Application of pulsed-field gel electrophoresis and binary typing as tools in veterinary clinical microbiology and molecular epidemiology of bovine and human *Staphylococcus aureus*

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Abstract

Thirty eight bovine mammary Staphylococcus aureus isolates from diverse clinical, temporal and geographical origins were genotyped by pulsed-field gel electrophoresis (PFGE) after Smal digestion of prokaryotic DNA, and by means of binary typing using 15 strain-specific DNA probes. Seven pulsed-field types and four subtypes were identified, as were 16 binary types. Concordant delineation of genetic relatedness was documented by both techniques, yet based on practical and epidemiological considerations, binary typing was the preferable method. Genotypes of bovine isolates were compared to 55 previously characterized human S. aureus isolates through cluster analysis of binary types. Genetic clusters containing strains from both human and bovine origin were found, but bacterial genotypes were predominantly associated with a single host species. Binary typing proved an excellent tool for comparison of S. aureus strains, including methicillin resistant S. aureus, derived from different host species and from different databases. For 28 bovine S. aureus isolates, detailed clinical observations in vivo were compared to strain typing results in vitro. Associations were found between distinct genotypes and severity of disease, suggesting strain specific bacterial virulence. Circumstantial evidence furthermore supports strain specific routes of bacterial dissemination. We conclude that PFGE and binary typing can be successfully applied for genetic analysis of S. aureus isolates from bovine mammary secretions. Binary typing in particular is a robust and simple method and promises to become a powerful tool for strain characterization, for resolution of clonal relationships of bacteria within and between host species, and for identification of sources and transmission routes of bovine S. aureus.

Abbreviation key: BT = binary type, MRSA = methicillin resistant S. aureus, MSSA = methicillin susceptible S. aureus, PFGE = pulsed-field gel electrophoresis, SCC = somatic cell count.
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Introduction

Infections due to staphylococci are of major importance to veterinary and human medicine. *Staphylococcus aureus* is one of the most significant pathogens causing intramammary infections in dairy cattle worldwide. In humans, *S. aureus* is a major cause of community acquired as well as nosocomial morbidity and mortality. In the last decades increasing prevalence of methicillin-resistant *S. aureus* strains has become an additional infection control problem in human medicine. Staphylococcal strains may vary considerably in virulence and epidemiological potential. To control spread of staphylococcal infections, sources of contamination and mechanisms of transmission must be identified. Detailed pathogenetic and epidemiological studies depend on the availability of typing systems that differentiate between strains belonging to the same bacterial species.

In veterinary microbiology, many techniques have been applied for characterization of bovine *S. aureus* strains. Phenotypic methods include phage typing and multilocus enzyme electrophoresis. Genotypic methods include single gene typing systems, such as detection of coagulase gene polymorphism and ribotyping, and whole genome typing systems, such as arbitrarily primed PCR. Furthermore, plasmid pattern analysis has been used to differentiate among *S. aureus* isolates of bovine origin, based on diversity of extrachromosomal DNA. In human microbiology, most of these procedures have been superseded by newer methods that have enhanced resolving powers, including pulsed-field gel electrophoresis (PFGE) of DNA macrorestriction fragments and, more recently, binary typing. PFGE is a reliable and reproducible method with high discriminatory power. Drawbacks of this method are that it is laborious and expensive, and that complex DNA patterns may be difficult to interpret, especially for large collections of isolates. For clinical laboratories processing great numbers of samples, these limitations may be impediments to routine use. Binary typing is a highly reproducible and stable library typing method with excellent discriminatory abilities. It has the additional advantage of producing a simple binary output, facilitating interpretation and comparison of typing results, and it lacks experimentally unstable parameters such as electrophoretic conditions. Recently, several authors have reported on the use of PFGE for characterization of bovine isolates, but so far binary typing has not been applied to *S. aureus* isolates of bovine origin.

