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Comparative Evaluation of Reagent Strip Tests for Cow-Side Diagnosis of Subclinical Endometritis in Dairy Cattle

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Research Article

Keywords: Subclinical endometritis, endometrial cytology, cytobrush, LE strip test, pH strip test, Serum NO and MDA

Posted Date: May 7th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9381915/v1

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Abstract

Subclinical endometritis (SCE) is a significant reproductive disorder in dairy cows that negatively affects fertility without overt clinical signs. The present study was conducted to evaluate the efficacy of different diagnostic methods in subclinical endometritis. Fifty-seven clinically healthy crossbred dairy cows (> 60 days in milk) presented for artificial insemination were included in the study. Endometrial cytology using cytobrush technique served as the reference diagnostic method, with ≥ 4% PMNs as the cut-off value. Based on endometrial cytology, 47 cows were positive and 10 were negative for subclinical endometritis. Cervico-vaginal mucus (CVM) collected aseptically from uterus was used for conducting White side test (WST), leukocyte esterase (LE), pH, protein and nitrate strip tests. LE, pH, protein and Nitrate strip tests were performed using multi-reagent urinalysis strips, with LE results read at 10 min and pH, protein and nitrate results at 60s as per manufacturer's instructions. WST, LE, pH, protein and Nitrate strip tests were evaluated and analyzed statistically using Receiver Operator Characteristic (ROC). WST at cut-off point > 0 (negative) showed Sensitivity (Se) of 74.5% and Specificity (Sp) of 70% (AUC 0.767; P < 0.001). Among individual multi-reagent strip tests, the LE strip test at cut-off value > 1(±/15 Leu/µl) showed better diagnostic performance (Se = 78.72%, Sp = 90%, AUC = 0.899; P < 0.001), followed by the pH strip test at cut-off value > 7 (Se = 85.11%, Sp = 80%, AUC = 0.810; P < 0.001). The LE + pH combination at the same cut-off values showed Se of 70.21% with 100% specificity, representing the best overall performance among the combined tests. While as the Protein and Nitrate tests showed lower diagnostic value. In current study, the level of agreement (kappa) of LE and pH with cytobrush cytology was moderate (0.51–0.544) and diagnostic accuracy was very good (80.70% and 84.21% and 84. Serum Nitric oxide (NO) and Malondialdehyde (MDA) levels were significantly (P < 0.05) higher in SCE positive animals (n = 27) that declined after recovery. In conclusion, LE and pH strip tests either alone or in combination can be used as non invasive, cowside diagnostic methods for detecting SCE under field conditions. In addition, elevated serum levels of biochemical parameters (NO & MDA) may be associated with subclinical endometritis in cows.

Introduction

A healthy reproductive tract is vital for proper reproductive performance. Postpartum uterine diseases, particularly endometritis is one of the major causes of reduced fertility and economic losses in dairy herds due to decreased milk yield, prolonged calving intervals, increased treatment costs, and higher culling rates. In addition, it has been demonstrated that subclinical endometritis has an impact on survival and quality of the embryo (Wagner et al. 2017). Their multifactorial and polymicrobial etiology, together with complex host immune responses, complicates diagnosis and management (Kasimanickam et al. 2025). Although a transient inflammatory response during early postpartum is physiological, persistence beyond 21 days postpartum is pathological and may affect up to 20% of cows (Pascottini et al. 2023). Subclinical endometritis (SCE), also termed cytological endometritis, is defined by an increased proportion of PMNs on endometrial cytology in the absence of visible vaginal discharge (Wagener et al. 2017; Krasniqi et al. 2024). Despite being asymptomatic, SCE significantly impairs fertility and is the most prevalent uterine disorder in lactating dairy cows (Kaufmann et al. 2009). Reported prevalence ranges from 19% to 74% globally (Plontzke et al. 2010), and from 3–25% in India (Parmar et al. 2021). Various techniques such as uterine biopsy, endometrial cytology, uterine lavage sample optical density test (ULSOD), white side test, leucocyte esterase test, biochemical analysis (nitric oxide, malondialdehyde, total antioxidant capacity, total protein, albumin, glucose, cholesterol, triglycerides etc.) Doppler and B-mode ultrasonography, bacterial and fungal culture, measurement of acute phase proteins (Heptoglobin, Serum amyloid-A) and inflammatory markers (IL-6, 10 and TNF-α) have been employed for the diagnosis of SCE (Heidarpour et al. 2012; Vinita et al. 2018). Endometrial cytology using the cytobrush technique is considered the reference diagnostic method (Kasimanickam et al. 2005; Vinita et al. 2018), because of easy sample collection, reliable sample quality, rapid outcomes, and good repeatability of the test (Wagener et al. 2017). but its field application is limited by the requirement for technical expertise and laboratory processing The main limitations of cytology-based methods are observer bias and variation in cut-off values used to differentiate affected from unaffected animals at different days postpartum. The reported sensitivity and specificity of the cytobrush technique for detecting subclinical endometritis (SE) range from 17% to 86% and 42% to 94%, respectively (Wagener et al. 2017). Sensitivity denotes the probability that a truly SE-affected cow tests positive, whereas specificity reflects the probability that a truly unaffected cow tests negative. In dairy practice, low sensitivity leads to false negatives and reproductive losses due to missed treatment, while low specificity results in false positives and unnecessary therapy. Therefore, rapid and economical cow-side alternatives with sensitivity and specificity comparable to endometrial cytology are the need of the hour, and leukocyte esterase (LE) reagent strip tests have gained increasing attention in this regard (Asfar et al. 2021). LE strips detect neutrophil activity through colorimetric reactions and have long been used in human medicine for rapid diagnosis of inflammation in body fluids such as urine, peritoneal fluid, and pleural fluid (Azoulay et al. 2000). Leukocyte esterase (LE), pH, protein and nitrate tests were performed using reagent strips based on colorimetric reactions. The LE test detects esterase activity released from neutrophils. Leukocyte esterase hydrolyses an indoxyl ester substrate, producing indoxyl which reacts with a diazonium salt to form a purple azo dye. The pH test is based on the double indicator system consisting of methyl red and bromothymol blue, which change colour according to hydrogen ion concentration. The protein test utilizes the protein error principle of pH indicators, where tetrabromophenol blue changes colour in the presence of proteins, primarily albumin, at a constant acidic pH. The nitrate (nitrite) test is based on the Griess reaction, in which nitrite formed from bacterial reduction of nitrate reacts with p-arsanilic acid (sulfanilamide) and N-(1-naphthyl)ethylenediamine to produce a pink azo dye (Strasinger and Lorenzo 2014). More recently, they have been applied for detecting endometrial inflammation using cervico-vaginal mucus (Hajibemani et al. 2016; Asfar et al. 2021). Accordingly, the present study aimed to evaluate the efficacy of different diagnostic methods in comparison with endometrial cytology and to assess the association of biochemical parameters with subclinical endometritis in dairy cattle.

