TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2021) 45: 540-546
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doi:10.3906/vet-2006-123

Leukocyte esterase reagent strips: rapid, economical and reliable cow side test for subclinical endometritis

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Received: 24.06.2020 • **Accepted/Published Online:** 25.04.2021 • **Final Version:** 29.06.2021

Abstract: This study was carried out in crossbred cows (>60 DIM) to evaluate leukocyte esterase (LE) strip test, protein strip test, pH strip test, white side test (WST) of estrual mucus, in comparison to endometrial cytology (EC) using cytobrush (CB) as reference diagnostic method for subclinical endometritis (SCE). Endometrial cytology was done in 78 cows with ≥4% PMNs as cut-off value for SCE. Leukocyte esterase strip test was done by contacting estrual mucus with multireagent urinalysis reagent strips and results recorded after 10 min. Similarly, protein and pH strip tests were conducted using the same strips and results recorded after 60 s. White side test of estrual mucus was conducted simultaneously. Leukocyte esterase, protein and pH strip tests were evaluated and analyzed statistically using receiver operator characteristic (ROC). LE strip test at cut-off value ≥ ± acquired from ROC analysis showed sensitivity (Se) of 86.67% and specificity (Sp) of 66.67%. Similarly, protein and pH strip tests showed Se of 53.33%, 66.67% and Sp of 66.67%, 77.78%, respectively. Strip tests were also combined in three combinations (LE + protein, LE + pH and LE + protein + pH) and overall decrease in Se was observed in all three combinations (46.67%, 60% and 33.33%, respectively); while, a slight increase in Sp (77.78%) was also observed. Sensitivity and Sp of WST were 63.33% and 55.56%; respectively, and these values for WST were lower compared to those in LE test. In conclusion, cow side LE test can be used as an economic and easy test with acceptable Se and Sp compared to WST instead of EC for SCE in cattle. However, no improvement in performance of LE strip test was observed when combined with pH and protein strip tests.

Key words: Subclinical endometritis, endometrial cytology, leukocyte esterase strip test

1. Introduction

The foundation for optimum fertility and high conception rates in dairy cows require healthy uterine milieu [1]. Corpus luteal function, decreased sperm motility and reduced embryonic viability are the impairments of blooming reproduction at various steps, leading to decline in pregnancy rate and occur due to inflammatory atmosphere being present in utero [2]. In this aspect, subclinical endometritis (SCE) is important as it produces negative outcome of reproduction and considerable economic sufferings by being the chief cause of repeat breeding and all this by being asymptomatic as well as immensely prevalent disease in cows [3,4].

SCE is known to occur when the uterine interior coating (endometrium) is inflamed. Term cytological endometritis has been used for SCE due to association with elevated fraction (proportion) of poly-morpho-nuclear cells (PMN) in samples of endometrial cytology (EC) acquired by cytobrush [5] or small-volume uterine lavage or from biopsy samples by histology [4,6]. Also bacteriology and ultrasonography [7,8] have been engaged to diagnose it. Although cytobrush (CB) technique is presently being considered the reference and most efficient method for cytological identification of SCE [9] but the practical applicability of this method for field situations is not realistic due to the reasons that this method is time

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consuming and most importantly demands technical expertise for collecting in utero samples and then to stain as well as to identify individual cells in samples.

Leukocyte esterase (LE) reagent test using LE coated strips is an alternative mode to detect inflammatory cells in the diseased uterus [10]. The leukocyte esterase activity of neutrophils' is mainly evaluated by this test and the number of leukocytes is related by the intensity of colour change [11]. Diagnoses of UTIs in humans is usually being done by this test but recently have been used for diagnosis of inflammation in the uterus using cervico-vaginal mucus [12] and projected as an alternative, innovative and creative cow-side test for identification of SCE [10].

A practical cow-side and rapid clinical diagnostic test to inspect and manage SCE under field conditions is very much desired. Therefore, the objective of this study was to determine the efficacy of various diagnostic tests (LE, protein, pH, or combination of reagent strip tests and white side test (WST) in comparison to reference test (endometrial cytology) in search of a potential cow side test for SCE.

