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Comparative analysis between the cytobrush and low-volume uterine flush techniques for endometrial cytology in clinically normal postpartum crossbred dairy cows

Comparação das técnicas de citologia endometrial escova citológica e lavado uterino de baixo volume no pós-parto de vacas leiteiras mestiças

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Abstract

This study compared two frequently employed techniques for the collection of endometrial and inflammatory cells and characterized postpartum endometrial cytology (EC) of clinically normal postpartum crossbred dairy cows in dairy farming system in Southwestern Brazil. Thirty-four crossbred, clinically healthy dairy cows with normal delivery and puerperium, complete uterine involution and without any treatment were monitored until 42 days in milk (DIM). All cows were evaluated by complete clinical and gynecological examinations at days 0, 7, 14, 21, 28, and 42 DIM. The gynecological examinations were done by transrectal palpation, ultrasonography, vaginoscopy, evaluation of the vaginal mucus and EC by using the cytobrush (CB) and low-volume uterine flush (LVF) techniques. The agreement (Kappa statistic) between the two technicians was good for CB (86%) and LVF (80.3%) for the counting of the percentage of neutrophils. The average number of neutrophils was significantly higher throughout the experiment for LVF, but a reduced percentage of neutrophils were observed during the postpartum period for both techniques. The amount of macrophages, lymphocytes, and eosinophils were not affected during postpartum and there was no significant difference relative to these cells when the two techniques were compared. There were significant differences in the percentage of cows with subclinical endometritis only at d28 by CB (22.2%) and LVF (59.3%).
Key words: Uterine diseases, clinical pathology, diagnostic cytology, cattle

Resumo

Objetivou-se comparar duas técnicas frequentemente usadas na colheita de células endometriais e inflamatórias e caracterizar a citologia endometrial (CE) de vacas leiteiras mestiças no pós-parto

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fisiológico, criadas em sistemas de fazendas leiteiras do sudoeste do Brasil. Foram utilizadas 34 vacas clinicamente sadias com parto e puerpério fisiológico, completa involução uterina e sem qualquer tratamento até os 42 dias pós-parto (dpp). Realizou-se o exame clínico e ginecológico no parto e aos 7, 14, 21, 28 e 42 dpp. O exame ginecológico foi executado pela palpação retal, ultrassonografia, vaginoscopia, avaliação de muco vaginal e CE realizada com escova citológica (CB) ou por lavado uterino de baixo volume (LVF). A concordância (Kappa statistic) entre os técnicos na contagem da porcentagem de neutrófilos foi boa para CB (86%) e para LVF (80,3%). A média de neutrófilos foi maior em todos os momentos na técnica de LVF, porém a redução de neutrófilos foi observada durante o pós-parto nas duas técnicas de citologia. A contagem de macrófagos, linfócitos e eosinófilos não variou durante o pós-parto e não houve diferença entre as técnicas CB e LVF. Houve diferença apenas aos 28 dpp, na porcentagem de vacas com endometrite subclínica, entre as técnicas CB (22.2%) e LVF (59.3%).

Palavras-chave: Doenças uterinas, patologia clínica, citologia, bovinos

Introduction

Fertility after parturition of dairy cows is considered as the principal economic factor of milk producing farms, where any alteration or extension to this phase results in progressive economic loss (PATEL et al., 2006). Puerperium is defined as the period between parturition and complete uterine involution, being approximately 40 days in milk (DIM) in cows. Four events occur concomitantly after calving and expulsion of the placenta: uterine involution, endometrial regeneration, resumption of the ovarian cyclicity, and elimination of bacterial contamination (SHELDON, 2004; SHELDON et al., 2008).

Although the uterus is sterile during gestation, relaxation of the vulva and cervical dilation soon after calving allow microorganisms from the environment, skin, and feces to contaminate the uterine lumen (SHELDON, 2004; SHELDON; DOBSON, 2004). Bacterial contamination of the genital tract of cows during postpartum is a dynamic situation, with alterations of bacterial populations and spontaneous recontaminations during the first weeks of postpartum (GRIFFIN; HARTGAN; NUNN, 1974). However, a few weeks thereafter, there is significant reduction of uterine infection and most animals might recuperate spontaneously (GRUNERT et al., 2005). Elliot et al. (1968) have demonstrated that the proportion of animals positively diagnosed with uterine bacterial infection is reduced from 93% at 15 DIM to 9% by 46% to 60 DIM. Although most cows would have eliminated bacteria during the first five weeks, a small percentage remains infected.

