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A comparative study of various techniques for diagnosing subclinical endometritis in repeat breeder cows

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ABSTRACT

In the absence of a single trustworthy test, the diagnosis of subclinical endometritis (SCE) is difficult, which leads to many afflicted cows being repeat breeders. This study in 60 repeat breeder cows examined during estrus, investigated twelve distinct laboratory and cow-side tests involving endometrium (uterine lavage cytology), genital discharge (consistency; Whiteside test; pH [pH paper]; qualitative and quantitative microbiology), ultrasonography (endometrial thickness; uterine fluid), and uterine lavage (urinary test strip-based – pH, leukocyte esterase, and protein; pH; uterine lavage sample optical density [ULSOD] at 450 and 620 nm). According to receiver operating characteristics, uterine lavage cytology with a cutoff of ≥2% polymorphonuclear (PMN) cells, sensitivity and specificity of 100% and 55.10%, respectively, was considered as the gold standard for assessing the effectiveness of alternative diagnostic tests. The cytological prevalence of SCE was 56.67%. All other diagnostic methods exhibited low sensitivity (26.5% to 73.5%) and a poor to moderate Cohen's kappa agreement amongst them (κ: -0.0967 to 0.4042); the greatest being between PMN% and microbial presence. Intrauterine fluid accumulation was absent in all cows. The Whiteside test, with a threshold of ≥2, showed the highest specificity (92.3%) and diagnostic accuracy (56.7%). The protein (test strip) (76.9%) and $ULSOD_{450}$ (65.4%) tests were the next two best tests. In conclusion, diagnosing SCE in repeat breeders should begin with the cow-side Whiteside test, which, due to its exceptional specificity, can be trusted to exclude the presence of SCE. Uterine lavage cytology is only necessary for cows' positive on the Whiteside test.

Keywords: Cows, Diagnosis, Repeat breeding, Subclinical endometritis

The lack of overt clinical symptoms in subclinical endometritis (SCE) makes it challenging to diagnose the disease in cows that go on to become repeat breeders (Denis-Robichaud and Dubuc 2015). According to Salasel *et al*. (2010), up to 52.7% of repeat breeder cows had SCE. Research on SCE diagnosis has predominantly focused on early postpartum cows (20 to 62 days after calving), whereby a polymorphonuclear (PMN) threshold range of 5-18% and the presence of mucopurulent or purulent discharge (Denis-Robichaud and Dubuc 2015, Van Schyndel *et al.* 2018) have been utilized. In contrast, the PMN threshold for SCE was 1% in the cows presented for insemination (Pascottini *et al*. 2017). Variations in the PMN cutoff can also impinge upon the results of other cow-side SCE diagnostic tests, like leukocyte esterase and the Whiteside reaction, which rely on the PMN load in the samples. It was therefore hypothesized that the diagnostic criteria established for SCE in early postpartum cows cannot be extrapolated to cyclic repeat breeder cows at the moment they can conceive. On the other hand, diagnostic studies on SCE in repeat breeders have been sparse and restricted to pH, Whiteside test, and genital microbiota (Bhat *et al*. 2014, Pothmann *et al*. 2015, Bedewy and Rahawe 2019). Furthermore, these studies lack interpretive criteria and validation due to the absence of cytological confirmation of SCE at estrus. The more popular cytotape or cytobrush methods, which only look at a small portion of the endometrium, are inferior to endometrial cytology employing uterine lavage (UL) that represents the complete endometrium (Sood 2024). Hence, the current study provides a comprehensive evaluation of 12 different laboratory and cow-side tests for detecting SCE in repeat breeder cows.

MATERIALS AND METHODS

The study adhered to the guidelines of the College Animal Ethical Committee. Sixty regularly cycling repeat breeder Jersey crossbred cows with no indication of pus in their estrual genital discharge (henceforth referred to as discharge), and absence of any other concomitant

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reproductive pathology were the subjects of the current investigation, which were investigated over the course of two consecutive estrous cycles. The cows were owned by different farmers, managed in a semi-intensive system, and received appropriate quantities of seasonal fodder, forages, and concentrates.

For the first recorded estrus, data on periparturient issues (e.g., prolapse, dystocia, retained placenta) and general parameters viz. age (in years), parity, body condition score (BCS) (Edmundson *et al.* 1989), days in milk (DIM), number of previous inseminations, estrus duration (Ruthwal 2010), and history of metrorrhagia, were obtained. A corpus luteum was confirmed in all cows 12 to 14 days after the end of estrus.