The purpose of this study was to determine whether PFGE and binary typing are suitable techniques for differentiation of isolates of *S. aureus* recovered from bovine mammary secretions. In addition, a collection of bovine isolates was compared to a collection of human isolates, including methicillin-resistant strains, to explore clonal relatedness of isolates from cattle and humans as determined by binary typing. Finally, associations of bacterial strains with clinical observations in cattle were examined to identify possible relations between genotypes and bacterial virulence or routes of spread.
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Materials and Methods

Bacterial isolates

This study included 38 bovine S. aureus isolates collected from eight dairy herds in The Netherlands between May 1997 and February 1999. Three herds (I, II and III) were involved in a longitudinal survey on population dynamics of intramammary infections. In those herds, milk samples were routinely collected from all four udder quarters of each cow at three-weekly intervals for 81 weeks. Samples from the other five herds were submitted to the diagnostic laboratory of the Animal Health Service, Deventer, The Netherlands, as part of a dairy health improvement scheme. Bacteria were cultured from milk samples according to National Mastitis Council standards and identified at the species level as described previously. Isolates were stored frozen until further use. Isolates were selected to represent different geographical, temporal and clinical origins (Table 1).

Binary typing data on 55 human S. aureus isolates from diverse geographic and temporal origins in the United States and The Netherlands were used (Table 2). Human collections include methicillin resistant S. aureus strains (MRSA, n=37) and methicillin susceptible S. aureus strains (MSSA, n=18) and have been described in detail before.

Clinical and subclinical disease characteristics

Detailed records of clinical observations were available for isolates 1 to 33 (Table 1). In addition, somatic cell counts (SCC) of milk samples yielding isolates 1 to 33, with exception of isolates 8-10, were determined by means of a Fossomatic cell counter (Foss Electric, Hillerød, Denmark). SCC is a measure of the leucocyte content of milk and is used as an indicator for infection. The threshold between non-infected and infected is commonly set at 200,000 cells/ml. Isolates 34 and 35 were cultured from bulk milk samples from farm III and disease classifications do not apply. For samples yielding isolates 36-40, SCC was determined but detailed clinical data were not available.

Based on clinical symptoms and SCC, isolates 1-33 were classified as belonging to one of four clinical groups in order of increasing severity of infection; (1) subclinical infection with non-elevated SCC (in 1000 cells/ml: median 97, range 11-152), (2) chronic subclinical infection with elevated SCC (in 1000 cells/ml: median 1278, range 210-7821), (3) short duration mild clinical disease or short duration subclinical disease with high SCC (in 1000 cells/ml: median 4560, range 411-8710), and (4) acute severe clinical disease (Table 1). SCC was not determined for group 4 samples, because clot formation in mammary secretions interfered with SCC measurement.
Table 1. Summary of epidemiologic data, PFGE typing data and binary typing results for 38 bovine *Staphylococcus aureus* isolates.

