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Use of reagent test strips for diagnosis of endometritis in dairy cows

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Abstract

The use of leukocyte esterase (LE), protein, and pH tests were evaluated on widely available urinary test strips (Multistix 10 SG; Bayer Corporation, Elkart, IN, USA) on uterine lavage samples as a potential cow-side test for the diagnosis of cytologic endometritis. Uterine lavage samples of 563 lactating Holstein cows between 40 and 60 days postpartum from 28 herds were evaluated. Endometrial cytology was used as the reference for endometritis, with a cutoff point of ≥10% neutrophils. All three (LE, protein, and pH) were increased in cows with cytologic endometritis and the associations were highly significant. Optimal cutoff points determined by receiver operating characteristic analysis for LE, protein, and pH were ≥++, ≥300 mg/dL, and ≥7.0, respectively. Combining the results for LE and pH improved the performance of the test strip, but this resulted in a group of cows (20.6% of cows) which were approximately equally likely (46% with endometritis and 54% without endometritis) to have cytologic endometritis or not, and therefore could not be accurately classified. The direct relationship between reagent strip test and reproductive performance was also evaluated. Reproductive impairment due to endometritis was restricted to multiparous cows; significantly decreased reproductive performance was observed for multiparous cows with lavage fluid LE ≥+++ (154 vs. 115 median days not-pregnant), as well as cows with pH ≥ 7.0 (150.5 vs. 111.5 median days not-pregnant), but not in cows with high protein, even at the highest cutoff point. In conclusion, reagent strip test results were strongly associated with cytologic endometritis and reproductive impairment; however, in comparison with conventional cytology, the performance of reagent strip as an alternative test was relatively poor and may require further refinement.

1. Introduction

Endometritis is an inflammatory uterine disease that persists beyond normal uterine involution and impairs reproductive performance [1], [2], [3] and [4]. Affected cows frequently have no external symptoms [5] and [6]. Diagnostic methods, such as ultrasonographic evaluation of the reproductive tract and uterine content are inferior to cytologic examination of uterine content [1] and [3], which lead to the proposed disease definition based on cytology as the presence of >18% neutrophils in uterine samples collected between 21 and 33 days postpartum, or >10% neutrophils between 34 and 47 days postpartum, in the absence of purulent vaginal discharge [5]. Cytologic evaluation of uterine samples is currently the best method to diagnose inflammatory disease of the uterus. In a farm setting, however, this method is inconvenient, as it involves collection of the sample, preparation of the slides and staining, followed by microscopic examination and identification and enumeration of cells. Uterine samples collected using the cytobrush method [1] and [7] allow easier slide preparation compared with samples collected using low-volume uterine lavage, but still require the time-consuming cell evaluation step. The lack of a practical cow-side test is a major reason endometritis is not monitored or managed in commercial herds.

A candidate cow-side test for the diagnosis of endometritis from uterine lavage fluid is leukocyte esterase (LE), for example on a reagent strip intended for urinalysis, such as Multistix 10 SG (Bayer Corporation, Elkart, IN, USA). In a smaller study, Santos et al. [8] reported high sensitivity (83%) and specificity (94%) when using the LE strip to diagnose endometritis. Multistix 10 SG (Bayer Corporation) is a reagent strip of 10 tests, namely: LE, nitrite, urobilinogen, protein, pH, blood, specific gravity, ketone (acetic acid), bilirubin, and glucose. The LE compound is present in neutrophils; therefore, a positive result of this test is the most direct indicator of inflammatory cells in urine using reagent strips. In addition, protein and pH reagent tests may be useful in the diagnosis of endometritis, as well as providing insight into the pathogenesis of the condition. Fluid accumulation in the uterine lumen is used as an indicator of inflammation [1] and [3] which, if present, could elevate the protein content of the recovered fluid of low volume uterine lavage, making protein concentration a potential diagnostic test. Furthermore, inflammation of the udder or vesicular glands elevates the pH of milk [9] and seminal fluid [10], respectively, but it is unknown if inflammation of the uterus is associated with an elevation of pH in uterine fluid.

The objectives of this study were to: (1) determine if LE, protein, pH, or a combination of reagent strip tests were associated with cytologic endometritis; (2) identify cutoff points for associated reagent tests based on cytology and reproductive outcome; and (3) identify other factors associated with LE, protein, and pH in uterine lavage samples.

