

**The advantages of using cytopins of uterine lavage fluid for the diagnosis of
equine endometritis**

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Abstract

Uterine lavage (UL) is a routine diagnostic procedure for endometritis. In UL the fluid is centrifuged and the sediment smeared. Samples prepared in cytocentrifuges, the so-called “cytospins”, are useful for evaluating cells in fluids, but never been used in UL. The aim of this study was to assess the usefulness of cytospins after UL, comparing automatic *versus* manual cytocentrifuges, and to determine their value for the diagnosis of endometritis. The study was divided in two parts. Firstly, UL was performed in 16 mares and a small part of the retrieved fluid was cytocentrifuged in an automatic (PreCyto) and manual (PreMan) cytocentrifuge, whereas the remaining fluid was centrifuged. After that, the sediment was divided into three quotas. One quota was smeared, one was processed in an automatic cytocentrifuge (PostCyto) and the last quota was cytospinned in a manual apparatus (PostMan). Cytospins obtained were scored for cellularity, cell preservation, presence of inflammatory cells, bacteria and contaminants; results were compared with sediment smears. Secondly in this study, the best cytospin method was compared with sediment smears in another group of 13 mares, which had endometrial biopsy after UL. Agreement between sediment smears and cytospins was poor to moderate. Compared to sediment smears, cytospins were more cellular, with better morphological details. Urine crystals and fecal contamination were detected more often in cytospins (especially PostCyto and PostMan). No differences in the percentage of inflammatory or epithelial cells existed. PostMan was considered the best method to evaluate UL fluid and it had higher sensitivity (80%), compared with sediment smears (60%), for diagnosing endometritis. Cytocentrifugation offers significant advantages over sediment smears and the manual cytocentrifuge is well suited for horse stable conditions.

1. Introduction

The evaluation of the reproductive tract in mares includes various procedures ranging from the simple observation of the genitalia, up to the uterine sampling. The latter can be achieved by a histological biopsy or by cytology, using either a cytobrush, a cotton swab or a uterine lavage (UL). An endometrial biopsy collects a fragment of tissue, whilst UL comprises epithelial cells (luminal/glandular) and inflammatory cells, as well as fluid that spreads over the entire uterus [1]. Therefore, it may provide a more accurate diagnosis of endometrial conditions, compared with cytobrushes or cotton swabs that only sample a few spots [1-3]. In fact, several authors consider UL more sensitive than swabs or cytobrushes for the diagnosis of mare endometritis [1,4,5]. Following the conventional UL procedure, used since the technique was first described, the recovered fluid is bulk centrifuged (i.e., centrifuged in large tubes) and the sediment is smeared over a slide [2]. It has been shown [6,7] that cell recognition is harder in sediment smears compared with cytocentrifuged preparations — the so-called “cytospins” — in different fluid samples from horses, such as bronchoalveolar lavage (BAL). In sediment smears, cells tend to be smaller and dark staining [6,7], and the differential cell count (namely the percentage of macrophages and lymphocytes) differs from cytospins [7]. In these latter, cells are concentrated and automatically appear in a monolayer over a circular area of the slide, enabling a fast and more reproducible observation. Cytospins are nowadays recommended for the analysis of BAL in horses [8,9,10], but their utility has never been assessed in UL.

Any new cytological diagnostic method for the evaluation of the reproductive tract of mares should be harmless to the endometrium and able to isolate a high number of cells that could be readily identified by optical microscopy. Moreover, in order to be accepted by daily practice, such new method should be quick and relatively straightforward to use

and, ideally, it should not be expensive or depend on heavy equipment, so that it can be easily carried out in horse stable settings [11]. Recently, a low cost and portable manual cytocentrifuge was developed for fluid samples of dogs and cats [12], but this equipment has never been tested for UL of mares. Considering the advantages of cytopins over sediment smears and the portability and low cost of a manual apparatus, we hypothesized that cytopins would be useful for cytological evaluation of uterine lavage fluid (ULF). This study had a dual aim: to compare sediment smears (i.e., smears made from the sediment after bulk centrifugation, which is the conventional method in UL) with cytopins of ULF and to determine their sensitivity and specificity in the diagnosis of endometritis. Considering that cytocentrifugation already concentrates the cells in fluids to a certain extent, we also assessed the potential utility of cytopins obtained directly from ULF (i.e., prior to bulk centrifugation of ULF), as well as of cytopins obtained after bulk centrifugation. In addition, we evaluated the feasibility of a manual cytocentrifuge, which is affordable and portable, suited to daily horse stable conditions. To achieve those goals, this study was divided in two parts. Firstly, we compared the various cytocentrifugation approaches with sediment smears in a group of mares to find out the best method. Secondly, we compared the best cytocentrifugation method with sediment smears in another group of mares that had endometrial biopsy specimen taken after UL to determine if they exhibited inflammation in histology.

