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Efficacy of Cytobrush, Uterine Lavage and Endometrial Biopsy Techniques for Cytological and Cultural Evaluation of Endometritis in Barren Mares

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Abstract

A study was carried out on 10 barren mares to evaluate comparative efficacy of 3 diagnostic techniques of endometritis, viz., endometrial biopsy (EB), cytobrush (CB) and low-volume uterine lavage (LVL). The samples collected were evaluated cytologically and bacteriologically. CB yielded significantly more PMNs per high power field (HPF) (57.33±0.74) than EB (35.50±1.54) and LVL (14.35±1.05). In LVL, the proportion of PMNs to uterine epithelial cells (1.20%) was higher than in CB (0.86%) and EB (0.48%). EB yielded significantly more intact cells (90%) than CB (60%) and LVL (42%). Distorted cells were significantly more in smears made from LVL (48%) than from EB and CB (9% each). Fragmented cells were significantly more in CB (31%) than EB (1%) and LVL (10%) samples. Inflammatory cells, other than PMNs, were detected in CB and EB, and vaginal epithelial cells only in LVL. According to criterion II (≥1% PMNs/300 cells), endometritis was diagnosed in 30% mares by EB and CB and 40% by LVL. None of the mares was positive as per criterion I (≥1 PMNs/HPF). Positive microbial growth was obtained in 7, 6 and 8 mares using EB, CB and LVL. All the cytologically positive mares were also positive for microbial cultures. Whereas, 30% positive cultures from CB and 40% from EB and LVL had negative cytology. The most common pathogenic bacteria cultured from CB and EB were β-hemolytic Streptococci and E. coli, whereas in LVL, E. coli was isolated more frequently. Staphylococcus spp., Pseudomonas spp. and Yeast were also isolated mostly in combination with other microbes. The combined infections were seen only in EB and LVL samples. Cloudy or mucoid effluxes of LVL were mostly associated with isolation of E. coli and β-hemolytic Streptococcus (5/10). The recovery of Streptococcus was associated with a rise in uterine pH (8.0), but not the E. coli (pH 6.5). In all three methods, number of samples culturally positive for β-hemolytic Streptococcus were always associated with a positive cytology, but not the E. coli.

Keywords: Bacteriology, Comparative Cytology, Cytobrush, Endometritis, Endometrial Biopsy, Low Volume Uterine Lavage, Mare

Introduction

Endometritis, an inflammation of the endometrium, is a common cause of infertility in mares leading to failure to conceive, early embryonic death, and a shortened luteal phase (LeBlanc and Causey, 2009). Endometritis has a significant economic impact due to irregular estrous cycles requiring intensive clinical breeding management and more cycles to become pregnant (LeBlanc, 2010). Endometritis occurs when foreign molecules, often spermatozoa and/or bacteria, are introduced into the reproductive tract, specifically at mating and/or due to general opportunistic Gram positive and Gram negative bacterial contamination (Watson 2000; Riddle *et al*., 2007; LeBlanc, 2008). The inflammatory response to uterine challenge is characterized by an influx of polymorphonuclear neutrophils (PMNs). Clinical signs of endometritis may include intrauterine fluid, endometrial edema, vaginal discharge, abnormal estrous cycles, and cervicitis. In addition, endometrial cytology positive for inflammation and a positive endometrial culture are features of endometritis. Clinical signs may vary depending on the chronicity of disease or type of bacteria involved. Bacteria such as *Pseudomonas aeruginosa* and some yeast and fungi are known to form biofilms (Ferris *et al*., 2014), resulting in persistent, chronic infections, yet such mares are often negative on culture and cytology (Beehan *et al*., 2015).

Endometrial cytology is a readily available diagnostic test to identify mares with endometritis. Low-volume uterine lavage (LVL) evaluates a larger endometrial surface area hence focal lesions will not be missed, but often results in the contamination from vaginal flora and a greater amount of distorted cells (LeBlanc *et al*., 2007, Christoffersen *et al* 2015), while swabbing the endometrium and cytobursh (CB) only samples a small focal area, potentially resulting in false negatives, but the quantity and quality of the cells obtained would be better (LeBlanc *et al*., 2007). On the other hand, chronic degenerative changes and deep inflammation can be diagnosed by uterine biopsy, which is often considered as the "gold standard" (LeBlanc and Causey, 2009). Endometrial biopsy (EB) is safe and useful, but not a practical technique as histopathology is characterized by a long time waiting for the results and the special tools needed for the biopsy collection. Bacteriological examination provides an indirect test for the diagnosis of endometritis in mares. The advantage of cytology is the ability to obtain results as early as the day of collection, while for bacteriological examination it takes 48-72 hrs from the time of collection. The literature on equine endometritis and diagnostic efficacy of different techniques in Indian context is meager. Hence, this study was carried out to compare the cytological and bacteriological status of uterine samples collected by CB, EB and LVL from mares having endometritis.

