Clinical Mastitis in Primiparous Holsteins: Comparisons of Bovine Leukocyte Adhesion Deficiency Carriers and Noncarriers

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ABSTRACT

The objective of this study was to determine the impact of bovine leukocyte adhesion deficiency on clinical mastitis incidence, severity, and duration in Holstein cows. Genomic DNA from milk of 847 Holstein cows in six Pennsylvania herds was used to determine bovine leukocyte adhesion deficiency genotypes (82 or 9.7% carriers). Data on clinical mastitis incidence, severity, duration, and pathogen involved were collected during first lactation for the project cows. One hundred ninety-four cows had one or more clinical mastitis episodes; milk samples from each quarter with clinical mastitis were collected at discovery of the episode and were cultured following National Mastitis Council recommendations. The overall incidence of clinical mastitis was significantly affected by sire and herd-year-season of calving. In addition, incidence of clinical mastitis tended to increase with age at first calving. Severity and duration of clinical mastitis were impacted by the pathogen involved.

Incidence of clinical mastitis from all pathogens, from coagulase-negative staphylococci, and from coliform bacteria was not significantly related to bovine leukocyte adhesion deficiency status. Carriers tended to have lower rates of mastitis from streptococci other than Streptococcus agalactiae when compared with noncarriers, but this result should be interpreted with caution because of the low frequency of mastitis from the streptococci. Bovine leukocyte adhesion deficiency status was unrelated to severity or duration of clinical episodes. Bovine leukocyte adhesion deficiency carriers are probably similar to noncarriers in resistance to clinical mastitis.

(Key words: bovine leukocyte adhesion deficiency, clinical mastitis)

INTRODUCTION

Genetic improvement in milk yield is associated with decreased mastitis resistance in dairy cattle (16). As a consequence, considerable current research has focused on traits that are correlated with mastitis and genetic differences in immunocompetence that influence mastitis (4, 5, 12, 14, 19).

The lethal, autosomal recessive disorder, bovine leukocyte adhesion deficiency (BLAD), is known to significantly impair immune function in homozygous recessive animals (3, 17). The disorder is not lethal in heterozygotes, but the impact of the BLAD allele on health traits is not fully understood. Recently, researchers have investigated the association between BLAD and mammary gland health (5, 13). Kelm et al. (5) did not find a significant relationship between BLAD and SCS or between BLAD and clinical mastitis. However, both studies (5, 13) revealed BLAD carriers had marginally lower estimates of SCS than noncarriers. Powell (13) reported a least-squares solution for PTA SCS of −0.1 (nonsignificant) for carrier bulls. Carrier animals could be more (or less) susceptible to mastitis than noncarriers. The direct effect of the BLAD locus on the immune system indicates that it could directly impact mastitis resistance in dairy cattle. Therefore, the objective of this research was to determine the impact of the D128G allele on clinical mastitis incidence, severity, and duration in heterozygous carriers versus homozygous normal, first lactation Holstein cows.

MATERIALS AND METHODS

Herdspersons in six cooperating Pennsylvania herds were instructed by research technicians to visually identify and classify clinical mastitis. Cows and their
milk were examined for clinical signs during regular milking of 847 first lactation Holstein cows from calving until the termination of the lactation. A scale from 1 (normal milk) to 5 (acute systemic mastitis) was defined to evaluate the absence or presence of a clinical episode and its severity. Herdpersons obtained quarter milk samples from 194 cows with clinical mastitis (score of 2 to 5 on severity scale) before treating with antibiotics or alternative therapy. Milk samples were aseptically collected in sterile, 7-ml polypropylene test tubes. Animal identification, quarter sample date, and quarter sample source (e.g., right front quarter) were recorded. Severity scores were recorded daily until the milk and cow returned to normal. Severity scores were also recorded if the quarter returned to normal and subsequently became abnormal again within 30 d of the initial clinical identification. Samples were frozen and transported weekly to the Animal Diagnostic Laboratory at The Pennsylvania State University for culturing.

Milk culture and BLAD testing procedures were as previously described (20). Quarters showing growth from two pathogens were reported as infected quarters for the organism that was less likely to be a contaminant as determined by guidelines from the National Mastitis Council (6). Milk culture results from the first clinical mastitis episode for the 194 cows with one or more recorded clinical mastitis episodes are in Table 1. Table 2 contains the number of cows per cooperating herd at risk for clinical mastitis and with BLAD genotypes available.