2. Materials and methods

This investigation was conducted at Veterinary Clinical Complex (VCC), FVSc and AH, Shuhama, Srinagar and Mountain Livestock Research Institute (MLRI), Manasbal, Ganderbal. Crossbred postpartum cows with more than 60 days in milk (DIM) presented for artificial insemination (AI) at VCC and MLRI were initially enrolled in the study. Investigation was then conducted in 78 clinically healthy cows with no apparent clinical signs of any disease, clear estrual mucus discharge and without any gross reproductive tract pathology detected perrectally. In the present study, diagnosis of SCE was done by: (i) endometrial cytology as reference method using cytobrush, (ii) leukocyte esterase strip test, (iii) protein and pH strip test, and (iv) white side test.

2.1. Endometrial cytology

Uterine cytology samples were collected by cytobrush (Care India Surgical, Mayfair Surgical Corporation, Ludhiana, PB, India) using cytobrush assembly as described by Singh et al. [13] with slight modification. Base of the CB was fixed on the stylette and retracted into the steel catheter. The CB assembly was then covered by sanitary sheath (IMV Technologies, Gurugram, HR, India) for protection against vaginal and cervical contamination. Before sampling, perineum including vulva was washed with water and soap and dried with soft absorbent cotton. The introduction of CB assembly into the uterine body through cervix and vagina was achieved while maintaining sleeved arm perrectally for cervical manipulation. CB progressed out of steel catheter by puncturing the sanitary sheath after crossing the cervical internal Os, approx 1–2 cm into uterine body. CB was rotated gently once and then again in opposite direction while being in contact with adjacent endometrium for cellular material collection. The whole apparatus was then removed from genital tract after retracting the CB into the steel tube.

Slides were prepared immediately by rolling the CB on glass slides. Slides were air dried, fixed in methanol (Merck Specialities Pvt Ltd, Vikhroli East, MB, India) for 5–10 min and after drying, staining was done with Giemsa stain (HiMedia Laboratories Pvt Ltd, Mumbai, India). Cytologic evaluation was made by light microscopy at magnification of 1000×. Cells were counted up-to 300 (endometrial cells and PMNs) and average percentage of PMN cells (neutrophils) was calculated and ≥ 4% of PMNs was taken as threshold level for positive SCE as per Singh et al. [13].

2.2. Leukocyte esterase reagent strip test

In LE strip test, about one ml of collected estrual mucus was contacted with multireagent LE test strip (Mission Urinalysis Reagent Strips ACON Laboratories, Inc., San Diego, CA, U.S.A) for 10–15 s. The results for LE strip test were then recorded based on the strip colour change (according to the manufacturer recommendations) after 10 min according to Hajibemani et al. [12].

2.3. Protein strip test

About one mL of collected EM was contacted with multireagent urinalysis reagent strip (Mission Urinalysis Reagent Strips ACON Laboratories, Inc.) for 10–15 s. Protein (mainly albumin) content of EM was recorded based on colour change after 60 s (according to the manufacturer recommendations).

2.4. pH strip test

About one mL of collected EM was contacted with multireagent urinalysis reagent strip (Mission Urinalysis Reagent Strips ACON Laboratories, Inc.) for 10–15 s. pH of EM was recorded based on colour change after 60 s (according to the manufacturer recommendations).

2.5. White side test

In WST, equal volume (1 mL each) of estrual mucus and 5% sodium hydroxide (Merck Specialities Pvt Ltd) was taken in a sterile test tube, properly mixed and then heated using spirit lamp to boiling point. The test tube was then cooled under tap water and colour change observed. Colour change from transparent towards yellow was taken as positive and no colour change was taken as negative as described by Bhattacharyya et al. [14].

2.6. Collection of estrual mucus

Collection of estrual mucus (EM) from study animals was carried out at approx 6–12 h postcommencement of behavioural oestrus as described by Bhat et al. [15] with slight modification. The study animals during EM collection were properly restrained in the service crate. Lubricated (with paraffin) full sleeve gloved left hand

was inserted per rectum for evacuation of rectal faeces. Perineal region including vulva was thoroughly washed with running tap water and then dried with soft absorbent cotton followed by swabbing with ethyl alcohol. While holding both the vulvar lips apart by an assistant, a sterile AI sheath over conventional AI gun was inserted into the vagina, then guided to the cervix and then to uterine body by the hand already introduced per rectum. The external end of the sheath after removing AI gun, was connected to a 20 mL disposable sterile syringe for aspiration of EM from body of uterus and deep cervix. After aspiration, syringe was detached and sheath wiped with ethyl alcohol soaked cotton swab after its removal from genital tract. Sheath containing EM was then transported immediately to lab for further processing.

