Specificity of two peripartum blood markers for early lactation acute uterine inflammation in pasture-fed, seasonal-calving dairy cows.

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Abstract: A delayed recovery of the reproductive tract from natural inflammatory processes associated with postpartum involution will compromise further reproductive function. Following a literature review, we selected serum amyloid A (SAA) and α1-acid glycoprotein (α1-AGP) to assess as potential circulating markers of acute uterine inflammation, as concentrations of these 2 acute phase proteins were reported to be elevated early postpartum in dairy cows with active uterine infection. Convenience serum samples from an induced model of uterine infection were used to measure concentrations of these markers. Infection was induced by infusing either $10^7$ or $10^9$ cfu of *Treperella pyogenes* (n = 9 cows each; bacteria group n = 18) or saline as a control (n = 18) into the uterus at 48 d postpartum. Although infection stimulated an increase in uterine polymorphonuclear neutrophils, SAA and α1-AGP concentrations in serum were not different between infusion groups. Cows were subsequently classified into uterine health groups based on the presence of endometritis, with or without the presence of *T. pyogenes* in uterine culture in response to uterine infection. Mean SAA concentrations were greater in cows that were either endometritis negative – infection positive (n = 9), endometritis positive – infection negative (n = 5), or endometritis positive – infection positive, compared with the endometritis negative – infection negative (n = 11) cows. There was no difference between uterine health groups for α1-AGP concentrations. Mean SAA and α1-AGP concentrations changed over time, reaching maximum concentrations on d 3 and 7 post-infusion, respectively, before decreasing thereafter to d 17 post-infusion.

In pasture-based, seasonal calving systems, reproductive efficiency is essential to maintain the 365-d inter-calving interval needed to synchronize feed demand with pasture supply and maximize profitability (Roche et al., 2017). Although compromised reproductive function is multifactorial and complex, postpartum uterine infection and inflammation are widely regarded as important elements (LeBlanc, 2020). Bacteria can be isolated from the uterus in over 90% of cows in the early postpartum period, illustrating that the reproductive tract is exposed to bacteria at calving (Sheldon et al., 2009). This exposure stimulates the immune response and associated pro-inflammatory processes as part of the acute phase response (APR) and acts to eliminate foreign entities and minimize the risk of infection (Sheldon et al., 2009). Furthermore, the bacterial exposure coincides with a period of peripartum immune dysregulation, and includes changes in neutrophil function, such as reduced neutrophil adhesion and myeloperoxidase activity (Crookenden et al., 2016, 2019). Dysregulation of immune, inflammatory, and metabolic pathways during the periparturient period have been implicated in the pathogenesis of postpartum diseases, including reproductive tract conditions such as endometritis (Huzzey et al., 2009; Bogado Pascottini and LeBlanc, 2020). Despite the necessity for and naturalness of the peripartum inflammatory processes, timely resolution is critical; a failure to resolve either infection or the inflammatory response associated with the infection negatively affects reproductive outcomes (Williams et al., 2005; Dubuc et al., 2010; Bradford et al., 2015).

Concentrations of acute phase proteins (APP) in circulation can increase by more than 25% following infection, inflammation, or trauma in response to pro-inflammatory cytokines (Eckersall and Bell, 2010). Based on a review of the literature, we proposed that serum amyloid A (SAA) and α1-acid glycoprotein (α1-AGP) be investigated for their specificity to uterine inflammation, as both markers have been associated with reproductive tract inflammation and bacterial presence. For example, increased concentrations of α1-AGP were associated with uterine *Escherichia coli* and *Treperella pyogenes*, and a high uterine bacterial growth score in the weeks following calving (Sheldon et al., 2001; Williams et al., 2005, 2007). We hypothesized that circulating concentrations of SAA and α1-AGP would be greater in cows with an induced uterine infection of *T. pyogenes* at 48 d postpartum.

Recognizing the increasing evidence that the inflammatory response to trauma or infection can present as various uterine disorders that may occur independently, cows were subsequently categorized into uterine health groups. Uterine PMN percentage and presence of *T. pyogenes* in culture from 3 to 17 d post-infusion were used to classify cows, regardless of bacterial infusion treatment, into these groups and compare profiles of circulating markers SAA and α1-AGP in cows with different immune responses to infection.

