

Use of a low-volume uterine flush for diagnosing endometritis in chronically infertile mares

Michelle M. LeBlanc^{a,*}, Joshua Magsig^a, Arnold J. Stromberg^b

^a Rood and Riddle Equine Hospital, Lexington, KY 40580, USA

^b Department of Statistics, University of Kentucky, Lexington, KY 40506-0027, USA

Abstract

Low-volume uterine flush ($n = 401$) was performed in 308 infertile mares to diagnose endometritis. Mares evaluated were either barren after three or more breedings or had two or more unsuccessful embryo recovery attempts during consecutive cycles. Culture results were compared with cytological and histological findings, efflux clarity and pH to substantiate that the micro-organisms recovered were truly pathogens. Cytological specimens were evaluated for presence of epithelial and inflammatory cells, bacteria, yeast and debris. Endometrial biopsies ($n = 110$) were examined for the presence of neutrophils in the stratum compactum. Micro-organisms were recovered in 282/401 (70%) of low-volume flushes; *E. coli* was most frequently isolated (42.2%), followed by β hemolytic *Streptococcus* (37.6%). Efflux clarity of 318 flushes was clear ($n = 109$), cloudy ($n = 149$), or mucoid ($n = 60$). Isolation of micro-organisms was highly associated with cloudy and mucoid effluxes ($P < 0.001$), debris on cytological specimens ($P < 0.001$), increased efflux pH ($P < 0.003$), and neutrophils on endometrial biopsy ($P < 0.01$). *E. coli* was associated with debris on cytological smear ($P < 0.002$), whereas β hemolytic *Streptococcus* was associated with increased efflux pH ($P < 0.002$). Using the presence of neutrophils in a tissue specimen as the “best standard” for diagnosing endometritis, the sensitivity of flush culture was 0.71 and for flush cytology was 0.8, whereas the specificity was 0.86 and 0.67, respectively. Neutrophils in uterine flushes under-reported inflammation; only 86/282 positive cultures were positive on cytology. The clinical estimate of a contaminated (false positive) flush culture was 11%, if a false positive was defined as positive culture with clear efflux and no debris or neutrophils on cytology (26/228). In conclusion, a low-volume uterine flush was a rapid, accurate method for identifying mares with chronic endometritis. When micro-organisms were recovered, endometritis was confirmed by efflux clarity, pH and cytological findings of debris, bacteria, or neutrophils. *E. coli* was most commonly isolated and it appeared to differ in pathogenicity from β hemolytic *Streptococcus*.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Mare; Endometritis; Diagnosis; Microbiology; Uterine cytology

1. Introduction

Bacterial uterine infections occur in 25–60% of barren mares [1–3] and inflict major losses on the equine breeding industry. An accurate diagnosis based

on history, clinical findings, uterine culture and cytology and, in some cases, histology is mandatory if mares are to be treated successfully. Correct interpretation of microbiological and cytological findings from the equine endometrium requires consideration of possible false positive and false negative culture results. False positive cultures have been associated with contamination of the culture instrument from the environment, external genitalia, and vagina [4–7], whereas false negative cultures have been associated with inadequate

* Corresponding author. Tel.: +1 859 233 0371;
fax: +1 859 255 5367.

E-mail address: Mleblanc@roodandriddle.com (M.M. LeBlanc).

sampling of the endometrium [4,8–12]. Although use of a guarded culture swab in combination with identification of neutrophils on cytological smear has decreased the number of mares treated inappropriately, not all mares with endometritis are identified, as the swab only comes in contact with a 1–2 cm area of endometrium directly cranial to the cervix. Neilsen (2005) recently reported that only 38 of 84 mares (45%) with bacteria isolated from the surface of an endometrial biopsy, had bacteria isolated from a uterine culture swab [8]. In that study, sensitivity of identifying bacterial growth from an endometrial surface swab was 0.34, whereas specificity was 1.0 when the presence of neutrophils in a tissue specimen was used as the best standard for diagnosing endometritis. Therefore, a major issue confronting clinicians are the false negatives associated with uterine swab cultures. Uterine flushes have been used for microbiologic and cytological examination of the mare's endometrium and represent an alternative to endometrial swabs [12–17]. In a small study, the low-volume uterine flush improved the ability to diagnose endometritis based on quantitative microbiologic and cytologic findings, relative to the guarded swab technique [12]. The increased frequency of diagnosis of endometritis with the flush technique was more evident in subfertile than normal mares.

Contamination of a uterine catheter with vaginal flora must be considered as a source of false positive culture results because contamination occurs more frequently in a semi-guarded (i.e. the flush technique) than in a completely guarded (i.e. swab technique) endometrial culture technique. False positives may be screened out by incorporating additional indices of inflammation, i.e. appearance of efflux, pH, or presence of debris or neutrophils on cytological specimens obtained from a low-volume flush. These indices of inflammation have a further use, the potential detection of false negative cultures. This study was conducted to determine if a low-volume flush of the uterine lumen improved the sensitivity of identifying endometritis in chronically infertile mares.