2. Materials and methods

All procedures were approved by the institutions' animal Welfare Committee (ORBEIA; Ref. P 211/2017). The investigation was divided in two parts (Fig. 1): in part I, we compared sediment smears with different cytocentrifugation approaches and in part II we

performed UL followed by endometrial biopsy, which is the gold standard for the diagnosis of endometritis in the mare [13,14].

2.1. Part I: Comparison of cytopins and sediment smears

Uterine samples from sixteen mares of various breeds, aged 4 to 24 years were included in this part of the study (Supplemental File 1). Seven mares were multiparous and nine were nulliparous mares subjected earlier to artificial insemination and embryo collection.

2.1.1. Uterine lavage

Samples were taken when the animals displayed estrous behavior after teasing with a stallion, and had a follicle ≥ 30 mm and edema of the endometrial folds, as detected by transrectal ultrasonography. The UL was carried out as described earlier [4,5]. Sterile Ringer's lactate (250 mL) was infused into the uterus and recovered by gravity flow in four sterile 50-mL conical tubes, after gentle massage of the uterus *per rectum*.

Generally, there was a variation in the opacity of the fluid recovered into the different tubes. A small volume (200 μ l for each cytopin) was retrieved directly from the tube containing the most opaque fluid and cytopin preparations were obtained using two methods: automatic cytocentrifugation (PreCyto) and an alternative manual spinner (PreMan). Afterwards, the four conical tubes with the effluent uterine fluid were spun in a bulk centrifuge (Sigma 2-16P®, Sigma Laborzentrifugen GmbH, Osterode, Germany) for 5 minutes at 1200 x g. The supernatant was aspirated and the sediment resuspended in ≈ 0.3 mL fluid. Generally, only a single tube formed a sediment, while in three mares a sediment existed in two tubes. In those cases, sediments were resuspended and mixed. Afterwards the resuspended sediments were divided in three quotas for: 1) sediment smear, where a small portion (two droplets) of the pellet were spread into a slide, which

represents the conventional procedure [4,15]; 2) cytopsin using an automatic cytocentrifuge (PostCyto); 3) cytopsin using the manual spinner (PostMan).

2.1.2. Cytopsin produced by the automatic cytocentrifuge (PreCyto and PostCyto)

Samples were centrifuged for six minutes in StatSpin Cytofuge 2® (Cytofuge 2® Inc, Norwood, Massachusetts, USA) at 140 x g (corresponding to 1,600 RPM), following manufacturer's recommendations. Reusable cell concentrators (VWR cat 720-1972, Fontenay-Sous-Bois, France), with disposable paper filters with a central hole of 7.25 mm (VWR cat 720-1973) fixed with metal holder clips (StatSpin® cat FFCL) were used. The paper filter was overlaid on glass slide and these were introduced in the plastic cell concentrator. The assembled set was fixed by the metal holder clip (Fig. 2). The concentrator conic funnel (Fig. 2) was loaded with 200 µl of UL in PreCyto and with 100 µl of resuspended sediment in PostCyto. After centrifugation, the metal holder clip was removed and the cell concentrator and paper filter were carefully detached without damaging the fresh cytopsin.

2.1.3. Cytopsin produced by the alternative manual spinner (PreMan and PostMan)

For the manual cytocentrifuge, a commercial salad spinner (Zyliss® cat 15201, Diethelm Keller brands, Zurich, Switzerland) was used as detailed elsewhere [12] (Fig. 2 and Supplemental File 2). The spinner, plastic made, includes an outer bowl with an inner removable wide-mesh basket. The cover contains a spinning mechanism operated by constantly pulling a handle. Styrofoam cushions hold the same material as the automatic cytocentrifuge (reusable cell concentrators, disposable paper filters, and metal holder clips), which is fixed to the basket of the spinner by rubber bands. Cell concentrators are aligned to guarantee centrifuge balancing. Up to six concentrators can fit in the basket.

The handle was pulled continuously for 5 minutes [127 x g equivalent to 1,150 RPM, as measured by a digital tachometer (DT-2234C, Rinch Industrial, China, accuracy \pm 1 RPM)]. In PreMan the funnel of the cell concentrators was loaded with 200 μ l of UL, whereas in PostMan 100 μ l of the resuspended sediment was used. To maximize cell recovery, samples were spun within few seconds after filling the concentrators.

