ABSTRACT

The relationship between plasma estradiol concentration at time of examination and prevalence of uterine disorders, agreement among methods, and associations of diagnosis with pregnancy hazard and milk yield was studied in 268 Holstein cows examined at 30 ± 3 (exam 1) and 44 ± 3 d in milk (DIM; exam 2). Purulent vaginal discharge was sampled using 2 methods: gloved hand and Metricheck (Simcro, Hamilton, New Zealand; PVD; score ≥3). Percentage of polymorphonuclear leukocytes was determined by endometrial cytology (CYTO; exam 1: ≥18%, exam 2: ≥10%); diameter of uterine horns (UTH; >20 mm), diameter of the inner layer of the cervix (CVX; >20.5 mm), presence of fluid in the uterine lumen (FL), and ovarian structures were evaluated by ultrasonography. A blood sample was collected at each exam for estradiol analysis. Prevalence at exams 1 and 2 was, respectively, 14.2 and 18.5% (PVD), 21.4 and 10.1% (FL), and 40.6 and 50.2% (CYTO). Prevalence of PVD at exam 1 was greater among cows with estradiol ≥2 pg/mL (19.4 vs. 8.2%). Agreement of all methods with CYTO was poor, the greatest being between CYTO and FL (exam 1; kappa = 0.19). Agreement between CYTO and PVD, and between CYTO and FL (exam 1; kappa = 0.15 and 0.35, respectively) was higher among cows with estradiol ≥2 pg/mL. Likelihood of PVD at exam 1 was greater if cows were positive for CVX (odds ratio (OR) = 3.0), FL (OR = 2.6) or had estradiol ≥2 pg/mL (OR = 2.7). Likelihood of CYTO increased with dystocia (OR = 2.3) and FL (OR = 2.5). Estradiol did not influence diagnosis at exam 2. Positive FL or CYTO at exam 1 was associated with reductions in milk yield of 59 to 180 kg by 45 DIM. Pregnancy hazard until 250 DIM was reduced by CYTO at exam 1 (hazard ratio = 0.74) and by PVD (hazard ratio = 0.68) at exam 2. However, FL and CYTO reduced pregnancy hazard only when estradiol was ≥2 pg/mL (exam 1), whereas PVD reduced pregnancy hazard when diagnosed at exam 2 with estradiol <2 pg/mL. Overall, agreement was poor and effects of positive diagnosis differed according to method and DIM at exam. Estradiol concentration influenced prevalence, agreement, likelihood of positive diagnosis, and its effects on days to pregnancy.

Key words: cytology, endometritis, estradiol, fertility

INTRODUCTION

Bacterial contamination of the uterus occurs in most cows during the early postpartum period. Inflammation, delayed uterine involution, and impairment of fertility result from the persistence of this contamination and its progression to infection (Sheldon et al., 2006). Subclinical or cytological endometritis (defined as elevated percentage of PMN in endometrial cytology, in the absence of purulent vaginal discharge, PVD; Kasimanickam et al., 2004) and clinical endometritis (diagnosed by detection of PVD; LeBlanc et al., 2002; Pleticha et al., 2009) have been associated with reduced pregnancy risk. Similarly, both conditions have been associated with reduced conception rates (Kasimanickam et al., 2004) and increased days open (Barlund et al., 2008).

Definitions for the various uterine pathologies that affect postpartum cows have been proposed by Sheldon et al. (2006). There is still controversy regarding PVD, because it could be of vaginal, cervical, or uterine origin. Detection and scoring of vaginal discharge is an indirect assessment of endometritis (Pleticha et al., 2009), as PVD has been observed in absence of endometrial inflammation (Dubuc et al., 2010) and uterine contamination (Westermann et al., 2010). There is weak (Metricheck, Simcro, Hamilton, New Zealand;
Dubuc et al., 2010) to moderate (vaginoscopy; Barlund et al., 2008) agreement between PVD and cytological endometritis. The terminology “PVD” is considered more appropriate than “clinical endometritis” (Dubuc et al., 2010) because this is a nonspecific indication of an inflammatory process (de Boer et al., 2014).

Barlund et al. (2008) observed approximately 90% specificity but low sensitivity when criteria such as PVD, presence of fluid in uterus, or increased endometrial thickness were compared with endometrial cytology. It has been suggested that presence of fluid in the uterine lumen could reduce recovery of PMN and influence diagnosis (Barlund et al., 2008). Presence of fluid is suggestive of defense through clearance mechanisms, whereas presence of PMN relates to the cellular response to infection (Kasimanickam et al., 2004; Barlund et al., 2008). Ultrasonography allows objective measurements of the uterus and cervix and detection of intraluminal fluid, although it does not seem to provide more accurate diagnosis, having cytology as the reference method, compared with evaluation of vaginal discharge (Sheldon et al., 2006; Barlund et al., 2008). Vaginoscopy and ultrasonographic evaluation of the reproductive tract showed similar sensitivity and specificity for prediction of pregnancy by 150 DIM (Barlund et al., 2008).

The agreement between methods for diagnosis of diseases of the reproductive tract could be impaired by the stage of the estrous cycle at examination. An endocrine milieu of high progesterone impairs immune function via reduced production of mucus by the cervix, myometrial contractions, uterine gland secretions, and PMN phagocytic activity, whereas a profile of high estradiol likely improves immunity (LeBlanc, 2008). Estradiol increases blood flow to the reproductive tract, synthesis of mucus, and PMN function (Hussain, 1989). The effects of estradiol and progesterone in the reproductive tract could affect diagnosis by altering the percentage of endometrial PMN, recovery of PMN in cytology due to presence of mucus, and detection of discharge from uterine or cervical origin due to degree of cervix openness. In support of this, Brodzki et al. (2015) reported that the percentage of PMN on endometrial cytology slides obtained from healthy cows during the follicular phase was 2 times greater than that observed on luteal phase samples.

