Université de Montréal

COMPARISON OF A LEUKOCYTE ESTERASE TEST WITH ENDOMETRIAL CYTOLOGY FOR THE DIAGNOSIS OF SUBCLINICAL ENDOMETRITIS AND CORRELATION WITH FIRST SERVICE PREGNANCY RATE IN POSTPARTUM HOLSTEIN COWS

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RÉSUMÉ

L'objectif de la présente étude était d'évaluer un test d'estérase leucocytaire (LE) pour le diagnostic de l'endométrite subclinique chez les vaches Holstein en période postpartum. Les tests effectués à partir d'échantillons provenant soit de l'endomètre (UtLE) ou du col utérin (CxLE) ont été comparés à la cytologie endométriale (CE). Par ailleurs, deux méthodes d'évaluation des lames ont été comparées. Deux cent quatre vingt-cinq vaches Holstein de 5 troupeaux laitiers commerciaux ont été évaluées entre 21 et 47 jours en lait (JEL). Soixante sept vaches ont été diagnostiquées avec une endométrite clinique suite à un examen transrectal et vaginoscopique et ont été exclues de l'étude. Deux cent dix-huit vaches ont eu des prélèvements pour la CE et le test LE. La fonction ovarienne a été déterminée à la palpation transrectale. La banque de données utilisée pour chacune des vaches a été effectuée à partir du logiciel DSA (Dossier de Santé Animale) laitier. Le pourcentage de neutrophiles était significativement corrélé avec les scores de LE utérin et cervical. L'activité de CxLE et UtLE diminuait significativement avec les JEL, mais n'était pas associée au risque de gestation à 90 JEL (n= 186). Le pourcentage de neutrophiles mesuré à la CE entre 32 et 47 JEL était associé significativement au risque de gestation à 90 JEL (n= 94, P=0.04). Pour la même période, selon une analyse de survie, les vaches avec >2,6% de neutrophiles à la CE étaient définies comme étant atteintes d'une endométrite subclinique avec une prévalence de 56%. Les résultats indiquent que le test

d’estérase utérin ou cervical a une bonne concordance avec le pourcentage de neutrophiles à la CE. Une endométrite subclinique diagnostiquée par cytologie endometriale entre 32 et 47 JEL est associée à une réduction du risque de gestation au premier service.

Mots clés: endométrite subclinique, cytologie endométriale, estérase leucocytaire, taux de gestation.

ABSTRACT

The point toward this study was to determine the diagnostic test characteristics of the leukocyte esterase activity test for subclinical endometritis in postpartum Holstein dairy cows. The objectives were 1) to compare uterine leukocyte esterase activity and the endometrial cytology (EC) 2) to compare leukocyte esterase activity of the cervix (CxLE) and the uterus (UtLE). 3) Compare two methods of assessing the slides (i.e. an exhaustive method and a rapid method). Two hundred eighty five post partum Holstein cows from 5 commercial dairy herds had a post partum evaluation between 21 and 47 days in milk (DIM). Sixty seven cows where diagnosed with clinical endometritis by transrectal and vaginoscopy examinations and were excluded from the study. Two hundred eighteen cows were enrolled for endometrial cytology and esterase activity test. The ovarian status was determined by transrectal examination. Computerized databank, dairy DSA (Dossier de Santé Animale) indexing all the cows was used to retrieve individual information for analysis. The percentage of neutrophils was significantly correlated with the LE from the uterus and cervix. The LE from cervix and uterus decreased significantly with DIM, however, they were not statistically associated with pregnancy risk at 90 DIM (n= 186). Between 32-47 DIM, the percentage of neutrophils and risk of pregnancy at 90 DIM were associated (n=94, P=0.04). For the same period, survival analysis identified cows with > 2.6 % neutrophils on EC as subclinical endometritis cows with a prevalence of 56%. The two methods for assessing the

slides were correlated by 81%. Subclinical endometritis diagnosed by endometrial cytology between 32 and 47 DIM was associated with reduced risk of pregnancy at first service.

Key words: subclinical endometritis, endometrial cytology, leukocyte esterase.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

C3	Complement 3
C3b	Complement Component 3b
C5	Complement 5
CxLE	Cervix leukocyte esterase
DIM	Days in Milk
DSA@	Dossier de Santé Animal
EC	Endometrial Cytology
ECF-A	Eosinophil Chemotactic Factor - A
Fc-component	Immunoglobulin Component
G	Group
GT	Group Total
IgG	Immunoglobulin G
JEL	Jour en Lait
LE	Leukocyte Esterase
LTB4	Leukotriene B4
PGE	Prostaglandin E
PGF2α	Prostaglandin F2α
PMN	Polymorphonuclear
RM	Rigorous Method

ROC	Receiver/Response Operating Characteristics
SM	Simple Method
TNF	Tumor Necrosis Factor
TRE	Trans-rectal Examination
UtLE	Uterine Leukocyte Esterase
VEx	Vaginal Examination
VWP	Voluntary Waiting Period

To my family and friends

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INTRODUCTION

The most common causes of uterine inflammation occur as the result of postpartum ascending contamination by nonspecific environmental organisms. At partum the uterus becomes vulnerable to bacterial contamination by the prolonged dilatation of one of its most important barriers, the cervix. Bacterial contamination is almost certain, and the reestablishment of a sterile uterine cavity depends on the cow's ability to reconstitute the reproductive tract after parturition to its pre-gravid condition in a few weeks with assistance of a competent immune system. The initial response to bacterial contamination is a neutrophilic influx, which may induce further inflammatory responses, which include mast cell activation, eosinophil chemotaxis, and serum extravasation with subsequent complement activation.

Uterine diseases after parturition have an important impact on the reproductive performance of dairy cows, affecting more then 50% of the dairy cattle, disrupting uterine and ovarian functions, increasing services per conception, calving to first service interval, calving to conception interval, culling rates (Borsberry and Dobson 1989; Heuwieser, Tenhagen et al. 2000) and decreasing conception rate (Fourichon, Seegers et al. 2000; LeBlanc, Duffield et al. 2002). It creates financial losses to the dairy industry that can be as much as US$ 285 per lactation cow (Bartlett, Kirk et al. 1986; Guard 1994; Drillich, Beetz et al. 2001). Since a certain degree of uterine inflammation is normally present during the physiological uterine involution of the uterus

during the post partum (Gier and Marion 1968)and a lack of rigorous characterisation of the process, the complex relationship between postpartum diseases and reproductive performance remains unclear (Fourichon, Seegers et al. 2000).

Even thought subclinical endometritis is a difficult condition to diagnose, it could still impair reproductive performance with few studies performed on cows (Kasimanickam, Duffield et al. 2004; Gilbert, Shin et al. 2005; Santos, Lamb et al. 2009). In cows, reasonable methods to appraise the inflammatory process of the endometrium comprise uterine lavage and cytobrush cytology (Kasimanickam, Duffield et al. 2004; Gilbert, Shin et al. 2005). Both methods have been described and accepted as diagnostic techniques. The cytobrush technique seems to be the most practical technique and the most reliable diagnostic test compared with uterine lavage (Kasimanickam, Duffield et al. 2005)and ultrasonography (Barlund, Carruthers et al. 2008).

Leukocyte esterase test has been used for a rapid diagnosis of inflammation in many body fluids such as urine, pleural fluid, peritoneal fluid, and cerebrospinal fluid (Levy, Tournot et al. 1989; Azoulay, Fartoukh et al. 2000; Braga, Souza et al. ; Rerknimitr, Rungsangmanoon et al. 2006) and could be used as an indirect method to detect neutrophils in the cow's uterus as suggested (Santos, Roman et al. 2006).

CHAPTER 1 - LITERATURE REVIEW

Parturition

The fetus initiates the end of pregnancy by signaling to the dam once it is ready to survive on its on. There are many hormonal changes associated with parturition concerned with maturation of the fetus lungs, softening of cervix and dilation of the birth canal, uterine contraction, milk synthesis and ejection. The hypothalamic-pituitary axis of the fetus is responsible for signaling for initiation of parturition (Wood 1999) with fetal ACTH and corticosteroids becoming elevated 1 to 2 days before parturition (Wood 1999). The conversion of progesterone to estrogens by cotyledon's enzyme is caused by the increased concentration of fetal corticosteroids (Wood 1999). Such an increase in placental steroidal activity is evidenced by the dramatic rise in prepartum concentrations of estrogens, estrone sulfate, and other estrogen precursors. Estrogen stimulates release of maternal PGF2α from the uterine endometrium, resulting in increase of receptor of oxytocin, which results in the regression of the corpus luteum of pregnancy (Garverick and Smith 1993; Wood 1999) and an influx of inflammatory cells into the uterine lumen. Furthermore, PGF2α, estrogen, and relaxin cause softening of the cervix and relaxation of the pelvic ligaments to facilitate birth. Inflammatory cells invade also the cervix at term with its cytokines being involved in cervical ripening (Kelly 2002) which, act

on fibroblasts and smooth muscle cells to release proteases (Sennstrom, Ekman et al. 2000). Interleukin 8 (IL-8) acts as a neutrophil chemotactic factor and is involved in the inflammatory cell invasion of the cervix at term. Its administration causes cervical softening in experimental animals (Chwalisz, Benson et al. 1994; Kelly 2002). The physical barriers composed of the cervix, the vagina and the vulva are compromised and providing a unique opportunity for bacteria to colonize the endometrium (Elliott, McMahon et al. 1968; Sheldon, Noakes et al. 2002).