2. Materials and methods

2.1. Sample collection

Uterine lavage samples used in this experiment were part of a larger study [4]. The present study was initiated after 10 herds had already been sampled. All samples collected from that point on were included in the present study. Selection of herds for the study was from a convenience sample of herds that were willing to participate in the study. The inclusion criteria for herds sampled were: located in New York State, large herd size (minimum of 400 milking cows), and used DairyComp 305 (Valley Ag Software, Tulare, CA, USA) for maintaining herd records. The inclusion criteria for cows sampled were: between 40 and 60 days postpartum, apparently healthy (by cursory visual examination), no external vaginal discharge observed by visual examination, not inseminated, and at least 2 days before the end of the voluntary waiting period for that specific farm (average 59 days; range 50 to 70). Herd records were obtained at the time of sampling and reproductive outcomes were obtained by follow-up herd records collected 4 and 6 mo after sampling.

Animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Uterine lavage samples were obtained as previously described [2]. Briefly, paper towels were used to cleanse the perineum of the cow, then a 63.5 cm sterile flex tip infusion pipette (Exodus Breeders Corporation, York, PA, USA) was introduced into the uterus through the cervix, and 20 mL sterile saline solution (0.9% Sodium Chloride Injection USP; Baxter Healthcare Corp., Deerfield, IL, USA) was infused into the uterus. Approximately 5 to 8 mL of fluid was recovered by aspiration. The samples were put on ice and transported to the laboratory for analysis. One drop of uterine lavage sample was added to each test on the Multistix 10 SG (Bayer Corporation) reagent strip. Protein and pH results were evaluated after 1 min and the LE result evaluated after 2 min, as per manufacturer instructions. Protein results were recorded in six categories which were: negative, trace, + (30 mg/dL), ++ (100 mg/dL), +++ (300 mg/dL), and ++++ (>2000 mg/dL); pH results were recorded in seven categories: 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5; and LE results were recorded in five categories: negative, trace, + (small), ++ (moderate), and +++ (large). Cytologic evaluation of the uterine lavage samples was performed after cytocentrifugation (105 × *g* for 3 min) and staining using Camco stain Pak stain (Cambridge Diagnostic Products, Inc., Fort Lauderdale, FL, USA) by counting 200 cells (neutrophils, lymphocytes, macrophages, and uterine epithelial cells, excluding erythrocytes) and results were expressed as the percentage of total cells. Cows were considered positive for endometritis if neutrophils were >10% of total cells [5].

2.2. Data management and statistical analysis

2.2.1. Association of reagent strip results with cytologic endometritis

All three end points (LE, protein, and pH) were recorded as ordered categories. The categories of LE 'negative' and protein 'negative' had less than five observations that were positive for endometritis and these categories were combined with 'trace' for all analyses. To test the hypothesis that the reagent strip results were associated with endometritis, a multivariable logistic regression model was produced using PROC GLIMMIX of SAS, Version 9.2 (SAS Institute, Cary, NC, USA) with cytologic endometritis as the dependent variable. Herd was included as a random effect. The association between LE, protein, and pH were tested individually with cytologic endometritis and a final model was built for each reagent strip test. In addition to reagent strip test results, fixed effects considered were: parity (primiparous or multiparous), body condition score (≥3.5 or <3.5), ketosis, metritis, retained placenta (disease or no disease), Log (first test-day somatic cell count), days postpartum at sampling, and two-way interactions. Disease occurrence data for ketosis, metritis, and retained placenta were according to herd records with the diagnosis made by the herdsman. The final model was built using manual backwards stepwise variable selection and variables were retained if $P < 0.05$.

To evaluate reagent strip as a diagnostic test for endometritis, the reagent strip test results were dichotomized (positive test or negative test) at all possible cutoff levels. A series of 2 × 2 tables was created for reagent strip test against cytologic evaluation. True positive, true negative, false positive and false negative results were recorded, and sensitivity (Se), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV) were calculated using those values at the apparent prevalence in these herds. The cutoff point with the highest sum of sensitivity and specificity was selected as optimal. Receiver operating characteristic (ROC) analysis was performed using MedCalc version 11.5 (MedCalc Software, Mariakerke, Belgium) and the area under the curve and P values are reported.

