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Staphylococcus aureus strains in primiparous and multiparous cows in six herds with a high prevalence of *Staph. aureus* intramammary infections

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The proportion of different strains of *Staphylococcus aureus* was tested in four groups of lactating dairy cows in six herds with a high overall prevalence of *Staph. aureus* using random amplified polymorphic DNA PCR. Group 1 included primiparous cows in early lactation (<50 days in milk, DIM). Group 2 consisted of primiparous cows in late lactation (>250 days in milk). Groups 3 and 4 were multiparous cows in the respective stages of lactation. Eight cows from each group on each farm were tested. Overall quarter prevalence of *Staph. aureus* ranged from 23·4 to 32·0% in the herds. Of the 130 isolates included in the analysis 86·9% were high prevalence strains (more than three isolates per herd), while 13·1% were strains that were only identified in one or two samples. Low prevalence strains were found in all six herds. The proportion of low prevalence strains was higher in multiparous than in primiparous cows (odds ratio, OR 4·4, 1·2–16·6). It is concluded that low prevalence *Staph. aureus* strains are common even in herds with a high prevalence of *Staph. aureus* and that their frequency is lower in primiparous cows than in older cows.

Keywords: *Staphylococcus aureus*, intramammary infection, mastitis, dairy cow.

Staphylococcus aureus is one of the most important mastitis pathogens in dairy cattle. Its overall prevalence in large German dairy herds has been estimated at 5·7% of all quarters, with a higher prevalence in late than in early lactation (Tenhagen et al. 2006). Similar data have been reported for other countries and herd sizes (Poelarends et al. 2001; Gianneecchini et al. 2002; Makovec & Ruegg, 2003; Østeras et al. 2006). *Staph. aureus* is generally characterized as a contagious mastitis pathogen that is predominantly spread during the milking process via milkers' hands, contaminated towels and milking clusters (Hoedemaker, 2001).

Recently several researchers have demonstrated that there are differences between *Staph. aureus* strains concerning their epidemiological features. There are strains with the typical features of contagious mastitis pathogens, while other strains seem to act in a way that is commonly attributed to environmental pathogens such as *Escherichia coli* or *Streptococcus uberis* (Lam et al. 1996; Sommerhäuser et al. 2003). However, these strains are usually far less prevalent in the herds than the contagious strains.

While the milking process is generally established as the primary source of intramammary infections (IMI) with *Staph. aureus*, this does not explain the regular isolation of *Staph. aureus* from secretions of primiparous cows immediately after parturition. Between 4% and 15% of udder quarters in these animals are reported to harbour *Staph. aureus* (Roberson et al. 1998; Edinger et al. 2000; Tenhagen et al. 2001). It has been assumed that the strains isolated from heifers may differ from those of older lactating dairy cows (Gillespie et al. 1999; Tenhagen et al. 2006). However, several studies found identical strains in heifers and in the lactating dairy herd (Roberson et al. 1998; Reppel et al. 2005).

It was the objective of this study to analyse the distribution of high and low prevalence *Staph. aureus*

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Table 1. Characteristics of the six study farms

	Farm no.						
	1	2	3	4	5	6	Mean
No. of cows	250	350	330	470	420	410	372
Milk test day SCC ($\times 10^{-3}$/ml)†	360	501	580	479	268	298	398‡
Prevalence							
Quarter	30·5	31·3	23·4	26·6	28·1	32·0	28·7
95% CI	22–38	23–39	16–30	19–34	20–36	24–40	25–32
Cow	62·5	56·3	46·9	62·5	46·9	59·4	55·8
95% CI	44–78	38–72	28–63	44–78	28–63	41–75	48–63
Dry cow therapy§	Select.	Blanket	Select.	Select.	Blanket	Blanket	
PMTD¶							
Type	Cup	Cup	Cup	Spray	Spray	Cup	
Product	Not known	Iodine	Iodine	Chlorhexidine	Peracetic acid	Peracetic acid	
Consistent	No	Yes	Yes	Yes	Yes	No	
Cluster disinfection consistent	Yes	No	No	Yes	No	Yes	
Grouping by infection status	Yes	No	Yes	Yes	Inconsistent	Inconsistent	
Common towel	No	No	No	No	No	No	

† Arithmetic mean somatic cell count/ml over the year before samples were taken (includes 11 milk test days)
‡ Geometric mean
§ Selective = only some cows of the herd receive antibiotic dry cow therapy; Blanket = all cows receive antibiotic dry cow therapy
¶ Post-milking teat disinfection

strains in primiparous and multiparous cows in early and late lactation in herds with a high prevalence of *Staph. aureus*.

