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Microarray based study on virulence-associated genes and resistance determinants of *Staphylococcus aureus* isolates from cattle

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Abstract

*Staphylococcus aureus* is a common pathogen which can colonise and infect not only man, but also domestic animals. Especially, infection of cattle is of high economic relevance as *S. aureus* is an important causal agent of bovine mastitis. In the present contribution, a DNA microarray was applied for the study of 144 different gene targets, including resistance genes and genes encoding exotoxins, in *S. aureus* isolated from cows. One hundred and twenty-eight isolates from Germany and Switzerland were tested. These isolates were assigned to 20 different strains and nine clonal complexes. The majority of isolates belonged either to apparently closely related clonal complexes 8, 25, and 97 (together 34.4%) or were related to the sequenced bovine strain RF122 (48.4%). Notable characteristics of *S. aureus* of bovine origin are the carriage of intact haemolysin beta (in 82% of isolates tested), the absence of staphylokinase (in 89.1%), the presence of allelic variants of several exotoxins such as toxic shock syndrome toxin and enterotoxin N, and the occurrence of the leukocidin *lukF*-P83/*lukM* (in 53.1%). Two isolates were methicillin-resistant *S. aureus* (MRSA). One of them was a clonal complex 8 MRSA related to the epidemic MRSA strain Irish 01. The other one belonged to ST398/spa-type 34 resembling a newly emerging MRSA strain which has been described to occur in humans as well as in domestic animals. The presence of these two strains highlights the possibility of transfers of *S. aureus* strains between different host species.

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1. Introduction

*Staphylococcus aureus* can cause a variety of pyogenic infections and toxin-mediated conditions. It harbours a complex system of exotoxins which facilitates the killing of phagocytes, lysis of erythrocytes, or manipulation of the T-cell response (Dinges et al., 2000). Many *S. aureus* isolates carry multiple exotoxins, and there is a high redundancy within classes of exotoxins. There are, at least, four distinct bicomponent leukocidins, approximately 20 different superantigenic toxins (TST and enterotoxins), three exfoliative toxins, and six haemolysins. This diversity could be a reason for the ability of *S. aureus* to colonise or to infect different host species. Infection of cattle is of high economic relevance as *S. aureus* is an important agent of bovine mastitis (Akineden et al., 2001; Nickerson et al., 1995; Rainard et al., 2003; Zschock et al., 2005). The knowledge of possible host specificities of certain virulence factors could be important for the understanding of the pathogenesis and for vaccine development.

Antimicrobial resistance of *S. aureus* is an increasingly important problem. The most important issue is resistance towards methicillin and all beta-lactam antibiotics (methicillin-resistant *S. aureus*, or MRSA). There are reports of veterinary hospitals suffering from MRSA epidemics (Seguin et al., 1999), and it has also been demonstrated that pets can become silent carriers of MRSA (Manian, 2003). The emerging epidemic of virulent, Panton-Valentine leukocidin carrying MRSA in humans appears to be mirrored by clinically similar infections in pets (Rankin et al., 2005).

For these reasons, research on *S. aureus* in veterinary medicine should, like in human medicine, focus on antimicrobial resistance and on the issue of exotoxins. In the present contribution, a diagnostic DNA microarray based assay was applied for the study of resistance genes and genes encoding exotoxins in *S. aureus* isolated from cows.

2. Materials and methods

2.1. Isolates and DNA preparation

One hundred and twenty-eight bovine *S. aureus* isolates were tested. Fifty of them were obtained by the Institute of Veterinary Bacteriology in Bern, Switzerland, in order to diagnose cases of bovine mastitis. Another 77 were sampled as a part of routine surveillance activities by the Animal Health Service of Thuringia and were sub-cultivated by the Friedrich Loeffler Institute (FLI), Germany. One isolate was found by local authorities in a case of septic abort in Thuringia. All isolates were cultured on Columbia blood broth (OXOID, Wesel, Germany) and incubated 12–48 h at 37 °C. Single colonies were picked for further sub-culturing. Genomic DNA was prepared after enzymatic lysis using 100 μl of a lysis solution containing 0.05 mg lysostaphin (AMBI Products, Lawrence, USA), 2 mg lysozyme (Sigma, Steinheim, Germany), 2 mg ribonuclease A (Sigma), 2 μl Tris/HCl (20 mM, pH 8.0), 2 μl EDTA (2 mM), and 1 μl Triton X-100. After incubation (45 min, 37 °C, 300 rpm), 10 μl proteinase K and 100 μl of buffer AL (both from Qiagen kit DNeasy™, Qiagen, Hilden, Germany) were added. After a second incubation (45 min at 56 °C) the sample was processed using the Qiagen device EZ1 according to the tissue lysis protocol supplied by the manufacturer.

