Assessment of diagnostic techniques for subclinical endometritis in repeat breeder cows

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Abstract

The diagnosis of subclinical endometritis (SCE), especially in repeat breeder cows, is challenging, as there is no single reliable test. The current study on 60 such cows examined during estrus twelve distinct laboratory and cow-side tests during estrus. The tests represented the endometrium (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 [pH paper]; uterine lavage sample optical density [ULSOD] at 450 nm and 620 nm). Endometrial cytology with a cutoff point of ≥1.00% polymorphonuclear (PMN) cells (sensitivity and specificity of 100.00% and 40.00%, respectively) was considered as the gold standard in evaluating the relative efficacy of alternative tests based on receiver operative characteristics. The cytological prevalence of SCE was 75.00%. All other diagnostic methods had a low sensitivity (33.30 to 62.20%) and a low to fair Cohen's kappa agreement (κ: -0.016 to 0.315; highest for microbial presence) with PMN%. Intrauterine fluid accumulation was absent in all the cows. At a threshold value of ≥1, the Whiteside test demonstrated the highest specificity (96.20%) and diagnostic accuracy (71.70%), but it was constrained by the highest false positive count. The $ULSOD_{620}$ and consistency of genital discharge were the next two most accurate tests (66.70% each). To summarize, Whiteside test is the first test to be used in diagnosing SCE diagnosis in repeat breeders. Its exceptional specificity reliably determines the absence of SCE. Endometrial cytology is only necessitated in Whiteside positive cows.

Keywords: Cows; diagnosis; repeat breeding; subclinical endometritis.

Introduction

Subclinical endometritis (SCE) lacks overt clinical manifestations and may therefore escape diagnoses and treatment in dairy animals that end up as repeat breeders (Denis-Robichaud and Dubuc 2015; Nischala and Sireesha 2018). The importance of diagnosing SCE is highlighted by the fact that up to 40.5% of cows have SCE (Villasenor-Gonzalez et al. 2024). The preponderance of rigorously designed research on SCE diagnosis has primarily restricted to early postpartum cows, specifically between 20 and 62 days after calving. The diagnostic criteria using endometrial cytology in these studies have been a polymorphonuclear (PMN) threshold range of 5.00 to 18.00% and the presence of mucopurulent or purulent discharge (Denis-Robichaud and Dubuc 2015; Van Schyndel et al. 2018). In contrast, the PMN threshold for SCE was 1.00% in the cows presented for insemination and having no evidence of pus in genital secretions (Pascottini et al. 2016). 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. Also to be noticed is that each of the aforementioned studies only examined a restricted number of SCE diagnostics, which varied between the studies. Consequently, their interpretive potential may be arguable. It was therefore hypothesized that the diagnostic criteria established for SCE in more extensively investigated 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 (Bedewy and Rahawy 2019; Bhat et al. 2014; Pothmann et al. 2015). Furthermore, these studies lack interpretive criteria and validation due to the absence of cytological confirmation of SCE at estrus. Hence, the present study aims to furnish a comprehensive and objective assessment of 12 distinct laboratory and cow-side tests for the detection of SCE in repeat breeding cows.

Materials and Methods

The current study was carried out over a period of one and a half years at Department of Veterinary Gynaecology and Obstetrics, DGCN COVAS, Palampur, Himachal Pradesh (32.1858° N, 76.8939° E; Fig 1), at an elevation of 1,250 meters above mean sea level.

Ethics approval

The procedures used in the study were as per the guidelines of the College Animal Ethical Committee.

Animals, location and management

The present study was conducted in Jersey crossbred cows with an exotic inheritance of up to 87.50%. The cows were owned by different farmers and were managed in a semi-intensive manner receiving adequate amounts of seasonal fodder, forages, and concentrates.

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Fig 1. Geographical map of area under study (Palampur, Himachal Pradesh), Red Map Pin (Left image) and Red Dotted area (Right image); Source (Google Maps).

