

Study of *Staphylococcus aureus* collected at slaughter from dairy cows with chronic mastitis

Renata Piccinini^{1*}, Riccardo Tassi¹, Valentina Daprà¹, Rachel Pilla¹, Jackie Fenner^{2,3}, Ben Carter⁴ and Muna F. Anjum^{2,3}

¹ Department of Veterinary Pathology, Hygiene and Public Health, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy

² Department of Bacteriology, Veterinary Laboratories Agency-Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK

³ Technology Transfer Unit, Veterinary Laboratories Agency-Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK

⁴ Department of Primary Care and Public Health, School of Medicine, Cardiff University, Gwenfro Unit 5, Wrexham Technology Park, Wrexham LL17 7YP, UK

Received 21 September 2011; accepted for publication 19 January 2012; first published online 28 February 2012

Staphylococcus aureus is one of the most important pathogens associated with bovine mastitis. Recent studies have shown that *Staph. aureus* strains may differ in virulence, and in their ability to disseminate across commercial dairy herds. The goal of this study was to determine whether *Staph. aureus* isolates differed in their ability to colonize mammary tissue, and whether such differences could be related to molecular characteristics. Quarter milk and mammary tissues of 22 cows from two dairy herds, were collected at slaughter and bacteriological analysis was performed. All *Staph. aureus* isolates were characterized by Pulsed Field Gel Electrophoresis (PFGE) and microarray. Overall 45 mammary quarters were infected and 20 *Staph. aureus* isolates were identified. The bacteria were mostly recovered from both milk and tissue of the same quarter in significantly higher numbers from herd A cows compared with herd B. Molecular characterization of the isolates showed distinct PFGE profiles for isolates from each herd. Differences in virulence factors between herds A and B isolates were evidenced. The genes for enterotoxin D, J and R were present in herd A, those for G, I, N, M, O and U were shown in herd B, whilst both components of the leukocidin *lukD/E* genes were only carried by herd A isolates. Furthermore, all herd A isolates showed β -haemolysin activity, which was absent in all but one isolate from herd B. Therefore our data indicate that *Staph. aureus* isolates showing differences in their ability to disseminate and colonize across quarters, also have significantly different virulence characteristics.

Keywords: *Staphylococcus aureus*, bovine mastitis, molecular characteristics, mammary localization.

Staphylococcus aureus is one of the most important pathogens associated with bovine intramammary infections. However, as most infections are subclinical, they are often undetected. Furthermore, the efficacy of control programmes differs broadly among herds (Zecconi et al. 2003; Moret-Stalder et al. 2009) probably as a consequence of the multifactorial pathogenesis of *Staph. aureus* mastitis. The microorganism enters the mammary gland via the teat canal, multiplying within the cistern, where adhesion to mammary epithelial cells represents an important step in the process of infection (Deگو et al. 2002).

Many studies performed in vitro have demonstrated the ability of the bacteria to penetrate and divide into various human, bovine, chicken and mouse cells, including

neutrophils (Chandler et al. 1974; Anderson & Chandler, 1975; Ogawa et al. 1985; Almeida et al. 1996; Lammers et al. 1999; Lowy, 2000). In experimental infections, *Staph. aureus* have been shown within the alveoli and lactiferous ducts (Nickerson & Heald, 1981; Gudding et al. 1984) and inside mammary epithelial cells (Hensen et al. 2000). Intramammary challenge using a high inoculum density has caused severe acute mastitis, characterized by cell necrosis and a wide distribution of bacteria in the gland (Chandler et al. 1974). In chronically infected cows, viable *Staph. aureus* have been demonstrated in macrophages and in some alveolar cells isolated from milk (Hebert et al. 2000). *Staph. aureus* produces several virulence factors, such as proteins promoting adhesion to mammary tissues, toxins that can damage the parenchyma and enterotoxins that interfere with the immune response of the udder, being superantigens.

*For correspondence; e-mail: renata.piccinini@unimi.it

Recent studies have shown that *Staph. aureus* strains may differ in virulence (Tollersrud et al. 2000; Zecconi et al. 2006; Fournier et al. 2008; Piccinini et al. 2009). Furthermore, the interactions between microorganism, host immune system and environmental factors can modulate the outcome of the disease, but the role of each factor in the pathogenesis of mastitis has not been investigated in commercial dairy herds, since most in-vivo studies are experimental challenges. Therefore, the goals of this study were to determine whether (a) the ability of *Staph. aureus* isolates to colonize mammary tissue differed among cows and herds in naturally infected animals and (b) such differences could be related to molecular characteristics of isolates.