Data collection started in July 1991 and ended in 1994. The first year-season of calving included all animals fresh from the commencement of the project until the end of October 1991. Thereafter, year-seasons were defined by 4-mo periods (November through February, March through June, and July through October). Because of limited comparisons within some herd-year-seasons, several animals were moved to an adjacent year-season (Table 3).

One hundred thirty-five bulls sired the 847 cows. Seventy-seven sires had daughters with at least one clinical mastitis episode during first lactation. The mean number of daughters per sire was 6.3, and the mode was one daughter per sire. The bull with the most daughters had 140 (16.5% of the cows on project), and another bull had 123 daughters (14.5% of project cows). All other sires had 49 or fewer daughters on the project.

The mean age at calving was 25 mo; the range was 22 to 33 mo. The median and mode were both 24 mo. Mean lactation length was 315 d (range 3 to 999). Median lactation length was 310 d. Seventy-five percent of all lactations ended before 365 d. Lactations longer than 365 d were truncated to 365 d. Clinical episodes after 365 DIM were ignored in the analyses.

The majority of first clinical episodes in first lactation were identified during the first 21 d postpartum. Nearly 37% were identified by 4 d after calving. The mean DIM when the first clinical mastitis episode was identified was 80.6 d. The median was 20 DIM, and the mode was 1 DIM when the clinical episode was reported. Two heifers with clinical episodes before calving were removed from the severity and duration data set.

Actual 305-d milk production or 305-d projections were available based on DHIA records for 838 cows. Mean, minimum, and maximum 305-d milk production values were 8557 kg, 3089 kg, and 12,351 kg, respectively.

### Variable Definitions

Various dependent variables were constructed to evaluate the impact of BLAD carrier status on clinical mastitis episodes.
TABLE 2. Number of cows in first lactation per herd that were at risk for clinical mastitis and with status of bovine leukocyte adhesion deficiency available.

<table>
<thead>
<tr>
<th>Herd</th>
<th>Carriers</th>
<th>Noncarriers</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>(%)</td>
<td>Project</td>
</tr>
<tr>
<td></td>
<td>Herd</td>
<td>Project</td>
<td>Herd</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td>39</td>
<td>7</td>
<td>47</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>9.7</td>
<td>765</td>
</tr>
</tbody>
</table>

mastitis incidence, severity, and duration. Binary (0/1) dependent variables were defined based on the condition of no clinically infected quarters (0) or the condition of one or more clinically infected quarters (1) during first lactation. Separate binary variables were constructed for the presence or absence of one or more clinical episodes considering all bacterial species, considering only coagulase-negative staphylococci (CNS), considering only streptococci other than Streptococcus agalactiae (SNA), and considering only coliform bacteria. Another binary variable was developed that considered clinical episodes from either coliform species or SNA; this variable represented the major environmental pathogens found in this study.

The total number of clinical episodes during the first lactation was also studied by creating a discrete dependent variable (0 = no clinical, 1 = one clinical episode, 2 = two clinical episodes, . . .). However, results are not presented for the analysis of the total number of clinical episodes because they were very similar to the results from the analysis of the binary variables.

TABLE 3. Number of cows at risk for mastitis and number of cows at risk with one or more clinical episodes by herd and year-season of calving.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cows at risk for mastitis</td>
<td>Cows</td>
<td>1 or more clinical episodes</td>
<td>Cows</td>
<td>1 or more clinical episodes</td>
<td>Cows</td>
</tr>
<tr>
<td>Herd</td>
<td>3</td>
<td>24</td>
<td>10</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>23</td>
<td>4</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>29</td>
<td>9</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>29</td>
<td>11</td>
<td>30</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>. . .</td>
<td>. . .</td>
<td>75</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>. . .</td>
<td>. . .</td>
<td>20</td>
<td>.</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>34</td>
<td>159</td>
<td>35</td>
<td>213</td>
</tr>
</tbody>
</table>

¹Year-season of calving consisted of 4-mo periods. Two year-seasons consisted of months in consecutive calendar years (November through February).