The innate immune system is mainly responsible for the elimination of uterine bacterial contamination during postpartum and consist of anatomic, physiological, inflammatory and phagocytic barriers. The vulva, vaginal vestibule, and cervix are physical barriers that prevent the ascension of bacteria into the genital tract. The main phagocytic barrier is composed of neutrophils that migrate to the uterine lumen in response to invading bacteria (GRIFFIN; HARTGAN; NUNN, 1974; ZERBE et al., 2000; SHELDON et al., 2002). However, it is known that the functional capacity of neutrophils is reduced during postpartum, although the exact mechanism has not been well elucidated (BAGGIOLINI, 1998).

Recently, subclinical endometritis (SE), as characterized by endometrial cytology (EC), has been the focus of many studies since it is known to adversely influence the reproductive efficiency due to reduced pregnancy rate and increasing time to pregnancy (BACHA; REGASSA, 2010; MCDOUGALL et al., 2010; CHEONG et al., 2011), increase the incidence of repeat breeder syndrome (SALASEL; MOKHTARI; TAKTAZ, 2010), and subclinical mastitis (BACHA; REGASSA, 2010). McDougall et al. (2010) have suggested that the percentage of polymorphonuclear (PMN) cells is a better predictor of reproductive performance than

either intra-uterine bacteriology or gross vaginal inflammation score.

The choice of the best technique for EC has not yet been well established and since the cut-off for the diagnosis of SE has been different in various studies it is difficult to interpret the results. Studies have compared the differences between the utilization of the cytobrush (CB) and low-volume uterine flush (LVF) techniques for endometrial cytology, but all have been done in high milk producing Holstein cows maintained in intensive housing conditions (KASIMANICKAM et al., 2005; BARLUND et al., 2008). However, no study has been located that compared the efficiency of these techniques in crossbreed dairy cows raised in semi-intensive conditions. In most States within Brazil cows are raised under these conditions; this condition occurs in the State of Minas Gerais, which owns the largest milk producing herd and is responsible for 26.2% (8.4/30.7 billion of Kilograms of milk /year) with an average production of 1540 Kilograms/cow/year (IBGE, 2010). Consequently, the understanding of the effects of these routine techniques of endometrial cytology in crossbreed cows is of fundamental importance not only for the local cattle industry, but for all other geographical locations where cattle might be raised under similar husbandry practices.

The objectives of this study were to compare two frequently used techniques for the collection of endometrial and inflammatory cells and to characterize the postpartum EC of clinically normal postpartum crossbred dairy cows in dairy farming system in Southwestern Brazil.

Materials and Methods

Animals, geographical location, diet and herd management

The experiment was done at the Glória Experimental Farm, Universidade Federal de Uberlândia (UFU), Minas Gerais, southwestern Brazil; this study was approved by the Ethics Committee for Animal Usage in Experiments (CEUA/UFUNº047/2011). Thirty-four crossbred dairy cows, having two or more parturitions, with parturitions occurring between 01/2011 and 08/2011, were used during this study. The study population consisted of healthy dairy cows with normal calving and puerperium, without any treatment, complete uterine involution and without uterine infections (puerperal metritis, clinical endometritis, and pyometra) as characterized by Sheldon et al. (2008). Both endometrial techniques were done in all cows during each period evaluated.

All animals were confined during the experiment and received a ration according to NRC (2001); water was administered *ad libitum*. These animals were annually vaccinated against rabies, clostridial diseases, leptospirosis, foot and mouth disease, and paratyphoid. Additionally, all females were vaccinated against brucellosis between three and eight months of age, and were annually evaluated for tuberculosis, leptospirosis, and brucellosis.

