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Araştırma Makalesi ***Research Article***

Vaginal Fornix Discharge Cellularity and Its Leukocyte Esterase Activity for Diagnosis of Endometritis in Dairy Cows

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Abstract

The objective of the present study was to evaluate the application of some strip test markers (i.e., leukocyte esterase (LE) activity, protein, nitrate and pH) for diagnosis of endometritis in dairy cows using vaginal fornix discharge. Also, the total white blood cell count (t-WBC/µl) of this secretion and degenerative changes of neutrophils in cervical cytology were used as alternative methods to predict progression of the endometritis severity. Holstein cows (n=215) between 30-40 days in milk (DIM) were included and examined. Giemsa-stained smear was prepared from cervical mucus. Cervical cytology test was considered as reference screening method for the detection of subclinical endometritis. The LE activity and t-WBC in the vaginal fornix discharge of subclinical endometritis cows were significantly higher than those from healthy cows. Sensitivity and specificity were 78% and 73% for LE10 activity (10 minutes after contacting with discharges) and 60% and 69% for t-WBC (cut off point=210 cells/µl) for diagnosis of subclinical endometritis, respectively. There was a good agreement between LE10 activity, t-WBC and cervical cytology test with a Kappa coefficient of 0.4 and 0.42, respectively (P<0.0001). Total WBC count in discharge and degenerative neutrophils (DN) percentages increase simultaneously with the degree and severity of endometritis. There was a highly significant (P<0.01) correlation between t-WBC and some reagent strip test markers (LE activity, protein and nitrate) in clear discharge of studied cows. In conclusion, the present results suggest the LE activity and t-WBC in vaginal fornix discharge could be used as non-invasive reliable and valid methods for screening of subclinical endometritis in postpartum dairy herds.

Introduction

Endometritis is an inflammation related to the endometrium. This is one of the most common uterine disorders in dairy cows, that is defined as the presence of purulent (>50 %pus) or mucopurulent (approximately 50 % pus, 50 % mucus) uterine exudate in the vagina, 21 days or more postpartum in the absence of systemic clinical signs (LeBlanc et al., 2002). Abnormal discharge reflects uterine infection (Williams et al., 2005). Clinical endometritis is diagnosed by transrectal palpation for delayed involution, the use of transrectal ultrasonography for measurement of the diameter of the uterine horns and cervix, observation of mucus and pus within the uterine lumen, and examination of the contents of the vagina for the presence of pus following manual vaginal examination or vaginoscopes or metricheck (Leblanc et al., 2002; Sheldon et al., 2002). In addition to this, sometimes subclinical endometritis is traditionally characterized by inflammation of the endometrium in cow that impaire reproductive performance in the absence of signs of clinical endometritis (Cheong et al., 2011; Gilbert et al., 2005; Kasimanickam et al., 2004). There are different techniques for diagnosis of inflammatory processes of the endometrium, including ultrasonography (Barlund et al., 2008), culture of uterine fluids (Bretzlaff, 1987), uterine biopsy (Bonnett et al., 1991) and uterine cytology (Gilbert et al., 1998). Uterine cytology samples can be provided using flushing the uterine lumen (Gilbert et al., 2005) or by cytobrush technique (Kasimanickam et al., 2004). In the cytological technique, percentage of neutrophils was measured for diagnosis of subclinical endometritis. Subclinical endometritis has been identified according to the presence of >18% neutrophils in uterine cytology samples collected at clean test (Sheldon et al., 2006). Ahmadi et al. (2005) and Yavari et al. (2009) reported that percentage of neutrophils from cervical and uterine fluid samples were not different significantly and

cervical sampling is simple and practical method in all herds.

Although uterine cytology sample is currently the reference method for diagnosis of subclinical endometritis (Barlund et al., 2008). This method is generally not practical in the field condition, assumes much time and requires technical proficiency for collection and identification of different cells in uterine samples. Accordingly, we require a practical cow-side and rapid clinical diagnostic test to inspect and manage subclinical endometritis in commercial herds.