The objectives of this study were (1) to evaluate the association of plasma estradiol concentration and ovarian structures at time of examination with diagnosis and agreement among methods, and (2) to study the effect of positive diagnosis by various methods and under different estradiol concentrations on days to pregnancy and milk yield.

### Materials and Methods

#### Animals and Management

This observational study took place at the University of British Columbia’s Dairy Education and Research Centre (Agassiz, British Columbia, Canada) following the guidelines of the Canadian Council on Animal Care (CCAC, 2009). The local institutional animal care committee approved all experimental procedures.

Lactating Holstein cows (n = 268) were enrolled at 27 to 33 DIM. Data were collected until dry off or culling. Cows were housed in a sand-bedded freestall barn and fed a TMR to meet or exceed the requirements of a 620-kg Holstein cow producing 40 kg of 3.5% FCM per day (NRC, 2001). Data related to AI, pregnancy diagnosis, and milk production were recorded for the entire experimental period. Calving difficulty was recorded on a 1 to 4 scale (1 = unassisted; 2 = assistance by one person with hands only; 3 = assistance from more than one person and using chains; and 4 = abnormal presentation); stillbirths were also recorded. Milk production is presented as kilograms of milk produced until 45 DIM (M45d) and kilograms of milk adjusted to 305 d (M305d).

The reproductive management protocols from the dairy were followed during the experiment. Cows were artificially inseminated upon detection of estrus (visual and automated activity monitor; Heatime, SCR Engineers, Netanya, Israel) after a voluntary waiting period of 60 d. Cows not detected in estrus by 90 DIM or diagnosed nonpregnant were enrolled in timed ovulation synchronization programs (Santos et al., 2004). Pregnancy diagnosis was performed at 35 ± 6 d after AI (positive in the presence of uterine fluid with amniotic vesicle containing a viable embryo). The number of days open was recorded for each cow.

#### Reproductive Tract Examination

Each cow was examined twice in the postpartum period, at 30 ± 3 DIM (exam 1) and at 44 ± 3 DIM (exam 2; n = 521 exams; Figure 1), to determine score of vaginal discharge, diameter of the inner layer of the cervix (CVX) and of each uterine horn (UTH; reported values correspond to the largest horn), presence of fluid in uterus (FL) and of corpus luteum (CL), and percentage of PMN among endometrial cells on cytology slides. One person performed all diagnostic procedures. Body condition score was measured at exam 1 (1 to 5 scale; Ferguson et al., 1994). The complete exam was performed following milking (morning or afternoon), before cows returned to their pens.
**Vaginal Discharge.** A sample of vaginal discharge was retrieved by manual examination with a gloved hand (lubricated hand inserted through cleaned vulvar lips and advanced to the cranial extent of the vaginal fornix), as described by Sheldon et al. (2006). Following the gloved hand exam, discharge was retrieved using Metricheck (Simcro), a device consisting of a 40-mm silicon hemisphere attached to a 50-cm-long stainless steel rod. The device was disinfected with iodine solution and inserted through cleaned vulvar lips, advanced to the cranial extent of the vaginal fornix, and then retracted caudally. The discharge adhering to the glove and to the Metricheck device was scored from 0 to 5 (0 = no discharge; 1 = clear mucus; 2 = mucus containing flecks of pus; 3 = discharge containing <50% purulent material; 4 = discharge containing >50% purulent material; and 5 = discharge with odor and containing >50% purulent material; McDougall et al., 2007). Diagnosis of PVD was considered positive when discharge score was equal to or greater than 3.

**Ultrasonography.** After evaluation of vaginal discharge, measurement of uterus and cervix, and diagnosis of FL were performed by rectal ultrasonography. The previously gravid horn was identified according to the difference in horn diameter at exam 1. Diameters of the inner layer of cervix, measured at the middle portion, and of the uterine horns (lumen and endometrium; approximately 2 cm past the intercornual ligament) were obtained using an ultrasound machine (Aloka SSD-500, Aloka Co. Ltd., Wallingford, CT) equipped with a 7.5-MHz linear rectal transducer. Intraluminal fluid was recorded as present or absent. Asymmetry between uterine horns (ASY) was calculated as the modulus of the difference between right and left horn. Both ovaries were scanned to determine presence of CL.

**Uterine Cytology.** Samples for endometrial cytology (CYTO) were collected at exams 1 and 2 from the previously gravid horn using a cytobrush (VWR Canlab, Mississauga, ON, Canada) threaded onto a steel rod and placed into a steel tube, following the procedure described by Kasimanickam et al. (2004). The use of a cytobrush has been reported to be a consistent and reliable technique for endometrial cytology sampling (Kasimanickam et al., 2005), being more repeatable within observer than sampling by uterine lavage (Barlund et al., 2008). After sample collection, the cytobrush was rolled onto a glass microscope slide, which was placed in a box and transported to the on-farm laboratory for staining.