2.2.2. Association of reagent strip results with reproduction

Reproductive outcome was examined by Cox proportion hazards model using days from calving to subsequent pregnancy as the event of interest. Incomplete observations were right censored when the cows were culled, designated "Do-Not-Breed" by the herdsman, or at 210 days-in-milk. Reagent strip results were dichotomized at all levels and were tested individually and sequentially from the lowest threshold to the highest for effect on calving-to-conception interval using Stata version 10 (Stata Corp.) including herd as a random (shared frailty) effect. The model controlled for the effects of parity (primiparous or multiparous), body condition score (≥3.5 or <3.5), ketosis, metritis, retained placenta, displaced abomasum (disease or no disease), Log (first test day somatic cell

count), days postpartum at first-insemination, and two-way interactions. Models were built by manual backward stepwise exclusion of variables. If the interaction with parity was $P < 0.05$, the analysis was stratified by parity. Variables were retained in the final model if $P < 0.05$.

2.2.3. Factors associated with reagent strip tests

The purpose of this analysis was to determine if there were factors that affected LE, protein, and pH results, other than cytologic endometritis. A multivariable logistic regression model was produced with PROC GLIMMIX of SAS, Version 9.2 (SAS Institute), with cumulative logit function and reagent strip results as the dependent variable. The variables tested methods for model building were the same as described in section 2.2.1, except endometritis was tested as a fixed effect, in addition to the described list of variables.

2.2.4. Determination of combination cutoff points

Reagent strip possible results were dichotomized to all possible combinations and evaluated for Se, Sp, PPV and NPV to detect cytologic endometritis. The optimal combination was evaluated for first-service conception rate and calving-to-conception interval, using the PROC GLIMMIX of SAS, Version 9.2 (SAS Institute) and Stata Version 10 (Stata Corp.), respectively, as described for individual reagent strip test.

3. Results

3.1. Descriptive statistics

In total, 563 cows from 28 herds were included in the study. The median herd size was 895 (range, 540 to 3000) milking cows and the average projected 305 days mature-equivalent milk production was 12 562 (SD = 620) kg of milk. Overall prevalence of cytologic endometritis was 27.7% (156/563), whereas the average within-herd prevalence was 27.8% (range, 5.3% to 52.6%).

3.2. Reagent strip compared with cytology

All three, LE, protein, and pH were strongly associated with cytologic endometritis and retained in the respective final models. Ketosis was retained in all three final models, and metritis was retained in the final model for protein reagent test association with cytologic endometritis. The proportions of cows with endometritis increased as LE, protein, and pH values increased. Herd was not significant ($P > 0.15$) as a random variable for any of the reagent strip test models.

Dichotomized reagent strip results categorized by endometritis disease status determined using lavage are summarized (Table 1). Based on receiver operator characteristics analysis, the optimal cutoff points were ≥ ++ for LE (area under the receiver operating characteristic curve [AUC] = 0.69; $P < 0.0001$), ≥ +++ for protein (AUC = 0.60; $P < 0.001$), and ≥7.0 for pH (AUC = 0.64; $P < 0.001$) to be used for the diagnosis of endometritis. At the optimal cutoff point, the LE test had Se = 76.9%, Sp = 51.8%, PPV = 38.0%, and NPV = 85.4%. At the optimal cutoff point, the protein test

had Se = 58.3%, Sp = 55.8%, PPV = 33.5%, and NPV = 77.7%. At the optimal cutoff point, the pH test had Se = 44.9%, Sp = 78.4%, PPV = 44.3, and NPV = 78.8.

Table 1
Performance of Multistix 10 SG (Bayer Corporation) LE, protein, and pH reagent strip for diagnosis of endometritis from uterine lavage samples collected from postpartum dairy cattle.

Test	Cutoff	TP (N)	TN (N)	FP (N)	FN (N)	Se (%)	Sp (%)	PPV (%)	NPV (%)
LE	≥ Trace	152	28	379	4	97.4	6.9	28.6	87.5
	≥+	145	74	333	11	92.9	18.2	30.3	87.1
	≥++	120	211	196	36	76.9	51.8	38.0	85.4
	≥+++	53	367	40	103	34.0	90.2	57.0	78.1
Protein	≥+	147	47	360	9	94.2	11.5	29.0	83.9
	≥++	133	98	309	23	85.3	24.1	30.1	81.0
	≥+++	91	227	180	65	58.3	55.8	33.6	77.7
	≥++++	31	367	40	125	19.9	90.2	43.7	74.6
pH	≥6.5	101	219	188	55	64.7	53.8	34.9	79.9
	≥7.0	70	319	88	86	44.9	78.4	44.3	78.8
	≥7.5	49	349	58	107	31.4	85.7	45.8	76.5
	≥8.0	30	373	34	126	19.2	91.6	46.9	74.7
	≥8.5	18	392	15	138	11.5	96.3	54.5	74.0

