Influence of Parity and Stage of Lactation on the Somatic Cell Count in Bacteriologically Negative Dairy Cows

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ABSTRACT

This study examines the influence of parity, stage of lactation, and single isolations (i.e., the isolation of a microorganism that could not be reisolated in the same quarter in the next sampling) of staphylococci other than Staphylococcus aureus (coagulase-negative staphylococci), Corynebacterium bovis, or esculin-positive cocci other than Streptococcus uberis (referred to as esculin-positive cocci throughout) on the monthly log_e-transformed somatic cell count (SCC) for 180 first, second, and third parity cows that were observed over a whole lactation. Repeated measures ANOVA was used to analyze the data. No significant effect was found for the infection variable. However, the results indicated that even single isolations of coagulase-negative staphylococci, C. bovis, or esculin-positive cocci resulted in a numerical or statistically significant increase in SCC. Least squares mean SCC (log_e-transformed) for bacteriologically negative cows and cows with single isolations of coagulase-negative staphylococci, C. bovis, or esculin-positive cocci were 3.90, 3.97, 4.08, and 4.17, respectively.

Significant effects of parity, stage of lactation, and the interaction of parity and stage of lactation could not be found when only bacteriologically negative cows were included. Therefore, these effects must be due to factors that were present in the infected groups. (Key words: somatic cell counts, bacteriologically negative cows, parity, stage of lactation)

Abbreviation key: CNS = coagulase-negative staphylococci, CSCC = cow SCC, lnSCC = log_e-transformed SCC, LSD_α=0.05 = least significant differences (α = 0.05).

INTRODUCTION

The measurement of SCC from Dairy Herd Improvement programs is used worldwide as an indicator of subclinical mastitis. Because bulk milk SCC is used as a surveillance tool for mastitis control on the herd level, cow SCC (CSCC) is used to trace subclinically infected cows.

Intramammary infections have been recognized as major factors that influence SCC (10, 22). Many researchers (4, 16, 22, 24) have examined the SCC in bacteriologically negative cows. In bacteriologically negative cows, factors such as parity, stage of lactation, and milk production have been associated with variation in SCC (12, 24). Apart from the variables mentioned previously, other factors, including the technique used to measure SCC, bacterial culture methods, and, importantly, the definition of an IMI, contribute to the variation in SCC of bacteriologically negative cows. Based on the SCC of bacteriologically negative cows, threshold values have been suggested to trace subclinically infected cows (1, 4, 5, 18, 21).

The effect of an IMI caused by coagulase-negative staphylococci (CNS) and Corynebacterium bovis on the SCC has been recognized (3, 7, 13, 17). However, the isolation of CNS and C. bovis from a single sample was considered to be a false-positive result (3, 17, 22). Although esculin-positive cocci other than Streptococcus uberis (referred to as esculin-positive cocci throughout) are considered major pathogens, these
Cocci are often evaluated together with Strep. uberis and Streptococcus dysgalactiae as one taxonomic group (1, 7). When these pathogens were classified separately, no further attention was paid to them (4).

The purpose of this study was to examine the influence of parity and stage of lactation on the log e-transformed (×10^3 cells/ml) SCC (lnSCC) of samples from cows that were bacteriologically negative over an entire lactation and to examine the influence of a single isolation of CNS, C. bovis, or esculin-positive cocci on the lnSCC during the entire observed lactation.

**MATERIALS AND METHODS**

**Herd Selection**

Twenty-five dairy herds that were located in the provinces of East and West Flanders, Belgium were followed during a 20-mo period (February 1993 to September 1994). Criteria for herd selection were 1) willingness of the farmer to cooperate, 2) participation in the DHI program that was organized by the Flemish Cattle Breeding Association, 3) minimum herd size of 25 cows, and 4) breed (Holstein Friesian and Red and White).