Materials and Methods

The present work was carried out from October 2018 to May 2019 after approval of IAEC, AAU, Anand on 10 numbers of randomly selected mares with the history of long barrenness/infertility, brought to the Veterinary Clinical Complex of the College in Anand, and from other stud farms of Police in Gujarat. Patient data and detailed history (breeding history) were recorded, following which the animals were palpated rectally and cytological and microbiological sampling of the endometrium was done. Majority of the mares were barren because of failure to conceive or exclusion from breeding for patrolling.

Endometrial Sample Collection

Under all aseptic precautions, a single-guarded CB technique (Mayfair Endocervical brush, Care India Surgicals, Ludhiana, India), EB (Nelson's bovine biopsy forceps) and LVL were performed sequentially under xylazine sedation and manual restraint on each animal. With a sleeved arm, the required instruments were introduced through the vagina and cervix into the uterus. All samples were processed for cytological and bacteriological evaluation.

Briefly, endometrial cytology samples were collected by rotating the CB in a clockwise direction keeping in contact with the uterine wall and then retracted into the outer tube prior to removal from the uterus. Cytology slides were prepared by gently rolling the CB onto a sterile microscope slide and then stored in a transport medium until bacteriological examination was done.

EBs were taken from the base of either uterine horns with a Nelson's biopsy forceps. After the forceps was placed in the uterine lumen, the sleeved arm was withdrawn from the vagina and inserted into the rectum to guide the forceps to the desired place and to put endometrial mucosa into the forceps. Following the collection of the biopsy sample, the jaws of the instrument were opened and the tissue was retrieved into the storage vial with the help of a

sterile artery forceps.

For performing LVL, a sterile i/v infusion catheter was passed per vaginum into the uterus 10-12 cm deep and 60-120 mL of 0.9 % normal saline was infused into the uterus (Plate 1a). The uterus was manipulated trans-rectum for a minimum of 30 sec to distribute the fluid throughout the uterine lumen, left for 2 minutes and was aspirated using a sterile AI sheath, connected to a sterile 60 mL syringe. The aspirated fluid was transferred into sterile 15-mL centrifuge tubes. The gross characteristic of the LVL fluid was recorded by holding the samples up to the light (Plate 1b). It was graded as clear, cloudy or clear with mucus strains (mucoid efflux) (Plate 1). Cloudy or mucoid efflux was graded as positive and clear efflux as negative (LeBlanc *et al*., 2007). The pH of samples was measured with a pH paper and compared to the pH of the 0.9 % normal saline used for the LVL (Plate 1c). After centrifugation of sample, the supernatant was removed and the remaining pellet was re-suspended in 1 mL of saline.

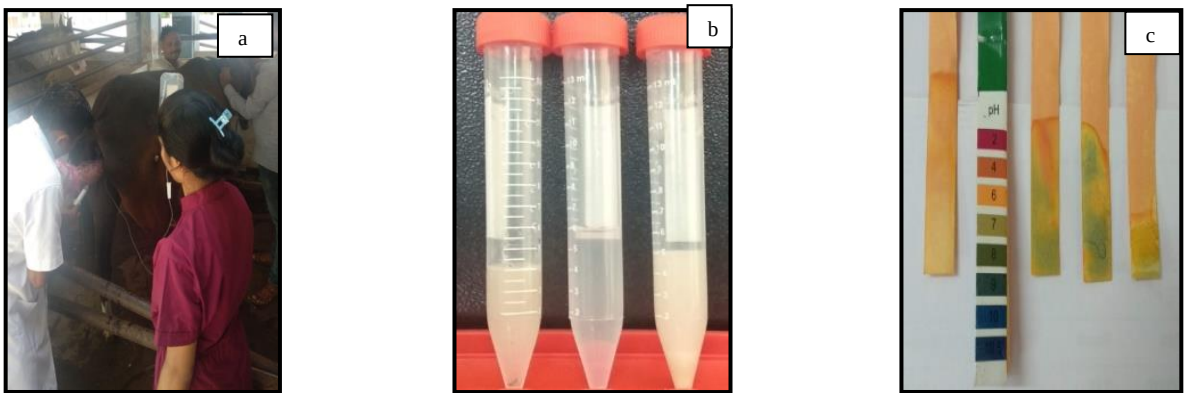

Plate 1: Performing low-volume uterine lavage (a) and Comparison of LVL fluid for cloudiness or mucus strands (b) and pH (c)