2.7. Statistical analysis

Receiver operator characteristic (ROC) was use to analyze the data of LE, protein and pH strip tests statistically and the area under the curve (AUC) and p values were reported as displayed on the graph generated by ROC analysis. The AUC was used to find optimal cut-off point. The cut-off with the highest sum of sensitivity and specificity was selected as optimal. Data of strip test combinations and data of WST were analysed using diagnostic test evaluation calculator to calculate sensitivity and specificity. ROC and diagnostic test evaluation calculator were both utilized from MedCalc Statistical Software version 19.0.4 (MedCalc Software bvba, Ostend, Belgium).

3. Results

3.1. Endometrial cytology

EC was used as a standard diagnostic method to estimate the diagnostic precision of other tests. Based on EC, sixty (60) cows (≥ 4% PMNs) were found to be positive and 18 cows (< 4% PMNs) were negative (control) for SCE.

3.2. Leukocyte esterase strip test

LE test results were recorded in five categories: – or (negative), ± or (15 Leu/µL), + or (70 Leu/µL), ++ or (125 Leu/µL), and +++ or (300 Leu/µL). Area covering the ROC curve (AUC) was 0.811 with significance level p (area = 0.5) value less than 0.0001 (Figure 1). Based on ROC analysis, the cut-off point selected was greater than 15 Leu/µL (> ±) to be used for positive diagnosis of SCE. At the cut-off point, LE test showed sensitivity (Se) of 86.67% and specificity (Sp) of 66.67%. Se and Sp of LE test based on ROC analysis at different cut-off valves is shown in Table 1.

3.3. Protein strip test

Protein results were recorded in five categories: - or (negative), ± or (15 mg/dL), + or (30 mg/dL), ++ or (100 mg/dL), and +++ or (300 mg/dL). Area covering the ROC curve (AUC) was 0.633 with significance level p (area = 0.5) value of 0.0587 (Figure 2). Based on ROC analysis, the

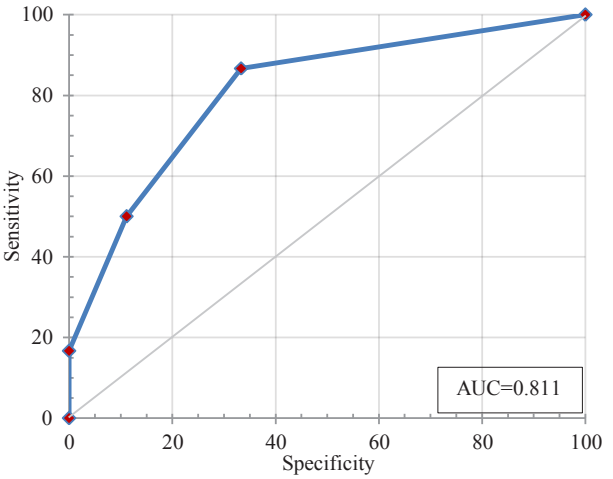

Figure 1. Area under curve (AUC) plot graph of leukocyte esterase strip test.

Table 1. Sensitivity and specificity of leukocyte esterase strip test at different cut-off values based on receiver operator characteristic analysis.

Cut-off's for number of leukocytes/µL in estrual mucus	Sensitivity (%)	Specificity (%)
≥15 Leu/µL (±)	100.00	0.00
>15 Leu/µL (±)	86.67	66.67
>70 Leu/µL (+)	50.00	88.89
>125 Leu/µL (++)	16.67	100.00
>500 Leu/µL	0.00	100.00

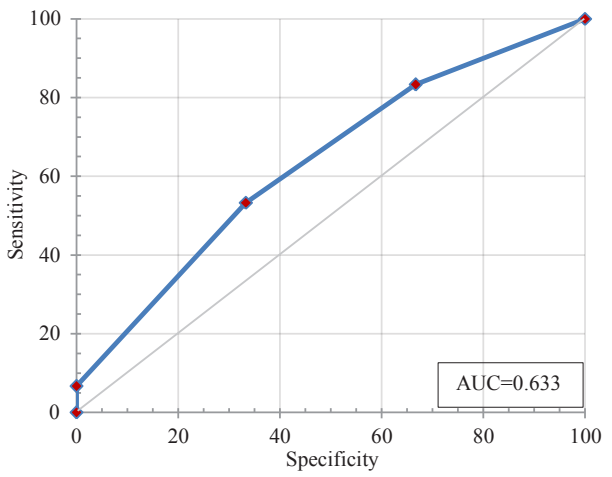

Figure 2. Area under curve (AUC) plot graph of protein.

cut-off point selected was greater than 30 mg/dL (> +) to be used for positive diagnosis of SCE. At this cut-off point, protein showed Se of 53.33% and Sp of 66.67%. Se and Sp of protein based on ROC analysis at different cut-off valves is given in Table 2.