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The list of standard abbreviations for JDS Communications is available at [adsa.org/jdsc-abbreviations-24](https://adsa.org/jdsc-abbreviations-24).
A better understanding of the profile of inflammatory markers in response to uterine trauma or infection may help identify the pathways involved in the failure to resolve inflammation. Therefore, we aimed to identify whether the profile of circulating concentrations of 2 inflammatory markers were similar between cows that had a) no endometritis or bacterial infection, b) had endometritis in the absence of T. pyogenes in uterine bacterial culture, c) did not develop endometritis but had T. pyogenes isolated in culture, and d) both endometritis and T. pyogenes isolated.

Serum samples used in the current study were a convenience sample from a previously completed ‘parent’ experiment (McDougall et al., 2021). The study protocol was not registered, and study data are not publicly available. The Animal Ethics Committee of AgResearch (Ruakura, Hamilton, New Zealand) approved all animal manipulations for the parent experiment (#13902) in accordance with the New Zealand Animal Welfare Act (Ministry for Primary Industries, 1999). During the experimental period, cows rotationally grazed fresh perennial ryegrass (Lolium perenne L.) and white clover (Trifolium repens L.) used for a subsequent sample that was placed in Amies transport medium (Copan, Fort Richard Laboratories, Auckland, NZ) for microbiological culture of T. pyogenes, the methods for which are outlined in McDougall et al. (2021). Proteins α1-AGP (Life Diagnostics, West Chester, PA, USA) and SAA (TriDelta Development Ltd., Maynooth, Co. Kildare, Ireland) were measured in serum from cows in the SAL and BACT groups at d 0, 3, 7, 11, and 17 post-infusion. Dilution of serum samples with assay buffer was optimized to 1:150 for SAA and 1:20,000 for α1-AGP using a 3-step dilution series. Intra-assay average CV were 10.9% and 4.7% for SAA and α1-AGP, respectively, and inter-assay CV were 12.1% and 12% for SAA and α1-AGP, respectively, with a lower limit of quantification of 1.5 μg/mL and 7 ng/mL, respectively.

Data were log_{10} transformed to achieve homogeneity of variance for serum SAA, α1-AGP, and uterine PMN and analyzed using appropriate procedures in SAS (SAS Studio 3.8, SAS release 9.04). The experimental unit was the cow; no cows or data points were excluded from the analyses. Parity was categorical (primiparous, parity 2–3, and 4+) in the models. A repeated measures mixed model (PROC MIXED) was undertaken to compare mean serum SAA and α1-AGP concentrations, and uterine PMN percentage between the T. pyogenes 10^7 cfu and 10^9 cfu infusion groups. A repeated measures mixed model with backward stepwise elimination was undertaken to determine the effect of day, infusion group, and their interaction on concentrations of serum SAA and α1-AGP, and uterine PMN percentage. The d 0 concentration of SAA and α1-AGP, and the d 0 uterine PMN percentage were included in the respective models as a fixed effect and retained in the final model regardless of significance. Fixed effects (parity and calving season day; number of days from the 1st June to actual calving date) were retained in the model if they were significant (P < 0.05). A separate autoregressive covariance structure for infusion group was chosen.

Effects retained in the final model for SAA were d 0 concentration (P < 0.001), infusion group (P = 0.27), day (P < 0.001), and infusion group by day (P = 0.18). For uterine PMN percentage, effects retained were d 0 uterine PMN (P = 0.31), parity group (P < 0.001), infusion group (P < 0.001), day (P = 0.0082), and infusion group by day (P = 0.18). Fixed effects retained in the final model for α1-AGP were d 0 concentration (P < 0.001), infusion group (P = 0.73), day (P = 0.036), and infusion group by day (P = 0.68).
Cows were subsequently classified into 4 uterine health groups based on uterine PMN and bacterial infection of T. pyogenes from the uterine swab culture. For uterine PMN, cows were classified as endometritis positive (POS) if there were ≥5% PMN at 2 or more time points, and otherwise classified as endometritis negative (NEG). For bacterial infection, cows were classified into infection positive if there were ≥3 colonies of T. pyogenes in the microbial culture at 2 or more time points, and otherwise infection negative. These were further combined into endometritis negative – infection negative (NEG-NEG; n = 11), endometritis negative – infection positive (NEG-POS; n = 9), endometritis positive – infection negative (POS-NEG; n = 5), and endometritis positive – infection positive (POS-POS; n = 11) groups.