2.2. Amplification, labelling, and array hybridisations

Diagnostic DNA microarrays based on the ArrayTube (AT) platform (CLONDIAG chip technologies, Jena, Germany) were used for genotyping of staphylococcal DNA. These microarrays were manufactured and processed as described previously (Korczak et al., 2005; Monecke and Ehricht, 2005; Monecke et al., 2003). A set of probes covering species markers of *S. aureus*, antibiotic resistance determinants and toxins, as well as probes for the discrimination of allelic variants of the accessory gene regulator (*agr*) cluster has already been described and evaluated (Monecke et al., 2007; Monecke and Ehricht, 2005). The array comprised the probes listed in Table S1 (online supplement) in twofold redundancy. The set of probes included all sequences which were available at the time of completion of bioinformatic work-up (early 2005). RF122 genome sequence data (GenBank NC_007622.1) were not covered as they were released only after the completion of the array.

Genomic target DNA was amplified in a linear manner and labelled using a multiplex primer
elongation reaction incorporating biotin-16-dUTP (Roche Diagnostics, Penzberg, Germany), which has been described previously in detail (Monecke et al., 2007; Monecke and Ehricht, 2005). Primers are listed in Table S1 (online supplement). Biotinylated single stranded amplicons were subsequently hybridised to the array and hybridisation was visualised using streptavidin–horseradish–peroxidase and a precipitating chromogenic substrate (Seramun green, Seramun, Woiziaig, Germany). The array tube reader ATR01 and the Iconoclust software package (CLONDIAG chip technologies) were used combined with a defined script according to manufacturers recommendations and to the procedure described in Monecke et al. (2007) and Monecke and Ehrich (2005) in order to record and to analyse hybridisation patterns.

2.3. Sequencing

PCR amplification and sequencing were performed when ambiguous hybridisation results, the presence of only one component of a bicomponent toxin, or the
absence of a toxin from a previously known pathogenicity island were observed.

For detection of the gene encoding toxic shock syndrome toxin ( tstI ) and enterotoxin gene N ( sen ) following primers were used, respectively: tstI-fw 5′-ACGTTTACACATTTGGAAGG-3′ and tstI-rv 5′-CGTTTAAAGGATATATGTTTAT-3′, sen-fw 5′-CTATTAATGTAGAAGGTTCTATTAA-3′ and sen-rv 5′-TCATGATTGAGTATAATC-3′.

Primers for lukE were lukE-fw 5′-AAGTGAAAATATACTAGAATAAATTTGT-3′ and lukE-rv 5′-GTTTTTGTAGTTACATTTTG-3′, and splA was amplified using primers splA-fw 5′-TGTATATAGTTAATGTTATCT-3′ and splA-rv 5′-TGATATAGTATCTAGAATTTGT-3′. PCR was performed using polymerase (1 µl per 100 µl reaction mixture) and 10 × PCR-buffer by Genaxxon BioScience GmbH (Biberach, Germany) adding 1.5 mM MgCl₂ and 1 µM of each primer. PCRs were uniformly performed with a denaturation step of 2 min at 95 °C, followed by 35 cycles: 30 s at 95 °C, 20 s at 55 °C, and 45 s at 72 °C, and by a final extension period of 5 min at 72 °C.

The amplicons were inserted into a commercially available cloning vector (pCR2.1-topo, Invitrogen, Germany), and recombinants were subjected to DNA sequencing using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Darmstadt, Germany), M13 primers, and an ABI PRISM 310 Genetic Analyser (Applied Biosystems) according to manufacturers instructions.

