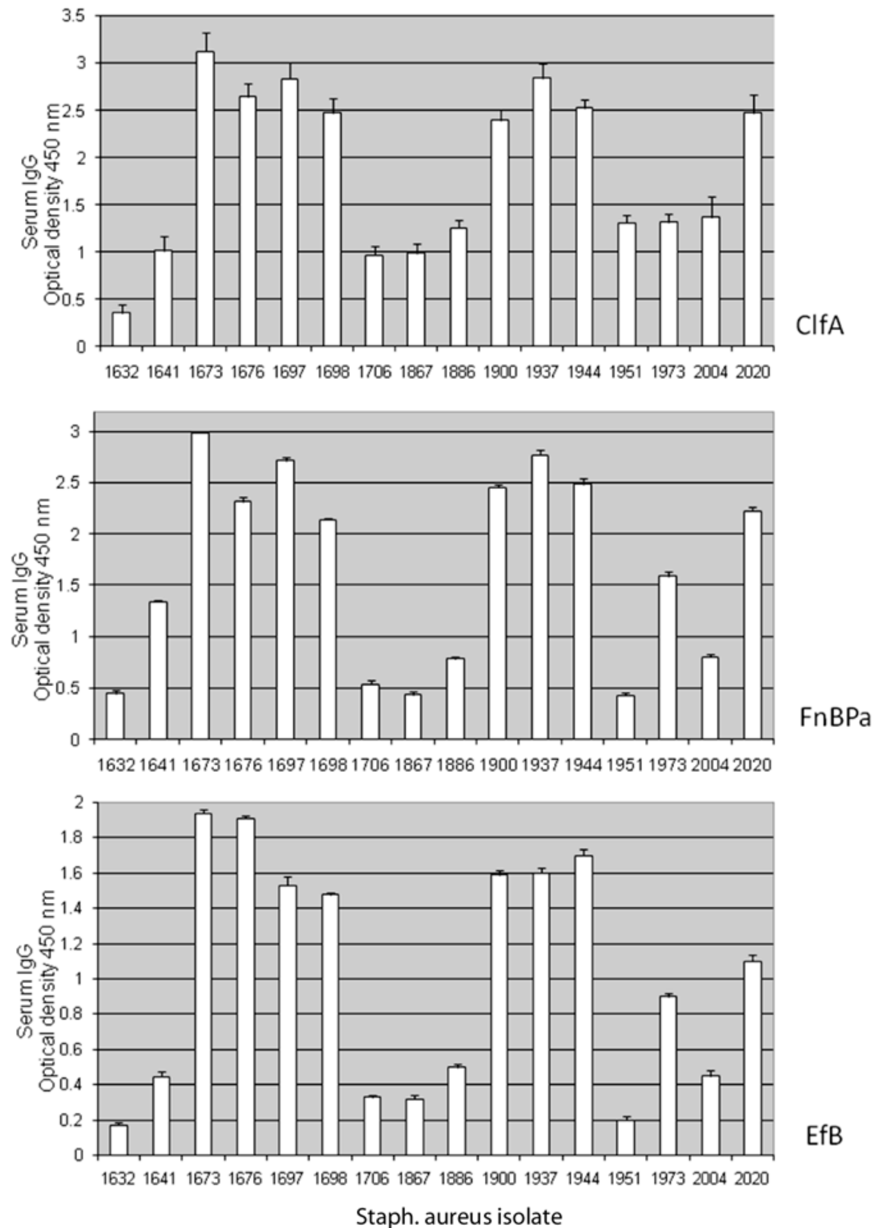

Fig. 1. Binding of *Staphylococcus aureus* isolates by serum IgG directed against single adhesins. Results are expressed as means of optical density (OD) at 450 nm obtained for each *Staph. aureus* strain probed with serum (dilution 1 : 5000) from mice ($n=3$ each adhesin) immunized with the specific adhesin ± SE subtracted the OD value of non-immune serum (dilution 1 : 5000, $n=5$).

titre against the various antigens present in the vaccine was comparable, suggesting the absence of significant antigenic interference (data not shown).