Protein results were recorded in six categories which were: negative, trace, + (30 mg/dL), ++ (100 mg/dL), +++ (300 mg/dL), and ++++ (>2000 mg/dL); and LE results were recorded in five categories: negative, trace, + (small), ++ (moderate), and +++ (large).
FN, false negative; FP, false positive; LE, leukocyte esterase; NPV, negative predictive value; PPV, positive predictive value; Se, sensitivity; Sp, specificity; TN, true negative; TP, true positive.

3.2. Reagent strip and reproduction

Elevated LE was significantly associated with decreased hazard of pregnancy (increased calving-to-conception interval) only at the highest cutoff point of ≥ +++ (144 median days not-pregnant for LE ≥ +++ and 117 median days not-pregnant for LE < +++ cows; hazard ratio [HR] = 0.76; 95% confidence interval [CI] 0.57 to 1.00; P = 0.05). The interaction of parity and LE was significant (P = 0.04), therefore, further analyses were stratified by parity. Stratification by parity showed the effects to be limited to multiparous cows where LE ≥ +++ (154 median days not-pregnant) had 39 longer median days not-pregnant compared with LE < +++ (HR = 0.66; 95% CI, 0.45 to 0.97; P = 0.03). Body condition score (HR = 1.52; 95% CI, 1.16 to 1.20; P = 0.002) and days postpartum at first-insemination (HR = 0.98; 95% CI, 0.97 to 0.99; P = 0.003) were retained in the final model. In primiparous cows, the HR for LE was not significant (P > 0.20) at the highest cutoff with any combination of covariates. The pregnancy survival curves for LE ≥ +++ effect stratified by parity

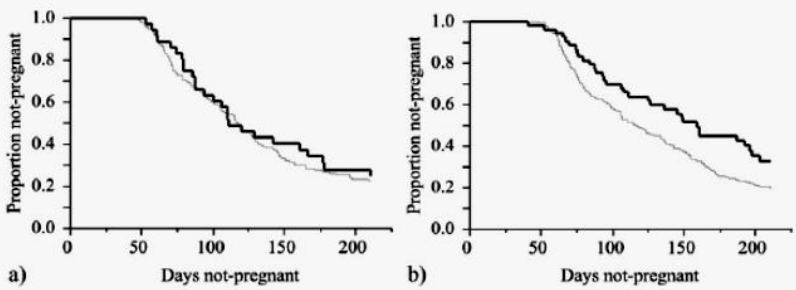

Fig. 1. Kaplan-Meier survival curves for leukocyte esterase (LE) ≥ +++ (thick line) effects on calving-to-conception interval. (A) Primiparous cows with LE ≥ +++ did not show impaired reproductive performance compared with cows with LE < +++ (median days not-pregnant 111 and 117 days; P = 0.57). (B) Multiparous cows with LE ≥ +++ had longer median days not-pregnant (154 days) compared with cows with LE < +++ (116 days; P = 0.02). LE results were recorded in five categories: negative, trace, + (small), ++ (moderate), and +++ (large).

are shown (Fig. 1).

Protein concentration was not significant at any cutoff point, combined or stratified by parity ($P > 0.20$) on calving to conception interval.

A significant effect on hazard of pregnancy was found for pH ≥ 7.0 and the interaction between pH ≥ 7.0 and parity was significant ($P = 0.04$). Stratification by parity revealed the effects to be limited once again to multiparous cows where the median calving-to-conception interval increased from 111.5 days in pH < 7 to 150.5 days in pH ≥ 7.0 (HR = 0.68; 95% CI, 0.50 to 0.92; $P = 0.01$) with body condition score (HR = 1.50; 95% CI, 1.14 to 1.96; $P = 0.003$) and days postpartum at first-insemination (HR = 0.98; 95% CI, 0.97 to 0.99; $P = 0.001$) also retained in the final model. In primiparous cows, pH was not significant ($P > 0.20$) at any cutoff and covariate combination.