2.4. spa-typing and MLST

spa-typing is based on sequence polymorphisms of repeating units within the staphylococcal protein A gene (spa). It was performed according to previously described protocols (Harmsen et al., 2003). Types were assigned using the code system described on Ridom SpaServer website (www.spa.ridom.de), and SpatypeMapper software (CLONDIAG) freely down-
MLST for selected isolates was performed as described by Enright et al. (2000).

### 2.5. Split network tree construction

The tree shown in Fig. 5 was constructed using the SplitsTree software (Huson and Bryant, 2006) on default setting. Some sequenced or other reference strains were also included for comparison. RF122 and the sequenced isolate of USA300 were not available for testing. For the latter one, hybridisation results of clinical isolates (Monecke et al., 2007) were used instead. Hybridisation results for every single gene (as shown in Figs. 1–4) were converted into a code (A = gene present, T = gene absent, G = gene yielding variable results within one strain; C = gene appearing to be present in a deviant allelic variant, i.e., being detectable only in experiments with high DNA load, or by PCR). The resulting “sequence”, which represented the complete hybridisation pattern of a given strain, was used subsequently for tree construction.

### 3. Results

#### 3.1. Typing and strain characteristics

Using microarray analysis, it was possible to assign the 128 isolates included into the study to 20 distinct strains belonging to seven clusters or clonal lines.
Clusters and strains primarily were distinguishable by hybridisation profile (Figs. 1–4), by spa-typing, MLST, and by comparison of hybridisation profiles to strains of known affiliation.

Following strains were discernible:

Strains 1 and 2 were agrI/clonal complex (CC) 97 strains. One representative isolate was subjected to MLST belonging to ST97. These isolates were very similar to the sequenced strain NCTC8325 (CC8), but genes ssl1, ssl3, and ssl11, respectively, ssl1, and ssl11 were absent.

The hybridisation profile of Strain 3 (agrI/CC8) was practically identical to NCTC8325, its spa-repeats were similar. Strain 4 was related to Strain 3, carrying the same virulence factors, but it was a MRSA harbouring mecA, the tobramycin resistance determinant aadD, and the macrolides/clindamycin resistance gene ermA. Strains 5 and 6 differed in their carriage of exotoxin genes harbouring, respectively, sed + sej + ser or sea + sed + sej + ser.

Strains 7 and 8 gave an overall similar hybridisation pattern, but contained the egc-enterotoxin gene cluster (seg + sei + sem + sen + seo + seu/y), or that cluster plus the bovine leukocidin lukF-P83/lukM. Strain 8 was subjected to MLST, yielding ST20.
Strain 9 belonged to agr-group I and spa-type t034, and strain 10 was an otherwise similar MRSA. It belonged to ST398 and spa-type t034.

Strain 11 belonged to agr-group I and spa-type t1403. According to MLST, it was ST132/CC133.

Strain 12 was agr-group II and, according to hybridisation pattern and spa-type (t002), it belonged to CC5. Strains 13–18 resembled RF122, a sequenced strain of bovine origin, with regard to spa-types and overall virulence gene content, including the presence of leukocidin lukF-P83/lukM. Some differences in exotoxin carriage allowed an assignment of isolates to strains 13 (sed), 14 (ssl4), 15 (tst1, sec, sel, and ssl4), 16 (ssl7), 17 (tst1, sec, sel, and ssl7), or 18 (sec, tst1, sec, and sel).

Strain 19 was agr-group II and spa-type t160. Strain 20 belonged to agr-group III and to CC1.

Representative isolates of every strain as defined by array hybridisation were spa-typed. Typing results are summarised in Table 1. Fig. 5 shows similarities between the strains in form of a split network tree.