<table>
<thead>
<tr>
<th>Herd</th>
<th>Isolate</th>
<th>Cow-quarter&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Collection period</th>
<th>Clinical characteristics</th>
<th>Chn. group&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PFGE type</th>
<th>Binary code&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Binary type&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>75-1</td>
<td>1997 (Aug)</td>
<td>long, high SCC</td>
<td>2</td>
<td>A</td>
<td>00111111111101</td>
<td>8189</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>75-2</td>
<td>1997 (Aug)</td>
<td>long, high SCC</td>
<td>2</td>
<td>B</td>
<td>00111111111101</td>
<td>8189</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>75-4</td>
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<td>B</td>
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<td>8189</td>
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<tr>
<td></td>
<td>5</td>
<td>78-3</td>
<td>1997 (Aug)</td>
<td>long, high SCC</td>
<td>2</td>
<td>B.1</td>
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<tr>
<td></td>
<td>6</td>
<td>63-3</td>
<td>1997 (June)</td>
<td>short, high SCC</td>
<td>3</td>
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<td>00111111111111</td>
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<tr>
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<td>77-4</td>
<td>1997 (Oct)</td>
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<td>8</td>
<td>67-3</td>
<td>1998 (Jan)</td>
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<td>4</td>
<td>D</td>
<td>10101001110111</td>
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<td></td>
<td>9</td>
<td>74-4</td>
<td>1997 (Dec)</td>
<td>severe disease</td>
<td>4</td>
<td>D</td>
<td>10101001110111</td>
<td>21747</td>
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<td>10</td>
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<td>4</td>
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<td></td>
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<td>14</td>
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<td>17</td>
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<td>2</td>
<td>E</td>
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<td>3283</td>
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<tr>
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<td>18</td>
<td>13-3</td>
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<td>2</td>
<td>E.1</td>
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<tr>
<td></td>
<td>19</td>
<td>99-2</td>
<td>1997 (July)</td>
<td>long, high SCC</td>
<td>2</td>
<td>E</td>
<td>00110010100111</td>
<td>3283</td>
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<tr>
<td></td>
<td>20</td>
<td>108-3</td>
<td>1997 (July)</td>
<td>long, high SCC</td>
<td>2</td>
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<tr>
<td></td>
<td>21</td>
<td>47-3</td>
<td>1998 (May)</td>
<td>mild clinical</td>
<td>3</td>
<td>D</td>
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<td>mild clinical</td>
<td>3</td>
<td>E</td>
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<td>3283</td>
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<tr>
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<td>25</td>
<td>25-3</td>
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<td>long, low SCC</td>
<td>1</td>
<td>C</td>
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<td>1235</td>
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<td></td>
<td>26</td>
<td>95-1</td>
<td>1997 (July)</td>
<td>mild clinical</td>
<td>3</td>
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<td></td>
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<td>31-3</td>
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<tr>
<td></td>
<td>32</td>
<td>86-2</td>
<td>1998 (May)</td>
<td>long, high SCC</td>
<td>2</td>
<td>E</td>
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<tr>
<td></td>
<td>33</td>
<td>86-2</td>
<td>1998 (July)</td>
<td>long, high SCC</td>
<td>2</td>
<td>E</td>
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<tr>
<td></td>
<td>34</td>
<td>n.a.</td>
<td>1998 (May)</td>
<td>bulk milk sample</td>
<td>-</td>
<td>E</td>
<td>00110101010111</td>
<td>3283</td>
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<tr>
<td></td>
<td>35</td>
<td>n.a.</td>
<td>1998 (July)</td>
<td>bulk milk sample</td>
<td>-</td>
<td>E</td>
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<td>3283</td>
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<tr>
<td>IV</td>
<td>36</td>
<td>Ada 126</td>
<td>1999 (Feb)</td>
<td>unknown</td>
<td>-</td>
<td>E</td>
<td>00110101010111</td>
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<tr>
<td></td>
<td>37</td>
<td>9363-3</td>
<td>1999 (Feb)</td>
<td>unknown</td>
<td>-</td>
<td>E.2</td>
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<td>V</td>
<td>38</td>
<td>205-4</td>
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<tr>
<td>VII</td>
<td>39</td>
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<tr>
<td>VIII</td>
<td>40</td>
<td>Klara-4</td>
<td>1999 (Feb)</td>
<td>unknown</td>
<td>-</td>
<td>G</td>
<td>001011111111101</td>
<td>6141</td>
</tr>
</tbody>
</table>

<sup>a</sup> udder quarter position: 1 = right front; 2 = left front; 3 = right rear; 4 = left rear

Continued overleaf
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b) 1 = subclinical infection with SCC \( \leq \) 152,000 cells/ml; 2 = chronic subclinical infection with SCC \( \geq \) 210,000 cells/ml; 3 = mild clinical disease or short subclinical disease with high SCC; 4 = acute severe clinical disease.

c) Overall results after hybridization with 15 strain specific DNA probes (AW-1 through AW-15)\(^{42}\).

d) Binary type is the binary code transformed to a decimal number.

e) Subclinical disease with occasional mild clinical flare-ups (clots in milk).

PFGE

PFGE was carried out as described by Struelens \textit{et al.}\(^{35}\). \textit{SmaI} (Boehringer, Mannheim, Germany) was used for digestion of genomic DNA. PFGE of DNA digests was performed with a CHEF Mapper (BioRad, Veenendaal, The Netherlands) through a 1\% SeaKem agarose gel (FMC, SanverTECH, Heerhugowaard, The Netherlands) under the following conditions: initial switch time 5 s to final switch time 15 s, run time 10 hr, followed by initial switch time 15 s to final switch time 45 s for 14 hr; linear ramping; 6 V cm\(^{-1}\); 120° angle (60°/-60°); 14°C; 0.5x TBE. A lambda DNA polymer (BioRad, Veenendaal, The Netherlands) was used as molecular size marker. Gels were stained with ethidium bromide for 1 hr, destained in water and photographed under UV light with a charged-coupled device (CCD) camera.