3.3. Factors associated with reagent strip tests

Cytologically diagnosed endometritis was significantly associated with the reagent strip tests and was retained in the final models of LE (odds ratio [OR] = 4.49; 95% CI, 3.07 to 6.56; $P < 0.0001$), protein (OR = 1.84; 95% CI, 1.26 to 2.67; $P = 0.0015$) and pH (OR = 2.67; 95% CI, 1.88 to 3.79; $P < 0.0001$). Herd was significant in all the final models. The final model for LE did not retain any other fixed variables, whereas the final model for pH retained parity (primiparous OR = 1.79; 95% CI, 1.29 to 2.49; $P = 0.001$) and in the final model for protein, retained placenta was retained (OR = 1.94; 95% CI, 1.02 to 3.69; $P = 0.045$).

3.4. Reagent tests used in combination

Although protein was associated with cytologic endometritis, the use of this test in combination did not improve the overall performance of the other reagent strip tests. The cutoff point of ≥ +++ for LE in combination with pH ≥ 7.0 had the best performance. Using this combination of cutoff points, NPV was 75.6% (394/521), whereas the PPV was 69.0% (29/42) with 18.6% Se and 96.8% Sp. A closer examination of the combined performance of LE and pH at each category to diagnose cytologic endometritis found the test to be much more accurate at the high and low end, whereas cows that had LE = ++ but pH ≥ 7.0 and cows that had LE = +++ but pH < 7.0 could not reliably be assigned an endometritis classification. Therefore, the results were stratified into the positive group (LE ≥ ++ and pH ≥ 7.0), negative group (LE < ++ and pH < 7.0), and the undetermined group (LE = ++ but pH ≥ 7.0; or LE = +++ but pH < 7.0). The PPV for positive group was 69.0% and the NPV for the negative group was 82.5%; however, there was a large group of undetermined cows (20.6% of cows).

Kaplan-Meier survival curves for calving-to-conception interval stratified by parity using combined LE and pH are shown (Fig. 2). Once again, reproductive impairment was restricted to multiparous cows where combination positive cows had 184 median days not-pregnant, whereas undetermined

cows had 129 median days not-pregnant and combination negative cows had 113 median days not-pregnant ($P < 0.001$).

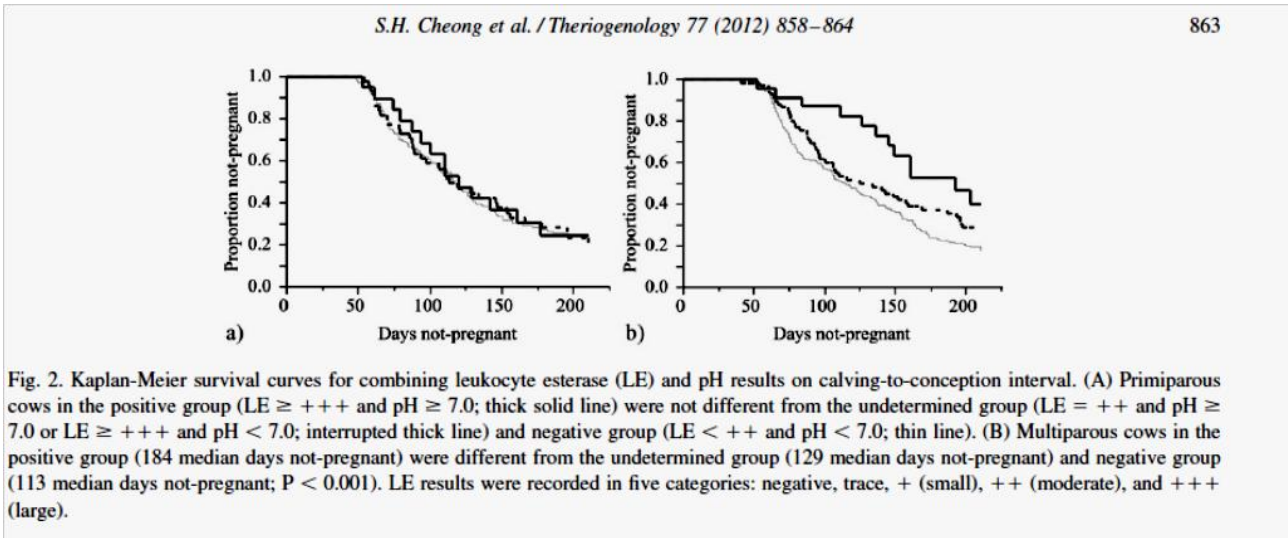

Fig. 2. Kaplan-Meier survival curves for combining leukocyte esterase (LE) and pH results on calving-to-conception interval. (A) Primiparous cows in the positive group (LE ≥ +++ and pH ≥ 7.0; thick solid line) were not different from the undetermined group (LE = ++ and pH ≥ 7.0 or LE ≥ +++ and pH < 7.0; interrupted thick line) and negative group (LE < ++ and pH < 7.0; thin line). (B) Multiparous cows in the positive group (184 median days not-pregnant) were different from the undetermined group (129 median days not-pregnant) and negative group (113 median days not-pregnant; $P < 0.001$). LE results were recorded in five categories: negative, trace, + (small), ++ (moderate), and +++ (large).