Endometrial smears were air-dried and stained with Romanowsky stain (Diff-Quick, Fisher Diagnostics, Middletown, VA). Slides were examined at 400× magnification using a light microscope (Nikon Eclipse E200, Nikon, Tokyo, Japan). A minimum of 100 cells (endometrial cells and PMN) were counted in each of 3 slide sections. The percentage of PMN was determined as the number of PMN relative to total counted cells (mean of the 3 fields). Diagnosis of positive CYTO was given when PMN percentage was ≥18% at exam 1 and ≥10% at exam 2 (Kasimanickam et al., 2004; Sheldon et al., 2006).

**Analysis of Plasma Estradiol.** Blood samples (7 mL) were harvested from the median coccygeal vein or artery utilizing Vacutainer tubes (Vacutainer system,
Becton Dickinson, Rutherford, NJ) with K₂EDTA at the beginning of each exam for analysis of plasma estradiol concentration. All samples were placed immediately on ice, transported to the laboratory, and centrifuged at 2,000 × g for 15 min for separation of plasma, which was stored at −80°C. Concentrations of estradiol were determined using a double-antibody 125I-based assay (Burke et al., 2003), with modifications. Standards were prepared by solubilizing purified 17β-estradiol (Sigma, St. Louis, MO) into PBS containing 0.1% gelatin (Sigma; 0.1% PBS gel; pH = 7.4) and then diluted according to the concentration of standards desired. Standard curve concentrations were determined using 200 μL of standard and 400 μL of charcoal-stripped steer plasma per tube. The tubes receiving pools and samples to be analyzed received 400 μL of the unknown sample or pools, and 200 μL of 0.1% PBS gel. All tubes were extracted by adding 4 mL of diethyl ether anhydrous (Sigma, St. Louis, MO), and then estradiol was extracted. Mean intra- and interassay CV were 4.2 and 5.2%, respectively; mean sensitivity was 1.1 pg/mL.

**Statistical Analyses**

Analyses were performed with SAS Studio University Edition (version 3.1; SAS Institute Inc., Cary, NC) and R (version 3.2.1; R Foundation for Statistical Computing, Vienna, Austria) via RStudio (version 0.99.442; RStudio Inc., Boston, MA). Significance was set at a probability of type I error of 5% and tendencies between 5 and 10%. Some continuous variables were categorized for further analysis: estradiol concentration (<2 or ≥2 pg/mL), BCS (low if ≤2.75; high if ≥3.0), UTH (small if ≤20 mm; large if >20 mm), ASY (asymmetric if difference >5 mm; symmetric if ≤5 mm), and CVX (small if ≤20.5 mm; large if >20.5 mm). The estradiol cut-point was determined as 2 pg/mL, given that this value approximates the minimum concentration observed during the follicular phase of the estrous cycle (Sartori et al., 2004). The BCS cut-point was set at the median of the distribution. Cut-points for UTH, ASY, and CVX were determined as the third quartile of the distributions. Parity effects were analyzed as primiparous versus multiparous; dystocia was analyzed as calving with no assistance versus calving with any level of assistance.

Prevalence of uterine disease at exams 1 and 2 was determined by calculating the frequency of positive diagnosis according to each method. Cohen’s kappa statistics (κ) for agreement among methods and its 95% confidence intervals were calculated with Proc FREQ (AGREE option). Prevalence and agreement between methods were also calculated according to categories of plasma estradiol concentration and presence or absence of a CL. Frequency distributions of PMN percentage were plotted using the hist() function in RStudio.

The effects of plasma estradiol, presence of CL, BCS, parity, and calving-related events on diagnosis of uterine disease were tested with logistic regression (LOGISTIC procedure) and backward elimination at P = 0.20. The effect of diagnosis on M45d and M305d was tested with the MIXED procedure. In addition to diagnosis, the models included effects of category of estradiol concentration, parity, BCS (high vs. low), and calving-related events (stillbirth and dystocia). Separate models were used for each diagnostic method and exam.

The association between diagnosis (PVD, FL, and CYTO) and pregnancy hazard by 250 DIM was tested with Cox proportional hazard models (PHREG procedure of SAS), controlling for effects of parity, BCS, stillbirth and dystocia. The data were analyzed by exam and by category of estradiol concentration. Data were right-censored at culling or at 250 DIM if pregnancy had not been previously confirmed. Only variables with P ≤ 0.20 were kept in the final models. The LIFETEST procedure was used to obtain median days open and the proportion of nonpregnant cows by time for construction of survival curves.

Sample size was calculated for detection of difference in prevalence of positive diagnosis among groups of 35 and 25%, with 80% power and 95% confidence, which yielded a sample size of 393 exams. Calculated sample size for pregnancy hazard was equal to 254 subjects for 80% power and 95% confidence, considering 30% of subjects diagnosed positive, correlation among diagnosis and covariate (i.e., parity) of 0.15, postulated hazard ratio of 1.5, and percentage of failure by censoring time of 90%.

**RESULTS**

Mean (SD) parity of the cows enrolled in the experiment was 2.8 (1.8) lactations. Primiparous and multiparous (2nd to 10th lactation) represented 29 and 71% of the cows, respectively. Median BCS at 30 ± 3 DIM was 2.75 (range: 1.75 to 3.75). Mean (SD) for M45d and M305d were 1,616 (454) kg and 10,763 (2,039) kg, respectively.
Prevalence

The prevalence of uterine disease according to each method is shown in Table 1. Because of the similar prevalence between PVD obtained with gloved hand and Metricheck, the presented PVD results will refer to Metricheck diagnosis unless noted otherwise. Reporting of uterine measurements is focused on ASY. References to gloved hand and UTH measurements are made when appropriate. The frequency distribution of PMN in each exam is presented in Figure 2. First quartile, median, and third quartile of PMN distribution were 2.2, 13.6, and 29.5% for exam 1, and 2.4, 10.0, and 18.2%, respectively, for exam 2.