Materials and Methods

Animals

Twenty-two Holstein cows from two dairy herds (herd sizes 100 and 180 lactating cows respectively), characterized by a high prevalence of *Staph. aureus* subclinical mastitis, were sampled at slaughter, according to the European regulation CE 1774/2002. Out of them, 6 cows from herd A and 13 from herd B had been diagnosed as infected by *Staph. aureus* during lactation, 3–4 months before slaughter, and showed somatic cell counts (SCC) continuously higher than 5×10^5 /ml of milk. The remaining 3 animals were chosen as negative controls, because all the bacteriological analysis performed during lactation were negative for *Staph. aureus*. All cows were free of clinical infections.

Bacteriological analysis

Just before culling, quarter milk samples were taken from each animal in accordance with National Mastitis Council (NMC) guidelines (NMC, 1999). Then one section of parenchymal tissue and one section of cisternal tissue were collected from each mammary quarter for bacteriological investigation. A total of 28 quarters were examined from herd A and 60 from herd B. All samples were kept at 4 °C until bacteriological analysis of milk and tissues was performed. Cisternal and parenchymal sections were sampled with a sterile swab that was rotated inside a 2-cm long cut, over a surface of 1 cm², and then plated on a blood agar plate. Ten µl of quarter milk were plated on blood agar plate (5% bovine blood; Oxoid, Cambridge, UK) and incubated overnight at 37 °C. Colonies of growth were isolated; the large and haemolytic ones that were catalase and coagulase positive were identified as *Staph. aureus* following Hogan et al. (1999) and thereafter were confirmed by API ID32 *Staph.* (Biomérieux, F).

The antibiotic susceptibility of all isolates to the drugs mostly used in mastitis therapy (penicillin, oxacillin, first generation cephalosporins, tylosin, streptomycin, rifaximin) was tested by the Minimal Inhibitory Concentration (MIC)

method in microplate, following Clinical and Laboratory Standards Institute (2008). The MIC value is defined as the lowest concentration that completely inhibits microbial growth. Finally, the production of α -, β - and δ -haemolysin was determined by cross-streaking the isolates perpendicularly to an only β -haemolytic *Staph. aureus* on a blood agar plate, following Traber et al. (2008). Subsequently, isolates were frozen in Brain Heart Infusion Broth (Oxoid) with 15% glycerol (v/v) until further molecular analyses were performed.

Molecular analysis of *Staph. aureus* isolates

Immediately after thawing, *Staph. aureus* isolates were subcultured on blood agar plate (5% bovine blood; Oxoid) and processed by PFGE, Multilocus Sequence Typing (MLST) and a *Staph. aureus* array.

PFGE analysis. Total DNA preparation, digestion with the *Sma*I restriction enzyme (MBI Fermentas, Vilnius, Lithuania) and PFGE were performed as described by Chung et al. (2000). To determine strain relatedness, DNA band patterns were analysed with Molecular Analyst software, version 1.6 (Bio-Rad Laboratories). A band position tolerance of 4% was used for comparison of DNA patterns.

DNA extraction. From each isolate, one colony was cultured in Nutrient Broth (Oxoid) at 37 °C overnight. The broth was then centrifuged at 15 000 g for 2 min, the pellet was resuspended in 50 mM EDTA and lysostaphin (5 mg/ml; Sigma-Aldrich, St. Louis MO, USA) was added for bacterial lysis. DNA was extracted using DNeasy kit (QIAGEN, Hilden, Germany); DNA amount and purity were tested with a ND-100 Spectrophotometer (NanoDrop Technologies Inc., Wilmington DC, USA), while DNA integrity was checked by electrophoresis on 0.8% agarose gel containing ethidium bromide (0.5 µg/ml) in TAE buffer.

MLST analysis. MLST was performed on two representative isolates from each herd. DNA extracted as indicated above, was processed following Bonura et al. (2010).

***Staph. aureus* array analysis and PCR.** DNA microarray (Alere Technologies, Jena, Germany) was used to genotype *Staph. aureus* isolates. A set of probes covering antibiotic resistance determinants, different toxins (enterotoxins, leucotoxins, haemolysins) and the allelic variants of the *agr* gene are included in the chip. The arrays were processed as previously described (Monecke & Ehrlich, 2005). Briefly, genomic DNA was employed for linear amplification and labelling of target genes with biotin-16-dUTP (Roche Diagnostics, Mannheim, Germany). The amplified products were added to the array tubes and any hybridization detected using streptavidin-horseradish-peroxidase and a chromogenic substrate (Seramun, Wolzlig, Germany). The