Selection of cows and preliminary information

The absence of pus in the genital discharge (hereafter referred to as discharge), as well as apparent reproductive pathology in regular cycling repeat breeder cows were the primary criteria for selection. A total of 60 such cows were investigated during two consecutive estrous periods

In the first reported estrus, the information on periparturient problems (prolapse, dystocia, retained placenta) and general parameters viz. age (years), parity, body condition score (BCS) (Manriquez et al. 2021), days in milk (DIM), number of previous inseminations, estrus duration (Ramadhana et al. 2022) and history of metrorrhagia, was recorded. Presence of corpus luteum was ensured in all the cows at 12 to 14 days after end of estrus.

Diagnostic investigations and grouping of cows

At second reported estrus, the interestrus interval (IEI) was recorded. The diagnostic investigations performed upon discharge, genital ultrasonography, endometrial cytology and uterine lavage were conducted in a sequential manner, 12 to 16 hr after estrus onset, as mentioned hereunder:

Using a sterile insemination sheath attached to a syringe, the discharge was aspirated aseptically from the uterine horn and cervix. Subsequently, the discharge was evaluated for consistency, Whiteside test, pH and microbiological parameters. The consistency of discharge was determined by facilitating its flow from the insemination sheath placed obliquely. The discharge breaking within 15 sec, within 15 to 30 sec or failing to break in > 30 sec, graded as stringy, viscous or highly viscous, was allocated, respectively, a numerical score of 1, 2 and 3. For conducting Whiteside test (Bhat et al. 2014), a small aliquot of the discharge was mixed with an equal volume of 5.00 to10.00% sodium hydroxide solution (Merck, Mumbai, India) and then subjected to heating until it reached its boiling point. Upon cooling, the changes in color intensity were recorded and designated as normal (turbid or no color), mild infection (light yellow color), moderate infection (yellow color) and severe infection (dark yellow color), with numerical scores of 0, 1, 2 and 3 allocated to each category, respectively. The pH of discharge was assessed utilizing pH indicator strips (pH range: 6.5-9; Hi Media Laboratories Private Limited, Mumbai). A portion of the discharge was transferred in a sterile 15 mL conical tube, which was kept in a cold and insulated box until return to laboratory for the investigations on spectrum of aerobic and anaerobic microbes (Whitman 2015) and bacterial load (colony forming units; CFU/mL) (Salfinger and Tortorello 2015). The cows that were infected were categorized as either having single or mixed infections.

The transrectal ultrasonography investigation (5MHz linear array transducer in a real time B mode; Exago, IMV, France) comprised of recording the size (mm) and location (right or left ovary) of the ovulatory follicle. Thereafter, three separate cross-sectional images of the entire thickness and lumen of each uterine horn at its mid-length were recorded. To obtain the mean endometrial thickness (mm), the two values were subtracted. The intrauterine fluid was identified by recording the uterine luminal diameter (mm) in the area of greatest fluid accumulation.

The endometrial cytology samples were collected aseptically from dorsal endometrial surface of the uterine body using a specially fabricated sterile 0.40 cm thick stainless steel “Bovine endometrial cytotaping catheter” (Fig 2). The endometrial smear was stained with Leishman’s stain (Sigma-Aldrich; EC Number 235-732-1) by standard procedure and a total of 200 cells were counted per slide in multiple fields to ascertain the PMN cell percentage. After statistical analysis, the latter information was used to categorize the cows as subclinical endometritis positive (SCE+) and subclinical endometritis negative (SCE-). Lastly, uterine lavage was collected using a sterile two-way Foley balloon latex catheter of 22FG size (Romson’s®; Romsons Scientific and Surgical Industries, Pvt. Ltd., India) in a manner adopted for embryo flushing. A 20 mL volume of 0.90% sterile NaCl physiological saline was slowly infused into the uterus, followed by a gentle massage of the uterine horns for a minute. Finally, 5 to 12 mL of the infusate was recovered in a sterile 15 mL

Fig 2. Bovine endometrial cytotaping catheter. Please take note of the left-hand portion that has paper taped to it. The taped portion can be tightly squeezed into the barrel and can move out when desired.