At exam 1, 6.8% of the cows were positive for PVD and FL, 8.4% for PVD and CYTO, and 12.9% for FL and CYTO. At exam 2, simultaneous prevalence of PVD and FL was 2.3%, PVD and CYTO 11.7%, and FL and CYTO 5.5%.

Prevalence of PVD at exam 1 was greater among cows with plasma estradiol concentration ≥2 pg/mL at the time of exam (P = 0.02; Figure 3). A similar effect was not observed for positive CYTO or FL on either exam (P > 0.10; Figure 3). Presence or absence of CL at the time of exam influenced only the prevalence of FL at exam 2, which was greater among cows that had no CL at examination (16.5 vs. 6.5%; P = 0.02).

Agreement Among Methods

Overall, the agreement was poor between CYTO and other methods (PVD, FL, ASY; Table 2) at both exams, but kappa statistics were greater for exam 1 than for exam 2. There was no agreement between CVX and CYTO. Table 3 describes the agreement among methods other than CYTO. Methods for evaluation of vaginal discharge had better agreement with FL than with UTH, ASY, and CVX.

We observed better agreement between CYTO and PVD or FL among cows with estradiol concentrations ≥2 pg/mL than among cows with estradiol <2 pg/mL at exam 1 (Table 4). This effect was not observed at exam 2 (κ < 0.15; P > 0.10). The agreement between CYTO and PVD or FL was also improved when cows had a CL at time of exam 1 compared with those without a CL (κ = 0.21 vs. 0.06 for PVD; κ = 0.25 vs. 0.15 for positive FL; CL vs. no CL, respectively).

Factors Affecting Positive Diagnosis of Reproductive Tract Disorders

Purulent vaginal discharge was more likely observed at exam 1 if cows presented large CVX (odds ratio (OR) = 3.0; P = 0.02), FL (OR = 2.6; P = 0.06), or estradiol ≥2 pg/mL (OR = 2.7; P = 0.03; Figure 3) in the same exam; PVD was also more likely to be observed in primiparous cows (OR = 2.8; P = 0.03). There was a tendency for increased likelihood of PVD at exam 2 when cows were also positive for CYTO (OR = 2.1; P = 0.08).

The likelihood of positive CYTO at exam 1 increased with dystocia (OR = 2.3; P = 0.006) and FL (OR = 2.5; P = 0.01). There was a tendency for greater likelihood of positive CYTO if PVD was observed (OR = 2.2; P = 0.07). The likelihood of positive CYTO diagnosis at exam 2 (PMN ≥10%) was influenced only by PVD (OR = 2.1; P = 0.08) and by large ASY (OR = 2.5; P = 0.03).

Intraluminal fluid was more likely to be observed at exam 1 if cows presented large ASY (OR = 3.6; P = 0.03), positive CYTO (OR = 2.3; P = 0.03), and PVD (OR = 2.3; P = 0.09). At exam 2, only large ASY increased the likelihood of positive FL (OR = 4.3; P = 0.008).

Effects of Positive Diagnosis on Milk Production and Hazard of Pregnancy

Cows diagnosed positive for FL or CYTO at exam 1 had lower M45d (P = 0.03; Figure 4) but PVD at exam 1 was not associated with reduced M45d (P > 0.05). In addition to positive diagnoses, first lactation and occurrence of dystocia were associated with reduced M45d (P < 0.05). At exam 2, PVD, presence of FL, or posi-

Table 1. Prevalence [% (no. positive/no. examined)] of positive diagnosis on 2 consecutive reproductive tract examinations according to various methods applied to lactating Holstein cows

<table>
<thead>
<tr>
<th>Diagnostic method</th>
<th>Exam 1 (30 ± 3 DIM)</th>
<th>Exam 2 (44 ± 3 DIM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVD (Metricheck)</td>
<td>14.2 (38/268)</td>
<td>18.5 (46/249)</td>
</tr>
<tr>
<td>PVD (gloved hand)</td>
<td>13.4 (30/224)</td>
<td>15.5 (32/207)</td>
</tr>
<tr>
<td>FL</td>
<td>21.4 (57/266)</td>
<td>10.1 (22/221)</td>
</tr>
<tr>
<td>CYTO</td>
<td>40.6 (101/249)</td>
<td>50.2 (113/225)</td>
</tr>
<tr>
<td>CVX</td>
<td>33.0 (88/267)</td>
<td>15.6 (35/225)</td>
</tr>
<tr>
<td>UTH</td>
<td>40.4 (107/265)</td>
<td>24.0 (54/225)</td>
</tr>
<tr>
<td>ASY</td>
<td>32.5 (86/265)</td>
<td>18.2 (41/225)</td>
</tr>
</tbody>
</table>

1Vaginal discharge; positive if discharge score ≥3 (0 to 5 scale, according to McDougall et al., 2007). Metricheck was from Simcro (Hamilton, New Zealand).
2Presence of fluid in uterine lumen detected with ultrasound.
3Endometrial cytology; positive if percentage of PMN in endometrial cytology slides was ≥18% at exam 1 and ≥10% at exam 2.
4CVX = diameter of cervix inner layer; UTH = diameter of the largest uterine horn–lumen and endometrium; ASY = asymmetry; modulus of the difference in diameter between uterine horns. Positive if greater than the third quartile: CVX >20.5 mm; UTH >20 mm; ASY >5 mm.
Positive CYTO did not influence M45d ($P > 0.05$). Positive diagnoses were not associated with M305d independent of method or exam ($P > 0.05$).

