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Bovine *Staphylococcus aureus*: Association of virulence genes, genotypes and clinical outcome

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Abstract

Based on our clinical experience on bovine mastitis, we hypothesized that subtypes of *Staphylococcus aureus* (*S. aureus*) exist which differ in their contagious and pathogenic properties. In order to investigate this hypothesis, we analyzed strains of *S. aureus* isolated from spontaneous intramammary infection (IMI) with their virulence gene patterns and genotypes obtained by PCR amplification of the 16S–23S rRNA intergenic spacer (RS–PCR). The genotypes were then associated with epidemiological and clinical data including 26 herds. The results demonstrated a high association between genotypes and virulence gene patterns as well as between epidemiological and pathogenic properties of *S. aureus*. In particular, genotype B was related to high contagiosity and increased pathogenicity whereas the other types (C, OG) were found with infection of single cows. Because of the high clinical relevance, our results indicate the need to subtype the IMI-associated strains of *S. aureus* in the future.

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Keywords: *Staphylococcus aureus*; Virulence factors; Contagiosity; Genotypes; PCR

1. Introduction

Staphylococcus aureus (*S. aureus*) is the most important etiologic agent of contagious bovine mastitis and is very common in Swiss dairy herds (Schällibaum, 1999). It is able to produce a number of virulence factors such as protein A or leukocidins (Kerro Dego et al., 2002). Protein A is located in the cell wall and captures antibodies (Abs) (Foster and McDevitt, 1994). Opsonizing Abs of the IgG2 class, together with neutrophils, play a major role in the defense against invading pathogens (Burton and Erskine 2003). Leukocidins are staphylococcal bicomponent exotoxins of the family of pore forming toxins (Miles et al., 2001). One of the several components is leukotoxin E. Leukocidins were shown to be cytotoxic for erythrocytes and leukocytes including bovine neutrophils and macrophages (Loeffler et al., 1986). These toxins are considered as virulence factors in bovine mastitis (Younis et al., 2005) and may play an important role in the development of this disease (Loeffler et al., 1988). Another virulence factor is coagulase (Sutra and Poutrel, 1994) which is an exoenzyme and clots plasma by conformational activation of prothrombin (Panizzi et al., 2004).

Recent studies suggest that staphylococcal enterotoxins (SETs) act as virulence factors in cattle (Chang et al., 2005). Among other effects, these superantigens induce in this species the production of interleukin 4 and 10 which activate T_H2 cells leading to reduced clearance of microbial pathogens (Burton and Erskine 2003). The five staphylococcal enterotoxins SEA to SEE as well as the toxic shock syndrome toxin-1 (TSST-1) are well known for several years,

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whereas the toxins SEG, SEH, SEI and SER and the staphylococcal enterotoxin-like superantigens SElJ to SElQ and SElU have been reported only recently (Omoe et al., 2005).

Various methods exist to subtype *S. aureus*. Phenotyping such as antibiotic susceptibility testing, or checking for production of SETs (Cenci-Goga et al., 2003) become more and more replaced by DNA based methods. They include pulsed-field gel electrophoresis (PFGE) (Anderson et al., 2006; Haveri et al., 2007), binary typing (Zadoks et al., 2000), multilocus sequence typing (MLST) (Enright et al., 2000) or DNA arrays (Monecke and Ehricht, 2005). In addition, PCR based methods (PCR: polymerase chain reaction) have been used. The latter include random or specific amplification of polymorphic DNA (Stepan et al., 2004). One method repeatedly used to genotype *S. aureus* is PCR amplification of the 16S–23S rRNA intergenic spacer (RS–PCR) as originally described by (Jensen et al., 1993). Its discriminatory power is very similar to the one of PFGE (Kumari et al., 1997), a proposed reference method in genotyping *S. aureus* (Weller 2000). In contrast to PFGE, the RS–PCR allows a high sample throughput.

Of particular clinical importance is the question concerning the existence of subtypes of *S. aureus* responsible for IMI, which differ with respect to their pathogenic and contagious properties, requiring different strategies towards prevention and treatment of the intramammary infection. In order to investigate this hypothesis, we first started a methodological study to obtain an overview of the different subtypes of *S. aureus* which can be isolated from milk samples of cows with spontaneous IMI. As pathogenicity and contagiosity of a certain strain might depend on its repertoire of virulence factors, we analyzed the isolates for their presence of various virulence genes/polymorphisms by multiple PCRs. Because this type of analysis is laborious and therefore not suitable for field studies requiring the examination of numerous samples, we associated the obtained gene patterns with those of the highly discriminatory and rapid RS–PCR. In a second step, the genotypes were then associated with clinical and epidemiological data from 26 herds to study the contagious and pathogenic properties of the subtypes.

2. Material and methods

2.1. Staphylococcus aureus isolates

In total, 291 strains of *S. aureus* were included in this study. All of them were isolated from milk samples of cows with spontaneous *S. aureus* intramammary infection using standard procedures proposed by the National Mastitis Council (NMC, 1999). Identification of *S. aureus* and other mastitis pathogens such as *Staphylococcus* spp. or *Streptococcus* spp. was done according to the guidelines of NMC (1999) which include morphology, biochemical properties, and detection of hemolysis. 81 strains were obtained from individual diagnostic analysis (G strains). Additional 210 strains were isolated from bovine milk samples collected during an epidemiological study on *S. aureus* in cow herds of Switzerland (M strains). In each of the 26 herds investigated, all the lactating cows ($n = 449$) were checked for udder health comprising clinical examination of udder, teats and visual milk inspection. Furthermore, milk of each quarter was checked by the California Mastitis Test (CMT) followed by aseptical sample collection for bacteriological testing and analysis of somatic cell counts (SCC). The samples were transported at 4 °C and analyzed for SCC within 24 h. Samples designed for bacteriology and PCR analysis were stored at −20 °C until further use.