graduated conical bottom test tube. The contents were placed on ice. In the lab, a drop of uterine lavage sample was added to each designated segment for pH, leukocyte esterase and protein analysis of the Urine Test Strips 10P (Aspen Diagnostics Pvt. Ltd., Delhi, India). The results of each were recorded after 1 min, as per the manufacturer's instructions. pH results were recorded in seven categories: 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5; leukocyte esterase results were recorded in five categories (Leuko/µL): negative (0), ca. 9.15 (0.5, trace), ca.70 (1, mild), ca.125 (2, moderate) and ca.500 (3, large); and protein results were recorded in six categories: 0 (negative), 0.5 (trace), 1 (30mg/dL), 2 (100 mg/dL), 3 (300 mg/dL) and 4 (>2000 mg/dL). The pH (pH paper) of lavage, as for discharge, was also recorded. A portion of the uterine lavage was also evaluated for uterine lavage sample optical density (ULSOD). It is a highly accurate, non-invasive diagnostic method used to assess SCE and uterine health in cows (Machado et al. 2012). The ULSOD evaluation was done at 450 nm and 620 nm using a 96-well microplate (Sigma-Aldrich) that was read in a Synergy HT microplate reader (BioTek Instruments, Winooski, VT, USA) (Machado et al. 2012). The diagnostic investigations in the laboratory were initiated within 1 to 2 hr of sample collection.

Statistical analysis

The data are presented as Mean±S.E.M. unless otherwise stated. The average values of general and diagnostic parameters between the SCE+ and SCE- groups were compared using a Student's *t*-test. A receiver operator characteristics (ROC) curve analysis was performed to determine the optimal cutoff point values for the PMN percentage in the endometrial cytology sample (as the gold standard) and thereafter for the other diagnostic parameters. The optimal cutoff point was selected based on the highest sum of sensitivity (Se) and specificity (Sp). To test the individual association of endometrial cytology with the different diagnostics, the test results were dichotomized (SCE+ or SCE-) at all possible cutoff levels. For microbiological analysis, the presence of microbes (yes/no) was considered. A series of 2x2 tables were created for different diagnostics against cytology evaluation. The true positive (TP), true negative (TN), false positive (FP) and false negative (FN) counts were recorded, which were also used to ascertain the positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy of each test. Cohen's kappa (κ) analysis was used to quantify the level of agreement between cytology and other tests. The κ value agreement was interpreted as follows: value of <0.21, 0.21 to 0.40, 0.41 to 0.60, 0.61 to 0.80, and > 0.80 as a poor, fair, moderate, substantial, and almost a perfect agreement, respectively (Thrusfield and Christley 2018). Furthermore, the concordance between certain diagnostic measures, such as ULSOD (450 nm *vs.* 650 nm), uterine lavage pH (pH paper *vs.* test strip), and pH (pH paper) of sample specimens (discharge *vs.* uterine lavage), was analyzed using the Bland-Altman scatter plot. A Pearson correlation was established between various diagnostic variables in the SCE+ and SCE-. Multiple correlations between the diameter of the ovulatory follicle on either ovary (independent variable) and the endometrial thickness of ipsilateral or contralateral horns (dependent variables) were also calculated in the SCE+ and SCE- groups. The entire statistical analysis was conducted using MedCalc-version 22.032 (MedCalc Software, Ostend, Belgium). A level of $p<0.05$ was considered to be statistically significant, and tendencies are reported at $0.05<p<0.10$.

Results

All the cows, except for one in each of the SCE+ and SCE- groups that had retained placenta at the last calving, experienced an uneventful periparturient period. The number of successive inseminations in the SCE+ and SCE- groups was 5.07±0.49 and 5.20±1.08, respectively. On ROC analysis, the PMN cell cutoff point, area under the ROC curve, significance level, Se and Sp were ≥1.00%, 0.9253, 0.0001, 100.00%, and 40.00%, respectively. The cytological prevalence of SCE was 75.00% (n = 45; average PMN cell percentage of 2.22±0.26). The average values of the general parameters for SCE+ and SCE- cows, respectively, were as follows: age (months), 101.70±6.90 *vs.* 90.80±13.40; parity, 3.11±0.40 *vs.* 2.30±0.80; DIM, 605.70±48.90 *vs.* 948.80±193.90 (p=0.016); and BCS, 2.70±0.00 *vs.* 2.70±0.00. The average values of general reproductive parameters did not differ between the SCE+ and SCE- groups, which were as follows: estrus duration (hr), 42.60±2.90 *vs.* 43.20±5.20; inter estrus interval (days), 19.40±0.30 *vs.* 18.80±0.70; and size of preovulatory follicle (mm), 13.70±0.3 *vs.* 14.60±0.50, respectively.