At exam 1, diagnosis of PVD or FL had no effect on hazard of pregnancy by 250 DIM ($P = 0.78$ and $P = 0.15$, respectively). However, positive CYTO at exam 1 was associated with reduced pregnancy risk [hazard ratio (HR) = 0.74, $P = 0.04$]. Median days open for CYTO positive and negative cows at exam 1 were 135.6 and 119.7, respectively. At exam 2, PVD (HR = 0.68, $P = 0.04$; negative vs. positive: 122.7 vs. 142.1 d open) was associated with reduced pregnancy hazard. There was no effect of CYTO or FL diagnosed at exam 2 on hazard of pregnancy ($P = 0.12$ and $P = 0.23$, respectively).

Figure 2. Frequency distribution of percentage of PMN in endometrial cytology slides from 268 lactating Holstein cows examined at 30 ± 3 (exam 1) and 44 ± 3 DIM (exam 2).

Figure 3. Prevalence of positive diagnosis by purulent vaginal discharge score (PVD; Metrichek; Simcro, Hamilton, New Zealand), PMN ≥18% on endometrial cytology (CYTO), and presence of intraluminal fluid (FL) at 30 ± 3 DIM (exam 1) according to plasma estradiol concentration ≥2 pg/mL (solid bars) and <2 pg/mL (open bars) categories. $P$-values refer to $2 \times 2$ $\chi^2$ test.
At exam 1, cows with plasma estradiol ≥2 pg/mL had reduced hazard of pregnancy until 250 DIM if diagnosed positive for FL (HR = 0.61; \( P = 0.04 \)) and for CYTO (HR = 0.58; \( P = 0.001 \)), whereas no difference in hazard of pregnancy was detected among cows with plasma estradiol <2 pg/mL diagnosed negative or positive according to FL (\( P = 0.52 \)) or CYTO (\( P = 0.61 \)). At exam 2, presence of PVD was associated with reduced hazard of pregnancy for cows with estradiol <2 pg/mL (HR = 0.64; \( P = 0.04 \)). Among cows with estradiol ≥2 pg/mL at exam 2, PVD had no effect on hazard of pregnancy by 250 DIM (\( P = 0.61 \)). Survival curves by diagnosis and estradiol category for each method are presented in Figure 5.

### DISCUSSION

Although PVD and CYTO are commonly used for diagnosis of reproductive tract disorders in cows, there is a lack of agreement among these methods. Sampling and interpretation of endometrial cytology slides are likely subjected to diverse sources of variation, including sampled area, number of rotations of the cytobrush against the endometrium, and methodology of cell sampling and interpretation.

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**Table 2.** Agreement between endometrial cytology (CYTO) and discharge score determined with Metricheck (PVD; Simcro, Hamilton, New Zealand), presence of intraluminal fluid (FL), asymmetry of uterine horns (ASY), and cervix diameter (CVX)

<table>
<thead>
<tr>
<th>Diagnostic method</th>
<th>Kappa (95% CI)</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exam 1 (30 ± 3 DIM); vs. CYTO (≥18% PMN)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVD(^1)</td>
<td>0.13 (0.02; 0.23)</td>
<td>0.006</td>
</tr>
<tr>
<td>FL(^2)</td>
<td>0.19 (0.08; 0.31)</td>
<td>0.0003</td>
</tr>
<tr>
<td>ASY(^3)</td>
<td>0.13 (0.01; 0.25)</td>
<td>0.02</td>
</tr>
<tr>
<td>CVX(^3)</td>
<td>0.00 (−0.13; 0.11)</td>
<td>0.45</td>
</tr>
<tr>
<td>Exam 2 (44 ± 3 DIM); vs. CYTO (≥10% PMN)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVD</td>
<td>0.09 (−0.01; 0.19)</td>
<td>0.04</td>
</tr>
<tr>
<td>FL</td>
<td>0.02 (−0.05; 0.09)</td>
<td>0.27</td>
</tr>
<tr>
<td>ASY</td>
<td>0.12 (0.02; 0.22)</td>
<td>0.01</td>
</tr>
<tr>
<td>CVX</td>
<td>−0.01 (−0.10; 0.08)</td>
<td>0.39</td>
</tr>
</tbody>
</table>

\(^1\)Vaginal discharge; positive if discharge score ≥3 (0 to 5 scale, according to McDougall et al., 2007).

\(^2\)Presence of fluid in uterine lumen detected with ultrasound.

\(^3\)CVX = diameter of cervix inner layer; ASY = asymmetry; modulus of the difference in diameter between uterine horns. Positive if greater than the third quartile: CVX >20.5 mm; diameter of the largest uterine horn—lumen and endometrium >20 mm; ASY >5 mm.