All the 291 isolates were checked by PCR (see below) for the presence of the *nuc* gene which codes for the thermonuclease and is known to be specific for *S. aureus* (Brakstad et al., 1992). Isolates lacking this gene were excluded from the present study. All the *nuc*-positive isolates were then subjected to genotype analysis (see below). The 291 isolates further served as a source to create a subgroup of epidemiologically independent strains ($n = 101$). It comprised all the G strains (all the strains were from different herds) and a set of M strains: for each of herd 1 to 59 (see Table 5), 1 representative of the 2 most frequently observed genotypes was included.

2.2. Extraction of nucleic acids

A single colony of *S. aureus* was cultured in 4 ml sterile TS (Trypticase Soy Broth; Becton, Dickinson and Company) at 37 °C overnight. 1 ml of the culture was then centrifuged at $18,000 \times g$ for 10 min. The pellet was resuspended in 950 μl sterile water and total nucleic acid (NA) containing both DNA and RNA were extracted according to (Chavagnat et al., 2002). The eluate was tested for the quantity and integrity of NA by electrophoresis using a 0.8% agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH = 8.3) containing ethidium bromide (0.5 μg/ml). The gels were visualized and photographed under UV light. The isolated NAs were stored at −20 °C until further use.

2.3. Primers

The PCR primers described by Monday and Bohach (1999) were used to amplify the SET genes *sea* to *sej*. For *tst*, we employed the primers according to Lovseth et al. (2004). The *coa* gene (coagulase) and the *x*-region of *spa* (protein A) were detected by the primers published by Akineden et al. (2001). Primers for the *nuc* and the *lukE* gene (coding for leukotoxin E) were designed with the OLIGO 6.0 software (National Biosciences Inc.) and are shown in Table 1. Finally, the G1 and L1 primers described by Jensen et al. (1993) were used for genotyping by RS–PCR.

2.4. Analysis of virulence genes and genotyping

The PCR reaction mix (total volume 25 μl) for the multiplex assays included 1x PCR buffer (Qiagen), 4.0 mM

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Table 1
Newly designed oligonucleotides used as primers in the present study

Gene protein	Primer	Sequence (5′–3′)	Amplicon size (bp)
nuc (PCR)	Nuc-S	CTG GCA TAT GTA TGG CAA TTG TT	664
Thermonuclease	Nuc-AS	TAT TGA CCT GAA TCA GCG TTG TCT	
lukE (PCR)	lukE-S	AAT GTT AGC TGC AAC TTT GTC A	831
Leukotoxin E	lukE-AS	CTT TCT GCG TAA ATA CCA GTT CTA	
nuc (Sequencing)	NucSeq-S	TGT ATG GCA ATT GTT TCA ATA TTA CTT	
	NucSeq-AS	TTT CGC TTG TGC TTC ACT TTT TC	

$MgCl_2$, 400 μM dNTPs, the primers (final concentration of each primer 300 nM) for *seb*-*sec, sec, seg, sei* and *sej* (first multiplex PCR) or *sea, sed, see* and *seh* (second multiplex PCR), 2.5 U HotStarTaq Polymerase (Qiagen), and ~6 ng of NA. To activate the polymerase, a pre-PCR step was run at 95 °C for 15 min, continued by the following PCR profile: 95 °C for 1 min, 68 °C (for the first 15 cycles) and 64 °C (for the last 20 cycles) for 45 s, 72 °C for 1 min. PCR was terminated by a final extension at 72 °C for 10 min followed by cooling down to 4 °C. Monoplex PCR for *tst* was done as described for the multiplex assays.

For the detection of *nuc*, *coa*, *spa* and *lukE* genes, the reaction mix contained 1x HotStarTaq Master Mix (Qiagen), 300 nM of each primer and ~6 ng of NA (total volume 25 μl). The following cycling program was applied: 95 °C for 15 min followed by 35 cycles (*spa*: 32 cycles) including 94 °C for 1 min, 60 °C (*lukE*: 62 °C) for 1 min, 72 °C for 1 min. PCR was terminated as described above.

For genotyping by RS–PCR, the method of Jensen et al. (1993) was used which is based on amplification of the 16S–23S rRNA intergenic spacer region. Each reaction contained (total volume 25 μl) 1x HotStarTaq Master Mix (Qiagen), 800 nM of each primer (G1 and L1 primer) and ~30 ng NA. The PCR profile was: 95 °C for 15 min, followed by 27 cycles comprising 94 °C for 1 min, followed by a 2 min ramp and annealing at 55 °C for 7 min. After a further 2 min ramp, extension was done at 72 °C for 2 min. PCR was terminated as described.

For all the different PCRs mentioned above, negative and positive controls were included in every run. For the negative control, sterile water was added instead of nucleic acids. As positive control, we used bovine strains positive for the corresponding genes.

Analysis of the PCR products for *nuc* was performed by agarose gel electrophoresis using a 1.3% gel and ethidium bromide. PCR products of the 16S–23S rRNA intergenic spacer region, SET genes, *coa*, and *spa* were analyzed by the miniaturized electrophoresis system "DNA 7500 LabChip" (Agilent Technologies). This system separates pieces of DNA according to their size resulting in a plot of corresponding peaks (electropherogram) which can be monitored online on a personal computer and can be evaluated and translated into a pseudo-gel (see Fig. 1) by a particular software (Agilent Technologies). For interpretation of the 16S–23S rRNA intergenic spacer region results, two patterns were considered different if two and more peaks of the electropherogram differed in size. Subtypes differed in only one peak from the main genotype. The genotypes were named consecutively with letters starting from A; the subtypes were indicated by doubling the corresponding letter of the main genotype (for example AA).