Clinical evaluation

A complete clinical evaluation of the cows was done on the day of parturition, and then on days 7, 14, 21, 28, and 42DIM as described by Saut et al. (2010). The following parameters were evaluated: rectal temperature; cardiac, respiratory and ruminal frequencies, and the color of the ocular mucous membranes. Thereafter, the following evaluations were done in all cows: gynecological examinations and collection and evaluation of the vaginal mucus and endometrial samples for cytological examination were obtained from all cows by using the CB and the LVF techniques. During this study the CB was performed before the LVF as recommended by Kasimanickam et al. (2005) and Cocchia et al. (2011).

Gynecological evaluation

This consisted of a combination of transrectal palpation, ultrasonographic evaluation, and

vaginoscopy. The diameters of the gravid and non-gravid uterine horns from the previous gestations were determined by ultrasonography (DP-2200VET®, Mindray, Shenzhen, China). The physical evaluation was done with a vaginal speculum to evaluate the vagina and the external cervical ostium for alterations of color, edema and lacerations.

Collection and evaluation of vaginal secretion

The collection was done as previously described (WILLIAMS et al., 2005); briefly, the vulvar region to be examined was cleaned and then a gloved hand was introduced to collect the vaginal secretion. The evaluation was modified and classified using a number scale based on the color and the proportion, with the aid at tubes (50 ml, non-skirted), of its content: 1, clear or translucent mucus; 2, bloody-mucus or chocolate-colored mucus; 3, mucus containing flecks of white or off-white pus; 4, < 50ml exudate containing ≤ 50% white or off-white mucopurulent material; and 5, > 50 ml exudates containing ≥ 50% purulent material, usually white or yellow, but occasionally sanguineous.

Cytobrush technique (CB)

EC was done by using the CB as described by Kasimanickam et al. (2005). The normal non-sterile cytobrush handle was cut to approximately 3cm in length, threaded onto a 65cm solid stainless steel rod, and placed in a stainless steel tube (50cm in length and 5mm in diameter) for passage through the cervix. The vulva was cleaned, and the instrument passed through the vagina into the external ostium, advanced through the cervix into the base of the larger horn, at which point the stainless steel tube was adequately retracted to expose the cytobrush. EC samples were collected by rotating the cytobrush in a clockwise direction when in contact with the uterine wall. The cytobrush was retracted into the stainless steel tube prior to removal from the uterus.

Slides for cytology examination were prepared by rolling the cytobrush onto a clean glass microscope slide and stained by the May Grunwald-Giemsa technique. Each slide was examined by light microscopy (magnification x400) to perform the differential cell count of 200 cells by two independent technicians. The agreement between the technicians was good (86.0% - Kappa statistic = 0.66) for counting the percentage of neutrophils.

Low-volume uterine flush technique

The EC for low-volume uterine flush technique was done as described by Gilbert et al. (2005). Sterile 0.9% sodium chloride solution (20ml) was infused into the uterus using a sterile, individually packed plastic infusion pipette. The vulva and perineum were cleaned, and the pipette inserted into the vagina. The pipette was then manipulated through the cervix and the saline solution infused into the uterus. The uterus was gently massaged for about 10s, and some of the infused fluid aspirated via the same infusion pipette. No special effort was made to retrieve the rest of the fluid if it did not flow freely.

The recovered fluid was transferred into a sterile plastic tube and transported refrigerated to the laboratory for further processing (within 4 h).The tube was vortexed to resuspend cellular material, and 100-250µL of the supernatant was transferred to the cytocentrifuge chamber (Serocito 2400®, FANEM LTDA, Brazil). The slides were then stained by the May Grunwald-Giemsa technique, and examined by light microscopy (magnification x400) to perform the differential cell count of 200 cells by two independent technicians. The agreement between the technicians was good (80.3% - Kappa statistic = 0.59) for counting the percentage of neutrophils.