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**Table 3.** Agreement (Cohen’s kappa statistic) between positive diagnoses by different methods based on vaginal discharge (PVD; gloved hand and Metricheck), uterine intraluminal fluid (FL) and measurements of cervix (CVX) and uterus [largest uterine horn (UTH) and asymmetry (ASY)] of lactating Holstein cows

<table>
<thead>
<tr>
<th>Diagnostic method</th>
<th>PVD(^1) (\text{(gloved hand)})</th>
<th>FL(^2)</th>
<th>ASY(^3)</th>
<th>UTH(^3)</th>
<th>CVX(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exam 1 (30 ± 3 DIM)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>PVD (Metricheck)</td>
<td>0.73(^*)</td>
<td>0.25(^*)</td>
<td>0.15(^*)</td>
<td>0.04(^{NS})</td>
<td>0.19(^*)</td>
</tr>
<tr>
<td>PVD (gloved hand)</td>
<td>—</td>
<td>0.39(^*)</td>
<td>0.20(^*)</td>
<td>0.10(^*)</td>
<td>0.20(^*)</td>
</tr>
<tr>
<td>FL</td>
<td>—</td>
<td>—</td>
<td>0.29(^*)</td>
<td>0.31(^*)</td>
<td>0.23(^*)</td>
</tr>
<tr>
<td>UTH</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.23(^*)</td>
</tr>
<tr>
<td>ASY</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.30(^*)</td>
</tr>
<tr>
<td>Exam 2 (44 ± 3 DIM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVD (Metricheck)</td>
<td>0.66(^*)</td>
<td>0.04(^{NS})</td>
<td>0.13(^*)</td>
<td>0.02(^{NS})</td>
<td>−0.05(^{NS})</td>
</tr>
<tr>
<td>PVD (gloved hand)</td>
<td>—</td>
<td>−0.05(^{NS})</td>
<td>0.06(^{NS})</td>
<td>0.01(^{NS})</td>
<td>−0.01(^{NS})</td>
</tr>
<tr>
<td>FL</td>
<td>—</td>
<td>—</td>
<td>0.24(^*)</td>
<td>0.33(^*)</td>
<td>0.08(^{NS})</td>
</tr>
<tr>
<td>UTH</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.15(^*)</td>
</tr>
<tr>
<td>ASY</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.24(^*)</td>
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</tbody>
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\(^1\)Vaginal discharge; positive if discharge score ≥3 (0 to 5 scale, according to McDougall et al., 2007). Metricheck was from Simcro (Hamilton, New Zealand).

\(^2\)Presence of fluid in uterine lumen detected with ultrasound.

\(^3\)CVX = diameter of cervix inner layer; UTH = diameter of the largest uterine horn—lumen and endometrium; ASY = asymmetry; modulus of the difference in diameter between uterine horns. Positive if greater than the third quartile: CVX >20.5 mm; UTH >20 mm; ASY >5 mm.

\(^*\)\( P < 0.05\); \(^{NS}\)\( P > 0.05\).
count. A gold standard test for reproductive tract disorders has not yet been defined (de Boer et al., 2014). Data reviewed by de Boer et al. (2014) documented the variation in cut-points, strategy for their determination, and DIM at examination. The PMN data in the present study had a fairly uniform distribution between 0 and 20% PMN, different from that in McDougall et al. (2011), for example, who observed more skewed data.

Results of this study indicated that plasma estradiol concentration and presence or absence of a CL at time of examination affect the apparent prevalence and agreement among methods for diagnosis of reproductive tract disorders. Our results indicate an association of FL and CYTO with reduced milk yield in the first 45 DIM and of PVD and CYTO with decreased hazard of pregnancy. Even though these associations were not consistent among different methods, exams, or category of estradiol concentration at the time of exam, the magnitude of reduction in pregnancy hazard for CYTO and PVD was similar to that reported by others (Denis-Robichaud and Dubuc, 2015: PVD and CYTO with cut-point of 6% PMN; Kasimanickam et al., 2004: CYTO with cut-point of 18% PMN in the absence of PVD).

Cut-points for %PMN are commonly determined based on their associations with reproductive performance or according to data distribution (de Boer et al., 2014). We acknowledge that the cut-points applied to our data have been used where clinical cases (PVD) have been excluded (Kasimanickam et al., 2004). However, given our %PMN distribution, cut points of 6 and 4% for exams 1 and 2, respectively, or based on third quartiles would not be representative classifications. Differences in sampling, processing, and reading techniques could result in variable adequacy of cut-points among studies. Consistency within study is an essential

Table 4. Agreement between positive diagnosis for endometrial cytology (CYTO) and vaginal discharge score determined with Metricheck (PVD) or presence of fluid in the uterine lumen (FL) in lactating Holstein cows examined at 30 ± 3 DIM (exam 1), according to plasma estradiol concentration at time of examination

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Estradiol &lt;2 pg/mL</th>
<th>Estradiol ≥2 pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVD vs. CYTO</td>
<td>0.13 (0.02)</td>
<td>0.15 (0.02)</td>
</tr>
<tr>
<td>FL vs. CYTO</td>
<td>0.03 (0.34)</td>
<td>0.35 (&lt;0.0001)</td>
</tr>
</tbody>
</table>

1Vaginal discharge; positive if discharge score ≥3 (0 to 5 scale, according to McDougall et al., 2007). Metricheck was from Simcro (Hamilton, New Zealand).
2Endometrial cytology: positive if percentage of PMN in endometrial cytology slides was ≥18% at exam 1.
3Presence of fluid in uterine lumen detected with ultrasound.