Involution

Following calving, the uterus must undergo extensive remodeling to reduce in size, remove cellular debris, and restore a normal histological architecture (Gier and Marion 1968; Leslie 1983; Sheldon and Dobson 2004). By 7 to 10 days postpartum, the uterine wall is still very thick and several hundred ml of fluid and lochia may still be present within the lumen. By the 10th to 14th day after calving, the capillary beds of the caruncles are exposed allowing slight hemorrhage to occur (Gier and Marion 1968) and neutrophils in great numbers enter the uterine lumen during this period given the lochia a somewhat purulent appearance which might lead to an incorrect diagnosis of endometritis and treatment of normal cows. During this process of involution the uterus loses a vast amount of weight and size, going from 5kg to 0.9kg and 25cm to 3cm in diameter in a 30-day post partum period; when the magnitude

of the uterus has come to its non gravid dimensions (Gier and Marion 1968; Vaillancourt 1987). A dynamic path of clearance and recontamination of bacteria continues for the first few weeks post partum (Griffin, Hartigan et al. 1974) with a wide range of bacteria species being established as uterine pathogens: *Arcanobacterium pyogenes, Escherichia coli, Fusobacterium necrophorum and Prevotella* spp (Studer and Morrow 1978; Miller, Kimsey et al. 1980; Dohmen, Lohuis et al. 1995; Bondurant 1999; Williams, Fischer et al. 2005). Indeed, *A. pyogenes, F. necrophorum* and *Prevotella* species act synergistically to enhance the odds and harshness of uterine disease (Griffin, Hartigan et al. 1974; Ruder, Sasser et al. 1981; Olson, Ball et al. 1984; Bonnett, Martin et al. 1991). The occurrence and severity of uterine disease during the post partum period depend on the balance between bacterial contamination and the animal's defense mechanisms. The uterine resistance depends on immediate defense against microorganisms (innate immunity) and mucosal defense systems rather than a long lasting defense (adaptive immunity) (King, Critchley et al. 2003; Sheldon, Lewis et al. 2006). Uterine diseases result from a breakdown of theses protective systems. Forty percent of cows have metritis in the first two weeks of post partum and 15% have clinical endometritis within the 3 to 6 weeks post partum (Lewis 1997; Sheldon, Lewis et al. 2006; Sheldon, Cronin et al. 2009). Subclinical endometritis is diagnosed only by the presence of neutrophils on cytology samples 3 weeks postpartum onward, with a prevalence of positive samples ranging from 0% to 74% (Kasimanickam, Duffield et al. 2004; Gilbert, Shin et al. 2005; Barlund, Carruthers et al. 2008).

By 21 DIM, the uterus has decreased in size, lies completely within the

pelvis and by 30DIM involution is normally complete. The inter-caruncular area is quickly repaired, however regeneration of the caruncular epithelium may only commence subsequent to the sloughing of the caruncles which begins about 15 DIM and is complete by 30 DIM (Gier and Marion 1968). However, complete microscopic involution (i.e. number of glands, dilatation of glands, fibrosis around glands, lymphocytic foci and number of inflammatory cells (Bonnett, Miller et al. 1991)) takes more time to occur than macroscopic involution measured by palpation. Up to 50 days are necessary for regression and re-epithelization of the endometrium to occur after parturition (Marion and Gier 1959).

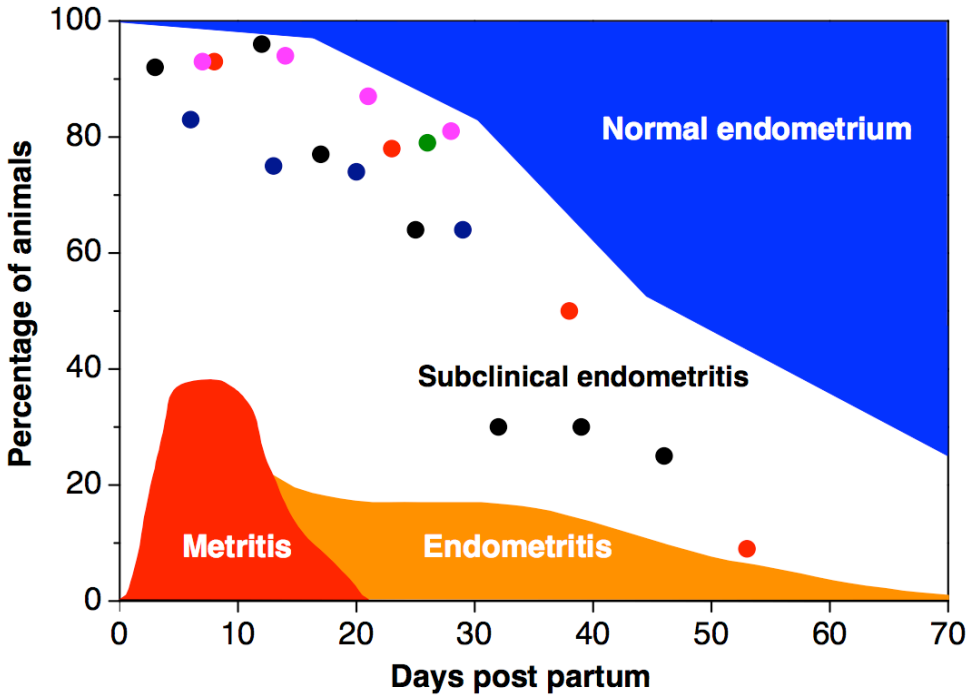

Figure 1.The incidence of uterine disease in postpartum dairy cattle (Sheldon, Cronin et al. 2009). Bacteria can be isolated from the uterus of most cows during the postpartum period; each marker color point indicates the percent of animals with bacteria isolated from the uterine lumen in different studies (Elliott, McMahon et al. 1968; Griffin, Hartigan et al. 1974; Bonnett, Martin et al. 1991; Sheldon, Noakes et al. 2002; Williams, Fischer et al. 2005). The colored areas represent the proportion of animals with metritis, clinical endometritis, sub-clinical endometritis and normal uterus during the postpartum period.

Uterine diseases

Metritis

Metritis is an inflammation of the endometrium and muscular layers of the uterus, which can extend to the serosa and ligaments (causing peri and parametritis) as well as to surfaces of other peritoneal viscera (Bondurant 1999). Histologically, evidence of severe edema, massive infiltration by leukocytes, and myometrial degeneration is shown, resulting in systemic signs of illness such as; fever, red-brown watery fetid vulvar discharge with a flaccid uterus, dullness, anorexia, increase of heart rate, and decrease milk production (Drillich, Beetz et al. 2001; Sheldon, Lewis et al. 2006). It is observed mostly during the first two weeks post partum. Dystocia, metabolic imbalances around parturition (Sandals, Curtis et al. 1979; Markusfeld 1987) and retained fetal membranes have been the major risk factors for metritis (Drillich, Pfutzner et al. 2003). There is also strong association between feed intake and cows' behaviour and the subsequent risk of development of metritis (Huzzey, Veira et al. 2007).

The bacterias most associated with metritis are; *E. coli, A. pyogenes, Fusobacterium necroforum*, *Provetella spp. Bacteroids spp* and a variety of anaerobic and gran-negative species that are eventually isolated (Drillich, Pfutzner et al. 2003). Incidence of metritis varies between studies and it can

reach up to 40%. (Etherington, Bosu et al. 1984; Bartlett, Kirk et al. 1986; Peeler, Otte et al. 1994; Sheldon, Cronin et al. 2009). In bovine metritis, there is an initial decrease in phagocytic activity of neutrophils followed by an increase 2–3 weeks later (Vandeplassche 1981; Frank, Anderson et al. 1983).

Some cases of metritis are less severe than the acute toxic form and occur after 3 weeks post partum and have been characterized by an enlarged uterus with fetid vaginal discharge and mild signs of systemic illness.

Pyometra

Pyometra is defined by accumulation of a changeable amount of purulent exudate within the endometrial lumen with persistence of a corpus luteum, consequentially, prolonging the estrous cycle. This condition is more likely to develop in cows that ovulate before the resolution of bacterial contamination. The destruction of endometrial cells and the switch of PGF production to PGE in the presence of bacterial contamination might prevent the luteolysis creating a continuous dominancy of the uterus by progesterone that is known to suppress the uterine defense mechanism (Sheldon, Cronin et al. 2009). The diagnosis of pyometra depends on transrectal palpation of a distended uterus and/or transrectal ultrasonography of a fluid-filled uterus with hyperechogenic content and the presence of a persistent corpus luteum, with a history of anestrus. The pyometra fluid can be shifted from one horn to the other, which cannot be done

with conceptus membranes, and often the uterine wall is thicker than that of a pregnant uterus. Treatment of pyometra is PGF2α or an analogue causing luteolysis and generating a switch in hormone dominancy (progesterone to estradiol) improving the uterine defense system to resolve the infection (Bretzlaff, Whitmore et al. 1982; Paisley, Mickelsen et al. 1986; Bretzlaff 1987; Gilbert and Schwark 1992).

Endometritis

The endometritis is an inflammation limited to the endometrium. This condition may follow parturition, copulation, artificial insemination, or infusion of irritants into the uterine lumen. Usually, the uterine dimension is normal and the presence of a purulent or mucopurulent discharge can be observed at the vulva and/or cervical os by vaginoscopy. The affected animal is not systemically sick.

In a study with a large number of dairy cows, the cervix with a diameter bigger than 7.5 cm had a greater association with endometritis than uterine dimension. Also, the presence of a purulent discharge at the vulva or cervical os after 21DIM or a mucopurulent discharge after 26DIM were considered positive signs of endometritis (LeBlanc, Duffield et al. 2002).

Historically endometritis has been diagnosed mainly by transrectal examination (TRE) 3 to 4 weeks after parturition. Diagnosis of endometritis by TRE is subjective, revealed only 16.9% of the affected cows, and has little

association with reproductive perfomance (Lewis 1997; LeBlanc, Duffield et al. 2002). Vaginoscopy, which is a simple and rapid method, revealed 23.5% of affected animals and has a better association to further reproductive performance (LeBlanc, Duffield et al. 2002), but it fails to detect 9.1% of clinically diagnosed cows (Kasimanickam, Duffield et al. 2004). Various studies used a scoring system of the vaginal secretion (Dohmen, Lohuis et al. 1995; Sheldon and Noakes 1998; Huszenicza, Fodor et al. 1999) and odor (Williams, Fischer et al. 2005) (Fig 2), which is correlated with bacterial culture and infertility. In one study, *Arcanobacterium pyogenes*, *Proteus* and *Fusobacterium necrophorum* were associated with mucopurulent or purulent vaginal secretion. *A. pyogenes*, *Escherichia coli*, non-hemolytic Streptococci, and *Mannheimia haemolytica* were associated with a fetid mucus odor (Williams, Fischer et al. 2005).