Statistical analyses

Statistical analyses were done by evaluating the means, standard deviations, medians and percentages

of the descriptive statistics by using the Minitab Release 16 (Minitab Inc, Pennsylvania, USA) program and Biostat 5.0 statistical software, the α level was set at 0.05 (VIEIRA, 2003). The variables were submitted to the Kolmogorov-Smirnov (K.S.) Test to evaluate their effects during puerperium. The variables with normal distribution (temperature, heart rate, respiratory rate and the diameter of the uterus) were submitted to variance analysis (ANOVA) with post-test Tukey-Kramer Multiple Comparisons Test. Additionally, those variables that did not demonstrate a normal distribution (ruminal frequency, neutrophils, endometrial epithelial cells, macrophages, lymphocytes and eosinophils) were analyzed by the Kruskal-Wallis test (non-parametric ANOVA) and post-test Dunn multiple comparison test. Comparison of the results of endometrial cytology between the LFV and CB techniques was done by using the Mann-Whitney Test. Kappa statistic was used to evaluate the agreement between the microscope evaluation done by the two technicians and the results of SE by the different techniques.

Results and Discussion

Only non-treated cows, without any alteration during the clinical and gynecological evaluations, and with all samples collected for evaluation by both techniques were included into this study. The values of the vital parameters (Table 1) of crossbred dairy cows were within reference limits (SAUT et al., 2010).

Table 1. Vital parameters of crossbreed dairy cows during physiological postpartum.

Parameters	Days in milk (DIM)					
	0	7	14	21	28	42
Temperature (°C)	39.0±0.6^{a}	38.1±1.5^{b}	38.2±0.5^{b}	38.3±0.6^{ab}	37.9±2.0^{b}	38.1±0.4^{b}
Heart rate (beats/min)	79.6±14.4^{a}	72.3±10.3^{ab}	71.5±8.7^{b}	73.1±9.6^{ab}	73.8±11.1^{ab}	71.7±12.1^{ab}
Respiratory rate (breaths/min)	35.7±14.2^{a}	33.8±9.5^{a}	30.6±7.6^{a}	33.0±11.1^{a}	32.1±7.7^{a}	31.7±8.2^{a}
Ruminal movements (mov/3min)	2.2±1.4^{a}	3.5±1.0^{b}	3.5±1.1^{b}	3.8±1.2^{b}	4.2±1.0^{b}	3.8±1.2^{b}

Note: different small letters in the same line indicate statistical difference. ANOVA with post-test Tukey-Kramer Multiple Comparisons Test for Temperature (p=0.0024), Heart rate (p=0.0433) and Respiratory rate (p=0.4032). Non Parametric ANOVA (Kruskal-Wallis) with post-test Dunn for Ruminal movements (p<0.0001).
Source: Elaboration of the authors.

The ultrasonographic examination of all cows at 42 DIM revealed that the uterus was within the pelvic cavity with diameter of 19.9 ± 3.9mm and 19.0 ± 3.7mm for the non-gravid and gravid uterine horn, respectively. By vaginoscopy there was no injury, edema or abnormal color of the vulva, vagina and external cervical ostium; these are manifestations of normal uterine involution at the end of the puerperium (SHELDON, 2004; SHELDON et al., 2008); similar results were described in crossbreed dairy cows raised in Southwestern Brazil (SAUT et al., 2010). Normally, there is tissue repair and regeneration of damaged vaginal structures during the puerperal period. However, bacterial infections are known to occur in 85% of cows at 15 DIM (GRIFFIN; HARTGAN; NUNN, 1974), and the severity of the infection might predispose the uterine layers to different degrees of lacerations with subsequent bacteremia and/or toxemia.

The results of EC for the two techniques are shown in Table 2. There was no difference in the

dynamics of the average decrease of neutrophils during the postpartum period for both techniques; similar results were described (KASIMANICKAM et al., 2005), where the decrease in the percentage of neutrophils occurred between 20 to 33 and 34 to 47 DIM.

Table 2. The dynamics of endometrial cytology observed during physiological postpartum in crossbred dairy cows by low-volume uterine flush and cytobrush techniques.