2.1.4. Qualitative and quantitative comparison between cytological samples

All cytological slides were air-dried, stained with a commercial Romanowsky-type stain (Hemacolor, Merck, Darmstad, Germany) and mounted with mounting media (Coverquick 2000, VWR Chemicals, Fontenay-Sous-Bois, France). For the qualitative comparison between methods, slides were coded and examined by a board-certified cytopathologist (MC) blinded to the method. Samples were assessed by scoring on a 1 to 3 scale the cellularity (1 = low, 2 = moderate, 3 = high) and cell preservation (1 = poor, 2 = moderate, 3 = good) of epithelial cells. The presence of neutrophils, eosinophils, lymphocytes, macrophages and erythrocytes was also assessed with a 1 to 2 scale (1 = absent, 2 = present). Likewise, the presence of contaminants (fecal and urinary material) and bacteria was also screened. For the latter, we also recorded if bacteria appeared phagocytized (1 = no, 2 = yes).

To further compare the methods, a differential count evaluating 400 cells was made to determine the number of neutrophils, eosinophils, lymphocytes, macrophages, erythrocytes and epithelial cells. The percentage of neutrophils in relation to epithelial cells (%N) was calculated by dividing the number of neutrophils by the number of neutrophils plus epithelial cells in the 400 cells differential. When the morphological identification of cells was not possible they were assigned as unclassifiable and their number recorded.

2.2. *Part II: Comparison of cytological methods with endometrial biopsy*

For this part of the study, a second and different group of 13 mares was used. Mares aged 4 to 26 years of various breeds had reproductive problems (Supplemental File 3) and the procedures (UL and biopsies) were included for the diagnosis of endometritis. In this case, UL was performed as previously described and cytological samples were obtained using two methods only (sediment smears and the best cytocentrifugation method as assessed in Part I) (Fig. 1). Endometrial biopsies were taken within 15 min of UL. It should be stressed that it was already shown that UL prior to endometrial biopsy does not affect the number of neutrophils in endometrial vessels or tissues [16].

2.2.1. *Endometrial biopsies*

Biopsies were obtained as detailed elsewhere [13,16]. The collected material was fixed in formalin, routinely processed and stained with Hematoxylin-Eosin. Specimens were evaluated by light microscopy by two observers (MS, RM) blinded to the cytological classification. The presence of neutrophilic infiltration of the luminal epithelium or stratum compactum was assessed: if three or more neutrophils occurred per five fields (400x magnification), the sample was considered positive for endometritis [1,4,5].

2.2.2. *Comparison of cytopspins and sediment smears with histopathology*

As mentioned, only cytopspins obtained by the best method of Part I (PostMan) and sediment smears were considered. All procedures were similar to those previously described, except that only a quantitative comparison was performed: 400 cells were also counted to determine the %N. When the morphological identification of cells was not possible they were assigned as unclassifiable and their number recorded. It is opportune

to mention that the researchers performing the cytological quantifications (RM, TR) were blinded to histopathology results. Afterwards, the cytological results were compared to the histopathology, which was taken as the gold standard for diagnosing endometritis [1,13,14], in order to determine the sensitivity and specificity of the methods.

2.3. Statistical analysis

The software SPSS18 (IBM, Armonk, USA) was used. The differences between scores were assessed using the Wilcoxon signed-rank test, with a Bonferroni correction (statistical significance set at $p < 0.05$). The agreement between the four cytopsin methods of Part I was assessed with kappa statistics. For interpreting the strength of agreement, the following standards were considered: ≤ 0.40 = poor, $0.41-0.60$ = moderate, $0.61-0.80$ = good and $0.81-1$ = almost perfect [17]. For the differences in the %N and in the percentages of other cells (eosinophils, lymphocytes, macrophages, erythrocytes and unclassifiable) the Mann-Whitney U test was applied. The sensitivity, specificity, and positive and negative predictive value for sediment smears and cytopsin (PostMan) were calculated.

In Part II, a receiver operating characteristic (ROC) curve analysis was performed to determine the best cut-off for those methods, the accuracy of the methods and to test for differences between them. These were done using R 3.6.1 (R Core Team, 2020) and the pROC package [18]. Unless stated otherwise, all data is presented as mean \pm standard deviation.

