Bacterial Genotype Affects the Manifestation and Persistence of Bovine Staphylococcus aureus Intramammary Infection

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Two-hundred seventeen Staphylococcus aureus isolates from 116 dairy cows with intramammary infections were analyzed by pulsed-field gel electrophoresis to study the association between symptom severity, persistence of infection, and bacterial genotype. Among five main genotypes infecting 90% of the cows, one was associated with severe clinical symptoms but reduced persistence.

Staphylococcus aureus is a common cause of bovine intramammary infection (IMI) worldwide. It is contagious and generally responds poorly to treatment. However, according to previous studies, virulence of this mastitis pathogen differs among strains (2, 3, 5, 11, 12). Those studies used a variety of methods of analysis based on phenotype and genotype, which makes comparisons difficult. Pulsed-field gel electrophoresis (PFGE), which is considered the best standard typing method, has been little used to investigate the possible associations between S. aureus genotypes and manifestation of bovine IMI (7, 12). Establishing a link between strain types and their pathogenicity would help to improve treatment and prevention of staphylococcal mastitis. The objective of our study was to investigate the genetic relationships among S. aureus strains from bovine IMI, using PFGE, and to examine whether the manifestation and persistence of S. aureus IMI were genotype dependent.

The study material included the history, clinical data, and bacterial isolates of 116 dairy cows (134 mastitic mammary quarters) requiring veterinary care due to S. aureus IMI in 1993 to 1997 in 70 herds located in southern Finland, in the practice area of the ambulatory clinic of the University of Helsinki. Only the first IMI episode of the same cow was included. The attending veterinarians of the clinic visited the farm, recorded the clinical symptoms of the cow, and collected quarter milk samples by routine methods (4). A cell lysosome-originated indicator for udder inflammation, N-acetyl-β-D-glucosaminidase (NAGase) activity, was determined from the milk samples by a fluorogenic method (6). Gram-, catalase-, and coagulase-positive cocci were confirmed as S. aureus by PCR amplification of the thermonuclease (nuc) gene as described previously (1). Susceptibility to amoxicillin-clavulanate, cloxacillin, and penicillin G was tested by the disk diffusion method (9), and production of β-lactamase was determined from all isolates by using nitrocefin (Becton Dickinson Microbiology Systems, Cockeysville, Md.). The isolates were preserved at -70°C in Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, United Kingdom) to await further studies.

The cows were treated for 5 days with penicillin G for IMIs caused by β-lactamase-negative S. aureus strains (n = 69) or with amoxicillin-clavulanate (n = 28) or cloxacillin (n = 19) for IMIs caused by β-lactamase-positive strains. The cows were revisited 2 and 4 weeks posttreatment for follow-up sampling and clinical examination. Isolated S. aureus strains were preserved for further studies. The symptoms of the cow were graded as subclinical (milk somatic cell count of >300,000/ml, no clinical symptoms), mild (visual abnormalities in milk and/or swelling or tenderness in the udder), or severe clinical, if elevated rectal temperature (>39.0°C) and other general

FIG. 1. Strains used in this study. From the left: control (NCTC 8325); pulsotype A, clone A3; pulsotype F; pulsotype F (same mammary quarter, first posttreatment sample); pulsotype D, clone D2; pulsotype J; pulsotype A, clone A1; control; pulsotype A, clone A1; pulsotype P; pulsotype A, clone A1; clone A1 (same cow, posttreatment infection in a new quarter); pulsotype C, clone C2; clone C2 (same cow, concomitantly infected adjacent quarter); control strain.

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Symptoms (anorexia, decreased rumen motility, and general attitude) were also observed. An infection was considered to be persistent if the original S. aureus genotype was isolated from the follow-up milk samples.

Chromosomal DNA was prepared from the isolates and cleaved with Smal (Boehringer, Mannheim, Germany) as described earlier (10). The DNA restriction fragments were separated according to the procedure described by Murchan et al. (8). PFGE fingerprints were stored, processed, and analyzed with BioNumerics software (version 10.1; Applied Maths, Kortrijk, Belgium) with Dice coefficients, using clustering by the unweighted pair group method with arithmetic mean. A position tolerance of 1.0 was used in the comparison of bands from different fingerprints. Genetic relatedness of the strains was defined visually as follows. Fingerprints with one to three band shifts were interpreted as being genetically unrelated and of a different pulsotype. The effect of pulsotype on severity of symptoms was tested by multilocus genomic regression (proportional odds model) by including time of infection (days from calving) in the model. The effects of pulsotype, time of infection, treatment (penicillin G versus other β-lactam antibiotics), and severity of symptoms on persistence of infection were tested by logistic regression. The effects of pulsotype and time of infection on milk NAGase activity were tested by two-way analysis of variance, using log-transformed values for NAGase. The analyses were carried out with Stata 7.0 (Stata Statistical Software, College Station, Tex.). P values of <0.05 were considered to be significant.