We next determined the ability of specific anti-adhesins IgG and IgA to recognize *Staph. aureus* isolated from different subclinical cases of mastitis. Three weeks after the second immunization dose, specific anti-adhesins IgG and IgA were detectable in the serum of all the immunized animals and their ability to react with different *Staph. aureus* isolates was assessed. As shown in Fig. 1, the ability of serum IgG to recognize specific *Staph. aureus* adhesins using total bacterial lysates from the different isolates, as capturing antigen, was strikingly different.

Indeed, similar results were obtained when we used hyperimmune serum raised against a mixture of adhesins (Fig. 2). Thus, taking as arbitrary cut-off an OD of 1, six *Staph. aureus* isolates (namely 1632, 1706, 1867, 1886, 1951, 2004) reacted less efficiently with the anti-adhesins IgG mixture, whereas the other ten isolates used in the study, reacted strongly with the immune serum.

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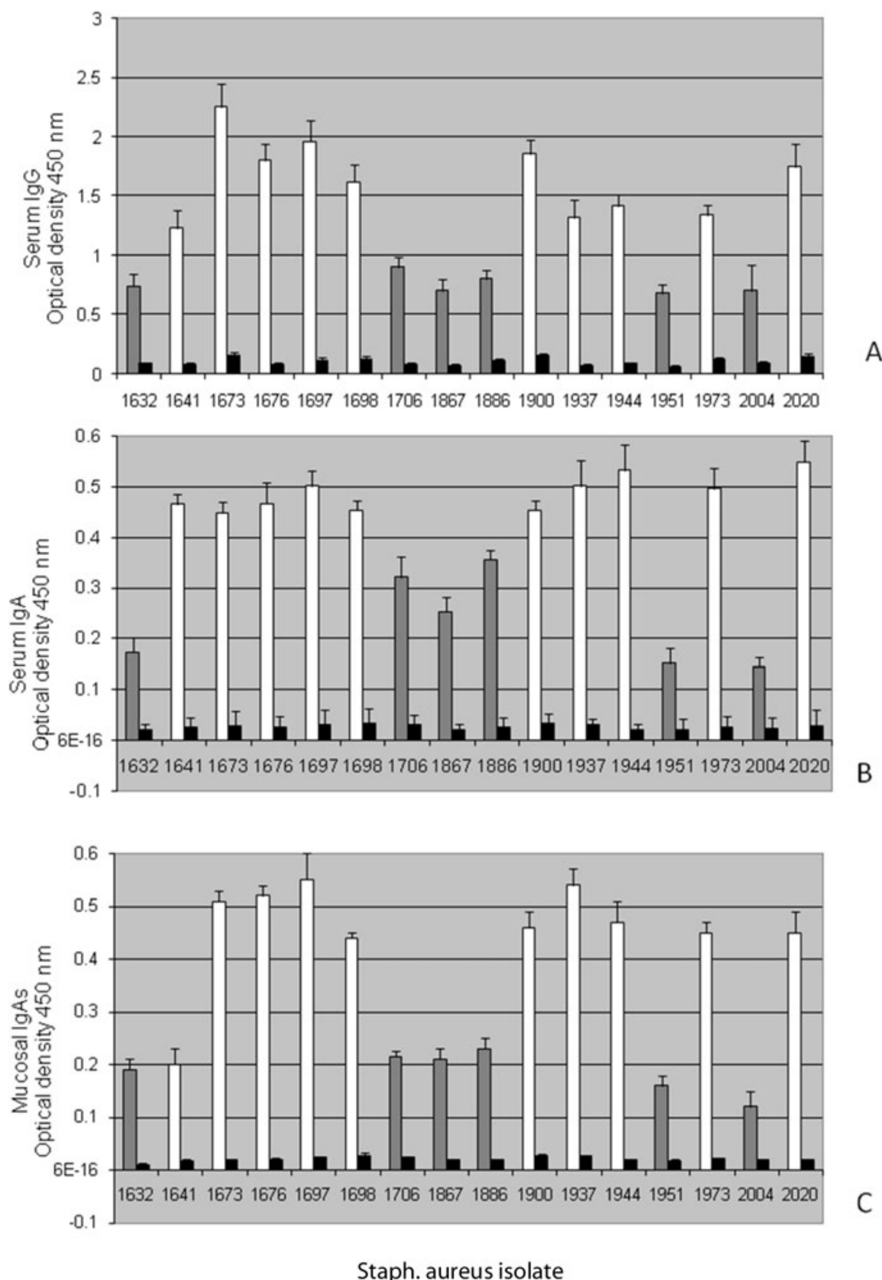