More recently, leukocyte esterase (LE) activity technique proposed as an alternative and creative cow-side test for the diagnosis of subclinical endometritis (Couto et al., 2013). This method primarily assesses neutrophils' LE activity in the uterine lavage fluid, uterine and cervical cytobrush samples (Cheong et al., 2012; Couto et al., 2013). Leukocyte esterase material was released by neutrophil cells and reacts with indoxil carbonic acid ester. The esterase reaction with diazoniun salt is released indoxil which is oxidized, yielding a violet azo dye (Kutter et al., 1987); the severity of the color is related with leukocyte counts. This test usually use for diagnosis of urinary tract infection, mainly may be applied for the rapid diagnosis of inflammation in pleural fluid, peritoneal fluid, and cerebrospinal fluid (Azawi 2008; Cheong et al., 2012; Drillich et al., 2010; Santos et al., 2006; Sheldon et al., 2006).

In a study, Santos et al. (2006) demonstrated high correlation between endometrial cytology and LE activity which was provided by uterine lavage samples, in which the LE test had sensitivity (83%) and specificity (94%) for diagnosis of subclinical endometritis. In another study, the LE test yielded a sensitivity and specificity of 77% and 52%, respectively (Cheong et al., 2012). Couto et al. (2013) reported a significant correlation between LE activity and endometrial cytology in cytobrush samples. Interestingly in their study, cervical LE activity strongly associated with percentage of neutrophils in endometrial cytology. In the three recently studies that were mentioned, reagent strip of Bayer Corporation was used for LE activity.

The objectives of the present study were to: (1) evaluate the association between strip test markers, i.e., LE, protein, nitrate, and pH and cervical cytology indices, (2) determine optimum time and cutoff points for LE using test strips (Combi-Screen Analyticon, Germany), (3) determine the association between total white blood cell count of vaginal fornix discharge (t-WBC of discharges) and strip test markers and its optimum cutoff points based on the cervical cytology results, (4) detect the correlation between the presence of neutrophil degenerative changes and degree of endometritis as well as strip test markers.

Materials and Methods

Animal and Samples Collection

Holstein cows (n=215) examined from registered primiparous and multiparous Iranian Holstein cattle at the farm of Farzis milk and meat producing complex in Shiraz, Fars province, south of Iran. This study began in November 2013 and continued until November 2014. The cows were kept under the same weather and management conditions in a similar manner. All cows were housed in open shed barns, milked thrice daily and artificially inseminated exclusively after a voluntary waiting period of approximately 55 days. Cows between 30-40 DIM were included and examined in the study. Manual vaginal examination, transrectal palpation and ultrasonography were used for inspection of genital tract. In the use of manual vaginal examination, the lateral, dorsal and ventral walls of the vaginal fornix and the external cervical os were palpated and the mucus contents withdrawn for examination. Clinical endometritis is characterised based on vaginal mucus character: score 0=clear discharge (including both healthy and subclinical endometritis); score 1=mucus containing flecks of white or off-white pus; score 2=discharge containing (approximately 50% pus, 50% mucus material); and score 3=discharge containing >50% purulent material (Williams et al., 2005). The cervical cytology preparation and recording of vaginal discharge characteristics were done for each studied cows (Yavari et al., 2009). Vaginal fornix discharges were collected within a container of 50 ml and were transferred to laboratory on ice in order to perform the LE test and t-WBC using hemocytometer.

Reagent Strip Test and Cell Counting

Discharges were strewed on reagent strip test (Analyticon Biotechnologies AG D-35104 Lichtenfels, Germany, Combi screen 11 sys plus). pH, protein, nitrate were evaluated base on color change after 1, 5, 10, 15 min and the LE result was evaluated after 2, 5, 10, 15 min; in order to achieve the optimal time with the highest sensitivity and specificity.