At the second reported estrus, the interestrus interval (IEI) was recorded. Sequential diagnostic tests were conducted using discharge, genital ultrasonography, and uterine lavage, approximately 12 to 16 h after estrus onset.

For discharge evaluation, a sterile insemination sheath was attached to a syringe, and the discharge was aspirated aseptically from the uterine horn and cervix. The discharge was then assessed for consistency, Whiteside test, and pH. The consistency of discharge was evaluated based on its flow from the insemination sheath, which was classified into stringy, viscous, or highly viscous, with numerical scores of 1, 2, and 3, respectively. The Whiteside test involved use of sodium hydroxide solution, whereas the pH was assessed using pH indicator strips.

Transrectal ultrasonography was performed using a 5-MHz linear array transducer to record the size and location of the ovulatory follicle, as well as endometrial thickness of each uterine horn that was used to calculate mean endometrial thickness. Uterine fluid accumulation was assessed by recording the uterine luminal diameter at the area with the greatest fluid.

Finally, using a sterile two-way 22 FG Foley balloon catheter, 20 mL of sterile saline was infused into the uterus. After a gentle uterine massage, 5-12 mL of UL was recovered in a sterile graduated conical test tube, which was straight away placed on ice. A portion of UL was immediately utilized to assess pH, leukocyte esterase, and protein using urine test strips. Another portion of the UL was sent for microbiological analysis to assess the spectrum of aerobic and anaerobic microbes and bacterial load (CFU/mL). Infected cows were classified as having either single or mixed infections. The remaining UL was centrifuged at 700×g for 5 min. The supernatant was used to analyze ULSOD at 450 and 620 nm in a microplate reader. On the other hand, the pellet was smeared onto a clear microscopic slide, which was subjected to Leishman's staining for cytological examination and counting 200 cells. The latter ascertained percentage of polymorphonuclear (PMN) cells so as to categorize the cows as subclinical endometritis positive (SCE+) or negative (SCE-).

All the aforesaid procedures on cows, their principles and interpretation were in accordance with Sood (2024).

The comparison of average (Mean ± S.E.M) values between the SCE- and SCE+ groups for general and diagnostic parameters was performed using a student's t-test. A receiver operator characteristic (ROC) curve analysis was carried out to determine optimal cutoff values for the PMN percentage in the UL endometrial cytology,

Table 1. Comparison of average (Mean ± S.E.M.) values of genital discharge and uterine lavage related diagnostic parameters in repeat breeding cows with (SCE+) and without (SCE-) subclinical endometritis (SCE)

Parameter	SCE+ (n=34)	SCE- (n=26)	*p*-value
Genital discharge			
Consistency	2.21 ± 0.13	2.08 ± 0.17	0.53
Whiteside test	1.24 ± 0.10	1.04 ± 0.09	0.15
pH (pH paper)	8.71 ± 0.27	8.54 ± 0.31	0.68
Ultrasonography			
Endometrial thickness (mm)			
Right uterine horn	8.38 ± 0.31	8.53 ± 0.68	0.93
Left uterine horn	8.64 ± 0.30	8.29 ± 0.43	0.26
Uterine lavage			
Test strip related			
pH	6.53 ± 0.15[a]	6.38 ± 0.19[a]	0.53
Leukocyte esterase	1.29 ± 0.16	1.27 ± 0.19	0.93
Protein	1.82 ± 0.23	1.31 ± 0.23	0.12
pH (pH paper)	8.57 ± 0.30[b]	8.60 ± 0.34[b]	0.94
$ULSOD_{450}$	0.18 ± 0.02	0.18 ± 0.04	1.00
$ULSOD_{620}$	0.08 ± 0.01	0.09 ± 0.02	0.63
Colony forming units /mL	$80.61 \times 10^4 \pm 38.52 \times 10^4$	$120.25 \times 10^4 \pm 103.82 \times 10^4$	0.69

[ab] values with different superscripts within a column differ at $p<0.00$

which was used as the gold standard. Sensitivity (Se) and specificity (Sp) of each test were used to select the optimal cutoff points. For microbiological analysis, the presence of microbes (yes/no) was considered. 2x2 tables were created to compare different diagnostic tests against cytology results, and diagnostic accuracy was determined using the positive predictive value (PPV), negative predictive value (NPV). Cohen's kappa (κ) analysis was undertaken to assess the extent of agreement (poor, fair, moderate, substantial, or almost perfect) between any two different tests. The concordance between various diagnostic measures, such as UL pH (pH paper vs. urine test strip), ULSOD (450 vs. 650 nm), thickness of uterine horn (left vs. right) and pH (discharge vs. UL), were analyzed using the Bland-Altman scatter plot. Pearson correlations were calculated between different diagnostic variables for both SCE- and SCE+ groups. Additionally, multiple correlations between the ovulatory follicle diameter (independent variable) and endometrial thickness of both horns (dependent variable) were assessed.