2. Materials and methods

2.1. Mares and sample collection

Low-volume uterine flushes ($n = 401$) were collected, regardless of stage of estrous cycle, from 308 mares presented for infertility at Rood and Riddle Equine Hospital between July 2004 and July 2006. Infertile mares consisted of naturally mated Thoroughbred mares, and artificially bred Saddlebred, Quarter Horse, Arabian,

or Warmblood mares, aged 5–24 years. Criteria for mares to be included in the study were that they had been bred three or more times unsuccessfully in the same breeding season, had a history of ≥ 2 years of reproductive failure, or had two or more unsuccessful embryo recovery attempts during consecutive cycles. The low-volume uterine flush was conducted after a complete reproductive assessment, including transrectal manual and ultrasonographic examinations of the reproductive tract, and vaginal speculum examination, were performed.

Mares were examined either restrained in stocks or backed out a stall door with a twitch applied to their nose. After the ultrasonographic examination, the vulva and perineum were scrubbed with providone-iodine scrub¹ rinsed three times with water, and dried with a paper towel. A vaginal speculum examination was performed and then a uterine catheter² was passed per vaginum into the uterus by an examiner whose arm was covered by a clear sterile sleeve and surgical glove. Ten to 12 cm of catheter were inserted into the uterus by manually passing it through the cervix. The balloon on the catheter was not filled with air or water. A sterile 60 mL catheter-tip syringe was used to infuse 60 mL of sterile physiological saline into the uterus. The uterus was manipulated by transrectal palpation for a minimum of 30 s to distribute the fluid throughout the uterine lumen. The uterine horn containing the catheter tip was then cradled by the veterinarian's hand (transrectal manipulation) and the saline drained into a sterile 50 mL conical tube³ by gravity flow. The volume recovered was recorded. Physiological saline was used in lieu of lactated ringers, because changes in efflux pH associated with bacterial isolation were greater with saline in preliminary trials.

An aliquot (4 mL) of the sample was placed in a 5 mL sterile tube for measuring pH. The remaining sample was centrifuged at 410 rpm for 10 min. All but 5 mL of the supernatant was decanted and a sterile culture swab was inserted into the pellet and placed in Amie's transport medium with charcoal⁴ for subsequent microbiologic culture. A second sterile swab was placed in the pellet and the swab rolled onto a glass slide and air-dried for cytological examination.

¹ Betadine surgical scrub (Butler Animal Health Supply; Lexington, KY 40511, USA).

² Bivona uterine catheter EUF 80 (Butler Animal Health Supply; Lexington, KY 40511, USA).

³ Fifty millilitre Tube (Fischer Scientific; Hanover Park, IL, USA).

⁴ Difco[®] Transport Medium Amies (Becton, Dickinson and Co.; Sparks, MD, USA).

2.2. Efflux clarity, pH and cytology

Efflux clarity of 318 samples was recorded by holding the sample up to the light. Efflux clarity was graded as clear, cloudy or clear with mucus strains (mucoid efflux). For statistical analysis, cloudy or mucoid efflux was graded as positive and clear efflux was graded as negative. The pH of 184 samples was measured from a 5 mL aliquot of efflux with a pH meter⁵ and compared to the pH of the saline contained in the 250 mL bottle used for the low-volume flush.

Cytological specimens were stained with Diff Quick stain⁶ and a minimum of 10 fields were evaluated microscopically under oil immersion (1000×) by one of the three medical technologists. Cytological smears were evaluated for the presence of epithelial cells, debris, inflammatory cells, bacteria, and yeast. Cytological specimens that contained scant epithelial cells, no neutrophils, debris or bacteria were classified as hypocellular. Smears were considered indicative of inflammation if there were an average of one or more neutrophils per 1000× magnification in 10 fields. The amount of debris in the smear was scored as: none = 0; mild = 1; moderate = 2 or heavy = 3. Debris was defined as follows: mild debris, <25% of the smear covered with debris; moderate debris, 25–75% of the smear covered with debris; heavy debris, >75% of the smear covered in debris.

2.3. Microbiology

Endometrial cultures were plated on blood and Levine Eosin-Methylene Blue plates at the medical laboratory at Rood and Riddle Equine Hospital within 6 h of sample procurement. After 24 h incubation in atmospheric air at 37 °C, plates were examined for growth and were re-examined at 48 and 72 h for the presence of bacteria or yeast. Bacteria were identified with BBL crystals.⁷ Antimicrobial sensitivities were determined for all organisms by the Kirby Bauer method. Primary identification of yeast was determined by dark field microscopy. Specific yeast or fungal cultures were further identified by the Microbiology Laboratory at Cornell University, Ithaca, NY, USA. Samples considered positive for growth had to have four or more colonies of a pathogenic organism isolated.

Culture plates with growth of more than three organisms were considered to be contaminated and recorded as negative growth. *Bacillus*, *Micrococcus* or α *Streptococcus* were recorded only if isolated in pure culture.

2.4. Histology

An endometrial biopsy was obtained for histopathologic examination from 110 of the 308 mares after the uterine flush. Endometrial biopsies were fixed in Bouin's solution and stained with haematoxylin and eosin. Slides were examined for the presence of neutrophils within the epithelial luminal epithelium, stratum compactum and stratum spongiosum [18]. Infiltration of three or more neutrophils per five fields of high magnification (400×) was considered as evidence of acute endometritis [19].

2.5. Prevalence of micro-organisms recovered by culture swab

Uterine culture swabs were collected from a population of Thoroughbred mares residing on farms visited daily by the author and a second veterinarian in the practice during the 2002 through 2006 breeding seasons in order to determine if the prevalence of micro-organisms recovered from farm mares in Central Kentucky was similar to findings previously published. Uterine swabs were obtained from maiden and barren mares before their first mating and from maiden, barren and foaling mares after their second natural mating. Swab cultures were collected with a Kalajian guarded culture instrument⁸ that was either passed through a sterile vaginal speculum into the cervix and uterus or manually inserted by a veterinarian wearing a sterile sleeve.