Protein results were categorized in several categories which included: code 0: negative, code 1: trace, code 2: 30, code 3: 100, code 4: 500 mg/dl based on the color of the reagent area. pH results were categorized in several categories which ranged from 5.0 - 9.0 based on the color of the reagent area. Nitrate results were categorized in several categories which included: code 0: negative, code 1:+, code 2:+ + based on the color change. Leukocyte esterase (LE) results

were recorded in two categories which included: code 0: negative, code 1:+ or higher on reagent strips based on the color change.

We used a Neubauer hemocytometer to manually count of t-WBC in the vaginal fornix discharges. Cells were counted on each side of the chamber, averaged, and multiplied by 10 to obtain a total cell count per cubic millimeter (micro liter).

Cervical cytology slides were prepared by smearing a drop of cervical mucus on a clean glass microscope slide (Ahmadi et al., 2006) and dried at room temperature and fixed with ethanol for 10 min and stained with a Giemsa stain for 20 min. Cytological assessment was determined base on the percent of neutrophils by counting 100-200 cells at 1000X magnification in each 20 microscopic field that were randomly selected. Cows were considered positive for subclinical endometritis if neutrophils' percentage were >18% of total cells (Kasimanickam et al., 2004). Cervical cytology was considered as reference method for evaluating the total WBC counting of vaginal fornix discharge and LE test. Also, types of neutrophils on cervical cytology were categorized in 2 class based on degenerative status of neutrophils in the slide; non-degenerate neutrophil (NDN) and degenerate neutrophil (DN). Neutrophils showing damage and degenerative changes characterized by loss of their characteristic lobulated and segmented appearance, rupture of the nuclear membrane and diffusion of chromatin materials considered as DN (Brownlow, 1983).

Statistical Analysis

The results were statistically analyzed using the SPSS statistical software (Version 15.0, SPSS Inc, Chicago, Illinois). Receiver operating characteristic (ROC) analysis was performed and the area under the curve (AUC) and P values were reported. The AUC was calculated to find optimal cutoff point for the number of neutrophils that was counted by hemocytometer slide. A similar analysis was performed to find optimal cutoff point for LE test of clear and total (clear and mucopurulent) discharges. The cutoff point with the highest sum of sensitivity and specificity was selected as optimum. The reagent strip test results were dichotomized (positive or negative test) at optimum cutoff level which calculated by ROC test. A series of 2×2 tables was created for reagent strip test against cytologic evaluation by crosstab procedure of SPSS software. In this procedure, an interrater reliability analysis using the Kappa statistic was performed to determine consistency among raters such as cytologic evaluation and LE of reagent strip test. Sensitivity (Se), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV) were calculated using true positive, true negative, false positive and false negative results and were all recorded.

The mean of cytology indices such as NDN, DN, total neutrophils and number of neutrophils were compared between cows that had ≤18% and >18% neutrophils by one-way ANOVA test. We used ANOVA test for analyzing the esterase activity and protein concentration at different time between cows had ≤18% and >18% neutrophils in the cytology samples. Correlation between neutrophil percentage, degenerative status of neutrophil (NDN and DN), t-WBC count and reagent strip test data were done by Pearson correlation test. For all analysis, statistical significance was defined as P≤0.05.

Results

The present study included 215 dairy cows and two cows were missed. In the study, prevalence of clinical endometritis was 51.2% (n=109); they were defined as score 1 (36.6%; n=78), 2 (11.3%; n=24) and 3 (3.3%; n=7). The prevalence of subclinical endometritis was 25.3% (n=54). Fifty (23.5%) cows with clear discharge were defined as healthy cow.

There was a significant difference between cows with various degrees of endometritis and healthy cows (had clear vaginal fornix discharge and ≤18% neutrophils in the cytology samples) in the percentage of degenerative neutrophils (DN) and t-WBC count. Total WBC count in discharge and DN percentages increased with the severity of uterine infection (Table 1).