Material and Methods

Experimental Animals

The present study was conducted on fifty seven postpartum crossbred dairy cows presented for Artificial insemination at the Veterinary Clinical Complex (VCC), FVSc & AH, Shuhama Jummu and Kashmir, India and Mountain Livestock Research Institute (MLRI), Manasbal, SKUAST-K from August 2018 to June 2019. The laboratory work was carried out in the postgraduate laboratory of the division of Animal Reproduction, Gynaecology & obstetrics, FVSc & AH,

Shuhama. Fifthy Seven (n = 57) crossbred postpartum dairy cows exceeding 60 days in milk (DIM) presented for artificial insemination (AI) at Veterinary Clinical Complex and Mountain Livestock Research Institute were included in the study. Cows with normal health, disease free status and optimum body condition score score between 2.50 to 3.0 (1–5 scale) (Roche *et al.*, 2009) were selected. The animals with history of dystocia, retained placenta, foul-smelling drainage or purulent/abnormal vaginal discharge, reproductive tract abnormalities (e.g., ovarian/uterine adhesions) detected per rectally and those within 60 days postpartum were excluded from the study. The study was approved by the Animal Experiments Local Ethics Committee of SKUAST-K, and all procedures were conducted in accordance with established animal welfare standards

Diagnosis of subclinical endometritis (SCE)

Diagnosis of SCE in cows was done by the following methods. Endometrial Cytology (EC) as reference method using Cytobrush technique, White side Test (WST) of Cervico-vaginal mucus (CVM), different reagent strip tests of CVM. First the animals were screened for SCE by endometrial cytology (EC) and those found positive were further subjected to WST, reagent strip tests test.

Endometrial cytology (EC) using cytobrush technique as reference method

Endometrial cytology (EC) samples were obtained using a modified cytobrush assembly for large animals (Mayfair Surgical Corporation, India) comprising a cytobrush affixed to a steel inner stylet and outer catheter. The brush was secured by heat fixation, and metallic components were sterilized in a hot air oven. The assembly was covered with a sanitary sheath (IMV Technologies Pvt. Ltd., India) to prevent contamination. Following rectal evacuation and aseptic preparation of the perineal region, the guarded cytobrush was introduced trans-cervically into the uterine body under rectal guidance. The brush was exposed and rotated gently against the endometrium to obtain cellular material, then retracted and withdrawn. Smears were prepared immediately, fixed in methanol (Merck Specialities Pvt. Ltd., India) for 5–10 min, and stained with Giemsa (HIMEDIA Laboratories Pvt. Ltd., India). A total of 100–300 cells were evaluated under 40× magnification, and ≥ 4% polymorphonuclear cells (PMNs) was considered diagnostic for subclinical endometritis (Singh et al. 2016).

Collection of cervico-vaginal mucus (CVM)

Cervico-vaginal mucus (CVM) was aseptically collected on the day of estrus using a sterile artificial insemination (AI) sheath attached to a 20 mL syringe. The perineal region was washed, dried, and disinfected with 70% alcohol prior to sampling. With a lubricated gloved hand inserted per rectum to guide the sheath, CVM was aspirated from the external os or mid-cervix, transferred to a sterile test tube, and immediately transported to the laboratory. Samples were subjected to white side test (WST) and different reagent strip test (LE, pH, Protein and Nitrate strip tests) (Bhat et al. 2014).