2.6. Statistical analysis

Categorical variables were compared using contingency tables and Pearson Chi-square tests, whereas continuous variables were compared using Student's *t*-tests; *P* < 0.05 was considered significant. All analysis was done using JMP (SAS, Inc., Cary, NC, USA). Data are presented as means ($\pm$ S.E.M.). Presence of neutrophils on endometrial biopsy was used as the best standard for predicting endometritis. Sensitivity was calculated as the proportion of mares with neutrophils in

⁵ Denver Instrument Benchtop pH Meter UB-5 Ultra Basic (Fischer Scientific).

⁶ Diff Quik (Hemal Stain Co. Inc.; Danbury, CT, USA).

⁷ BBL crystals (Becton Dickinson and Co.; Sparks, MD, USA).

⁸ Kalajian culture instrument (Kalajian Industries, Inc. Signal Hill, CA, USA).

the endometrial tissue and a positive result from the compared test or tests (flush culture or neutrophils on cytological specimen). Specificity was calculated as the proportion of mares without neutrophils in the endometrial tissue and a negative result from the compared test.

3. Results

3.1. Microbiology

Bacteria were isolated from 282 of 401 uterine flushes (70%). *E. coli* was isolated most frequently, either alone (27.6%) or in combination with another micro-organism (14.6%; Fig. 1). Beta hemolytic *Streptococcus* was the second most frequently isolated organism (20.9% alone; 16.7% in combination with a second organism). The two organisms were isolated from 79.8% of the positive flush cultures. Mixed infections were present in 71/282 (25%) uterine flushes (Fig. 1). Isolation of β hemolytic *Streptococcus* and *E. coli* (22/282, 7.8%) was most common. Beta hemolytic *Streptococcus* was isolated with a pathogen other than *E. coli* in 25 flushes (8.9%), whereas *E. coli* was isolated

with a pathogen other than β hemolytic *Streptococcus* in 19 of 282 (6.7%) uterine flushes. Micro-organisms considered not to be pathogens of the genital tract (α *Streptococcus*, *Micrococcus* and *Bacillus*) were recovered from approximately 10.5% of the uterine flushes (Fig. 1). Yeast was isolated from 17 flushes (alone $n = 8$; with β hemolytic *Streptococcus*, $n = 5$; *E. coli*, $n = 3$; and with both organisms, $n = 1$). *Klebsiella* was recovered with proteus or *Staphylococcus aureus* once.

The most frequently isolated micro-organism from culture swabs was β hemolytic *Streptococcus* (39%; Fig. 2), with *E. coli* being second most common (16%). Together, the two organisms represented 55% of the positive culture swabs. Two or more organisms were recovered from culture swab in <2.3% of the positive cultures.

3.2. Efflux clarity, pH and isolation of micro-organisms

Mean volume recovered was 33.6 ± 0.3 (range, 7–60 mL). Efflux clarity of 318 flushes was clear ($n = 109$), cloudy ($n = 149$) or clear with mucus strains ($n = 60$). Cloudy and mucoid effluxes were highly

