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Short Paper

Rapid leukocyte esterase detection of cytological endometritis in dairy cows

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Abstract

Background: Certain limitations are associated with all kinds of diagnostic techniques used for the identification of bovine cytological endometritis under field conditions. Leukocyte esterase (LE) tape of urinary test strips has been used to detect cytological endometritis with an acceptable level of agreement with endometrial cytology in lactating dairy cows; however, the sampling procedure of LE tape strips is still time-consuming and not advisable for use at the farm level. **Aims:** The present study was designed to provide a simpler and faster LE test to improve the field applicability of LE tape strips for the detection of bovine cytological endometritis. **Methods:** Endometrial samples were collected using cytobrush and modified LE tape techniques from slaughtered (n=74) and live dairy cows (n=43). The urinary strip's LE tape was affixed onto the cytobrush rod head using a hot glue adhesive. This modification created the possibility of rapid reading of the intensity of color change of LE tapes. **Results:** Considering the cytology as the reference method, the sensitivity and specificity of the LE method for slaughterhouse samples were 82% and 73%, respectively. The agreement between endometrial cytology and the modified LE technique, considering the combination of slaughterhouse and clinical samples, was found to be moderate (κ=0.51; P<0.0001) in the present study. **Conclusion:** A rapid leukocyte esterase test can be used as a cow side and potential alternative to endometrial cytology; however, the efficacy of this method should further be evaluated based on the conception rates in dairy cows.

Key words: Cytobrush, Cytological endometritis, Dairy cows, Leukocyte esterase

Introduction

In many instances, subclinical endometritis (SE) or cytological endometritis is the main carryover effect of postpartum uterine infections in lactating dairy cows (Ribeiro *et al*., 2016; Horlock *et al*., 2020). Cows with a calving problem or one or two reproductive tract diseases including metritis or clinical endometritis did show more pregnancy loss and lower fertility after first pregnancy diagnosis at 30 d and second diagnosis of gestation (Ribeiro *et al*., 2013; Pinedo *et al*., 2020; Bruinjé *et al*., 2024). In herds where the prevalence of SE is high, the potential economic losses could be significant mainly due to a higher return to estrus, an increase culling rate (Cheong *et al*., 2011; Paiano *et al*., 2023). Different methods including uterine histopathology, ultra-sonography, and cytology have been applied to detect SE in dairy cows (Bogado Pascottini, 2016; Wagener *et al*., 2017; Van Schyndel *et al*., 2018). Presently, uterine histopathology is considered the gold standard method for diagnosis of SE; however, its routine usage at the farm level is questionable (Bogado Pascottini, 2016). In general, there are certain drawbacks associated with all of the aforementioned techniques, including uterine cytology. These limitations encompass factors like expenses, the amount of time required, and the potential for inaccuracies (Bogado Pascottini, 2016). Further, most of these methods are not easy to use under field conditions. Santos (2006) was the first to use leukocyte esterase (LE) tape on urinary test strips to detect SE in lactating dairy cows. Leukocyte esterase is an enzyme found in neutrophils that could react with agents within LE tapes and produce a color change. Therefore, a positive reaction could indicate the presence of neutrophils (Kutter *et al*., 1987). Denis-Robichaud and Dubuc (2013 and 2015) also reported an acceptable level of agreement between uterine cytology and LE and then suggested it as an alternative to endometrial cytology in field conditions. Although the LE test is a method that can be applied in the field (Denis-Robichaud and Dubuc,

2013; Asfar *et al*., 2021), the sampling procedures remain time-consuming and impractical for farm-level use due to the requirement for uterine flushing or collecting the uterine mucus. Therefore, the following study was designed to provide a simpler and faster LE test to improve the field applicability of this technique for the detection of SE in dairy cows.

Materials and Methods

Ethical approval

This study was approved by the Committee of Research and Law for Animal Experiments at the School of Veterinary Medicine, Shiraz University (approval No.: 1GCB2M143807).