Fig. 2. Binding of *Staphylococcus aureus* isolates by serum IgG (A) and serum IgA (B) and mucosal IgA (C) anti-*Staph. aureus* adhesins. Results are expressed as means of optical density (OD) at 450 nm obtained for each *Staph. aureus* strain probed with serum from control non-immune mice ($n=5$) (black bars) and mice immunized with the adhesins mixture ($n=3$) ±SE, grey bars indicate poor reactive lysates and open bars reactive lysates. Serum dilutions: 1:5000 for IgG and 1:1000 for IgA, intestinal washes dilution: 1:20.

As shown in Fig. 2, also serum or mucosal derived specific anti-adhesins IgA and mucosal IgA recognized with varying ability different *Staph. aureus* isolates. Indeed, applying an arbitrary cut-off OD of 0·4 we were able to separate *Staph. aureus* isolates in two groups. Thus, serum IgA and mucosal IgA again recognized efficiently isolates 1641, 1673, 1676, 1697, 1698, 1900, 1937, 1944, 1973 and 2020, whereas IgA reacted poorly with isolates 1632, 1706, 1867, 1866, 1951 and 2004, further suggesting a different level of adhesins immunoreactivity in specific isolates.

Genetic polymorphism in Staph. aureus *isolates and reactivity to anti-adhesin antibodies*

To explore the variable reactivity of different *Staph. aureus* isolates to immune serum, we first confirmed the presence

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Table 3. Statistical analysis by GLM model applied to estimate the influence of *Staph. aureus* gene polymorphism on immune response to a recombinant protein vaccine

		Factors				
Immunoglobulin	Result	*spa*	*efb*	*coa*	*cna*	Replicates (*n* of mice)
IgG	*F* value	4·29	2·49	1·92	0·02	0·38
	P	0·0209	0·0963	0·1605	0·9060	0·6869
IgA	*F* value	1·11	1·54	2·03	0·19	0·29
	P	0·3414	0·2273	0·1452	0·6686	0·7498
Mucosal IgA	*F* value	3·68	3·81	4·07	0·47	0·26
	P	0·0345	0·0311	0·025	0·4990	0·7348

Table 4. Statistical analysis by GLM model applied to estimate the influence of *efb* and *spa* gene polymorphisms on immune response to a recombinant protein vaccine

		Factors			
Immunoglobulin	Result	*spa*	*efb*	*efb* × *spa*	Replicates (*n* of mice)
IgG	*F* value	3·74	1·59	5·37	0·44
	P	0·0328	0·2168	0·0087	0·6484
IgA	*F* value	1·74	2·26	6·75	0·18
	P	0·1894	0·1176	0·0030	0·8347
Mucosal IgA	*F* value	0·30	1·04	4·87	0·23
	P	0·7455	0·3636	0·0062	0·7954

of genes coding for *fnbpA*, and *clfA* by PCR analysis on genomic DNA extracted from highly and poor responsive isolates. The reactions produced an amplification product of the expected size from the various isolates for the different genes, and the sequences for these adhesins from high and poor responder isolates did not show any significant polymorphism for *fnbpA*, and *clfA* (data not shown).

The results of the general linear model including the polymorphisms of the four genes considered and the mouse showed that this latter one had no significant influence on mean OD (Table 3). Moreover, none of the factors considered had any significant influence on serum IgA means, while significant differences were observed for IgG and mucosal IgA.

Isolates with >9 repetitions of *spa* X regions showed a significant lower reactivity to anti-adhesins IgG and mucosal IgA as opposed to isolates with a 7–9 repetitions. Indeed, IgG OD values were 0·97±0·23 in isolates with >9 repetitions *v.* 1·40±0·52 in 7–9 repetition isolates. When IgA were considered, isolates with >9 repetitions showed OD values of 0·28±0·11 *v.* 0·38±0·14 of 7–9 repetition isolates. Furthermore, a significant difference was observed for IgG values between *efb* cluster I and III, with OD values of 1·14±0·47 and 1·25±0·14, respectively. Isolates in *efb* cluster II showed significantly higher mucosal IgA reactivity when compared with cluster I, respectively 0·37±0·16 and 0·30±0·14. Isolates in *coa* cluster I showed a significant lower mucosal IgA response (data not shown). Finally, the presence of *cna* gene was not associated with any difference in reactivity to serum antibodies.