Endometrial cytology	Diagnostic Technique	Days in Milk (DIM)			
		14	21	28	42
Neutrophils	LVF	46.9±38.0 ^{aA} (47.0)	53.1±39.4 ^{aA} (61.7)	41.2±37.6 ^{abA} (45.0)	12.2±20.6 ^{bA} (4.0)
	CB	23.3±32.6 ^{aB} (5.2)	22.3±27.9 ^{aB} (11.0)	12.5±23.5 ^{abB} (2.5)	3.3±6.6 ^{bB} (0.0)
Endometrial epithelial cells	LVF	52.3±38.5 ^{aA} (52.0)	45.4±39.4 ^{aA} (37.2)	57.5±38.1 ^{abA} (55.0)	87.0±21.3 ^{bA} (94.0)
	CB	75.7±32.7 ^{aB} (92.2)	77.3±27.8 ^{aB} (88.2)	87.1±23.6 ^{abB} (97.0)	95.9±8.0 ^{bB} (100.0)
Macrophages	LVF	0.2±0.4 ^{aA} (0.0)	0.2±0.4 ^{aA} (0.0)	0.3±0.8 ^{aA} (0.0)	0.1±0.4 ^{aA} (0.0)
	CB	0.2±0.4 ^{aA} (0.0)	0.2±0.4 ^{aA} (0.0)	0.1±0.1 ^{aA} (0.0)	0.1±0.3 ^{aA} (0.0)
Lymphocytes	LVF	0.5±0.7 ^{aA} (0.0)	0.9±2.0 ^{aA} (0.0)	0.5±1.2 ^{aA} (0.0)	0.6±1.6 ^{aA} (0.0)
	CB	0.8±1.2 ^{aA} (0.0)	0.2±0.3 ^{aA} (0.0)	0.4±0.8 ^{aA} (0.0)	0.6±1.9 ^{aA} (0.0)
Eosinophils	LVF	0.1±0.3 ^{aA} (0.0)	0.4±2.0 ^{aA} (0.0)	0.4±1.5 ^{a*} (0.0)	0.1±0.3 ^{aA} (0.0)
	CB	0.0±0.1 ^{aA} (0.0)	0.0±0.1 ^{aA} (0.0)	0.0±0.0 ^{a*} (0.0)	0.1±0.2 ^{aA} (0.0)

Mean ± SD (Median). Different small letters within the same line indicate significant difference of days in milk (DIM) by ANOVA with post-test Tukey-Kramer Multiple Comparisons Test. Different large letters in the same column indicate significant statistical differences between the techniques by the Mann-Whitney Test. * Evaluation not done between the techniques due to null value of the SD.
Source: Elaboration of the authors.

When the two techniques were compared, the LVF technique demonstrated comparatively higher percentage of neutrophils during the entire experiment (Table 2). Similar results had been described in chronically infertile mares that demonstrated elevation in the percentage of neutrophils by LVF (10.67%) relative to CB (3.66%) (COCCHIA et al., 2011). However, no differences were observed in involved Holstein cows between 37 and 47 DIM (KASIMANICKAM et al., 2005). Nevertheless, in that same study, the percentages of neutrophils were more elevated in cows by CB relative to LVF during 20 and 33 DIM, and this elevation was associated with the difficulty of recovering infused fluid from the was not fully involuted uterus, resulting in the recuperation of fewer cells (KASIMANICKAM et al., 2005).

During this study the CB was performed before the LVF, as recommended (KASIMANICKAM

et al., 2005; COCCHIA et al., 2011) to prevent irritation caused by the LVF and the accumulation of remnant unrecovered fluid in the uterus, although the authors understand that the CB could also cause irritation. This sequence of events was maintained during this study so that the results could be efficiently compared.

Kasimanickam et al. (2005) and Cocchia et al. (2011) demonstrated that the time required to obtain samples by the CB was consistent and faster relative to LVF, during which there was considerable variation in the time spent to obtain samples. Similar results were found in this study, but variables were not statistically evaluated. Barlund et al. (2008) have elected the CB as the reference cytological diagnostic test because of greater repeatability. Moreover, within the CB technique only one trained professional was required to obtain a sample, while with LVF there was always the need to have at least two people present to obtain a single sample; to assist with the introduction and aspiration of the sterile sodium chloride solution.