Material and Methods

The dairy herds were selected from herds included in a larger study on the prevalence of mastitis pathogens in dairy herds in Brandenburg, Germany (Tenhagen et al. 2006). Of the 80 herds included in that study, the 6 herds with the highest estimated prevalence of *Staph. aureus* were chosen to participate in this study. The quarter prevalence of IMI with *Staph. aureus* ranged from 23·4 to 32·0%. Some characteristics of the herds are summarized in Table 1.

During one farm visit, quarter milk samples were collected aseptically from 32 cows of each herd showing no signs of clinical mastitis. Four groups of cows were chosen for sampling: primiparous cows during the first 50 d of lactation (Group 1) and at the end of lactation (>250 days in milk (DIM), Group 2) and multiparous cows at the respective stages of lactation (Groups 3 and 4). Apart from parity, stage of lactation and absence of clinical mastitis, no further selection criteria were applied. Cows meeting the criteria and entering the parlour were sampled until the target number of eight cows per group was reached. No further information on the individual cows was collected.

Milk samples were cooled, shipped to the laboratory and cultured on the same day. In brief, 0·01 ml of milk were plated on one half of blood agar (Blood agar base No. 2, Oxoid, Wesel) containing 5% sheep blood and 0·1% aesculin. *Staph. aureus* was identified by colony morphology, haemolysis, a positive tube coagulase test and anaerobic fermentation of mannitol (Roberson et al. 1992). Quarters were considered infected when a minimum of one *Staph. aureus* colony was identified on the agar dish, to account for limited shedding of the pathogen in chronic cases as reported by Østeras et al. (2006). Somatic cell counts (SCC) of the samples were not determined. *Staph. aureus* isolates were stored frozen at −80 °C for further analysis.

Determination of genetic differences among Staph. aureus *isolates*

Genetic differences among isolates within a herd were determined by random amplified polymorphic DNA (RAPD) analysis. RAPD analysis is inexpensive, efficient and well suited for investigations incorporating large sample numbers. This technique has been used successfully for the characterization of numerous organisms including *Staph. aureus* (*e.g.* Lee, 2003; Smyth et al. 2006).

Staph. aureus, 20–22 isolates per herd, were analysed by RAPD analysis. The isolates were selected from the available isolates per herd by three criteria: 1, include a maximum number of cows; 2, include the same number of isolates from the four groups (if possible); and 3, if necessary, include randomly selected additional isolates. The last criterion was valid if there were only few isolates from primiparous cows. In that case, more isolates from older cows were randomly chosen.

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Chromosomal DNA preparation

DNA was purified using the commercial isolation kit Wizard Genomic DNA purification kit (Promega, Madison WI, USA) as described by the manufacturer. For DNA isolation the strains were thawed and replated on blood agar and then transferred to LB broth (LB-Bouillon, Merck, Darmstadt, Germany) for 24 h at 37 °C. Lysozyme and lysostaphin was used as recommended by the manufacturer in order to improve the cell wall lysis. DNA was stored for a maximum of 2 weeks in DNA rehydration solution at 4 °C until further use. Concentration of DNA was measured against a reference solution using the photometer DyNA Quand 200 and the respective kit DyNA Quant 200 (Hoefer, San Francisco CA, USA).

RAPD analysis

RAPD reaction was done using the 'Ready to go RAPD analysis Kit' (Amersham Biosciences, Piscataway NJ, USA) containing ready to go analysis beds and six RAPD analysis primers. In preliminary studies with a limited number of *Staph. aureus* isolates it was shown that the primer 2 (5′-d[GTTTCGCTCC]-3′ and the primer 5 (5′-d[AACGCGCAAC]-3′) had the highest discriminatory capacity. Therefore all further experiments were done with these primers.