Cytological Evaluation

The samples collected by all three techniques (CB, EB, LVL) were immediately smeared onto sterile glass slides, air dried, and fixed with methanol. These smears were then stained with Field's stain and were evaluated microscopically at a high-power magnification (400x or 1000x). Minimum of 10 fields were examined. The smears were evaluated for the presence of epithelial cells, debris, inflammatory cells, bacteria, and yeast. Smears were evaluated as indicative of inflammation using two criteria, (I) ≥ 1 PMNs per HPF in 10 fields, (II) ≥ 1% PMNs of the total 300 epithelial cells (Kozdrowski *et al*., 2013). The total cellularity, presence of debris and the quality of cells collected (% intact cells, distorted cells and fragmented cells) in the cytological smears were also evaluated.

Cultural/Microbiological Evaluation

Samples from each of the three techniques were streaked onto blood agar with 5 % sheep blood, MacConkey agar, Sabouraud dextrose agar and eosin-methylene blue agar within 6 hrs for bacterial culture. After 24 and 48 hrs of incubation at 37°C, plates were examined for growth/presence of bacteria or yeast. Bacteria were identified by their cultural characteristics, Gram staining and basic tests. Mixed cultures of more than three pathogens were considered as contamination. The percentage frequencies and means ± SDs of various observations on different cellular traits studied were calculated and analyzed using descriptive statistics. The findings obtained have been presented in different tables.

Results and Discussion

Clinical Findings

The mean age of mares in the study was 12.6±4.6 years. Out of 10 mares examined, majority of them did not show signs of clinical endometritis as determined by the vaginal discharge or by the presence of intrauterine fluid in ultrasonography. Majority of the animals were barren for more than 8 years. The relationship between age of barren mares and the incidence of endometritis is shown in Table 1. The incidence of endometritis was higher in mares

with abnormal perineal conformation as 83.33 % of mares histological positive for endometritis had abnormal perineal conformation (Table 2).

Table 1: Relationship between age of barren mares and incidence of endometritis (n=10)

Years barren	Endometritis positive	Endometritis negative
<8 years	1 (16.66 %)	1 (25.00 %)
>8 years	5 (83.33 %)	3 (75.00 %)
Total	6 (60.00 %)	4 (40.00 %)

Table 2: Relationship between perineal conformation and incidence of endometritis

Category	Endometritis positive	Endometritis negative
Abnormal perineal conformation	5 (83.33 %)	2 (50.00 %)
Normal perineal conformation	1 (16.66 %)	2 (50.00 %)
Total	6 (60.00 %)	4 (40.00 %)

Operating Speed of the Techniques

Endometrial cytological samples were collected from all mares, first with the CB, then with EB technique, and finally with LVL to avoid irritation of the endometrium caused by the lavage fluid (Roszel and Freeman, 1988). Also, the volume of fluid left in the uterus after the LVL could affect the degree of irritation and thereby the result of EB and CB sampling (Kasimanickam *et al*., 2005). It is arguable that the cytobrush technique may also cause irritation to the endometrium.

All sampling methods were performed by the same operator on the same animal. Sampling by the EB and CB were found to be easy and quick (5 minute on an average) to perform by a single operator in all cases. On the contrary, LVL took 8-12 minutes (10 minutes on an average) to collect the uterine samples. On average, 80 mL (range 60 to 120 ml) of 0.9 % normal saline was used in the process and 25 mL of the fluid was recovered (20-35 mL). Sampling by LVL required the assistance of 2-3 persons. Further, time and laboratory instrumentations were also needed for centrifugation and processing of the LVL samples. The variability in time, quantity of fluid used and aspirated in LVL concurred with the observations made by Cocchia *et al.* (2012), but it took a longer time to perform in mares with a pendulous uterus and the fluid recovery rates were also less.

Cytological Findings of the Smears

1. Background of the Cytological Smears

Slides prepared for the endometrial cytological examinations were all readable and were assessed successfully. The background content of the slides prepared from EB appeared contaminated with red blood cells, while slides prepared from CB appeared clear, whereas slides prepared from LVL appeared contaminated with red blood cells and debris. The presence of RBCs were higher in the lavage technique compared with the CB sampling. This could be due to the trauma induced by the manipulation of the uterus and the action of the infusion catheter while attempting to recover the fluid (Kasimanickam *et al*., 2005). Also, infusion fluid could have even yielded haemorrhage induced by the bristles of the cytobrush, or biopsy instrument previously used. Bourke *et al.* (1997) and Kasimanickam *et al.* (2005) reported the presence of larger amount of proteinacious material and red blood cells in CB samples than either in double guarded swab smears or in LVF smears in mares and cows, and the presence of large amount of erythrocytes was not indicated as typical of LVL smears (LeBlanc *et al*., 2007).