3. Results

Part I: Comparison of cytopsin and sediment smears

Processing samples in automatic and manual cytocentrifuges resulted in good quality cytopspins. The cellular distribution was homogeneous over the circular area, which roughly corresponds to the area covered by the X 4 objective. This contrasted with sediment smears, in which cell distribution was heterogeneous, as the cells were packed preferably over the leading edge of the slide (Fig. 2). As expected, cytopspins obtained prior to bulk centrifugation (*i.e.*, PreMan and PreCyto) were much less cellular compared to those obtained after bulk centrifugation (*i.e.*, PostMan and PostCyto). Still, the cytopspins obtained by the automatic and manual methods were roughly similar (Fig. 3). Cell lysis was less frequent in cytopspins compared with sediment smears and more cells were available for evaluation and counting, with better cellular detail. In epithelial cells, the chromatin was crisper and nuclear details enhanced in cytopspins comparing with sediment smears; there, chromatin tended to be smudged, with less nuclear details. In addition, some epithelial cells were difficult to recognize as such (Fig. 4). Unclassifiable cells were more frequent in sediment smears than in cytopspins. In these, the amount of unclassifiable cells was similar between the manual and automatic methods (data not shown). Curiously, the epithelial cells in cytopspins often tended to lose their ciliated tufts — the so-called ciliocytophthoria. Therefore, detached ciliated tufts and individual cilia often appeared free in the background (Figs. 4-5). With regard inflammatory cells, neutrophils appeared well spread over the background in cytopspins, and their recognition was easy.

The recognition of macrophages and lymphocytes was sometimes difficult, particularly in sediment smears, where macrophages should be differentiated from poorly preserved non-ciliated epithelial cells (Fig. 5A). Lymphocytes should be differentiated from basal epithelial cells (Fig. 5B). Eosinophils were easily recognized in cytopspins, by their typical large round and orange granules (Fig. 5C).

Contamination was recognized in cytospins, mostly on PostCyto and PostMan samples. Pollen grains (Fig. 6A) and fungal elements, namely of *Alternaria* spp. (Fig. 6B), were assumed to be fecal contaminants and were only observed in PostCyto (3/16) and PostMan (4/16). Calcium carbonate crystals (Fig. 3) were also observed in three mares. In one, crystals were detected in all samples (including sediment smears), while in the other two mares, they appeared only in PostCyto and PostMan.

The agreement between smears and cytospins for morphological details was poor to moderate (Supplemental File 4). The average scores for the parameters assessed are depicted in Table 1. The total score was calculated by adding up the scores of individual parameters. This was significantly lower in sediment smears (11.9 ± 1.4) comparing with PreCyto (14.8 ± 1.7) ($P < 0.001$) and with PreMan (13.7 ± 1.4) ($P = 0.001$). This difference in total score was more noticeable when sediment smears were compared with PostCyto (16.1 ± 1.3) ($P < 0.001$) and with PostMan (15.7 ± 1.7) ($P < 0.001$) samples. By comparing the total scores, differences existed between the pairs PreCyto and PostCyto ($P = 0.002$) and PreMan and PostMan ($P = 0.005$), but not between the pairs of samples obtained prior (PreCyto and PreMan) and after bulk centrifugation (PostCyto and PostMan).

The percentage of cells observed in sediment smears and in cytospin samples is depicted in Supplemental File 5. No differences in the percentage of cells were observed between methods. The %N in sediment smears was different from that on PreMan ($P = 0.03$), but no differences existed for other methods (Supplemental File 5). Considering the threshold of 5% for the %N [1], seven mares (out of 16) would have the cytological diagnosis of endometritis after assessing the sediment smears. Using the same threshold for cytocentrifugation methods, a diagnosis of endometritis would be reached in 11, 12, 12 and 10 in PreCyto, PreMan, PostCyto and PostMan, respectively. As such, the agreement

between sediment smears and other methods for a cytological diagnosis of endometritis was poor for PreCyto [$\kappa = 0.28$ IC95% (0.12-0.69)], moderate for PreMan and PostCyto [$\kappa = 0.41$ IC95% (0.66-0.76)] and good for PostMan [$\kappa = 0.64$ IC95% (0.29-0.98)]. Considering the agreement between PostCyto and PostMan together with a similar total score, PostMan was elected as the best cytopsin method and used in the Part II of this study.

Part II: Comparison of cytopsin (PostMan), sediment smears and histopathology

No differences in the percentage of epithelial cells, neutrophils, lymphocytes, macrophages and %N existed between cytopsin (PostMan) and sediment smears in this group of animals. Still, a difference existed in the amount of unclassifiable cells [$13.0\% \pm 6.6\%$ and $25.2\% \pm 8.8\%$ in PostMan and sediment smears, respectively ($P = 0.01$)].