The average values of diagnostic parameters in the SCE+ and SCE- groups (Table 1) are presented. As was evident, leukocyte esterase differed significantly, whereas protein tended to differbetween the groups. The endometrial thickness of the horns within and between the groups did not differ. However, the categorization of endometrial thickness according to the ovary bearing the ovulatory follicle (n=22/23 and 7/8 for right/left ovary in SCE+ and SCE-, respectively) revealed a greater thickness of the ipsilateral (7.8±0.6 to 10.1±1.9 mm) than the contralateral (7.2±0.7

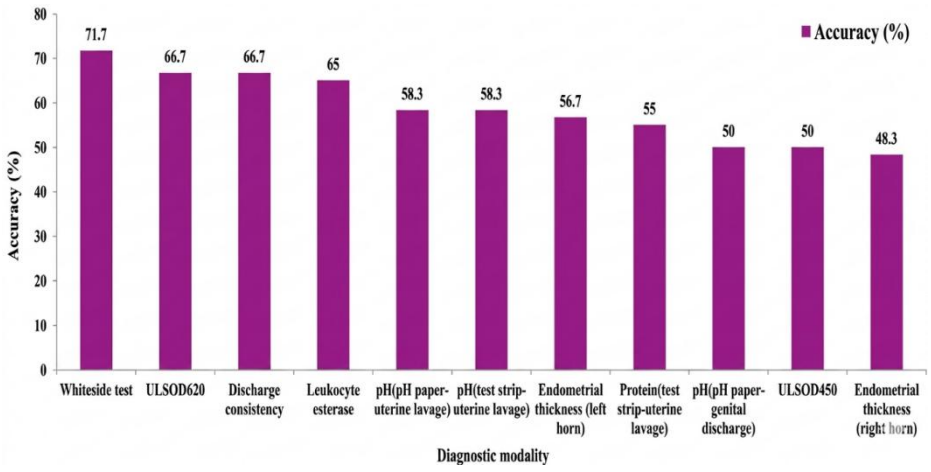

Fig 3. Comparison of the diagnostic accuracy of various modalities (in descending order) for subclinical endometritis in repeat breeder cows.

