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Responses and factors affecting intramammary infection rates resulting from infusion of a *Streptococcus uberis* strain in Friesian-Jersey crossbred cows

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**ABSTRACT**

The objective of this study was to investigate responses to an intramammary infusion of a *Streptococcus uberis* strain in 264 Friesian-Jersey cows, and identify factors contributing to clinical mastitis risk in the week following infusion. Factors examined for their contribution to risk include sire, days in milk at the time of infusion, SCC on the morning of infusion, previous mastitis treatment history, and the presence of any major pathogens at the time of infusion. Prior to infusion, milk samples were collected from all glands for bacteriology assessment, and antibiotic treatments were administered to clear infections. A single gland from each cow was infused with an average 10⁴ colony-forming units (sd=22) of *Streptococcus uberis*, and milk samples were collected at each milking for the subsequent 13 milkings, or until diagnosis of clinical mastitis. Logistic regression and survival analysis were used to determine risk factors. Findings showed that cows with a somatic cell count at infusion of ≥100,000 cells/ml had a lower incidence of clinical mastitis (18.3% vs. 81.5%; p<0.0001). Results also indicated that the presence of *Staphylococcus aureus* at infusion for cows that had no pre-infusion mastitis treatment(s) was associated with a lower incidence of clinical mastitis (29.0% vs. 82.8%; p<0.0001).

**Keywords:** Streptococcus uberis; clinical mastitis; SCC.

**INTRODUCTION**

Mastitis control is of particular interest to the dairy industry because of the major impact it has on farm management and profitability. High economic costs associated with mastitis are due to a number of factors including discarded milk, losses in production, reduced milk quality, increased health costs and involuntary culling costs. The causes of mastitis are complex, but it is usually a consequence of bacterial invasion of the teat canal resulting in infection of the mammary gland. Invasive bacteria range in pathogenicity and exhibit variation in the progression to clinical infection, with a significant proportion of potentially mastitic infections being overcome by the immune response before any clinical symptoms are observed. The ability to overcome infection is dependant on a number of factors including the bacterial species and the immune competence of the infected individual. Clinical cases are characterised by visible signs such as abnormal texture or discoloration of the milk, swelling and discoloration of the udder, and increased temperature or pain in the quarter (de Haas, 2003). Pathogen presence unaccompanied by visible signs is generally referred to as sub-clinical mastitis and is usually diagnosed using somatic cell count (SCC) and/or bacteriology screening.

*Streptococcus uberis* (SU) is the most common pathogen causing clinical mastitis in New Zealand (McDougall, 1998), and has been shown to have a bacteriological cure rate following therapy to naturally acquired infections of approximately 75 to 80% (McDougall, 1998). Natural exposure to SU can result in non-uniform risk across cows, but intramammary infusion of bacteria directly into glands results in consistent and high infection rates (Hill *et al.*, 1994; Finch *et al.*, 1997). Many studies have investigated the impact of intramammary SU infusion into dairy cattle glands (Hill *et al.*, 1994; Lacy-Hulbert *et al.*, 1996; Finch *et al.*, 1997), and findings have shown that isolates vary in pathogenicity and virulence (Hill, 1988; Leigh *et al.*, 1990). Studies from experimental challenge models have also shown that prior intramammary infection (IMI) can result in reduced clinical response rates (Hill *et al.*, 1994; Finch *et al.*, 1997), and that there is an inverse relationship between pre-challenge SCC and severity of clinical mastitis (Shuster *et al.*, 1996).

In this paper, we investigate responses to an intramammary infusion of a SU strain into a single gland, and identify factors affecting the subsequent immune response. Factors examined for their contribution to clinical infection risk include sire, days in milk at the time of infusion, SCC on the morning of infusion, previous mastitis...
treatment history in prior seasons as well as the season of infusion, and the presence of any major pathogens at the time of infusion.

MATERIALS AND METHODS

Animals used in the study were four year-old (3rd lactation) Friesian-Jersey second-cross cows (n=264) born in 2000 as part of the Friesian-Jersey Crossbred trial (Spelman et al., 2001). These animals were sired by one of six sires. They were on average 109 days (sd=21) postpartum at the time of infusion.