The gold standard for the diagnosis of endometritis (endometrial biopsy) allowed us to assess the %N by ROC curve analysis and to determine the area under the curve (AUC) (Table 2). Considering the best thresholds for sediment smears and cytopsin (PostMan), six mares (out of 10) were correctly diagnosed with the former, whereas eight mares (out of 10) were correctly diagnosed with the latter (Table 3). It is opportune to mention that no statistically significant difference was detected between the two ROC curves.

4. Discussion

Cytopsin have been used for the analysis of BAL in horses [8,9,10], but this method has never been used in the assessment of UL. The results of our study suggest that cytopsin should be used as first choice in UL since all types of uterine cells are recovered and significant gains in morphological preservation and diagnostic sensitivity are obtained. Cytopsin (especially when used after bulk centrifugation) recover more cells and

improve the recognition of urinary and fecal contamination. Notably, it has been established that endometrial cytology is a valuable method for diagnosing urine pooling in mares [19]. According to our results, such assessment can be further improved by the use of cytopsin preparations, which allow an almost immediate recognition of crystals since all the sample is confined into a small circular spot.

Recognizing fecal contamination is another advantage of cytopsin obtained after bulk centrifugation. It is well established that bacterial contamination from the caudal genital tract can occur with all sampling methods: swabs, cytobrushes and UL [1]. It is important to recognize contamination, as it may explain positive culture results, particularly when three or more species grow [1]. Remarkably, it has been shown that ULF generates more positive cultures (and more contamination) than swabs or biopsies [5]. In part I of this study, two out of four mares with fecal contamination had a non-inflammatory cytology (i.e., low numbers of neutrophils). Although microbiology was not done, it seems reasonable to suggest that the culture of ULF in those animals would probably have yielded growth of multiple bacterial species (i.e., false positive results). It has been shown that combining multiple tests increases the accuracy of the diagnosis of endometritis [1]. Herein, we described the presence of *Alternaria* spp. and pollen grains in UL. These are rare contaminants, occasionally described as contaminant in cervicovaginal smears of women [20,21]. To the best of our knowledge, these contaminants have never been described in ULF of mares. In women, pollen grains in cervicovaginal smears have been associated with genital lavage with vegetal components [20]. Pollen grains should be differentiated from parasite ova by their larger size, refractive appearance and thick wall [22]. As to *Alternaria* spp. it has typical septate conidia and a brownish color, being a common plant pathogen [22]. We hypothesized that *Alternaria* spp. and pollen grains were probably ingested during grazing, and their appearance in ULF was likely due to

fecal contamination since their presence was restricted to mares that had this contamination. If it was due to the environment, we would expect to see *Alternaria* spp. and pollen grains in more mares, namely in those without fecal contamination.

It has been established for long that smears of ULF prepared directly from the liquid (i.e., without bulk centrifugation) contain insufficient cells [19]. Our results suggest that cytopins prepared directly from the ULF liquid could be a choice, since a sufficient number of cells with better morphology (compared with sediment smears) is still recovered. In this sense, bulk centrifugation can be obviated, and this faster procedure may be relevant for veterinarians working in horse stable conditions, looking for a quick assessment of ULF. Still, we recommend the use of cytopins obtained after bulk centrifugation (PostCyto or PostMan), since more cells are recovered and there is a significant gain in morphological details. It has been reported that sediment smears of ULF produce many distorted cells [5,15] — as we also observed — more than in cytobrush samples or endometrial swabs [15], which makes sediment smears more difficult to evaluate [4,5]. Our results suggest that cytopin preparations allow an easier assessment of ULF, due to good cellularity and better morphology, with few unclassifiable cells.

The only disadvantage of cytopins is the ciliocytophthoria, an artifact of the preparation method. This has no clinical relevance, since ciliated tufts or individual cilia stain pink, making them impossible to be interpreted as bacterial rods (which are blue stained with any Romanowsky-type stain). Herein, ciliocytophthoria has no pathologic or physiologic significance and should not be confused with the loss of ciliated tufts from epithelial cells in the fall transition of mares (related with changing of hormonal status) [19]. Another potential disadvantage could be the cost of a cytopin centrifuge, but we showed that the preparations obtained by manual and automatic methods were comparable, and we