A repeated measures mixed model with backward stepwise elimination was undertaken to determine the effect of day, uterine health group, and their interaction on concentrations of serum SAA and α1-AGP. The d 0 concentration of SAA and α1-AGP, and the infusion group (i.e., SAL or BACT) were used as fixed effect covariates for the respective models and retained in the final model regardless of significance. Fixed effects (parity and calving season day) were retained in the model if they were significant (P < 0.05). A separate autoregressive covariance structure for uterine health group was chosen. Effects retained in the final models for SAA were d 0 concentration (P = 0.051), infusion group (P = 0.13), day (P < 0.001), uterine health group (P < 0.001), and uterine health group by day (P = 0.95). For α1-AGP, fixed effects retained were parity group (P = 0.050), d 0 concentration (P < 0.001), infusion group (P = 0.64), day (P = 0.015), uterine health group (P = 0.11), and uterine health group by day (P = 0.78). For each model, the Tukey-Kramer method for multiple comparisons adjustment was applied. Least squares means (LSM) and the 95% upper and lower confidence limits were back-transformed by raising 10 to the respective values. The back-transformed LSM (i.e., geometric mean) and the 95% confidence interval (CI) are presented in figures, tables, and text.

The first analysis involved comparing uterine PMN percentage and concentrations of circulating markers in saline and bacterial infusion groups. Following infusion, uterine PMN in the BACT group was greater than the SAL group (Table 1; mean [CI]: 5.4% [3.49 – 8.04%] and 1.6% [0.94 – 2.41%], respectively; P < 0.001). Uterine PMN initially increased with days post-infection, from a mean of 3.7% [2.12 - 5.98%] at d 3 post-infection to 5.2% [3.14 - 8.36%] at d 11 post-infection, then decreased to 1.3% [0.53 - 2.60%] at d 17 post-infection (Table 1; P < 0.01). There was no effect of infusion group, or an infusion group by day interaction, however, there was an effect of day post-infection on circulating concentrations of both SAA and α1-AGP (Table 1; P < 0.05). Concentrations of SAA were greatest at d 3 post-infection, at 58.7 µg/mL [41.63 – 82.54 µg/mL], decreasing to 15.8 µg/mL [11.03 – 22.57 µg/mL] at d 7 post-infection, to 6.5 µg/mL [11.03 – 22.57 µg/mL] at d 11 post-infusion, and increasing to 13.3 µg/mL [8.91 – 19.52 µg/mL] at d 17 post-infusion. The profile of serum concentrations of α1-AGP was slightly different, increasing from 0.72 mg/mL [0.606 – 0.834 mg/mL] at d 3 post-infection to 0.95 [0.823 – 1.082 mg/mL] at d 7 post-infection, and decreasing thereafter to 0.77 mg/mL [0.660 – 0.895 mg/mL] and 0.73 mg/mL [0.610 – 0.859 mg/mL] at d 11 and 17 post-infusion, respectively.

It was subsequently hypothesized that the presence or absence of endometritis and concurrent presence or absence of T. pyogenes may affect the profile of inflammatory markers. When cows were classified into uterine health groups based on uterine PMN percentage (i.e., endometritis positive or negative) and presence of T. pyogenes at 2 or more time points post-infection (i.e., infection positive or negative), there was an effect on the circulating concentrations of SAA. Mean concentrations for NEG-NEG, POS-NEG, NEG-POS, and POS-POS groups were 8.0 µg/mL [5.35 – 11.65 µg/mL], 18.2 µg/mL [12.73 – 25.87 µg/mL], 22.3 µg/mL [14.14 – 34.73 µg/mL], and 30.6 µg/mL [19.90 – 46.77 µg/mL], respectively (P < 0.001; Table 2; Figure 1). There was also an effect of day post-infection on both SAA and α1-AGP concentrations. With the uterine health group included in the model, SAA and α1-AGP concentrations were similar to models with the bacterial infusion group, with an effect of day post-infection. Concentrations of SAA were greatest at d 3 post-infection, with a mean of 62.1 µg/mL.