3.4. pH strip test

pH results were recorded in six categories: 6.5, 7.0, 7.5, 8.0, 8.5, and 9. Mean pH was found to be 8.23 ± 0.84. Area covering the ROC curve (AUC) was 0.694 with Significance level p (area = 0.5) value of 0.0027 (Figure 3). Based on ROC analysis, pH > 8 was used as cut off point for positive diagnosis of SCE. At this cut-off point, pH had Se of 66.67% and Sp of 77.78%. Se and Sp of pH based on ROC analysis at different cut-off values is displayed in Table 3.

Combination of LE test (cut-off value >±) and pH (cut-off value > 8) showed Se of 60.00% and Sp of 77.78%. Similarly LE test and protein when used in combination with cut-off value > ± for LE test and > + for protein, showed Se of 46.67% and Sp of 77.78% in comparison with EC. When all the three reagent strip test results i.e LE, protein and pH were used in combination using > ± for LE, > + for protein and > 8 for pH as cut-off values, showed Se of 33.33% and Sp of 77.78% in comparison with EC (Table 4).

Table 2. Sensitivity and specificity of protein at different cut-off values based on receiver operator characteristic analysis.

Cut-off's for amount of estrual mucus protein	Sensitivity (%)	Specificity (%)
≥15 mg/dL (±)	100.00	0.00
>15 mg/dL (±)	83.33	33.33
>30 mg/dL (+)	53.33	66.67
>100 mg/dL (++)	6.67	100.00
>300 mg/dL (+++)	0.00	100.00

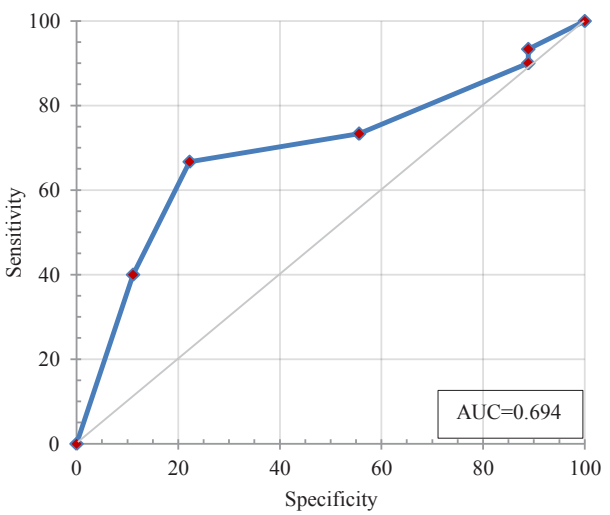

Figure 3. Area under curve (AUC) plot graph of pH.

3.5. White side test

WST results were recorded in four categories: transparent (no infection), light yellow (mild infection), yellow (moderate infection), and dark yellow (severe infection). Out of 78 animals, 46 (59%) showed positive reaction and 32 (41%) showed negative reaction to WST. However, out of 46 positive samples for WST, 39.1% (18) showed light yellow, 43.5% (n = 20) showed yellow and 17.4% (n = 08)

Table 3. Sensitivity and specificity of pH at different cut-off values based on receiver operator characteristic analysis.

Cut-off's for pH levels of estrual mucus	Sensitivity (%)	Specificity (%)
≥6.5	100.00	0.00
>6.5	93.33	11.11
>7	90.00	11.11
>7.5	73.33	44.44
>8	66.67	77.78
>8.5	40.00	88.89
>9	0.00	100.00

Table 4. Sensitivity and specificity of leukocyte esterase strip test, white side test, protein, pH, combination of leukocyte esterase and pH, leukocyte esterase and protein, and leukocyte esterase, protein and pH in comparison to endometrial cytology.