reaction was read on an array tube reader ATR01 and the image interpreted with the Iconoclust software package (Alere Technologies, Jena, Germany). Genes showing a signal intensity above 0.4 were considered to be present, those below 0.3 were considered absent and those between 0.3 and 0.4 were considered ambiguous. To confirm the presence of *lukD*, a PCR for detection of the whole gene was also designed. Each reaction mixture (25 µl) contained 1 µl of the purified genomic DNA, 2 U of Taq DNA polymerase (Promega, Madison WI, USA), 200 µM dNTPs (Promega, Madison WI, USA), 2.5 mM MgCl₂, and a 0.1 µM concentration of each of the following primers: *fw* (5′–3′) ATGAAAATTGAAAATTAGGCAAATCATCA and *rv* (5′–3′) TTATACTCCAGGATTAGTTTCTTTAGAATC. Amplicon size was 984 bp. Amplifications were carried out in a thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) through the following temperature program: 95 °C for 5 min; 40 cycles including 95 °C for 30 s and 50 °C for 30 s; 72 °C for 1 min. The products were electrophoresed in a 0.8% agarose gel containing ethidium bromide and visualized by transillumination under u.v. Finally, the amplicons were submitted to sequencing, that was performed on an Applied Biosystems 3730 DNA Analyzer, following the manufacturer's instructions (Applied Biosystems, Forster City CA, USA).

Statistical analysis

Both Fisher's exact test and Chi-square test of association were carried out to compare both the molecular data and the number of quarters infected in the cattle. The genetic markers were tabulated by the number of samples categorized as present or absent for both A or B groups. The Fisher's exact test compared the number of present genes with absent or ambiguous genes, between the two groups and calculated the odds ratio with an associated 95% exact confidence interval (Fleiss, 1981). The infected quarters data were tested using the same methodology.

Results

Bacteriological analysis

Overall 88 quarter milk samples, 88 samples of quarter parenchyma and 88 samples of cisternal tissues were analysed. Bacteriologic results confirmed that the 19 cows culled because of *Staph. aureus* mastitis were infected, while the 3 negative controls were free from contagious microorganisms (Table 1). Infection was defined as positive counts of *Staph. aureus*, expressed as colony-forming units (CFU) per ml of milk, and CFU in 1 cm² of parenchyma. All cisternal samples showed the same results as the milk (data not shown).

There were milk or tissue infections in 16 out of 24 quarters in herd A and 29 of 52 quarters in herd B, and both milk and tissue infection in 10 of 24 quarters in herd A, and

12 of 52 in herd B. Twenty *Staph. aureus* isolates, 6 in herd A and 14 in herd B, were identified. Five isolates infected only the milk (*n.* 2145, 2146, 2148, 2160) or only the parenchyma (*n.* 2164) in herd B, a factor not seen for any of the isolates in herd A, all of which infected both milk and parenchyma. Interestingly, two *Staph. aureus* isolates, with different status for harbouring the collagen-binding adhesin gene (data not shown), were recovered from cow B-11 but from different quarters (one from front right and rear left quarters, the other from front left and rear right quarters) (Table 1).

Although differences were observed in the rates of infection between the two herds, the statistical analysis could not demonstrate this ($P > 0.1$). However, an association was found between the spread of the infection from a single quarter to multiple quarters, and the infection being located in both the tissue and the milk, compared with a single location ($P = 0.0369$). Furthermore, evidence of this association can still be seen after accounting for herd differences (Table 2, $P = 0.07$). Therefore it was more likely for a cow from herd A to have both milk and tissue infection in multiple quarters compared with a cow from herd B. From the microarray data it was found that many markers were associated with isolates gathered from either herd A or B (see next section).

False negative results of milk microbiological analysis occurred in both herds: in herd A, 1 out of 16 infected quarters was *Staph. aureus* positive only in the tissue (6.25%) while in herd B, 3 of 29 (10.3%) infected quarters were *Staph. aureus* positive only in the tissue i.e. milk false negative.

All 20 *Staph. aureus* isolates were tested for their susceptibility to a number of antimicrobial compounds that are commonly used in the treatment of mastitis. The results showed that the isolates were highly susceptible to most antibiotics tested, although less so to streptomycin (Table 3).

Molecular analysis of *Staph. aureus* isolates

The molecular differences between the 20 *Staph. aureus* isolates from herds A and B were characterized by PFGE, MLST and microarray. Major results from array analysis are summarized in Table 4. Presence or absence of *lukD* gene was further confirmed by PCR set up for the whole gene, since a product was obtained only in the strains from herd A. All amplicon sequences matched with GenBank accession number Y13225.1 with 98% homology. Inter-herd differences were evidenced by PFGE and MLST patterns, as well as by array profiles. PFGE revealed two patterns differing in 5 bands, which could be considered as distinct, in accordance with Tenover et al. (1995). One pattern was exclusively displayed by all *Staph. aureus* isolates in herd A, and the other was characteristic of all herd B isolates (data not shown). Also, a single profile was identified by MLST in herd A and belonged to ST8, while the isolates from herd B were characterized by ST20.