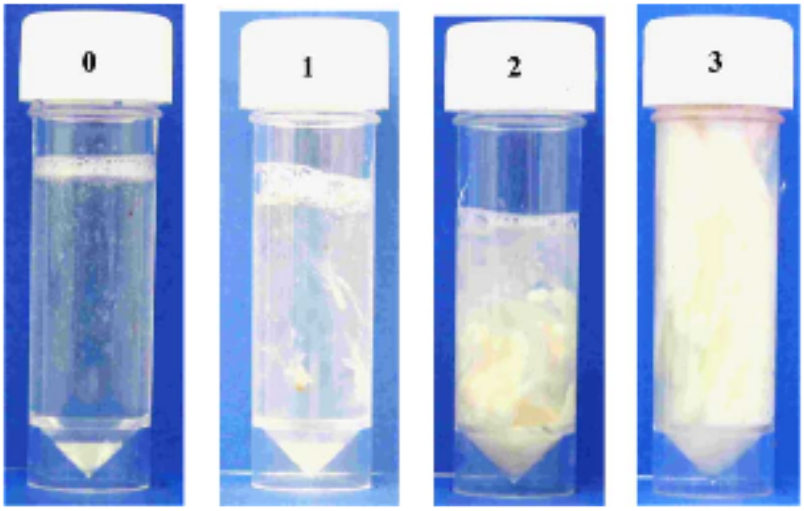

Figure 2. Vaginal discharge from cows at post partum scored by its visual characteristics with 0 - clear vaginal mucus, 1 - mucus with flecks of pus, 2 - 50% mucus 50% pus and 3 - >50% pus (Williams, Fischer et al. 2005)

Ultrasonography is another diagnostic method that can improve the sensitivity and specificity when applied with another method (Kasimanickam, Duffield et al. 2004; Barlund, Carruthers et al. 2008), however when used alone it is able to detect only severe endometritis. (Mateus, Lopes da Costa et al. 2002).

Bacteriology can be used to diagnose endometritis but the cost, the urgency to treat and the predominance of *A. pyogenes* and anaerobic bacteria as pathogens; are reasons that do not justify this diagnostic practice. The definitive diagnosis of endometritis is based on the histological examination of endometrial biopsies. However, biopsies are expensive, time consuming, and have been reported to be detrimental to future fertility when applied early on postpartum. (Etherington, Bosu et al. 1984; Bonnett, Martin et al. 1993)

Cows with endometritis have their blood neutrophils counts augmented two weeks before parturition and up to 4 weeks post partum when compared with cows clear of uterine disease (Kim, Na et al. 2005). Also, neutrophils from cows with endometritis have a decrease in phagocytic activity (Vandeplassche and Bouters 1983; Anderson, Hemeida et al. 1985; Kim, Na et al. 2005), suggesting that cows which develop endometritis are susceptible even before parturition by a poorly understood mechanism where the immune system is debilitated probably by internal or external stress factors like concomitant diseases, malnutrition, poor housing, management, negative energy balance, submissive behavior (Kim, Na et al. 2005).

Subclinical endometritis

Subclinical endometritis is defined as endometrial inflammation of the uterus determined by cytology, in the absence of signs of endometritis (LeBlanc, Duffield et al. 2002). The diagnosis of subclinical endometritis is based on observations of neutrophils (fig.3) after endometrial cytology based on uterine lavage with a small volume or using a cytobrush (Kasimanickam, Duffield et al. 2004; Gilbert, Shin et al. 2005; Hammon, Evjen et al. 2006; Barlund, Carruthers et al. 2008; Galvao, Greco et al. 2009). However the cytobrush has delivered better results with improved cellular quality and superior repeatability is the better the choice for the diagnosis of subclinical endometritis. (Kasimanickam, Duffield et al. 2005; Barlund, Carruthers et al. 2008)

There are some studies that assessed the effect of subclinical endometritis on reproductive performance but the parameters used to pose a positive diagnosis of subclinical endometritis varied greatly between studies: (i. e. percentage of neutrophils, methodology of sampling, small volume flush versus cytobrush, DIM of sampling, DIM as time limit to become pregnant) make it very difficult to compare results (Barlund, Carruthers et al. 2008; Galvão, Frajblat et al. 2009). Nonetheless, in one study the diagnosis of subclinical endometritis was based on a ratio of >18% neutrophils in uterine cytology samples collected 20–33 days post partum or >10% neutrophils at 34–47 days

post partum using 132 DIM to calculate the risk of pregnancy cutoff (Kasimanickam, Duffield et al. 2004). In another study which used uterine lavage, a count of >5% of neutrophils between 40 and 60 DIM was considered positive, with 120DIM for the time limit for getting pregnant (Gilbert, Shin et al. 2005).

The incidence of subclinical endometritis varies between 0 and 73% and is associated with longer intervals to conception and a greater likelihood of culling (Gilbert, Shin et al. 2005; Kasimanickam, Duffield et al. 2005; Barlund, Carruthers et al. 2008). Studies on subclinical endometritis are at its beginning though intrauterine infusion of cephapirin benzathine, has shown improvement on reproductive performance of cows declared positive for subclinical endometritis (Kasimanickam, Duffield et al. 2005).

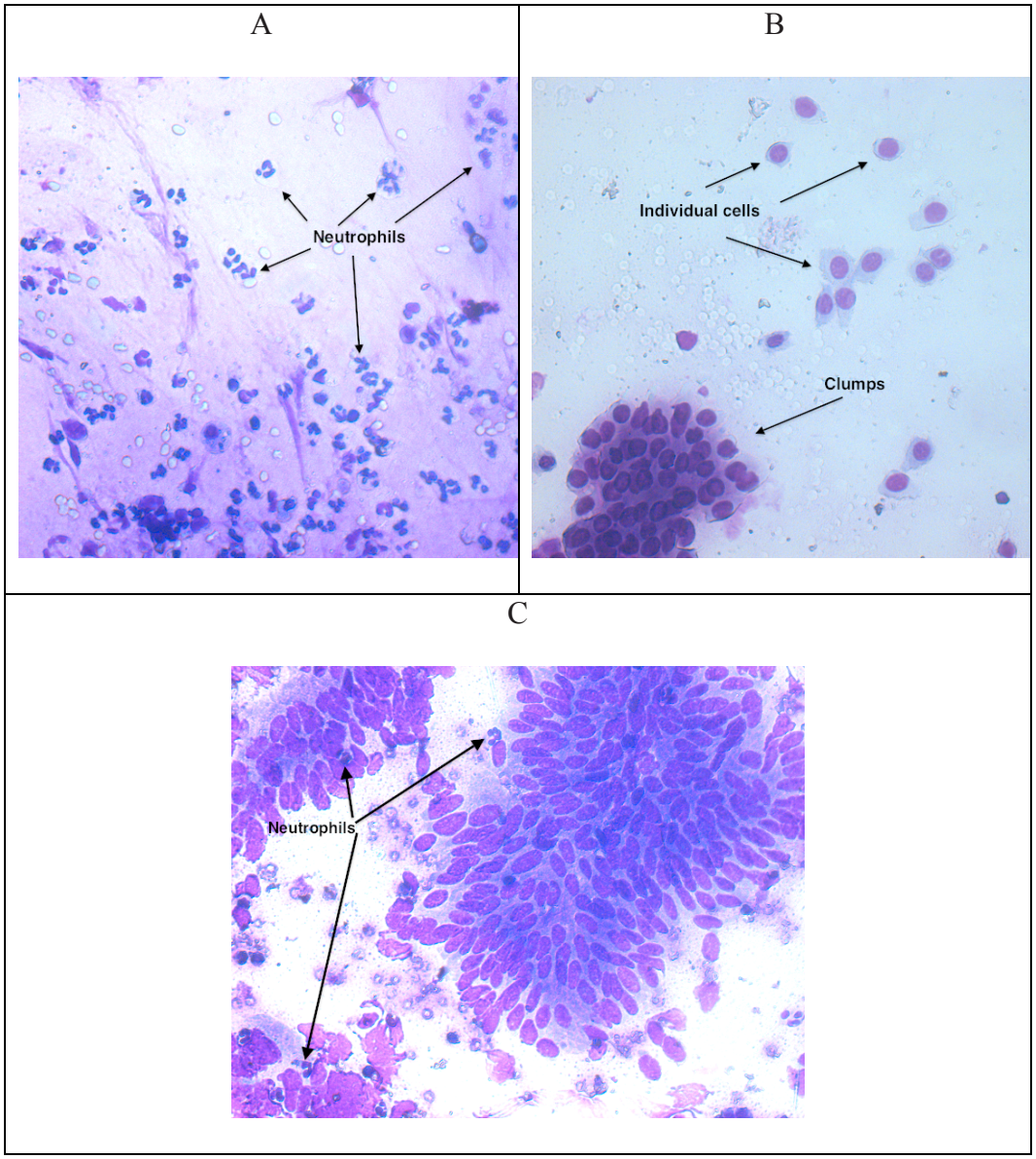

Figure 3. Endometrial cytology slides. Picture A shows a large quantity of neutrophils. Picture B shows endometrial cells without white blood cells. Picture C shows endometrial cells with neutrophils scattered within the slide.

Uterine defense

The first response of the uterus against foreign bodies (bacteria, sperm) is a neutrophilic influx into the endometrium and uterine lumen (Klucinski, Dembele et al. 1995). Experimental replication of this event has been achieved by several investigators (Kluciński, Targowski et al. 1990; Butt, Senger et al. 1991; Zerbe, Schuberth et al. 1996) utilizing an extended variety of substances and microorganisms, inducing a significant influx of neutrophils into the uterine lumen. Recruitment and activation of neutrophils was presumed to be the result of cytokine release from lymphocytes induced by the prior immunization. However, when nonspecific inflammation was induced in the endometrium, intrauterine neutrophils influx was present, but with reduced activity when compared to circulating neutrophils (Kluciński, Targowski et al. 1990; Klucinski, Dembele et al. 1995). There are several mechanisms by which neutrophils phagocytize and kill microorganisms. They can be directly attracted to microbial products or they can be stimulated by complement chemotactic substance like the LTB4 that is a potent leukotriene largely present in inflamed uteri and by complement component C5a (Belluzzi, Galeotti et al. 1994; Bondurant 1999). The opsonization (i.e., complement component C3b and specific antibodies) of microorganisms in the intrauterine lumen improves neutrophil phagocytosis due to the presence of receptors for the Fc component of the antibody and receptors for C3b on the neutrophil. Such binding and engulfing of opsonized microorganism is greatly enhanced (Watson 1985;

Watson 1989; Rysanek, Babak et al. 2001). The neutrophils then kill the microorganisms by a mechanism of oxygen dependent generation of superoxide anions(Küther, Audigè et al. 1998), lysozymes and proteolytic enzymes. Neutrophils flushed from normal uteri on postestrus had a greater degree of expression of Fc receptors for IgG1 and IgG2 antibodies than did peripheral blood neutrophils. Neutrophils from cows with experimental endometritis showed a decrease in the expression of Fc receptors for IgG antibodies and a lower index of Fc-mediated phagocytosis (Klucinski, Niemialtowski et al. 1994).