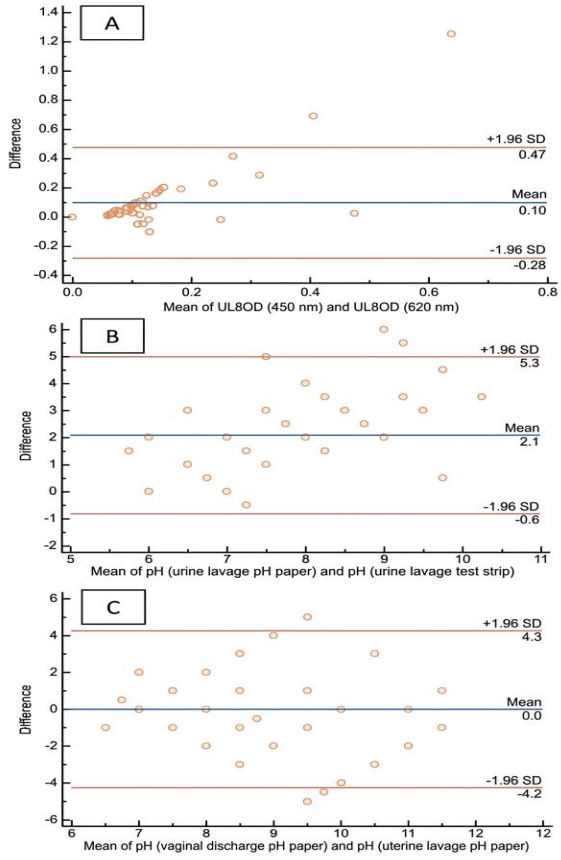

Fig 4. Bland-Altman scatter plots evaluating the concordance between various diagnostic modalities and alternative methods employed to assess the same modality for the diagnosis of subclinical endometritis (SCE) in repeat breeder cows: Panel A depicts the concordance of uterine lavage sample optical density (ULSOD) at 450 nm and 620 nm, respectively ($p=0.0002$); Panel B depicts the concordance of pH of uterine lavage measured by pH paper and test strip ($p<0.0001$); Panel C depicts the concordance of genital discharge and uterine lavage measured by pH paper ($p=0.9293$).

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)

Parameters	SCE+	SCE-	p-value
Genital discharge			
Consistency	2.20 ± 0.12	2.00 ± 0.22	0.41
Whiteside test	1.20 ± 0.09	1.00 ± 0.10	0.23
pH (pH paper)	8.69 ± 0.24	8.47 ± 0.38	0.64
Colony forming units /mL	$11.0x10^4 \pm 6.3x10^4$	$5.9x10^4 \pm 5.9x10^4$	0.65
Ultrasonography			
Endometrial thickness (mm)			
Right uterine horn	8.38 ± 0.32	8.53 ± 0.99	0.85
Left uterine horn	8.64 ± 0.28	8.29 ± 0.58	0.55
Uterine lavage			
Test strip related			
pH	6.51 ± 0.14^a	5.90 ± 0.49^a	0.10
Leukocyte esterase	1.47 ± 0.13	0.73 ± 0.23	0.00
Protein	1.78 ± 0.20	1.06 ± 0.30	0.06
pH (pH paper)	8.52 ± 0.25^b	8.20 ± 0.76^b	0.60
$ULSOD_{450}$	0.20 ± 0.03	0.13 ± 0.02	0.19
$ULSOD_{620}$	0.09 ± 0.01	0.06 ± 0.01	0.10

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

Table 3: Cohen's Kappa (κ) score and extent of agreement between different diagnostic modalities in detection of subclinical endometritis (SCE) among repeat breeding cows

Variables	κ-score	Extent of agreement
PMN (%) * genital discharge consistency	0.111	Slight
PMN (%) * Whiteside test	0.000	Poor
PMN (%) * pH (pH paper) genital discharge	0.077	Slight
PMN (%) * microbial presence	0.315	Fair
PMN (%) * endometrial thickness (left horn)	0.174	Slight
PMN (%) * endometrial thickness (right horn)	0.114	Slight
Endometrial thickness (left horn) * Endometrial thickness (right horn)	0.330	Fair
PMN (%) * pH (test strip)	0.107	Slight
PMN (%) * leukocyte esterase	0.275	Fair
PMN (%) * protein	0.181	Slight
PMN (%) * pH (pH paper) uterine lavage	-0.016	Poor
PMN (%) * $ULSOD_{620}$	0.212	Fair
PMN (%) * $ULSOD_{450}$	0.012	Slight
Leukocyte esterase * protein	0.324	Fair
Leukocyte esterase * pH (test strip) uterine lavage	-0.210	Poor
Protein * pH (test strip)	0.350	Fair

to 8.9±1.0 mm) horn in both the groups. Consequently, the presence of an ovulatory follicle on the left ovary was correlated with a greater endometrial thickness (p=0.059) of the ipsilateral (8.80±0.41 mm) than the contralateral (7.79±0.34 mm) uterine horn in the SCE+ cows. The variation in endometrial thickness was not associated with the size of the ovulatory follicle on either ovary in both the groups. The cows showed no evidence of intrauterine fluid accumulation.

The attributes associated with ROC (Table 2) and the diagnostic accuracy for diverse diagnostic modalities (Fig 3) with reference to endometrial cytology is presented. Whiteside test (highest overall Sp) followed by $ULSOD_{620}$/consistency, and leukocyte esterase were found to be most accurate. Among the latter, leukocyte esterase ($\kappa = 0.275$) and $ULSOD_{620}$ ($\kappa = 0.212$) had a fair degree of agreement with PMN cell percentage (Table 3).