Figure 4. Mean ± SEM milk production until 45 DIM by Holstein cows examined at 30 ± 3 DIM (exam 1), according to (A) presence of intraluminal fluid (FL) detected by ultrasonography: solid bars = negative; open bars = positive (P = 0.02); and (B) percentage of PMN on endometrial cytology (CYTO): solid bars = PMN <18%; empty bars = PMN ≥18% (P = 0.05). Within chart and parity, asterisks indicate effect of diagnosis. Parity effect: P < 0.0001.
Figure 5. Survival curves for DIM to pregnancy until 250 DIM for cows diagnosed positive or negative for presence of intraluminal fluid detected by ultrasonography (FL; exam 1), endometrial cytology using a cytobrush, positive if PMN ≥18%; (CYTO; exam 1), and examination of vaginal discharge with Metricheck (Simcro, Hamilton, New Zealand), positive if score ≥3 (PVD; exam 2), according to plasma estradiol concentration (<2 or ≥2 pg/mL) at the moment of examination. Effect of diagnosis on hazard of pregnancy, for low and high estradiol, respectively: \( P = 0.52 \) and \( P = 0.04 \) (FL), \( P = 0.61 \) and \( P = 0.001 \) (CYTO), and \( P = 0.04 \) and \( P = 0.48 \) (PVD). Asterisks (*) indicate statistical difference between positive and negative diagnosis.
The greater prevalence of FL and CVX, UTH, and ASY above the cut-point at exam 1 versus exam 2 is in accordance with the normal process of uterine involution, which should be completed at approximately 6 wk postpartum (Sheldon et al., 2006) and coincides with DIM at exam 2. On the other hand, PVD and CYTO were more prevalent at exam 2. This is contrary to our initial hypothesis and to results from others. Although different criteria were applied, Kasimanickam et al. (2004) observed similar prevalence of CYTO between 2 examinations 14 d apart, and Dubuc et al. (2010) reported greater prevalence of PVD and CYTO at 35 DIM than at 56 DIM. The greater prevalence of PVD and CYTO at 44 DIM compared with 30 DIM could be associated with the large variability in prevalence of cytological endometritis (LeBlanc et al., 2011). This could also reflect consequences of manipulation of the reproductive tract increasing the observation of an inflammatory response at exam 2. Sheldon et al. (2002) did not observe bacterial contamination or increased inflammatory response in the uterus after gloved hand examination; data on contamination or induction of local inflammation after use of Metricheck or cytobrush are not available. Prevalence of PVD sampled with gloved hand or Metricheck was similar at both exams but lower than reported by McDougall et al. (2007) and Pleticha et al. (2009), likely due to our choice of a more severe score as the cut-point for positive diagnosis and to our second examination at greater DIM.

Metricheck and endometrial cytology are likely more specific diagnoses for infection and inflammation than detection of uterine fluid or measurements of uterus and cervix, which also quantify postpartum involution and thus are nonspecific indicators of endometritis (Sheldon et al., 2006). Vaginoscopy has been reported to be more specific and sensitive for diagnosis of endometritis than measurements of the uterus by palpation (LeBlanc et al., 2002). It is likely that our decision for positive diagnosis by ASY and CVX with cut-point at the third quartile identified cows that experienced the slowest process of involution within our sample, even though it has been suggested that presence of fluid at 20 to 33 DIM could be a normal condition (Kasimanickam et al., 2004). The greatest agreement was observed between FL and ASY or CVX. Compared with CYTO, FL was the method with the greatest agreement and it showed fair agreement with PVD. Accumulation of intraluminal fluid is increased in cows with severe endometritis and correlated with bacterial growth from uterine swabs (Mateus et al., 2002). Therefore, FL at 30 DIM could be indicative of uterine pathology and a common factor among cows presenting PVD and CYTO simultaneously.

At exam 2, the risk of CYTO or PVD was increased by the presence of each other, suggesting reciprocal effects even if these methods diagnose distinct pathologies. This is in agreement with the notion that PVD can be a clinical symptom of cytological endometritis or of other disorders, such as vaginitis (de Boer et al., 2014). The increased risk of FL at both exams and of CYTO at exam 2 in cows with large ASY further supports the relationship between these measurements and uterine involution. Fluid and increased PMN percentage on endometrial cytology have been regarded as different defense mechanisms in the uterus—clearance and cellular response, respectively. Differences in apparent prevalence among methods and exams, in addition to overall poor agreement, indicate diagnosis of distinct pathologies that additively impair reproductive performance (Barlund et al., 2008; Dubuc et al., 2010).

Prevalence of PVD at exam 1 was greater among cows with plasma estradiol ≥2 pg/mL. Greater secretion by the endometrial glands, myometrial contractions, and opening of the cervix observed in periods of estradiol predominance (Hussain, 1989; LeBlanc, 2008) could contribute to greater likelihood of PVD detection. Estradiol predominance has also been associated with increased blood flow to the uterus and enhanced PMN function (Hussain, 1989). Subandrio et al. (2000) observed an increase in phagocytic capacity of PMN during such periods. The PMN percentage on endometrial cytology of healthy cows has been reported to be greater at the follicular phase than at the luteal phase (2.8 and 1.3%, respectively; Brodzki et al., 2015). It is possible that a greater concentration of estradiol induces accumulation and discharge of fluid. It has been suggested that the probability of PVD diagnosis is increased during estrus (Kasimanickam et al., 2004); our results show that estradiol at concentrations expected during proestrus and estrus levels increased the likelihood of PVD diagnosis.

Like estradiol, progesterone could have effects on the reproductive tract and interfere in diagnosis, but evaluation of progesterone concentrations was not possible in this study. The effect of the presence of CL, as in indirect indication of progesterone predominance, was associated with reduced prevalence of FL at exam 2, in agreement with suppression of endometrial gland secretion under such conditions (LeBlanc, 2008). Progesterone also reduces synthesis of cervical mucus,
phagocytic activity by PMN, and myometrial contractions (LeBlanc, 2008). Prevalence of PVD or CYTO, however, was not affected by the presence or absence of a CL. The observed effects of estradiol and presence of CL on prevalence of positive diagnoses suggest facilitated diagnosis at exam 1 when concentrations of estradiol were higher, whereas presence of a CL at exam 2 induced physiological changes related to the estrous cycle, likely confounding the diagnosis.

