Evaluation of reticuloruminal temperature for the prediction of clinical mastitis in dairy cows challenged with Streptococcus uberis

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ABSTRACT

Automated monitoring devices have become increasingly utilized in the dairy industry, especially for monitoring or predicting disease status. While multiple automated monitoring devices have been developed for the prediction of clinical mastitis (CM), limitations in performance or applicability remain. The aims of this study were to (1) detect variations in reticuloruminal temperature (RRT) relative to an experimental intramammary challenge with Streptococcus uberis and (2) evaluate alerts generated automatically based on variation in RRT to predict initial signs of CM in the challenged cows based on severity of clinical signs and the concentration of bacteria (cfu/mL) in the infected quarter separately. Clinically healthy Holstein cows without a history of CM in the 60 d before the experiment (n = 37, parity 1 to 5, ≥120 d in milk) were included if they were microbiologically negative and had a somatic cell count under 200,000 cells/mL based on screening of quarter milk samples 1 wk before challenge. Each cow received an intra-reticuloruminal automated monitoring device before the trial and was challenged with 2,000 cfu of Strep. uberis 0140J in 1 rear quarter. Based on interrupted time series analysis, intramammary challenge with Strep. uberis increased RRT by 0.54°C [95% confidence interval (CI): 0.41, 0.66] at 24 h after the challenge, which remained elevated until the end of the study. Alerts based on RRT correctly classified 78.3% (95% CI: 65.8, 87.9) of first occurrences of CM in the challenged quarter reached 5.0 log10 cfu/mL, reaching a sensitivity of 73.5% (95% CI: 55.6, 87.1) and a specificity of 87.5% (95% CI: 71.0, 96.5). Overall, the results of this study indicated that RRT was affected by the intramammary challenge with Strep. uberis and the RRT-generated alerts had similar accuracy as reported for other sensors and algorithms. Further research that includes natural infections with other pathogens as well as different variations in RRT to determine CM status is warranted.

Key words: precision dairy, automated monitoring device, reticuloruminal temperature, clinical mastitis detection

INTRODUCTION

Automated monitoring devices (AMD) have become increasingly available in the dairy industry (Bell and Tzimiropoulos, 2018). Adoption of AMD is expected to grow as the size and consolidation of dairy herds continue to increase (Barkema et al., 2015; Gonzalez-Mejia et al., 2018; Luby et al., 2020). Multiple AMD have been developed to aid in early detection of clinical mastitis (CM) as it represents a major concern for dairy farmers (Delgado et al., 2017; Liang et al., 2017; USDA, 2018). Worldwide, 20 to 40% of lactating dairy cows develop CM throughout lactation and Streptococcus uberis is responsible for about 5 to 17% of those cases (Oliveira et al., 2013; Ruegg et al., 2015; Tomazi et al., 2018). Previous research evaluating the pathogenesis of Strep. uberis (Archer et al., 2020) and intramammary antimicrobial efficacy (Hillerton and Kliem, 2002; McDougall et al., 2019; Ruegg and Erskine, 2020) have reported that most cases benefit from antimicrobial therapy because of its relatively low spontaneous cure rate. For herds that have automatic milking systems, use of AMD to detect IMI can be of major importance given the inability to visually assess milk quality (Hogeveen et al., 2021).
Among the attributes frequently utilized to determine IMI are included milk yield, milk composition, SCC, milk electrical conductivity, and milk flow rate (Sørensen et al., 2016; Khatun et al., 2017; Fadul-Pacheco et al., 2021). In addition, activity-recording devices that measure number of steps or allocation of time (lying, standing, walking, or ruminating) have also been used with varying degrees of success (Stangaferro et al., 2016; Khatun et al., 2018). Previous researchers have reported body site temperature to be an efficient tool to determine IMI given the febrile state generated by the immune response upon infections (Rambeaud et al., 2003; Tassi et al., 2013). However, most evaluations have focused on udder skin surface or rectal temperature, the latter being the most utilized technique in dairy farms (Sathiyabarathi et al., 2016; Machado et al., 2021). The use of intra-reticuloruminal AMD offer an alternative to manually monitoring rectal temperature, providing automated and frequent monitoring without the need to restrain cows (Adams et al., 2013). The purposes of this study were to (1) determine variations in reticuloruminal temperature (RRT) relative to an experimental intramammary challenge with Strep. uberis and (2) evaluate alerts generated automatically based on variation in RRT to predict initial signs of CM in challenged cows based on severity of clinical signs and concentration of bacteria (cfu/mL) in the infected quarter. We hypothesized that the intramammary challenge with Strep. uberis will generate a variation in RRT and the alerts generated would have good performance to identify cows with IMI in early stages before the occurrence of clinical signs. In addition, we hypothesized that the performance of the alerts based on RRT would increase with severity of clinical signs and as the number of bacteria in the infected quarters increased.