### 3.2. Superantigen carriage

Strains 15, 17, and 18 (23 isolates) harboured the pathogenicity island SaPIbov (Fitzgerald et al., 2001) comprised of enterotoxin genes C and L as well as gene for toxic shock syndrome toxin 1 (tst1). Enterotoxin genes sec and sel were detected by array hybridisation, but tst1 was not detectable. According to the description of the bovine pathogenicity island (Fitzgerald et al., 2001), these three genes were expected to occur together. Therefore, a PCR for the detection of tst1 was developed (for primer sequences, see above), and tst1 was indeed found in strains 15, 17, and 18. Sequencing of PCR products from two strain 18 isolates (GenBank entries EF531614 and EF531615) confirmed the presence

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**Table 1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Strain definition</th>
<th>Detected spa-types</th>
<th>Isolates from Switzer-land</th>
<th>Isolates from Thuringia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain 1</td>
<td>agr I/clonal complex 97, ssl1,3,11-neg.</td>
<td>04-17; 07-16-12-21-17-34-13-33-34-33-34; 07-16-12-21-17-34-33-34-33-34</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>Strain 2</td>
<td>agr I/clonal complex 97, ssl5-pos.</td>
<td>t028 (-04-); (07-23-21-17-34-33-34-33-34-33-34)</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Strain 3</td>
<td>agr I/clonal complex 8, ssl1,3,11-pos.</td>
<td>04-20-21-17-34-22-25; 11-12-21-17-34-22-25-25</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Strain 4</td>
<td>agr I/clonal complex 8 MRSA</td>
<td>t068 (11-19-19-12-21-17-34-24-34-22-25)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Strain 5</td>
<td>agr I/clonal complex 8, sed, sel, ser-pos.</td>
<td>11-12-21-17-34-24-34-22-25-25</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Strain 6</td>
<td>agr I/clonal complex 8, sea, sed, sel, ser-pos.</td>
<td>11-12-21-17-34-24-34-22-25-25</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Strain 7</td>
<td>agr I/clonal complex 20, ege-pos.</td>
<td>26-06-17-21-34-24-22-13-13</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Strain 8</td>
<td>agr I/clonal complex 20, ege-pos., lukF-P83/M-pos.</td>
<td>07-06-17-21-34-22-24-34-13</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Strain 9</td>
<td>agr I/ST398/spa t034</td>
<td>t034 (08-16-02-25-02-25-34-24-25)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Strain 10</td>
<td>agr I/ST398/spa t034, MRSA</td>
<td>t034 (08-16-02-25-02-25-34-24-25)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Strain 11</td>
<td>agr IV clonal complex 133</td>
<td>Two isolates typed, both t1403 (03-23-24)</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Strain 12</td>
<td>agr II/clonal complex 5</td>
<td>t002 (26-23-17-34-17-20-17-12-17-16)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Strain 13</td>
<td>agr II/RF122-like, sed-pos.</td>
<td>04-20-17-25-17</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Strain 14</td>
<td>agr II/RF122-like, ssl4-pos.</td>
<td>Two isolates typed, both 04-20-17-31-24</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Strain 15</td>
<td>agr II/RF122-like, sec, sel, ssl4-pos.</td>
<td>04-20-17-31-24</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Strain 16</td>
<td>agr II/RF122-like, ssl7-pos.</td>
<td>Four isolates typed, all t529 (04-34)</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>Strain 17</td>
<td>agr II/RF122-like, sec, sel, ssl7-pos.</td>
<td>Four isolates typed, three times t529 (04-34) and once-34</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Strain 18</td>
<td>agr II/RF122-like, seh, sec, sel-pos</td>
<td>t529 (04-34)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Strain 19</td>
<td>agr II/spa t160</td>
<td>t160 (07-23-21-24-33-22-17)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Strain 20</td>
<td>agr III/clonal complex 1</td>
<td>t127 (07-23-21-16-34-33-13) t174 (14-21-16-34-33-13)</td>
<td>0</td>
<td>13</td>
</tr>
</tbody>
</table>
of a variant of \textit{tst1} which differed in three positions within the probe binding site from the \textit{tst1} sequences of human isolates (AB084255.1, AY074881.1, BA000017.4 [2137855:2137879], BA000018.3 [2061422:2061446], J02615.1, U93688.2). Regarding this region, both sequences were identical to previously published sequences from bovine isolates (AF217235.1 and NC_007622.1 [399750:400454]; see Fig. 6).

The gene encoding enterotoxin A was found in six isolates, all of which were positive for \textit{sak} and negative for \textit{hlb}. Five isolates belonged to two \textit{agr}-type I clusters. One isolate, which belonged to \textit{agr}-type II, carried a variant of \textit{sea} (as in sequence strain N315; BA000018.3). The enterotoxin B gene was found in strain 18, which also harboured SaPIbov.