It is questionable whether the effect on immune function is due to presence of estradiol or absence of higher progesterone concentrations (Rowson et al., 1953; Hussain, 1989; LeBlanc, 2008). The present study indicates an important effect of estradiol, but further analysis of effects of estradiol and evaluation of progesterone’s role should be performed in controlled studies. Cows with PVD are less likely to have palpable ovarian structures (LeBlanc et al., 2002) and more likely to have abnormal ovarian dynamics and abnormal progesterone profiles than healthy cows (Mateus et al., 2002). This observational study is the first to focus on effects of stage of the cycle and endocrine profile on diagnosis of reproductive tract disorders. Future research should address the effects of progesterone concentration and of estradiol in the context of progesterone, which were limitations of this study.

The importance of stage of the cycle, or the effect of estradiol, is further supported by the increased apparent prevalence of PVD and increased likelihood of detection of PVD among cows with estradiol ≥2 pg/mL. In this situation, PVD could be due to greater secretion of mucus from vagina, cervix, and uterus, but also due to an open cervix, where PVD would likely be of uterine origin. Our hypothesis is that estradiol induces changes in the reproductive tract that facilitate detection of discharge in the vagina. This hypothesis is supported by the improved agreement of PVD and FL with CYTO among cows with estradiol ≥2 pg/mL at exam 1, as well as by increased likelihood of PVD diagnosis when FL and large CVX were present.

Although estradiol ≥2 pg/mL was a risk factor for increased PVD diagnosis, it reduced the likelihood of CYTO diagnosis at exam 1. These results could be due to a combined effect of estradiol on greater discharge of intraluminal uterine content, increasing the likelihood of PVD but reducing recovery of PMN with the cytobrush technique (Barlund et al., 2008). Migration of PMN to the uterus might be enhanced during periods of estradiol predominance, but presence of fluid could impair collection of cells adhered to the endometrium. The reduced likelihood of diagnosis when estradiol ≥2 pg/mL is in agreement with the decrease in pregnancy hazard for CYTO-positive cows observed only when estradiol was also ≥2 pg/mL. This suggests different effects of positive diagnosis, with the same cut-point, when circumstances of examination were different. Nevertheless, the effects of estradiol concentration at time of exam on prevalence and agreement and its different effects on pregnancy hazard support the concept that PVD, CYTO, and FL represent different pathologies.

Although the greater prevalence and agreement with CYTO observed for PVD when estradiol was ≥2 pg/mL suggest improved diagnosis, PVD only reduced pregnancy hazard when estradiol at time of exam was <2 pg/mL, suggesting that the PVD diagnosed when concentrations of estradiol were higher could be less detrimental to fertility. Diagnosis by CYTO and FL at exam 1 reduced the pregnancy hazard significantly only when diagnosed at periods of estradiol ≥2 pg/mL, in contrast to PVD observations. Diagnosis by FL and CYTO could be facilitated by higher circulating estradiol or the methods used failed to identify cows at risk of reproductive failure when estradiol was <2 pg/mL. Plasma estradiol concentrations were dichotomized with the objective of studying its effects as part of 2 distinct cycle phases, although we acknowledge that this practice might lead to some information loss or, potentially, to misclassification of data points near the cut-point (Dawson and Weiss, 2012).

Effects of positive diagnosis on pregnancy hazard varied among methods and time of examination. Previously, Kasimanickam et al. (2004) reported reduced pregnancy at first AI if, in the absence of PVD, endometrial cytology was positive at 34 to 47 DIM but not at 20 to 33 DIM, whereas days open were greater for cows diagnosed positive in either exam. The different effects of method by time of examination on pregnancy hazard might be related to proximity to breeding. It should be investigated whether treatment at this point effectively reduces losses in reproductive performance compared with earlier diagnosis. The practice of 2 examinations seems to provide an opportunity to combine early diagnosis and avoidance of confounding effects due to incomplete uterine involution (Kasimanickam et al., 2004; LeBlanc, 2008). Alternative methods for diagnosis of reproductive tract disorders should be considered regarding their agreement with CYTO but also considering the effects of diagnosis on reproductive performance.

Although M305d was not associated with positive diagnosis by any method, M45d was significantly lower (1.7 to 3.3 kg/d) when cows were positive for FL or CYTO at 30 DIM (exam 1). It is difficult to establish a cause–consequence relationship between production and disease; reduced milk production has been associated with clinical metritis (Fourichon et al., 1999), but
its relationships with PVD and cytological endometritis have not been investigated.

CONCLUSIONS

The agreement between PVD or FL and CYTO was poor but improved among cows with plasma estradiol ≥2 pg/mL, indicating that, in such conditions, vaginal discharge could represent a more specific measure of uterine disease. Because examination at estradiol concentrations that are characteristic of follicular phases resulted in increased apparent prevalence and greater likelihood of diagnosis by some methods, awareness of estrous cycle phase could improve accuracy of diagnosis. Moreover, intraluminal fluid presented the best agreement with endometrial cytology and had better, albeit still poor, agreement with other methods. Diagnoses of CYTO and FL were associated with reduced milk yield in the first 45 DIM, and detection of PVD at 44 ± 3 DIM increased time to pregnancy. Given the effects of estradiol on diagnosis, and diagnosis associations with milk yield and pregnancy hazard, effects of progesterone concentration should be similarly evaluated, as well as the effects of estradiol in conjunction with progesterone.

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