**MATERIALS AND METHODS**

**Study Design and Enrollment Criteria**

This study was nested within a randomized clinical trial that was evaluating the effects of a feeding additive (NutriTek; Diamond V) on mammary gland immune responses after an intramammary challenge with Strep. uberis. Results of that study will be separately reported. Cows from the Michigan State University Dairy Teaching and Research Center were eligible if they were >120 DIM at the beginning of the study, had 4 functional teats, did not have CM or other major health events during the previous 60 d, and their 2 previous DHI tests had SCC ≤200,000 cells/mL. To manage data collection, cows were enrolled sequentially in 4 cohorts of 9 to 12 cows each and were randomly assigned to receive the feed additive (19 g/cow per d) or to a control group. The study periods included 7 d of acclimatization to the basal diet (phase 1), 45 d of the feeding trial (phase 2), 5 to 7 d of intramammary challenge with Strep. uberis (phase 3), and 45 d of follow-up (phase 4; Figure 1). For our study, an intra-reticuloruminal AMD (Herd-Strong; DVM Systems) was orally administered to 37 enrolled cows 16 d before the bacteriological challenge. Each intra-reticuloruminal AMD recorded RRT every 30 min and transmitted the data to a cloud-based data repository where it was automatically processed by the manufacturer to generate an alert when RRT departed 1 standard deviation from the baseline temperature. All procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University (protocol 20-21-000-19).

**Data Collection and Intramammary Bacterial Challenge**

During phase 1, milk samples from all 4 quarters were aseptically collected and cultured to ensure eligibility for the trial. During phase 2, quarter-level milk samples for SCC analysis were collected once a week during the first 5 wk. During wk 6 (i.e., week before challenge), quarter milk samples were collected twice, d −3 and d −0 (immediately before challenge) for microbiological analysis and SCC. On d 0 (i.e., the first day of the challenge), after the morning milking was completed, 2,000 cfu of Strep. uberis 0140J suspended in 2 mL of PBS (i.e., 1,000 cfu/mL) were infused into a randomly assigned rear quarter of each cow. The detailed procedure to generate the inoculum has been previously described (Finch et al., 1997; Swartz et al., 2021). During phase 3, milk from the challenged quarter was collected for microbiological analysis and SCC from d 1 to 7 (cohorts 1 and 2) and 1 to 5 (cohorts 3 and 4) post-challenge. In phase 4, quarter milk was collected for microbiological analysis and SCC once weekly through d 95. All milk microbiological procedures were performed in the Ruegg laboratory at Michigan State University following National Mastitis Council guidelines (NMC, 2017).

Once daily during the morning milking, researchers evaluated cows for clinical signs of mastitis. When CM was detected, cases were categorized for severity (Pinzón-Sánchez and Ruegg, 2011). Mild indicated only abnormal milk; moderate indicated evidence of mammary gland inflammation (e.g., udder swelling, redness, pain); and severe indicated the presence of systemic signs of illness (e.g., depression, anorexia, dehydration, fever). Rectal and vaginal temperatures were measured daily after the first milking. Fever, used to determine cows with a CM severity score of 3 (systemic symptoms), was defined as rectal temperature ≥39.7°C. On
d 7 (cohorts 1 and 2) or d 5 (cohorts 3 and 4), cows were initially treated once daily for 5 (cohorts 1 and 2) or 8 d (cohorts 3 and 4) using an intramammary antibiotic (Ceftiofur; Spectramast LC, Zoetis) and then monitored for 45 d. Treatments were initiated earlier in cohorts 3 and 4 to limit progression of cases. For analysis, data were used from 1 wk before challenge until treatment was administered.