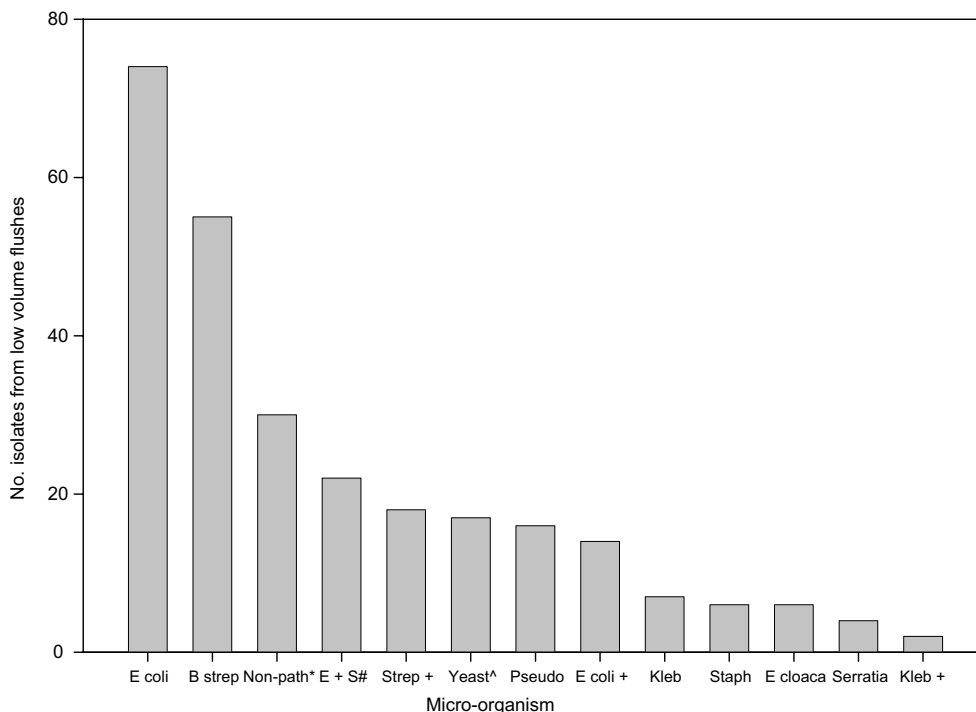


Fig. 1. Prevalence of micro-organisms isolated from 401 low-volume uterine flushes from mares. *Non-pathogens include α *Streptococcus*, *Bacillus*, and *Micrococcus*; E + S#: number of cultures with *E. coli* and β hemolytic *Streptococcus* isolated; +: micro-organism was isolated with an organism other than *E. coli* or β hemolytic *Streptococcus*; Yeast^: yeast was isolated alone in 8 of 17 cultures, with β hemolytic *Streptococcus* ($n = 5$), *E. coli* ($n = 3$) or with both organisms ($N = 1$). Pseudo, *Pseudomonas*; Kleb, *Klebsiella*; Staph, *Staphylococcus aureus*.

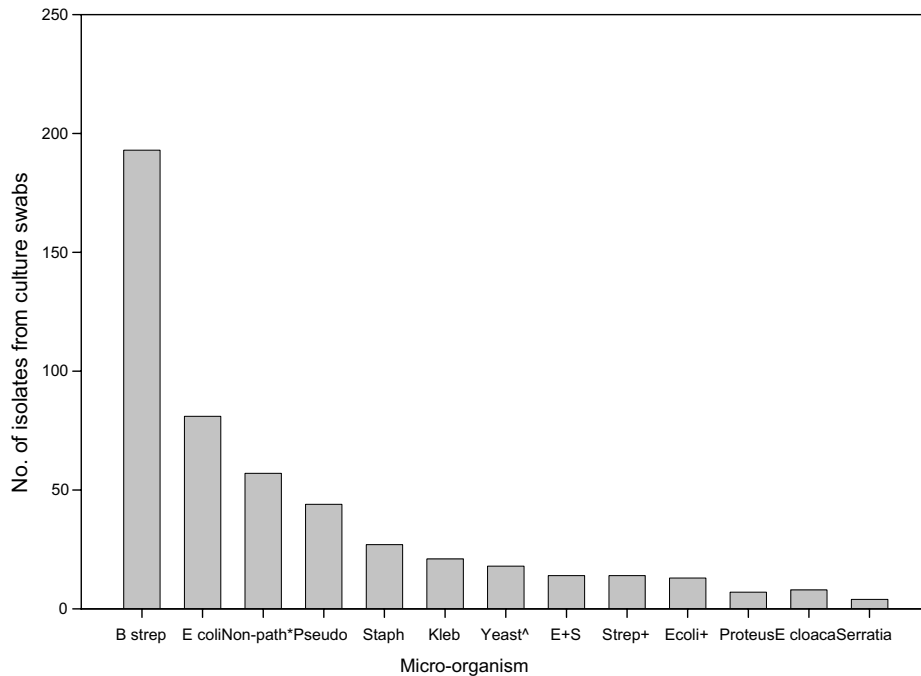


Fig. 2. Prevalence of micro-organisms isolated from culture swabs obtained from Thoroughbred mares residing on farms routinely visited in central Kentucky ($n = 498$). *Non-pathogens include α *Streptococcus*, *Bacillus*, and *Micrococcus*; E + S: number of cultures with *E. coli* and β hemolytic *Streptococcus*; +: micro-organism was isolated with an organism other than *E. coli* or β hemolytic *Streptococcus*. Pseudo, *Pseudomonas*; Kleb, *Klebsiella*; Staph, *S. aureus*.

associated with isolation of micro-organisms ($P < 0.0001$; Fig. 3). Micro-organisms were isolated from 86% (179/209) of cloudy or mucoid effluxes. *E. coli* and β hemolytic *Streptococcus* were isolated most frequently from cloudy or mucoid effluxes (144/209; 69%). Eighty-one of 100 *E. coli* positive flushes (81%)

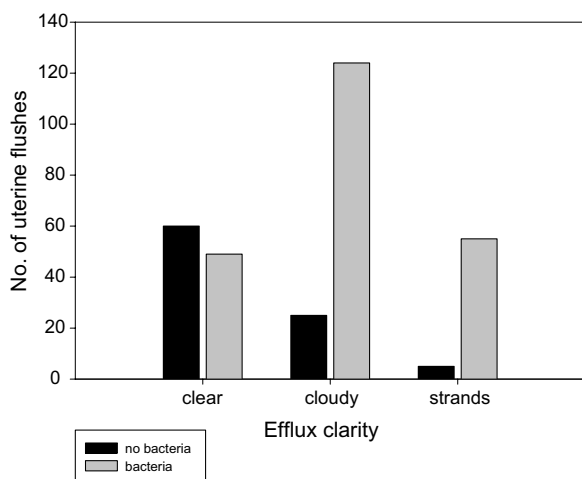


Fig. 3. Relationship between efflux clarity and isolation of micro-organisms from low-volume uterine flushes of mares. More cloudy or mucoid effluxes (strands) contained micro-organisms than clear effluxes ($P < 0.001$).

and 64 of 81 β hemolytic *Streptococcus* positive flushes (79%) were cloudy or mucoid ($P < 0.005$). Isolation of β hemolytic *Streptococcus* from the efflux was highly associated with a rise in pH (5.99 ± 0.05 ; $P < 0.003$; Table 3), whereas, recovery of *E. coli* was not associated (Table 1).