Table 1. Geometric means and 95% lower (LCI) and upper (UCI) confidence intervals of uterine PMN percentage and concentrations of serum amyloid A and α1-acid glycoprotein for cows in the saline (SAL; n = 18) and bacteria (BACT; n = 18) infusion groups from d 3 to 17 post-infusion

<table>
<thead>
<tr>
<th></th>
<th>SAL</th>
<th>BACT</th>
<th>Infuse</th>
<th>P-value&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>LCI</td>
<td>UCI</td>
<td>Mean</td>
</tr>
<tr>
<td>Uterine PMN (%)</td>
<td>3</td>
<td>2.2</td>
<td>0.93</td>
<td>4.40</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.4</td>
<td>0.46</td>
<td>3.07</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1.8</td>
<td>0.69</td>
<td>3.74</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>1.0</td>
<td>0.15</td>
<td>2.39</td>
</tr>
<tr>
<td>Serum amyloid A (µg/mL)</td>
<td>3</td>
<td>46.8</td>
<td>30.28</td>
<td>71.98</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>16.5</td>
<td>10.45</td>
<td>25.71</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>4.3</td>
<td>2.45</td>
<td>7.06</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>15.4</td>
<td>9.46</td>
<td>24.69</td>
</tr>
<tr>
<td>α1-acid glycoprotein (mg/mL)</td>
<td>3</td>
<td>0.77</td>
<td>0.626</td>
<td>0.917</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.90</td>
<td>0.749</td>
<td>1.063</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0.76</td>
<td>0.619</td>
<td>0.909</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.70</td>
<td>0.556</td>
<td>0.854</td>
</tr>
</tbody>
</table>

<sup>1</sup>P-values presented for effect of uterine infusion group (infuse), day post-infection, and the interaction of infusion group and day post-infection in the repeated measures mixed models.
[44.11 – 87.13 µg/mL], decreasing to 16.5 µg/mL [11.51 – 23.42 µg/mL] at d 7 post-infusion and 6.6 µg/mL [4.46 – 9.66 µg/mL] at d 11 post-infusion, and increasing to 14.1 µg/mL [9.53 – 20.53 µg/mL] at d 17 post-infusion (Table 2; *P < 0.001). At d 3 post-infusion, α1-AGP concentrations were 0.79 mg/mL [0.618 – 0.898 mg/mL], increasing to 1.04 mg/mL [0.901 – 1.184 mg/mL] at d 7 post-infusion, and decreasing again to 0.84 mg/mL [0.721 – 0.977 mg/mL] at d 11 post-infusion and 0.79 mg/mL [0.660 – 0.927 mg/mL] at d 17 post-infusion (Table 2; *P < 0.05).

There is increasing evidence of the distinct pathogenesis of uterine diseases, including endometritis and purulent vaginal discharge (PVD). For example, one can be diagnosed in the absence of the other condition (Dubuc et al., 2010; Pascottini and LeBlanc, 2020), and diagnosis of either is associated with negative effects on conception and pregnancy outcomes (McDougall et al., 2009). Furthermore, when coincidental, they can be synergistic in their negative effects on reproduction (Dubuc et al., 2010). By inducing a uterine infection at 48 d postpartum in otherwise healthy cows, we aimed to provide a model of infection and the resultant acute inflammation without the homeorhetic changes associated with parturition and any associated metabolic and immune disturbances.

We hypothesized that circulating markers of inflammation would be increased in the BACT group, however, there was no difference for either SAA or α1-AGP concentrations between uterine infusion groups. The lack of difference between uterine infusion groups for SAA and α1-AGP concentrations appear contradictory to previous studies (Sheldon et al., 2001; Williams et al., 2005, 2007). In these studies, circulating concentrations of SAA and α1-AGP increased in the first 5 wk following calving, which was associated with reproductive tract inflammation and bacterial infection, including *T. pyogenes*. One reason for the apparent contradiction may have been the use of different antimicrobials.