**Statistical Analysis**

All statistical analyses were done using R 3.4.4 software (R, RStudio Inc.). Cow was the experimental unit. Cows were included in the analysis when quarter milk samples collected 1 wk before challenge were microbiologically negative and had a SCC under 200,000 cells/mL. Of 37 cows, 4 received an additional intra-reticuloruminal AMD because of technical issues. Of 37 AMD, 35 recorded RRT every 30 min while 2 recorded RRT every 30 s. To standardize analysis of data obtained from cows with the later 2 AMD, a random temperature measurement was selected every 30 min for each AMD. Pearson correlation was calculated between RRT measured every 30 min and the randomly selected RRT measured every 30 s.

**Temperature Variations After Challenge**

Correlations of vaginal and rectal temperatures with RRT collected after challenge until treatment were done using Pearson correlation. Effects of intramammary challenge on RRT were evaluated using interrupted time series analysis. This approach allowed us to evaluate the effect of an intervention (i.e., intramammary challenge) on a given outcome (i.e., RRT) immediately after the intervention and over time (Wagner et al., 2002; Soumerai et al., 2015; Bernal et al., 2017). The model was built as a segmented regression, which divides a time series into pre- and post-challenge segments and is based on the following algorithm:

\[
Y_{it} = \beta_0 + \beta_1 \times \text{time}_{it} + \beta_2 \times \text{challenge}_{it} + \beta_3 \times \text{time after challenge}_{it} + \nu_i + \epsilon_{it},
\]

where \(Y_{it}\) is the RRT for cow \(i\) in time \(t\) (measured every 30 min), \(\beta_0\) estimates the baseline level of the RRT at the beginning of the time series, \(\beta_1\) estimates the pre-challenge trend where \(\text{time}\) is a continuous variable indicating the time at time \(t\) from the start of the study period for cow \(i\), \(\beta_2\) estimates the variation in RRT post-challenge for cow \(i\), \(\beta_3\) estimates the change in RRT post-challenge trend for cow \(i\) where “time after challenge” is a continuous variable indicating the number of measurements after the start of the challenge at time \(t\), \(\nu_i\) represents the random effect of cow \(i\), and \(\epsilon_{it}\) is an error term. As error terms of consecutive observations are often correlated because time is a predictor in segmented regression analysis (Wagner et al., 2002), the model included an autoregressive term. The cohort and feeding treatment were included and kept in the model as potential confounders independently of statistical significance. In addition, a Fourier term was included in the model to smooth within-day variations (Bhaskaran et al., 2013). Given that the intramammary challenge could have a lagged effect on RRT measurements, a potential delayed effect was explored.

**Calculation of Test Characteristics**

Sensitivity (Se), specificity (Sp), positive predictive values (PPV), negative predictive values (NPV), and accuracy were calculated (Dohoo, 2009). For each calculation, each cow served as its own control. Thus, Sp was calculated based on the days before challenge as...
cows were culture negative and free of IMI, while Se was calculated based on days after challenge as cows were culture-positive indicating occurrence of IMI. The timeframe used for calculations varied among cows depending on outcome.

Test characteristics of the RRT alerts to predict clinical signs of mastitis based on severity scores (mild, moderate, severe) were calculated using the following definitions: Disease negative and disease positive referred to cows before and after the challenge, respectively. Test positive and test negative referred to the presence or absence of an RRT alert, respectively. Calculations were done separately for each severity score. The timeframe for calculation of Se ranged from the time of intramammary challenge until 24 h before the first occurrence of clinical signs of the given severity. An RRT alert generated during this period was deemed as a true-positive, while the lack of an RRT alert was deemed as a false-negative. The Sp was calculated during the period before the challenge and used the same duration as Se. For example, if a cow had initial clinical signs of a given severity on d 4 post-challenge, the first 3 d post-challenge were used to calculate Se and the 3 d pre-challenge were used to calculate Sp. Contrary to Se, an RRT alert for Sp during the pre-challenge time period was deemed a false-positive as the cow was healthy, while the lack of an RRT alert was deemed as true-negative. Test characteristics for each severity score were calculated separately.

Similarly, test characteristics of the RRT alerts to predict initial signs of CM were calculated using the period from the time of intramammary challenge until 24 h before the first occurrence of clinical signs but independent of severity score. The defined period used for each cow was applied to calculations of Sp during the days before the challenge.