DNA amplification was performed in a Perkin-Elmer cycler (Gene Amp, PCR-Systems 2400, Perkin-Elmer, Göttingen, Germany) using 25 pmol of primer 2 or 5, 10 ng of template DNA and the analysis beads in a final volume of 25 µl. The cycler was programmed for one cycle of 5 min at 95 °C followed by 45 cycles of 1 min at 95 °C, 1 min at 36 °C and 2 min at 72 °C.

The amplicons were analysed on a 1·5% agarose gel containing 0·5 µg of ethidium bromide/ml at 90 V of 4–5 h. Analysis of all isolates was done in duplicate.

Data analysis

Fingerprints were compared within herds only. It was the purpose of this study to analyse the distribution of strains within herds but not to identify strains that could be isolated from different herds. To facilitate the analysis of genetic differences among isolates within a herd, all tested isolates of a herd were run in the same agarose gel.

Isolates were regarded as identical when RAPD patterns obtained with both primers were identical. Any difference in the bands was regarded as a genetic difference (Sabat et al. 2006). Isolates with identical fingerprints for both primers were defined as a strain. This very strict definition was chosen because all samples were collected during the same visit and therefore there was no time for mutations to occur within the population. However, we cannot rule out mutations occurring after sampling. No attempt was made to classify genetic relatedness between the strains that differed.

Two types of strains were classified. High prevalence strains, *i.e.* more than three identical isolates from the same farm were distinguished from low prevalence strains (only one or two identical isolates from the same farm).

Effect of herd, age group and stage of lactation on the frequency of single isolates was tested using binary logistic regression (SPSS, Version 12.0, SPSS Inc., Munich) with type of strain (high prevalence *v.* low prevalence) as binary outcome and herd (1 to 6, categorial), age group (0|1) and stage of lactation (0|1) as covariates.

Results

A total of 130 *Staph. aureus* isolates were analysed using RAPD analysis. Owing to the limited number of *Staph. aureus* isolates that were found in primiparous cows, nearly all isolates from Groups 1 and 2 were included. Primer 2 differentiated 20 different genotypes, primer 5 found 19 different genotypes. The combined analysis of the results of both primers yielded 25 different strains.

The number of different strains identified on the farms varied between 2 and 6. In 3 herds, there was one predominant strain accounting for 82–95% of the isolates. In the other three herds, there were two strains that together contributed 82–90% of the isolates. In herd 1, there was one strain with 54·5% of the isolates and another with 27·3% of the isolates. In herd 2, the two predominant strains contributed 63·6 and 18·2% of the isolates, respectively. In herd 6, the proportions were 55·0 and 35·0%, respectively. Overall, the predominant strains contributed 86·9% of all isolates in this study (Table 2).

Besides the predominant strains, there were low prevalence strains in all herds. The single isolates were predominantly found in multiparous cows (20·6 and 17·1% of all isolates in Groups 3 and 4, respectively). In primiparous cows, the proportion of single isolates was lower (5·6 and 5·4% in Groups 1 and 2, respectively).

More than one isolate per cow was analysed in 30 cows. Of those, 21 cows (70%) had the same strain in all infected quarters, while 9 (30%) had more than one strain. One of these cows had three different strains in the four infected quarters (Table 3).

Logistic regression revealed that the difference in the proportion of isolates of low prevalence strains between the age groups was significant (P=0·03). The odds ratio (OR) for a strain to be a single isolate was 4·4 in multiparous compared with primiparous cows (95% confidence interval, CI,: 1·2–16·6). There was no significant effect of stage of lactation (Table 4).