The entire statistical analysis was conducted using MedCalc software (Version 22.032; Ostend, Belgium), and a significance level of $p<0.05$ was considered statistically significant, with tendencies between 0.05 and 0.10.

RESULTS AND DISCUSSION

Except for one cow each in SCE+ and SCE- groups having retained placenta during their last calving; all others experienced an uncomplicated periparturient period. The ROC analysis of endometrial cytology revealed a PMN cell cutoff point, area under the ROC curve, significance level, Se, and Sp of ≥2%, 0.774, 0.004, 100%, and 55.10%, respectively. At 2% PMN threshold, a previous study cited 88% Se and 45% Sp, respectively, in postpartum cows (VanSchyndel *et al.* 2018). The cytological prevalence of 56.67% SCE in current study was slightly higher than the 53% (3% PMN, Salasel *et al.* 2010) in repeat breeder cows. The difference in Se, Sp, and SCE prevalence among different studies stem from variations in the PMN% cutoff thresholds.

None of the diagnostic parameters differed between the SCE+ and SCE- groups (Table 1).

Table 2 outlined the ROC analysis attributes for various diagnostic methods. Based on UL endometrial cytology diagnostics, the methods with the greatest area under the curve and most significant values, in descending order, were $ULSOD_{450}$ and Whiteside test/protein (test strip). However, Whiteside test had the highest Sp and diagnostic accuracy, which were followed by $ULSOD_{450}$, and protein (test strip). In contrast to the Whiteside based prevalence of 16.7% in the current study, Bedewy and Rahaway (2019) observed all 42 repeat breeder cows to be Whiteside positive. The discrepancy *per se* can be linked to much higher PMN% of 12-18 in the latter study. A correlation ($p<0.01$) between PMN% and Whiteside test in our study agrees to that of Bedewy and Rahaway (2019). In light of aforesaid, the Whiteside test by virtue of its highest Sp, which was even

Table 2. Comparative performance and apparent prevalence of subclinical endometritis (SCE) among repeat breeders using different diagnostic modalities[#]

Sample / parameter	Cutoff value	Area under curve	*p*-value	True positive (n)	True negative (n)	False positive (n)	False negative (n)	Sensitivity (%)	Specificity (%)	Positive likelihood ratio	Positive predictive value (%)	Negative predictive value (%)	Overall accuracy (%)	Apparent prevalence of SCE (%)
Genital discharge														
Consistency	≥2	0.42	0.86	28	9	17	6	38.2	57.7	0.90	62.2	60.0	43.0	46.67
Whiteside test	≥2	0.60	0.06	10	24	2	24	29.4	92.3	3.81	83.3	50.0	56.7	16.7
pH (pH paper)	≥9.0	0.51	0.43	14	15	11	20	47.1	61.5	1.22	56.0	42.9	39.0	23.33
Endometrial thickness (mm)														
Right uterine horn	≥7.5	0.57	0.16	24	13	13	10	70.6	50.0	2.40	64.9	56.5	45.7	40.00
Left uterine horn	≥8.8	0.42	0.83	15	14	12	19	44.1	53.9	0.92	55.6	42.4	38.3	25.00
Uterine lavage														
Test strip related														
pH	≥6.5	0.57	0.16	22	14	12	12	64.7	57.7	1.52	64.7	53.8	45.3	36.67
Leukocyte esterase	≥1	0.50	0.47	25	8	18	9	73.5	30.8	1.06	58.1	47.1	38.3	41.67
Protein	≥3	0.60	0.06	14	20	6	20	41.2	76.9	1.78	70.0	50.0	47.3	23.33
pH (pH paper)	≥9	0.51	0.43	16	15	11	18	47.0	61.5	1.22	59.3	45.5	41.0	26.67
ULSOD450	≥0.12	0.61	0.06	20	17	9	14	58.8	65.4	1.69	69.0	54.8	48.3	33.33
ULSOD620	≥0.06	0.20	0.20	24	9	17	10	67.7	50.0	1.35	58.5	47.4	39.0	40.00

[#] In reference to uterine lavage-based cytology cutoff values of ≥ 2% polymorphonuclear cells

higher than 55.10% of UL cytology, can be trusted to rule out SCE. Moreover, Whiteside test had a fair κ agreement with PMN% (0.2177).