### Table 2. Geometric means and 95% lower (LCI) and upper (UCI) confidence intervals of concentrations of serum amyloid A and α1-acid glycoprotein from d 3 to 17 post-infusion for cows classified into uterine health groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Day</th>
<th>Serum amyloid A (µg/mL)</th>
<th>Mean</th>
<th>LCI</th>
<th>UCI</th>
<th>α1-acid glycoprotein (mg/mL)</th>
<th>Mean</th>
<th>LCI</th>
<th>UCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEG-NEG</td>
<td>3</td>
<td>30.3</td>
<td>17.0</td>
<td>24.2</td>
<td>33.0</td>
<td>0.74</td>
<td>0.59</td>
<td>0.60</td>
<td>0.70</td>
</tr>
<tr>
<td>NEG-POS</td>
<td>7</td>
<td>7.4</td>
<td>3.80</td>
<td>5.23</td>
<td>10.58</td>
<td>0.83</td>
<td>0.67</td>
<td>0.71</td>
<td>0.95</td>
</tr>
<tr>
<td>POS-NEG</td>
<td>11</td>
<td>2.2</td>
<td>1.32</td>
<td>1.39</td>
<td>2.21</td>
<td>0.69</td>
<td>0.54</td>
<td>0.60</td>
<td>0.90</td>
</tr>
<tr>
<td>POS-POS</td>
<td>17</td>
<td>0.9</td>
<td>0.54</td>
<td>0.70</td>
<td>1.16</td>
<td>0.74</td>
<td>0.59</td>
<td>0.60</td>
<td>0.70</td>
</tr>
</tbody>
</table>

*P-values presented for effect of uterine health group, day post-infection, and the interaction of uterine health group and day post-infusion in the repeated measures mixed models.
lie in the experimental approaches. The previous studies used a naturally occurring peripartum infection model. In such a model, it is not possible to ascertain causation; the infection was associated with changes to the APR, characterized by increases in circulating inflammatory markers (Sheldon et al., 2001; Williams et al., 2005, 2007). However, it is unknown whether this is a coincidental effect of peripartum immune dysregulation, and the metabolic changes associated with the transition to lactation on the APR, or whether the homeorhetic changes associated with parturition contribute to the pathogenesis of uterine infection and postpartum diseases (Crookenden et al., 2016, 2019; LeBlanc, 2020).

Although there was an increase in uterine PMN in the days following infusion for the BACT group indicating a localized inflammatory reaction, some cows in the SAL group also exhibited an inflammatory response, with an increase in uterine PMN. It is possible that taking endometrial samples and the infusion of saline, or a combination of both, caused trauma to the endometrial lining, which triggered an inflammatory cascade and the influx of PMN. Cows were subsequently classified by the inflammatory response to the infusion, and the presence of T. pyogenes in bacterial culture. These 4 uterine health groups were used to compare concentrations of SAA and α1-AGP to investigate whether different responses to endometrial lining damage and bacterial ingress also had differences in the profile of inflammatory markers. Although the profile of inflammatory markers was not different between uterine health groups during the post-infusion period, i.e., a lack of group by time interaction, mean concentrations of SAA were greater in groups with an inflammatory response, or bacterial infection, or both, compared with cows that had no apparent inflammatory response to bacterial ingress or resolved the infection rapidly. Therefore, we hypothesize that there were differences in the immune response to the treatment protocol, that were reflected in the elevated concentrations of SAA in cows that had active inflammation or infection.

It is important to note the infection model in the current study was undertaken in healthy cows at d 48 postpartum, and the uterine environment created by the process of the treatment, and the presence of SAA. Increased concentrations of inflammatory markers peripartum or in very early lactation could be indicative of a peripartum immune dysregulation that increases the risk of endometritis (Pascottini and LeBlanc, 2020; Spaans et al., 2023). Although there was no difference over the post-infusion period between uterine health groups, mean SAA concentrations for groups with uterine infection-induced endometritis, or bacterial infection with T. pyogenes, or both, were greater than cows with no inflammatory response to treatment protocols. Therefore, the degree and duration of the acute phase response to uterine infection can be characterized by increased concentrations of circulating inflammatory markers, such as SAA.

References


NOTES

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