Discussion

The overall quarter prevalence of *Staph. aureus* in the six herds studied here was much higher than the prevalence reported in other studies (Poelarends et al. 2001;

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Table 2. Distribution of different *Staphylococcus aureus* genotypes within 6 herds with a high prevalence of *Staph. aureus* intramammary infection

Parity		Primiparous		Multiparous		
Days in milk	Strain No.	<50	>250	<50	>250	Total
Total No. of isolates		18	37	34	41	130
High prevalence strains						
Herd 1 (Isolates/cows)	1A	5/4	1/1	4/3	4/4	14/12
	1B	0/0	3/3	1/1	0/0	4/4
Herd 2 (Isolates/cows)	2A	1/1	7/4	2/1	2/2	12/8
	2B	3/2	0/0	1/1	2/2	6/5
Herd 3 (Isolates/cows)	3A	1/1	7/3	5/3	8/6	21/13
Herd 4 (Isolates/cows)	4A	2/2	7/6	5/4	6/4	20/16
Herd 5 (Isolates/cows)	5A	1/1	6/3	3/1	8/5	18/10
Herd 6 (Isolates/cows)	6A	3/3	1/1	3/2	4/3	11/9
	6B	1/1	3/2	3/3	0/0	7/6
Total (Isolates/cows)		17/14	35/24	27/17	34/26	113/80
High prevalence strains (%)		94·4	94·6	79·4	82·9	86·9
Low prevalence strains						
Herd 1 (Isolates/cows)	1C,D,E,F	1/1	1/1	1/1	1/1	4/4
Herd 2 (Isolates/cows)	2C,D,E	0	0	2/2	2/2	4/4
Herd 3 (Isolates/cows)	3B	0	0	1/1	0	1/1
Herd 4 (Isolates/cows)	4B,C	0	0	1/1	1/1	2/2
Herd 5 (Isolates/cows)	5B,C,D,E	0	0	2/2	2/2	4/4
Herd 6 (Isolates/cows)	6C,6D	0	1/1	0	1/1	2/2
Total (Isolates/cows)		1/1	2/2	7/7	7/7	17/17
Low prevalence strains (%)		5·6	5·4	20·6	17·1	13·1

Table 3. Homogeneity of strains isolated from different quarters of the same cow

No. of quarters	No. of animals	No. of different strains		
		1 strain	2 strains	3 strains
2 quarters	23	16	7	—
3 quarters	5	5	0	0
4 quarters	2	0	1	1

Gianneecchini et al. 2002; Makovec & Ruegg, 2003; Østeras et al., 2006; Tenhagen et al. 2006). The results of our study show that low prevalence *Staph. aureus* strains are a common finding also in large herds with a high general prevalence of *Staph. aureus*. This is in line with reports from other studies in smaller herds with lower prevalences and lower SCC (Lam et al. 1996; Sommerhäuser et al. 2003). In a recent Canadian study, only one genotype was isolated from the majority of herds (58·6%). However, this study used fewer isolates per herd. Therefore the less prevalent types of *Staph. aureus* may have gone undetected (Sabour et al. 2004).

Most isolates were from one or two predominant strains in the herd. This is in accordance with the general classification of *Staph. aureus* as a contagious pathogen that is transmitted between cows and quarters during the milking process (Neave et al. 1969; Hoedemaker, 2001). The consistent use of control measures for contagious pathogens would most likely reduce the prevalence of these strains effectively. None of the six herds implemented all elements of a contagious mastitis control programme consistently. The weak spot in the management, *i.e.* the preventive measure that was not implemented differed between herds (Table 1). However, there was a plausible explanation for the high *Staph. aureus* prevalence in all of the herds. Inconsistent use of control measures has recently been associated with high SCC in large dairy herds (Köster et al. 2006).

Thirteen percent of the isolates were from low prevalence strains. It has been assumed that low prevalence strains cannot be controlled by programmes to reduce contagious mastitis pathogens as they probably have features commonly found in environmental mastitis pathogens (Sommerhäuser et al. 2003). However, in the herds studied here, these isolates were a small minority and the consistent implementation of a control programme for contagious pathogens could reduce the prevalence of *Staph. aureus* by 80–90°% if it was effective against the contagious strains.