Restriction enzyme analysis of *lukE* was performed as follows: 10 μl *lukE* PCR product in a total volume of 20 μl was incubated at 37 °C for 1 h containing 1x Restriction buffer L (Roche Diagnostics) and 0.5 U/μl *Rsa*I (Roche Diagnostics). Then 10 μl of the cleaved PCR product was analyzed by electrophoresis through a 1.8% (w/v) agarose gel and 0.5 μg/ml ethidium bromide.

To verify the specificity of the *nuc* and *lukE* PCR, the corresponding amplicons (single bands) were purified using the "QIAquick PCR Purification Kit" (Qiagen) according to the protocol of the manufacturer and were sequenced on both strands using the primers listed in Table 1. The sequencing procedure was done at Microsynth GmbH, Balgach, Switzerland. Compared to the bovine *S. aureus* strain RF122, the similarity for both the *nuc* and the *lukE* gene was 99%.

2.5. Analysis of somatic cells

To analyse the SCC, the milk samples were prewarmed at 37 °C for 10 min before analysis. The somatic cells were then counted with a Fossomatic 5000, Integrated Milk Testing (FOSS, Hillerod, Denmark).

2.6. Cluster analysis

In order to investigate the interrelationship among the various *S. aureus* isolates, the Wagner parsimony method with binary states was performed. For the current analysis, 11 variables were included and coded as 1 if the gene/polymorphism or restriction site for *Rsa*I was present or coded as 0 if they were absent. To test the tree, we analyzed an additional set of 65 most parsimonious trees which had been generated from the original data matrix. The analyses were done using the PHYLIP 3.6 software package obtained from J. Felsenstein (Department of Genome Sciences, University of Washington, Seattle, USA).

2.7. Statistical analysis

Data were expressed as frequencies or %. SCC values were $\log_{10}$-transformed. Associations for binary data were

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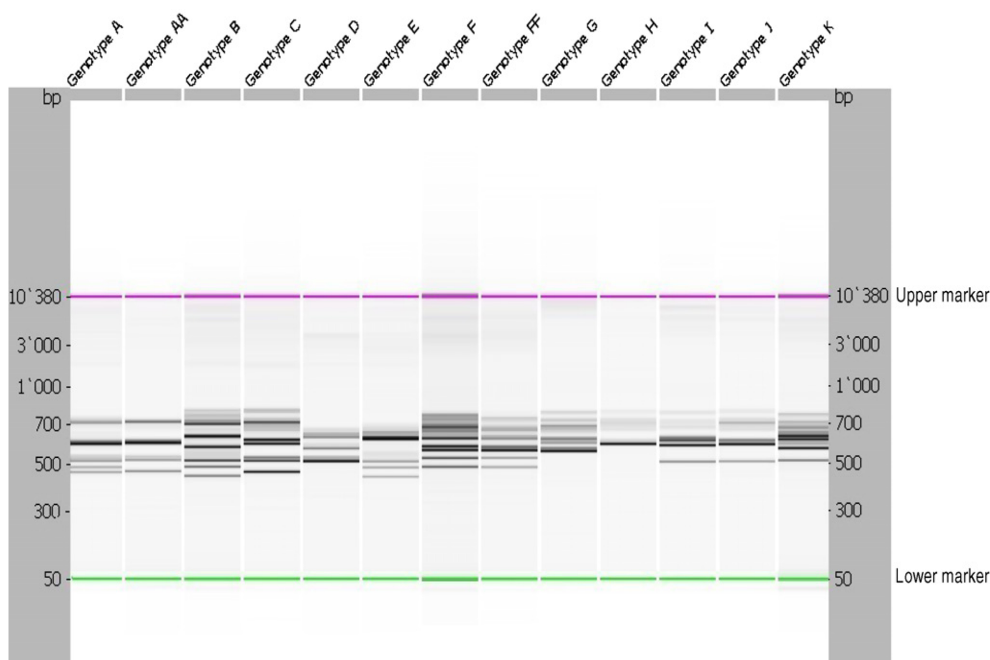

Fig. 1. Genotypes. Electrophoresis of various PCR products of the 16S–23S rRNA intergenic spacer region run in the DNA 7500 LabChip.

analyzed by computing the simple matching dichotomy coefficients (S4). Perfect and lacking associations result in a value of 1 and 0, respectively. The Chi-square test was used to analyze whether the number of infected quarters per cow depended on the genotype of *S. aureus* and whether coinfection with other mastitis-relevant pathogens is associated with particular genotypes. Single-factor analysis of variance was done by the Kruskal–Wallis test. Comparison between two groups was performed using the Mann–Whitney U test.

For analyzing the pathogenicity of *S. aureus* subtypes, the following procedure was applied to exclude a herd bias: if more than one isolate was found for a particular subtype in a herd, the mean of the $\log_{10}$SCC value over all the quarters with identical subtype was used. If there was only one value, the observed $\log_{10}$SCC value was chosen. As a consequence, for a particular genotype only one $\log_{10}$SCC value for each herd and genotype was used to calculate the geometric mean, minimum and maximum of SCC.

