Validation of a simple method for the interpretation of uterine cytology in cows

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ABSTRACT: One of the main drawbacks of using endometrial cytology in cows is the time required for sample collection and interpretation. It is recommended to count a large number of polymorphonuclear neutrophils (PMN) and to calculate their overall percentage. However, since counting a large number of cells is a laborious method, it would be preferable to simplify the analysis by counting the number of PMN in few microscopic fields. Therefore, the aim of this study was to assess whether a simple test, based on calculating the average number of PMN in 10 fields at 1000×, could be a reliable technique for the diagnosis of endometritis. Two hundred and sixty endometrial samples were taken from Holstein cows at different postpartum stages using an adapted cytobrush. Smears obtained were air-dried for fixing and stained with a Romanowsky-type procedure. To evaluate the counting method, the percentage of PMN in 150 cells was calculated as well as the average number of PMN in 10 fields at 1000×. Receiver operating characteristic (ROC) curves was constructed to evaluate both methods, the percentage of PMN (used as reference) and the average number of PMN. It was observed that the area under the curve is (regardless of cut-off used) higher than 0.99 and the correspondence between both methods were 1.58 PMN/field for the cut-off value of 15% and 2.40 PMN/field for the cut-off value of 20%. These results show that this simple method could be used to determine the percentage of PMN in endometrial cytological samples and to diagnose endometritis in cows.

Keywords: subclinical endometritis; dairy cattle; polymorphonuclear neutrophils; ROC curves

Subclinical endometritis is defined as an inflammation of the endometrium without systemic illness or signs, and it is associated with delayed uterine involution (Kasimanickam et al. 2004). There could be several causes for an increase in the susceptibility of the uterus to trauma and infections resulting in syndromes ranging from mild endometritis to toxic metritis (Kasimanickam et al. 2005). Numerous risk factors are implicated in the occurrence of uterine disease in dairy cows, including retained placenta, dystocia, twins, parity, management, environment, and genetic influence (Coleman et al. 1985). Such pathologies have a negative effect on reproductive performance because they increases services per conception, the calving to first-service interval and the calving to conception interval, thereby reducing the conception rate (Fourichon et al. 2000; Heuwieser et al. 2000; Leblanc et al. 2002).

There are several techniques for the diagnosis of the subclinical endometritis that will be discussed here briefly, but only some of these can be applied to the collection of the endometrial cells. In these techniques, endometrial and inflammatory cells may be collected by using a guarded cotton swab (Studer and Morrow 1978), uterine biopsy (Bourke et al. 1997), uterine lavage (Hammon et al. 2001), or cytobrush (Glenthoj et al. 1986). Both uterine lavage and cytobrush are less invasive techniques than uter-

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ine biopsy (Kasimanickam et al. 2005). Cytobrush is a less harmful technique for the endometrium than the uterine lavage, since the fluid produces endometrial irritation (Brook, 1993). Saline solution also increases the time required to obtain samples, causes a 17% of failure in attempts to recover fluid and increases the distortion of cells harvested by the lavage technique (Kasimanickam et al. 2005)

Endometritis in dairy cows is one of the most controversial topics among practitioners due to the lack of a diagnostic gold standard (Kasimanickam et al. 2005). Multiple bacterial species have been isolated from more than 90% of cows during the early postpartum period, but the prevalence of endometritis decreases over time (Elliot et al. 1968). Neutrophils constitute the first defensive barrier against invading pathogenic organisms postpartum, resulting in an increase in the PMN population within the uterine lumen (Butt et al. 1993). Investigators have reported differing findings about the percentage of neutrophils that indicates subclinical endometritis in cows. Hammon et al. (2006) established as subclinical endometritis-positive those cows with counts of PMN > 25% performed at 28 ± 3 days postpartum, while others established percentages higher than 18% or 10% in endometrial samples collected between days 21 to 33 or 34 to 47 after calving, respectively (Kasimanickam et al. 2004; Sheldon et al. 2006). On the other hand, it has been reported that more than 5% of PMNs is a significant cut-off point for the endometrial inflammatory response in cows sampled using uterine lavage between 40 and 60 days postpartum (Drillich et al. 2005; Gilbert et al. 2005). Differences have been observed when samples are evaluated by different technicians (Santos et al. 2009), and it could be recommended to control these between-technician variations.