In addition to neutrophil migration, other cellular components that are eventually activated include macrophages, lymphocytes, eosinophils, and mast cells. Inflammatory mediators are then released, such as tissue necrosis factor (TNFα), histamine and prostaglandins, interleukins, and chemotactic factors for neutrophils and eosinophils, including LTB4 and eosinophil chemotactic factor A (ECF-A) (Miller 1996). The eosinophils also release inflammatory mediators and chemicals such as superoxides and enzymes that kill microorganisms. Uterine mast cell degranulation releases tryptase and other proteases that can activate complement components C3 and C5 to generate anaphylatoxins. They also release kallikreins that generate kinins (Küther, Audigè et al. 1998) whish are potent vasoactive agents that increase vascular permeability.

It is important to understand that estrus alone is associated with a slight migration of neutrophils into the endometrium (Studer and Morrow 1978) with a maximum number of cells in the normal uterine lumen shortly after estrus and ovulation. The great majority of these cells are neutrophils, which shows

augmented phagocytic and killing ability during estrus over diestrus (Watson 1985). Lymphocytes are generally present in the stratum compactum of the endometrium. Their numbers change during the estrous cycle, being highest in the periestrus period and lowest at diestrus (Vander Wielen and King 1984).

Leukocyte esterase

Leukocyte esterase (LE) is used to test for the presence of white blood cells in the urine as indication of an inflammation process of the urinary tract. In humans, it has been used for a rapid diagnosis of inflammation in many body fluids such as urine, pleural fluid, peritoneal fluid, and cerebrospinal fluid (Levy, Tournot et al. 1989; Azoulay, Fartoukh et al. 2000; Braga, Souza et al. ; Rerknimitr, Rungsangmanoon et al. 2006). The principle of leukocyte esterase (LE) test is that the esterase released from activated neutrophil cells reacts with indoxil carbonic acid ester; indoxil is released by the esterase and reacts with diazoniun salt and is oxidized yielding a violet azo dye. The intensity of the color is correlated to leukocyte counts according to the fabricant (Kutter, Figueiredo et al. 1987). Positive test results are clinically significant (Herlihy, Wilkerson et al. 1984; Romero, Emamian et al. 1988; Levy, Tournot et al. 1989; Braga, Souza et al. 2006; Rerknimitr, Rungsangmanoon et al. 2006). The LE test is also used to screen for gonorrhea. The combination of the LE test with the urinary nitrite test provides an excellent screen for establishing the

presence of a urinary tract infection. Urine samples that test positive for both nitrite and leukocyte esterase should be cultured for pathogenic bacteria.

In the dog where the esterase leukocyte test activity have been studied in cases of pyuria, the correlation with esterase activity and the disease has not been significant and the sensitivity and specificity appears to be low, suggesting that canine urine may contain a esterase inhibitor or canine neutrophils do not express the same quantity or type of esterase expressed by human leukocytes. (Vail, Allen et al. 1986; Bauer, Rettig et al. 2008) In the cow, investigators have used a commercial leukocyte esterase strip test in uterine lavage for diagnosis of endometritis and showed a high correlation between endometrial cytology and leukocyte esterase with 96% sensitivity and 98% specificity. (Santos, Roman et al. 2006).

The objectives of this study are to:

1) Compare uterine leukocyte esterase activity and the endometrial cytology (EC).

2) Compare leukocyte esterase activity of the cervix (CxLE) and the uterus (UtLE).

3) Compare two methods of assessing the slides

We hypothesized that the diagnosis of subclinical endometritis can be accomplished using the leukocyte esterase activity.

CHAPTER 2

VALIDATION OF LEUKOCYTE ESTERASE ACTIVITY FOR THE DIAGNOSIS OF SUBCLINICAL ENDOMETRITIS IN POSTPARTUM DAIRY COWS

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Abstract

The aim of this study was to compare leukocyte esterase activity and the endometrial cytology (EC) for the diagnosis of subclinical endometritis in Holstein cows (n=218) between 21 and 47 days in milk (DIM). The relationship between the uterine and cervical leukocyte esterase activity was also determined. In addition, two methods for assessing endometrial cytology were compared. Cows from five commercial dairy herds were allocated to the study based on the absence of vaginal discharge at vaginoscopy or genital anomalies after transrectal examination. Cows included in the study were monitored biweekly for at least 200 days. The percentage of polymorphonuclear cells (% neutrophils) was correlated with esterase score measured either from the uterus (UtLE, P=0.0001) or the cervix (CxLE, P=0.002). Both CxLE and UtLE (P=0.0009 and P=0.0001 respectively) decreased with DIM, however they were not statistically associated with pregnancy risk at 90 DIM. Between 32-47 DIM, the % neutrophils and risk of pregnancy at 90 DIM were associated (P=0.04). For the same period, survival analysis identified cows with > 2.6 % neutrophils at EC as subclinical endometritis cows. In conclusion, the results indicate that uterine leukocyte esterase activity is correlated to percentage of neutrophils at endometrial cytology but is not reliable to predict the risk of pregnancy. Subclinical endometritis (> 2.6 % neutrophils) diagnosed by EC between 32 and 47 DIM was associated with reduced risk of pregnancy.

Introduction

In the last decade, decrease of the fertility of dairy cows has gathered considerable attention in veterinary science (Bousquet, Bouchard et al. 2004). As the reproductive performance is associated with the health status of the uterus at the end of the voluntary waiting period (VWP) (Ferguson and Galligan 2000), assessment of uterine condition in postpartum cows has been the focus of research. In cows, lochia is normally expelled from the reproductive tract during the first two or three weeks after parturition (Gier and Marion 1968). Discharges can persist longer depending on the virulence of the causative organism and predisposing factors to the disease (Azawi 2008). Uterine disease affects half of dairy cattle (Markusfeld 1987; Clay, Welper et al. 2004) and causes infertility by disrupting uterine and ovarian functions (Mateus, Lopes da Costa et al. 2002). As inflammation is normally present during the physiological uterine involution in postpartum cows, rigorous characterization of pathologic conditions become intricate. Therefore, the complex relationship between postpartum diseases and reproductive performance remains unclear (Fourichon, Seegers et al. 2000).

Clinical endometritis is defined as inflammation of the endometrium with vaginal purulent discharge after 21 DIM or mucopurulent discharge after 26 DIM in absence of systemic clinical signs (LeBlanc, Duffield et al. 2002; Sheldon, Lewis et al. 2006; LeBlanc 2008). Characterization of the vaginal discharge is associated with uterine pathogens and a prognosis on the cow's

future fertility (Sheldon, Cronin et al. 2009). The clinical endometritis has a negative impact on reproductive performance increasing services per conception, calving to first service interval, calving to conception interval (Borsberry and Dobson 1989; Heuwieser, Tenhagen et al. 2000), risk of pregnancy (LeBlanc, Duffield et al. 2002) and conception rate (Fourichon, Seegers et al. 2000). Postpartum uterine disease represents a leading cause of reproductive inefficiency (Sheldon, Lewis et al. 2006) and could mean significant economic loss for the dairy industry (Bartlett, Kirk et al. 1986; Guard 1994; Drillich, Beetz et al. 2001). The financial impact is driven by infertility, excessive culling, reduced production, and cost of treatment. Contrary to clinical endometritis, subclinical endometritis does not present any clear evidence of endometrial inflammation at the genital examination (LeBlanc, Duffield et al. 2002). As the more severe inflammation (clinical endometritis) subsides, the drainage of abnormal uterine fluid is not obvious (Sheldon and Dobson 2004). Even though the condition is much more difficult to diagnose, subclinical endometritis could still impair reproductive performance (Sheldon, Lewis et al. 2006). The incidence of subclinical endometritis depends on the diagnostic technique and the day of the postpartum period when the diagnosis is made, and may reach 74% in a herd (Gilbert, Shin et al. 2005). In other studies, subclinical endometritis has been reported as high as 43% for cows between 20 and 33 DIM, 45% for cows between 34 and 47 DIM and 53% for cows between 40 and 60 DIM. (Kasimanickam, Duffield et al. 2004; Gilbert, Shin et al. 2005)

Once the endometrial cells recognized pathogens present in the uterus (innate immune system), they secrete peptides like cytokines and chemokines which attract inflammatory cells like neutrophils (Sheldon, Cronin et al. 2009). Neutrophils are recruited from the circulation into the lumen, eliminate bacteria and become an excellent indication of an inflammatory process (Tizard 1996; Wade and Lewis 1996). Blood-derived neutrophils are the main effector cells for removing bacteria from the uterus. After elimination of the pathogenic organisms, inflammation subsides and neutrophils will be limited to the uterine lumen fluid (Kluciński, Targowski et al. 1990). Persistent neutrophils in the endometrium in the absence of bacteria may be an important characteristic of subclinical endometritis and cause of infertility in cattle (Sheldon, Cronin et al. 2009). The neutrophils are the predominant inflammatory cells in the uterine lumen and their relative proportion has been shown to be predictive of future reproductive performance. In absence of clinical signs, subclinical endometritis could be cytologically defined as > 8.0% neutrophils of total cells collected by cytobrush in dairy cows between 28 and 41 DIM (Barlund, Carruthers et al. 2008) or >10% between 34 and 47 DIM (Kasimanickam, Duffield et al. 2004). However, high incidence of a transient inflammation response in the postpartum uterus, time variation of sampling the uterus and the lack of standardization of different techniques sustain the controversy concerning the case definition of subclinical endometritis.

Diagnosis of subclinical endometritis should depend on direct methods that will reveal the presence of inflammatory cells in the lumen or within the

endometrium. In cows, moderately invasive methods to evaluate the inflammatory process of the endometrium include uterine lavage and cytobrush cytology. Both methods have been described and accepted as diagnostic techniques. The cytobrush technique seems to be the most useful technique and the most reliable diagnostic test compared with uterine lavage (Kasimanickam, Duffield et al. 2005)and ultrasonography (Barlund, Carruthers et al. 2008). Cytobrush cytology is considered the reference of cytological diagnosis for subclinical endometritis because of the quality of cells and better repeatability. However, it is not a cow-side test. Although neither complex nor expensive, endometrial cytology requires special instruments and expertise and lacks of immediacy. From a point of view of practicality and efficiency, the diagnostic test for subclinical endometritis needs to be performed rapidly and the results analysed on farm. With these characteristics, a treatment decision can be made and executed at the time of the visit by the veterinarian.