The method of generalized estimating equations (GEE) was used to compare the genotypes among each other based on the analyzed genes. GEE was introduced by Liang and Zeger (1986) and has become an important tool in analyzing longitudinal data or repeated measures as in the present case. If a *S. aureus* isolate showed a positive PCR result for a particular gene/polymorphism or a *Rsa*I restriction site, it was classified as present (=1); otherwise it was considered as absent (=0). For this study, 11 of these binary variables were included into the GEE analysis. Computation was performed using the 'proc genmod' of the SAS 8.0 statistical software package (SAS Institute, 2002). For all the other statistical analyses, Systat 10 (Systat Software, 2000) was applied. Significance was defined as values of $p < 0.05$. For all statistical analyses in which multiple testing was necessary, values of p were adjusted according to Holm.

3. Results

Besides the required presence of the *nuc* gene, all the 291 *S. aureus* strains proved positive for *spa* and *coa* confirming their identity.

For SET gene analysis, a subset of 101 epidemiologically independent isolates was used. Out of them, 96 (95.0%) were positive for at least one SET gene, whereas five isolates (5.0%) were completely negative for any of the tested genes. Eighteen strains showed 1 toxin gene, 21 strains 2, 42 strains 3 genes. Fifteen isolates presented 4 SET genes. The PCR products of the genes *sea*, *sec*, *sed*, *seg*, *sei*, *sej* and *tst* were in accordance with the predicted length in each case. The *seb*, *see* and *seh* genes were never detected in our isolates. As Table 2 demonstrates, there were high and low associations between various SET genes. In particular, *sec* and *tst* always occurred simultaneously (S4 = 1.0). In addition, there were high associations between *sed* and *sej* (S4 = 0.960), and *sea* and *sed* (S4 = 0.852). In contrast, the coefficients between *sea* and *seg* (S4 = 0.129) as well as between *seg* and *sea*/*sed* were remarkably small (S4 = 0.139). Interestingly, there was no association between *sei* and all the remaining SET genes (S4 ≈ 0.5).

Analysis of the *spa* *x*-region produced different sized amplicons (100 bp, 150 bp, 180 bp, 250 bp, 270 bp, 290 bp and 320 bp). The same was the case for the *coa* products: 560 bp, 640 bp, 730 bp, 850 bp and 940 bp. For *lukE*, 96.0% of the strains were positive with an amplicon of 830 bp. Restriction enzyme analysis with *Rsa*I revealed a

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Table 2
Association between the occurrences of staphylococcal enterotoxin genes

SET genes	*sea*	*sec*	*sed*	*seg*	*sei*	*sej*	*tst*
sea	1.0	0.426	0.852	0.129	0.594	0. 852	0.426
sec		1.0	0.356	0.683	0.515	0.396	1.0
sed			1.0	0.138	0.584	0.960	0.356
seg				1.0	0.475	0.139	0.683
sei					1.0	0.604	0.5185
sej						1.0	0.396
tst							1.0

The binary associations are expressed by the simple matching dichotomy coefficient (S4) based on the results obtained from 101 epidemiologically non-related strains of *S. aureus.* SET: Staphylococcal enterotoxin.

polymorphism near nucleotide 130 (position according to the bovine RF122 strain) producing two or three fragments. Further analysis by sequencing showed a silent mutation at nucleotide 132, whereby deoxythimidine was exchanged by deoxyadenosine leading to the loss of the *Rsa*I restriction site.

Analysis of the 16S–23S rRNA intergenic spacer region by RS–PCR revealed 17 different genotypes and two subtypes (a selection of 13 types is shown in Fig. 1). Out of them, type B and C were the most frequent ones as they made up 80.2% of all the isolates (genotype B: 30 isolates; genotype C: 51 isolates). The other 15 genotypes occurred only rarely ranging between 1.0% and 4.0% (equivalent to 1 to 4 strains). For further analysis, the rare genotypes were grouped together and were named ‘other genotypes’ (OG).

The patterns generated by RS–PCR and the miniaturized electrophoresis were well reproducible. To demonstrate this, three different strains were evaluated by 10 (pattern C and F) and 15 (pattern B) independent experiments, respectively. The typical patterns were always found and definite identification was possible at all the times.

As shown in Table 3, the genotypes B, C and OG differed clearly in their virulence gene pattern tested. Most genotype B strains were positive for the enterotoxin genes *sea*, *sed* and *sej*. All of them were negative for *sec, seg* and *tst*. The amplicon for the *x*-region of protein A gene was 250 bp or longer for all the genotype B strains, the *lukE* amplicon was cut by *Rsa*I into three fragments, and the *coa* amplicon was 640 bp. In contrast, all the genotype C strains were positive for enterotoxin gene *seg*. The majority of them were also positive for *sec* and *tst*. The amplicon for the *x*-region of protein A was only 100 bp long and *Rsa*I digestion of the *lukE* amplicon produced two fragments. The size of the *coa* amplicon was always 640 bp.

The results of the 20 OG are listed in detail in Table 4. Except for *sea*, all enterotoxin genes could be detected among these 20 strains. Again, *sec* and *tst* occurred together. Five genotypes (5 strains) were negative for all enterotoxin genes tested. For *spa*, amplicons of 100 bp to 320 bp could be detected. Amplicons of the coagulase gene were 640 bp, 730 bp, 850 bp or 940 bp long. The *lukE* amplicon was cut by *Rsa*I into two or three fragments. Five strains of the genotypes D, K, O and P were negative for the leukotoxin E gene.