Table 1. Bacterial isolation of *Staph. aureus* from milk and parenchyma samples from mammary quarters. The number of cow and bacterial isolate from each herd is also given

Cow N.	Mammary quarter								Isolate N.
	Front left		Front right		Rear left		Rear right		
	Milk (CFU/ml)†	Parenchyma (CFU/cm ²)	Milk (CFU/ml)	Parenchyma (CFU/cm ²)	Milk (CFU/ml)	Parenchyma (CFU/cm ²)	Milk (CFU/ml)	Parenchyma (CFU/cm ²)	
A-1	>1000	–	>1000	1	–	–	500	–	2141
A-2	–	–	>1000	3	–	–	–	–	2142
A-3	>1000	–	>1000	1	–	1	300	1	2143
A-4	>1000	–	–	–	>1000	19	–	–	2166
A-5	>1000	23	–	–	>1000	2	>1000	18	2167
A-6	–	–	800	17	>1000	–	>1000	20	2168
A-7‡	–	–	–	–	–	–	–	–	
B-1	–	–	–	–	>1000	1	–	–	2144
B-2	–	–	–	–	–	–	100	–	2145
B-3	–	–	–	–	>1000	–	–	–	2146
B-4	400	4	–	–	–	–	>1000	3	2147
B-5	200	–	–	–	–	–	600	–	2148
B-6	–	–	>1000	–	–	–	300	5	2157
B-7	–	–	>1000	19	900	–	>1000	3	2158
B-8	–	5	500	15	–	–	>1000	–	2159
B-9	–	–	>1000	–	100	–	–	–	2160
B-10	>1000	4	>1000	18	–	9	–	5	2161
B-11	>1000	–	>1000	25	>1000	–	>1000	24	2162, 2163
B-12	–	–	–	–	–	11	–	–	2164
B-13	>1000	5	>1000	–	–	–	>1000	–	2165
B-14‡	–	–	–	–	–	–	–	–	
B-15‡	–	–	–	–	–	–	–	–	

† Colony forming units (CFU)/ml

‡ Negative control cow (cullled for reasons other than chronic *Staph. aureus* infection)**Table 2.** Differences in *Staph. aureus* infection between herds A and B

	Herd A, numbers of cows with:		Herd B, numbers of cows with:	
	Multiple-quarter infection	Single-quarter infection	Multiple-quarter infection	Single-quarter infection
Milk and tissue	5	1	7	1
Milk or tissue	0	0	2	3

Analogously, the presence of enterotoxin and leukocidin genes also differentiated the herds. In fact using the Fisher's exact test on our array data, we found strongly significant genetic differences between herds A and B (Table 4). The genes *seD*, *seJ*, *seR*, and *lukD* were more likely to be present in herd A [$P=0.0004$; 95% exact CI (4.3, Inf*)] whereas *seC*, *seI*, *seN*, *seM*, *seO*, and *seU* were more likely to be present in herd B [$P=0.0002$; 95% exact CI (5.0, Inf*)]; (*Inf, because in cases where no present strains were found in either group an upper 95% exact CI cannot be calculated). In contrast, the allelic variants of *agr* genes BCD group-I, were identified in all the isolates from both herds, while group-II, III, IV variants were overall absent. All four haemolysin genes (*hla*, *hly*, *hld*

and *hlgA*) were carried by all strains. Despite their presence, production of β -haemolysin was highly specific to herd A ($P=0.0002$; 95% exact CI [5.0, Inf¹], but not to herd B, where β -haemolysin was never demonstrated except in one isolate (Table 4).

Discussion and Conclusion

To our knowledge, the present study represents the first investigation involving 22 udders collected at slaughter from two herds. Nineteen of the 22 udders were from infected glands of dairy cows culled because of subclinical *Staph. aureus* mastitis, whilst 3 were negative controls. The results confirm that different *Staph. aureus* strains can be isolated from cattle suffering chronic subclinical mastitis in different herds, as previously described (Zecconi et al. 2006; Fournier et al. 2008; Graber et al. 2009; Piccinini et al. 2010). Furthermore, we documented in the field the ability of *Staph. aureus* to infect mammary parenchyma and contaminate milk. Analogous results had been shown by Hebert et al. (2000) who isolated the bacteria from milk macrophages and alveolar cells of chronically infected cows. An interesting finding from our results was the difference in the rate of infection between herds A and B, and a statistically

Table 3. Minimal inhibitory concentration (MIC) of *Staph. aureus* isolates to a number of antimicrobial agents commonly used for treatment of mastitis

Antibiotic	Herd A		Herd B	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
Penicillin	0.05	0.05	0.25	0.25
Oxacillin	0.125	0.5	0.125	0.125
Cephalosporin	0.125	0.125	0.125	0.125
Tylosin	0.5	1	0.5	0.5
Streptomycin	1	2	2	2
Rifaximin	>0.06	>0.06	>0.06	>0.06

†MIC is defined as the lowest concentration that completely inhibits microbial growth and is expressed as µg/ml

significant association being found of *Staph. aureus* present in milk and tissue from multiple quarters in cattle from herd A compared with herd B. This result could have important consequences in the field, as in multiple-quarter infections the cure rate is lower at the cow level than for single-quarter infections (Osteras et al. 1999).