The cutoff values for test strip-based leukocyte esterase (≥1), protein (≥3), and pH (≥6.5) disagreed with prior studies on SCE or clinical endometritis citing the corresponding cutoff values of both leukocyte esterase and protein as ≥2 (Cheong *et al.* 2012, Denis-Robichaud and Dubuc 2015, Van Schyndel *et al.* 2018). Early postpartum stage (21 and 60 DIM) and much higher PMN% (≥5 to 18) are the two reasons for the said discrepancy between the earlier and present investigation on cyclic cows. This also justified the 15% to 33% underestimation of SCE prevalence and a slight agreement (κ = 0.04 to 0.18) between test strip-based parameters and PMN% in present study compared to Cheong *et al.* (2012) citing an overestimated SCE

The cutoff values: consistency pertains to the genital discharge breaking in ≥15 to 30 sec, Whiteside test with a color shade of yellow or higher; leukocyte esterase (Leuko/μL) with a sample value of ≥ ca.125, protein with a sample content of ≥300 mg/dL prevalence. For the said reasons, a significant correlation between the test strip parameters and PMN% (Santos *et al.* 2006) was absent in the SCE+ cows.

The endometrial thickness of both uterine horns lacked in any differences within and between the SCE+ and SCE- cows, probably suggesting a mild inflammation. However, the presence of an ovulatory follicle on the left ovary, rather than its size, was correlated with the endometrial thickness of the ipsilateral than the contralateral horn in the SCE+ cows, which contributed to numerically greatest thickness of the left horn (Table 1). Also, presence of ovulatory follicle on left ovary was associated with a significant correlation ($p<0.01$) between the endometrial thickness of the two horns in the SCE+ cows. Although without taking into account the location and size of ovulatory follicle, an earlier study on slaughtered cows (Fuentes *et al.* 2017) also revealed left horn to be more reliable rather than the right for diagnosing SCE. Collectively, the findings *ut*