**Sample Collection and Analysis**

**CSCC.** At monthly intervals 11 times a year (herds were not visited during either July or August), a composite milk sample was collected with an off-line milk meter (Tru Test Ltd., Auckland, New Zealand) or an on-line milk meter (Fullflow™; Packo-Fullwood, Zedelgem, Belgium) from all cows lactating for two successive milkings. One-half of the milk sample was collected during the first milking, and the second half was collected during the next milking. Samples were preserved with 0.02% bronopol (The Boots Co. PLC, Nottingham, England) at 4°C. The CSCC was measured on these samples with the Somascope (Delta Instruments, Drachten, The Netherlands) (14).

**Subclinical mastitis.** At monthly intervals, quarter foremilk samples from all cows were taken by trained technicians, usually within 2 d after the collection of the samples to determine CSCC. The teats were cleaned with dry udder cloths. Dirty teats were washed and dried. Before milk samples were taken, teats were disinfected with cotton moistened with a solution of ethyl alcohol (70%) and chlorhexidine (200 mg/100 ml). The milk samples were transported immediately after collection to a laboratory and streaked for initial isolation within 2 to 3 h after collection.

**Clinical mastitis.** Foremilk samples were taken by the farmer from every quarter that showed clinical signs of mastitis. The same procedure was followed as described previously to collect the clinical milk samples, although these milk samples were stored at −20°C until the next visit of one of the technicians who collected the monthly quarter foremilk samples. The samples were then transported to the laboratory for bacteriological culture.

**Isolation Procedure and Identification**

Quarter milk samples were streaked onto a 90-mm Petri dish with a blood agar base (Oxoid, Basingstoke, England) supplemented with 5% bovine blood; samples were also streaked onto an Edwards medium (Oxoid) supplemented with 5% bovine blood. Agar plates were incubated at 37°C and read after 24 and 48 h.

Isolates were classified as described by the National Mastitis Council (9): Staph. aureus, CNS, Streptococcus agalactiae, Strept. dysgalactiae, Strep. uberis, esculin-positive cocci, C. bovis, corynebacteria excluding C. bovis, Arcanobacterium pyogenes, Escherichia coli, Klebsiella spp., Serratia spp., other Enterobacteriaceae, Pseudomonas aeruginosa, yeasts, and fungi.

**Selection of Cows**

Monthly CSCC for cows in their first, second, or third parity that had been observed over an entire lactation were selected for statistical analysis when no microorganisms were isolated and when no clinical mastitis was observed during the observed lactation. Cows in first, second, or third lactations were also selected when 1) CNS, C. bovis, or esculin-positive cocci could be isolated in a single sample (i.e., isolation of CNS, C. bovis, or esculin-positive cocci in one of the monthly samplings could not be reisolated in the same quarter in the next sampling) and 2) no clinical mastitis was observed during the observed lactation. Lactation was defined as the period from parturition to drying off or the period from parturition to the end of the observation period when this period was longer than 250 d, whichever came first. Other parities and microorganisms were not considered because of the limited number of observations.
TABLE 1. Total number of cows and observations per parity and per class of infection status.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Parity</th>
<th>Infection status¹</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Cows, no.</td>
<td>96</td>
<td>66</td>
<td>18</td>
</tr>
<tr>
<td>Observations, no.</td>
<td>817</td>
<td>579</td>
<td>155</td>
</tr>
</tbody>
</table>

¹ 0 = Bacteriologically negative, 1 = infection caused by coagulase-negative staphylococci, 2 = infection caused by Corynebacterium bovis, and 3 = infection caused by esculin-positive cocci (other than Streptococcus uberis).

Statistical Analysis

Data file 1, including all selected cows, was analyzed with repeated measures ANOVA (2) using the following model:

\[ Y_{ijkl} = \mu + PAR_i + INF_j + (PAR \times INF)_{ij} + COW_{k(ij)} + DIM_l + (PAR \times DIM)_{il} + (INF \times DIM)_{jl} + (PAR \times INF \times DIM)_{ijl} + \epsilon_{ijkl} \]