In cows with clear discharge, the percentages of NDN, DN, neutrophil and t-WBC count were significantly higher (P<0.05) in subclinical endometritis cows than in healthy cows (Table 1). Dichotomized reagent strip test results categorized into two groups according to the cervical cytology methods (>18% neutrophils as endometritis disease and ≤18% as healthy cows) in order to find optimum time with highest sensitivity and specificity for using strip test markers. The optimum time for LE evaluation was considered as 10 minutes based on receiver operator characteristics (ROC) analysis, area under the curve (AUC), and Kappa coefficient.

Area under the curve for the cutoff point of + for LE test was calculated as 0.8 in order to differentiate the diagnosis of subclinical endometritis in clear discharge based on the ROC analysis (P<0.0001). According to the above-mentioned cutoff point, the LE10 test had Se=78%, Sp=60%, PPV=69%, and NPV=70%. There was a good relation between LE10 and cervical cytology test with a Kappa coefficient of 0.4 (P<0.0001). Also, the optimal cutoff point for t-WBC was determined as 210 cells/μl in the vaginal fornix discharge (AUC=0.786;

P<0.0001). At the optimal cutoff point, the t-WBC test had Se=73%, Sp=69%. There was a good relation between t-WBC and cervical cytology test with a Kappa coefficient of 0.42 (P<0.0001).

As shown in Figure 1, the LE activity was significantly lower (P<0.05) in healthy cows compared to cows with subclinical endometritis at time of 5, 10 and 15 minutes (Figure 1). The LE activity and protein concentration were increased in all times in all group of disease (both subclinical and clinical endometritis) compared to healthy group (P<0.05, Figures 2 and 3). There were no significant differences in nitrate and pH of discharge in disease cows compared with those in healthy cows.

Table 2 displays correlation between NDN %, DN % and neutrophils percentage (N%), t-WBC and reagent strip test markers in cows with clear discharges (including healthy and subclinical endometritis) and total studied cows (with clear discharge and clinical endometritis). Interestingly, there was highly significant (P < 0.01) correlation between t-WBC and some reagent strip test markers in cows with clear discharge (Table 2).

Table 1. Comparison of cytological indices (percentage of NDN, DN and neutrophil) and t-WBC count (Mean ± SE) between cows with different types of endometritis and healthy cows.

Endometritis Condition	NDN%	DN%	Neutrophil%	t-WBC (/μl)
Healthy Cow	3.5±0.6^{a}	1.1±0.4 ^{a}	4.6±0.8^{a}	267.4±65.6^{a}
Subclinical	41.1±3.1^{b}	12.1±2.9^{b}	53.1±3.7^{b}	1522.4±337.5^{b}
Type 1 Clinical	41.9±3.6^{b}	18.9±2.8^{b}	60.9±3.9^{c}	10201.3±1287.1^{c}
Type 2 Clinical	45.2±4.4^{b}	41.0±4.9^{c}	86.7±4.9^{d}	37725.4±4830.3^{d}
Type 3 Clinical	46.3±10.6^{b}	48.2±11.5^{c}	94.5±3.2^{d}	58177.1±13377.5^{e}

NDN=Non degenerative neutrophil, DN=Degenerative neutrophils, Neutrophils=Percentage of total neutrophils, and t-WBC=Total WBC count of vaginal fornix discharge; ^{a,b,c,d,e} Different superscript in columns indicate significant difference (P<0.05).

Table 2. Correlation coefficients (r) between reagent strip test markers and NDN%, DN%, N% and t-WBC in cows with clear discharges (including healthy and subclinical endometritis) and total studied cows (with clear discharge and clinical endometritis) at different times.