White Side Test (WST)

For conducting WST, 1ml of CVM was heated with equal volume of 5% sodium hydroxide (Merck Specialities Pvt. Ltd., India) up to the boiling point. After cooling with the tap water, the intensity of color changes were studied and graded as normal (turbid or no colour), mild infection (light yellow colour), moderate infection (yellow colour) and severe infection (dark yellow colour) (Bhat et al., 2014).

Regent Strip Tests

For conducting different reagent strip tests, one ml of collected CVM was transferred into a sterile petridish and multi-reagent strip (Mission Urinalysis Reagent Strips, ACON Laboratories, Inc., U.S.A) was contacted with petridish for a period of 10 seconds. The results of leukocyte esterase strip test were then recorded based on the strip colour changes (according to the manufacturer recommendations) after a period of 10 minutes and change in colour was taken as positive (Hajibemani et al. 2016). pH, Protein and Nitrate strip test results of the CVM were recorded based on the colour change being observed after 60 seconds, according to the manufacturer's instructions, using the same urinalysis reagent strips (Asfar et al. 2021).

Blood Sampling and Biochemical Analysis

Blood (8 mL) was collected by jugular venipuncture in plain tubes at estrus before treatment (E) and at subsequent estrus before artificial insemination (SE) from SCE-positive animals. Animals negative on the White Side Test at SE were considered recovered. Accordingly, serum samples were categorized as pre-treatment (SCE positive; n = 27) and post-treatment (recovered based on WST negative; n = 27). After clotting, samples were centrifuged (3000 rpm, 10 min), and serum was stored at − 20°C until analysis. Serum nitric oxide (NO) was estimated by the modified Griess reaction (Sastry et al. 2002). Samples were reduced using copper–cadmium alloy, reacted with sulfanilamide and N-naphthylethylenediamine dihydrochloride, and absorbance was recorded at 545 nm using a Cytation-3 Imaging Reader (Biotek, USA). NO concentration was calculated from a potassium nitrate (KNO_3) standard curve. Serum malondialdehyde (MDA), an index of lipid peroxidation, was estimated by the thiobarbituric acid method (Draper and Hadley 1990). Absorbance was measured at 535 nm using a Shimadzu UV Spectrophotometer (Japan), and MDA concentration was calculated using the molar extinction coefficient ($1.56 \times 10^5\ M^{-1}\ cm^{-1}$).

Statistical analysis

Statistical analysis for evaluation of different diagnostic methods was performed using MedCalc statistical software (version 23.4.9; Ostend, Belgium). Data from endometrial cytology, WST, LE strip test, pH strip test, protein strip test, nitrate strip test and their combinations were analyzed using receiver operating characteristic (ROC) curve analysis and area under the curve (AUC) to assess diagnostic accuracy. AUC values ranged from 0.5 (no discrimination) to 1.0

(perfect discrimination). The optimal cut-off point was determined as the value with the highest Youden Index (Se + Sp-1). Agreement between the diagnostic test and the gold standard was assessed using Cohen's kappa coefficient. Kappa values were interpreted as follows: <0.21 poor agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 good agreement, and 0.81–1.00 very good agreement. A post-hoc power analysis was performed using G*Power 3.1. Based on an observed distribution of 82.5% positive and 17.5% negative cases in a sample of 57 animals, the study achieved a statistical power of 0.998 at an alpha level of 0.05, with a large effect size (w = 0.65). NO and MDA levels were analyzed using the paired t test in IBM SPSS statistical software (version 27.0.1.0). Differences were considered statistically significant at $P < 0.05$ (Snedecor and Cochran 1994).

Results

Endometrial cytology

EC using CB technique was done in 57 cows by counting 300 cells (endometrial cells and PMNs) and ≥4% PMNs was taken as cut-off for positive diagnosis. Based on EC, 47 cows were positive (≥ 4% PMNs) and ten cows were negative (< 4% PMNs). EC by CB technique was used as reference diagnostic method to evaluate the diagnostic accuracy of other tests.

White Side Test

Out of screened crossbred cows (n=57), 38 (66.67%) tested positive, while 19 (33.33%) were negative. Among the WST positive samples, yellow coloration indicating moderate infection was most frequent 16 (42.11%), followed by light yellow 14 (36.84%) and dark yellow reactions 8 (21.05%). Area under the ROC curve (AUC) was 0.767 with P value less than 0.001 with Youden index of 0.44. Based on ROC analysis, the optimal cut-off point selected was > 0 (negative) to be used for positive diagnosis of SCE. At the optimal cut-off point, WST showed Se of 74.47%, Sp of 70% and accuracy of 73.6% with k= 0.33(fair agreement). (Table 1; Fig. 1)

Leukocyte Esterase Strip Test (LEST)

LE test results were recorded in five categories *viz.* - (negative), ± (15 Leu/µl), + (70 Leu/µl), ++ (125 Leu/µl) and +++ (500 Leu/µl) indicating nil, trace, small, moderate and large number of leucocytes, respectively. Area under the ROC curve (AUC) was 0.899 with P value less than 0.001 with Youden index of 0.69(good/substantial). Based on ROC analysis, the optimal cut-off point selected was greater than > 1(15 Leu/µl (±) to be used for positive diagnosis of SCE. At the optimal cut-off point, LE test showed Se of 78.72% and Sp of 90% with k= 0.50(moderate agreement) (Table 2; Fig. 2).

pH Strip Test

pH strip test results were recorded under seven categories *viz.* 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. Mean pH of CVM was found to be 7.84 ± 0.77. Area under the ROC curve (AUC) was 0.810 with P value less than 0.001 with Youden index of 0.65 (good/substantial). Based on ROC analysis, the optimal cut-off point selected was greater than > 7 to be used for positive diagnosis of SCE. At the optimal cut-off point, LE test showed Se of 85.11% and Sp of 80% with k=0.544 (moderate agreement) (Table 3; Fig. 3).