Virulence gene patterns presented in Table 3 revealed obvious differences between the genotypes B, C, and OG. To further confirm these results, the three groups were compared by the statistical GEE procedure. According to this analysis, genotypes B and C ($p < 0.001$) as well as genotypes B and OG ($p < 0.001$) differed from each other in a highly significant manner. A significant difference was also observed for the genotypes C and OG ($p < 0.01$).

For cluster analysis, only epidemiologically independent isolates ($n = 101$) of *S. aureus* were included. The dendrogram (Fig. 2) confirmed the differences between the genotypes. In particular, the strains of genotype B and C formed two distinct groups whereas the OG strains were located in between, with a closer relation, however, to the C than to the B genotypes. The same topology was also found when an additional set of 65 most parsimonious trees was tested.

Considering the 26 cow herds tested, bacteriological analysis of all the single quarter milk samples revealed a total of 218 *S. aureus* strains (Table 5). There were 13 herds with one isolate and 6 herds with two isolates of *S. aureus*. In addition, we discovered 8 herds with 3, 7, 9, 10, 11, 47, 51 or 55 isolates, respectively. Furthermore, we found by non-parametric analysis of variance that the cow prevalence for *S. aureus* was highly affected by the genotype ($p < 0.001$). Indeed, there was a large difference between

Table 3
Distribution of virulence genes and their properties among epidemiologically independent strains of genotype B, C and other genotypes

Genotype	Number of strains	Number and percentage of strains positive for a particular gene/polymorphism										Amplicon size (bp)	
		sea	*sec*	*sed*	*seg*	*sei*	*sej*	*tst*	*lukE*	*Rsa*I *lukE* 2	*Rsa*I *lukE* 3	*spa*	*coa*
B[a,b]	30	23 77%	0 0%	26 84%	0 0%	10 33%	24 80%	0 0%	30 100%	0 0%	30 100%	⩾ 250	640
C[a,c]	51	0 0%	31 61%	0 0%	51 100%	17 33%	0 0%	31 61%	51 100%	51 100%	0 0%	100	640
Other[b,c]	20	0 0%	4 20%	4 20%	14 70%	5 29%	2 10%	4 20%	16 80%	7 35%	9 45%	⩾ 100	⩾ 640
Total	101	30	35	54	65	32	50	35	97	58	39		

Genotypes with identical superscripts differ significantly:
[a,b] $p < 0.001$.
[c] $p < 0.01$.

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Table 4
Distribution of virulence genes and their properties among rare genotypes (OG)

Genotype	Number of strains	Number of strains positive for a particular gene/polymorphism										Amplicon size (bp)	
		sea	*sec*	*sed*	*seg*	*sei*	*sej*	*tst*	*lukE*	*Rsa*I *lukE*2	*Rsa*I *lukE*3	*spa*	*coa*
A	1	0	1	0	1	0	0	1	1	1	0	100	640
AA	3	0	2	0	3	0	0	2	3	3	0	100	640
D	1	0	0	0	0	0	0	0	0	Negative		250	850
E	1	0	0	1	1	1	1	0	1	0	1	290	730
F	1	0	0	0	1	1	0	0	1	1	0	150	640
FF	1	0	0	0	1	1	0	0	1	1	0	250	640
G	1	0	1	0	0	0	0	1	1	0	1	320	940
H	1	0	1	0	1	1	0	1	1	1	0	100	640
I	1	0	0	0	0	0	0	0	1	0	1	100	940
J	1	0	0	0	1	0	0	0	1	1	0	100	640
K	2	0	0	0	2	2	0	0	0	Negative		270	730
L	1	0	0	0	0	0	0	0	0	0	1	100	940
M	1	0	0	0	0	0	0	0	1	0	1	290	640
N	1	0	0	1	1	0	1	0	1	0	1	290	850
O	1	0	0	1	1	0	0	0	0	Negative		180	640
P	1	0	0	1	1	0	0	0	0	Negative		180	640
R	1	0	0	0	0	0	0	0	1	0	1	290	940
Total	20	0	5	4	14	6	2	5	14	8	7		