Pre-trial microbiology and treatments

Seven to 10 weeks prior to infusion, IMI status was determined by taking a single milk sample (~5 ml) from each quarter following aseptic teat-end preparation. From this screening, those cows with a significant infection (i.e. Staphylococcus aureus (SA), Streptococcus uberis (SU), Streptococcus dysgalactiae (DY), or Coagulase negative staphylococci (CNS) were present) were treated with antibiotics. Of the 56 cows treated, 38 (67.9%) were for SA, 9 (16.1%) were for unidentified pathogens, 5 (8.9%) were for CNS, 3 (5.4%) were for combined DY and SA, and the remaining 1 was a case of DY. Those requiring treatment were infused intramammarily 3 times at 48-hour intervals with 200mg of cloxacillin sodium (Orbenin LA; Pfizer New Zealand Ltd, Auckland, New Zealand).

Assignment of challenge week and gland for infusion

Cows were assigned for infusion to 1 of 4 consecutive weeks based on expected oestrus dates, previous mastitis treatments and teat abnormalities. Mating dates and progesterone samples were used to identify expected oestrus dates over the challenge period, and cows were excluded from a week where oestrus was likely to occur. Cows that had teat abnormalities, or had been treated for mastitis previously during the season, were assigned to weeks 3 or 4 where possible. This gave these cows more opportunity to clear the infection, and more importantly minimised the risk of any residual antibiotic effects. Outside these constraints, random assignment to weeks resulted in 65, 66, 65 and 68 cows allocated to weeks 1, 2, 3 and 4 respectively.

The choice of which gland to infuse was made two weeks prior to infusion, and was based on whether or not there had been previous clinical treatments or incidences of significant major infection in the gland during the season of infusion. Wherever possible, the right rear gland within each cow was assigned, however, where there had been previous infection in this gland, the left rear gland was assigned instead.

Pre-infusion sampling

Foremilk samples were collected from each quarter at the 5th milking prior to infusion for subsequent bacteriology assessment. At the morning milking immediately prior to infusion, foremilk samples from all quarters were assessed visually for rapid mastitis test (RMT) score (0=none, T=trace, 1, 2 and 3 scale) and the presence of flecks or clots (0=none, 1=few, 2=many). Duplicate milk samples from the infusion quarter were also collected for assessment of bacteriology (~5 ml) and SCC (~30 ml).

Infusion and post-infusion sampling

The nominated gland was infused intramammarily after a morning milking with a single SU strain that had been stored at –70 °C in 0.85% w/v saline and 10% v/v glycerol. The average CFU infused were 77.6 (sd=10.0), 100.4 (sd=14.9), 112.4 (sd=19.8) and 126.8 (sd=5.8) in challenge weeks 1, 2, 3 and 4 respectively. The SU isolate was from a clinical case of mastitis from a cow in a herd near Morrinsville, New Zealand (McDougall et al., 2004).

Post-infusion sampling was carried out using the same protocols as for pre-infusion sampling, except samples were taken from the challenge quarter only. Post-infusion samples were collected for the subsequent 13 milkings following infusion or until diagnosis of clinical mastitis.

Clinical diagnosis and challenge treatments

Glands were defined as having clinical mastitis when clots were detected and the RMT score was ≥ 2. Upon diagnosis, cows were infused 3 or 4 times at 12-hour intervals with 250mg cefuroxime sodium (Spectrazol® Milking Cow; Schering-Plough Animal Health Ltd, Upper Hutt, New Zealand).

Bacteriology

Milk samples (10 µl) were streaked onto a ¼ of a 5% blood agar plate containing 0.1% esculin (Fort Richard, Auckland, New Zealand) and were incubated at 37 °C for 48 hours. For samples from which SU was isolated, the second sample was used to enumerate the number of CFU present. The number of CFU was determined by counting the number of colonies (between 25 and 250) present on one plate in a series of 10-fold serial dilutions of milk in peptone broth (Fort Richard, Auckland, New Zealand). A gland was defined as
infected where ≥ 3 colonies were present, and defined as contaminated where > 3 distinct colony types were present.

**Classification of infection status at infusion**

Cows were defined as infected at infusion (i.e. when bacteria were isolated in any gland from samples collected on the 5th milking prior to infusion or bacteria were isolated in the infusion gland from samples collected immediately prior to infusion) or clear at infusion (i.e. no bacteria were isolated in any gland from samples collected on the 5th milking prior to infusion and no bacteria were isolated in the infusion gland from samples collected immediately prior to infusion). Where one or more of the samples was either contaminated or had no result and the other samples were clear, the cow was defined as being clear at infusion.