recommend the use of the manual method (PostMan) for the general assessment of ULF. The salad spinner costs about 100 times less than a professional cytopspin centrifuge and the cost of each analysis is small, limited to the price of the slide and filter. All other material (such as cell concentrators and metallic clips) are reusable and affordable. Sediment smears and cytopspins have been compared in other types of horse fluids, such as BAL [7]. Apart from the benefits in cell morphology, it has been shown that cytopspins tend to lower the percentage of lymphocytes, whilst increasing the percentage of macrophages [7]. This does not seem to have occurred in our study because lymphocytes were always observed in cytopspins. Nevertheless, lymphocytes and macrophages have less clinical importance than neutrophils and %N, which are the cornerstones to identify acute endometritis [1]. It should be emphasized that the %N can vary with the sampling methods [1,23], being reported to be higher in sediment smears of ULF, comparing with swabs and cytobrushes [15]. Our results suggest that the percentage of neutrophils and the %N of cytopspins are similar to that of sediment smears of ULF.

The thresholds for cytological diagnosis of endometritis have been debated for long, being tuned over the years as a percentage (%N) or number of neutrophils per high power field (HPF) [1,23]. Kozdrowski et al. [14] reported that the %N enabled a diagnosis of more cases of endometritis, with higher sensitivity, of mares in anestrus, diestrus and estrus comparing with neutrophils per HPF. The use of the latter is impracticable in cytopspin samples, because cells become crowded over the circular area of the slide. In cytopspins, the %N seems more reasonable and the threshold for this percentage has ranged from ≥ 0.5 to $>5\%$ [1], since it is generally accepted that normal mares have a low percentage of neutrophils in ULF [1]. In our case, we further refined the thresholds for %N in sediment smears (5%) and cytopspins (4%), with 60% and 80% sensitivity, respectively. It should be stressed that the sensitivity obtained in our study (for the

cytological assessment of sediment smears of ULF) is comparable to other reports (using cytology as a single diagnostic method) [1]. According to our data, a threshold of 4% (i.e., lower than the conventional 5%) should be adopted for cytopins in daily clinical practice. Nevertheless, further studies using a larger number of mares (and particularly more normal, endometritis-free mares), and coupling endometrial cytology, microbiology and biopsy are needed to further elucidate the best threshold and the sensibility/specificity of cytopins in ULF.

The accuracy of a diagnostic test can be evaluated by the AUC. This varies between 0.5 (that represents a worthless test not capable of discriminating normal from affected cases) to 1 (a perfect test that would have 100 % sensitivity without false-positives, across all thresholds). An AUC of 0.93 [IC95% (0.77-1.00)] for cytopins (Table 3) means that there is a 93% chance that the method will distinguish normal mares from those with endometritis [24]. This can be considered as a diagnostic method with excellent discrimination [25]. By contrast, sediment smears had an AUC of 0.77 [IC95% (0.46-1.00)] and can be considered as a diagnostic method with fair discrimination [25]. However, no statistical significant difference was detected between the AUC of the two tests, but this might be due to the small sample size.

In conclusion, the use of cytopins provides samples with better cell morphology, with fewer unrecognizable cells, and grants higher sensitivity for detecting equine endometritis. We recommend the use of cytopins after bulk centrifugation as a first choice in UL. Considering the simplicity and low-cost of a manual spinner, this should be included in the toolbox of veterinarians devoted to equine reproduction, especially those working in horse stable conditions.

5. Conflict of interest

The authors declare that they have no conflict of interest.

References

[1] Katila T. Evaluation of diagnostic methods in equine endometritis. *Reprod. Biol* 2016;16:189-196.

[2] Ball BA, Shin SJ, Patten VH, Lein DH, Woods GL. Use of a low-volume uterine flush for microbiologic and cytologic examination of the mare's endometrium. *Theriogenology* 1988;29:1269-1283.

[3] Riddle WT, LeBlanc MM, Stromberg AJ. Relationships between uterine culture, cytology and pregnancy rates in a Thoroughbred practice. *Theriogenology* 2007;68:395-402.

[4] LeBlanc MM, Magsig J, Stromberg AJ. Use of a low-volume uterine flush for diagnosing endometritis in chronically infertile mares. *Theriogenology* 2007;68:403-412.

[5] Christoffersen M, Brandis L, Samuelsson J, Bojesen AM, Troedsson MH, Petersen MR. Diagnostic double-guarded low-volume uterine lavage in mares. *Theriogenology* 2015;83:222-227.

[6] Lapointe JM, Vrins A, Lavoie JP. Effects of centrifugation and specimen preparation technique on bronchoalveolar lavage analysis in horses. *Equine Vet J* 1994;26:227-229.