²No cows calved during this year and season.
normal for all days of the clinical episode (possible scores of 2 to 150). Scores for severity on the first d of the clinical episodes were distributed as follows: 19% were scored 2, 46% were scored 3, 29% were scored 4, and 6% were scored 5. Highest severity scores during the first clinical episode had a distribution similar to the severity scores on the first d of the clinical episode. For the highest severity score, 9% were scored 5, 29% were scored 4, 44% were scored 3, and 18% were scored 2. Ten percent of the first clinical episodes lasted only 1 d, 15% lasted 2 d, 17% lasted 3 d, 14% lasted 4 d, 10% lasted 5 d, and 8% lasted 6 d. About 25% of the first clinical episodes lasted more than 6 d, but only 6 episodes lasted more than 13 d.

Statistical Analysis

The following logistic regression model was used to describe clinical mastitis incidence and to evaluate the effects of the BLAD allele on clinical mastitis incidence. The analysis was done using the PROBIT procedure of SAS (15).

\[ Y_{ijkl} = F + S_i + H_j + L_k + b_1A_{ijkl} + b_2T_{ijkl} + e_{ijkl} \]  

where \( Y_{ijkl} \) = clinical mastitis incidence with 0 indicating no clinical episodes during first lactation and 1 indicating one or more clinical episodes during first lactation, \( F \) = overall mean, \( S_i \) = sire i, \( H_j \) = herd-year-season of calving j, \( L_k \) = BLAD status k, \( A_{ijkl} \) = age at calving, \( T_{ijkl} \) = lactation length, \( b_1 \) and \( b_2 \) = regression coefficients, and \( e_{ijkl} \) = unexplained residual effects.

Severity and duration analyses were conducted using the following linear model.

\[ Y_{ijkl} = F + O_i + H_j + L_k + bA_{ijkl} + e_{ijkl} \]  

where \( Y_{ijkl} \) = severity on the first d of clinical episode, highest severity during the first clinical episode, days of duration of the first clinical episode, or sum of daily severity codes that were above normal for all days of the clinical episode; \( F \) = overall mean; \( O_i \) = organism type i; \( H_j \) = herd-year-season of calving j; \( L_k \) = BLAD status k; \( A_{ijkl} \) = age at calving; \( b \) = regression coefficient; and \( e_{ijkl} \) = unexplained residual effects distributed as \( N(0, \sigma^2) \).

For the analysis of the severity and duration measures, all 194 cows with at least one clinical episode were included. Organisms were categorized as follows for this analysis: SNA, CNS, coliform infections, \textit{Staphylococcus aureus}, yeasts, contaminated or missing samples, samples with no bacterial growth, and all other pathogens. Effects for sire and DIM at the first clinical episode were initially included in the model for severity and duration analyses but were not significant (\( P > 0.10 \)) and were dropped from the model.

RESULTS AND DISCUSSION

Clinical Mastitis Incidence

One hundred ninety-four of the 847 cows (22.9%) had at least one clinical mastitis episode (Table 1). Of the 194 first clinical episodes, 27 samples were contaminated or missing (13.9%). Thirty-five clinical samples (18%) showed no bacterial growth. Positive culture results with identified organisms were obtained from the remaining 132 cows (68%). The most prevalent infections were caused by SNA; 54 cows (27.8%) were infected with SNA. Thirty-six cows (18.6%) were positive for CNS. Coliforms were cultured from 26 cows (13.4%). All other pathogens combined were responsible for less than 10% of the first clinical episodes.

The incidence of cows with one or more clinical mastitis episodes (22.9%) was consistent with incidence levels reported by Smith et al. (18) but were lower than 32 and 39% incidence reported by Lescourret et al. (7) and Morse et al. (9), respectively. The lower incidence has been attributed to strict milking hygiene practices, non-lactating antibiotic therapy, and low prevalence of \textit{Streptococcus agalactiae} and \textit{Staphylococcus aureus} in all project herds.

Herd-year-season of calving was significant for all clinical mastitis incidence variables. Others (2, 18) have reported significant herd and season effects. Seasonal differences have resulted in variable levels of bacteria supported by organic bedding (2). Mammary infection, by coliform bacteria in particular, was proportional to exposure of the teat to mastitis-causing pathogens. Seasonal variation is also influenced by the temperature, humidity, and other weather conditions, thereby affecting the level of stress on the cow and her immune status (18).