Micro-organisms were isolated from 45% (49/109) of clear flushes. *E. coli* and β *Streptococcus* were recovered most frequently (35/49; 71%; $n = 19$ and 16, respectively). The remaining 14 cultures grew the following: bacteria not considered to be a pathogen ($n = 7$), *S. aureus* ($n = 3$), *Enterobacteria cloaca* ($n = 2$) or *Pseudomonas aeruginosa* ($n = 2$). Twenty three of the 49 clear effluxes (47%) with micro-organisms recovered had additional indices of inflammation, i.e.

Table 1
Relationship between efflux pH and isolation of micro-organisms from low-volume uterine flushes of mares

Category	Mean $\pm$ S.E.M.	95% CI*
No bacteria	5.64 $\pm$ 0.04 a	5.55–5.72
Bacteria	5.83 $\pm$ 0.02 b	5.78–5.89
β <i>Streptococcus</i>	5.99 $\pm$ 0.05 b	5.81–5.99
<i>E. coli</i>	5.78 $\pm$ 0.03 a	5.69–5.85

Within a column, means with different letters (a, b) differ ($P < 0.002$).

* CI: confidence interval.

Table 2

Relationship between neutrophils on cytological specimens and isolation of bacteria from low-volume flushes and efflux clarity from low-volume uterine flushes of mares

Number of neutrophils on cytological smear ^a	Number of positive cytologies	Number of positive cultures	Efflux clarity ^b
0–2	53	40	39/39
2–5	12	11	10/10
≥5	40	35	28/28
Total	105	86	77/77

^a Number of neutrophils/field at a magnification of 1000×. A minimum of 10 fields were evaluated.

^b Efflux clarity—number of cloudy or mucoid effluxes divided by the total number of samples graded for clarity. Presence of neutrophils on cytological specimens was associated with positive culture results ($P < 0.001$) and with a cloudy or mucoid efflux ($P < 0.001$).

heavy debris ($n = 17$), a pH > 5.8 ($n = 3$), or both ($n = 3$).

3.3. Cytological findings—neutrophils, micro-organisms and debris

Presence of neutrophils on cytological specimens was associated with positive culture results ($P < 0.001$) and with a cloudy or mucoid efflux ($P < 0.001$). However, neutrophils were present in only 105 of 401 cytological smears (26%; Table 2). Eighty-six of the 105 positive cytologies had micro-organisms isolated from the paired culture. All but three effluxes containing neutrophils were cloudy or mucoid. Bacteria or yeast were identified in 49 of 401 (12%) cytological

specimens and were isolated from 45 of the 49 (92%; $P < 0.003$) paired cultures.

Debris on cytological specimens was associated with isolation of micro-organisms in flushes ($P < 0.0001$). Fifty percent (141/282) of flushes with micro-organisms isolated had moderate or heavy debris on cytological specimens while 26% (31/119) of flushes with no micro-organisms recovered had debris. *E. coli* was highly associated with debris (70/119 positive *E. coli* flushes; $P < 0.0002$), whereas, recovery of β *Streptococcus* was not associated. Debris was present in 119 of 209 (57%) cytological smears obtained from cloudy or mucoid effluxes, whereas only 3 of 60 clear flushes (5%) without bacteria had debris. Debris was most common in cytological specimens with 0–2 neutrophils/field (142/245 cytological specimens; 58%; $P < 0.003$) and was observed in only 29% of the smears with > 2 neutrophils/field (15/52).

3.4. Histological findings

Isolation of bacteria from the uterine flush was highly associated with the presence of neutrophils in the stratum compactum ($P < 0.007$; Table 3). Micro-organisms were recovered from the uterus of 70 of the 110 mares from which an endometrial biopsy was procured. Sixty-eight of the 70 endometrial biopsies (97%) had histological evidence of acute endometritis. Five of the 40 mares with negative cultures had eosinophils and neutrophils in endometrial samples. Three of the five mares had urine in the vagina. Relationships between clear efflux, histology and culture results are presented in Table 3. All culture

Table 3

Relationships between histological findings, flush culture, flush cytology and clear efflux from low-volume uterine flushes of mares

	Histology+ (PMN+)	Histology– (PMN–)	Total	Sensitivity	Specificity
Culture+	68 (62%)	2 (2%)	70 (64%)	0.71	0.86
Culture–	28 (25%)	12 (11%)	40 (36%)		
Total	96 (87%)	14 (13%)	110 (100%)		
Cytology+	81 (74%)	3 (3%)	84 (76%)	0.80	0.67
Cytology–	20 (18%)	6 (5%)	25 (23%)		
Total	101 (92%)	9 (8%)	110 (100%)		
Clear efflux					
Culture+	16 (48%)	0 (0%)	16 (48%)	0.60	1.0
Culture–	11 (33%)	6 (18%)	17 (52%)		
Total	27 (82%)	6 (18%)	33 (100%)		

Growth of micro-organisms (culture+) or no growth (culture–) from uterine flushes and the presence of neutrophils on cytology (+ is ≥ 1 neutrophils/field at 1000×) tested against histological examination of endometrial biopsy specimen (+: > 3 neutrophils/5 fields at high magnification 400×). Results from histological examination were used as the best standard.