During this study there were unsuccessful attempts to recover fluids via LVF in a few cows; however, these animals were eliminated from the study. Frustrated attempts were also described with this technique (BACHA; REGASSA, 2010; KASIMANICKAM et al., 2005) with descriptions of 17% of all unsuccessful attempts to recover fluid in Holstein cows. Kasimanickam et al. (2005) used 60 ml of saline, three times more than that used in our study, which was based on the technique of Gilbert et al. (2005). The failure to recover 100% of the samples associated with the maintenance of the applicator within the uterus for longer periods are the negative features for the LVF technique, since the combination of these practices might result in damage to the endometrium. No studies that evaluated the risk associated with EC were located, suggesting that these risks should be evaluated. Alternatively, difficulties relative to sample collection were not described by others (GILBERT et al., 2005; BARLUND et al., 2008).

Additionally, the LVF technique affected the integrity of the cells, resulting in a larger percentage of distortions and degenerated cells when compared with CB. Although we were unable to identify the actual cause of these alterations, the method of sample collection, transport or processing could have contributed to the difficulty associated with the interpretation of approximately 200 cells. The samples were processed within four hours on LVF. Cellular lesions have been associated with the solutions used during the processing and centrifuging (BARLUND et al., 2008). Cellular distortions have also been described in the EC of mares and have been attributed to the time spent during sample processing and the centrifugation process (COCCHIA et al., 2011).

Other inflammatory cells (macrophages, lymphocytes, and eosinophils) were present in relatively low concentrations, but did not demonstrate variation between the periods evaluated and did not present differences between the LVF and CB techniques (Table 2). The effects of these inflammatory cells were not described in similar studies (KASIMANICKAM et al., 2004; GILBERT et al., 2005; KASIMANICKAM et al., 2005; BARLUND et al., 2008; BACHA; REGASSA, 2010; MCDOUGALL et al., 2010); however, during routine cytological diagnosis at our laboratory in cows with puerperal metritis and endometritis, a greater percentage of these cells are observed by both techniques, particularly with the LVF technique (SAUT et al., 2010; unpublished results). Therefore, we recommend that these cells should be included during the evaluation of EC of cows, and that the evaluation should not be restricted to neutrophils. Further, other inflammatory cells have been described in mares with endometritis by CB, since the structure of the brush, with nylon fibers at the tip of handle and fibers perpendicular to handle allows the collection of cells more deeply in the thickness of the endometrium (COCCHIA et al., 2011).

The prevalence of SE decreased with the increasing postpartum period (Table 3); similar

results have been described (KASIMANICKAM et al., 2004; GILBERT et al., 2005; PLONTZKE et al., 2010). A similar study that was done in Ethiopia with crossbred dairy cows where SE was considered as being ≥ 5% neutrophils by LVF, related a prevalence of 47.5 and 30.5% in the fourth and eighth weeks with a significant decrease between these periods (BACHA; REGASSA, 2010).

Table 3. Incidence of subclinical endometritis using >18% neutrophils (d21, d28) and >10% (d42) as cut-off point.

Diagnostic Technique	Subclinical endometritis (%)		
	21DIM	28DIM	42DIM
LVF	65.4 ^{aA}	59.3 ^{abA}	26.1 ^{bA}
CB	42.3 ^{aA}	22.2 ^{bB}	14.8 ^{bA}

Different small letters in the same line and large letters within the same column indicate statistical difference by the Fisher's Exact Test ($p \leq 0.05$).
Source: Elaboration of the authors.