Test characteristics of the RRT alerts to determine IMI based on bacterial count of quarter milk samples were also calculated. The Se was defined as the correct identification of IMI based on generated RRT alerts when bacterial concentration was less than or equal to a defined bacterial concentration (calculated for each 1.0 log₁₀ cfu/mL unit from 1.0 log₁₀ cfu/mL to 12.0 log₁₀ cfu/mL). Given that some cows had high bacterial concentrations since the first sampling day after challenge, not all cows were included in the analysis of each bacterial concentration. For example, when evaluating the ability of the RRT alerts to determine CM with a bacterial count ≤4.0 log₁₀ cfu/mL, a cow with a bacterial concentration of 5.0 log₁₀ cfu/mL at the first sampling day post-challenge was not included. As bacterial concentration increased, more cows were included, which is reflected in the increasing sample size. The Sp was defined as the correct identification of non-IMI status based on the lack of RRT alerts based on bacterial concentration of 0 (before the challenge). Thus, for the calculation of Sp, the same duration was used as for Se. For example, to calculate Sp with a threshold of 5.0 log₁₀ cfu/mL, if bacterial count in a milk sample exceeded 5.0 log₁₀ cfu/mL on d 4 after challenge, an RRT alert registered within those 4 d was considered true-positive, while absence of an RRT alert was deemed as false-negative. Using the same duration for the same cow to calculate Sp, an RRT alert registered in the 4 d preceding the challenge was deemed false-positive, while a lack of an RRT alert was deemed true-negative.

RESULTS

The correlation between average RRT recorded by AMD every 30 min and a randomly selected RRT recorded by AMD that recorded every 30 s was 0.91 (95% CI: 0.90, 0.92). Data from an AMD that recorded 33 consecutive observations between 20.0 and 24.2°C during a 16.5 h period (0.12% of the data set) were excluded. In addition, 99 consecutive observations between 20.7 and 24.7°C, recorded by another AMD over a 49.5 h period (0.38% of data), were excluded. No other data were removed.

Of enrolled cows (n = 37), 20 were primiparous and 17 were multiparous. Average milk yield at enrollment was 40.9 ± 1.3 kg/cow per d (±SE). Bacteriological evaluation of each quarter milk sample (n = 148) collected the week before the challenge (d −3 and d 0) showed no bacteriological growth and no clinical signs of mastitis, indicating cows had no IMI the week before the challenge. Of experimentally infected cows (n = 37), *Strep. uberis* O140J was recovered from the challenged quarter of 36 cows. During the week of the challenge, 32 cows exhibited clinical signs of mastitis, from which 2 cows only had mild signs, 23 cows reached moderate signs, and 7 cows reached severe signs. In addition, *Strep. uberis* was still recovered by d 5 to 7 after the challenge in all the 36 cows that developed IMI, indicating a lack of spontaneous cure during this period.

Pearson correlations with RRT were 0.64 (95% CI: 0.55, 0.70) for vaginal temperature and 0.50 (95% CI: 0.39, 0.59) for rectal temperature. Based on use of interrupted time series, the estimated marginal mean of RRT during the week before challenge was 39.1°C (95% CI: 38.9, 39.3; Figure 2). After intramammary challenge, we observed an increase in RRT of 0.54°C (95% CI: 0.41, 0.66; P < 0.001). The increased RRT was observed 24 h after intramammary challenge and remained constant until the end of the follow-up period (5 to 7 d post mastitis challenge) at 39.7°C (95% CI: 39.4, 39.9).
Parity was not kept in the final model. Feeding additive treatment was not significant in the model ($P = 0.64$) but was kept to prevent potential residual confounding. After intramammary challenge with *Strep. uberis*, the frequency of RRT alerts increased as compared with RRT alerts recorded before challenge (Figure 3; $P < 0.001$). Of all RRT alerts, 13.6% occurred in healthy cows (the week before the challenge), 86.4% occurred in intramammary-infected cows (the week after the challenge). Like the increase in RRT, the peak number of RRT alerts occurred 24 hr after the challenge.