Studied Cows	Reagent Strip Test	NDN%	DN%	N%	t-WBC
Clear discharges (n=104)	LE 2	0.27**	0.33**	0.32**	0.31**
	LE 5	0.45**	0.44**	0.52**	0.48**
	LE 10	0.54**	0.37**	0.57**	0.55**
	LE 15	0.52**	0.36**	0.56**	0.52**
	Pro 1	0.07	0.22*	0.13	0.35**
	Pro 5	0.32	0.20*	0.08	0.37**
	Pro 10	0.03	0.14	0.06	0.32**
	Pro 15	0.38	0.20*	0.08	0.33**
	Nit 5	0.002	0.067	0.08	0.28**
	Nit 10	0.32	0.04	0.09	0.32**
	Nit 15	0.004	0.006	0.01	0.28**
Total (n=213)	LE 2	0.40**	0.50**	0.60**	0.68**
	LE 5	0.50**	0.55**	0.68**	0.75**
	LE 10	0.52**	0.45**	0.69**	0.78**
	LE 15	0.50**	0.53**	0.67**	0.76**
	Pro 1	0.24**	0.34**	0.41**	0.60**
	Pro 5	0.21**	0.34**	0.37**	0.60**
	Pro 10	0.17**	0.28**	0.32**	0.55**
	Pro 15	0.20**	0.30**	0.34**	0.54**
	Nit 1	0.03	0.03	0.02	0.05
	Nit 5	0.02	0.03	0.06	0.13
	Nit 10	0.08	0.10	0.15*	0.23**
	Nit 15	0.08	0.10	0.14	0.22**

NDN=non degenerative neutrophil, DN=degenerative neutrophils, N=percentage of total neutrophils, and t-WBC=total WBC count of vaginal fornix discharge, LE 2, 5, 10 and 15= esterase activity of strip test at time of 2, 5, 10 and 15 minutes, Pro 1, 5, 10 and 15=protein concentration at time of 1,5,10 and 15 minutes and Nit 1, 5, 10 and 15=nitrate results of strip test at time of 1, 5, 10 and 15 minutes.** indicate significant difference (P<0.01);* indicate significant difference (P<0.05).

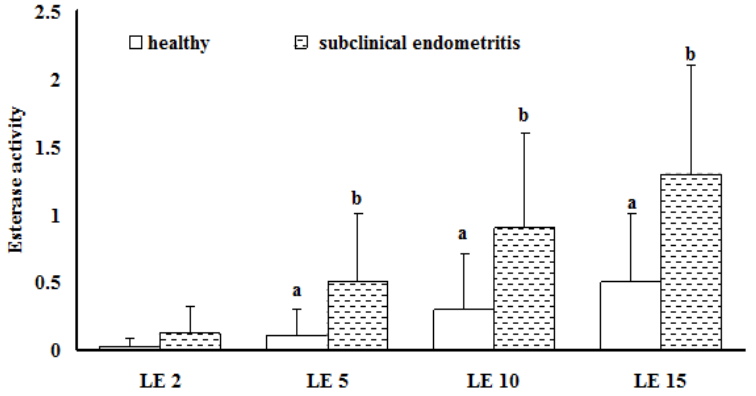

Figure 1. Comparison of LE activity between healthy (≤18%) and subclinical endometritis (>18% neutrophils in cytology) cows with clear discharge at different times (2, 5, 10, 15 minutes (LE 2-15)); a and b, Different letters above bar indicate significant difference in each time (P<0.05).

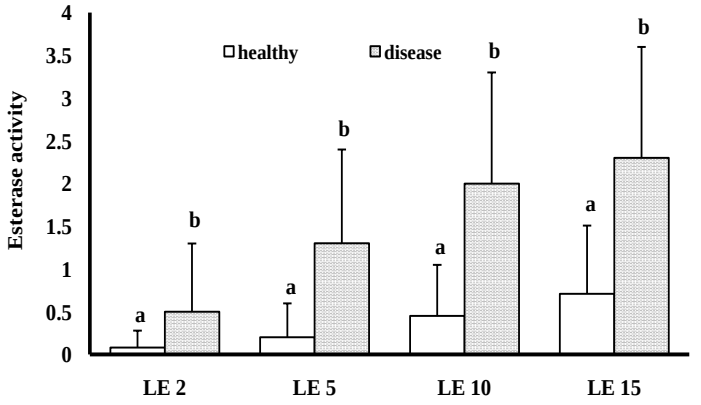

Figure 2. Comparison of LE activity between healthy (≤18%) and disease (>18% neutrophils including both subclinical and clinical endometritis) cows at different times (2, 5, 10, 15 minutes (LE 2-15)); a and b, Different letters above bar indicate significant difference in each time (P<0.05).