A review of recent manuscripts has revealed that there is no consensus between authors relative to threshold for the percentage of neutrophils that characterizes SE, ≥3% (SALASEL; MOKHTARI; TAKTAZ, 2010), ≥ 5% (GILBERT et al., 2005; PLONTZKE et al., 2010; SANTOS et al., 2009; BACHA; REGASSA, 2010) and ≥10% (KASIMANICKAM et al., 2004) of neutrophils, nor for the time interval and period of days stipulated for the formation of groups. Consequently, we decided to compare the two techniques at 21, 28, and 42 DIM. Additionally, we established a different cut-off point to determine the percentage of neutrophils required to characterize SE (Table 3), as defined by Kasimanickam et al. (2004). Additionally, during post-partum of dairy cows the inflammation caused by contamination due to microorganisms within the environment, skin, and faeces (SHELDON, 2004; SHELDON; DOBSON, 2004) as well as the significant reduction in the frequency of uterine infection in the preceding weeks (ELLIOT et al., 1968; GRUNERT et al., 2005) should be considered.

Barlund et al. (2008) indicated a cut-off of 8% for neutrophils (CB) between 28 and 41 DIM with the reduced value being associated with pregnancy status at 150 DIM, and reported an incidence of endometritis in 11.1% of cows evaluated. Similar results occurred in this study where 14.8% of subclinical endometritis was observed at 42 DIM.

When the values of LVF were compared to those of CB, used as the reference diagnostic test for subclinical endometritis, a moderate, poor, and fair agreement was observed at 21, 28, and 42 DIM, respectively (Table 4). These agreement should be considered in comparative studies of different techniques to characterize SE, since studies have utilized either LVF (GILBERT et al., 2005; SANTOS et al., 2009; BACHA; REGASSA, 2010; SALASEL; MOKHTARI; TAKTAZ, 2010) or CB (KASIMANICKAM et al., 2004; PLONTZKE et al., 2010) but not both techniques. Barlund et al. (2008) described a more elevated agreement relative to the CB and LVF techniques (Kappa statistic 0.72), and suggested that LVF has a sensibility of 92.3% and specificity of 93.9% for the diagnosis of SE in cows.

Table 4. Comparison of Low-volume uterine flush technique using >18% neutrophils (d21, d28) and > 10% neutrophils (d40) cut-off with cytobrush technique as the reference diagnostic test for subclinical endometritis

Diagnostic Technique	DIM	Sensitivity %	Specificity %	Kappa (*P* value)	Fisher Exact
Low-volume uterine flush technique	21	100.0	53.3	0.48 (<0.01)	<0.01
	28	50.0	35.0	-0.09 (0.25)	0.64
	42	66.7	78.9	0.32 (0.05)	0.17

Low-volume uterine flush technique- cut-off = >18% (d21, d28) and >10% (d42).
Source: Elaboration of the authors.

Gilbert et al. (2005) observed 53% of SE (>5% neutrophils – LVF) in cows between 40 and 60 DIM (*n*=141), with prevalence varying from 37% to 74% between five herds. The values (26.1%) of SE obtained (>5% neutrophils – LVF) during this study, at 42 DIM, were lower than previously reported (GILBERT et al., 2005) probably due to a more frequent clinical evaluation during post-partum which resulted in the diagnosis and early exclusion of cows with puerperal metritis, clinical endometritis, or other systemic disease. Maybe the exclusion of animals with purulent secretion at d21 and those with mucopurulent secretion at day 28 might have an effect on the percentage of neutrophils observed. Additionally, the cows during this study demonstrated a relatively low milk production (20.8 liters/day) and the adequate diet during this phase reduced the risk of negative energetic balance. Dietary factors such as energy balance might be important for neutrophil function and the immune response (HERATH et al., 2006).

Additional studies are required to establish adequate criteria for the interpretation of SE in crossbred dairy cows by CB and LVF techniques and to evaluate the effects of SE on the reproductive performance of crossbreed cows. These data already exist for Holstein cows maintained in intensive housing conditions.

In conclusion, the average number of neutrophils was significantly higher throughout the experiment for LVF, but a reduced percentage of neutrophils were observed during the postpartum period for both techniques. The amount of macrophages, lymphocytes, and eosinophils were not affected during postpartum and there was no significant difference relative to these cells when the two techniques were compared. There were significant differences in the percentage of cows with subclinical endometritis only at d28 by CB (22.2 %) and LVF (59.3 %). The cytological endometritis incidence in crossbred dairy cows was 26.1% at low-volume uterine flush technique and 14.8% at cytobrush technique at 42 DIM.

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