It is worth noting that this study found ~6–10% of instances with false negative results of milk bacteriological analysis, where *Staph. aureus* was isolated from the parenchyma of infected quarters but the microorganism was not shed in the milk. Also, bacterial counts in the parenchyma appeared always at a much lower level than in the milk, supporting the results of numerous histopathological studies of the gland, which barely demonstrated the presence of *Staph. aureus* in infected udder tissues (Chandler & Reid, 1973; Sordillo et al. 1989).

Molecular analysis of *Staph. aureus* isolates from herds A and B showed differences in their profile which may account for the differences seen in colonization between the herds. Firstly, both PFGE and MLST profile of isolates from herd A were distinct from herd B although the intra-herd PFGE isolate profile was identical, suggesting infection and proliferation with a different clonal type for each herd. Secondly, the virulence pattern of the isolates from each herd was virtually identical and statistically significant differences in carriage of several virulence determinants were evidenced between the herds. These differences included the enterotoxin and leukocidin genes. The *seD*, *seJ*, and *seR* enterotoxin genes and both *lukD* and *lukE* leukocidin genes were identified in all isolates from herd A, whilst herd B isolates harboured *seG*, *seI*, *seN*, *seM*, *seO* and *seU* enterotoxin genes and only the *lukE* gene. The *seDJR* genes are encoded on a large penicillinase plasmid (Osteras et al. 1999) while the *seGINMOU* genes are commonly found in the enterotoxin gene cluster (*egc*) on a genomic island (Lindsay & Holden, 2006). The differences between isolates from each herd were probably reflected in the PFGE and MLST profiles. Interestingly, all herd A isolates carrying enterotoxin genes on plasmids, *lukD/E* and only *agrI*, belonged to ST8 and shared the genetic background of MRSA lineage as reported by Argudin et al. (2011). On the

Table 4. Major results of PCR and microarray analysis of *Staph. aureus* isolates. Unless indicated otherwise, (+) denotes presence, whilst (–) denotes absence in all isolates within each herd. The values from the Fisher's exact test are also given where applicable

Genes	Herd A isolates	Herd B isolates	Fisher's exact test
<i>β-lactamase</i>	+	+	ns†
<i>resistance to methicillin and other antibacterial agents</i>	–	–	ns
<i>sak</i>	–	–	ns
<i>seA, seB, seC, seE</i>	–	–	ns
<i>seD, seJ, seR</i>	+	–	<i>P</i> =0.0004
<i>seG, seI, seM, seN, seO, seU</i>	–	+	<i>P</i> =0.0002
<i>tsst</i>	–	–	ns
<i>hla, hlb, hld</i>	+	+	ns
<i>hlg</i> locus (<i>lukS, lukF, hlgA</i>)	+	+	ns
<i>lukS-PV</i>	–	–	ns
<i>lukF-PV</i>	–	–	ns
<i>lukD</i>	+	–	<i>P</i> =0.0004
<i>lukE</i>	+	+	ns
<i>lukM</i>	–	–	ns
<i>lukF</i>	–	–	ns
<i>agrB-I, C-I, D-I</i>	+	+	ns
<i>agrB-II, B-III, B-IV; C-II, C-III, C-IV; D-II, D-III</i>	–	–	ns
<i>β</i> -haemolysin production	+	–‡	<i>P</i> =0.0002
<i>α</i> -, <i>δ</i> -haemolysin production	+	+‡	ns

† Not significant

‡ Isolate 2163 produced *β*-haemolysin, but neither *α*- nor *δ*-haemolysin

contrary, herd B isolates belonged to ST20, which is rarely detected in the dairy cow (Monecke et al. 2007; Bystron et al. 2010). In accordance with the literature, all ST20 isolates detected in herd B carried *egc* and *β-lactamase* (*blaZ*) gene. Both MLST profiles identified could be grouped with human clones, demonstrating that host-association is not absolute, as reported by Holmes & Zadoks (2011).

Even though *lukE* was carried by all the strains, the active toxin could be produced only in herd A, since the *D* component of *lukD/E* gene was demonstrated only in that group. Owing to the possibility of a sequence variant undetectable by array, the results were further confirmed by PCR and sequence of the amplicons.

It could be hypothesized that the statistically significant greater ability of *Staph. aureus* isolates from herd A to infect multiple quarters of cows was related to the presence of *lukD/E* and *seDJR* genes for three reasons: firstly, this leukocidin is known to strongly interfere with mammary response by damaging the leukocytes (Rainard et al. 2003; Younis et al. 2005); secondly, *seDJR* genes have been related to higher *Staph. aureus* prevalence in infected herds (Fournier et al. 2008; Piccinini et al. 2010); thirdly, the *egc* superantigens are significantly more frequent in commensal strains than in invasive isolates, probably because the concentration of these toxins does not increase with increasing bacterial counts. In contrast, plasmid toxins are

able to orchestrate an efficient adaptive immune response (Grumann et al. 2008).