Characteristics of background may be indicative of uterine inflammation. The debris may represent mucus, degenerated neutrophils, damaged epithelial cells, and inflammatory residue and its presence on cytological specimens was associated with isolation of microorganisms similar to the studies conducted by LeBlanc *et al* (2007). Debris were present in a higher percentage of LVL smears and only this technique, which yielded diluted sample of luminal contents, seems to give information about some effects of the inflammation, such as a change in the mucus. On the other hand, clear smears, such as those prepared by CB, facilitated the cells evaluation compared to those

with a proteinaceous background.

2. Total Cellularity of the Cytological Smears

The number of cells (endometrial epithelial cells, neutrophils, other inflammatory cells, vaginal epithelial cells) recovered by the three techniques are shown in Table 3. All smears showed good cellularity. Cytobrush yielded significantly more cells/HPF (57.33 ±0.74) than EB (35.50±1.54) and LVL (14.35±1.05). Also, EB produced significantly more cells than LVL in 10 HPFs.

Martin-Hirsch *et al.* (2005) reported that in cytological studies, the number of cells collected is critical, as it influences the threshold level used to define inflammation. In the present study, all smears showed a good total cellularity. The CB yielded higher number of cells compared with EB or LVL. Fibers from a brush can scrape the endometrial surface and can penetrate deeper, thus enabling collection of a greater number of cells (Overbeck *et al*., 2011). The CB technique is quick, safe, relatively inexpensive, easy to obtain under field conditions and enables the diagnosis of subclinical endometritis. Smears obtained from EB were characterized by the lowest number of harvested cells; nevertheless, all smears showed a good total cellularity. This finding was consistent with the report of Cocchia *et al.* (2012), that the smears with similar or higher numbers of cells/HPF are characterized by a good total cellularity.

Table 3: Total cells/HPF, total PMNs/HPF and ratio of PMNs to uterine epithelial cells recovered from the mare by three diagnostic techniques (Mean ± SD)

Sampling method	Number of cells/HPF	Number of PMNs/HPF	PMN/Epithelial cells ratio (%)	No. of uterine epithelial cells
Endometrial biopsy	35.50^{a} ± 1.54	0.11 ± 0.10	0.48 ± 0.54	298.5
Cytobrush	57.33^{b} ± 0.74	0.17 ± 0.18	0.86 ± 0.73	297.3
Low-volume uterine lavage	14.35^{c} ± 1.05	0.22 ± 0.24	1.20 ± 0.96	296.4

Means bearing uncommon superscript within the column differ significantly ($p<0.05$).

3. PMNs per HPF and PMNs to Uterine Epithelial Cells in Cytological Smears

The LVL and CB produced more PMNs/HPF (0.22±0.24; 0.17±0.18) than EB (0.11 ±0.10). PMNs/HPF collected by CB and LVL did not differ statistically. The proportion of PMNs/uterine epithelial cells (1.20 %) was found to be higher in LVL than in smears made from CB (0.86 %) and EB (0.48 %). CB collected higher proportion of PMNs than EB (Table 3). None of the smears revealed severe inflammation (> 5 PMNs/HPF). Smears without any PMNs were also present in our study.

In order to evaluate the inflammation, two criteria were assumed. According to criterion I (≥ 1 PMN/HPF), none of the smears was found to be positive for endometritis by any of the three methods. According to criterion II (≥ 1% PMN/300 epithelial cells), endometritis was diagnosed in 30 (3/10) % of the mares by EB and CB samples and 40 (4/10) % by LVL. Regardless of the method used for sample collection, endometritis was diagnosed more frequently if smears were evaluated according to criterion II. This finding was in agreement with the observations made by Kozdrowski *et al.* (2015).

All, but one of the 4, mares positive for endometritis by LVL were also positive by CB; one of these was negative by EB. Of the 7 mares negative for endometritis by EB, 6 were also negative by CB and LVL. These data were, however, insufficient to conclude. No consensus exists in the literature as to what constitutes equine endometritis based on endometrial cytology. The cut-off values used to classify mares based on cytologic findings vary between 0.5 % and 5 % neutrophils depending on the study, making comparison across studies difficult (Card 2005, Riddle *et al*., 2007). Riddle *et al.* (2007) used a cut-off value of >2 PMNs per HPF (×400) to diagnose mares with endometritis by endometrial swab cap cytology wherein the mares had a significant decrease in pregnancy rate when > 2 PMNs/HPF were identified on cytology. However, LeBlanc (2011) used a cut-off of ≥1 neutrophil per field (×1,000) in LVL fluid.