Macrorestriction patterns were analyzed both visually and by computer-aided methods. Visual interpretation of banding patterns was done following guidelines suggested by Bannerman \textit{et al.}\(^{5}\) and Tenover \textit{et al.}\(^{38, 39}\). Isolates with identical restriction profiles were assigned the same type and identified with a capital letter. Isolates that differed from main types by one to three band shifts consistent with a limited number of genetic events were assigned subtypes, indicated with a numeral suffix. Isolates with more than three such differences were considered to be different types. Banding patterns were digitized with a Hewlett-Packard Scanjet IIcx/T scanner and stored as TIFF files. Patterns were analyzed using GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium) to calculate Dice coefficients of correlation and to generate a dendrogram by UPGMA clustering (Unweighted Pair Group Method using Arithmetic averages).

Binary typing

Macrorestriction fragments obtained through PFGE were Southern blotted onto Hybond N\(^{+}\) membranes (Amersham, Buckinghamshire, United Kingdom). Cloned DNA fragments designed for binary typing of human \textit{S. aureus} strains were used as probes\(^{32}\). Labeling, hybridization and detection of the probes were performed with enhanced chemiluminescence (ECL) direct labeling and detection systems, according to the manufacturer's protocols (Amersham Life Science, Buckinghamshire, United Kingdom). Hybridization of 15 DNA probes was scored with a 1 or a 0 according to the presence or
absence of a hybridization signal, resulting in a 15 digit binary code for each *S. aureus* isolate. Binary codes were transformed into decimal numbers to define binary types (BT) and a dendrogram was constructed using hierarchical cluster analysis (SPSS 8.0 for Windows, SPSS Inc.).

**Table 2.** Characterization of human *Staphylococcus aureus* collections from which binary types are used in this study.

<table>
<thead>
<tr>
<th>Collection</th>
<th>Geographic origin</th>
<th>Isolate numbers</th>
<th>Description of collection</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>United States</td>
<td>41-66</td>
<td>Community acquired MRSA strains from a New York City hospital (n=26)</td>
<td>41</td>
</tr>
<tr>
<td>2</td>
<td>United States</td>
<td>67-80</td>
<td>Selection of geographically diverse strains from multicenter collection of MRSA strains (n=5) and MSSA strains (n=9)</td>
<td>38, 42</td>
</tr>
<tr>
<td>3</td>
<td>The Netherlands</td>
<td>81-85</td>
<td>MSSA strains isolated from healthy persistent nasal carriers (n=5)</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>The Netherlands</td>
<td>86-95</td>
<td>MRSA strains (n=6) and MSSA strains (n=4) from outbreaks in Dutch hospitals and nursing homes</td>
<td>42</td>
</tr>
</tbody>
</table>

a) Isolate numbers as used in figure 4.

b) Center for Disease Control and Prevention.

**Statistical analysis**

Log-normalized SCC for clinical groups 1, 2 and 3 was compared by means of One-way ANOVA (SPSS 8.0 for Windows, SPSS Inc.)

Fisher’s exact test of the relationship between clinical groups of origin and strains was performed using analytical software (StatXact version 2.05, CYTEL Software Corporation, Cambridge, MA). Isolates 34-40 were excluded from this analysis because insufficient clinical data were available. Isolates 29, 31 and 33 were excluded because they represent the same infectious episodes as isolates 28, 30, and 32, respectively. For analysis of the association between clinical groups and PFGE types, types that occurred only once (A, F) were excluded from analysis and subtypes (B.1, B.2, and E.1) were grouped together with their respective main types. For analysis of the association between clinical groups and binary types, BT-clustering at 90% genetic similarity was used to define separate groups.
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Results

PFGE

All bovine isolates were typable by PFGE. Among 38 isolates seven pulsed-field types and four subtypes were identified through visual interpretation of gels (Figure 1, Table 1). Three PFGE types (A, F and G) and all subtypes (B.1, B.2, E.1 and E.2) were identified only once, while PFGE types C, D and E were found in two, three and four herds, respectively.

Figure 1. Example of pulsed-field gel electrophoresis of SmaI macrorestriction fragments of bovine Staphylococcus aureus isolates, showing isolates 17 to 40. Molecular sizes are indicated on the right.