Protein Strip Test

Protein strip test results were recorded in five categories *viz.* - (negative), ± (15mg/dl), + (30 mg/dl), ++ (100 mg/dl) and +++ (300 mg/dl). Area under the ROC curve (AUC) was 0.713 with P value of 0.002 with Youden index of 0.4(fair/moderate). Based on ROC analysis, the optimal cut-off point selected was greater than > 0(negative) to be used for positive diagnosis of SCE. At the optimal cut-off point, LE test showed Se of 59.57% with Sp 80% with k=0.24 (fair agreement) (Table 4; Fig. 4).

Nitrate Strip Test

+Nitrate strip test results were recorded in two categories *viz.* Negative (-) and + (different degrees of pink color changes based on manufacturer's recommendations). Area under the ROC curve (AUC) was 0.520 with P value of 0.810 and Youden index of 0.04(poor). Based on ROC analysis, the optimal cut-off point selected was greater than >0 to be used for positive diagnosis of SCE. At the optimal cut-off point, Nitrate test showed Se of 34.04% and Sp 70% with k=0.19 (poor agreement) (Table 5; Fig. 5).

Combination of Strip Tests

The results of sensitivity (Se) and Specificity (Sp) for combination of strip tests are depicted in (Table 6; Fig. 7-10) and Combination of LE test (cut-off value >±) and pH (cut-off value >7) showed Se of 70.21% and Sp of 100% with k=0.45(moderate agreement) and Youden index of 0.70(Good/Substantial). Similarly LE test and protein when used in combination with cut-off value > ± for LE test and > 0(-) for protein, showed Se of 55.32% and Sp of 90% in comparison with EC with k= 0.25(fair agreement) with Youden index of 0.45 (Fair). When all the three reagent strip test results *i.e.,* LE, protein and pH were used in combination using > ± for LE, > 0(-) for protein and > 7 for pH as cut-off values, showed Se of 48.94% and Sp of 100% in comparison with EC with k= 0.25(fair agreement) with Youden index of 0.48(fair) (Table 7). In SCE cows (n = 27), the mean serum nitric oxide level was significantly ($P < 0.05$) higher before treatment (46.76 ± 4.55 µM) compared to after treatment (10.72 ± 1.14 µM). Similarly, malondialdehyde concentration was significantly ($P < 0.05$) elevated before treatment (69.83 ± 7.18 nmol/ml) and declined following treatment (30.84 ± 4.42 nmol/ml).

Discussion

The study investigated the diagnostic performance of different reagent strip tests and conventional white side test against endometrial cytology in dairy cows. Endometrial cytology is a reliable and widely used method for diagnosing subclinical endometritis, based on increased polymorphonuclear cells (PMNCs) during uterine inflammation (Dubuc et al. 2010). It is semi-invasive and cost-effective, performed using Cytobrush (CB), Cytotape (CT) or Low Volume Uterine Lavage (LVUL). The cytobrush technique is suitable around artificial insemination (AI) and offers good accuracy and high specificity, however, it is not a cow-side test and is associated with high red blood cell contamination, low representative sampling area (1.5–2.5 cm^2), low PMN collection, moderate cellular detail with cell distortion, reduced sensitivity due to limited area and the need for skilled personnel. Therefore, rapid, non-invasive and easily applicable alternative cow-side diagnostic tests are required for early diagnosis of SCE. Leukocyte esterase (LE) in particular along with pH strip testing, has been recognized as a rapid, non-invasive and practical cow-side diagnostic approach for detecting subclinical endometritis. Recent studies have also evaluated leukocyte esterase (LE) urinalysis strips as practical cow-side tests for subclinical endometritis using cytobrush technique. In the present study, the Leukocyte Esterase Strip Test (LEST) demonstrated a sensitivity of 78.72% and specificity of 90%, indicating a good diagnostic balance with high accuracy (80.70%) in identifying non-affected animals under field conditions.