De Amorim *et al.* (2016) reported a ratio of 1 % or more neutrophils to epithelial cells to be the most sensitive cytologic assessment for diagnosing endometritis in mares. The PMNs/HPF collected by CB and LVL did not differ

and were found to be more than PMNs/HPF yielded by EB. We found that the method used for smear evaluation could have a great impact on results obtained, because regardless of the method used for sample collection, endometritis was diagnosed more frequently if smears were evaluated according to criterion II. During estrus, even in healthy mares a small amount of PMNs in the uterus is considered to be normal (Overbeck *et al*., 2011), because neutrophil migration into the uterine lumen is higher during maximal estrogen dominance than during progesterone dominance. It was shown that in cases in which 0.5-5.0 % PMNs were found in samples collected in advanced estrus, 37.5 and 17.0 % of mares became pregnant if the specimens were collected using CB or CS, respectively (Kozdrowski *et al*., 2013) indicating that at least some of those mares in fact did not suffer from endometritis (false-positive results).

Martin-Hirsch *et al.* (2005) reported that the structure of the brush, with nylon fibers at the tip of handle and fibers perpendicular to handle, increases collection surface. Compared with both EB and LVL techniques, it allows the collection of cells in more depth within the thickness of the endometrium. Fibers are relatively rigid, and can scrape the endometrial surface and penetrate into the gland, collecting inflammatory cells infiltrating the endometrium. In contrast, the lavage technique allows an increase in the area of endometrium sampled, collecting a diluted sample of luminal contents, mucus and/or exudates, from a large uterine surface (Kasimanickam *et al*., 2005).

4. Quality of Cells Harvested in Cytological Preparations

The EB yielded significantly more intact cells (90 %) than CB (60 %) and LVL (42 %). Distorted cells were significantly more frequent in smears made from LVL than in those collected by EB and CB. The CB harvested significantly more fragmented cells than EB and LVL (Table 4, Plate 2). The cytobrush technique resulted in less distortion of cells compared with the lavage technique. This finding is similar to that reported in the cows by Kasimanickam *et al* (2005) and Barlund *et al* (2008), but differed from the results of LeBlanc *et al* (2007) in the mare. In the lavage technique, the procedure, from sample collection to the preparation of the slide for cytologic examination, took variable time affecting the total nucleated cell count and their appearance, apart from cellular distortion by centrifugation. Cellular fragmentation was frequently observed in CB smears. A disadvantage reported for brush is that rigid fibers can be traumatic (Martin-Hirsch *et al* 2005) and could be responsible for cells fragmentation. A gentle technique in preparing smears is mandatory.

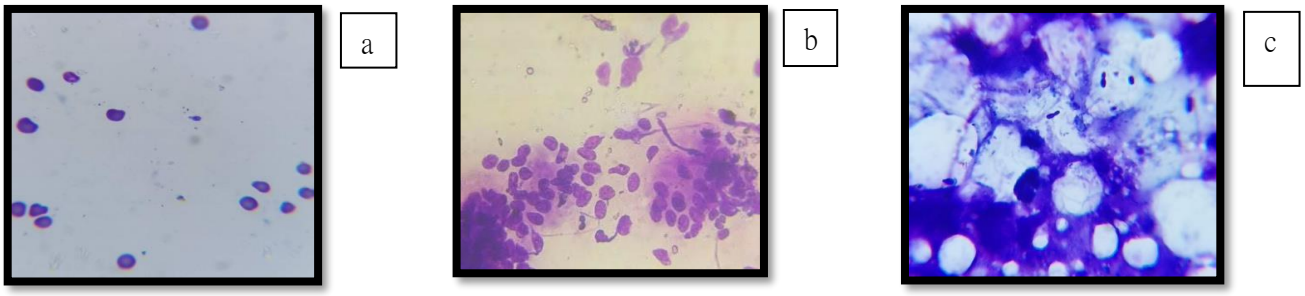

Plate 2: Cytology smears prepared from EB (a), CB (b) and LVL fluid (c) showing different cellularity

Table 4: Quality of cells harvested (mean ± SD) from endometrium of mare by three diagnostic techniques

Sampling method	Intact cells (%)	Distorted cells (%)	Fragmented cells (%)
Endometrial biopsy	$90.00^{a} \pm 0.94$	$9.00^{a} \pm 0.67$	$1.00^{a} \pm 0.82$
Cytobrush	$60.00^{b} \pm 0.82$	$9.00^{a} \pm 0.94$	$31.00^{b} \pm 1.41$
Low-volume uterine lavage	$42.00^{c} \pm 0.82$	$48.00^{b} \pm 0.67$	$10.00^{c} \pm 0.82$

Means bearing uncommon superscript with the column differ significantly ($p<0.05$).