where

\[ Y_{ijkl} = \text{observed value (lnSCC of the monthly cow milk samples);} \]
\[ \mu = \text{overall mean;} \]
\[ PAR_i = \text{fixed effect of parity (} i = 1, 2, \text{ or } 3; \]
\[ INF_j = \text{fixed effect of a single isolation (} j = 0, 1, 2, \text{ or } 3; \]
\[ COW_{k(ij)} = \text{random effect of cow nested within } PAR_i \text{ and } INF_j \text{ (} k = 1 \text{ to } 180; \]
\[ DIM_l = \text{fixed effect of stage of lactation (} l = 1 \text{ to } 10; \]
\[ (PAR \times INF)_{ij} = \text{interaction of parity and infection status;} \]
\[ (PAR \times DIM)_{il} = \text{interaction of parity and stage of lactation;} \]
\[ (INF \times DIM)_{jl} = \text{interaction of parity, infection status, and stage of lactation;} \]
\[ \epsilon_{ijkl} = \text{random error term.} \]

The lnSCC (×10³ cells/ml) was used as the dependent variable. Three parity classes (first, second, and third) were considered. The stage of lactation variable (DIM_l) was coded in 30-d periods (i.e., 1 = 0 to 30 DIM to 10 = 271 to 300 DIM). When no microorganisms were isolated during the entire observed lactation, the variable INF_j was coded 0. Codes 1 to 3 were given to cows from which CNS, C. bovis, or esculin-positive cocci could be isolated in single samples, respectively. When two or more of these microorganisms were isolated in a single sample during the observed lactation, the INF_j variable was coded according to the microorganism listed first in the following series: esculin-positive cocci, CNS, and C. bovis.

To examine whether a single isolation of CNS, C. bovis, or esculin-positive cocci only affected the corresponding test day measure of the lnSCC, data file 2 was analyzed using the same model as described previously. Data file 1 was reduced to data file 2 by deleting the test day data corresponding to a positive bacteriological isolation.

The least significant difference multiple comparison method (15) (α = 0.05) was calculated for every paired comparison.

RESULTS

During the 20-mo study period, 1312 cows were followed for an entire lactation, and 180 cows (13.7%) met the selection criteria for further statistical analysis. Data file 1 consisted of 1551 observations and a mean of 8.6 CSCC measurements per lactation. No microorganisms were isolated from 44 cows (24.4%) during the observed lactation (Table 1). Coagulase-negative staphylococci, C. bovis, and esculin-positive cocci were isolated in single samples from 35.6, 21.1, and 18.9% of the cows, respectively. The proportion of positive bacteriological isolations per class of infection status at a given time during lactation is presented in Figure 1. The mean numbers of milk samples from which CNS, C. bovis, and esculin-positive cocci were isolated per lactation were 1.8, 1.5, and 1.2, respectively.

Results of the statistical analysis of data file 1 are shown in Table 2. The overall mean lnSCC was 3.95 (51.9 × 10³ cells/ml). The whole-plot error term (0.1161) was one-third the size of the subplot error term (0.3521). No overall effect was found for the infection status variable (P = 0.0989), but paired comparisons showed a difference |P = 0.0026; least
The proportion of positive bacteriological isolations of coagulase-negative staphylococci (solid bar), Corynebacterium bovis (open bar), and esculin-positive cocci (other than Streptococcus uberis) (patterned bar) at a given time during lactation and per class of infection status.

Figure 2. Geometric mean cow SCC of bacteriologically negative cows in first (○), second (▼), and third (▲) parity at various stages of lactation.

### Table 2. Repeated measures ANOVA using parity and infection status as the whole-plot fixed effects, cow as the whole-plot random effect, and stage of lactation as the subplot fixed effect.