The 217 PFGE-typed isolates were divided into 22 different pulsotypes (A to V). Pulsotypes A to E were representative of those strains infecting the majority of the cows (104 of 116 [90%]) in multiple herds; they were found during consecutive years and comprised two to five closely related clones (A1 to A5, B1 and B2, C1 and C2, D1 and D2, and E1 to E4). Pulsotypes F to V were sporadic, being isolated from individual cows only. Spreading of pulsotypes A, D, and E from the original infected quarter to a previously healthy quarter in seven cows was observed. The same clone was isolated if the cow had several infected quarters (Fig. 1).

Associations between pulsotypes and IMI severity were noted here, which supported the observations of Zadoks et al. (12). Infections caused by pulsotype B were related to severe symptoms. Compared with this genotype, infection of pulsotype A decreased the odds of severity of IMI by a factor of 0.12 (95% confidence interval [CI], 0.021 to 0.70), infection of pulsotype C by a factor of 0.012 (95% CI, 0.00052 to 0.28), and infection of pulsotype E by a factor of 0.037 (95% CI, 0.0043 to 0.32). However, pulsotype B was invariably eliminated from the udder (Table 1). These IMIs may have developed quickly with obvious clinical symptoms, whereas the others progressed more slowly, often being chronic when becoming clinical. It is likely that this affected the treatment response. The response among IMIs of pulsotype A was more favorable if penicillin was used (infections caused by β-lactamase-negative strains), compared with other treatments (infections caused by β-lactamase-positive strains) (P < 0.05), and if the symptoms were subclinical rather than mild clinical (P < 0.05).

The symptoms of the cows were more severe (P < 0.05) and milk NAGase values were higher (P < 0.05) within 1 month from calving than in later lactation. Unlike in the study by Middleton et al. (7), inflammation of the udder, as indicated by milk NAGase, was here pulsotype dependent and it supported the associations between pulsotypes and severity of symptoms.

NAGase levels of IMIs promoted by pulsotype B were significantly higher (mean, 427 to 441 U/ml; standard error [SE], 45.7 to 48.0) compared with those promoted by pulsotype C (mean, 124 to 127 U/ml; SE, 27.0 to 28.2) (P < 0.05), and they tended to be higher than those associated with pulsotypes A (mean, 307 to 317 U/ml; SE, 32.5 to 33.3) (P < 0.05) and E (mean, 282 to 307 U/ml; SE, 46.0 to 54.6). The NAGase values associated with pulsotype D (mean, 420 to 433 U/ml; SE, 60.5 to 62.8) were close to those for pulsotype B.

In conclusion, our findings provide evidence of differences in virulence potential between S. aureus genotypes causing bovine IMI, but further evaluation of the virulence requires more research.

We wish to thank Elina Siren and other laboratory personnel in the National Public Health Institute for skillful technical assistance. We also thank all the veterinarians and laboratory personnel of the ambulatory clinic at the Faculty of Veterinary Medicine, University of Helsinki, for collecting the study material. The Ministry of Agriculture and Forestry and Valio, Ltd., supported this study financially.

### Table 1. Manifestation and persistence of IMI among 103 cows infected with five main S. aureus pulsotypes

<table>
<thead>
<tr>
<th>Pulsotype</th>
<th>No. of:</th>
<th>Severity of IMI</th>
<th>Persistence of IMI/no. infected (OR; 95% CI for OR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Herds</td>
<td>Cows</td>
<td>Subclinical</td>
</tr>
<tr>
<td>A</td>
<td>34</td>
<td>49</td>
<td>15</td>
</tr>
<tr>
<td>B</td>
<td>19</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>12</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>103</td>
<td>28</td>
<td>49</td>
</tr>
</tbody>
</table>

*Pulsotypes A, C, D, and E relative to pulsotype B. One cow with concomitant mild and subclinical IMI of pulsotype A was excluded.

Subclinical IMI is the comparison level. *, P < 0.05; **, P < 0.01.

Values for NAGase were compared with other treatments (infections caused by NAGase-positive strains) (95% CI, 0.021 to 0.70), infection of pulsotype C by a factor of 0.012 (95% CI, 0.00052 to 0.28), and infection of pulsotype E by a factor of 0.037 (95% CI, 0.0043 to 0.32). However, pulsotype B was invariably eliminated from the udder (Table 1). These IMIs may have developed quickly with obvious clinical symptoms, whereas the others progressed more slowly, often being chronic when becoming clinical. It is likely that this affected the treatment response. The response among IMIs of pulsotype A was more favorable if penicillin was used (infections caused by β-lactamase-negative strains), compared with other treatments (infections caused by β-lactamase-positive strains) (P < 0.05), and if the symptoms were subclinical rather than mild clinical (P < 0.05).

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