To assess the role of the interaction between the polymorphisms of the two genes (*efb* and *spa*) showing a significant influence on immune reactivity, a simplified GLM model was applied. This model included only the two genes as factors, their interactions and the replicates (mouse). The results of the statistical analysis (Table 4) showed that the interaction between the two genes had a significant influence on IgG, IgA and mucosal IgA response, while *spa* gene influenced significantly only the IgG response. In the other cases the immune response was not influenced by single gene clusters (*efb* or *spa*).

The data presented in Table 5 compare the OD values observed when isolates where classified by both *efb* and *spa* gene clusters. Isolates with >9 repetitions (*spa* gene) and in *efb* cluster I showed significantly lower OD values when compared with isolates in the other clusters. When IgA OD response was considered (Table 6) a similar pattern was observed. However, the differences between isolates with >9 repetitions and in *efb* cluster I reached statistical significance when compared with isolates in *efb* cluster II, while there were no significant differences with other *spa* clusters. Finally, the analysis of mucosal IgA response (Table 7) confirmed the results observed for IgG with a significantly lower mean OD

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Table 5. Mean IgG optical density (±SD) observed for sixteen *Staph. aureus* isolates classified by *efb* gene clusters and by *spa* repetitions clusters

spa gene repetitions	*efb* cluster I	II	III
⩽6	1·72±0·17[a,1,†]	1·03±0·38[b,1]	1·25±0·49[b,1]
7–9		1·71±0·38[a,2]	1·25±0·52[b,1]
>9	0·85±0·18[a,2]	1·20±0·10[b,1]	

† Different letters show statistically significant difference among rows; different numbers show statistically significant difference among columns; α=0·05

Table 6. Mean IgA optical density (±SD) observed for sixteen *Staph. aureus* isolates classified by *efb* gene clusters and by *spa* repetitions clusters

spa gene repetitions	*efb* cluster I	II	III
⩽6	0·43±0·05[a,1,†]	0·32±0·19[a,1]	0·38±0·03[a,1]
7–9		0·47±0·06[a,2]	0·36±0·09[b,1]
>9	0·29±0·01[a,1]	0·46±0·01[b,2]	

† Different letters show statistically significant difference among rows; different numbers show statistically significant difference among columns; α=0·05

in isolates with >9 repetitions (spa gene) and in *efb* cluster I.

Discussion

Staph. aureus is a unique pathogen, responsible of a variety of acute and chronic diseases in man and animals (Honeyman et al. 2001; Zecconi et al. 2006a). The vast array of hosts infected by this pathogen is made possible by the large number of virulence factors carried and its ability to adapt to different environments. Indeed, in recent years several reports describe relevant strain variability not only among *Staph. aureus* isolates adapted to diverse species but also between subjects of the same species, supporting a major role of gene polymorphism in the virulence and the ability to evade the immune system (Voyich et al. 2005). To address the relevance of field isolate variability, in this study we used a multidisciplinary approach to evaluate the ability of antibodies directed against surface adhesion factors to recognize *Staph. aureus* isolated from quarter milk samples of dairy cows with subclinical mastitis and to determine how this response correlates with the genetic heterogeneity of *Staph. aureus* isolates.

We used as target antigens multiple bacterial proteins involved in microbe adhesion to host tissues, since recent advances in human and veterinary medicine have underscored the role of adhesins in *Staph. aureus* virulence

Table 7. Mean mucosal IgA optical density (±SD) observed for sixteen *Staph. aureus* isolates classified by *efb* gene clusters and by *spa* repetitions clusters

spa gene repetitions	*efb* cluster I	II	III
⩽6	0·50±0·01[a,1,†]	0·30±0·18[a,1]	0·34±0·10[a,1]
7–9		0·49±0·03[a,2]	0·34±0·15[a,1]
>9	0·21±0·04[a,2]	0·42±0·01[b,2]	