<table>
<thead>
<tr>
<th>Variable</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parity (P)</td>
<td>2</td>
<td>5.0405</td>
<td>0.0075</td>
</tr>
<tr>
<td>Infection status (I)</td>
<td>3</td>
<td>2.1251</td>
<td>0.0989</td>
</tr>
<tr>
<td>P × I</td>
<td>6</td>
<td>0.9786</td>
<td>0.4414</td>
</tr>
<tr>
<td>Cow</td>
<td>168</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage of lactation (L)</td>
<td>9</td>
<td>12.1780</td>
<td>0.0001</td>
</tr>
<tr>
<td>P × L</td>
<td>18</td>
<td>5.3154</td>
<td>0.0001</td>
</tr>
<tr>
<td>I × L</td>
<td>27</td>
<td>1.1356</td>
<td>0.2882</td>
</tr>
<tr>
<td>P × I × L</td>
<td>54</td>
<td>1.0749</td>
<td>0.3331</td>
</tr>
<tr>
<td>Error</td>
<td>1263</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Significant difference (α = 0.05) (LSD_{α=0.05} = 0.24) in the lnSCC between cows infected with esculin-positive cocci (lnSCC = 4.17) and bacteriologically negative cows (lnSCC = 3.90). The lnSCC of cows infected with C. bovis (lnSCC = 4.08) were borderline significant (P = 0.0589; LSD_{α=0.05} = 0.23) compared with the lnSCC of bacteriologically negative cows. No differences (P = 0.3581; LSD_{α=0.05} = 0.20) were observed among cows infected with CNS (lnSCC = 3.97) and bacteriologically negative cows.

The lnSCC differed by parity (P = 0.0075). The interaction of parity and infection status was not significant (P = 0.4414). Least squares means lnSCC of all cows (infected as well as bacteriologically negative) were 3.89 (48.9 × 10^3 cells/ml), 4.08 (59.1 × 10^3 cells/ml), and 4.11 (60.9 × 10^3 cells/ml) for first, second, and third parity cows, respectively. The least squares means of lnSCC for bacteriologically negative cows in first, second, and third parity, respectively, were 3.80 (44.7 × 10^3 cells/ml), 3.93 (50.9 × 10^3 cells/ml), and 3.97 (53.0 × 10^3 cells/ml). Paired comparison of bacteriologically negative cows in second lactation versus bacteriologically negative cows in first lactation was borderline significant (P = 0.0537; LSD_{α=0.05} = 0.26). Paired comparison of bacteriologically negative cows in third lactation versus bacteriologically negative cows in first lactation was not significant (P = 0.42).

Stage of lactation and the interaction of stage of lactation and parity affected (P = 0.0001) lnSCC. Least squares means lnSCC of all cows (infected as well as bacteriologically negative) started at 4.02 (55.9 × 10^3 cells/ml) in the 1st mo of lactation, decreased between 31 and 60 DIM to 3.71 (40.8 × 10^3 cells/ml), and increased toward the end of the lactation period [lnSCC = 4.50 (90.0 × 10^3 cells/ml)]. No interaction (P = 0.2882) of stage of lactation and infection status or three-way interaction (P = 0.3331) of parity, infection status, and stage of lactation was observed. Least squares means lnSCC by parity and by stage of lactation in bacteriologically negative cows are shown in Table 3 and Figure 2. Least squares means lnSCC in bacteriologically negative cows in first lactation remained constant during the whole lactation. Least squares means lnSCC of bacteriologically negative cows in second and third lactation were comparable with those of the bacteriologically negative cows in first lactation during the first 240 DIM. Beyond 240 DIM, least squares means lnSCC increased (Figure 2).

Differences in lnSCC during lactation between bacteriologically negative cows in second and third lactation versus bacteriologically negative cows in first lactation were evaluated. The difference in the lnSCC between bacteriologically negative cows in second and third lactation and bacteriologically negative cows in first lactation at 61 to 90 DIM was used as a refer-
TABLE 3. Least squares means of the loge-transformed SCC (lnSCC) (×10^3 cells/ml) by parity number and stage of lactation for cows without IMI throughout the observation period.