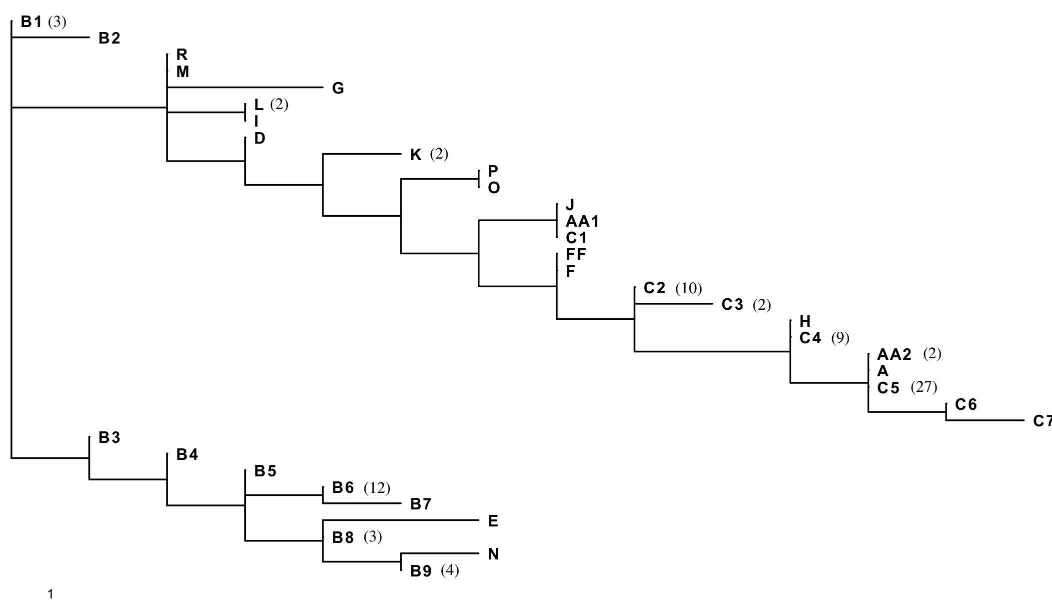

Fig. 2. Dendrogram. Tree obtained by the maximum parsimony method based on 11 genetic criteria. Each external node is a representative of a cluster containing 1 or more epidemiologically independent isolates with identical constellation of virulence factors. If there was more than 1 observation per cluster, the observed quantity is given in parenthesis. The letters of the external nodes specify the genotype. The following numbers indicate the different clusters of each genotype.

the medians of genotype B and C ($p < 0.001$) and between B and OG ($p < 0.001$; see Table 6 and Fig. 3). No difference, however, was observed between genotype C and OG ($p > 0.05$). On quarter level, the results were very similar (Table 6, Fig. 4). Again, the prevalence depended strongly on the genotype of *S. aureus* ($p < 0.001$): there was a marked difference between genotype B and C ($p < 0.001$), whereas the medians for genotype C and OG were almost equivalent ($p > 0.05$).

IMI caused by OG was always restricted to a single cow and quarter of a herd, respectively, (see Table 5). The same results were also obtained for the majority of IMIs caused by genotype C: in 64.7% of the herds ($n = 11$) where C types were found, always a single cow was infected; two and three positive cows were found in 5 and 1 herds, respectively. Constantly one quarter per cow was infected. Considering genotype B, always a considerable number of cows per herd was affected with a minimum of 2 and a maximum of 22 animals (median = 8.5). IMI was observed in 189 quarters ranging between 1 to 4 infected quarters per cow. 51 cows had a genotype B infection in 1 quarter, 49 cows in 2 to 4 quarters. Frequency table analysis showed

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Table 5
Epidemiological data and the associated genotypes of *S. aureus*

Herd	Cows total	Infected cows	Prevalence cow (%)	Quarters total	Infected quarters	Prevalence quarter (%)	Genotype (number of isolates)
1	24	2	8.3	96	2	2.1	C (1), A (1)
2	11	2	18.2	44	2	4.5	C (2)
4	18	7	38.9	72	7	9.7	B (7)
6	22	1	4.5	88	1	1.1	C (1)
7	17	1	5.9	68	1	1.5	C (1)
12	14	1	7.1	56	1	1.8	C (1)
16	14	1	7.1	56	1	1.8	C (1)
18	8	1	12.5	32	1	3.1	C (1)
20	14	1	7.1	56	1	1.8	E (1)
21	15	1	6.7	60	1	1.7	H (1)
22	9	2	22.2	35	2	5.7	B (2)
23	8	2	25.0	32	2	6.3	C (2)
26	25	1	4.0	99	1	1.0	C (1)
31	15	1	6.7	59	1	1.7	C (1)
33	16	2	12.5	62	2	3.2	C (1), D(1)
39	17	9	52.9	67	11	16.4	B (11)
40	10	2	20.0	40	2	5.0	C (2)
41	12	1	16.7	48	2	4.2	C (2)
42	4	1	25.0	16	1	6.3	R (1)
43	9	3	33.3	33	3	9.1	C (3)
48	10	1	10.0	40	1	2.5	C (1)
52	16	10	62.5	64	10	15.6	B (8), C (2)
54	45	20	44.4	180	47	26.1	B (48)
57	39	22	56.4	155	55	35.5	B (54), C (1)
58	34	23	67.6	135	51	37.8	B (50), J(1)
59	23	7	30.4	92	9	9.8	B (9)

Table 6
Cow and quarter prevalences depending on the genotype of *S. aureus* obtained by RS-PCR. B: genotype B. C: genotype C. OG: other genotypes (A, D, E, H, J, R)

	Cow prevalence (%)			Quarter prevalence (%)		
	B	C	OG	B	C	OG
Median	$47.2^{a,b}$	7.1^{a}	6.3^{b}	$14.5^{c,d}$	1.8^{c}	1.6^{d}
Maximum	64.7	33.3	7.1	37.0	9.1	1.8
Minimum	22.2	2.6	2.9	5.7	0.6	0.7

$^{a–d}$ Medians with identical superscripts differ with a value of $p < 0.001$.

that the number of infected quarters per cow depended strongly on the genotype ($p < 0.001$).

In all of the herds, only one or two different genotypes were observed (Table 5). In 21 herds there was 1 (81%), in 5 herds there were 2 subtypes (19%). If a B genotype was accompanied by another one, the B type was always the predominant one. The virulence gene pattern of B genotypes was very stable within a particular herd as all the B types of the herd showed the identical constellation (herds 4, 22, 39). The same was true for herd 52, 54, 57, 58 and 59 (data not shown).