Two CC8 strains (strain 5 and 6; seven isolates) contained genes for enterotoxins D, J, and R. These three genes always were found together.

The \textit{egc}-cluster (comprising of enterotoxins genes G, I, M, N, O, and U/Y) was found in CC20 strains 7 and 8, in strain 12, and in the RF122-like strains 13–18. In the latter, the enterotoxin N gene was not reliably detectable by probe hybridisation yielding positive results only in samples with very high DNA content. PCR products were obtained for strain 16, strain 17, and strain 18 isolates. Sequencing of these products (GenBank entries EF531604, EF531605, and EF531606) showed identity to the RF122 sequence (NC_007622.1 [1839538:1840293]). Compared to the Mu50 sequence (NC_002758.2 [1955162:1955938]), two bases within the probe binding site were exchanged (Fig. 6).

### 3.3. \textit{ssl}-gene carriage

\textit{ssl}-genes were used for assignment of isolates to strains and clonal complexes. Results are summarised in Fig. 2.

### 3.4. Leukocidin carriage

The Panton-Valentine leukocidin (\textit{lukS/F}-PVL) was not found, but the bicomponent leukocidin \textit{lukF-P83/lukM} was detected in 68 out of 128 isolates (53.1%) belonging to strains 8, 11, and 13–18. Notably, 61 of them (90%) were \textit{agr}-type II.

Leukocidin \textit{lukD/E} was found in all but two related strains (strain 9 and 10) although some isolates gave variable, weak, or only PCR-positive results. Because weak or variable signal intensity was regarded as an indicator for sequence variation, PCR primers were
designed, and sequencing of cloned PCR products was performed. PCR products of one strain 16 isolate, two strain 17 isolates and one strain 18 isolate were sequenced. GenBank entries are for strain 16, EF531607; for strain 17, EF531609 and EF531610; and for strain 18, EF531608. Compared to COL and Mu50 sequences (NC_002951.2 [1935656:1936591] and NC_002758.2 [1948025:1948960]), two base exchanges within the probe binding sites of these isolates were found. Additionally, the primer binding sites of these isolates differed in one or two (strain 16) positions from RF122 (NC_007622.1 [1831611:1832546]) and Mu50 sequences (Fig. 6).

A putative bi-component leukocidin (SAV2004 and 2005, SA1812 and 1813, MW1941 and 1942; "lukX/Y" in Fig. 3) was also found in the majority of isolates.

3.5. Haemolysin carriage and staphylokinase

Genes for haemolysins alpha, delta, and gamma (hla, hld, and the hlg-locus comprised of lukS, lukF, and hlgA) were present in all isolates. All isolates also contained an unnamed putative haemolysin (BA000017.4 [963137:963165], CP000046.1 [927983:928011, hl in Fig. 3). The haemolysin gene hllIII was not detectable in the RF122-like clonal complex (strains 13–18) as well as in strain 11.

As staphylokinase (sak) and enterotoxin A carrying phages can integrate into the haemolysin beta gene hlb (Coleman et al., 1989, 1991; Iandolo et al., 2002), the relations of these genes were analysed. Primer and probe were designed to engulf that insertion site. Therefore, a positive signal can be interpreted as indicator for the presence of untruncated hlb and for the ability to express haemolysin B (Monecke et al., 2007; Monecke and Ehricht, 2005). One hundred and five out of 128 isolates (82%) were positive for hlb. Staphylokinase (sak) was rather rare, being detectable in 14 isolates (10.9%). Nine out of 23 hlb-negative isolates were positive for sak, while only five out of 105 hlb-positive isolates harboured that gene, all of these being strain 20.
Six out of 23 hlb-negative isolates contained sea, and this gene was not found among hlb-positive isolates.

3.6. Other virulence genes

Genes coA (although some isolates showed only a weak coagulase reaction), katA, sbi, and spa always were present. Exfoliative toxin genes (eta, etB, etD) and epidermal cell differentiation inhibitor genes (ednA–C) were not found.