More isolates originated from older cows than from primiparous cows. This is in line with the higher risk of *Staph. aureus* IMI that has been reported repeatedly (Zadoks et al. 2001; Tenhagen et al. 2006). The

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Table 4. Summary results of logistic regression on the probability of a strain being high prevalence (0) or low prevalence (1) with respect to herd, age group (0/1) and stage of lactation (early (0) *v.* late (1))

	B	SE	Wald	df	*P* value	Odds Ratio	95 % CI
Herd				5	0·611		
Herd 1	0·794	0·952	0·696	1	0·404	2·213	0·34–14·31
Herd 2	0·813	0·954	0·727	1	0·394	2·255	0·35–14·62
Herd 3	−0·947	1·283	0·545	1	0·460	0·388	0·03–4·79
Herd 4	−0·143	1·071	0·018	1	0·894	0·867	0·11–7·07
Herd 5	0·596	0·956	0·388	1	0·533	1·814	0·28–11·81
Age group	1·476	0·681	4·703	1	0·030	4·376	1·15–16·62
Stage of lactation	−0·164	0·553	0·088	1	0·767	0·849	0·29–2·51
Constant	−4·447	1·701	6·837	1	0·009	0·012	

contribution of low prevalence strains to the total number of isolates per group was higher in older cows. It has been proposed that the *Staph. aureus* strains that are isolated from primiparous cows might be of environmental origin, as the time at risk for an infection via the milking process has only been rather short in these animals (Tenhagen et al. 2006). The results of our study do not support this hypothesis as the same strains were found in primiparous and multiparous cows. However, Tenhagen et al. (2006) included far more herds and also herds with a low prevalence of *Staph. aureus*. This difference in the study population may have contributed to the different findings in this study.

The samples in this study were not taken before the first milking after parturition. Therefore the primiparous cows may have been infected during the milking process. However, if strains originating not from the mammary glands of the lactating dairy cows were of major importance in the primiparous cows at calving, they should have a higher proportion in the group of cows sampled before 50 DIM and then be replaced by strains originating from the lactating cows further into lactation. Changes in the proportion of ampicillin-resistant *Staph. aureus* in the course of the first lactation indicated such a shift in the population of *Staph. aureus* (Tenhagen et al. 2006). In fact, the numbers of infections by high prevalence strains nearly doubled in the course of the first lactation indicating new infections with high prevalence strains in the course of lactation. However, the ratio of infections with low and high prevalence strains did not change.

Our results are in line with other studies that demonstrated that the *Staph. aureus* strains in dairy herds isolated from heifers at calving are often related to the strains isolated from lactating older cows or older cows at parturition (Roberson et al. 1998; Reppel et al. 2005). The role of infection routes other than milking time transmission cannot be analysed with our data.

In the majority of cases (21/30) different quarters of the same cow harboured the same strain. However, thirty percent of the cows that contributed more than one isolate had different isolates in different quarters. In one case even three different strains were identified in the same cow, two being high prevalence and one a low prevalence strain. This has also been reported in a Canadian study; however, that study did not report the number of animals that contributed more than one isolate to the samples included in the study (Sabour et al. 2004). The isolation of different strains from the same cow was rather unexpected, as it does not support the contagious nature of *Staph. aureus* as a mastitis pathogen. It has been shown that the risk of a quarter for an IMI with *Staph. aureus* increases if other quarters of the same cow are infected (Zadoks et al. 2001). With a contagious pathogen it would be expected that this increased risk refers to infection from quarter to quarter during milking.

There are several reports that put the discriminatory power of RAPD-PCR into question (Grundmann et al. 2002; Sabat et al. 2006). Especially the use of a single primer has been associated with limitations in discriminatory power (Grundmann et al. 2002). We tried to minimize the disadvantages in the technology by using two primers and thereby could effectively increase the discriminatory power. Furthermore we did only compare isolates that had been run on the same agarose gel to avoid inconsistencies associated with the comparison of different gels. Other methods may have been able to discriminate further between genotypes or elucidate their degree of genetic relatedness. However, this remains to be investigated in further studies.

Conclusions

The results of our study show that the majority of isolates from high prevalence *Staph. aureus* herds belong to one or two strains, but other strains can nevertheless be isolated in all herds and that various strains can also be identified in the same cow. The contribution of the low prevalence strains is greater in multiparous than in primiparous cows. Strategies to reduce the prevalence of *Staph. aureus* as a contagious mastitis pathogen in dairy herds will most likely reduce the number of infections substantially.

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