<table>
<thead>
<tr>
<th>Stage of lactation (DIM)</th>
<th>Parity number</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no.</td>
<td>lnSCC</td>
<td>no.</td>
<td>lnSCC</td>
</tr>
<tr>
<td>0–30</td>
<td>16</td>
<td>3.89</td>
<td>0.64</td>
<td>11</td>
</tr>
<tr>
<td>31–60</td>
<td>26</td>
<td>3.74</td>
<td>0.66</td>
<td>13</td>
</tr>
<tr>
<td>61–90</td>
<td>23</td>
<td>3.77</td>
<td>0.67</td>
<td>15</td>
</tr>
<tr>
<td>91–120</td>
<td>24</td>
<td>3.51</td>
<td>0.68</td>
<td>14</td>
</tr>
<tr>
<td>121–150</td>
<td>25</td>
<td>3.71</td>
<td>0.70</td>
<td>13</td>
</tr>
<tr>
<td>151–180</td>
<td>24</td>
<td>3.77</td>
<td>0.68</td>
<td>11</td>
</tr>
<tr>
<td>181–210</td>
<td>23</td>
<td>3.84</td>
<td>0.67</td>
<td>15</td>
</tr>
<tr>
<td>211–240</td>
<td>23</td>
<td>3.66</td>
<td>0.67</td>
<td>9</td>
</tr>
<tr>
<td>241–270</td>
<td>19</td>
<td>3.92</td>
<td>0.65</td>
<td>14</td>
</tr>
<tr>
<td>271–300</td>
<td>14</td>
<td>3.84</td>
<td>0.67</td>
<td>6</td>
</tr>
</tbody>
</table>

1Number of observations.
2SEM × √n0.

ence. Although lnSCC increased numerically toward the end of the lactation in the bacteriologically negative cows in second and third lactations, differences were not significant (P = 0.1055 for the differences between bacteriologically negative cows in second lactation vs. bacteriologically negative cows in first lactation and P = 0.2382 between bacteriologically negative cows in third lactation vs. bacteriologically negative cows in first lactation, respectively).

The evolution of the differences in lnSCC during lactation between the lnSCC of cows infected with CNS, C. bovis, or esculin-positive cocci minus the lnSCC of the bacteriologically negative cows was evaluated; difference in lnSCC between cows infected with CNS, C. bovis, or esculin-positive cocci versus the lnSCC of bacteriologically negative cows at 61 to 90 DIM was used as the reference (Figure 3). The shapes of the difference curves were similar for the three infection classes, including positive values for all three infection groups between 121 and 240 DIM. The differences in lnSCC between cows that were infected with esculin-positive cocci versus bacteriological negative cows varied (P = 0.0070) over lactation. For isolates of CNS and C. bovis, these differences did not vary (P = 0.1309 and P = 0.5737, respectively) according to stage of lactation. In Figures 4, 5, and 6, the geometric mean SCC per class of infection status during lactation are given for cows in first, second, and third lactations, respectively.

When the classes of cows infected with CNS, C. bovis, or esculin-positive cocci were combined, the interaction of infection status and parity became statistically significant (P = 0.0004). The interaction of infection status and stage of lactation and the three-way interaction were both borderline significant (P = 0.0667 and P = 0.0605, respectively).

In data file 2, the overall mean lnSCC of 1342 observations was 3.94 (51.3 × 10^3 cells/ml). The whole-plot error term was 0.1186, and the subplot error term was 0.3484. Least squares means lnSCC for cows infected with CNS was 3.95 (51.9 × 10^3 cells/ml). Least squares means lnSCC for the cows infected with C. bovis and least squares means lnSCC for the cows infected with esculin-positive cocci were not estimated because of empty data fields.

**DISCUSSION**

Because no international standard exists for the definition of an IMI, methods used to define udder health status vary (3, 4, 17, 24), and the guidelines
for diagnosis of an IMI have changed over time. In a 1967 International Dairy Federation bulletin (11), an udder was considered normal when no pathogenic bacteria were isolated and when the SCC was less than 500 × 10³ cells/ml. In another International Dairy Federation bulletin, in 1987, Griffin et al. (8) concluded that, if diagnosis of an IMI caused by major pathogens was based on the agreement of two consecutive samples tested bacteriologically or with a third if the first two were inconsistent, the accuracy was 99%, and the repeatability exceeded 95%. Using this definition of an IMI, the isolation of a major pathogen in a single sample was considered to be a false-positive result. Analogous studies on the diagnosis of IMI caused by minor pathogens have not been conducted. Therefore, guidelines for the diagnosis of an IMI caused by major pathogens have generally been adopted for minor pathogens; the isolation of CNS and C. bovis in a single sample is considered to be a false-positive result.