Moreover expression of haemolysins was characteristic of each herd. The array data showed that the genes for the different haemolysins were present in all the *Staph. aureus* isolates considered, as would be expected from previous reports (Aarestrup et al. 1999; Haveri et al. 2007). Nevertheless, in contrast to Aarestrup et al. (1999), 95% of our isolates expressed α -haemolysin, but only 35% expressed β -haemolysin. It is noteworthy that 86% of the isolates expressing β -haemolysin were from herd A. As suggested by Larsen et al. (2002), β -haemolysin may be an active factor in the pathogenesis of bovine mastitis, contributing to the highest invasiveness of herd A isolates. Such differences in expression are probably due to the *hlyB* gene becoming inactivated by integration of lysogenic phages, as reported by van Wamel et al. (2006), but this requires further investigation.

Finally, the same allelic variants of the *agr* group were present in all the isolates. The *agr* locus is important for virulence in a variety of animal models of infection and it regulates the production of both α - and δ -haemolysin (Traber et al. 2008), which were used in this study as a surrogate to determine *agr* expression (Shopsin et al. 2010). The results indicated that 95% of the isolates expressed both haemolysins and hence will be more likely to express plasmid enterotoxins, which are under control of the *sar* and *agr* response regulators (Cheung et al. 2004). The only isolate with an anomalous result in haemolysin production was strain 2163, which was one of two different isolates recovered from two quarters of the same cow in herd B.

The carriage of genes encoding antibiotic resistance was rare amongst these isolates, as previously reported (Monecke et al. 2007) and was reflected in the very low MIC values. This result was similar to that reported by Rubin et al. (2011) who detected resistance only to trimethoprim/sulphamethoxazole. Minor differences were observed in herd A for oxacillin, tylosin and streptomycin, while all herd B isolates demonstrated identical susceptibility. A clear difference between herds was shown for penicillin, since MIC values in herd B were 5-times higher than in herd A, even though *blaZ* was carried by all isolates. The sensitivity of herd B strains was consistent with the literature (Haveri et al. 2005; Kaase et al. 2008) reporting MIC values as low as 0.06 $\mu\text{g/ml}$ for isolates proved to possess *blaZ*. To the contrary, lower values were always related to the absence of the gene. The MIC of 0.05 $\mu\text{g/ml}$ observed in the present study could be considered as a borderline value, still consistent with *blaZ* carriage.

In conclusion, our data indicate that *Staph. aureus* isolates have different abilities to colonize mammary tissue and disseminate across mammary quarters. These differences are likely to be due to virulence factors which influence the genetic characteristics and probably the diffusiveness of the different strains. Further studies are required to determine whether the virulence factors identified in this study are the only or even the key components involved in infection and

dissemination across herds or whether they are part of a complex interaction of various factors involved in pathogenesis of mastitis in naturally infected animals.

We thank Dr C Bonura of the Department of Health Promotion Sciences, 'G. D'Alessandro', University of Palermo, Italy, for MLST of *Staph. aureus* isolates collected in the study.

References

- Aarestrup FM, Larsen HD, Eriksen NH & Elsborg CS 1999 Frequency of alpha- and beta-haemolysin in *Staphylococcus aureus* of bovine and human origin. A comparison between pheno- and genotype and variation in phenotypic expression. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **107** 425–430
- Almeida RA, Matthews KR, Cifrian E, Guidry AJ & Oliver SP 1996 *Staphylococcus aureus* invasion of bovine mammary epithelial cells. *Journal of Dairy Science* **79** 1021–1026
- Anderson JC & Chandler RL 1975 Experimental Staphylococcal mastitis in the mouse. Histological, ultrastructural and bacteriological changes caused by a virulent strain of *Staphylococcus aureus*. *Journal of Comparative Pathology* **85** 499–510
- Argudín MA, Mendoza MC, Méndez FJ, Martín MC, Guerra B & Rodicio MR 2011 Clonal complexes and diversity of exotoxin gene profiles in methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates from patients in a Spanish hospital. *Journal of Clinical Microbiology* **47** 2097–2105
- Bonura C, Plano MRA, Di Carlo P, Calà C, Cipolla D, Corsello G, Mammina C & EPI-MRSA Working Group 2010 MRSA ST22-IVa (EMRSA-15 clone) in Palermo, Italy. *Journal of Infection and Public Health* **3** 188–191
- Bystron J, Podkowik M, Korzekwa K, Lis E, Molenda J & Bania J 2010 Characterization of borderline oxacillin-resistant *Staphylococcus aureus* isolated from food of animal origin. *Journal of Food Protection* **73** 1325–1327
- Chandler RL & Reid IM 1973 Ultrastructural and associated observations on clinical cases of mastitis in cattle. *Journal of Comparative Pathology* **83** 233–241
- Chandler RL, Reid IM, Harrison R & France BR 1974 Ultrastructural, morphometric and associated observations on experimental mastitis in cattle. *Journal of Comparative Pathology* **84** 517–531
- Cheung AL, Bayer AS, Zhang G, Gresham H & Xiong YQ 2004 Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. *FEMS Immunology and Medical Microbiology* **40** 1–9
- Chung M, de Lencastre H, Matthews P, Tomasz A, Adamsson I, Aires de Sousa M, Camou T, Cocuzza C, Corso A, Couto I, Dominguez A, Gniadkowski M, Goering R, Gomes A, Kikuchi K, Marchese A, Mato R, Melter O, Oliveira D, Palacio R, Sa-Leao R, Santos Sanches I, Song JH, Tassios PT & Villari P 2000 Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. *Microbial Drug Resistance* **6** 189–198
- Clinical and Laboratory Standards Institute 2008 Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standards (M31–A3). Clinical and Laboratory Standards Institute, Wayne, PA, USA
- Dego OK, Van Dijk JE & Nederbragt H 2002 Factors involved in the early pathogenesis of bovine *Staphylococcus aureus* mastitis with emphasis on bacterial adhesion and invasion. *Veterinary Quarterly* **24** 181–198
- Fleiss JL 1981 *Statistical Methods for Rates and Proportions*, 2nd edn. New York, NY, USA: John Wiley
- Fournier C, Kuhnert P, Frey J, Miserez R, Kirchhofer M, Kaufmann T, Steiner A & Graber HU 2008 Bovine *Staphylococcus aureus*: association of virulence genes, genotypes and clinical outcome. *Research in Veterinary Science* **85** 439–448