Table 4 contains the regressions of mastitis incidence during the first lactation on age at calving and lactation length from the logistic model (Model 1). Age at calving significantly influenced the incidence of clinical mastitis by all organisms (\( P < 0.05 \)). Positive b-values indicated older heifers at calving were more likely to have a clinical infection. Increased heifer age at first calving appeared to increase the risk of clinical mastitis because of longer periods of exposure. This age at calving effect on clinical mastitis agreed with other reports that animal environment influences heifer intramammary infections and mastitis (11).
Regression estimates for BLAD status for clinical mastitis incidence from the various mastitis-causing pathogen groups are in Table 5 [Model 1]. Odds ratios for noncarriers compared with carriers are also in Table 5. The impact of BLAD carrier status on the clinical mastitis incidence when all organisms were considered was not significant ($P > 0.05$). These results were consistent with results from Wanner et al. (20), which showed no difference between carriers and noncarriers in intramammary infections near first parturition when infections were caused by all pathogens. Approximate Type II error rates indicated that our experiment was fairly conclusive when evaluating the impact of BLAD on overall clinical mastitis incidence. Assuming a Type I error rate of .05 (equivalent to $P = 0.05$), the probability of a Type II error was approximately 20% when trying to detect a 2% difference in clinical mastitis incidence between carriers and noncarriers.

Carriers of BLAD and noncarriers had similar rates of clinical mastitis incidence from CNS or coliform bacteria. However, BLAD carriers tended to have lower rates ($P < 0.05$) of clinical mastitis incidence when only clinical mastitis from SNA was considered (3 of 82 carriers compared with 62 of 765 noncarriers had clinical incidence caused by SNA). Odds ratios indicated that noncarriers were 9.55 times as likely as carriers to have clinical mastitis caused by SNA. Results involving clinical mastitis caused by individual pathogen groups should be viewed more cautiously than mastitis involving all pathogen groups because of the low frequency of clinical episodes when only individual pathogen groups are considered. The ability to test for differences was lower when frequencies were as small as the frequencies found when only individual pathogen groups were considered.

### TABLE 4. Logistic regression of clinical mastitis incidence on lactation length and age at calving in months.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Lactation length</th>
<th>SE</th>
<th>Age at calving</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>All species</td>
<td>$-0.0026^*$</td>
<td>0.0014</td>
<td>0.13*</td>
<td>0.07</td>
</tr>
<tr>
<td>Coagulase-negative staphylococci</td>
<td>0.0025</td>
<td>0.0034</td>
<td>0.21</td>
<td>0.13</td>
</tr>
<tr>
<td>Streptococci other than <em>Streptococcus agalactiae</em></td>
<td>$-0.0069^*$</td>
<td>0.0020</td>
<td>0.14</td>
<td>0.10</td>
</tr>
<tr>
<td>Coliforms</td>
<td>$-0.0030$</td>
<td>0.0033</td>
<td>0.16</td>
<td>0.15</td>
</tr>
<tr>
<td>Coliforms and streptococci other than <em>Strep. agalactiae</em></td>
<td>$-0.0051^*$</td>
<td>0.0018</td>
<td>0.13</td>
<td>0.09</td>
</tr>
</tbody>
</table>

$^*P < 0.10.$

$^*P < 0.05.$

Lactation length was important ($P < 0.05$) in explaining clinical incidence by SNA and the common environmental pathogens represented by the combination of coliform bacteria and SNA (Table 4). The regression coefficients suggest that a shorter lactation may be related to greater incidence of clinical mastitis by these common environmental organisms. Early dry-off to begin dry-cow therapy and culling could account for part of this effect.

Sire was included in the model because it improved the fit of the models. The log likelihood quantifies the overall fit of a logistic model. The difference in log likelihoods between the models with and without sire multiplied by $-2$ is distributed as chi-squared. Sire reduced the log likelihood ($P < 0.05$) when the dependent variable was clinical mastitis incidence by all organisms (lower log likelihood is desirable). Heritability estimates for clinical mastitis incidence from a larger data file that included the data from this project are in Nash (10).