Although endometrial cytology in cows is not a complicated technique, an easier procedure could be implemented. In the present study, the correlation between the number of inflammatory cells (PMN) using two different procedures is evaluated, and it is hypothesised that the average number of PMN in 10 fields could be as efficient as the total percentage of PMN.

MATERIAL AND METHODS

For this study, 260 endometrial samples were obtained from apparently healthy, mature cows. All animals were subjected to an exhaustive examination including rectal palpation, vaginoscopy and ultrasonography to rule out clinical endometritis. Samples were taken from milking cows around day 35 to 45 postpartum.

The endometrial cytology was taken with an adapted cytobrush over a stainless rod with a sanitary plastic sleeve. It was introduced throughout the cervix as far as the uterus corpus. Then, the brush was rotated over the uterine wall and withdrawn. Samples were prepared by rolling the brush onto a clean prelabelled glass slide, followed by air-drying. The smears were stained with a Romanowski stain (fixant, eosin and blue solutions for fast staining, Panreac, Barcelona, Spain) before microscopic examination.

The evaluation was carried out following two different procedures:

(A) Determination of a total of 150 cells at 1000× and assessment of the percentage of PMNs. This procedure was considered as the reference proof.

(B) Determination of PMNs in 10 fields at 1000× and assessment of the average number of PMN per field. This was considered as the procedure for validation.

A pearson correlation between both procedures was carried out. The cut-off points corresponding to 5% and 30% of two differential PMN counts for subclinical endometritis diagnosis were determined using the receiver operating characteristic (ROC) curve.

ROC curves were used to determine similarities of the different cut-off values. Only one specific cut-off value was not used because it varied according to the interval from calving to sample collection.

ROC curves are an important and unifying tool in the process of assessment and diagnosis in dichotomic variables (prevalence or absence of disease). The equivalence between the test method (percentage PMN in the sample) and the specificity and sensitivity of the cut-off point of the test to validate (mean PMN per field in 10 fields) for the different cut-off points was evaluated. All statistical analyses were performed using the SPSS 15.0 statistical software package.

RESULTS AND DISCUSSION

The correlation between the reference (percentage of PMNs) and the proposed (average number

Table 1. Cut-off points corresponding to two different PMN counts, obtained with ROC (receiver operating characteristic) analyses

Percentage of PMN (procedure A)	Area under the curve	Mean of PMN in 10 fields (procedure B)	Sensitivity (%)	Specificity (%)
30	0.995	7.70	100	98.4
25	0.993	2.40	100	96.8
20	0.994	2.40	100	97.2
15	0.997	1.58	100	98.0
10	0.996	1.13	100	97.5
5	0.992	0.52	100	95.3

of PMN) procedures was significant (r = 0.843, $P < 0.01$).

As shown in Table 1, the highest sensitivity was always obtained, and the specificity was also high (over 0.95). The obtained results demonstrate that all positive samples could be diagnosed and negative samples were undetected only in a very low proportion. The reference procedure (A) needed a longer time period for smear evaluation, due to the high number of cells for counting. However, less time was necessary for cell recounting when the proposed procedure (B) was used. In spite of the time required for making the diagnosis, the critical point is the establishment of a procedure that can be carried out in farms. Procedure B has been demonstrated to be a fast, easy and accurate technique for subclinical endometritis diagnosis.

Other attempts to simplify the evaluation of endometrial cytology consisted in the subjective assessment of subclinical endometritis, used by Santos et al. (2009) in beef cattle. A subjective technique using 5.5% of PMN as a cut-off point was validated, obtaining a correlation of 0.83, specificity of 100%, sensitivity of 78% and area under the curve of 0.951. The mentioned values were lower than the ones obtained in the present study. The time required for a subjective assessment used by Santos et al. (2009) and for the count of PMN in 10 fields is very similar. The better results and similar time necessary in the procedure proposed here affirm the suitability of the method for the diagnosis of endometrial cytology in cows. Moreover, subjective assessments should be validated with each observer, and results could be biased.

In summary, it can be concluded that endometritis (clinical or subclinical) diagnosis can be carry out determining the average of PMN in 10 fields, which results in the same efficiency as that obtained by calculating the total percentage of PMN. Importantly, the proposed method gives results faster and is easier to carry out.

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