The Se of RRT alerts to predict CM at least 24 h before occurrence was greatest for cows with severe CM (100%; 95% CI: 59.0, 100), while the Sp was greatest for cows with moderate CM (92.6; 95% CI: 75.7, 99.1; Table 1). However, estimates of test characteristics are not precise and have wide and overlapping confidence intervals. The Se to predict first signs of CM independent of severity was 70.0% (95% CI: 50.6, 85.3), while Sp was 86.7% (95% CI: 69.3, 96.2). The PPV was 84.0% (95% CI: 63.9, 95.5), while the NPV was 74.3% (95% CI: 56.7, 87.5). Accuracy of classification was 78.3% (95% CI: 65.8, 87.9). The bacterial concentration in milk samples increased after challenge. The Se of the RRT alerts to identify IMI when bacterial count was $\leq 4.0 \log_{10} \text{cfu/mL}$ in milk samples was 66.7% (95% CI: 69.3, 96.2) and the Sp was 86.7% (95% CI: 69.3, 96.2). After a bacterial count of $4.0 \log_{10} \text{cfu/mL}$, Se increased numerically until $6.0 \log_{10} \text{cfu/mL}$, remaining...
stable until 12.0 log_{10} cfu/mL (Table 2, data shown until 7.0 log_{10} cfu/mL). Meanwhile, Sp measured with no bacterial growth was stable at 86.1 (95% CI: 70.5, 95.3) to 88.2% (95% CI: 63.6, 98.5). The combined ability of RRT alerts to correctly classify IMI and healthy cows reached 81.8% at a minimum bacterial count of 5.0 log_{10} cfu/mL. After this level, there was a modest improvement in classification.

**DISCUSSION**

In this experiment, we observed that RRT of dairy cows was modified in response to IMI caused by *Streptococcus uberis* after 24 h post-infection. This variation in RRT was enough to generate alerts based on 1 standard deviation of the baseline temperature. The ability to identify IMI was related to severity score and bacterial concentration in the infected quarter and improved with bacterial concentration and severity of clinical signs.

The RRT during the week before challenge was within the normal temperature range of 38.0–39.4 °C previously reported (Bewley et al., 2008b) and increased after intramammary challenge, remaining constant until the end of the study period. AlZahal et al. (2011) reported that RRT increased by 2.4°C in quarters experimentally infected with *Escherichia coli*. We observed a smaller increase in RRT, likely due to differences in pathogenicity between *Strep. uberis* and *E. coli*, which highlights pathogen-specific characteristics of temperature variations after IMI. Increased RRT may also be related to the concentration of bacteria in the infected quarters, as a correlation between bacterial concentration and body temperature has been previously reported (Rambaud et al., 2003; Tassi et al., 2013). Similar to our observation of an RRT of 39.7°C when bacterial count reached 3.0 log_{10}, Swartz et al. (2021) reported average vaginal temperature of 39.5°C with bacterial count of 2.0 log_{10} after challenging cows with 1 × 10^4 cfu/mL of *Strep. uberis*. Nevertheless, in agreement with other studies, correlations of RRT with vaginal and rectal temperatures are relatively low (Bewley et al., 2008a; Lees et al., 2019). Multiple factors have been shown to influence the strength of the correlations including ambient temperature, temperature of drinking water, feed intake, and composition of the diet (Timsit et al., 2011; Ammer et al., 2016; Lees et al., 2019).

The prediction of CM using milk-related attributes (e.g., conductivity, SCS, lactate dehydrogenase, and milk yield), alone or in combination, has been widely evaluated showing diverse performance (Ankinakatte et al., 2013; Khatun et al., 2018; Fadul-Pacheco et al., 2021). Still, none has met acceptable values for the prediction of CM defined as 80% Se and 99% Sp (ISO, 2007; Hogeveen et al., 2021). The use of RRT to predict CM has been rarely evaluated. Adams et al. (2013) used a different intra-reticuloruminal AMD that measured