An alternative method to assess inflammatory cells in the lumen of the uterus is the leukocyte esterase test. It has been used for a rapid diagnosis of inflammation in many body fluids such as urine, pleural fluid, peritoneal fluid, and cerebrospinal fluid (Levy, Tournot et al. 1989; Azoulay, Fartoukh et al. 2000; Braga, Souza et al. ; Rerknimitr, Rungsangmanoon et al. 2006) and could be used as an indirect method to detect neutrophils in the cow's uterus. Santos et al., (Santos, Roman et al. 2006) investigated the use of a commercial strip test, used for urinary neutrophils, in uterine lavage for diagnosis of endometritis and showed a high correlation between endometrial cytology and leukocyte

esterase activity with 96% sensitivity and 98% specificity. The leukocyte esterase released from neutrophil cells reacts with indoxil carbonic acid ester. Indoxil is released by the esterase reaction with diazoniun salt and is oxidized yielding a violet azo dye (Kutter, Figueiredo et al. 1987). The intensity of the color correlates to leukocyte counts according to the fabricant.

The aim of this study is to compare leukocyte esterase activity to the endometrial cytology to diagnose subclinical endometritis in clinically normal postpartum dairy cows. The relation between the uterine and cervical leukocyte esterase activity was assessed and the impact of subclinical endometritis was evaluated. We hypothesized that the diagnosis of subclinical endometritis can be achieved using the leukocyte esterase activity.

Materials and Methods

Holstein cows (n=285) from 5 commercial herds monitored bimonthly by the ambulatory clinic of the Faculty of veterinary medicine of l'Université de Montréal (Quebec, Canada) were enrolled in the research project. Herd records were compiled in a databank DSA@HR (Dossier de Santé Animal: DS@HR, 2725 boul. Western Casavant, St-Hyacinthe, QC, J2S 0E5) and validated for reproduction. All herds were housed in tie-stall stanchion barns, milked twice daily and artificially inseminated exclusively after a voluntary waiting period of approximately 60 days and on heat detection. The total mixed ration diet is

composed mainly of corn silage, alfalfa/grass silage and corn meal and protein supplements. A genital exam was performed by transrectal palpation and vaginoscopy for all cows. Only cows without evidences of clinical endometritis according to the criteria of Leblanc (LeBlanc, Duffield et al. 2002) and between 21-47 DIM were included in the study.

Cows were subjected to different diagnostic tests in succession. Reproductive examination included measurement of the cervical size and uterine size (cm) at the base of the horn, (left and right) and assessment of the ovarian activity (presence of a corpus luteum, follicles and cysts) as determined by transrectal palpation. Vaginal secretion was classified according to Williams and al. (Williams, Fischer et al. 2005) with some variations. The vaginal examination (VEx) was done with a disposable vaginoscope to visualize the cervical os and secretion which were scored as; VEx0, no mucus, VEx1, clear mucus, VEx2, mucus containing flecks of white or off-white pus, VEx3, ≤50% white or off-white mucopurulent material, VEx4, purulent material, usually white, occasionally yellow. Cows with VEx2, VEx3 and VEx4 were considered as having clinical endometritis.

Once the cow met the enrollment criteria, the endometrial cytology and the leukocyte esterase test were performed. For the leukocyte esterase test in the cervix (CxLE), the reagent strip was partially introduced into a uterine infusion pipette, (Continental Plastic Corp. Delavan, WI, USA) with the reactive pad protruding outside the pipette. Through a disposable and sterile lubricated 50 cm long vaginoscope, the cervix was visualized and the pipette with the

leukocyte esterase pad Uristix® (Bayer Health Care L.L.C., Elkhart, IN) was introduced into the first ring of the cervix for 5 seconds and the color pad determined after two minutes according to the fabricant recommendations.

Lastly, uterine leukocyte esterase activity (UtLE) was measured and cellular material were harvested for cytological evaluation by cytobrush (VWR Canlab, Mississauga, ON, Canada) technique as previously described (Kasimanickam, Duffield et al. 2004). Briefly, the cytobrush was screwed onto a solid stainless steel rod and placed in a stainless steel tube of 65 cm long for passage through the cervix. To protect the instrument from vaginal contamination, the apparatus was inserted into a double pipette (Continental Plastic Corp., Delavan, WI, USA). The instrument was introduced into the vagina as the vaginoscope was removed, a sleeved arm was introduced into the rectum to facilitate passage of the instrument and it was pushed through the double pipette. The tube was advanced through the cervix into the body of the uterus where the cytobrush was pushed out (1.0 cm) of the stainless steel tube and exposed the lumen of the uterus. The cytobrush in contact with the endometrium was gently rotated clockwise approximately one turn to obtain cellular material from the adjacent endometrium. The cytobrush was retracted into the stainless tube before removing the whole apparatus from the genital tract.

Slides were prepared by rolling the cytobrush on a predetermined surface area of a clean glass microscope slide and left to dry. After the slides were made, the cytobrush was plunged into a 3ml glass tube containing 1ml of saline

0.9% and gently shaked for 30s. The leukocyte esterase strip was then inserted into the glass tube. The strip was removed from the tube and the result (UtLE) recorded after 2 minutes. The slides were transported to the laboratory where they were stained with a modified Giemsa stain (PROTOCOL HEMA 3 STAIN SET, Fisher Diagnostics, Ficher Scientific Company L.L.C. Valley Pike, Middletown, VA, USA). Once stained and dried, cover slip was applied using Histofluid ®mounting medium (Paul Mareinfield GmbH & Co., Lauda Koenigshofen, Germany).

Slide evaluation

Two methods were used to evaluate slides; a first rigorous method (RM) of reading the slides was done followed by a second simple assessment method (SM) more likely to be used in the field. The RM consisted in drawing imaginary lines of 0.5cm apart starting in the middle (line #1) of the slide. Line #2 was drawing on the right side of line #1 and line #3 on the left side. Following the same pattern, line #4 was the following right of the line #2 and line #5 the following left to line # 3. Slides were assessed by reading the five straight lines, bottom to top, starting from the central line (#1) to the exterior one (1 to 5), under a 400X magnification by the same examiner (figure 1). A differential count of all neutrophils, lymphocytes, macrophages and epithelial cells using a minimum of 500 cells or all five lines was obtained to provide a

quantitative assessment of endometrial inflammation. Also, the number of clumps of epithelial cells and the number of fields assessed were recorded. The SM method consisted of assessing first the slide at 200X magnification to localize the area with a reasonable quantity of recognizable cells (independent of each kind). From these areas, ten microscopic fields were randomly selected and total neutrophil counts were recorded at 400X.

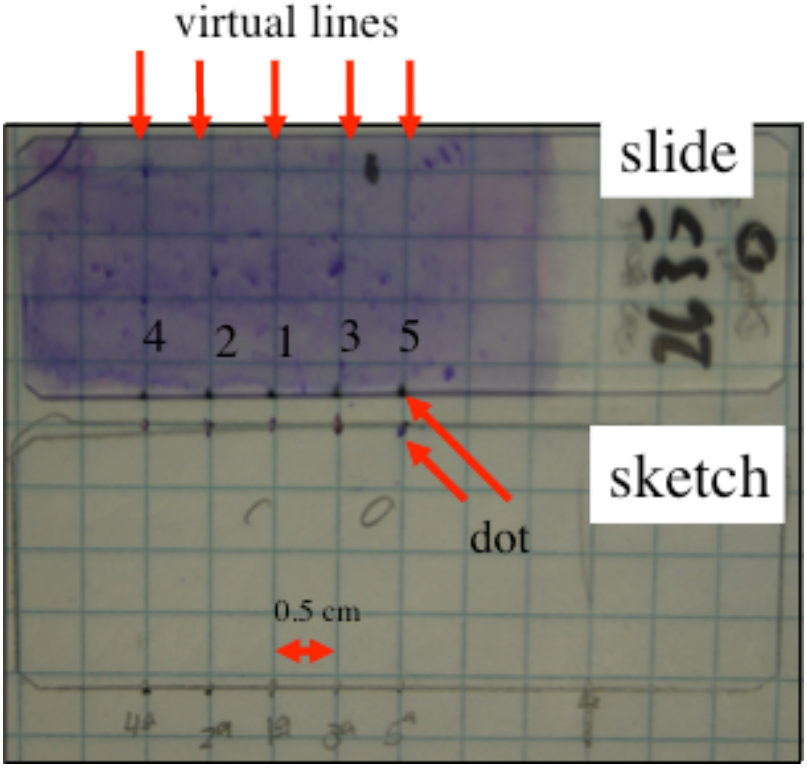

Figure 1. Methodology of RM slide evaluation.

Slides were placed on a squared paper (0.5cm) and small dots were made at the bottom of the slide following the dots represented on the sketch previously made on the square paper. Following a virtual strait line bottom to top starting on 1 to 5, every 3rd field was assessed and the cells present were counted until a total of 500 cells were counted or until the top end of the 5th line was reached.

Criteria of exclusion

Cows that received systemic antibiotics, intrauterine infusion or reproductive hormone administration from a period of 10 days prior to the sampling were excluded from the experiment. Furthermore, cows, with a history of twins, metritis, clinical endometritis, displacement of abomasum and retained placenta were also excluded. Cows identified to be culled by the owner before 200 days in milk were not included in the study. Cows that were not bred before 90 DIM were excluded for the analysis of conception rate. This study did not control for any other treatment for which ever cause (i.e. mastitis, pneumonia etc.) that may have been administered between the time of the test and the time of pregnancy diagnosis.

Data analysis and Statistics

Data analysis was conducted with SAS v 9.1.3. A linear mixed model with the site of ULE as fixed effect, dairy herd as a random effect and the DIM as a cofactor was used to estimate a correlation between the UtLE or CxLE and percentage of neutrophils. A Weighted kappa was plotted to measure the correlation between UtLE and CxLE. To estimate the correlation between the percentage of neutrophils in RM and SM a linear regression was used. Using a mixed logistic regression with dairies as random effect and DIM as cofactor,

the percentage of neutrophils, UtLE and CxLE were analysed to predict pregnancy on cows at risk to become pregnant at 90DIM (i.e inseminated between 43-90 DIM) to all cows (GT), and the cows were divided in two groups for further analysis; group 1 (G1) cows from 21 to 31 DIM and group 2 (G2) cows from 32 to 47 DIM. A Receiver/Response Operating Characteristics (ROC) curve was plotted using the sensitivity and specificity for each possible percentage of neutrophils in the G2 group against prevalence of gestation at 90DIM to find a cut off for the percentage of neutrophils. We used 90DIM as the limit for calculating the success of insemination because it gives a majority of cows (85%) the chance to be inseminated at least once if we consider a first AI at 60DIM and a heat detection rate at 50%. Pregnancy diagnosis was made by palpation after 30 days and reconfirmed after 45 days after AI.