- Graber HU, Naskova L, Studer E, Kaufmann T, Kirchhofer M, Brechbühl M, Schaeren W, Steiner A & Fournier C 2009 Mastitis-related subtypes of bovine *Staphylococcus aureus* are characterized by different clinical properties. *Journal of Dairy Science* **92** 1442–1451
- Grumann D, Scharf SS, Holtfreter S, Kohler C, Steil L, Engelmann S, Hecker M, Völker U & Bröker BM 2008 Immune cell activation by enterotoxin gene cluster (egc)-encoded and non-egc superantigens from *Staphylococcus aureus*. *Journal of Immunology* **181** 5054–5061
- Gudding R, McDonald JS & Cheville NS 1984 Pathogenesis of *Staphylococcus aureus* mastitis: bacteriologic, histologic, and ultrastructural pathologic findings. *American Journal of Veterinary Research* **45** 2525–2531
- Haveri M, Roslof A, Rantala L & Pyorala S 2007 Virulence genes of bovine *Staphylococcus aureus* from persistent and nonpersistent intramammary infections with different clinical characteristics. *Journal of Applied Microbiology* **103** 993–1000
- Haveri M, Suominen S, Rantala L, Honkanen-Buzalski T & Pyorala S 2005 Comparison of phenotypic and genotypic detection of penicillin G resistance of *Staphylococcus aureus* isolated from bovine intramammary infection. *Veterinary Microbiology* **106** 97–102
- Hebert A, Sayasit K, Senechal S, Dubreuil P & Lagace J 2000 Demonstration of intracellular *Staphylococcus aureus* in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk. *FEMS Microbiology Letters* **193** 57–62
- Hensen SM, Pavicic MJ, Lohuis JACM, de Hoog JAM & Poutrel B 2000 Location of *Staphylococcus aureus* within the experimentally infected bovine udder and the expression of capsular polysaccharide type 5 *in situ*. *Journal of Dairy Science* **83** 1966–1975
- Hogan JS, Gonzales RN, Harmon RJ, Nickerson SC, Oliver SP, Pankey JW & Smith KL 1999 *Laboratory Handbook on Bovine Mastitis*, revised edition, p. 222. Madison, WI: National Mastitis Council Inc.
- Holmes MA & Zadoks RN 2011 Methicillin resistant *S. aureus* in human and bovine mastitis. *Journal of Mammary Gland Biology and Neoplasia* **16** 373–382
- Kaase M, Lenga S, Friedrich S, Szabados F, Sakinc T, Kleine B & Gatermann SG 2008 Comparison of phenotypic methods for penicillinase detection in *Staphylococcus aureus*. *Clinical Microbiology and Infection* **14** 614–616
- Lammers A, Nuijten PJM & Smith HE 1999 The fibronectin binding proteins of *Staphylococcus aureus* are required for adhesion and invasion of bovine mammary gland cells. *FEMS Microbiology Letters* **180** 103–109
- Larsen HD, Aarestrup FM & Jensen NE 2002 Geographical variation in the presence of genes encoding superantigenic exotoxins and β -hemolysin among *Staphylococcus aureus* isolated from bovine mastitis in Europe and USA. *Veterinary Microbiology* **85** 61–67
- Lindsay JA & Holden MT 2006 Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. *Functional and Integrative Genomics* **6** 186–201
- Lowy FD 2000 Is *Staphylococcus aureus* an intracellular pathogen? *Trends in Microbiology* **8** 341–343
- Monecke S & Ehrlich R 2005 Rapid genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates using miniaturised oligonucleotide arrays. *Clinical Microbiology and Infection* **11** 825–833
- Monecke S, Kuhnert P, Hotzel H, Slickers P & Ehrlich R 2007 Microarray-based study on virulence associated genes and resistance determinants of *Staphylococcus aureus* isolates from cattle. *Veterinary Microbiology* **125** 128–140
- Moret-Stalder S, Fournier C, Miserez R, Albini S, Doherr MG, Reist M, Schaeren W, Kirchhofer M, Graber HU, Steiner A & Kaufmann T 2009 Prevalence study of *Staphylococcus aureus* in quarter milk samples of dairy cows in the Canton of Bern, Switzerland. *Preventive Veterinary Medicine* **88** 72–76