Table 4

Contingency tables showing relationships between flush culture, flush cytology and efflux clarity from low-volume uterine flushes of mares

	Culture+	Culture–	Total	Clinical estimate of false +
Flush PMN+	86 (22%)	19 (5%)	105 (26%)	70% ^a
Flush PMN–	196 (49%)	100 (25%)	296 (74%)	
Total	282 (70%)	119 (30%)	401 (100%)	
Efflux+	179 (56%)	30 (9%)	209 (66%)	21% ^b
Efflux–	49 (15%)	60 (19%)	109 (34%)	
Total	228 (72%)	90 (28%)	318 (100%)	
Efflux+; debris+	202 (64%)	30 (9%)	232 (73%)	11% ^c
Efflux–; debris–	26 (8%)	60 (19%)	86 (27%)	
Total	228 (72%)	90 (28%)	318 (100%)	

Growth of micro-organisms (culture+) or no growth (culture–) from uterine flushes tested against the presence of neutrophils (PMN) in cytological specimens (+: one or more neutrophils/field evaluated at 1000×) efflux clarity (+: cloudy or mucoid, –: clear) or efflux clarity and cytological debris (moderate or heavy).

^a 196/282 = 70%.

^b 49/228 = 21%.

^c 26/228 = 11%.

positive mares with clear efflux had neutrophils on biopsy. Six of 17 culture negative mares had negative biopsy results.

3.5. Sensitivity and specificity

Using the presence of neutrophils in a tissue specimen from the uterus as the “best standard” for diagnosing endometritis, the sensitivity of bacterial growth from uterine flush was 0.71 and the sensitivity for neutrophils on cytology was 0.80. The specificity for diagnosing endometritis on flush culture or neutrophils on cytology was 0.86 and 0.67, respectively (Table 3). Table 4 shows relationships between flush culture and cytology results and efflux clarity. The clinical estimate of the number of contaminated (false positive) flush cultures was 70% (196/282), if a false positive was defined as culture positive/cytology negative. The clinical estimate of contaminated (false positive) flush cultures was 21% (49/228) if a false positive was defined as positive culture/clear efflux. The clinical estimate of contaminated (false positive) flush cultures was 11% if a false positive was defined as positive culture/clear efflux and no debris.

4. Discussion

Using the presence of neutrophils in a tissue specimen as the “best standard” for diagnosing endometritis, Neilsen reported the sensitivity of bacterial growth from an endometrial biopsy was 0.82, whereas

growth from an endometrial guarded culture swab was only 0.34 [8]. In the present study, flush culture was twice as sensitive as swab culture estimated by Neilsen (0.71 versus 0.34) when the same “best standard” was used. Therefore, flush culture doubled the ability to detect infected mares based on culture alone. The improved sensitivity appeared to result from improved detection of gram negative organisms as recovery of β hemolytic *Streptococcus* from uterine flush did not differ from previous reports nor from swab results presented here (38% of positive flush cultures; 39% of positive swab cultures; Figs. 1 and 2 [2,4,6,11,20–22]). Recovery of *E. coli*, however, was significantly higher from uterine flushes (42%) than from culture swabs taken in this (16%) and previous studies [2–4,6,11,20–22]. Differences in pathogen–host relationships and types of mares presented for infertility may have contributed to the findings.

Using the biopsy data, our best estimate of the number of false positives (contaminated effluxes) was 3% (2/70; Table 3). However, clinicians would identify a disproportionately high number of positive cultures as contaminated (70%) if the presence of neutrophils detected in the efflux cytology was used as the only index of inflammation (Table 4). By using additional flush indices, the number of false positives can be dropped to 21% using flush appearance, or 11% using a combined index of flush appearance and debris on cytology (Table 4). A flush sample should be regarded as contaminated if bacteria are isolated but the efflux is clear, there is no change in pH, cytology is either

hypocellular or non-inflammatory, and no neutrophils are present in the endometrial tissue (if biopsy is obtained).

Only 86 of 282 positive cultures (22%; Table 4) had neutrophils on cytology. Therefore, endometrial cytology from a low-volume uterine flush may not reflect the degree of inflammation in the endometrium. Palm recently reported a pronounced rise in neutrophil numbers within the stratum compactum when endometrial biopsies were obtained 12 h after uterine infusions with semen extenders, saline or seminal plasma, however, neutrophil numbers in cytological smears obtained from low-volume uterine flushes did not change [17]. 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. Debris was most commonly seen in cytological specimens that had either no neutrophils or 0–2 neutrophils/field (142/245 cytological specimens; 58%; $P < 0.003$; Table 2); however, it was uncommon in smears with >2 neutrophils. 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 highly associated with debris, whereas β hemolytic *Streptococcus* was not associated. Type of organism recovered by culture swab in a large clinical investigation also influenced cytology results [22]. In that study, positive cytologies were less common when gram negative bacteria were isolated (52–55%) from culture swab than when gram positive bacteria were isolated (67–82% [22]).

E. coli and β hemolytic *Streptococcus* appeared to vary in their pathogen–host relationships. Streptococcal uterine infections in the mare are watery [1] 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 [23–26]. Little is known about the uterine inflammatory response to *E. coli* in the mare. *E. coli* colonizes the genitourinary tract in humans and tenaciously adheres to the epithelium, preventing its physical removal [27]. In chronic infections, it is known to secrete a biofilm, a hydrated matrix of polysaccharide and protein, providing an adhesive matrix for micro-colonies [28–32]. 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 [29–32]. Uterine exudate in *E. coli* infections may be tenacious, similar to that reported for *Klebsiella* infections [1], making it difficult to isolate on a culture swab. *E. coli* may also be associated with focal infection as endoscopic evaluation of the uterine lumen of two affected mares revealed focal, granulomatous plaques (LeBlanc M, unpublished observations).