The findings of the present study are in consistent with earlier studies (Santos et al. 2006; Dodiya et al. 2024). However, some studies have reported comparable Se, but lower Sp ranging from 74%% to 30.8% (Cheong et al. 2012; Hajibemani et al. 2016; Sajidi et al. 2025; Sood et al. 2025). Similarly, Van Syndel et al. (2018) and Asfar et al. (2021) have documented comparable Se, but lower Sp (65% and 66.67%) respectively. The differences in specificity in results with Cheong et al. (2012) from the current study may be due to the use of a different reagent strip (Multistix 10 SG; Bayer Health Care L.L.C., Elkhart, IN, USA), higher cut-off values for endometrial cytology (≥ 10% PMN) and the LE test (≥ ++), uterine lavage technique and cows between 40 and 60 DIM. The differences with Sajidi et al. (2025) may be due to use of modified LE tape technique where in LE tape was affixed onto the cytobrush rod head using a hot adhesive glue compared to use of direct contact of CVM with the LE strips in present study. While as others employed uterine lavage sampling technique and a lower EC cut-off (≥ 2% PMN) (Sood et al. 2025). The lower specificity reported by Van Syndel et al. (2018) compared to our study may be attributed to indirect LE testing using Multistix 10 SG strips dipped in cytobrush saline suspension prepared with 40 mL of 0.9% NaCl physiological saline, sampling between 29 and 35 DIM with a > 5% PMN cut-off, and color interpretation after 2 minutes. In contrast, Arango-Sabogal et al. (2019) observed markedly lower sensitivity (42.6%) despite having same specificity (90%).The lower sensitivity may be due to indirect LE assessment using 0.9% saline rather than direct mucus contact as done in present study. Similarly, Bartolome et al. (2025) reported 100% sensitivity with lower specificity (58.33%) in cows with clinical endometritis, be attributed to the use of a different LE strip (Combur10 Test®) and a higher cytological cut-off (≥ 5% PMN) compared with the ≥ 4% PMN criterion used in the current study.

Examination of cervico-vaginal mucus for appearance, consistency and pH is useful for identifying subclinical endometritis (SCE) as the condition alters its physico-chemical properties. In the present study, the pH strip showed satisfactory diagnostic performance for SCE detection, with a sensitivity of 85.11% and specificity of 80.0% at a ROC derived cutoff of > 7. The mean CVM pH was recorded 7.84 ± 0.77, indicating an alkaline environment associated with SCE and demonstrating a good discrimination between affected and non-affected animals under field conditions. The results of Se observed in present study are consistent with those reported by Bartolome et al. (2025) who recorded a Se of 82.61%. The results of Sp 78.4% and 77.8% as reported by others (Cheong et al. 2012; Asfar et al. 2021), respectively are also in agreement with the current study. Elevated CVM or uterine fluid pH in SCE has been consistently reported in the previous studies. Bhat et al. (2015) and Kumar et al. (2017) documented higher pH values (8.19 ± 0.06 and 7.95 ± 0.096) of CVM.

In repeat breeding cows, CVM pH ranged from 6.5 to 9.0 with a mean of 7.95 as reported by Kumar et al. (2017) while Kumar et al. (2014) reported narrower range of pH values between7.5–8.0. Previous studies also reported higher pH values with proposed diagnostic cutoffs of ≥ 7.0 (Cheong et al. 2012; Bedewy and Rahaway (2019); Raval *et al.*, 2018; Parikh et al., 2022). Further, Barakat et al. (2020) also reported that pH values of CVM ranged between 8.53–8.57 before treatment, supporting the diagnostic relevance of CVM pH measurement using pH strip test in cattle.The difference in Sp results with those of Bartolome et al. (2025) may be due to direct assessment of vaginal discharge rather than CVM, inclusion of clinical endometritis cases, use of a different LE strip (Combur10 Test®) and a higher cytological cutoff value (≥ 5% PMN) in their study. The differences in Se with those of Cheong et al. (2012) may be attributed to use of a different reagent strip (Multistix 10 SG), a higher EC threshold (≥ 10% PMN), and uterine lavage sampling technique, whereas with those of Asfar et al. (2021) may be due to comparatively larger sample size compared to our study. Contrarly to others, Sood et al. (2025) reported lower sensitivity (64.7%) and specificity (57.7%) of pH strip test possibly due to use of different pH strip, uterine lavage technique and a lower EC cutoff (≥ 2% PMN) in their study. Elevated CVM pH in cows with SCE could be due to bacterial contamination and bacterial toxins in uterine fluids, which creates an unfavorable uterine environment for spermatozoa and embryo survival and disrupts endometrial endocrine function and luteolysis of the ovarian corpus luteum (Sheldon et al. 2006; Kumar *et al.*, 2014).

In the present study, protein estimation in CVM using reagent strip testing demonstrated a sensitivity of 59.57% and specificity of 80%, indicating its poor diagnostic accuracy (63.16%) compared to cytobrush, WST, LEST and pH. These findings are in agreement with earlier studies showing considerable variability in the diagnostic performance of protein reagent strip tests for SCE. Cheong et al. (2012) documented a sensitivity of 58.3% and specificity of 55.8% while as Asfar et al. (2021) reported a sensitivity and specificity of 53.3% and 66.7%, respectively. However, Sood et al. (2025) observed lower sensitivity (41.2%) but similar specificity (76.9%) to the current study. The above studies indicate an overall weak association between protein concentration and SCE. In contrast, Ricci et al. (2017) reported a markedly higher sensitivity (100%) but extremely low specificity (12.5%), reflecting a high rate of false-positive results. These contrasting findings may be attributed to several methodological differences including uterine lavage technique, using different strips (Combur Test®), evaluation at different postpartum periods (30 and 60 days in milk) and the application of variable PMN cut-off values (6.5% at 30 DIM and 2.5% at 60 DIM) compared to the ≥ 4% cut-off used in the present study. Increased total protein concentration in uterine fluid in SCE is due to inflammatory exudation and fluid accumulation, however despite higher protein levels in affected cows, protein estimation alone has demonstrated poor diagnostic reliability (Cheong et al. 2012; Hajibemani et al. 2016).