† Different letters show statistically significant difference among rows; different numbers show statistically significant difference among columns; α=0·05

(Sutra & Poutrel, 1994; Foster & Hook, 1998; Peacock et al. 2002; Zecconi et al. 2006b). Several reports show that recombinant bacterial adhesins are effective antigens and can be used to trigger significant immune responses (Shkreta et al. 2004; Castagliuolo et al. 2006). Preliminary studies using recombinant *Staph. aureus* adhesins, such as Efb, FnbpA and ClfA have shown that they are effective in inducing specific antibody responses. Indeed, using recombinant proteins expressed in *Esch. coli* we also induced a strong and specific humoural and mucosal antibody response. In addition, the amplitude of the immune response towards the different antigens present in the vaccine mixture was comparable, therefore confirming the absence of relevant antigenic interferences between these proteins.

We decided to see how a number of *Staph. aureus* isolated from mammary glands affected by subclinical mastitis would react with immune serum obtained from mice immunized with a mixture of recombinant *Staph. aureus* adhesins. We observed striking differences in the reactivity of the field isolates to the antibodies present in the serum and mucosal surfaces, suggesting the existence of strains poorly recognized by the antibodies produced. Several factors could be behind these differences; among them, we explored the role of four different virulence factors. The results obtained were partly unexpected. Indeed, both *coa* polymorphism and *cna* presence did not show a consistent influence on isolate reactivity with immune serum, whereas the polymorphism of *efb* and *spa* genes seemed to be associated to a different reactivity of the isolates. Thus, our results further support the view that anti-adhesins immunoglobulin, although able to recognize field isolates, show a different reactivity against isolates with different gene patterns (Brennan et al. 1999; Brouillette et al. 2002; Shkreta et al. 2004; Castagliuolo et al. 2006). This study suggests that *spa* gene pattern of *Staph. aureus* isolates plays a significant role in the efficacy of the immune response induced by a proteic subunit vaccine. These observations emphasize furthermore the importance of the association between *spa* gene polymorphism and *Staph. aureus* virulence (Frenay et al. 1994; Dalla Pozza et al. 1999; Palmqvist et al. 2002). On the

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contrary *efb* polymorphism was reported to be of modest relevance in the immune response when used as antigen (Palma et al. 2001). Also, the analysis of the interaction between *efb* and *spa* polymorphisms suggests that the *spa* gene plays a more relevant role than the *efb* gene.

Although this study confirmed that it is possible to induce an immune reaction against several *Staph. aureus* adhesins, the functional relevance of antibodies produced with immunization to prevent adhesion to mammary gland epithelial cells and to induce opsonization by phagocyte cells might be hampered by the variable reactivity of the field isolates. Indeed, the different reactivity of these isolates, which may contribute to their ability to escape from the immune response, can be predicted on the basis of the genetic profile (Voyich et al. 2005).

Conclusions

This study has several implications for the further development of *Staph. aureus* vaccines. *Spa* gene polymorphism has been confirmed to play a relevant role in *Staph. aureus* infection both in human and veterinary medicine (Frenay et al. 1994; Moodley et al. 2006; Hallin et al. 2007). Moreover, our study suggests that it plays an important role also in the immune response to specific adhesins. The reasons for these differences should be explored further, even though the role of *spa* expression on *Staph. aureus* adhesion to platelets and on lymphocyte function has been already shown (Nguyen et al. 2000; Palmqvist et al. 2005). This result supports the use of *spa* gene polymorphism not only as an epidemiological criterion to classify isolates (Moodley et al. 2006; Hallin et al. 2007) but also to identify the most suitable strains to be used in vaccine development. Indeed, the results of this study and the biological properties of *spa* gene products support the importance of this gene in the immune response.

The overall results further confirm the importance of a disease- and environment-specific analysis of isolates (Lammers et al. 2000; Fux et al. 2005; Scherl et al. 2005; Josefsson et al. 2008). Indeed, the poor success of field trials can be a consequence of the huge diversity of the prevalent strains among different environments. Thus, as opposed to other pathogens, to enhance the chances of developing an effective vaccine against *Staph. aureus* mastitis the identification and characterization of prevalent strains in a certain area might be a strategy to implement in future studies.

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