4. Discussion

Reagent strip results of LE, protein, and pH reagent strip were highly associated with cytologic endometritis. However, the performance of LE in this study was not as accurate as described by Santos et al. [8]. There are three important differences between Santos et al. and the present study. First, the Santos study set the LE cutoff point at + and endometritis cutoff point at 5.5% neutrophils. The analysis was repeated using data from this study at these cutoff points and found the LE test to have a Se of 48.8% and Sp of 73.3%, which was still lower than the 83% Se and 94% Sp reported by Santos et al. Secondly, cows were sampled between 1 and 7 wk postpartum by Santos et al., whereas cows were sampled later postpartum between 40 and 60 days in the present study. Cows in the early postpartum period tend to have very high proportions of neutrophils in endometrial cytology [2], which may have improved the performance of the reagent strip test. Finally, the uterine lavage samples for the current study were only tested at the laboratory and the time interval between sampling and testing was large in some cases, as the farms sampled were an average 3 h drive from the laboratory. The reactivity of LE in human urine with reagent strip is decreased in approximately 25% of samples after 24 h of refrigeration [11] increasing false negative results. In the present study, the false negative results remained low for LE, despite the long transport time. Inflammatory conditions in cattle have been reported to cause an increase in the pH of fluid excretions [9] and [10]. In the present study, pH of uterine fluid was increased in cows with endometritis relative to normal cows and the correlation with endometritis was even higher than the LE test. The optimal cutoff point of pH ≥ 7.0 was similar to the recommended cutoff point for semen from bulls with seminal vesiculitis [10]. To our knowledge, this is the first report of increased pH in uterine fluid of cows with endometritis. The Se and Sp of pH to diagnose

endometritis at the optimal cutoff point ≥7.0 was still relatively poor, at only 44.9% and 78.4%, respectively.

The protein reagent strip test had the weakest association with cytologic endometritis and was not predictive of future reproductive performance. Storage of refrigerated human urine samples for 24 h increased the false positive results in reagent strips [11]. The observed PPV for the protein test was low, even at the highest cutoff point (43.7%), which may be attributed to the long transport time between sample collection and testing.

Multistix 10 SG (Bayer Corporation) is a reagent strip designed as a rapid test for human urinalysis. The LE reagent strip is a highly sensitive test for human pyuria and is an excellent screening test [12]. Conversely, the LE reagent strip test is not very sensitive and is not recommended as a screening test for pyuria in small animal veterinary medicine [13] and [14]. The different source of samples tested could affect the performance of the test. There are no published studies evaluating the repeatability of LE, protein, or pH on the Multistix 10 SG (Bayer Corporation) reagent strip. The LE and protein reagent strips were not designed for uterine lavage and the available increments of reference results at the critical concentrations for endometritis are too large. The overall prevalence of endometritis by cytology was 27.7%, but the prevalence at the optimal cutoff points for LE and protein were 56.1% and 48.1%, respectively, which is a gross overestimation. The next category for LE and protein grossly underestimated the prevalence at 16.5% and 12.6%, respectively. At the optimal cutoff point for pH, the overall prevalence was 28.1%, which was close to the cytologically derived prevalence in this study. The categorical increment for pH around the critical point for cytologic endometritis was 0.5, which appeared to be narrow enough, whereas the available categories for LE and protein did not appear to have sufficient resolution for optimal performance. Combining LE and pH results improved the PPV to 69.0% and the NPV to 82.5% however; there was a group of undetermined cows (20.6%).

In summary, reagent strip results were significantly associated with cytologic endometritis and predicted poor reproductive performance. However, the Se and Sp of reagent strip tests were relatively poor. Modification of the test strips to optimize diagnostic categories for bovine endometritis seems to offer the potential for an accurate and convenient cow-side diagnostic tool.

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