<table>
<thead>
<tr>
<th>CM severity score</th>
<th>Test characteristic</th>
<th>Ability to predict CM at least 24 h before onset of clinical signs, % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild (severity 1)</td>
<td>Se</td>
<td>90.9 (58.7, 99.8)</td>
</tr>
<tr>
<td></td>
<td>Sp</td>
<td>90.9 (58.7, 99.8)</td>
</tr>
<tr>
<td></td>
<td>PPV</td>
<td>90.9 (58.7, 99.8)</td>
</tr>
<tr>
<td></td>
<td>NPV</td>
<td>90.9 (58.7, 99.8)</td>
</tr>
<tr>
<td></td>
<td>Accuracy</td>
<td>90.9 (70.8, 98.9)</td>
</tr>
<tr>
<td>Moderate (severity 2)</td>
<td>Se</td>
<td>77.8 (57.7, 91.4)</td>
</tr>
<tr>
<td></td>
<td>Sp</td>
<td>92.6 (75.7, 99.1)</td>
</tr>
<tr>
<td></td>
<td>PPV</td>
<td>91.3 (72.0, 98.9)</td>
</tr>
<tr>
<td></td>
<td>NPV</td>
<td>80.6 (62.5, 92.5)</td>
</tr>
<tr>
<td></td>
<td>Accuracy</td>
<td>85.2 (72.9, 93.4)</td>
</tr>
<tr>
<td>Severe (severity 3)</td>
<td>Se</td>
<td>100 (59.0, 100)</td>
</tr>
<tr>
<td></td>
<td>Sp</td>
<td>85.7 (42.1, 99.6)</td>
</tr>
<tr>
<td></td>
<td>PPV</td>
<td>87.5 (47.3, 99.7)</td>
</tr>
<tr>
<td></td>
<td>NPV</td>
<td>100 (54.1, 100)</td>
</tr>
<tr>
<td></td>
<td>Accuracy</td>
<td>92.9 (66.1, 99.8)</td>
</tr>
</tbody>
</table>

1Based on severity score at least 24 h before the occurrence of clinical signs of a given severity in 37 Holstein dairy cows.
2Se = sensitivity: correct prediction of a CM status based on a generated RRT alert.
3Sp = specificity: correct prediction of a CM absence status based on the lack of an RRT alert.
4PPV = positive predictive value: probability that, given an RRT alert, the cow will develop CM of a given severity.
5NPV = negative predictive value: probability that, given a lack of an RRT alert, the cow will not develop CM of a given severity.
6Accuracy includes 22 observations for mild severity, 54 for moderate, and 14 for severe severity.
Table 2. Performance of the reticuloruminal temperature (RRT) alerts to predict clinical mastitis (CM) based on a maximum concentration of *Streptococcus uberis* in milk equal to or less than:

<table>
<thead>
<tr>
<th>Test characteristic, % (95% CI)</th>
<th>4.0 log&lt;sub&gt;10&lt;/sub&gt; cfu/mL</th>
<th>6.0 log&lt;sub&gt;10&lt;/sub&gt; cfu/mL</th>
<th>8.0 log&lt;sub&gt;10&lt;/sub&gt; cfu/mL</th>
<th>10.0 log&lt;sub&gt;10&lt;/sub&gt; cfu/mL</th>
<th>12.0 log&lt;sub&gt;10&lt;/sub&gt; cfu/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Se&lt;sup&gt;1&lt;/sup&gt;</td>
<td>66.7 (47.2, 82.7)</td>
<td>77.1 (59.9, 89.6)</td>
<td>78.4 (61.8, 90.2)</td>
<td>83.8 (68.0, 93.8)</td>
<td>86.5 (71.2, 95.5)</td>
</tr>
<tr>
<td>Sp&lt;sup&gt;2&lt;/sup&gt;</td>
<td>86.7 (69.3, 96.2)</td>
<td>88.6 (73.3, 96.8)</td>
<td>91.9 (78.1, 98.3)</td>
<td>91.9 (78.1, 98.3)</td>
<td>81.1 (64.8, 92.0)</td>
</tr>
<tr>
<td>PPV&lt;sup&gt;3&lt;/sup&gt;</td>
<td>83.3 (62.6, 95.3)</td>
<td>87.1 (70.2, 96.4)</td>
<td>90.6 (75.0, 98.0)</td>
<td>91.2 (76.3, 98.1)</td>
<td>82.1 (66.5, 92.5)</td>
</tr>
<tr>
<td>NPV&lt;sup&gt;4&lt;/sup&gt;</td>
<td>72.2 (54.8, 85.8)</td>
<td>79.5 (63.5, 90.7)</td>
<td>81.0 (65.9, 91.4)</td>
<td>85.0 (70.2, 94.3)</td>
<td>85.7 (69.7, 95.2)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>76.7 (64.0, 86.6)</td>
<td>82.9 (72.0, 90.8)</td>
<td>85.1 (75.0, 92.3)</td>
<td>87.8 (78.2, 94.3)</td>
<td>83.8 (73.4, 91.3)</td>
</tr>
<tr>
<td>Sample size&lt;sup&gt;5&lt;/sup&gt;</td>
<td>30</td>
<td>35</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
</tbody>
</table>

2. Sp = specificity: correct identification of non-IMI status based on the lack of an RRT alert.
3. PPV = positive predictive value: probability that, given an RRT alert, the cow has IMI.
4. NPV = negative predictive value: probability that, given the absence of an RRT alert, the cow does not have an IMI.
5. Sample size indicates participating cows in the analysis. Sample size for accuracy was double what was reported for each bacterial concentration level because it includes 2 observations per cow (before and after challenge).