The Bland-Altman scatter analysis (Fig 4) revealed a significant bias in $ULSOD_{450}$ *vs.* $ULSOD_{620}$ (Panel A) and uterine lavage pH (pH paper *vs.* test strip) (Panel B), which was absent for pH (pH paper) (discharge *vs.* uterine lavage) (Panel C).

The discharge in eight of the 60 (13.33%) repeat breeder cows, five (33.33%) in SCE- and three (6.66%) in SCE+, was negative for microbial growth. A total of 9 different bacterial genera dominated by four different *Bacillus* spp. in 23 (44.23%) cows, were recorded as single or mixed infection. The presence of microbes showed a fair degree of κ agreement (greatest in present study) with the PMN cell percentage (Table 3).

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 (%)	Apparent prevalence of SCE (%)
Genital discharge													
Consistency	≥2	0.52	0.39	35	5	10	10	42.2	66.7	1.26	77.7	33.3	58.3
Whiteside test	≥1	0.64	0.00	42	1	14	3	33.3	96.2	8.76	75.0	25.0	70.0
pH (pH paper)	≥9.0	0.49	0.52	20	10	5	25	44.4	66.7	1.33	80.0	28.6	33.3
Endometrial thickness (mm)													
Right uterine horn	≥8.7	0.57	0.20	17	12	3	28	46.7	80.0	1.19	85.0	30.0	28.3
Left uterine horn	≥8.7	0.58	0.22	23	11	4	22	47.8	81.0	1.20	85.2	33.3	38.3
Uterine lavage													
Test strip related													
pH	≥6.5	0.60	0.12	27	8	7	18	46.7	80.0	1.50	79.4	30.8	45.0
Leukocyte esterase	≥2	0.71	0.00	28	11	4	17	62.2	73.3	2.33	87.5	39.3	46.7
Protein	≥2	0.65	0.02	21	12	3	24	46.7	80.0	2.33	87.5	46.2	35.0
pH (pH paper)	≥9	0.51	0.43	27	8	7	18	47.0	61.5	1.22	79.4	30.8	45.0
$ULSOD_{450}$	≥0.14	0.61	0.03	21	9	6	24	46.7	60.0	1.66	77.7	27.3	35.0
$ULSOD_{620}$	≥0.06	0.67	0.00	33	7	8	12	62.2	66.7	1.87	80.5	36.8	55.0

[#] In reference to cytotape based cytology cutoff values of ≥ 1.00% polymorphonuclear cells; The cutoff values: consistency pertains to the genital discharge breaking in ≥ 15 to 30 sec; Whiteside test with a color shade of light yellow or higher; leukocyte esterase (Leuko/µL) with a sample value of ≥ca.125; protein with a sample content of ≥100 mg/dL

Table 4: Significant correlates among different diagnostic variables in repeat breeder cows with (SCE+) and without (SCE-) subclinical endometritis (SCE)

Variables	Group	R-score	p-value
PMN (%) * Whiteside test	SCE+	0.42	<0.01
Whiteside test * pH (pH paper) genital discharge	SCE+	0.30	<0.05
Endometrial thickness: Right uterine horn * left uterine horn (ovulatory follicle on left ovary)	SCE-	0.51	<0.00
Endometrial thickness: Right uterine horn * left uterine horn (ovulatory follicle on left ovary)	SCE+	0.02	<0.00
Endometrial thickness: Right uterine horn * left uterine horn (ovulatory follicle on right ovary)	SCE+	0.51	<0.00
PMN (%) * pH (pH paper) uterine lavage	SCE+	0.48	<0.01
pH (test strip) uterine lavage * pH (pH paper) uterine lavage	SCE+	0.39	<0.01
pH (test strip) uterine lavage * protein uterine lavage	SCE+	0.38	<0.01
pH (test strip) uterine lavage * $ULSOD_{620}$ uterine lavage	SCE+	0.46	<0.01
pH (test strip) uterine lavage * pH (pH paper) uterine lavage	SCE-	0.87	<0.01
pH (test strip) uterine lavage * $ULSOD_{620}$ uterine lavage	SCE-	0.79	<0.01
pH (pH paper) uterine lavage * $ULSOD_{620}$ uterine lavage	SCE-	0.86	<0.01
pH (test strip) uterine lavage * endometrial thickness (left horn)	SCE-	0.54	<0.05
Leukocyte esterase * protein	SCE+	0.40	<0.01
Leukocyte esterase * $ULSOD_{620}$	SCE+	0.32	<0.05