GelCompar analysis of PFGE results defined more clusters than visual interpretation. Depending on the level of genetic relatedness 13, 11 or 8 clusters were identified for 95%, 90%, and 80% similarity, respectively (Figure 2). The visually identified PFGE type B was divided in four (95%) or two (80%) separate clusters, while PFGE type D was divided into three (95%), two (90%), and one (80%) cluster(s). In the GelCompar analysis, visual PFGE types E and E.1 were grouped together at 95% similarity, and E, E.1 and E.2 at 90% genetic similarity.
Figure 2. Dendrogram showing the level of similarity between *Sma*I macrorestriction patterns of 38 bovine *Staphylococcus aureus* isolates as determined by PFGE and subsequent GelCompar analysis of digitized photographs. Scale indicates level of genetic relatedness within this set of strains. Capital letters indicate PFGE types as based on visual interpretation of PFGE results.
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Binary typing
All bovine isolates were typable using the binary method (Table 1). Out of 15 probes designed for typing of human S. aureus strains, four hybridized to all bovine isolates (AW-5, AW-8, AW-9 and AW-15), while all other probes hybridized to at least one bovine isolate. Genetic relatedness of isolates based on binary typing was depicted in a dendrogram (Figure 3.a).

Figure 3a. Dendrogram showing the grouping of 38 bovine Staphylococcus aureus strains on the basis of hybridization scores after binary typing with probes AW-1 to AW-15. Isolate number, visual PFGE type and binary code are given for all isolates. Scale indicates level of genetic relatedness within this set of strains.
For 95%, 90%, and 85% genetic similarity, respectively, eight, six and three clusters of strains were identified. Binding of probe AW-14 showed a low level of reproducibility among epidemiologically related isolates. Therefore, a separate dendrogram was constructed excluding AW-14 (Figure 3b), reducing the number of clusters to six at 95% similarity.

**Figure 3b.** Dendrogram showing the grouping of 38 bovine *S. aureus* strains after omission of probe AW-14 that is associated with hypervariable regions on the bovine staphylococcal genome. Isolate number, visual PFGE type and binary code are given for all isolates. Scale indicates level of genetic relatedness within this set of strains.
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Concordance between PFGE and binary typing

PFGE types assigned were compared with binary types. General agreement was found between the techniques, but with some discrepancies. Several visually identified PFGE types were grouped together by binary typing (e.g. A, three B isolates, B.1 and G at 95% binary similarity; E, E.1 and two out of three C isolates at 95% similarity; B, B.2 and E.2 at 90% similarity). Other PFGE types were divided into multiple binary clusters that differed by two or three probes (e.g. B into two binary clusters at 90% similarity) (Figure 3.a). Concordance of delineation of genotypically related clusters as determined by PFGE and binary typing improved when probe AW-14 was excluded (Figure 3.b).

Within-herd and between-herd heterogeneity

Genetic heterogeneity among S. aureus isolates recovered from bovine mammary secretions was observed within and between herds. Isolates from herd I (n=16) were divided into four PFGE types (A-D) and subclonal variation was observed in PFGE type B (subtypes B.1 and B.2). In herd II (n=8) three PFGE types (D-F) were identified, with subclonal variation in PFGE type E (subtype E.1). In herd III (n=11) two PFGE types (C, E) occurred. In isolates obtained from five herds that were not related to each other or herd I, II and III, three PFGE types and one subtype were identified (D, E, E.2, G), demonstrating that both heterogeneity and homogeneity between herds exists. Heterogeneity based on binary typing parallels heterogeneity of PFGE types for all herds.

Comparison of bovine and human strains

Binary types of bovine isolates from this study were compared to binary types from human S. aureus isolates that had been typed before with the same method. Most isolates clustered as host specific clones and full identity of the 15 digit binary code of bovine and human isolates was never observed (Figure 4). At 95% similarity, human isolate 61 clustered together with bovine isolate 6 (one-digit difference at probe AW-11), and human isolate 62 clustered with bovine isolates 1-5 (one-digit difference at probe AW-1) and bovine isolate 40 (two-digit difference). At 90% similarity, these bovine and human isolates formed one cluster that also included human isolate 53. Human isolates within this cluster differed from bovine isolates in the same cluster by three digits at most, with differences associated with six out of 15 DNA probes used. Human isolate 82 clustered together with all bovine type D isolates at 90% similarity, as did human isolate 50 with bovine isolate 20. Human isolates 50, 53, 61 and 62 were community acquired MRSA strains from a New York City hospital (Table 2). Human isolate 82 was an MSSA strain isolated from a persistent nasal carrier in The Netherlands. Bovine isolates that clustered with human isolates originated from four Dutch dairy herds that were epidemiologically unrelated to each other or the human sources of S. aureus included in the comparison.
Figure 4. Dendrogram showing the grouping of 55 unrelated human Staphylococcus aureus strains described previously and 38 bovine S. aureus strains on the basis of hybridization scores after binary typing with 15 DNA probes. Isolate numbers and binary codes are shown for all isolates. Scale indicates level of genetic relatedness within this collection of strains.
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**Association with clinical characteristics**