5. Other Inflammatory Cells in Cytological Smears

Inflammatory cells (lymphocytes, macrophages, eosinophils), other than PMNs, were collected by CB and EB from the same mare. They were evident in 3 cases. It is hypothesized that poor mechanical drainage leads to fluid accumulation in susceptible mares. A pendulous uterus, impaired lymphatic or cervical drainage and atrophy of

endometrial folds may contribute to free fluid accumulation (LeBlanc and Causey, 2009). If inflammation persists, there can be a long-standing influx of lymphocytes and plasma cells into the endometrium, possibly contributing to chronic degenerative changes and impairment of endometrial function (Causey, 2006). In the present study, epithelial cells from vagina were detected only in a few of LVL slides due to contamination of the uterine catheter with vaginal flora.

Cultural/Microbiological Findings of the Samples

In this study, positive growth was obtained from EB in 7 (70 %) mares, from CB in 6 (60 %) and from LVL fluid in 8 (80 %) mares (Table 5). In 3 of 10 (30 %) cytological smears obtained from EB and CB and in 4 of 10 (40 %) cytological smears obtained from LVL, the percentage of PMNs was ≥ 1. In EB, CB and LVL, all the cytological positive mares also had positive cultures, whereas 30 % positive cultures from CB and 40 % positive cultures from EB and LVL had negative cytology. The most common pathogenic bacteria cultured from EB was β-hemolytic *Streptococci* either alone (30 %) or in combination with other micro-organism, and from CB, β-hemolytic *Streptococci* and *E. coli* were isolated in equal proportions, whereas, in LVL, *E. coli* was isolated most frequently either alone (40 %) or in combination with other micro-organisms (10 %) and β-hemolytic *Streptococci* was the second most frequently isolated organism (20 % alone; 10 % in combination with other organisms) (Plate 3). This was in agreement with the study conducted by LeBlanc *et al.* (2007). In studies conducted by earlier researchers (Nielsen, 2005, Riddle *et al*., 2007, Frontoso *et al*., 2008), the most common pathogenic bacteria cultured from both EB and CB was β-hemolytic *Streptococci*. Also, mixed infections were found in EB and LVL techniques, among which 10 % of the mixed growth from LVL was found to be *E. coli* (50 %) + β-hemolytic *Streptococci* (50 %).

Table 5: Prevalence of microorganisms isolated from 10 mares using endometrial biopsy (EB), cytobrush (CB) and low volume uterine lavage (LVL) techniques

Micro-organisms	EB	CB	LVL
β hemolytic *Streptococcus*	3 (30%)	3 (30%)	2 (20%)
E. coli	3 (30%)	3 (30%)	4 (40%)
E.coli+ β hemolytic *Streptococcus*	0	0	1 (10%)
β hemolytic *Streptococcus*+Yeast	1 (10%)	0	0
Staphylococcus spp.+*Pseudomonas* spp.	0	0	1 (10%)
Total	7 (70%)	6 (60%)	8 (80%)