Protease genes splA and B were present in all strains but strain 9 and 10. Deviant sequence variants of splA in RF122-like strains were detectable by PCR. Isolates from strains 13, 16, and 17 were subjected to sequencing (GenBank entries EF531611, EF531612, and EF531613). All isolates showed identical primer binding sites as the RF122 sequence (AJ938182 [1818275:1818967]), which differed from the Mu50 sequence (NC_002758.2 [1942203:1942910]) in two positions. Concerning the probe binding site, RF122 and Mu50 sequences were identical, but strain 13 differed in one, and strain 16 and 17 in another position (Fig. 6).

3.7. Antibiotic resistance

Two isolates, strains 4 and 10 were mecA-positive. strain 4 belonged to agr-type I, CC8, and spa-type t068. It also carried the tobramycin resistance determinant aadD and the macrolide resistance gene ermB. Strain 10 was agr-type I, ST398, t034, and appeared to be closely related to strain 9 (see Fig. 5). It carried two macrolide/clindamycin resistance genes (ermA and ermC), as well as tetM which confers resistance to tetracyclines.

Among the other isolates, resistance genes were rather rare. Twenty-seven isolates carried the beta-lactamase gene blaZ. Among the methicillin-susceptible isolates, one harboured the macrolide/clindamycin resistance gene ermB, and another two contained ermC. The clindamycin/lincomycin resistance gene linA was found twice. The tetracycline resistance determinant tetM was present in one MRSA (strain10) and in six methicillin-susceptible isolates. None of the other resistance genes represented on the array was detected (see Fig. 4).

4. Discussion

S. aureus is an important agent of infection in man as well as in cattle. The aim of the study was to get insight into the virulence and antibiotic resistance traits of cattle isolates and to find possible differences in virulence factors between S. aureus isolates of human and bovine background. The geographic origin of the isolates from two rather distant areas, the Bern region, Switzerland, and Thuringia, Germany, allowed to exclude the theoretical possibility of confusing the features of predominant regional outbreak strains with host specific properties. The most conspicuous regional difference was the presence of a CC1 strain in Thuringia, which was not observed in Bern.

A remarkable difference between human and bovine S. aureus was the presence of leukokidin lukF–P83/lukM in a great proportion of bovine isolates (53.1%). This phage-borne bi-component leukokidin (Kaneko and Kamio, 2004; Kaneko et al., 1997; Yamada et al., 2005; Zou et al., 2000) has previously been found to be associated with mastitis in cows, ewes, and goats (Rainard et al., 2003). There is apparently no publication describing that toxin from host organisms other than ruminants, and the authors also were not able to detect its genes in a large number of human isolates (Monecke et al., 2007). On the other hand, S. aureus sampled from humans carry related, but discernible leukokidin genes (lukS/P–PVL). This allows to assume some host specificity which, ultimately, might lead to the evolution of distinct human and bovine populations of S. aureus.

Another striking difference between human and bovine isolates was observed regarding the presence of the staphylokinase gene sak. In human strains, sak is highly prevalent (Monecke et al., 2007) and haemolysin beta usually is inactivated by the insertion of phages carrying sak or sak and sea (Coleman et al., 1989, 1991; Iandolo et al., 2002). Some sak-positive bovine isolates also carried sea, of which two distinguishable variants were detected. Therefore, it can be assumed that at least three different phages were prevalent within the studied strains. Some ST1 isolates contained both, hlb and sak possibly indicating an integration of the phage at an unusual site. In some RF122-like strains hlb was disrupted although no sak was detectable. This could either indicate a fourth phage, or a possible truncation of one of the
phages which integrate into hlb. Generally, only a minority of bovine S. aureus carried sak, and these cases could possibly indicate human–cow-transmissions. For instance, the CC8 MRSA (strain 4) resembles a strain which is known to be prevalent in humans. It can be speculated that the presence of sak in staphylococci which colonise or infect humans gives a selective advantage which is more pronounced than the disadvantage caused by the loss of haemolysin beta. For the colonisation/infection of cows, staphylokinase could be less important than hlb. Another possibility is that sak-carrying phages emerged rather recently and simply had no time to spread widely in a bovine environment. This could be in accordance to the observation that the pandemic “phage pattern 80/81” strain from the 1940s did not carry sak (Monecke et al., 2007).