Of the 45 cows with IMI at infusion, one did not have SA isolated (*Escherichia coli* was the isolated pathogen) and was excluded from the analysis. Although glands had been assigned to avoid infusion into a previously infected gland, there were cases of new infections being established within the two-week window between gland assignment and infusion. This resulted in 18 of the 44 cows with SA isolated at infusion (41%), having SA isolated in their challenge quarter.

### Statistical Analysis

Initially, raw clinical infection rates and the hours to clinical infection were evaluated. It was hypothesised that some measure of exposure to IMI, prior to, or at the time of infusion, may alter the probability of clinical mastitis infection. To test this hypothesis, cows were classified as having (n=55) or not having had (n=208) antibiotic treatment(s) for mastitis in lactation 1, and having (n=40) or not having had (n=223) antibiotic treatment(s) for mastitis in lactation 2. A second model was formulated which incorporated an effect for SCC as a measure for exposure to infection prior to or at the time of infusion (rather than the untreated/treated and clear/SA binary indicators used in the first model). The decision was made to use SCC in a second model (rather than adding it as an effect in the first model) due to the high levels of collinearity with other factors already included in the first model. Cows were classified into two SCC groups based on SCC concentrations in the challenge quarter on the morning of infusion: 0-100,000 cells/ml (n=229) and ≥ 100,000 cells/ml (n=30). Note that for 4 cows there was no valid SCC recorded on the morning of infusion, and of those with SCC ≥100,000 cells/ml, 8 were in the range 100-200,000 cells/ml; 5 were in the range 200-400,000 cells/ml and 17 were ≥400,000 cells/ml. For each model, the significance of all fixed effects or any corresponding interaction terms were evaluated using type III \( \chi^2 \) statistics, and non-significant terms were excluded. Once the parameterisation of the models was finalised, probabilities of clinical diagnosis in an infused gland were evaluated as \( \frac{1}{1 + e^{-x}} \) where \( x \) was the fitted value estimated from the model. Wald-chi square tests were used to compare differences between these clinical diagnosis probabilities for fixed effect levels.

Survival analysis was carried out for this study with the limitation that it assumes that a clinical diagnosis will eventually be observed for all animals. Initial univariate survival (and failure) functions were evaluated using the non-parametric product-limit (Kaplan-Meier) method in JMP (version 5.0.1.2) software. These survival functions were stratified by sire, challenge week, whether or not there were treatments for mastitis prior to infusion, whether or not SA was present at infusion and SCC group. A set of survival functions stratified by the interaction between untreated/treated and clear/SA indicators were also generated. Hazard functions were examined independently for each fixed effect, and several parametric distributions were investigated to determine whether there was an appropriate parametric proportional hazard model to apply to the data. However, diagnostic plots for the
Weibull, lognormal and exponential distributions were non-linear, thus indicating that the semi-parametric Cox model was more appropriate. Two Cox models were formulated to estimate differences in clinical mastitis risk between levels of fixed effects. Both models included terms for sire and challenge week. The first model also incorporated binary indicators for whether or not the cow was treated prior to infusion and whether or not SA was present at infusion, whilst the second model incorporated a categorical effect for SCC group. Log-rank \( \chi^2 \) statistics were used to test for homogeneity of survival functions across fixed effects, and the significance of risk-ratio movements was assessed using standard normal z-tests.

**RESULTS**

Overall, the cumulative incidence of clinical diagnosis was 71.9% (189/263) and the mean interval to clinical diagnosis was 66.2 (sd=36.5) hours. The \( \log_{10} \) SCC on the morning of infusion were 1.67 (sd=0.86) and 1.20 (sd=0.54) for previously treated and untreated cows, respectively (p<0.0001) and 1.91 (sd=0.99) and 1.17 (sd=0.46) for cows having or not having a SA intramammary infection at infusion, respectively (p<0.0001).

Final parameter estimates from the logistic regression models for estimating the probability of clinical diagnosis in an infused gland are presented in Table 1. Fixed effects for days in milk at the time of infusion were not significant and were excluded from both models, as were all other non-significant interaction terms. Binary indicators for antibiotic treatment(s) in previous lactations were also assessed for the first model but were non-significant and were thus excluded. In the first model, the probability of clinical diagnosis was significantly associated with sire (p=0.005) and challenge week (p=0.004). The probability of clinical diagnosis for cows infused in week 4 was lower than for all other weeks (p=0.013, 0.008 and 0.002 compared to weeks 1, 2 and 3 respectively). The probability of clinical diagnosis was also significantly associated with the interaction between treatments prior to infusion and infection status at infusion (p=0.006). Clinical diagnosis probabilities did not differ within the treated cows, but they did differ within the untreated cows (29.0% vs. 82.8% for SA and clear cows respectively; p<0.0001).