- Nickerson SC & Heald CW 1981 Histopathologic response of the bovine mammary gland to experimentally induced *Staphylococcus aureus* infection. *American Journal of Veterinary Research* **42** 1351–1355
- NMC 1999 *Laboratory Handbook on Bovine Mastitis*. Madison, WI, USA: National Mastitis Council Inc.
- Ogawa SK, Yurberg ER, Hatcher VB, Levitt MA & Lowy FD 1985 Bacterial adherence to human endothelial cells *in vitro*. *Infection and Immunity* **50** 218–224
- Osteras O, Edge VL & Martin SW 1999 Determinants of success or failure in the elimination of major mastitis pathogens in selective dry cow therapy. *Journal of Dairy Science* **82** 1221–1231
- Piccinini R, Borromeo V & Zeconi A 2010 Relationship between *S. aureus* gene pattern and dairy herd mastitis prevalence. *Veterinary Microbiology* **145** 100–105
- Piccinini R, Cesaris L, Daprà V, Borromeo V, Picozzi C, Secchi C & Zeconi A 2009 The role of teat skin contamination in the epidemiology of *Staphylococcus aureus* intramammary infection. *Journal of Dairy Research* **76** 36–41
- Rainard P, Corrales JC, Barrio MB, Cochard T & Poutrel B 2003 Leucotoxic activities of *Staphylococcus aureus* strains isolated from cows, ewes, and goats with mastitis: importance of LukM/LukF-PV Leukotoxin. *Clinical and Diagnostic Laboratory Immunology* **10** 272–277
- Rubin JE, Ball KR & Chirino-Trejo M 2011 Antimicrobial susceptibility of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* isolated from various animals. *Canadian Veterinary Journal* **52** 153–157
- Shopsin B, Eaton C, Wasserman GA, Mathema B, Adhikari RP, Agolory S, Altman DR, Holzman RS, Kreiswirth BN & Novick RP 2010 Mutations in agr do not persist in natural populations of methicillin-resistant *Staphylococcus aureus*. *Journal of Infectious Diseases* **202** 1593–1599
- Sordillo LM, Nickerson SC & Akers RM 1989 Pathology of *Staphylococcus aureus* mastitis during lactogenesis: relationship with bovine mammary structure and function. *Journal of Dairy Science* **72** 228–240
- Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH & Swaminathan B 1995 Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology* **33** 2233–2239
- Tollersrud T, Kenny K, Caugant DA & Lund A 2000 Characterisation of isolates of *Staphylococcus aureus* from acute, chronic and subclinical mastitis in cows in Norway. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **108** 565–572
- Traber KE, Lee E, Benson S, Corrigan R, Cantera M, Shopsin B & Novick RP 2008 Agr function in clinical *Staphylococcus aureus* isolates. *Microbiology* **154** 2265–2274
- Van Wamel WJB, Rooijackers SHM, Ruyken M, van Kessel KPM & van Strijp JAG 2006 The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on β -hemolysin-converting bacteriophages. *Journal of Bacteriology* **188** 1310–1315
- Younis A, Krifucks O, Fleminger G, Heller ED, Gollop N, Saran A & Leitner G 2005 *Staphylococcus aureus* leucocidin, a virulence factor in bovine mastitis. *Journal of Dairy Research* **72** 188–194
- Zeconi A, Cesaris L, Liandris E, Daprà V & Piccinini R 2006 Role of several *Staphylococcus aureus* virulence factors on the inflammatory response in bovine mammary gland. *Microbial Pathogenesis* **40** 177–183
- Zeconi A, Piccinini R & Fox KL 2003 Epidemiologic study of intramammary infections with *Staphylococcus aureus* during a control program in nine commercial dairy herds. *Journal of the American Veterinary Medical Association* **223** 684–688