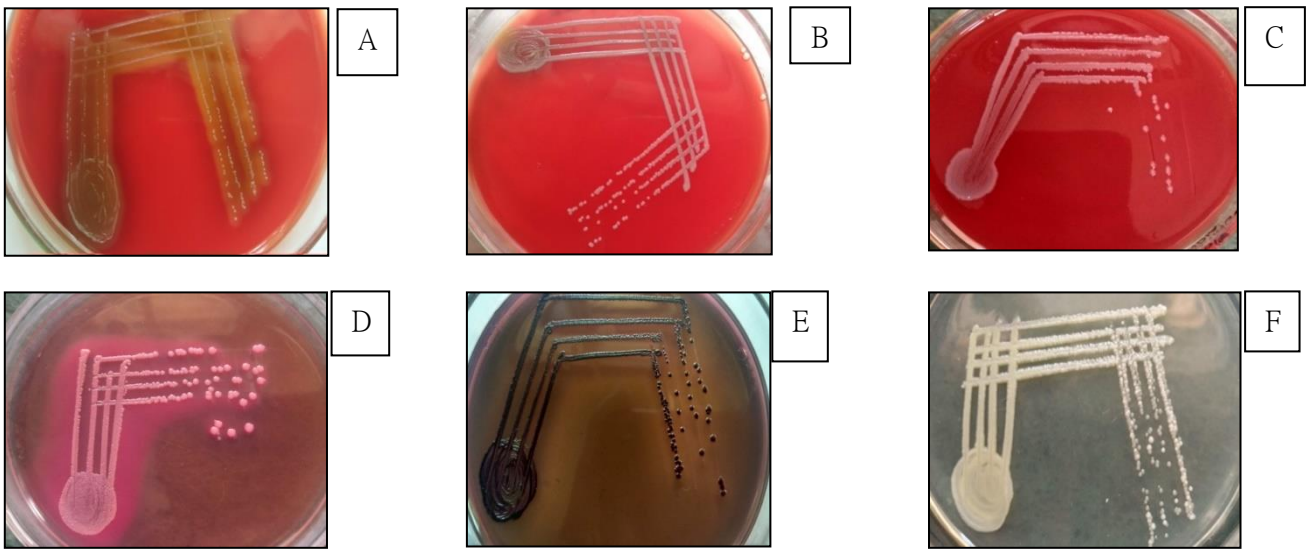

Plate 3: Microbial isolates obtained from uterine samples; (A) Growth of β-hemolytic *Streptococcus*, (B) *Staphylococcus* spp. and (C) *E. coli* on blood agar, (D) Growth of *E. coli* on MacConkey agar, (E) Growth of *E. coli* on EMB agar, (F) Growth of yeast organisms on SDA

The types of the microorganisms isolated from the uterus of the mares in present study were similar to those reported in earlier studies (Riddle *et al*., 2007, Nielsen, 2005, LeBlanc *et al*., 2007, Frontoso *et al*., 2008). Katila (2016) identified the colonies as potentially pathogenic or non-pathogenic and the most common pathogens were β-hemolytic *Streptococcus* and *E. coli*, but *Staphylococcus* spp., *Pseudomonas* spp., Klebsiella, fungi and yeasts were also seen in equine endometritis.

Efflux Clarity, pH, and Isolation of Micro-Organisms from LVL Samples

Mean volume recovered by LVL was 25±6.32ml (range, 20-35 mL). Efflux clarity of 5 flushes was clear, 3 were cloudy and 2 were clear with mucus strains. Cloudy or mucoid effluxes were associated with isolation of micro-organisms. *E. coli* and β-hemolytic *Streptococcus* were isolated most frequently from cloudy or mucoid effluxes (5/10). Two of the five *E. coli* positive flushes (40 %) and all three β-hemolytic *Streptococcus* positive flushes (100 %) were mucoid or cloudy. Micro-organisms were also isolated from 60 (3/5) % of clear flushes. *E. coli* 2/3 (66.67 %) and Staphylococcus spp. + Pseudomonas spp. 1/3 (33.33 %) were isolated from them.

Isolation of β-hemolytic *Streptococcus* from the efflux was highly associated with a rise in pH (mean pH 8.0), whereas, recovery of *E. coli* was not associated with pH alteration (pH 6.5). In general efflux with no bacteria had pH 6.0 and those with bacterial isolates had pH 7.0. These observations were in agreement with those made by LeBlanc *et al* (2007). Nielsen (2005) reported that *E. coli* and β-hemolytic *Streptococcus* appeared to vary in their pathogen-host relationships. Streptococcal uterine infections in the mare are watery and intra-uterine fluid is commonly visualized on ultrasonographic examination of the uterus of infected mares. Increased intra-luminal fluid and a rise in pH may be due to *Streptococcus* releasing super-antigens, streptokinases and other toxins, which attract pro-inflammatory mediators into the uterine lumen (Troedsson *et al*., 1993, Causey *et al*., 1995). Little is known about the uterine inflammatory response to *E. coli* in the mare. In chronic infections, it is known to secrete a biofilm, a hydrated matrix of polysaccharide and protein, providing an adhesive matrix for micro-colonies (Emody *et al*., 2003, Soto *et al*., 2006). Biofilms provide inherent resistance to antibiotics and both cellular and humoral immune defenses. The heavy debris on cytological smears seen in *E. coli* infections in this study was likely associated with biofilms, endometrial mucus and uterine inflammatory by-products.