In the second model, the probability of clinical diagnosis was significantly associated with sire (p=0.005), challenge week (p=0.005) and SCC group (p<0.0001). Cows infused in week 4 had a significantly lower probability of clinical diagnosis compared to those infused in all other weeks (p=0.041, 0.007 and 0.002 for weeks 1, 2 and 3 respectively). The clinical diagnosis probability was also significantly lower for those with a SCC of \( \geq 100,000 \) cells/ml on the morning of infusion compared to those with <100,000 cells/ml on the morning of infusion (p<0.0001).

**TABLE 1:** Parameter estimates for the probability of clinical diagnosis in an infused gland (based on logistic regression models).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>Estimate (std. error)</th>
<th>Probability of clinical diagnosis</th>
<th>Estimate (std. error)</th>
<th>Probability of clinical diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sire</strong></td>
<td></td>
<td></td>
<td>Model 1</td>
<td></td>
<td>Model 2</td>
</tr>
<tr>
<td>1</td>
<td>45</td>
<td>1.29 (0.44)</td>
<td>0.784</td>
<td>0.64 (0.49)</td>
<td>0.654</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>-0.75 (0.37)</td>
<td>0.321</td>
<td>-1.22 (0.41)</td>
<td>0.227</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>0.60 (0.44)</td>
<td>0.646</td>
<td>-0.17 (0.48)</td>
<td>0.457</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>0.47 (0.40)</td>
<td>0.616</td>
<td>0.00 (0.45)</td>
<td>0.501</td>
</tr>
<tr>
<td>5</td>
<td>59</td>
<td>0.60 (0.42)</td>
<td>0.645</td>
<td>0.44 (0.42)</td>
<td>0.608</td>
</tr>
<tr>
<td>6</td>
<td>42</td>
<td>0.59 (0.44)</td>
<td>0.643</td>
<td>0.29 (0.51)</td>
<td>0.571</td>
</tr>
<tr>
<td><strong>Challenge week</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>65</td>
<td>0.69 (0.38)</td>
<td>0.665</td>
<td>-0.05 (0.39)</td>
<td>0.487</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>0.77 (0.41)</td>
<td>0.684</td>
<td>0.37 (0.44)</td>
<td>0.591</td>
</tr>
<tr>
<td>3</td>
<td>65</td>
<td>0.87 (0.33)</td>
<td>0.705</td>
<td>0.59 (0.38)</td>
<td>0.644</td>
</tr>
<tr>
<td>4</td>
<td>47</td>
<td>-0.47 (0.30)</td>
<td>0.385</td>
<td>-0.93 (0.39)</td>
<td>0.283</td>
</tr>
<tr>
<td><strong>Treated prior to infusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Infection status at infusion</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. untreated/clear</td>
<td>185</td>
<td>1.57 (0.22)</td>
<td>0.828</td>
<td>1.48 (0.19)</td>
<td>0.815</td>
</tr>
<tr>
<td>2. untreated/SA</td>
<td>23</td>
<td>-0.90 (0.49)</td>
<td>0.290</td>
<td>-1.49 (0.50)</td>
<td>0.183</td>
</tr>
<tr>
<td>3. treated/clear</td>
<td>34</td>
<td>0.69 (0.41)</td>
<td>0.665</td>
<td>0.14 (0.50)</td>
<td>0.183</td>
</tr>
<tr>
<td>4. treated/SA</td>
<td>21</td>
<td>0.50 (0.50)</td>
<td>0.623</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SCC on morning of infusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. 0-100,000 cells/ml</td>
<td>229</td>
<td></td>
<td></td>
<td>1.48 (0.19)</td>
<td>0.815</td>
</tr>
<tr>
<td>2. &gt; 100,000 cells/ml</td>
<td>30</td>
<td></td>
<td></td>
<td>-1.49 (0.50)</td>
<td>0.183</td>
</tr>
</tbody>
</table>