The significant correlates among different diagnostic variables in SCE+ and SCE- cows are presented in Table 4. The endometrial thickness of the two uterine horns was related ($p<0.00$) to whether the ovulatory follicle was present on either ovary in SCE+ or only the left ovary in SCE. The test strip pH of uterine lavage had a widespread correlation with some diagnostics, with $ULSOD_{620}$ being common in both SCE+ and SCE- groups.

Discussion

At 1.00% PMN threshold, a Se and Sp of 100.00% and 40.00% revealed a divergence from a previous study citing the corresponding values to be 33.80% and 88.60%, respectively in cows at insemination (Pascottini et al. 2016). The cytological prevalence of 75.00% SCE in the present study was higher than the 53.00% (3.00% PMN) (Villasenor-Gonzalez et al. 2024) and 12.70% (5.00% PMN) recorded in repeat breeder cows (Pothmann et al. 2015). An altered Se, Sp and SCE prevalence can be linked to variations in PMN percentage cutoff metrics.

The consistency of discharge was the second most trustworthy cow-side surrogate test. Against 58.30%, thick discharge during estrus has been reported in 39.29% of repeat breeder cows (Raval et al. 2018) and 30.00% of normal cows (Gohel et al. 2012) at estrus. A reduced number of endometrial glands, altered intertwined mucoproteins, increased total proteins, or a modified composition of glycoprotein molecules in discharge due to persistent uterine inflammation may increase its viscosity (Siregar et al. 2019).

A significant correlation between PMN% and Whiteside test in SCE+ cows was in agreement with a prior study (Bedewy and Rahawy 2019) which recorded all 42 repeat breeder cows, but with a much higher PMN of 12.00 to 18.00%, testing positive for Whiteside. Conversely, in the present study, the Whiteside test at 1.00% PMN threshold demonstrated the highest Sp, making it a very promising modality for cow-side diagnosis in ruling out SCE. However, a high FP count decreased the Se of Whiteside test. An FP Whiteside reaction can arise from the physiological neutrophil infiltration and metrorrhagia, respectively, observed in 20.00% and 40.00% of the estrual histological specimens from normal cows (Raja et al. 2012). This justifies the positive test in 15.00% of healthy and fertile cows (Bhat et al. 2014). The similar number of cows with and without metrorrhagia in the SCE+ and SCE- groups ($\chi^2 = 0.17$) and the absence of an overt metrorrhagia at sampling rule out its potential impact on the FP reaction. Instead, we can link the prolonged estrus (42.60±2.90 hr in SCE+; 43.20±5.20 hr in SCE-) to the high Whiteside FP count. In cows with prolonged estrus, the number of leukocytes in genital mucus gradually increased for the first 18 hours and accelerated thereafter (Jakupov et al. 2024). Hence, it is strongly suggested that Whiteside test should be performed in the early part of estrus to prevent FPs arising due to prolonged estrus and metrorrhagia. Given the current findings, cows that test positive for Whiteside reaction should undergo a cytological confirmation, which has a Se of 100.00% for the diagnosis of SCE. As a result, it is crucial to acknowledge that a cytological examination in Whiteside FP cows may be counterproductive.

A pH (pH paper) greater than 7.8 confirms SCE (Bedewy and Rahawy 2019). However, metabolites from bacterial origin render the pH of genital contents to be alkaline (Rosales and Ametaj 2021; Jakupov et al. 2024), which justifies an alkaline pH in SCE-. There was a discrepancy in the pH measured by pH paper and test strip (Panel B, Fig 4). When compared to pH paper and pH meter, the urinary dipsticks underestimated the pH, and the extent of underestimation increased as the medium went from acidic to alkaline (Athanasiou et al. 2018). This disparity confirms that dipsticks perform poorly in diagnosing SCE (Cheong et al. 2012).