The relationship between flush culture, cytology results and efflux clarity is shown in Table 6. The clinical estimate of contaminated (false positive) flush cultures was 50.00 (4/8) %, if a false positive was defined as culture positive/cytology negative, while it was 37.50 (3/8) %, if a false positive was defined as positive culture/clear efflux, and 33.33 (1/3) %, if a false positive was defined as positive culture/clear efflux and no debris.

Table 6: Contingency table showing relationships between flush culture, flush cytology and efflux clarity from low-volume uterine flushes of mares

	Culture positive	**Culture negative**	**Total**	**Clinical estimate of false positive**
Flush PMN +	4 (40%)	0	4 (40%)	50.00% (4/8)
Flush PMN –	4 (40%)	2 (20%)	6 (60%)	-
Total	8 (80%)	2 (20%)	10 (10%)	-
Efflux +	5 (50%)	0	5 (50%)	37.50% (3/8)
Efflux –	3 (30%)	2 (20%)	5(50%)	-
Total	8 (80%)	2(20%)	10 (10%)	-
Efflux+;debris+	2 (20%)	0	2 (20%)	33.33% (1/3)
Efflux-; debris-	1 (10%)	2 (20%)	3 (30%)	-
Total	3 (30%)	2 (20%)	5 (50%)	-

Growth of microorganisms (culture+) or no growth (culture-) from uterine flushes tested against the presence of neutrophils (PMN) in cytological specimens (+: ≥1 PMN/field evaluated at 400x); efflux clarity (+: cloudy/mucoid, -: clear) or efflux clarity and cytological debris (moderate or heavy).

We could identify a higher number of positive cultures as contaminated (50.00 %) if the presence of neutrophils detected in the efflux cytology was used as the only index of inflammation. However, using biopsy data, our best estimate of false positive (contaminated effluxes) was 25.00 (2/8) %. A flush sample should be regarded as

contaminated if bacteria are isolated but the efflux is clear, with no change in pH, cytology being either hypocellular or non-inflammatory, and absence of neutrophils in the endometrial tissue.

In the present study, only 40.00 % positive cultures had neutrophils on cytology. Therefore, endometrial cytology from LVL flush may not reflect the degree of inflammation in the endometrium. Palm *et al.* (2006) reported a pronounced rise in neutrophil numbers within the stratum compactum when endometrial biopsies were obtained 12 hr after uterine infusions with semen extenders, saline or seminal plasma, however, neutrophil numbers in cytological smears obtained from LVL flushes did not change. The poor association between endometrial inflammation and cytological inflammation may result from debris in cytological specimens, dilution and centrifugation of the efflux, type of micro-organism recovered, or the duration of the infection. Debris contains mucus, damaged epithelial cells, and inflammatory by-products. Centrifugation of the flush may have disrupted cell walls, thereby increasing cellular debris. The type of micro-organism isolated may have influenced cytology results; *E. coli* was associated with debris, whereas β-hemolytic *Streptococcus* was not associated. Riddle *et al* (2005) reported that type of organism recovered by culture swab in a large clinical investigation also influence the cytology results. In that study, positive cytology was less common when Gram negative bacteria were isolated (52-55 %) from culture swab than when Gram positive bacteria were isolated (67-82 %).

Conclusion

This study on 10 barren mares evaluated the comparative efficacy of endometrial biopsy (EB), cytobrush (CB) and low-volume uterine lavage (LVL) for diagnosis of endometritis through cytology and bacteriology. CB yielded significantly ($p<0.05$) more PMNs/HPF than EB and LVL. The LVL and CB produced more PMNs/HPF than EB. In smears from LVL, the proportion of PMNs to uterine epithelial cells (1.20%) was higher. EB yielded more intact cells (90%) than CB and LVL. Distorted cells were significantly more frequent in LVL (48%), while fragmented cells were significantly more in CB (31%). According to criterion II (≥1% PMNs/ 300 epithelial cells), endometritis was diagnosed in 30% of the mares by EB and CB techniques and 40% by LVL. In contrast, none of the mares was found to be positive for endometritis according to criterion I (≥1 PMNs/HPF). Positive microbial growth was obtained in 7, 6 and 8 mares using EB, CB and LVL, respectively. All the cytologically positive mares were also positive for microbial cultures. Whereas, 30% positive cultures from CB and 40% positive cultures from EB and LVL had negative cytology. The most common pathogenic bacteria cultured from CB and EB were β-hemolytic *Streptococci* and *E. coli*, *w*hereas in LVL technique, *E. coli* was isolated more frequently. Cloudy or mucoid effluxes of LVL were mostly associated with isolation of *E. coli* and β-hemolytic *Streptococcus*, and the later was highly associated with a rise in pH.

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Conflict of Interests

There is no conflict of interest.

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