1Parameter included in Model 1 only.
2Parameter included in Model 2 only.
Kaplan-Meier failure distribution functions stratified by the interaction between indicators for treatments prior to infusion and infection status at infusion are shown in Figure 1. In the corresponding Cox model, the hazard of clinical mastitis varied among sires (p=0.005) and challenge weeks (p=0.002). Variation in hazard was also significant for the interaction between treatments prior to infusion and infection status at infusion (p=0.019). Using cows that were not treated, and had SA present at infusion (untreated/SA) as a reference level, hazard increased for all other treatment/pathogen status interactions. Corresponding risk ratios were 1.42, 1.17 and 1.11 for untreated/clear, treated/clear and treated/SA cows respectively, relative to the untreated/SA cows. P-values corresponding to these risk ratios were 0.004, 0.257 and 0.221 respectively.

FIGURE 1: Failure distribution functions for probability of clinical diagnosis in a gland from the challenge sample taken at time t (stratified by indicators for treatments prior to infusion and infection status at infusion).}

1. untreated/clear (cows that were not treated prior to infusion, and were clear of major pathogen IMI at infusion); 2. untreated/SA (cows that were not treated prior to infusion, and had SA present at infusion); 3. treated/clear (cows that were treated in season of infusion, and were clear of major pathogen IMI at infusion); 4. treated/SA (cows that were treated in season of infusion, and had SA present at infusion).

Kaplan-Meier failure distribution functions stratified by SCC classification are shown in Figure 2. In the corresponding Cox model with SCC class as a fixed effect, the hazard of clinical mastitis varied among sires (p=0.014), challenge weeks (p=0.002) and SCC class (p=0.0001). Using cows with SCC of ≥100,000 cells/ml, with a corresponding risk ratio of 0.414 (p<0.0001).

FIGURE 2: Failure distribution functions for probability of clinical diagnosis in a gland from the challenge sample taken at time t (stratified by SCC classification on the morning of infusion).

DISCUSSION

In this paper, a number of factors potentially affecting the risk of clinical mastitis after infusion of a SU strain have been investigated. Differences in clinical risk were identified between sires, challenge weeks, whether or not there were previous treatments for mastitis, whether or not SA was present at infusion, and SCC concentrations on the morning of infusion. Differences in clinical mastitis risk between sires in this study were attributed to one sire only and were consistent across all challenge weeks. The significance in differences between challenge weeks was attributed to a lower clinical infection rate for challenge week 4, and was consistent across all sires. Weeks 3 and 4 were assigned a higher proportion of cows with prior mastitis treatments (of the 55 cows with prior treatments, 23 were assigned to week 3, and 27 were assigned to week 4), so these weeks were expected to potentially have lower infection rates. However, week 4 was still significantly different from week 3, and an effect for mastitis treatments prior to infusion had already been removed from the models. Variation in the number of CFU infused in each week is not expected to have caused differences in weekly infection rates either (McDougall et al., unpublished), thus indicating that the difference was likely to be as a result of some undefined variation such as cow nutrition, weather or other environmental factors.

Epidemiological studies have suggested that low SCC cows or glands are at increased risk of subsequent clinical mastitis (Shuster et al., 1996; Suriyasathaporn et al., 2000; Beaudeau, et al.,
The current study supports this hypothesis, with increased risk of clinical mastitis in cows with a SCC of <100,000 cells/ml on the morning of infusion. The mechanism for this has not been fully resolved as large numbers of somatic cells alone do not necessarily confer resistance to mastitis (Coffey et al., 1986). Identification of the mechanism behind this effect will be the subject of a subsequent study.

Reduced clinical response rates have been shown where there is prior IMI in experimental challenge models (Hill et al., 1994; Finch et al., 1997), but differences in risk of new IMI with a major pathogen where there is a 'minor' pathogen already present are varied (Hogan et al., 1988; Matthews et al., 1991; Nickerson et al., 1994; Lam et al., 1997). Whether an existing IMI is protective or not appears to be dependent on the pathogen initially present and whether the study was undertaken under natural field conditions or using a challenge model. Little data is available about the effect that the presence of a major pathogen has on the risk of new infection with another major pathogen. In the present study, confounding between whether or not a cow had previous treatment(s) for mastitis, and whether or not SA was present at infusion meant that it was not possible to draw conclusions about these effects in isolation from one another. However, it was possible to conclude that the presence of SA at the time of infusion for cows that were not treated for mastitis (during the season of infusion) was associated with lower clinical mastitis risk.

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