Mixed infections with either β hemolytic *Streptococcus* or *E. coli* were two to four times more common from culture of low-volume flushes than from culture swabs in this or previous reports [8,22]. It is unlikely that these mixed infections were contaminants as they were associated with a rise in pH, cloudy or mucoid effluxes and heavy debris on cytological smears. A low-volume flush technique may identify more pathogenic organisms than the swab technique, as the saline comes in contact with a larger surface area. Mixed infections may be more common with β hemolytic *Streptococcus* and *E. coli* infections, as they have developed pathogenic mechanisms that promote mixed infections. *Streptococcus* resists phagocytosis by a combination of antigenic variation [33], anti-phagocytic M-like proteins [34], the hyaluronic acid capsule [35] and Fc receptors [36]. The Fc receptors on the streptococcal cell wall disrupt the complement cascade, which may provide opportunities for Gram negative organisms to also colonize the uterus [37]. *E. coli* produces a biofilm in chronic infections that protects itself and other micro-organisms from the host's inflammatory response [29–31].

Bacillus, *Micrococcus*, and α *Streptococcus* are not considered pathogens of the equine uterus [4,6,8,11, 12,20–22,38]. However, α *Streptococcus* and *Bacillus* have been associated with decreased 28-d pregnancy rates (22% per cycle pregnancy rate [22]), positive cytologies (56% [22]), and abortion due to both Mare Reproductive Loss Syndrome [39] and allantocentesis in the mare [40]. In this study, *bacillus*, α *Streptococcus* and *Micrococcus* were isolated from 10.5% of the low-volume flush cultures and the swab cultures. Seventy-eight percent of the cytological specimens had debris or neutrophils, both indices of inflammation. There are indications that these organisms, in some cases, may be associated with endometritis [22,39,40].

5. Conclusions

In summary, isolation of micro-organisms from low-volume uterine flushes was associated with cloudy

or mucoid effluxes, an increase in efflux pH, debris, bacteria or neutrophils on cytological specimen, and neutrophils in endometrial biopsies. The technique was a rapid, accurate and practical method for diagnosing endometritis in chronically infertile mares based on sensitivity and specificity estimates approaching 0.75 and 0.9, respectively.

Acknowledgements

This work was supported by Rood and Riddle Equine Hospital. The authors thank Dr. T. Riddle and Ms. Marianne Swintoski for technical assistance.