For two toxins, tst1 and enterotoxin N (sen), divergent sequences were found which appear to be specific for S. aureus of bovine origin as the recently released genome sequence of the bovine strain RF122 also contained similar variants of tst1 and sen. Human isolates of S. aureus should be screened in order to find, or to rule out, the occurrence of these variants. Both, bovine tst1 and sen, could not be detected directly using the array as sequence variations affected probe binding sites. Array hybridisation patterns, however showed signals for sec and sel, which form a pathogenicity island with tst1 or, respectively, for sem and seo which accompany sen. Because of these reactions, the presence of variants of tst1 and sen was suspected, and eventually confirmed by PCR and sequencing. The detection of these genes shows an advantage of array-based genotyping compared to other detection methods. Although some genes were not detectable because of yet unrepresented allelic variants, the array facilitated “proofreading” by generating enough information to check, e.g., for genes missed from known clusters or pathogenicity islands. The putative pathogenicity island SaPlbov comprising tst1, sec, and sel was found in strain 15, 17, and 18, but it was missed in closely related strains 13, 14, and 16. This underscores its mobility as already observed by Fitzgerald et al. (2001).

The haemolysin gene hllIII was not detectable in the RF122-like clonal complex. However, RF122 sequence data show that it was not completely absent, but that this strain contained a deviant allelic variant. Whether or not this was related to host specificity needs still to be determined.

Antibiotic resistance determinants appeared to be rather rare. While some isolates contained beta-lactamase, macrolide/clindamycin (ermA, ermC) or tetracycline resistance genes, only two mecA-positive isolates were found. Two isolates contained the clindamycin/lincomycin resistance gene linA, which could be related to a selective pressure by the occasional use of lincomycin in animal husbandry. Another interesting result was the absence of the genes aphA3 (neo- and kanamycin resistance) and sat (streptothricin resistance). These genes together form a transposon (Boerlin et al., 2001; Derbise et al., 1997; Werner et al., 2001) that was shown to be very common among human MRSA isolates (Monecke and Ehricht, 2005) from Dresden University Hospital (in Saxony, the neighbouring Federal State to Thuringia). Its absence in veterinary isolates could indicate that this high prevalence was rather a result of selective pressure due to the historical use of streptomycin preparations contaminated with streptothricin in human medicine (W. Witte, personal communication) than to the use of streptothricin in East German agriculture prior to 1989 (Teale, 2002; Werner et al., 2001).

Generally, we found some differences between S. aureus of human and bovine origin. These include the presence of sak- and sea-carrying phages which disrupt hlb, the presence of allelic variants of tst1, sen and, according to RF122 sequence data, also of hllIII, as well as the occurrence of the leuokcocidin lukF-P83/ lukM. None of these differences allows to distinguish bovine from human strains with certainty. Nevertheless, some clonal lines appear to be host-specific. The RF122-like clonal complex apparently does not occur in humans, and CC30 which is extremely important in men (including EMRSA-16 as well as the pandemic lukS/F-PVL-positive strains Phage Pattern 80/81 and WSPP-MRSA) was not found in cows. Specialisation to hosts appears not to be stringent enough to make transmissions of S. aureus from one host species to another impossible. Some clonal lines, such as CC1 (represented by Sanger 476, MW2, and strain 20) and CC8 (COL, NCTC8325, USA300, Irish 01, strains 3–6), seem to be rather promiscuous although some specific properties (presence of sak, disruption of hlb, presence of lukF-P83/lukM) often indicate host adaptation of a given isolate. However, in
some cases a direct transmission can be assumed. The CC8 MRSA isolate (strain 4) from this study had an overall hybridisation pattern and a spa-type (t068) related to a human healthcare-associated strain (Irish 01 (Monecke et al., 2007)). The other MRSA (strain 10) is related to an emerging MRSA which was shown to occur in humans, pigs (de Neeling et al., 2007; Huijsdens et al., 2006), dogs, and horses (Cuny et al., 2006), and one of the methicillin-susceptible bovine isolates described above (strain 9: agr I/spa t034) could represent a methicillin-susceptible ancestor.

Especially, the presence of the two MRSA strains among the isolates tested highlights the possibility of transmissions between humans and domestic animals. This warrants close surveillance and cooperation between clinical and veterinary microbiologists.

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