The limited performance of reagent strip tests can be explained by their high sensitivity to albumin and relatively low sensitivity to other proteins like hemoglobin, globulin and mucoprotein, resulting in false-positive results in alkaline samples (pH > 9) and false-negative results in samples with high specific

gravity (Sacks 2001). Hajibemani et al. (2016) further emphasized that although protein concentrations were increased in endometritis, but its assessment could not be recommended as a diagnostic tool for evaluation of CVM. In addition, Albumin, a negative acute-phase protein, decreases during inflammatory and metabolic stress conditions and has been reported to be lower in cows affected with SCE (Al-Albsy et al. 2023). Collectively, these findings indicate that while reagent strip based protein estimation may reflect uterine inflammatory changes, methodological variability and inconsistent diagnostic performance limit its usefulness as a stand-alone diagnostic tool for SCE. Instead systemic biomarkers such as serum albumin may provide more reliable insight into disease susceptibility.

The nitrite strip test has been used as a screening method for possible infections caused by nitrate reducing bacteria such as *E. coli* and *Trueperella pyogenes* (Hajibemani and Mirzaei 2022). These bacteria are the most important pathogens involved in the subclinical endometritis of cows. Based on ROC analysis, at optimal cut-off value of > 0, the nitrate strip test demonstrated a sensitivity of 34.04% and specificity of 70% which was lowest as compared to other reagent strip test like LE, Protein and pH, suggesting its limited diagnostic utility for detecting SCE under field conditions. The lower sensitivity of the nitrate strip test may be attributed to a higher rate of false negative results, as some non-nitrate reducing bacteria may be present in the CVM of cows with subclinical endometritis. Although nitrate test was significantly higher ($P < 0.05$) before treatment in the unresponsive cows than the responsive cows but had no significant ($P > 0.05$) differences after treatment (Hajibemani and Mirzaei 2022). There were non-significant differences ($P > 0.05$) in nitrate strip test using vaginal fornix discharge in SCE cows compared with those in healthy cows (Hajibemani et al. 2016).

In the present study, the LE and pH combination showed the best diagnostic performance with a sensitivity of 70.21%, specificity of 100% and accuracy of 75.44% comparable to those of Bartolome et al. (2025), who observed Se (65.22%) and Sp (87.5%) with an accuracy of 76.6%. In contrast, other studies reported very low sensitivity (18.6%) but similar specificity with current study (96.8% *vs*. 100%) for LE and pH combination (Cheong et al. 2012). Similarly, Asfar et al. (2021) reported (Se 60%, Sp 77.78%) of LE and pH combination in the detection of SCE. The LE and protein combination in the present study showed decreased sensitivity (55.32%) and specificity (90%) comparable to that of Asfar et al. (2021) in Se (46.67%) but not in Sp (77.78%). When LE, pH, and protein were combined, sensitivity decreased which was in close agreement to those of Asfar et al. (2021) in Se (33.3% *vs.* 48.94%) but with high specificity (77.8% *vs.* 100%). The findings indicate that combining parameters, particularly LE with pH, enhances diagnostic performance, while inclusion of additional parameters increases specificity with decrease in sensitivity.

In the current study, the diagnostic efficacy of the WST for the detection of SCE, when compared with endometrial cytology (EC) as the reference standard, revealed a sensitivity of 74.47% with a specificity of 70%. Previous studies have reported WST positivity ranging from 71.25–77.5% in repeat breeding cows which is comparable to the present study (Raja *et al.*, 2012; Bhat et al. 2014; Parikh, 2022). In contrast, Sood et al. (2025) reported a markedly lower sensitivity (29.4%) with a high specificity (92.3%), suggesting that although WST was effective in minimizing false-positive results, it failed to detect a substantial proportion of truly infected animals. The higher specificity in the said study may be attributed to the use of a stricter cut-off value (≥ 2) for WST interpretation, corresponding to a yellow or darker colour reaction. Additionally, the diagnostic accuracy reported in their study was relatively low (56.7%) compared to our study (73.68%), wherein optimal cut-off value of > 0 (light yellow /mild or higher), was employed, thereby improving the detection of infection. Contrary to our study, Asfar et al. (2021) reported lower sensitivity (63.3%) and specificity (55.6%) values. These differences may be attributed to variation in sample size, being more in their study compared to the present study. Similarly, Dodiya et al. (2024) reported a sensitivity of 50% with 100% specificity for WST, however, their diagnostic criteria differed substantially from this study. In their study, sensitivity was defined as the percentage of cows positive to endometritis by WST. However, current study used EC as reference method for detecting Se and Sp of WST. In addition, their study was conducted on cows with clinical endomeritis.