The RRT alerts showed a clear improvement in Se of prediction of CM when the bacteriological concentration of *Strep. uberis* in milk reached or exceeded 5.0 log<sub>10</sub> cfu/mL. The improvement in Se to predict CM as concentration of bacteria increased is likely associated with occurrence of greater severity (Wenz et al., 2001). However, it needs to be considered that experimentally induced mastitis likely differs in the magnitude and speed of bacterial growth as compared with naturally occurring infections, which could alter the performance of RRT-generated alerts under natural exposure conditions (Pedersen et al., 2003; Tassi et al., 2013; Swartz et al., 2021). This different rate of bacterial growth can be observed by looking at the length of the subclinical stage. The average duration between intramammary challenge and occurrence of clinical signs was 4.1 d. In naturally occurring IMI caused by *Streptococcus* spp., the subclinical stage has been reported to be 12.3 d (Todhunter et al., 1995). Another indication of the fast bacterial growth in the cows challenged is the rapid evolution of clinical signs as only 27.6% (8/29) of the moderate cases were preceded by a mild score on a previous day, indicating that in most cases, abnormal milk as the sole clinical sign lasted for <24 h.

The value of a screening test is based on its ability to identify animals that may benefit from an effective intervention. However, the benefit of the intervention such as antimicrobial treatments or isolation of cows is dependent on etiology (Oliver et al., 2004; Fuenzalida and Ruegg, 2019a). In our study, the RRT alerts were evaluated based on experimental challenge with *Strep. uberis*, and results should not be extrapolated to CM...
caused by other pathogens. Differences in host responses may be observed based on natural or experimental IMI (e.g., deposition of bacteria in the teat cistern via a cannula; Tassi et al., 2013), which limits the generalization of our results. Another limitation is that we used a prevalence of IMI of 50% to calculate Sp (each cow served as her own control). Given that predictive values are a function of Se, Sp, and prevalence, our decision to use 50% prevalence must be considered when extrapolating both positive and negative predictive values. Strep. uberis has been isolated in 13% to 28.2% of CM samples (Olde Riekerink et al., 2008; Verbeke et al., 2014; Ruegg et al., 2015). By applying Bayes’ theorem (Patterson and Horowitz, 1989) in a herd with a prevalence of CM caused by Strep. uberis of 18% (Royster et al., 2014) and a Se and Sp of 70.0% and 86.7% respectively, the PPV is expected to decrease to 53.6% as the NPV is expected to increase to 92.9%. These estimates suggest that while most cows with an RRT alert would not have CM, most of the cows without an RRT alert do not have an IMI. Although it is advantageous to have confidence that cows without alerts are likely to be not infected, it would be beneficial to follow cows with alerts by performing a confirmatory test such as forestripping or bacteriological culture to increase the confidence that cows without alerts are likely to not have CM, most of the cows without an RRT alert suggests that while most cows with an RRT alert would have CM status, the increase in RRT after intramammary challenge with Strep. uberis was enough to generate alerts based on 1 standard deviation of the baseline temperature. However, test characteristics of the RRT alerts to predict CM were less than optimal for CM prediction. The RRT alerts had numerically better precision to detect CM in cows with severe clinical signs. Sensitivity improved after bacterial counts in milk reached 4.0 log₁₀ cfu/mL. Further research that includes natural infections with other pathogens as well as different variations in RRT to determine CM status is warranted.

CONCLUSIONS

Our results indicate that the increase in RRT after intramammary challenge with Strep. uberis was enough to generate alerts based on 1 standard deviation of the baseline temperature. However, test characteristics of the RRT alerts to predict CM were less than optimal for CM prediction. The RRT alerts had numerically better precision to detect CM in cows with severe clinical signs. Sensitivity improved after bacterial counts in milk reached 4.0 log₁₀ cfu/mL. Further research that includes natural infections with other pathogens as well as different variations in RRT to determine CM status is warranted.

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REFERENCES

Dohoo, I. R. 2009. Veterinary Epidemiologic Research. 2nd ed. VER.


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