Study design and sample collection

CombiScreen® 11SYS PLUS (Analyticon Biotechnologies AG, Muehlenberg, Lichtenfels, Germany) commercial urinary test strip was employed in this study. The head of a cytobrush was cut with scissors and the rest of it was used as a probe. Subsequently, the urinary strip's LE tape was cautiously removed and affixed onto the probe using a hot glue adhesive (Fig. 1). Immediately after preparation, the probes were placed in the original containers, along with desiccant packs, to minimize humidity exposure during transport and storage. We initially performed this experiment using reproductive tracts collected from the abattoir and in the later stage on live dairy cows. A total number of 74 Holstein female reproductive tracts were collected between March 2023 and July 2023 from Shiraz slaughterhouse, Shiraz, Iran. The uteri were all non-pregnant from dairy cows and previously calved at least once, as evidenced by the presence of corpora albicantia. The uteri used were all in normal size and completely involuted (Noakes, 2019). All samplings were conducted at the slaughterhouse within 1 h after the collection of uteri specimens. Initially, the surface of the uterus was cleansed using a paper towel, followed by a precise incision made at the uteri bifurcation with a sharp and clean scalpel. While the opening of one uterine horn was held open with a forceps. An LE probe was carefully inserted into the initial portion of that horn (2-3 cm) when it was in contact with the endometrial surface, rotated one turn clockwise to be well weltered with superficial discharge. The intensity of color change was documented within 1, 3, and 5 min and results were classified into five groups. The color change of the probe was classified into five groups. These groups were categorized as follows: 0 (no color change), 0.5+ (trace), 1+, 2+, and 3+. A color change ≥1+ was considered a positive LE test. Following the retraction of the LE probe, a clean cytobrush was promptly introduced into the same horn close to the same place where the LE sample had been obtained. The cytobrush was rotated one turn clockwise to obtain cellular materials (Paiano *et al*., 2022). Subsequently, it was rolled onto a clean glass microscopic slide and then air-dried. Slides were then fixed using methanol and stained with Giemsa.

Fig. 1: Preparation of the LE probe for uterine sampling. (**a**) The urinary strip's LE tape was affixed onto the cytobrush probe head using a hot glue adhesive, (**b**) The LE probe was mounted on a stainless-steel rod (length of 65 cm, diameter of 4 mm) and placed in a uterine infusion pipette, and (**c**) The whole instrument was inserted into a plastic guard to prevent vaginal contamination

Next, uterine samplings were carried out on 43 live Holstein cows between 25-35 days postpartum from one dairy herd from August to September 2023, in Shiraz. The cows were selected based on the absence of abnormal discharge on the vulvar lips after massaging the uterine horns. The LE probe was mounted on a stainless-steel rod (length of 65 cm, diameter of 4 mm) and placed in a uterine infusion pipette (length of 50 cm, diameter of 6 mm) for passage through the cervix. The whole instrument was inserted into a plastic guard to prevent vaginal contamination (Fig. 1). The probe with a protective sheath was guided through the vagina to the external cervical os, the plastic sheath was punctured and the infusion pipette advanced further. The probe was brought out to be weltered with the superficial discharge of the initial portion of one uterine horn. Finally, the probe was retracted back into the infusion pipette and the whole instrument was removed from the reproductive tract. Results were recorded and categorized as previously described for slaughterhouse samples. To obtain cytological samples, instead of an LE probe, a cytobrush was mounted and the entire procedure was conducted similar to LE sampling. The slide preparation process also was done like what had been done for slaughterhouse samples. Following slide staining, a clinical pathologist evaluated all of samples. The proportion of neutrophils was determined by considering both neutrophils and endometrial cells, with a total count of 300 cells at a magnification of ×1000. Data were analyzed using SPSS Statistics® 27.0 (version 27.0 released 2020, SPSS Inc., Chicago, IL, USA). To assess the LE test as a diagnostic method for identifying SE, the results obtained were dichotomized; an LE value ≥1+ within 3 min was considered positive while any remaining values were deemed negative. With regarding cytology as the reference method, a 2 × 2 square was

created to compare LE results against cytology results, and the agreement and reliability between the two methods were determined using kappa statistics. The cutoff point for cytology was determined as neutrophils ≥3% in slaughterhouse samples (Diaz-Lundahl *et al*., 2022) and neutrophils ≥8% in field samples (Barlund *et al*., 2008). The ≥3% cutoff point for cytology in slaughterhouse samples was regarded as SE because the uterine samples were obtained from cows that were more than 50 days postpartum, as determined by the size and appearance of the uteri samples (Sheldon, 2019).