The results of the present study showed significantly ($P < 0.05$) higher serum nitric oxide (NO) and malondialdehyde (MDA) levels in SCE cows before treatment, which declined markedly after recovery. The elevated NO levels are in accordance with earlier reports (Li et al. 2010; Janowski et al. 2013; Kaya et al., 2017; El-Sayed et al. 2024), indicating increased inflammatory response and reactive nitrogen intermediate production during uterine infection, although Islam and Kumar 2015 reported non-significant differences. Similarly, higher MDA concentrations before treatment reflect enhanced lipid peroxidation and oxidative stress, as reported by (Heidarpour et al. 2012; Kaya et al. 2017; El-Sayed et al. 2024). The post-treatment decline in both parameters suggests reduction in inflammation, oxidative stress and restoration of uterine health (Sheldon et al. 2009; Kizil et al. 2010).

Conclusions

In conclusion, individually, WST (>0) showed moderate sensitivity and specificity, while LE >1(±/15 Leu/µl) and pH (>7) demonstrated a better balance of sensitivity and specificity. In contrast, protein and nitrate tests had lower sensitivity. Among combined tests, LE + pH, LE + protein and LE + pH + protein achieved 100% specificity but with reduced sensitivity compared to individual tests. Thus, higher TP and TN with minimal FP and FN improved sensitivity and specificity, whereas increased FN mainly reduced sensitivity in combined tests. Overall, LE and pH strip tests, either alone or in combination, can serve as convenient, non-invasive, point-of-care and affordable diagnostic tools for detecting subclinical endometritis under field conditions when evaluated against endometrial cytology using the CB technique.Elevated serum levels of biochemical parameters (NO & MDA) may be associated with subclinical endometritis i

In summary, reliable cow-side diagnostic methods can support early identification and timely management of subclinical endometritis (SCE), ultimately improving reproductive outcomes and economic returns in dairy herds. While LE and pH strip tests demonstrate field applicability, their diagnostic performance can be further strengthened through technical refinements. Since these strips are not originally developed for cervico-vaginal mucus evaluation, targeted modifications are essential to enhance their specificity and overall validity for SCE detection. Future large-scale studies across varied management systems, along with improved and optimized strip formulations, may help establish these

Declarations

Data Availability Statement:

The data will be made available on a reasonable request.

Acknowledgements

The authors would like to thank Incharge Mountain Livestock Research Institute and Division of Veterinary Clinical Complex, FVSc & AH, Shuhama, Srinagar for providing animals and the facilities of their divisions for this study.

Compliance with ethical standards

Statement of animal rights

Animal rights were considered in this study and ethical guidelines of institutional animal ethics committee were followed for conducting the experiment.

Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Tables 1 to 7 are available in the Supplementary Files section.