[7] Pickles K, Pirie RS, Rhind S, Dixon PM, McGorum BC. Cytological analysis of equine bronchoalveolar lavage fluid. Part 2: comparison of smear and cytocentrifuged preparations. *Equine Vet J* 2002;34:292-296.

[8] Zinkl JG . Lower respiratory tract, in: Cowell, R.L., Tyler, R.D. (Eds.). *Diagnostic Cytology and Hematology of the horse*. St. Louis: Mosby; 2002, p. 73-86.

- [9] Hermange T, Le Corre S, Bizon C, Richard EA, Courouc  A. Bronchoalveolar lavage fluid from both lungs in horses: Diagnostic reliability of cytology from pooled samples. Vet J 2019;244:28-33.
- [10] Allen KJ, Tennat KV, Franklin SH. Effect of inclusion or exclusion of epithelial cells in equine respiratory cytology analysis. Vet J 2019;254:105405.
- [11] Ley WB, Digra  ie WA, Holyoak GR, Slusher SH. Endometrim, in: Cowell RL, Tyler RD, editors. Diagnostic Cytology and Hematology of the horse, St Louis: Mosby; 2002, p. 180-186.
- [12] Marcos R, Santos M, Marrinhas C, Correia-Gomes C, Caniatti M. Cytocentrifuge preparation in veterinary cytology: a quick, simple, and affordable manual method to concentrate low cellularity fluids. Vet Clin Pathol 2016;45:725-731.
- [13] Nielsen JM. Endometritis in the mare: a diagnostic study comparing cultures from swab and biopsy. Theriogenology 2005;64:510-518.
- [14] Kozdrowski R, Sikora M, Buczkowska J, Nowak M, R  s A, Dzieciol M. Effects of cycle stage and sampling procedures on interpretation of endometrial cytology in mares. Anim Reprod Sci 2015 ;154 :56-62.
- [15] Cocchia N, Paciello O, Auletta L, Uccello V, Silvestro L, Mallardo K, Paraggio G, Pasolini MP. Comparison of the cytobrush, cottonswab, and low-volume uterine flush techniques to evaluate endometrial cytology for diagnosing endometritis in chronically infertile mares. Theriogenology 2012;77:89-98.
- [16] Linton JK, Stertich PL. The impact of low-volume uterine lavage on endometrial biopsy classification. Theriogenology 2016;86:1004-1007.
- [17] Vieira AJ, Garrett JM. Understanding interobserver agreement: the kappa statistic. Fam Med 2005;37:360-363.

443 [18] Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, Müller M. pROC:
444 an open-source package for R and S+ to analyze and compare ROC curves. BMC
445 Bioinformatics 2011;12:77.

446 [19] Roszel JF, Freeman K. Equine endometrial cytology. Vet Clin North Am Equine
447 Pract 1988;4:247-262.

448 [20] Accorsi CA, Mazzanti MB, Forlani L, Rivasi F. Pollen grains in human cytology.
449 Grana 1991;30:102-108.

450 [21] Hoda RS, Hoda SA. Fundamentals of Pap Test Cytology. New Jersey; Humana
451 Press, 2007.

452 [22] Pantanowitz L, Goulart RA, Martínez-Giron R. Mimics and contaminants, in
453 Cytopathology of infectious diseases. New York: Springer Science; 2011, p. 351-357.

454 [23] Card C. Post-breeding inflammation and endometrial cytology in mares.
455 Theriogenology 2005;64:580-588.

456 [24] Obuchowski NA. Receiver operating curves characteristic curves and their use in
457 radiology. Radiology 2003;229:3-8.

458 [25] El Khouli R, Macura KJ, Barker PB, Phil D, Habba MR, Jacobs MA, Bluemke DA.
459 The relationship of temporal resolution to diagnostic performance for dynamic contrast
460 enhanced (DCE) MRI of the breast. J Magn Reson Imaging 2009;30:999-1004.

Figure legends

Fig. 1. Experimental design.

Fig. 2. (A) Conventional cytocentrifuge and the material used to produce a cytospin. The filter (2) is inserted in the cell contractor (1), closer to the funnel (arrowhead); after inserting the glass slide (3), the set is fixed with a metal clip (4). The assembled set (detail) goes into the cytocentrifuge and the uterine lavage is poured in the funnel (block arrow); this model holds four cell concentrators per run. (B) In the manual cytocentrifuge, the same material is used. The assembled cell concentrator is fixed to Styrofoam pads and to the plastic basket by rubber bands and fluid is poured into the funnel (block arrow); this apparatus holds 6 cell concentrators per run.