Mean SCC of quarter milk samples differed with clinical group for groups 1, 2 and 3 (F-value=45.63, p<0.001, df=2). Subclinical infection with low SCC (clinical group 1) was associated with PFGE type C (Table 1; three C isolates in three group 1 samples). Binary typing discriminated between type C isolated from herd I (BT 1217) and herd III (BT 1235), in agreement with geographical clustering. Chronic subclinical infection with high SCC (clinical group 2) was associated with PFGE types A and B in herd I. PFGE types A and B were not isolated from any samples belonging to clinical groups 1 or 4 and only once from group 3. In herds II and III, clinical group 2 was associated with PFGE type E. Type E was also isolated from a group 3 sample, while one group 2 sample yielded PFGE type F. Clinical group 3 yielded several strains, categorized as B (herd I), D (herd I and II) or E (herd II and III). Acute severe clinical mastitis (clinical group 4) was associated with PFGE type D.

Associations between clinical groups and visually identified PFGE types were statistically significant (Fisher statistic=26.00, p=0.002, df=9). Associations between clinical groups and binary clusters were of borderline statistical significance when all probes were included in the analysis (Fisher statistic=24.70, p=0.05, df=15). Associations were significant after exclusion of probe AW-14 (Fisher statistic=19.10, p-value=0.02, df=15).

**Discussion**

**PFGE and binary typing**

Variation in gene content of staphylococcal chromosomes may be associated with presence of non-essential but clinically or epidemiologically relevant genes (e.g. virulence genes, resistance genes). PFGE is a well-known and powerful method for detection of genetic variation in *S. aureus* populations. Binary typing is a recently developed high-resolution molecular typing system that produces simple binary output and has the potential to become a technically simple and fast library typing system for *S. aureus* strains. To our knowledge, PFGE and binary typing had not been applied to *S. aureus* isolates of bovine origin. In this study, PFGE-profiles and binary codes for 38 isolates derived from bovine mammary secretions were determined and compared. After PFGE of *SmaI* macrorestriction fragments, seven main PFGE types and four subtypes were identified visually. Computer-aided cluster analysis identified more distinct types, depending on the level of genetic similarity chosen as cut-off value. What level of discrimination between clusters of strains is desired depends on the purpose of genotyping and results of PFGE must be analyzed in light of the epidemiological background. One visually identified PFGE type, type B, was subdivided over multiple clusters after UPGMA analysis, even at low genetic similarity levels (Figure 2). Isolates were from similar clinical and geographical
background, but subdivision may be related to different temporal origins of the samples (Table 1). Discrepancies between visual and computer aided interpretation are a drawback of pulsed-field typing, and limit its usefulness as a routine diagnostic technique for large numbers of samples.

In this study binary typing was preceded by PFGE typing, but binary typing can be performed as a single typing technique. Binary typing yielded 16 binary codes, clustered in three to eight clones, depending on levels of genomic similarity. The relevant level of discrimination and suitability of individual probes are subject of further study. However, interpretation of probe binding results is unequivocal. Probes AW-12 and AW-13 gave identical results, while probes AW-5, 8, 9 and 15 hybridized to all bovine strains and did not contribute to the discriminatory power of the typing system. A larger collection of bovine isolates should be studied to determine the informative value of these probes for differentiation of bovine S. aureus strains.