Figures

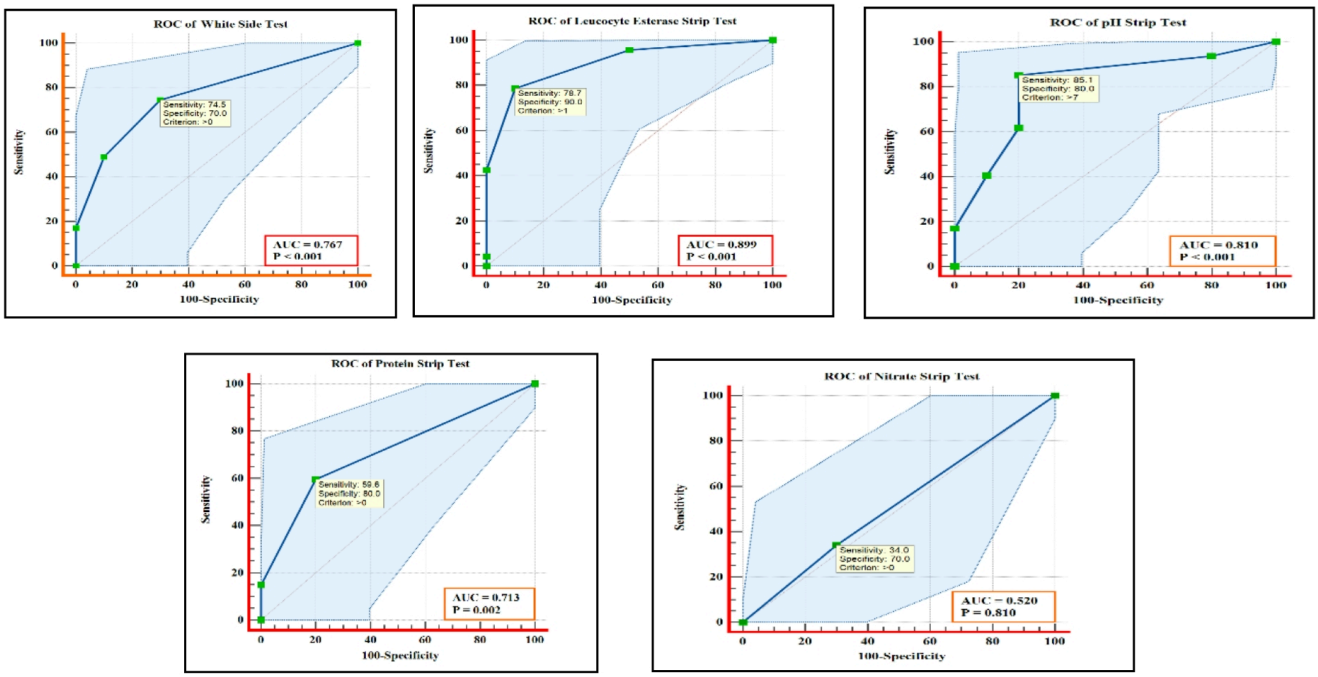


Figure 1

Fig.1 to 5: ROC outcome of WST, LE, pH, Protein, Nitrate strip tests

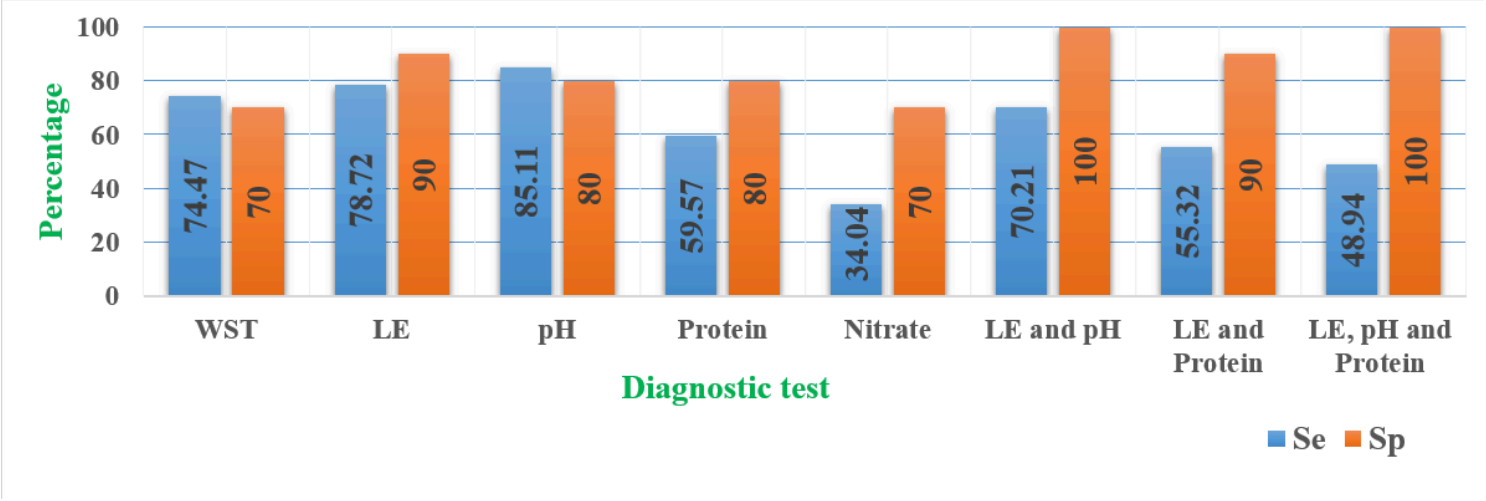


Figure 2

Fig. 6: Graphical representation of sensitivity (Se) and specificity (Sp) of different diagnostic tests in comparison to Endometrial Cytology

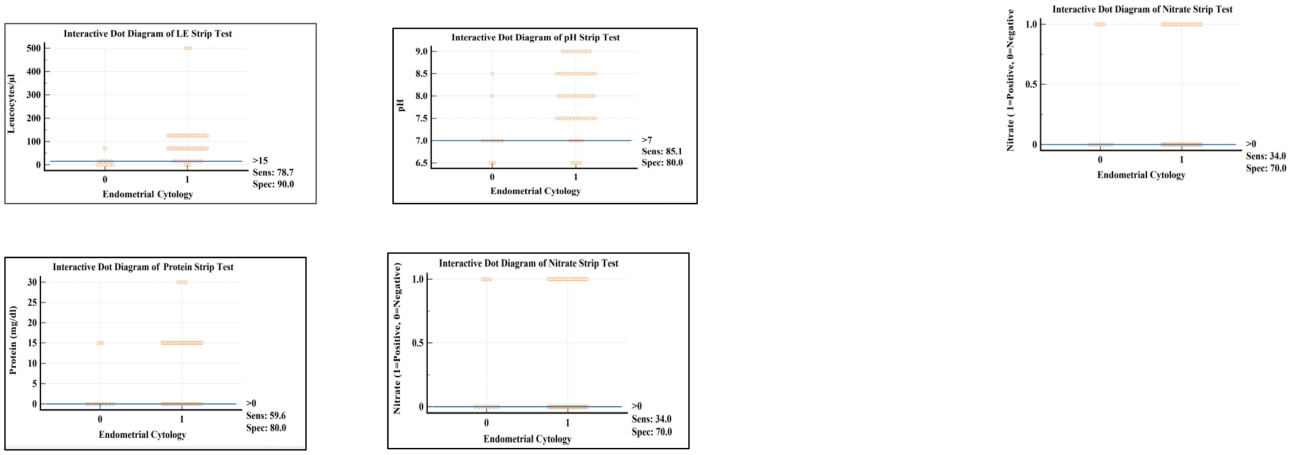


Fig. 7-10: Interactive dot diagrams of different reagent strip tests

Figure 3

Fig. 7-10: Interactive dot diagrams of different reagent strip tests (LE, pH, Prptein and Nitrate)

Supplementary Files

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- Tables.docx