Fig. 3. (A) General appearance of sediment smear and cytospins of uterine lavage prior to bulk centrifugation in a manual spinner (PreMan), automatic cytocentrifuge (PreCyto) and obtained after bulk centrifugation in a manual spinner (PostMan) and automatic cytocentrifuge (PostCyto); samples are from the same mare. In PreMan (B) and PreCyto (C), epithelial cells were seen both individually and in small clusters, neutrophils and a few erythrocytes were observed. Many more cells were recovered in PostMan (D) and PostCyto (E). Besides epithelial and neutrophils, calcium oxalate crystals (urinary contamination) could be observed. (E) In PostCyto the same cell types and contaminants were identified [Hemacolor, 100x and 1000x (inset)].

Fig. 4. Detail of epithelial cells in sediment smears (A) and cytospins (B) (obtained in a manual spinner, PostMan). Nuclear detail is better preserved in cytospins, even if the ciliated tuft is less evident, with more dispersed cilia in the background (arrows) (Hemacolor, 1000x).

Fig. 5. Detail of inflammatory cells in cytospins obtained in a manual spinner [PreMan (A), (B) and PostMan (C)]. A macrophage (arrowhead) and neutrophil (arrow) appear in

(A), along with a ciliated tuft (curved arrow). A lymphocyte and neutrophil (arrow) appear in (B). An eosinophil and neutrophils (arrows) are depicted in (C) (Hemacolor, 1000x).

Fig. 6. Detail of contaminants observed in cytopins [PostMan (A) and PostCyto (B)]. Pollen grains (A) were recognized by their large size and rounded shape, whilst *Alternaria spp.* (B) had a typical septate conidia and brownish color (Hemacolor, 1000x).

Supplemental File 1. Details (breed and reproductive status) of mares included in Part I of the study.

Supplemental File 2. Detail of the procedures needed to convert a salad spinner into a manual cytocentrifuge. For this conversion, rubber bands and rectangular styrofoam pieces are needed, apparat from the specific material of the cytocentrifuge (reusable cell concentrators, disposable filters, and metallic clips).

Supplemental File 3. Details (breed and reproductive status) of mares included in Part II of the study.

Supplemental File 4. Agreement (Cohen kappa) between sediment smears and cytopins obtained by manual apparatus prior to bulk centrifugation (PreMan), conventional cytocentrifuge prior to bulk centrifugation (PreCyto), manual apparatus after bulk centrifugation (PostMan), conventional cytocentrifugation after bulk centrifugation (PostCyto) for the different scored parameters. **Only a single case was detected by sediment smears and PreMan and PreCyto.

Supplemental File 5. Average percentage of neutrophils, eosinophils, lymphocytes, macrophages and epithelial cells in sediment smears and in cytopins obtained directly from the uterine lavage fluid using a manual (PreMan) and automatic cytocentrifuge (PreCyto) and obtained from the pellet (after bulk centrifugarion) using a manual (PostMan) and automatic cytocentrifuge (PostCyto). The percentage of neutrophils in

relation to epithelial cells (%N) is also included. Values are presented as percentages (mean \pm standard deviation). (*) Significant differences to sediment smears.

Table 1. Mean scores for cellularity, cell preservation, presence of neutrophils, eosinophils, lymphocytes, macrophages, erythrocytes and of contamination (urinary and fecal) and presence of bacteria in sediment smears and in cytospins. These were obtained with manual and automatic methods prior (PreMan and PreCyto, respectively) and after bulk centrifugation (PostMan and PostCyto). Except for the first two parameters (1 to 3 scale), all parameters were assessed with a 1-2 scale (1= absent/2= present). (*) Significant differences to sediment smears. (Ψ) Significant differences between PreMan and PostMan. (ϕ) Significant differences between PreCyto and PostCyto.

Table 2. Optimal cut-off of the percentage of neutrophils (%N) in sediment smears and cytospins obtained with the manual apparatus after bulk centrifugation (PostMan). The sensitivity, specificity, positive and negative predictive values obtained by Receiver Operating Characteristic (ROC) curves analyses are also presented.

Table 3. Percentage of neutrophils in relation to epithelial cells (%N) obtained by the evaluation of sediment smears, cytospins (after bulk centrifugation using a manual cytocentrifuge, PostMan) and the histopathological assessment of endometritis (positive/negative) in thirteen mares (Part II of the study).