Results

The study began in September 2007 and continued until October 2008. From all 285 cows examined during this study, 85 (29.8%) cows had on vaginal examination (VEx) a score VEx0; 133 (46.6%) cows had score VEx1; 27 cows (9.47%) had score VEx2; 22 (7.7%) cows had score VEx3 and 18 (6.3%) cows had score VEx4. Scores VEx2, VEx3 and VEx4 where considered clinical endometritis resulting in an overall of 23.5% of positive cases of endometritis

that varied within herds from 11.5% to 38.6% (11.5; 16.6; 27.2; 38.2; 38.6). Cows with clinical endometritis were treated and enrolled in another study.

Table 1 describes the prevalence of ovarian findings relevant to endometritis (n=285). Clinical endometritis cows showed less active ovaries with less cows having corpus luteum (15 cows, 22%) compared to cows without clinical endometritis (87 cows, 40%, $P<0.05$). Similarly, more cows had normal follicular activity with follicles between 10 and 20 mm (131 cows, 60%, $P<0.0003$) on the ovaries compared to diseased ones. Among the remaining 218 cows diagnosed without clinical endometritis, herd size ranged from 40 to 110 cows in milk with an average milk production of 8328kg per lactation and 2.4 lactations. The mean age was 3.9 years old (Table 2).

	Clinical Endometritis		
***Ovarian structures**	Positive (n=67)	Negative (n=218)	P
CL	15 (22%)	87 (40%)	0.048
CH	13 (19%)	27 (12%)	0.23
Cyst	1 (2%)	16 (7%)	0.02
Follicle (10-20mm)	24 (36%)	131 (60%)	0.0003

Table 1. Prevalence of ovarian structures in cows with and without clinical endometritis.

*Observations from the transrectal palpation.

N=218	Average	Range
Age	3.88 years	1.72 to 12.2
Milk Production	8328kg/year	4264 to 14341
Lactation	2.54	1 to 10
Herd size	66	40 to 110
MDFS	73	43 to 188
FSCR	31%	
Culling rate	12%	

Table 2. Characteristics of herds.

Median days to first service (MDFS), First service conception rate (FSCR).

Leukocyte esterase activity and endometrial cytology

The percentage of neutrophils diminished with the number of DIM (fig. 2). The esterase activity for both UtLE ($P<0.0001$) and CxLE correlates with the increase of neutrophils (fig.3). For UtLE, the percentage of neutrophils was significantly lower in cows with a score of 0 than in cows with score of 2 and 3 and significantly higher in cows with a score of 3 than in cows with a score of 0, 0.5, 1, and 2 (fig.3). For CxLE, neutrophil counts in cows with score 0 were significantly lower than in cows with score 1, 2 and 3 and percentage of neutrophils were not significantly different between cows with scores of 0.5, 1, 2 and 3 (fig.3). There was a slight agreement between UtLE and CxLE with a weighted Kappa of 0.37.

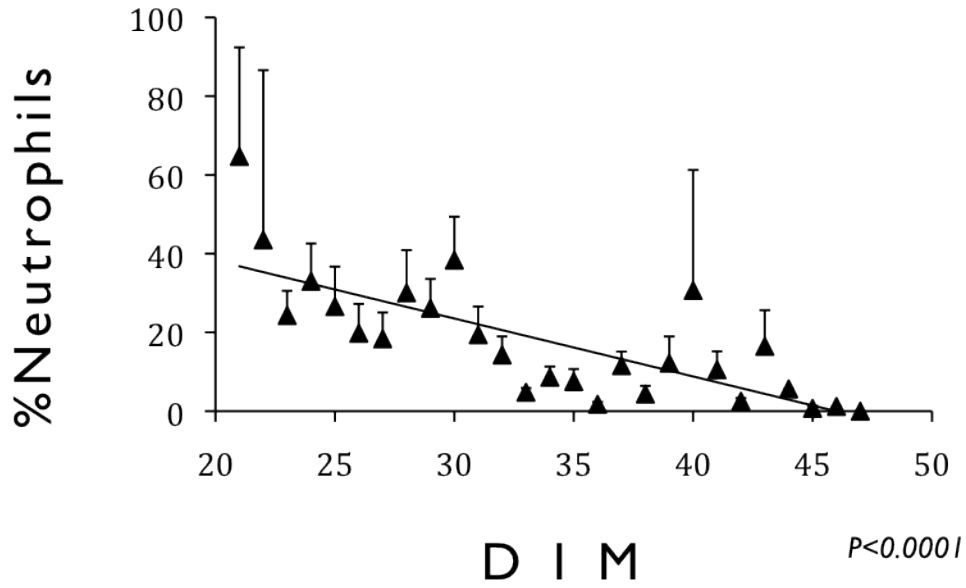

Figure 2. Correlation between endometrial cytology percentage of neutrophils and Days in milk (DIM) (n=218)

Data represents least-square means ± SEM

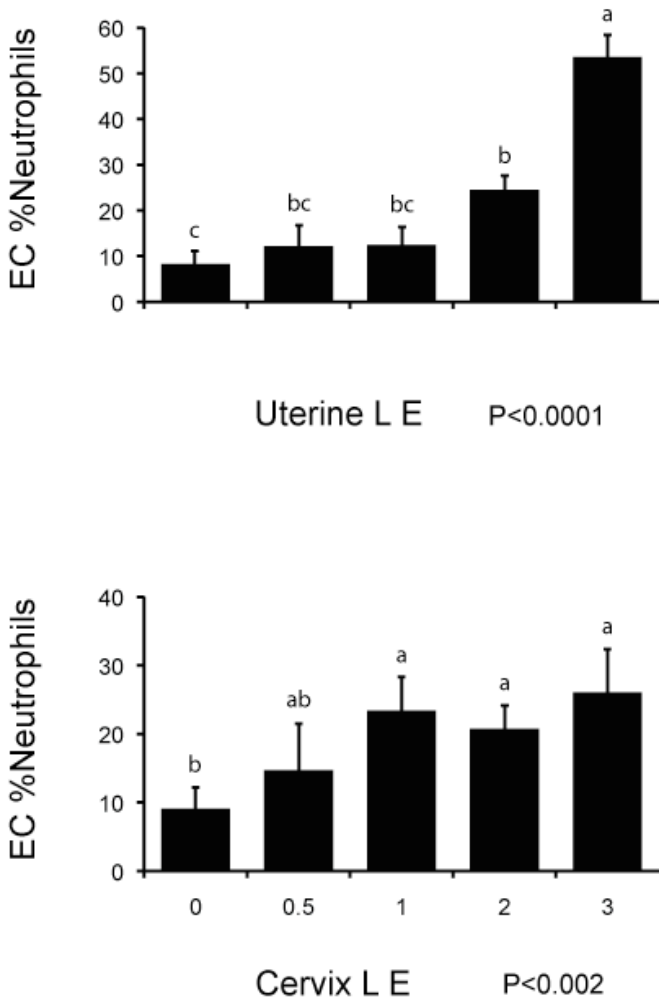

Figure 3. Relationship between endometrial cytology (EC %neutrophils) and leukocyte esterase activity scores in the uterus (n=218) and cervix (n=204).

Data represents least-square means ± SEM

Bars with different superscript differ (P<0.05)

Impact of subclinical endometritis

Among the 218 cows used in this study, 32 cows were bred after 90 DIM and were thus excluded from subsequent statistical analysis. The analyses demonstrated that the risk of pregnancy by 90DIM was not significantly associated with the UtLE and CxLE for any group GT, G1 and G2. Similarly, the risk of pregnancy at 90DIM for all cows (GT, n=186) and the group G1 (n=92) was not associated (P=0.7) with the percentage of neutrophils on endometrial cytology. However, in G2 (n=94) there was an association (P=0.04) between endometrial percentage of neutrophils and pregnancy risk at 90DIM (fig. 4). The risk of getting the cows of group G2 pregnant was diminished by 0.93 point (95%CI 0.89-0.98) each time the percentage of neutrophils was increased by 1.0 point in G2. The ROC curve demonstrated that the percentage of neutrophils that are considered positive for subclinical endometritis in the G2 group was 2.6 which gives a prevalence of subclinical endometritis of 56% for cows in G2 (32-47 DIM). (Table 3)

Table 4 shows the prevalence of ovarian structures found by transrectal palpation for cows in group G2. Statistical analysis demonstrated that there were no differences for any of the ovarian structures (i.e.corpus hemorrhagicum, corpus luteum, follicles and cysts) between the cows diagnosed with subclinical endometritis and cows without subclinical endometritis.

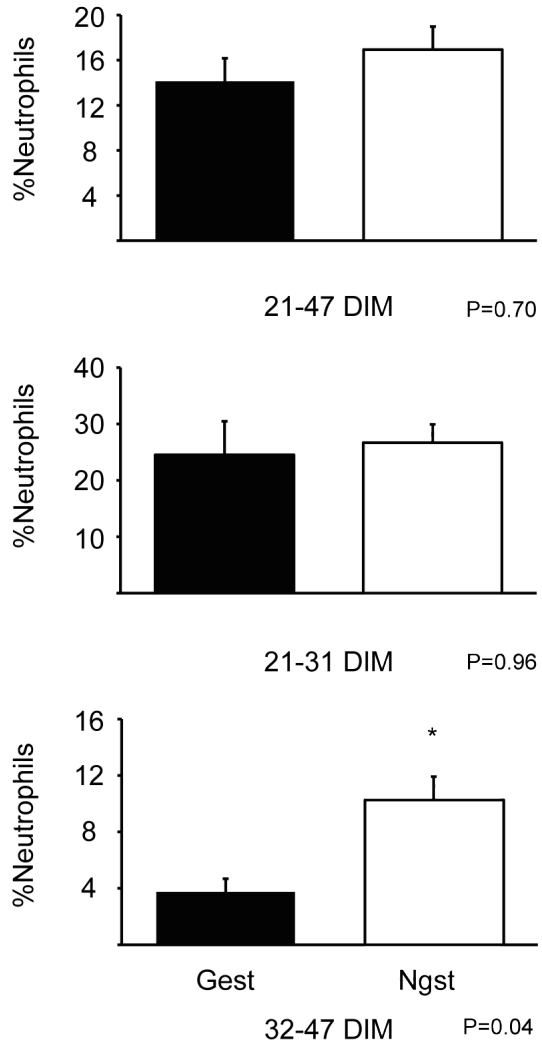

Figure 4. Percentage of neutrophils in pregnant (Gest) and open (Ngst) cows for the group 21-47 DIM (GT, n=186), group 21-31 DIM (G1, n=92) and 32-47 DIM (G2, n=94).

Data represents Least-square means ±SEM

* significant difference.