References

- [1] Dimock WW, Edwards PR. The pathology and bacteriology of the reproductive organs of mares in relation to sterility. KY Agricultural Exper Station Bulletin no. 286;1928:157–237.
- [2] Bain AM. The role of infection in infertility in the Thoroughbred mare. Vet Rec 1966;78:168–73.
- [3] Collins SM. A study of the incidence of cervical and uterine infection in Thoroughbred mares in Ireland. Vet Rec 1964;76: 673–6.
- [4] Blanchard TL, Cummings MR, Garcia MC, Hurtgen JP, Kenney RM. Comparison between two techniques for endometrial swab culture and between biopsy and culture in barren mares. Theriogenology 1981;16:541–52.
- [5] Knudsen O. Endometrial cytology as a diagnostic aid in mares. Cornell Vet 1964;54:415–22.
- [6] Wingfield Digby NJ, Ricketts SW. Results of concurrent bacteriological and cytological examinations of the endometrium of mares in routine stud farm practice. J Reprod Fertil Suppl 1982;32:181–5.
- [7] Ricketts SW. Bacteriological examinations of the mare's cervix: techniques and interpretation of results. Vet Rec 1981;108:46–51.
- [8] Nielsen JM. Endometritis in the mare: a diagnostic study comparing cultures from swab and biopsy. Theriogenology 2005;64:510–8.
- [9] Brook D. The diagnosis of equine bacterial endometritis. Compend Contin Educ Pract Vet 1984;6:S300–6.
- [10] Kenney RM. Cyclic and pathologic changes in the mare's endometrium as detected by biopsy, with a note on early embryonic death. J Am Vet Med Assoc 1978;241–62.
- [11] Ricketts SW, Mackintosh ME. Role of anaerobic bacteria in equine endometritis. J Reprod Fertil Suppl 1987;35:343–51.
- [12] Ball BA, Shin SJ, Patten WH, Lein DH, Woods GL. Use of a low-volume uterine flush for microbiologic and cytologic examination of the mare's endometrium. Theriogenology 1988;29:1269–83.
- [13] Strzemiński PJ, Benson CE, Acland HM, Kenney RM. Comparison of uterine protein content and distribution of bacteria in the reproductive tract of mares after intrauterine inoculation of *Haemophilus equigenitalis* or *Pseudomonas aeruginosa*. Am J Vet Res 1984;45:1109–13.
- [14] Williamson P, Dunning A, O'Connor J, Penhale WJ. Immunoglobulin levels, protein concentrations and alkaline phosphatase activity in uterine flushings from mares with endometritis. Theriogenology 1983;19:441–8.
- [15] Slusher SH, Freeman KP, Roszel JF. Infertility diagnosis in mares using endometrial biopsy, culture and aspirate cytology. In: Proceedings of the 43rd Annual Convention of the American Association of Equine Practitioners; 1985. p. 171–81.
- [16] Card C. Post-breeding inflammation and endometrial cytology in mares. Theriogenology 2005;64:580–8.
- [17] Palm F, Walter I, Budik S, Aurich C. Influence of different semen extenders and seminal plasma on the inflammatory response of the endometrium in estrous mares. Theriogenology 2006;94: 286–9.
- [18] Ricketts SW. The treatment of equine endometritis in studfarm practice. Pferdeheilkunde 1999;6:588–93.
- [19] Ricketts SW, Alonso S. Assessment of the breeding prognosis of mares using paired endometrial biopsy techniques. Equine Vet J 1991;23:185–8.
- [20] Shin SJ, Lein DH, Aronson AI, Nusbaum SR. The bacteriological culture of equine uterine contents, in vitro sensitivity of organisms isolated and interpretation. J Reprod Fertil Suppl 1979;27:307–15.
- [21] Allen WE, Newcombe JR. Aspects of genital infection and swabbing techniques in the mare. Vet Rec 1979;104:228–31.
- [22] Riddle WT, LeBlanc MM, Pierce SW, Stromberg AJ. Relationships between pregnancy rates, uterine cytology and culture results in a Thoroughbred practice in central Kentucky. In: Proceedings of the 50th Annual Convention of the American Association of Equine Practitioners; 2005. p. 51–4.
- [23] Causey RC. Making sense of equine uterine infections: the many faces of physical clearance. Vet J 2006;172:405–21.
- [24] Causey RC, Paccamonti DL, Todd WJ. Antiphagocytic properties of uterine isolates of *Streptococcus zooepidemicus* and mechanisms of killing in freshly obtained blood of horses. Am J Vet Res 1995;56:321–8.
- [25] Asbury AC, Gorman NT, Foster GW. Uterine defense mechanisms in the mare: serum opsonins affecting phagocytosis of *Streptococcus zooepidemicus* by equine neutrophils. Theriogenology 1984;21:375–85.
- [26] Troedsson MHT, Liu IKM, Thurmond M. Immunoglobulin (IgG and IgA) and complement (C3) concentrations in uterine secretion following an intrauterine challenge of *Streptococcus zooepidemicus* in mares susceptible to versus resistant to chronic uterine infection. Biol Reprod 1993;49:502–6.
- [27] Beachey EH. Bacterial-adherence: adhesion–receptor interactions mediating the attachment of bacteria to mucosal surfaces. J Infect Dis 1981;143:325–45.
- [28] Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. Ann Rev Microbiol 1995;711–45.
- [29] Soto SM, Smithson A, Horcajada JP, Martinez JA, Mensa JP, Vila J. Implication of biofilm formation in the persistence of urinary tract infection caused by uropathogenic *Escherichia coli*. Clin Microbiol Infect 2006;12:1034–6.
- [30] Emody L, Kerenyi M, Nagy G. Virulence factors of uropathogenic *Escherichia coli*. Int J Antimicrob Agents 2003;22(Suppl 2):29–33.
- [31] Shapiro JA. Thinking about bacterial populations as multicellular organisms. Ann Rev Microbiol 1998;52:81–104.
- [32] Freeman KP, Roszel JF, Slusher SH, Castro M. Variation in glycogen and mucins in the equine uterus related to physiologic and pathologic conditions. Theriogenology 1990;33: 799–808.

- [33] Walker JA, Timoney JF. Molecular basis of variation in protective SzP proteins of *Streptococcus zooepidemicus*. Am J Vet Res 1998;59:1129–33.
- [34] Timoney JF, Mukhtar MM. The protective M proteins of the equine group C streptococci. Vet Microbiol 1993;37:389–95.
- [35] Wibawan IWT, Pasaribu FH, Utama IH, Abdulmawjood A, Lammler C. The role of hyaluronic acid capsular material of *Streptococcus equi* sub sp. *zooepidemicus* in mediating adherence to HeLa cells and in resisting phagocytosis. Res Vet Sci 1999;38:88–95.
- [36] Jonsson H, Linkmark H, Guss B. A protein G-related cell surface protein in *Streptococcus zooepidemicus*. Infect Immun 1995;63:2968–75.
- [37] Lyle SK, Asbury AC, Boyle MDP, Reis KJ, Roudebush BA. Pathogenesis of equine endometritis: proposed interaction between streptococcal immunoglobulin-binding proteins and other uterine pathogens. J Reprod Fertil Suppl 1991;44:741.
- [38] Hinrichs K, Cummings MR, Sertich PL, Kenney RM. Clinical significance of aerobic bacterial flora of the uterus, vagina, vestibule, and clitoral fossa of clinically normal mares. J Am Vet Med Assoc 1988;193:72–5.
- [39] Donahue J, Sells S, Bolin D. Bacteria associated with mare reproductive loss syndrome: late fetal losses. In: Proceedings of the 1st Workshop on Mare Reproductive Loss Syndrome; 2002. p. 80–1.
- [40] Murchie TA, Macpherson ML, LeBlanc MM, Luznar S, Vickroy TW. Detection of gentamicin and penicillin concentrations in allantoic fluid of pregnant pony mares by in vivo microdialysis. Equine Vet J 2006;38:520–5.