The present study demonstrated that single isolations of CNS, C. bovis, or esculin-positive cocci resulted in a higher lnSCC. Cows for which no microorganisms were isolated during the entire observed lactation had the lowest least squares means lnSCC [lnSCC = 3.90 (49.4 × 10³ cells/ml)]; compared with reported values (1, 4, 5, 6, 12, 16, 19, 20, 21, 22, 24), only the results of Brolund (4) and Schepers et al. (20) were similarly low.

For cows that were bacteriologically negative at the time of sampling, Brolund (4) reported that the geometric mean SCC for the Swedish Red and White breed increased from 28 × 10³ cells/ml for cows in first lactation to 72 × 10³ cells/ml for cows in fourth or higher lactations. A similar increase was found using the Swedish Friesian breed (43 × 10³ cells/ml for cows in first lactation and 96 × 10³ cells/ml for cows in fourth and higher lactations). The least squares means lnSCC presented by Schepers et al. (20) were even smaller than our results. Although the criterion used in that study to classify a quarter that was free of an IMI allowed single isolations, SCC were measured on quarter foremilk samples (versus composite milk samples in our study) with the Fossomatic (versus Somascope), and the milk production of the herds was much higher than the milk production of the herds of our study (14).

In the present study, the influence of both parity and stage of lactation on the lnSCC was not obvious when only bacteriologically negative cows were considered. In bacteriologically negative cows in first lactation, no effect of stage of lactation was observed, and
the evaluation of the differences in the least squares means lnSCC over lactation between bacteriologically negative cows in second and third lactation versus bacteriologically negative cows in first lactation was not statistically significant. Eberhart et al. (6) and Natzke and Everette (16) were also unable to find an effect of parity or stage of lactation for uninfected cows. Possibly, the effects of parity and stage of lactation were mostly due to cows with IMI that had been classified as bacteriologically negative in previous studies (12, 19, 22, 24).

Infection status was entered as a whole-plot effect rather than as a subplot effect because Brolund (4) found that the prediction of the log10-transformed SCC was more precise when infection status was defined on a lactation basis than on a test day basis. This result was supported after deleting the test day lnSCC that corresponded with a positive bacteriological isolation (data file 2). The overall mean lnSCC, both the whole-plot and the subplot error, and the least squares means lnSCC of cows with single CNS infections were similar to the results shown by the analysis of data file 1.

Although a statistically significant influence on the lnSCC was only found in cows that were infected in a single sample with esculin-positive cocci, the influence on the lnSCC of cows infected in a single sample with C. bovis was borderline significant. The course of the least squares means lnSCC during lactation was similar for all three groups of infection status. The shape of the difference curves (Figure 3) could not be related to the time of isolation of CNS, C. bovis, and esculin-positive cocci (Figure 1). The proportion of CNS that were isolated during lactation was equally divided over the lactation. Corynebacterium bovis was more frequently isolated at the end of the lactation period, but esculin-positive cocci were more frequently isolated during the first half of the lactation period. This finding supports the poor correlation between udder health status and test day SCC (4). Differences in SCC were observed at the beginning of lactation (0 to 30 DIM) and between 121 and 240 DIM (Figure 3). The highest least squares means lnSCC at the beginning of lactation were more pronounced for cows in first lactation (Figure 4) than for cows in second (Figure 5) and third lactation (Figure 6). This result may be an indication that more cows in first lactation calved with spontaneously curing IMI than did multiparous cows, which generally received intramammary antibiotic treatment at dry-off (23).

CONCLUSIONS

The present study showed that the least squares means lnSCC in bacteriologically negative cows were low (lnSCC = 3.90). No effect of parity and stage of lactation on the lnSCC was found for these bacteriologically negative cows.

The isolation of esculin-positive cocci in a single sample during lactation showed a significant influence on the lnSCC for the entire observation period. The influence of a single isolation with C. bovis was borderline significant; CNS isolated from a single sample during lactation had no statistically significant effect on lnSCC.

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