	Subclinical endometritis		
	Negative	Positive	
N	41	53	56%
FSCR	39%	21%	0.08
MDO	128.6	158.5	0.34
MDFS	68.7	71.8	0.34
MAI	2.5	3	0.22

Table 3. Reproductive performances in G2 cows (32-47 DIM) with and without subclinical endometritis determined with a cut off of 2.6% neutrophils.

FSCR: First service conception rate

MDO: Median days open

MDFS: Median days to first service

MAI: Mean of artificial inseminations

	Subclinical endometritis		
	Positive	Negative	P
N	53	41	56%
CH	5	4	0.93
Follicle	30	24	0.74
Cyst	6	5	0.86
CL	30	18	0.26

Table 4. Ovarian structures found by transrectal palpation for G2 cows (32-47 DIM) with and without subclinical endometritis.

CH: Corpus Hemorrhagicum

CL: Corpus Luteum

Slide evaluation

A correlation ($P<0.0001$) was found between endometrial percentages of neutrophils obtained by both methods (RM and SM). The SM method appeared as effective as the RM method. With the SM method, 81% of the variation of number of neutrophils was explained by the variation of the percentage of neutrophils within RM. (Fig. 5).

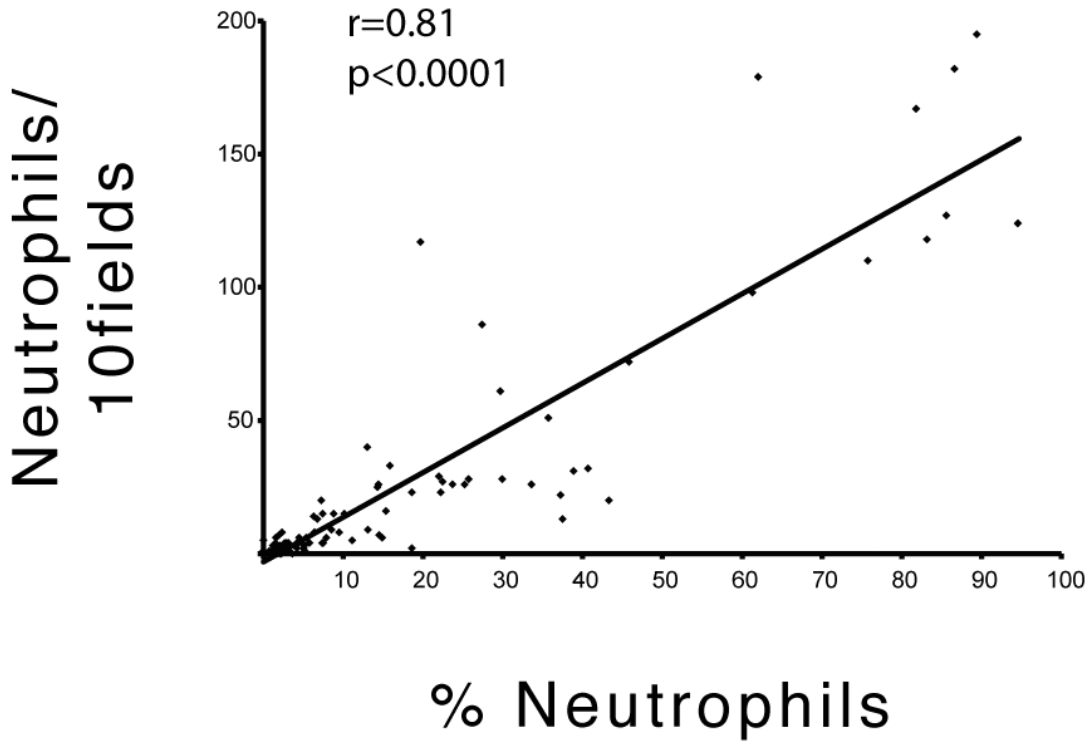

Figure 5. Correlation between two methods of slides evaluation, percentage of neutrophils in endometrial cytology (RM) and a total number of neutrophils per 10 high power (400X) fields (SM).

Discussion

Subclinical endometritis is characterized by inflammation of the endometrium without clinical signs and results in a significant reduction in reproductive performance (Kasimanickam, Duffield et al. 2004; Gilbert, Shin et al. 2005; Barlund, Carruthers et al. 2008; Santos, Lamb et al. 2009). The prevalence of the disease is very variable, and depends on the diagnosis technique; the DIM of the genital examination and the statistical method used to determine the cut-off point of the neutrophil ratio obtained from endometrial cytology (Guidry, Paape et al. 1976; Fourichon, Seegers et al. 2000; Kasimanickam, Duffield et al. 2005; Santos, Lamb et al. 2009). In all cases, the diagnosis is based on relative proportion of neutrophils. Cytobrush cytology appeared to be the most consistent and reliable technique to diagnose subclinical endometritis (Kasimanickam, Duffield et al. 2005; Barlund, Carruthers et al. 2008). However, cytobrush cytology is not a cow-side test and does not allow a rapid result and a treatment decision on the farm. Preliminary data on leukocyte esterase activity of uterine neutrophils proposed the technique as a cow-side test for subclinical endometritis allowing rapid decision making at the farm (Santos, Roman et al. 2006).

In the present study, the correlation between subjective scores of uterine leukocyte esterase activity and the percentage of neutrophils in the uterus determined by cytobrush cytology was high ($P<0.0001$). Santos et al., (Santos,

Roman et al. 2006) had similar results with a high correlation ($P<0.0001$) between leukocyte esterase score and the percentage of neutrophils from samples obtained by uterine lavage. This was supported by a correlation between vaginal discharge score and leukocyte esterase activity (data not shown). The CxLE was also correlated to the percentage of neutrophils on endometrial cytology (fig. 3). This is a good indication of uterine drainage of exudate from the uterus and is in agreement with another study comparing cytology from the cervix and uterus (Yavari, Haghkhah et al. 2009). The cervix is easier to reach; the technique could mean a practical advantage to diagnose subclinical endometritis on farm. However, even though the high correlation between the leukocyte esterase activity (UtLE and CxLE) and the percentage of neutrophils (EC), the pregnancy rate at 90DIM was not correlated to neither of them in any of the groups (GT, G1 and G2) of postpartum cows. With a high correlation between subjective score of UtLE and the percentage of neutrophils in the uterus for G2 (32-47 DIM), one would have expected that leukocyte esterase activity could be a predictor of pregnancy or infertility. Specially, that a significant negative effect of the presence of neutrophils on fertility was measured with the endometrial cytology for the same time window. The small number of cows in G2 (n=94) and the lower capacity of the test to detect esterase activity within an environment with a potentially smaller number of neutrophils could explain the lack of significant effect on fertility. The fact that two slides were produced with the same cytobrush before it was plunged in 1 ml of saline could probably explain the discrepancy of results between the EC and esterase activity in terms of pregnancy risk. With the methodology, many

of the neutrophils, cellular debris and uterine fluids stayed behind on the slides compromising the esterase activity assessment.

Because the presence of neutrophils and fluids in the cervix may be due mostly to drainage from the uterus (Wray 1982; Földi, Kulcsár et al. 2006; Azawi 2008) a high level of agreement was expected between UtLE and CxLE However, only a slight agreement between UtLE and CxLE was observed in the present study. The descrepency between UtLE and CxLE may be due to the following. During postpartum uterine involution, occasional reduced contractibility of the myometrium could temporally result in a poor clearance of the uterus (Rigby, Barhoumi et al. 2001) and maintain a large amount of neutrophils and fluid in the uterus, and not in the cervix. A good uterine clearance may push a great amount of neutrophils into the cervix and result in a relatively small quantity in the uterus increasing the disagreement between the UtLE and CxLE. Stage of the estrous cycle can affect the myometrial contractility and the neutrophil influx into the endometrial lumen and cervical mucus (Bondurant 1999). Furthermore, the cervix has its own inflammatory cell response to the presence of bacteria, which may not reach the uterus, with influx of neutrophils to the cervical lumen (Kelly 2002). Finally, as the leukocyte esterase test is based on hydrolytic reaction (Kutter, Figueiredo et al. 1987), a smaller amount of mucus or fluid in the cervix compared to the uterus could inhibit or slow down the chemical reaction and could be responsible for false negative results. During the clearance between the uterine lumen and the outer cervical os, the esterase enzyme could be potentially degraded.

In the present study, the mean percentage of endometrial neutrophils, when it was measured before 32DIM, did not predict pregnancy in cows at 90DIM. During that time, spontaneous resolution of uterine diseases occurs and it is associated with neutrophils increasing their killing capacity (L Mateus 2002), suggesting that subclinical endometritis cows might spontaneously recover. The fact that the present study was performed on several herds (n=5) could have reduced the effects of certain confounding variables like management strategies, body condition score, milk production and selected herds with high prevalence of subclinical endometritis (Gilbert, Shin et al. 2005; Barlund, Carruthers et al. 2008) and it is not in agreement with another study using a similar sampling technique (Kasimanickam, Duffield et al. 2004). Survival analysis based on a statistical model showing a significant effect of percentage of neutrophils on pregnancy risk is more likely to reflect physiological events.

Evaluation of the cow reproductive tract is based on breeding history, reproductive status, and physical examination findings, creating a more complete database from which to make a better diagnosis and prognosis. For more subtle disease like subclinical endometritis, additional and more invasive diagnostic methods are required. Endometrial cytobrush appears to be a reliable sampling technique showing more distinctively neutrophils, debris, mucus and endometrial cells (Kasimanickam, Duffield et al. 2005; Barlund, Carruthers et al. 2008). In mares, endometrial cytology is also useful to screen for an active inflammatory response (Bowen, Ley et al. 1987; LeBlanc, Magsig et al. 2007).

In addition to the technique of sampling, the method of assessment of the cytological smear could be important. The presence of mucus, debris, proteins and clumps or aggregates of endometrial cells could potentially complicate and influence the results and affect the interpretation. In the present study, the slides were evaluated according to two different approaches. The RM was an exhaustive method compared to the SM method, which is a more rapid and simple approach that could appeal to practitioners. Previous studies did not precisely and meticulously describe the way endometrial smears were analyzed. With an agreement of 81% between the two methods, SM did not show any disadvantages over the RM. Other researchers reported similar results in cows (Santos, Lamb et al. 2009) and mares (Bourke, Mills et al. 1997). Therefore, evaluation of endometrial slides should be initiated using low magnification under bright-field microscope for a first evaluation of the quality of the smear and to determine the areas for further analysis. A sufficient number of well preserved and adequately stained endometrial cells and evenly dispersed clumps or aggregates of endometrial cells would be a good indication of a good quality smear. Analysis of ten representative microscopic fields was enough to perform a good slide evaluation.