Parameter (cut-off)	Se and Sp[1]	%
LE (> ±)[2]	Se	86.67
	Sp	66.67
WST[3]	Se	63.33
	Sp	55.56
Protein (> +)	Se	53.33
	Sp	66.67
pH (> 8)	Se	66.67
	Sp	77.78
LE and pH in combination	Se	60.00
	Sp	77.78
LE and Protein in combination	Se	46.67
	Sp	77.78
LE, protein and pH in combination	Se	33.33
	Sp	77.78

[1] Se: sensitivity; Sp: specificity.
[2] LE: leukocyte esterase.
[3] WST: white side test.

showed dark yellow reaction. In comparison to EC, WST showed Se of 63.33% and Sp of 55.56% (AUC = 0.594).

In this study, against EC, LE strip test at cut-off > ± showed highest Se (86.67%) and 2nd highest Sp (66.67%) among all the diagnostic tests used. Maximum Sp (77.78%) was shown by pH test at cut-off > 8 and its Se (66.67%) was also 2nd highest after LE test. WST also exhibited a near similar Se (63.33%) as that of pH strip test but the Sp (55.56%) of WST was much lower than that of pH strip test as well as LE strip test. Lowest Se (53.33%) was shown by protein at cut-off > + among all the tests used but Se (66.67%) was similar to LE (Figure 4).

When the three tests (LE, protein and pH) were used in combination, improvement was seen in Sp (77.78%) and was found to be same in all three combinations; however a severe decline in Se was observed an all combinations. LE strip test and pH in combination showed Se (60.00%) while combination of LE, protein and pH showed lowest Se (33.33%) as illustrated in Figure 4.

4. Discussion

Endometrial cytology using CB is considered as the best minimally invasive diagnostic method for the detection of SCE. This method is believed to be without any negative effect on ensuing conception rate, even if uterine cytology sample is taken after few hours of insemination [16]. EC is neither complex nor costly but it is time consuming and requires equipment and expertise [17], thus cannot act as cow side test, therefore, need for an efficient practical cow side test arises having Se and Sp at par with cytology that can be utilized at field level.

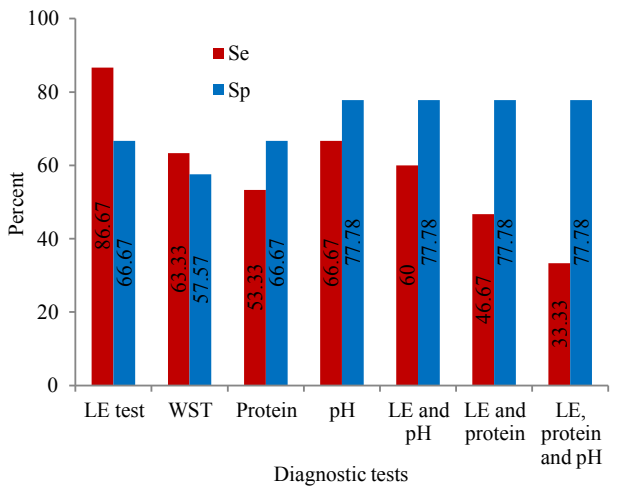

Figure 4. Sensitivity and specificity of diagnostic tests in comparison to endometrial cytology.
Se: sensitivity; Sp: specificity; LE: leukocyte esterase; WST: white side test.

LE strip test is one such test which has shown promising results. In this study, LE strip test results showed Se of 86.67% and Sp of 66.67% in comparison to EC and nearly parallel results were stated by Hajibemani et al. [12], with Se of 78% and Sp of 60% for LE strip test with respect to EC in dairy cows. Santos et al. [18] reported similar Se (83%) and contrasting higher Sp (94%) than the present study, which could be due to use of different LE strips (Multistix 10 SG, Bayer Health Care L.L.C., Elkhart, IN, USA) and sampling method i.e. uterine lavage by the investigator. In contrast to the present findings, slightly weak performance of LE strips was reported by Cheong et al. [19] with Se of 77% and Sp of 51.8%. Couto et al. [10] also reported lesser Se of 68.8% but demonstrated higher Sp of 72.6% in comparison to present study. These differences may be owing to use of different reagent strip (Multistix 10 SG, Bayer Health Care L.L.C.), higher cut-off for EC (≥ 10% PMN) and LE test (≥ ++) compared to the present study (cut-off for EC ≥ 4% PMN and for LE > ±). Further, Couto et al. [10] contacted LE test strips with 0.9% saline in which CB was plunged after rolling it on slides in contrast with estrual mucus in the present study.