Concordance between PFGE and binary typing
Several PFGE types were subdivided by binary typing. Binary codes within a PFGE type often differ by no more than one digit and in many cases this is the digit associated with probe AW-14 (Table 1, Figure 3.a). The observed discrimination within PFGE types may therefore be related to the detection of hypervariable domains on the genome of bovine S. aureus strains with probe AW-14. Similar “hypervariability” or inconsistent presence of probe-binding sequences has been described for epidemiologically and genetically related human S. aureus strains. The results could imply that probe AW-14, that is stable for typing of human S. aureus strains, is not stable for typing of S. aureus of bovine origin. On the other hand, probe AW-14 could be used to study short-term genome evolution in bacterial populations from bovine origin.

When ignoring binary code differences caused by probe AW-14, closer agreement between binary typing and pulsed-field typing is obtained, but some one-digit differences within PFGE types remain (Figure 3.b). PFGE types C isolated from herds I and III differ by one digit, associated with probe AW-11. This genotypic difference can be related to different geographical origins, but not to a difference in clinical course of infection. For PFGE type D, differences exist in geographical origin and in clinical course. Whether severity of disease is a herd effect (herd I vs. herd II), a strain effect (BT 21745/21747 vs. BT 21713/21715), a cow effect (mild cases in older animals, severe cases in heifers) or a chance effect is unknown.

Some binary clones are subdivided by PFGE (e.g. B and B.2 within BT 8177 and A, B and B.1 within BT 8189). Since isolates within these binary types where from similar geographical, temporal and clinical origin, binary typing seems the epidemiologically superior technique in these cases.
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Within-herd and between-herd heterogeneity

PFGE and binary typing differentiated strains within and between herds. Similar results were obtained by means of PCR-based DNA-fingerprinting in the USA 24 and the Netherlands 21, through multilocus enzyme electrophoresis of a worldwide collection of strains 17, by coagulase gene typing of European, American and Asian isolates 2, 36, with a combination of techniques for bovine isolates from the USA and Ireland 12, and by PFGE of German isolates 3a. In all studies, including the present one, a limited number of predominant types was found both within herds, in agreement with the contagious nature of S. aureus mastitis 21, and between herds, suggesting that certain variants present in the environment may have predilection for causing intramammary infections 2, 12, 36.

Subclonal heterogeneity within herds may be due to temporal evolution. Herds were selected for inclusion in the longitudinal survey, based on a history of presence of the pathogen in the herd for more than one year. The study period covered an additional 18 months, allowing for further genetic diversification 41. Similar subclonal genetic variation over time has been described for DNA macrorestriction patterns from human S. aureus isolates 27.

Comparison of bovine and human strains

Out of 55 human isolates and 38 bovine isolates, five human and 16 bovine isolates belonged to clusters sharing 90% to 95% similarity as determined by binary typing. At higher similarity levels, all clones were host species specific. Similar results were obtained by Kapur et al. 17 and by Lopes et al. 22. The results are consistent with the concept of host specificity among S. aureus clones and imply that successful transfer of bacteria between humans and cattle is not a frequent event 17. However, several studies are available that suggest that transfer of bacteria between humans and cows is possible 15, 30, 37. Those studies mostly focus on the role of humans as source of infection for dairy cattle. Another reason to be concerned about interspecies transfer of S. aureus is the routine use of antibiotics in dairy herd management 15, 33, 34. In farms with S. aureus mastitis problems oxacillin is used as dry cow treatment for all animals 8. Resistance to the closely related antibiotic methicillin is rare in bovine S. aureus 22 but has been reported from New York State 29, Europe 9 and Japan (cited in 18). Widespread use of oxacillin could promote selection of resistant clones 7. If interspecies transfer occurs, methicillin resistance in bovine strains may contribute to increasing prevalence of MRSA in humans. Since binary typing is a library system that can be applied to S. aureus isolates originating from humans and cattle, it is a useful tool in monitoring origins of MRSA strains and interspecies transfer of S. aureus. Addition of probes to test for presence of meca gene in the bovine typing system would furthermore allow monitoring of MRSA prevalence in veterinary diagnostic laboratories.
Association with clinical characteristics