Discussion

Endometritis is an inflammation related to the endometrium, and subclinical endometritis is characterized by inflammation of the endometrium in cow that impairs reproductive performance in the absence of signs of clinical endometritis (Cheong et al., 2011; Gilbert et al., 2005; Kasimanickam et al., 2004; LeBlanc et al., 2002). It is one of the most common reproductive disorder in postpartum dairy cattle leading high economic losses due to decreased milk yield, reduced fertility and increased treatment cost (Sheldon et al., 2008 and 2009). So, an early and accurate diagnosis of endometritis has been recommended due to its deleterious effect on the reproductive performance and serious economic impact on dairy herds. Neutrophil percentage measured in the uterine cytology method has been accepted as the reference technique for diagnosis of subclinical endometritis. Yavari et al. (2009) reported that the neutrophil percentage obtained from cervical and uterine sources was not different significantly. In the present study, we used cervical cytology as a simple and practical approach for evaluation of subclinical endometritis.

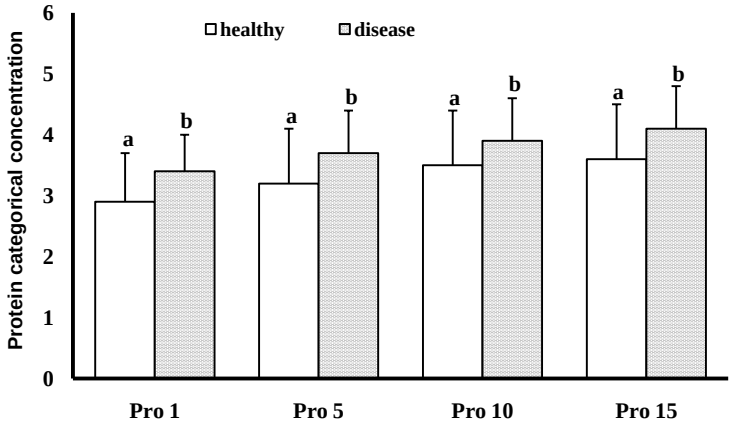

Figure 3. Comparison of protein levels between healthy (≤18%) and disease (>18% neutrophils including both subclinical and clinical endometritis) cows at different times (1, 5, 10, 15 minutes (pro 1-15)); a and b, Different letters above bar indicate significant difference in each time (P<0.05).

The reported lactational incidence rate of endometritis varies from 7.8 to 61.6% (Kasimanickam et al., 2004). Carneiro et al. (2014) reported the incidence rate of 26% for subclinical endometritis in crossbred dairy cows from 32 to 70 days after calving. In this study, the prevalence of subclinical and clinical endometritis was 25.3% and 51.2%, respectively. The results of the present study showed that the LE activity of vaginal fornix discharge in cows with subclinical endometritis was significantly higher compared to the healthy cows. Correlation between LE activity index of strip test and the neutrophil percentage detected in the cervical cytology was significant. Similarly, Couto et al. (2013) reported that leukocyte esterase score was correlated with the neutrophil percentage from samples obtained by uterine cytobrush and cervix. Also, Santos et al. (2006) reported a correlation between leukocyte esterase score and the percentage of neutrophils from samples of uterine lavage. Furthermore, Cheong et al. (2012) reported that LE activity was associated with subclinical endometritis and LE activity was significantly increased in cows with subclinical endometritis. They suggested that LE activity test on samples with uterine lavage source could be used for the diagnosis of subclinical endometritis with a sensitivity of 77%, and specificity of 51% using a reagent strip test (Multistix®10 SG; Bayer Corporation, Elkart, IN, USA). In addition, Santos et al. (2006) reported that using of LE test (Multistix®) on uterine lavage samples could predict subclinical endometritis with sensitivity of 83% and specificity of 94%.