Fluid accumulation during SCE increases in uterus which could lead to increase in protein concentration [19] and could be used in SCE diagnosis. EM from cows with SCE had markedly elevated intensity of total protein at 21 DPP compared to healthy cows [20]. In the current study, protein strip test results showed Se of 53.33% and Sp of 66.67% in comparison to EC and comparable Se of 58.3% was reported by Cheong et al. [19] but reported lower Sp of 55.8% for protein strip test with respect to EC. It was also reported by Cheong et al. [19] that there is weak association between protein and SCE. This low performance for protein strip test in both studies could be due to reagent strips being highly sensitive for albumin but much less sensitive for other proteins and in addition false positive results may be obtained from samples with high alkalinity and false negative outcome may arise due to high specific gravity [21]. Hajibemani et al. [12] reported that protein concentrations were increased in SCE but protein assessment of discharges could not be recommended for evaluation as a diagnostic means for SCE.

pH strip test results of the present study showed Se of 66.67% and Sp of 77.78% with mean pH of 8.23 ± 0.84 in SCE. Results reported by Cheong et al. [19] showed similar Sp (78.4%) but very low Se (44.9%) which could be due to use of different reagent strip (Multistix 10 SG, Bayer Health Care L.L.C.), higher threshold level of EC (≥ 10% PMN) and uterine lavage as sampling method. Bhat et al. [22] and Kumar et al. [23] reported increased pH (8.19 ± 0.06 and 7.95 ± 0.096, respectively) in cows with subclinical uterine infection which was in concurrence with this study. Cheong et al. [19] also reported increase in pH of uterine fluid in cows with SCE.

Multireagent strip tests were used in three different combinations (LE + pH, LE + protein and LE + protein + pH). In all the three combinations Se decreased drastically (60.0%, 46.7% and 33.3%, respectively) while Sp increased to 77.8% in comparison to LE test (Se of 86.67% and Sp of 66.67%) alone. The only probable reason that we were able to consider for the relation between the combination of the three tests and the decrease of Se was that pH and protein tests had much lower Se than LE. When the data of the 3 tests was combined and subjected to statistical analysis against standard test (EC), it further decreased the Se due to presence of low number of true positives than true negatives and false negatives concurrently. However, on comparing the three combinations with each other, better performance was shown by combination of LE and pH strip tests with Se of 60.0% and Sp of 77.8%. Cheong et al. [19] reported that LE and pH strip tests in combination increased overall Sp (96.8%) but drastically decreased Se (18.6%) in comparison to LE strip test which was in harmony with this study. Cheong et al. [19] also reported that on combining protein with LE only or with both LE and pH had no effect on increasing the performance of the strip test results, which was in accord with the outcome of present study.

In this present study, 59% animals showed positive reaction to WST with Se of 63.3% and Sp of 55.6% in comparison to EC. Bhat et al. [15] reported bacteriological growth present in 75% (60/80) animals in which 71.25% (57/80) were positive for WST. Similarly, Gupta et al. [24] used WST for detection of SCE and reported 45.8% (44/96) asymptotic animals positive to WST.

5. Conclusion

Leukocyte esterase strip test using estrual mucus showed acceptable Se and Sp in comparison to EC by cytobrush; thereby, it can be used as noninvasive, simple, inexpensive and reliable cow side method for screening SCE in cattle under field conditions. However, inclusion of protein and pH strip tests along with LE strip test does not improve the performance of strip tests. WST showed less sensitivity and specificity than LE test. EC may be replaced by LE test in the field for screening SCE in cows.

Faster intervention and treatment of SCE is possible by accurate cow-side diagnoses. Further modifications and refinements are required in the field to increase the validity of the test. Moreover, modification of the test strips is necessary to optimize them for diagnosis of SCE as these strip tests are not specific for uterine discharge testing.

Acknowledgments/funding/conflict of interest

The authors are thankful to all those involved in successful completion of this investigation at MLRI Manasbal and FVSc and AH, SKUAST-K, J and K, India**.**

This research did not receive any specific grant from funding agencies in public, commercial, or not-for-profit sectors. There are no benefits in any form from a commercial party or any of the authors related directly or indirectly to the subject of this manuscript.

The authors declare that they have no conflict of interest.

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