A limited number of isolates were included in statistical analyses and interdependence of within-herd observations was not taken into account. Thus, results of the analyses must be interpreted with care. However, in this study there was a significant correlation between \textit{S. aureus} strains and disease characteristics observed \textit{in vivo}. Such information is rarely available because most studies focus on clinical or subclinical mastitis only\textsuperscript{21,21a,37}, or don’t contain information on clinical background of samples\textsuperscript{2,12,36}. Matthews \textit{et al.}\textsuperscript{24} observed heterogeneity between subclinical and clinical isolates based on arbitrarily primed PCR, but heterogeneity within the group of subclinical isolates and overlap between genotypes isolated from both groups precluded firm associations. Kenny \textit{et al.}\textsuperscript{19} reported enterotoxin production by bovine mammary isolates of \textit{S. aureus} and suggest that enterotoxin production may be associated with clinical course of disease. Matsunaga \textit{et al.}\textsuperscript{23} attempted to relate toxin production and other virulence factors to severity of clinical disease. They concluded that the properties of \textit{S. aureus} isolated from peracute cases were different from those of acute and chronic isolates. No obvious differences between acute and chronic isolates were observed. The first conclusion is in agreement with our finding that all group 4 cases (peracute cases) are attributable to a specific PFGE type and binary type. In addition, our results suggest a difference between acute (clinical) and chronic (subclinical) cases, as shown by the associations between PFGE type C and clinical group 1, PFGE types B and E and group 2, and PFGE type D and group 3, respectively.

PFGE types C and E differed in binding of probe AW-4 only, but were associated with clearly distinguishable leucocyte response \textit{in vivo} (low vs. high SCC). Differences in leucocyte response \textit{in vitro} have been described by Aarestrup \textit{et al.}\textsuperscript{1} for different coagulase types isolated from cases of subclinical mastitis. Probe AW-4 has been shown to be homologous to a mobile genetic element, IS\textsuperscript{257}. IS\textsuperscript{257}, also known as IS\textsuperscript{431}, is a common insertion sequence in the staphylococcal chromosome and plasmids and can be associated with several resistance determinants, including methicillin resistance\textsuperscript{7}.

It must be emphasized that associations between clinical outcome of disease, PFGE types, binary types and specific probes in the binary typing system are as yet speculative and more typing needs to be done. If associations are confirmed, binary typing can be used for identification of unusual and more virulent strains, allowing for further pathogenetic studies and for tailored advice to farmers on management of specific cases.

An aspect of the association between genotype and epidemiological background that merits attention is the relation between PFGE type D and its origin. PFGE type D was isolated from all group 4 samples, all of which were obtained from heifers before first calving. Occurrence of \textit{S. aureus} mastitis in preparturient heifers is a widely reported phenomenon\textsuperscript{14,26}. Based on biotyping, antibiograms and phage typing, Roberson \textit{et al.}\textsuperscript{31} concluded that milk from the dairy herd and heifer body sites are the most likely sources of infections. In their study, the environment was a possible source of infection in 17 out of 61 cases but never the sole possible source. In contrast, our results show that the predominant \textit{S. aureus} genotype in the milking herd (PFGE type B, BT 8177/8189) is different from the
genotype found in heifer mastitis isolates (PFGE type D, BT 21745/7). This implies that the dairy herd is not the most likely source of heifer infections. In herd II, all type D cases occurred at a time that no other infected animals were present in the milking herd, as determined by three-weekly routine samplings (data not shown). Though not conclusive, this observation also suggests the environment as a more likely source of infection than the dairy herd. Determination of reservoirs, including environmental sources, is considered an important step when attempting to control *S. aureus* in a dairy herd. The genotyping techniques presented in this paper can be helpful in elucidating the relative importance of environmental sources in the farm level ecology of *S. aureus*.

**Conclusion and future developments**

This study shows that both PFGE and binary typing can be successfully applied to characterize *S. aureus* isolates of bovine mammary origin. Binary output was easier to interpret than banding patterns generated by PFGE and binary typing seemed superior to PFGE in clustering isolates from similar epidemiological background. As a library typing system, binary typing facilitates comparison of *S. aureus* isolates from bovine and human origin from world wide collections, analysis of clonal relatedness and host specificity, and monitoring of interspecies transfer. In this study, genetically related clusters of strains from human, bovine and mixed origin occurred. For isolates obtained from bovine mammary secretions, associations between bacterial strains and clinical characteristics of infection *in vivo* were observed, as was a tentative association between strains and sources of infection. Those observations need further validation through the study of larger strain collections or infection experiments. We conclude that binary typing in particular is a technique that is suitable for use in veterinary clinical microbiology and may contribute to development of case specific and farm specific recommendations for management of *S. aureus* problems in bovine medicine.

**References**


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