### TABLE 5. Impact of bovine leukocyte adhesion deficiency (BLAD) status on clinical mastitis incidence in 847 cows with BLAD status known. Data included 82 BLAD carriers and 765 noncarriers.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Cows with clinical mastitis</th>
<th>Estimate</th>
<th>SE</th>
<th>Odds ratio$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cows with clinical mastitis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n)</td>
<td>(n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All species</td>
<td>0.23</td>
<td>18</td>
<td>176</td>
<td>0.208</td>
</tr>
<tr>
<td>Coagulase-negative staphylococci</td>
<td>0.05</td>
<td>5</td>
<td>39</td>
<td>$-0.906$</td>
</tr>
<tr>
<td>Streptococci other than <em>Streptococcus agalactiae</em></td>
<td>0.08</td>
<td>3</td>
<td>62</td>
<td>2.257$^*$</td>
</tr>
<tr>
<td>Coliforms</td>
<td>0.04</td>
<td>4</td>
<td>31</td>
<td>0.013</td>
</tr>
<tr>
<td>Coliforms and streptococci other than <em>Strep. agalactiae</em></td>
<td>0.11</td>
<td>6</td>
<td>88</td>
<td>0.907</td>
</tr>
</tbody>
</table>

$^1$Odds of a noncarrier having one or more clinical episodes compared with a carrier.

$^*P < 0.10.$
TABLE 6. Impact of bovine leukocyte adhesion deficiency (BLAD) status on clinical mastitis severity and duration (194 cows with one or more clinical episodes).

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>R²</th>
<th>X</th>
<th>Range</th>
<th>BLAD status</th>
<th>Least squares mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severity on first day</td>
<td>0.28</td>
<td>3.22</td>
<td>2–5</td>
<td>Carrier</td>
<td>3.22</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Noncarrier</td>
<td>3.35</td>
<td>0.10</td>
</tr>
<tr>
<td>Highest severity</td>
<td>0.27</td>
<td>3.29</td>
<td>2–5</td>
<td>Carrier</td>
<td>3.24</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Noncarrier</td>
<td>3.42</td>
<td>0.11</td>
</tr>
<tr>
<td>Days of duration</td>
<td>0.23</td>
<td>5.29</td>
<td>1–29</td>
<td>Carrier</td>
<td>5.96</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Noncarrier</td>
<td>5.34</td>
<td>0.58</td>
</tr>
<tr>
<td>Total of severity codes[^1]</td>
<td>0.20</td>
<td>15.49</td>
<td>2–116</td>
<td>Carrier</td>
<td>17.24</td>
<td>4.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Noncarrier</td>
<td>16.13</td>
<td>2.15</td>
</tr>
</tbody>
</table>

[^1]: Total of severity codes is sum of daily severity codes that were above normal over all days of the clinical episode.

Severity and Duration

Organism type was a highly significant and important explanatory variable [Model 2] for all measures of severity and duration (P < 0.05). Clinical mastitis caused by yeast organisms was limited to 1% of all infections, but these clinical episodes were severe and lengthy. Coliform bacteria and SNA had above average severity and duration scores. *Staphylococcus aureus* infections combined the lowest severity scores of any organism type with one of the longest durations yielding an above average total of severity scores for a clinical episode. Coagulase-negative staphylococci had a very low number of severity days and the lowest total of severity scores for a clinical episode of all major organism types. Milk samples that showed no growth at the outset of a clinical episode tended to have low severity and duration scores. More details of the effect of organism type on severity and duration can be found in Nash (10).

Herd-year-season of calving and age at calving [Model 2] tended to influence severity score on the first d of a clinical episode and highest severity code given during the clinical episode (P < 0.10) (results not shown). However, herd-year-season of calving and age at calving were not important predictors of the duration or total of severity scores for clinical infections.

Sire did not significantly (P > 0.05) impact severity and duration of the first clinical mastitis episode [Model 2]. In addition, BLAD status did not significantly affect (P > 0.05) severity and duration of clinical mastitis (Table 6). Carriers and noncarriers with one or more clinical episodes tended to have clinical episodes that were similar in severity and duration.

Milk Yield

Milk yield was not impacted by BLAD status. Least squares means for first lactation 305-d milk yield were 8309 kg for carriers and 8276 kg for noncarriers. Heritability of first lactation 305-d milk yield in this study was 0.24. Carriers and noncarriers of BLAD appear to have similar milk yields (1, 8, 13).

CONCLUSIONS

Herd-year-season of calving and sire influenced clinical mastitis incidence during first lactations. In addition, clinical mastitis incidence increased as age of first calving increased.

Overall clinical mastitis incidence did not differ between heterozygous BLAD carriers and homozygous normal cows. Carriers tended to have lower rates of clinical mastitis because of streptococci other than *Streptococcus agalactiae*. Also, severity and duration of clinical mastitis episodes were not impacted by BLAD status. In general, BLAD carriers and noncarriers appeared to have similar resistance to clinical mastitis.

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REFERENCES