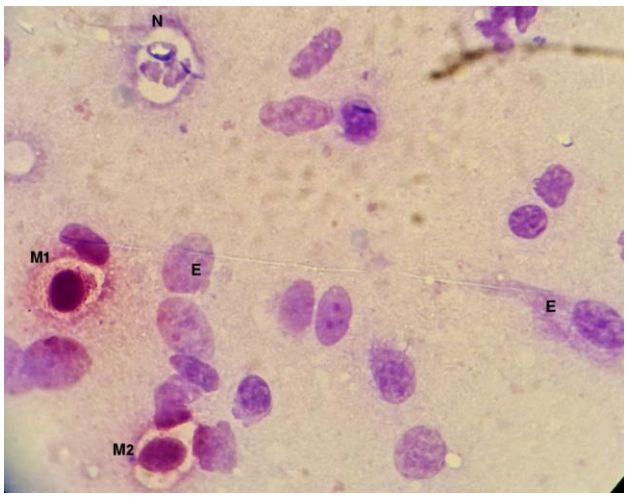

Fig. 2: Endometrial cytology sample stained with Giemsa (×1000). M1: Mast cell, M2: Poorly granulated mast cell, N: Neutrophil, and E: Epithelial cell

Results

Out of 74 reproductive tracts collected from the abattoir, 32 (43.2%) uteri tested were positive for LE. Endometrial cytology showed that 29.7% (22 out of 74) of the samples were detected as positive (Table 1). Considering the cytology as the reference method, the sensitivity and specificity of the LE method for slaughterhouse samples were 82% and 73%, respectively. The positive predictive value was 0.56 and the negative predictive value was 0.91. The agreement between the two methods was moderate (κ=0.49; P<0.0001) (Table 2). In this study, we considered the presence of mast cells as an indicator of inflammation owing to their extensive presence and the point that they contain leukocyte esterase enzyme (Fig. 2). Due to contamination of the esterase test pad with abundant red blood cells, three samples were excluded from the study to prevent color interference of LE test with severe red blood cell contamination. Then, the results of 40 cows were recorded in which 19 uteine samples tested positive for LE (47.5%). Sixteen samples tested positive for cytology (40.0%) in which the percentage of neutrophils ranged between 10% to 90% and only in one sample percentage of mast cells was higher than 3% (Table 1). When compared to cytology, the LE method had a sensitivity of 81% and specificity of 75% for field samples. Positive predictive and negative predictive values were 0.68 and 0.86, respectively. The agreement between the two methods was moderate (κ=0.55; P<0.0001) (Table 2).

Discussion

The results of the present study showed that using endometrial cytology, the prevalence of SE in uterine samples of the abattoir and live cows were 29.7% and 40.0%, while with the LE test (cutoff point; LE ≥1+) were 43.2% and 47.5%, respectively. The difference in the prevalence of SE using these two techniques can be attributed to the occurrence of neutrophil lysis in cytology samples while LE could still be present in the samples, causing positive LE results. Similarly, it has been observed that positive results in studying cellular elements in the urine samples may occur due to cell lysis caused by high pH and low specific gravity (Kutter *et al*., 1987). In addition, the absence of purulent discharges in the uteri samples could be another reason for detecting a lower SE by the cytology in the present study.

Using the LE test, Cheong *et al*. (2012) detected cytologic endometritis in postpartum dairy cows which was strongly associated with the results obtained using cytobrush. Cows detected with SE using the LE test had lower fertility after the first artificial insemination (Cheong *et al*., 2012). Denis-Robichaud and Dubuc (2015) reported a prevalence of 48.4% SE but Cheong *et*

Table 1: Number of samples and percentage of cows with endometritis diagnosed by each method (cytology and LE)

Sample		Cytology test	
		Positive (%)	Negative (%)
LE test	Slaughterhouse (n=74)		
	Positive (%)	18 (82)	14 (27)
	Negative (%)	4 (18)	38 (73)
	Clinical (n=40)		
	Positive (%)	13 (81)	6 (25)
	Negative (%)	3 (19)	18 (75)
	Total (n=114)		
	Positive (%)	31 (81.6)	20 (26.3)
	Negative (%)	7 (18.4)	56 (73.7)

Table 2: Leukocyte esterase (LE) test characteristics including sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), and the agreement between LE test and endometrial cytology