Examination for IMI caused by coinfection with *S. aureus* and other mastitis-related pathogens (*Staphyloccus* spp., *Streptococcus* spp.) revealed the following results: out of the 189 quarters infected with genotype B, a complete bacteriological status was obtained in 185 cases. Monoinfection with *S. aureus* was observed in 155 quarters (83.8%), coinfection in 30 quarters (16.2%). Considering

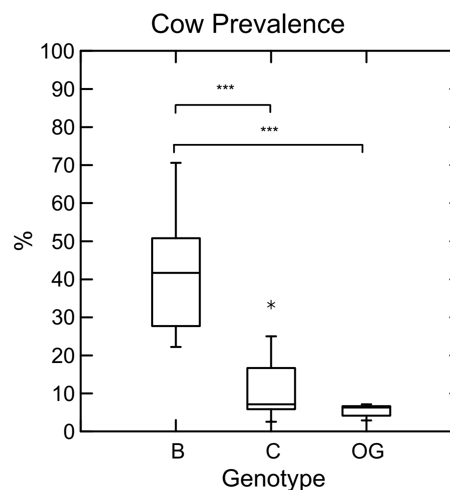

Fig. 3. Cow prevalences depending on the genotype of *S. aureus* obtained by RS-PCR. B: genotype B. C: genotype C. OG: other genotypes (A, D, E, H, J, R). $^{***}p < 0.001$.

the combined genotypes C and OG, 17 quarters showed a monoinfection with *S. aureus* (54.8%), 14 (45.2%) quarters a coinfection. Chi-square analysis for these data demonstrated that the number of monoinfected quarters depended strongly on the genotype ($p < 0.001$).

Analysis of SCC for IMI caused by monoinfection with *S. aureus* revealed rather small differences among the genotypes (see Fig. 5). For genotype B including data from 8 herds, the geometric mean was 7.85×10^5 cells/ml

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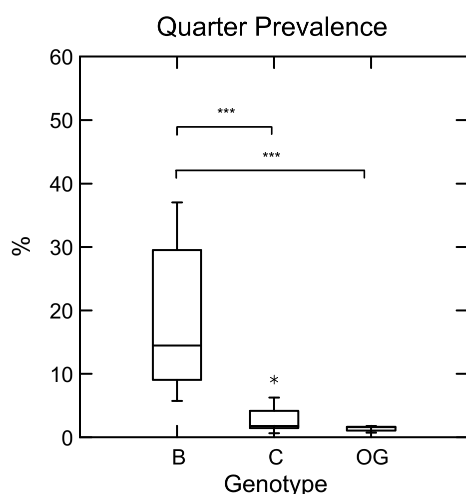

Fig. 4. Quarter prevalences depending on the genotype of *S. aureus* obtained by RS-PCR. B: genotype B. C: genotype C. OG: other genotypes (A, D, E, H, J, R). $^{***}p < 0.001$.

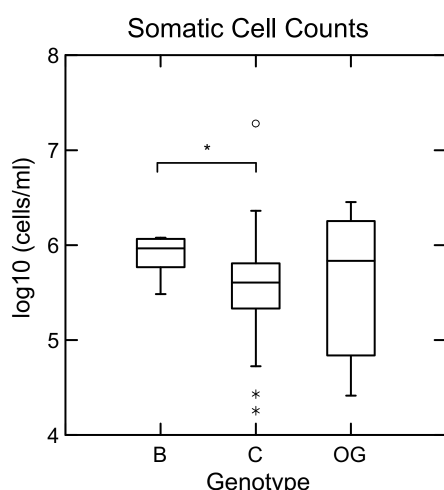

Fig. 5. SCC depending on the genotype of *S. aureus* obtained by RS-PCR. B: genotype B. C: genotype C. OG: other genotypes (A, D, E, H, J, R). $^{*}p = 0.025$.

(minimum = 3.05×10^5 cells/ml, maximum 1.20×10^6 cells/ml); for genotype C ($n = 19$), the geometric mean was 3.48×10^5 cells/ml (minimum = 1.80×10^4 cells/ml, maximum 1.91×10^7 cells/ml). Considering OG ($n = 6$), a geometric mean of 4.05×10^5 cells/ml was obtained (minimum = 2.60×10^4 cells/ml, maximum 2.84×10^6 cells/ml). A significant difference was observed between the means of genotype B and C ($p = 0.025$) but not between the other combinations.

4. Discussion and conclusions

The present study shows that the genotypes of bovine *S. aureus* causing IMI are highly associated with the virulence gene patterns and the epidemiological and pathogenic properties of this agent. The results further demonstrate that *S. aureus* isolated from IMI form a heterogeneous group requiring subtyping in the future.

The virulence genes were frequently linked. In particular, there was a high to perfect association between *sec*, *seg* and *tst* (linkage 1) as well as between *sea*, *sed* and *sej* (linkage 2). According to bioinformatic genome analysis of the bovine *S. aureus* strain RF122 as well as according to Fitzgerald et al. (2001), *sec* and *tst* are located close together (less than 2.2 kilobases (KB) apart) on the bovine staphylococcal pathogenicity island (SaPIbov) whereas *seg* is more than 1.4 megabases (MB) apart. This explains on one hand the perfect association of the *sec* and *tst* genes and on the other hand the lower, but still high association between the *sec*/*tst* and *seg* genes (S4 = 0.752). Based on our results, we also expect a close genetic association between *sed* and *sej*. Indeed, both genes are located on plasmid pIB485, and are separated by an intergenic region of less than 1 KB (Zhang et al., 1998).

Interestingly, the 2 gene linkages could be clearly attributed to the 2 main genotypes. Linkage 1 was observed with genotype C, linkage 2 with genotype B. Furthermore, it was possible to associate the identified virulence gene polymorphisms with particular genotypes (see Table 3). Combining these findings, genotype B was associated with *sed* (84%), *sej* (80%) and with *sea* (77%), as well as with a long amplicon of *spa* (100%) and a *lukE* amplicon cut into three fragments by *Rsa*I (100%). Genotype C was associated with *seg* (100%), *sec* and *tst* (61% each), a short amplicon of *spa* (100%) and a *lukE* amplicon cut into two fragments (100%). The other genotypes were heterogeneous in the presence of enterotoxin genes, length of *spa* amplicon, the presence and the number of restriction sites in *lukE* as well as in length of coagulase gene amplicon.