Conclusion

The present results support that the leukocyte esterase activity from

samples obtained by cytobrush cytology of the endometrium may have potential utility for the diagnosis of subclinical endometritis in postpartum dairy cows. Because of lack of power, further studies are required to test the full potential of leukocyte esterase activity for the diagnosis of subclinical endometritis in post partum cows. Cytobrush cytology assists in identifying animals with subclinical endometritis after 32DIM and the assessment of slides by a simple and more practical method provides a rapid and simple means of assessing endometrial cytology slide of individual cows.

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CHAPTER 3 – GENERAL DISCUSSION

The leukocyte esterase activity test followed closely the cytology exams as demonstrated on figure 2 and is in agreement with the study of Santos (Santos, Roman et al. 2006). However, in our study we were not able to find an esterase activity threshold score that could predict pregnancy risk and subclinical endometritis even on G2 where the EC showed a difference in pregnancy rate diagnosed at 90DIM with a cut off of 2.6% of neutrophils. From the present study, it would appear that the esterase activity measurements by commercial stick esterase test was not able to detect such low percentage of neutrophils for assessing subclinical endometritis. This finding can be explained by the lack of power or to details on the methodology (i.e., the esterase activity was only verified after the production of two slides for cytology evaluation) that may or may not have contributed to the esterase ability to detect such low percentage of neutrophils.

In our study, we demonstrated that the presence of neutrophils in the uterine lumen of cows after 32 DIM only, have a significant impact on subsequent fertility. This seems reasonable since many cows diagnosed with clinical endometritis are able to recover from this disease before 30 DIM (L Mateus 2002) suggesting that cows diagnosed with subclinical endometritis before 30 DIM can recover spontaneously. This study reported 2.6% of leukocytes as a cut off for the diagnosis of subclinical endometritis sampled between 32-47 days postpartum against a diagnosis of pregnancy at 90DIM.

The percentage of neutrophils used as a cut off in this study was much lower than the 10 and 8% reported by others (Kasimanickam, Duffield et al. 2004; Barlund, Carruthers et al. 2008). In these studies, the cytology samples were collected at a similar interval postpartum than the present study, but the pregnancy status time against which the cut off values were calculated from was much longer (132 and 150 DIM) compared to 90 DIM in the present study. Using 90 DIM as the limit for the diagnosis of pregnancy, we did not allow cows to cycle naturally or by hormonal manipulation several times before getting pregnant which could explain the lower cut off of neutrophils measured in the present study to identify cows with subclinical endometritis.

The first service conception rate between endometritis positive and endometritis negative cows sampled on G2 in this study was not significantly different and had only a tendency with a P value of 0.08. It was expected to see a significant difference in FSCR between endometritis positive and endometritis negative cows sampled on G2 since the correlation between the percentage of neutrophils measured on G2 and the pregnancy risk was significant. Other studies demonstrated a significant difference in FSCR between cows with or without subclinical endometritis without showing if there was a significant correlation between neutrophil count and pregnancy risk before defining the cut off that characterizes positive subclinical endometritis (Kasimanickam, Duffield et al. 2004; Barlund, Carruthers et al. 2008). Nevertheless, the number of cows used for analysis on our study was much smaller and could have contributed to the lack of statistical power.

A high level of agreement was expected between UtLE and CxLE, which did not occur. Sporadically contractions during uterine involution may be seen and may lead to a poor clearance of the uterus (Rigby, Barhoumi et al. 2001) keeping a large amount of fluid and neutrophils in the uterus. On the other hand a good uterine clearance may push a great amount of neutrophils into the cervix and result in a relatively small quantity in the uterus increasing the disagreement between the UtLE and CxLE. Stage of the estrous cycle can affect the myometrial contractility and the neutrophil influx into the endometrial lumen and cervical mucus (Bondurant 1999). Furthermore, the cervix has its own inflammatory cell response to the presence of bacteria, which may not reach the uterus, with influx of neutrophils to the cervical lumen (Kelly 2002). Finally, as the leukocyte esterase test is based on hydrolytic reaction (Kutter, Figueiredo et al. 1987), a smaller amount of mucus or fluid in the cervix compared to the uterus could inhibit or slow down the chemical reaction and could be responsible for false negative results.

A systematic approach to assess the slides performing a thorough scanning of slides and counting several fields and up to 500 cells (epithelial, neutrophils, macrophages and lymphocytes) was used in parallel with a simple method used routinely by clinical pathologists to diagnose the presence of inflammatory cells on cytology. The correlation between the systematic approach and the simple approach was high which indicates that a simple method can be used for the diagnosis of subclinical endometritis.

A great variation in methodology, statistical analysis and long period between the disease occurrence and the impact on fertility, make it difficult to draw definitive conclusions. One study that today is a reference for many others has found a cut off for the percentage of neutrophils without showing if the mean percentage of neutrophils for pregnant and non-pregnant cows were statistically significant, using this cut off for further analysis on fertility parameters between positive and negative subclinical endometritis cows (Kasimanickam, Duffield et al. 2004). Another study used a cut off based on previous experience without further explanation (5% neutrophils) (Gilbert, Shin et al. 2005). However in another study by the same research group, a different cut off (5.5% neutrophils) was used (Santos, Lamb et al. 2009). The point is; if a number of cows are divided into two groups, differences in reproduction parameters (days open, first service conception rate) between the two groups might be found. There are also many others variables that can be implicated in this scenario such as; nutrition, stress, milk production, management, etc.

Studies of subclinical endometritis are somewhat recent and have generated some data that is, unfortunately, not yet comparable. There is not, up untill now, a clear definition of subclinical endometritis and a ”gold standard test”.

The leukocyte esterase activity followed the percentage of neutrophils present in the samples, but was not able to predict pregnancy risk at first service between 60 to 90 DIM.

This study confirms the link between the percentage of neutrophils measured from the uterine lumen between 32 and 47 days post partum and the risk of pregnancy. However, there was no statistical difference in days open and only a tendency to decrease first service conception rate in cows where subclinical endometritis was diagnosed using a 2.6% cut off of neutrophils.

Future studies on subclinical endometritis should be concentrated in a post partum period where the uterine involution (macroscopic and microscopic) may have completely occurred. A much large number of cows should be used for the experiment; blood may be withdrawn for differentiation between cows with high and low concentration of progesterone and for analyses of *in vitro* neutrophil activity that may differ between cows with and without subclinical endometritis. As far as comparing leukocyte esterase and endometrial cytology using cytobrush for the diagnosis of subclinical endometritis an exclusive cytobrush should be used for the purpose of esterase activity measurements.

CONCLUSION

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ANNEX

Comparison of 4 commercial leukocyte esterase tests used with purulent discharge

Materials and Methods

Four esterase tests {Uristix® (Bayer Health Care L.L.C., Elkhart, IN, USA), Multistix® (Bayer Health Care L.L.C., Elkhart, IN, USA), Chemstrip® (Boehringer Mannheim Corp., Indianapolis, IN, USA) and Rapid Response®(BTNX Inc., Markham, ON, Canada)} were evaluated with purulent vulvar discharge from 5 different cows. All tests are based on the same colorimetric classification: grade 0, 0 PMN cell/ mm^3; grade 0.5, 15 PMN cells/mm^3; grade 1, 70 PMN cells/ mm^3; grade 2, 125 PMN cells/ mm^3; and grade 3, 500 PMN cells/mm^3. The purulent secretions were diluted as followed: pure, 1:500, 1:1000, 1:2000 and saline. The samples were unknown to the readers. The four esterase tests were done in triplicate for each dilution factor with 20µl sample and the color change was read in 2 minutes by two independent readers.

Data Analysis and Statistics

A non-parametric analysis of Kruskal-Wallis and a post-hoc test was conducted to estimate the best leukocyte esterase test.

Results

There was a difference (P=0.0001) between the leukocyte esterase tests with a purulent solution. The median score of the triplicates was significantly lower for the RapidResponse® than for the 3 other tests and when the group pure was considered positive and group saline negative, the sensitivity was only 21.4% (vs 100% for the other 3). At the dilution 1:1000, a difference (P=0.002) between the leukocyte esterase tests indicated that the median score of the RapidResponse® was significantly lower than the Multistix® and Uristix® and there was no difference between the RapidResponse® and Chemstrip®. This first experiment showed that 2 leukocyte esterase tests (Uristix® and Multistix®) were similar in their responses and the leukocyte esterase test Chemstrip® and RapidResponse® did not have a constant response. The leukocyte esterase test Uristix® was chosen in this study.

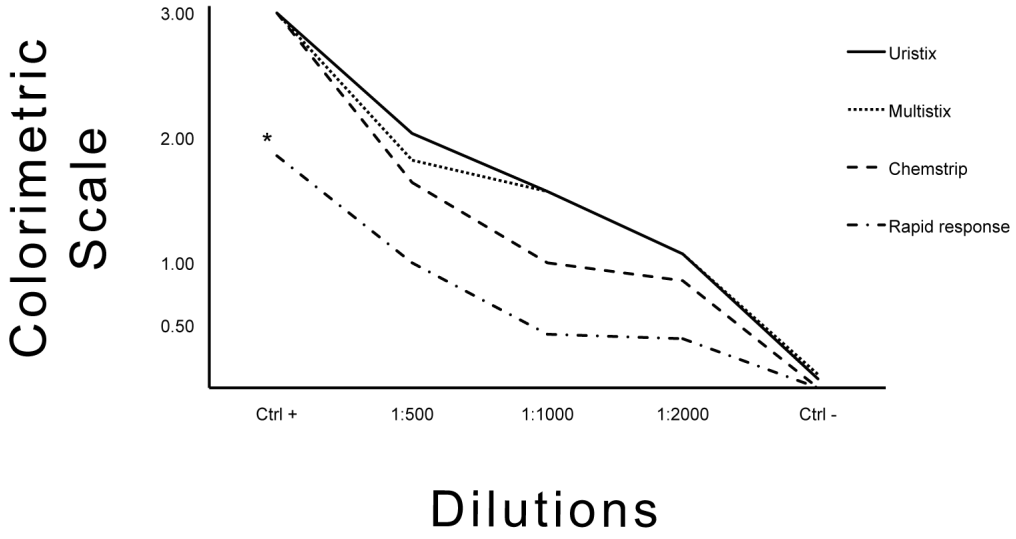

Figure 1. Correlation between the 4 commercial leukocyte esterase tests.

* Significant difference

Ctrl+ Control positive

Ctrl- Control Negative