In the present study, sensitivity and specificity of LE10 activity test were 78% and 60%, respectively, in the vaginal fornix discharge for diagnosis of subclinical endometritis. Cows with subclinical and clinical endometritis had higher t-WBC than healthy cows; also, t-WBC count increase with the severity of uterine infection. Total WBC count based on the cut off point = 210/μl could be used for diagnosis of subclinical endometritis with sensitivity of 73% and specificity of 69%. When cervical cytology test was considered as gold standard test, the interrater reliability for the LE10 and t-WBC count tests were found to be Kappa = 0.4 and 0.42, respectively. Combining the results for LE10 and t-WBC improved the specificity (75%) for the diagnosis of subclinical infections.

It seems that the difference in results from various reported studies for sensitivity and specificity may be related to the type of used reagent strip test, postpartum sampling schedule, different source of collected samples and sampling methods. In the present study, we used the Analyticon (Biotechnologies AG D-35104 Lichtenfels. Germany) reagent strip for vaginal fornix discharge at 30-40 days postpartum. Cheong et al. (2012) and Santos et al. (2006) used the Multistix® 10 SG (Bayer Health Care L.L.C., Elkhart, IN, USA) for uterine lavage samples between 40-60 and 7-49 days postpartum, respectively; whereas, Couto et al. (2013) used the pad uristix (Bayer Health Care L.L.C., Elkhart, IN, USA) reagent strip for uterine cytobrush and cervical samples at 21-47 postpartum DIM. Based on the results of different studies leukocyte esterase activity proposed the technique as a practical cow-side and rapid clinical

diagnosis test to inspect and manage subclinical endometritis in commercial herds.

In the present study, DN% of the cows was proportionally increased in cervical cytology with the degree and severity of endometritis. It seems that endometritis causes degenerative changes of neutrophils due to their exposure to toxic condition, especially in higher degree of endometritis. So, t-WBC and DN percentage may be associated with the severity of uterine infection. Therefore, t-WBC count and DN percentage in the vaginal fornix discharge can be used as complementary and as an alternative approach for more accurate determination of endometritis severity. To the best of our knowledge, there is no published data evaluating the degenerative changes of neutrophils in the slide and vaginal fornix cellularity as endometritis indicators. Endometrial cytology that described by previous researchers needs special instruments and proficiency to obtain the uterine lavage and cytobrush samples; it is also time-consuming and less practical for diagnosis of subclinical endometritis under field condition.

In the present study, protein concentration was markedly increased in cows with clinical endometritis. Protein concentration was also associated with percentage of neutrophils in the cytology and t-WBC at various times in total studied cows. Cheong et al. (2012) reported that, the protein reagent strip test had the weakest association with cytologic endometritis and was not predictive of future reproductive performance. The reagent strip test for evaluating protein is especially sensitive in the presence of albumin and other proteins (immunoglobulins) are indicated with less sensitivity as per manufacturer instructions. In addition, false positive results are possible in highly alkaline samples (pH>9) and in the presence of high specific gravity. Accordingly, protein assessment could not be recommended for evaluating of discharges as a diagnostic tool for subclinical endometritis.

In conclusion, the present results suggest the LE activity and t-WBC in vaginal fornix discharge could be used as non-invasive reliable and valid methods for screening the subclinical endometritis in postpartum dairy herds. However, further studies are essential to optimize LE activity as a cow-side diagnostic tool and discharge cellularity for estimating the degree of postpartum endometritis in dairy cows.

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