Comparison of genotypes B and C as well as genotypes B and OG by GEE showed highly significant differences in there virulence gene patterns ($p < 0.001$). The patterns were also different between genotype C and OG ($p < 0.05$); however, the difference was less evident. The high degree of association between genotyping by RS-PCR and the gene patterns indicates that the overall genetic information generated by the two approaches is very similar.

The obtained GEE results could be confirmed by cluster analyses. Although we examined only a limited number of criteria disabling a detailed interpretation of the most parsimonious tree in Fig. 2, the results demonstrated that the types B and C were located most apart from each other. In between them, the OG were positioned from which the C types had evolved. Our results further showed that the B types formed a homogenous cluster which was clearly separated from most of the OG. To confirm these findings, we used an additional set of most parsimonious trees. This analysis always resulted in the same topology: B genotypes – OG – C genotypes. The results of the cluster analyses were in good agreement with the statistical ones. In addition, cluster analysis explained the fact that the observed statistical difference between the C and OG was minor compared to the difference between the B and OG. For a

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more accurate phylogenetic statement, however, more criteria need to be included.

The fact that the enterotoxin genes *see* and *seh* were never detected was in concordance with the results of a German (Zschöck et al., 2005) and a Swiss (Stephan et al., 2001) study on bovine *S. aureus.* Considering the *seb* gene, Zschöck et al. (2005) found one positive isolate in 61 analyzed strains, whereas we could never detect this SET gene in our samples. Interestingly, *sea* was repeatedly discovered in some studies (ours, Stephan et al., 2001; Stephan et al., 2002; Haveri et al., 2007) while this gene or its product was absent in others (Srinivasan et al., 2006; Cenci-Goga et al., 2003). *sea* is carried by a family of temperate bacteriophages whose genomes incorporate and replicate with that of *S. aureus* (reviewed by Le Loir et al., 2003). The reason for the observed discrepancy is unclear. May be the geographical distribution of these phages is irregular so that the rate of infected *S. aureus* is changing or infection is even lacking.

The size polymorphisms in the *x*-region of *spa* that we found in the present study were consistent with the results of other studies (Frenay et al., 1996; Stephan et al., 2001). The polymorphism found in *lukE* was expected to have no impact on the function of leukotoxin E, but it was a strong discriminator.

According to our study, the cow and quarter prevalence depended strongly on the genotype ($p < 0.001$). Indeed, genotype B was exclusively observed in herds having real problems with IMI caused by *S. aureus*, as the prevalences on cow (up to 65%) and quarter level (up to 37%) were always very high. Frequently more than 1 quarter per cow was infected. These observations are in clear contrast to those made for genotype C and OG. In the latter cases, IMI was restricted to 1 or very few cows per herd resulting therefore in low prevalences. Constantly 1 quarter per cow was infected. Based on these results we conclude that *S. aureus* of genotype B is much more contagious than the C or OG types; genotype B affects whole herds, genotype C and OG single animals. IMI caused by type B *S. aureus* is almost exclusively a monoinfection whereas in half of the cases infection with genotype C or OG is frequently associated with additional mastitis-relevant pathogens. These findings, however, do not proof that the latter types are apathogenic. Actually SCC analysis of pure *S. aureus* IMI demonstrated that most of the C and the majority of the OG genotypes were associated with counts above 100,000 cells/ml, the upper limit of physiological SCC (Hamann 2003). Interestingly, for all the genotypes there were a few quarters with SCC below 100,000 cells/ml. May be, these isolates originated from contaminated teat skin or cellular shedding was transiently low at the day of sampling. Further studies, however, are necessary to elucidate these results. Genotype B is not only highly contagious, it is also associated with increased pathogenicity as SCC for this type is significantly higher ($p = 0.025$) than for C or OG. Compared to contagiosity, however, this property is less evident. Due to the different epidemiological properties of the subtypes, genotyping mastitis-related isolates of *S. aureus* is clinically very helpful, even for routine purposes: in the case of a milk sample positive for genotype B, further quarters of the same herd will be infected by this pathogen (herd problem). Under these circumstances, clinical treatment requires the classical sanitation program which is extensive, costly and long lasting. Genotypes C and OG, however, include single quarters within a herd so it is assumed that these types are to be treated as coagulase-negative *staphylococci.* In this case, the treatment will be simple and cheap.

The applied genotyping methodology is well characterized and its discriminatory power is very similar to the one of PFGE (Kumari et al., 1997), the proposed gold standard to genotype *S. aureus* (Weller 2000). Indeed, we found 17 different genotypes by the RS-PCR demonstrating its excellent discriminating properties. Furthermore, the RS-PCR was highly reproducible and allowed a high throughput (per day, up to 50 samples can be analyzed by a single person). In addition, RS-PCR is easy to perform and rather cost-efficient. These are prerequisites for future clinical and epidemiological studies on *S. aureus* mastitis strains as these types of investigations require a high sample throughput.

In conclusion, our results demonstrate that the genotypes of bovine *S. aureus* causing IMI are highly associated with the virulence gene patterns and the epidemiological and pathogenic properties of this pathogen. Genotyping by RS-PCR represents an accurate, rapid, and cheap tool for future field studies on *S. aureus* mastitis strains and generates clinically relevant results.

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