Against 86.66% cows positive for microbial growth, bacterial infection in discharge of 62.20 to 100% repeat breeding cows has been reported (Bhat et al. 2014; Pothmann et al. 2015). A fair κ agreement (highest in current study) between PMN cell percentage and bacterial presence (Table 3) suggests the microbial involvement in causing SCE. The observation of a similar microbial count between SCE+ and SCE- cows, the dominance of *Bacillus* spp., and more cows with a single microbial infection is consistent with earlier findings (Bhat et al. 2014; Wagener et al. 2015).

A similar endometrial thickness of either horn in the SCE+ and SCE- cows suggested mild inflammation. Interestingly, left uterine horn was more proactive in discerning a greater endometrial thickness. Regardless of the size of the ovulatory follicle, presence of ovulatory follicle on left ovary was associated with a significantly thicker endometrium of left than the right horn in SCE+ cows. Despite a comparable number of SCE+ cows possessing an ovulatory follicle on either the left (n=23) or the right (n=22) ovaries, the left horn alone exceeded the endometrial thickness diagnostic cutoff limit of ≥8.7 mm in ten cows against five for right horn. Additionally, there were 14 other instances where, despite a greater endometrial thickness of the right horn, the left horn exceeded the cutoff limit and was also diagnostic of SCE. In support of the present findings, a study on slaughtered cows observed that the left compared to the right horn was a better representative in diagnosing SCE (Fuentes et al. 2017). The above findings show that the two horns in the same cows have different pathophysiological dynamics.

The cutoff values for leukocyte esterase, protein, and pH, which were ≥2, ≥2, and ≥6.5, respectively, and leukocyte esterase' superiority over the other two, are in close agreement with a number of prior studies on SCE or clinical endometritis in postpartum cows between 29 to 60 DIM (Cheong et al. 2012; Denis-Robichaud and Dubuc, 2015; Van Schyndel et al. 2018). Interestingly, the similarity across the thresholds occurred despite a significant variation in the PMN percentage cutoff of ≥1.00% in this study as opposed to ≥5.00 to 10.18% in the postpartum cows. It is obvious that a greater PMN cell count raises the esterase content. The latter reason probably accounted for a 10.00 to 17.00% lower Se, an underestimation of SCE prevalence by 28% compared to its overestimation by 6.00 to 20.00%, and a fair (κ = 0.275) compared to a moderate to high (κ = 0.49to 0.62) agreement between leukocyte esterase and PMN percentage in the investigated repeat breeders compared to postpartum cows. Cheong et al. (2012) observed pH rather than leukocyte esterase to exhibit a stronger correlation with endometritis. This supports a significant and widespread correlation of pH (test strip/pH paper) with $ULSOD_{620}$, protein, and endometrial thickness in both SCE+ and SCE- cows (Table 4).

Reduced Se, Sp, and overall diagnostic accuracy of $ULSOD_{450}$, along with a strong bias between $ULSOD_{620}$ and $ULSOD_{450}$ (Panel A; Fig 4) and a relatively poor κ agreement with PMN cell percentage (Table 3), all pointed to $ULSOD_{620}$ superiority. Regarding the use of $ULSOD_{620}$ in the SCE diagnosis, no report is currently available. Alternatively, the $ULSOD_{620}$ threshold of 0.06 in the current study closely resembled to 0.059 in the postpartum cows with clinical endometritis having ≥18.00% PMN (Machado et al. 2012).

Conclusion

Reduced sensitivity and Cohen's kappa agreement between different diagnostics and PMN percentage sufficed subclinical uterine infection in repeat breeder cows. Nonetheless, the first test that should be run is the Whiteside reaction, a cow-side test that has the highest specificity and can be trusted to rule out subclinical endometritis. Conversely, the cytological confirmation (lab-test) may be required in repeat breeder cows with a positive Whiteside test.

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Author contributions

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Conflict of Interest Statement

No potential conflict of interest was reported by the authors.

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