Samples	Se (95% CI)	Sp (95% CI)	PPV (95% CI)	NPV (95% CI)	Kappa (P-value)
Slaughterhouse	82 (66-98)	73 (61-85)	56 (39-73)	90 (81-99)	0.485 (P<0.0001)
Clinical	81 (62-100)	75 (58-92)	68 (47-89)	86 (71-100)	0.545 (P<0.0001)
Total	81.5 (69.5-93.5)	74 (64-84)	61 (47-74)	88 (80-96)	0.51 (P<0.0001)

al. (2012) considering LE ≥2+ as a positive result, reported a higher prevalence of 56% SE. Different sampling procedures and definitions could be the possible explanations. Our study lacks the assessment of the association between the rapid LE results and with reproductive performance of SE dairy cows.

In a study conducted by Denis-Robichaud and Dubuc (2013), a method for performing the LE test was employed that differed from our approach. Denis-Robichaud and Dubuc (2013) collected uterine samples using a cytobrush and then rolled the samples onto a microscope slide for cytological evaluation. At the same time, the cytobrush was immersed in 1 ml of sterile water to facilitate the LE colorimetric testing. A drop of this solution was subsequently placed on an esterase strip to observe the reaction. In contrast, in our study, both tests were performed separately, allowing for a more accurate comparison between the two methodologies. Furthermore, our method exhibited a higher speed, as one additional step was eliminated from the procedure. It was also decided that the endometrial sample would not be mixed with sterile water, thereby preserving the integrity of the sample and potentially enhancing the accuracy of the results. This distinction in the experimental design may provide valuable insights into the efficacy and reliability of the LE test when applied to uterine samples.

The sensitivity of the rapid LE test in the present study (82%) were relatively similar to that of Santos (2006) (sensitivity=83%). While, the specificity of our study (73%) is considerably lower than that reported in this reference (94%). Potential reasons for the observed lower specificity could be the differences in the experimental design, the manufacturer company and batch to batch variations of the LE strips. Also, we used a modified method with different way of sampling. Further, the cows used for uterine sampling were in different clinical conditions. Other researchers with either similar or different cutoff points from LE ≥1+ to LE ≥2+ observed different sensitivity and specificity (Couto *et al*., 2013; Denis-Robichaud and Dubuc, 2015). In the study conducted by Arango-sabogal *et al*. (2019), with the cutoff of LE ≥2+, a decrease in sensitivity but an increase in specificity was observed (Arango-Sabogal *et al*., 2019). The agreement between the two methods in the present study, considering the combination of slaughterhouse and clinical samples, was found to be moderate (κ=0.51). Previous studies similarly documented moderate agreement between the LE test and endometrial cytology ($0.6 \geq \kappa \leq 0.41$). Santos (2006) and Denis-Robichaud and Dubuc (2015) reported kappa values of 0.62 and 0.43, respectively. One important finding of the present study was the presence of mast cells either in abattoir or live cows' endometrial samples. Out of 22 samples from the cows with SE, 12 (54.5%) samples had neutrophils as well as mast cells greater than 3%. Mast cells are detected in normal and abnormal healing processes and in particular, with increased numbers in abnormal healing environments (Monument *et al*., 2013; Hart, 2015). These cells, with their diverse roles, also contribute to chronic inflammation; whether by attracting cells such as macrophages and T lymphocytes that represent chronic inflammation or by releasing compounds such as tryptase and chymase which are involved in chronic inflammatory reactions (Metz *et al*., 2007). On the other hand, mast cells similar to neutrophils possess the enzyme chloroacetesterase, and the positive LE result may be attributed to the release of the enzyme from mast cells rather than neutrophils or both cells. In the present study, we used Giemsa staining which can easily identify mast cells by their metachromatic intracytoplasmic granules. It has been shown, however, that the intracytoplasmic granules of the mast cells may not stain properly when aqueous rapid stains such as Diff quick are used (Leclere *et al*., 2006; Sabattini *et al*., 2018). Accordingly, the neglected role of mast cells in bovine endometritis in previous cytological studies may be due to the method of rapid staining that is routinely used for cytological evaluation. The role of mast cells in the endometrial inflammatory conditions as well as its value for the diagnosis of uterine inflammation requires further investigation. In conclusion, the rapid LE test shows promise as a practical substitute for endometrial cytology in cow-side applications. Further research is required to evaluate the applicability of the rapid LE test for the prediction of the fertility of postpartum dairy cows.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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