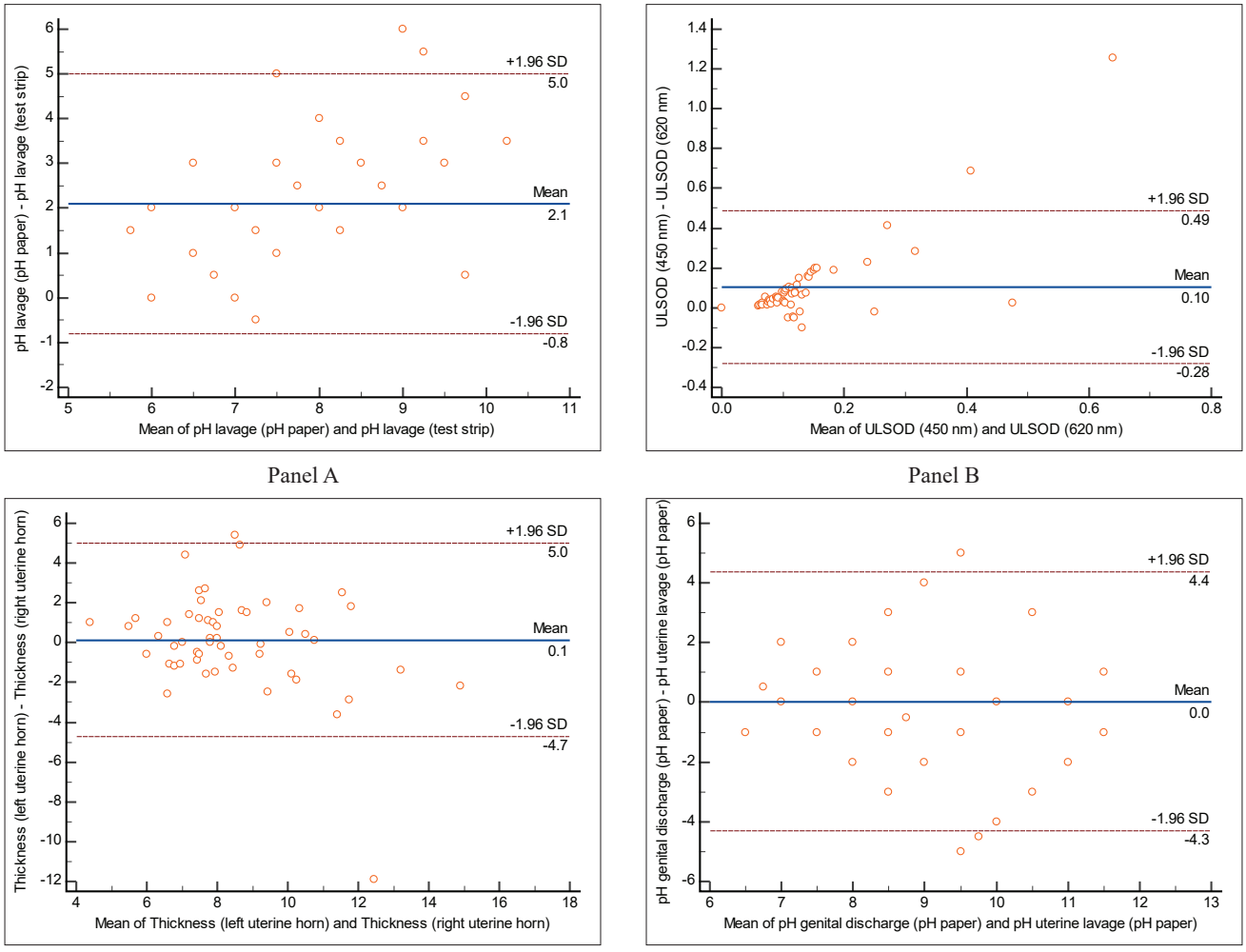

Fig. 1. Bland-Altman scatter plots evaluating the concordance between various diagnostic modalities and alternative methods employed to assess the same modality for the diagnosis of subclinical endometritis (SCE) in repeat breeder cows: Panel A depicts the concordance of uterine lavage pH (pH paper vs. test strip) ($p<0.0001$), Panel B depicts the concordance of $ULSOD_{450}$ and $ULSOD_{620}$ ($p<0.0001$), Panel C depicts the concordance of thickness of right and left uterine horn ($p= 0.6796$), Panel D depicts the concordance of pH (pH paper) of discharge and uterine lavage ($p=0.9293$)

supra suggested different pathophysiological dynamics in the two horns of the same cow. Hence, it is plausible to assume that any horn related discrepancy in diagnosing SCE can be avoided by using UL than cytobrush/cytotape as a diagnostic tool.

Overall, 83.33% (n=50) of the cows (82.35%; n=28 in SCE+ and 84.61%; n=22 in SCE-) were positive for microbial growth. Comparatively, bacterial infection in discharge of 62.2 to 100% repeat breeding cows has been reported (Bhat *et al*. 2014, Pothmann *et al*. 2015). Nine different bacterial genera, primarily *Bacillus* spp., were found in 23 cows (38.33%) as single or mixed infections, which agree with Bhat *et al*. (2014) and Wagener *et al*. (2015). Importantly, a moderate κ agreement (0.4042) between PMN % and bacterial presence, highest in the current study, suggests involvement of microbes in causing SCE. Bacterial metabolites can make genital contents alkaline (Salphale *et al.* 1993), which probably explained the alkaline pH in SCE- cows as well.

Bland-Altman scatter analysis (Fig. 1) revealed a significant bias between UL pH (pH paper vs. test strip) (panel A) and $ULSOD_{450}$ and $ULSOD_{620}$ (panel B) which, however, was absent for thickness of right and left uterine horn (Panel C) and pH (pH paper) of discharge and uterine lavage (panel D). The bias in UL pH garners support from the study of Nappert and Naylor (2001), who found that urinary dipsticks underestimated pH compared to both pH paper (also true in current study; Table 1) and pH meter, with the aberration increasing as the medium shifted from acidic to alkaline. Considering the $ULSOD_{450}$ vs. $ULSOD_{620}$ bias, the former compared to latter was superior in diagnosing SCE on account of relatively higher Sp (65.4 vs. 50.0%) and overall accuracy (48.3 vs 39.0%), as also a greater κ agreement with PMN% (0.2367 vs. 0.0537). In contrast, $ULSOD_{620}$ with a threshold of 0.059 (resembles to 0.06 in the current study) rather than $ULSOD_{450}$ was found superior in the postpartum cows; the variation *per se* could be due to higher grade of infection (clinical endometritis) as well as higher PMN% (≥18%) (Machado *et al.* 2012).

In conclusion, the lower sensitivity and Cohen's kappa agreement between different diagnostic methods and PMN% suffices mild uterine infection. However, the superior Sp of Whiteside reaction (a cow-side test) makes it reliable to exclude subclinical endometritis. Conversely, cytological confirmation (a lab test) may be required for repeat breeder cows yielding a positive Whiteside reaction.

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