SUBCLINICAL ENDOMETRITIS IN DAIRY CATTLE:

A PRACTICAL APPROACH

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Simplicity is the ultimate sophistication

Leonardo da Vinci

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AI	Artificial insemination
AI-CT	Artificial insemination-Cytotape
AI-CYTO	Artificial insemination-Cytological endometritis
BCS	Body condition score
CB	Cytobrush
CB-CIAE	Cytobrush-Naphtol-AS-D-chlorazetat-esterase
CB-DQ	Cytobrush-Diff Quick
CCC	Concordance correlation coefficient
CE	Clinical endometritis
CI	Confidence interval
CIAE	Naphtol-AS-D-chlorazetat-esterase
CL	Corpus luteum
CM	Clinical metritis
CS	Cotton swab
CT-DQ	Cytotape-Diff Quick
CY-CB	Cytology-Cytobrush
CYTO	Cytological endometritis
DIM	Days in milk
DPP	Days postpartum
EB	Endometrial biopsy
EB-HE	Endometrial biopsy-Haematoxylin eosin
EV-CB	*Ex vivo*-Cytobrush
HDL	High density lipoprotein
HPF	High power field
IL	Interleukin
IV-CB	*In vivo*-Cytobrush
IV-LVL	*In vivo*-Low volume lavage
k	Kappa value

LDL	Low density lipoprotein
LES	Leukocyte esterase strip
LVL	Low volume lavage
NPV	Negative predictive value
NSAID	Non-steroidal anti-inflammatory drug
OR	Odds ratio
PGF2α	Prostaglandin F2-alfa
PMN	Polymorphonuclear
PMN-EP	Polymorphonuclear-Epithelium
PMN-SC	Polymorphonuclear-*Stratum compactum*
PPV	Positive predictive value
PVD	Purulent vaginal discharge
r	Pearson correlation
RBC	Red blood cell
RFM	Retention of fetal membranes
ROC	Receiver operating characteristic
RPM	Rotation per minute
SCC	Somatic cell count
SCE	Subclinical endometritis
Se	Sensitivity
SE	Standard error
Sp	Specificity
TNF	Tumor necrosis factor
VD	Vaginal discharge
VWP	Voluntary waiting period

GENERAL INTRODUCTION

ROLE OF CATTLE IN GLOBAL FEEDING

The perceived limits to producing food for a growing global population have been a source of debate and preoccupations for ages [1]. A growing world population requires more food and nutrition of better quality. According to the latest United Nations estimates, the global population is set to rise from close to 7 billion in 2010 to about 9.5 billion by 2050 [2]. An increase of about 30 % (from 7 to 9.5 billion) will require increased food production of a similar amount. It has been estimated that crop yields will have to double by 2050 to meet the increasing demand for food [2]. Enhancing agriculture productivity while improving nutrition is, therefore, essential to meet our future food requirements [1].

Bovine milk and dairy products have long traditions in human nutrition [3]. Milk provides an easily accessible matrix, rich in a large variety of essential nutrients such as minerals, vitamins and easily digestible proteins with balanced amino acid profiles **(Table 1)**. However, in western societies, the consumption of milk has decreased during the last decades [3]. This trend may partly be explained by the claimed deleterious health effects that have been attributed to milk and dairy products. This criticism has arisen especially because milk fat contains a high fraction of saturated fatty acids assumed to contribute to heart diseases, weight gain and ultimately obesity [4]. Even though other studies have shown an inverse association between milk intake and overall cardiovascular disease risk [5]. It is scientifically proven that milk components take part in the metabolism in several ways; by providing essential amino acids, vitamins, minerals and fatty acids, or by affecting absorption of nutrients [3]. Daily consumption of 0.5 liter milk supplies a significant amount of many of the nutrients that are required on a daily basis.

Table 1. Milk composition and percent contribution to the daily dietary reference intakes of some nutrients in 0.5 liter whole milk, and their main health effects (Haug, Høstmark [3]).

Milk Component	Concentration in 1 liter whole milk	% contribution of 0.5 liter whole milk to reference intake	Health effects
FAT	33 g/l		Energy rich
SATURATED FATTY ACIDS	19 g/l		Increases HDL, small dense LDL, and total cholesterol. Inhibition of bacteria, virus
OLEIC ACID	8 g/l		Prevents CHD, gives stable
LAURIC	0,8 g/l		Antiviral and antibacterial
MYRISITC	3,0 g/l		Increases LDL and HDL
PALMITIC	8 g/l		Increases LDL and HDL
LINOLEIC	1,2 g/l		Omega-6 fatty acid
ALPHA	0,75 g/l		Omega-3 fatty acid
PROTEIN	32 g/l	30–40%	Essential amino acids, bioactive proteins, peptides. Enhanced bioavailability
LACTOSE	53 g/l		Lactosylation products
CALCIUM	1,1 g/l	40–50%	Bones, teeth, blood pressure, weight control
MAGNESIUM	100 mg/l	12–16%	For elderly, asthma treatment
ZINC	4 mg/l	18–25%	Immune function. Gene expression
SELENIUM	37 ug/l	30%	Cancer, allergy, CHD
VITAMIN E	0,6 mg/l	2 %	Antioxidants
VITAMIN A	280 ug/l	15–20%	Vision, cell differentiation
FOLATE	50 ug/l	6 %	DNA synthesis, cell division, amino acid metabolism
RIBOFLAVIN	1,83 mg/l	60–80%	Prevent ariboflavinosis
VITAMIN B_{12}	4,4 ug/l	90%	Key role in folate metabolism

Beef is regularly linked with a negative health image due to its “high" fat content and also as a cancer-promoting food [6]. However, this discussion overlooks the fact that beef is a major source of micronutrients such as essential amino acids and vitamins, which are either not present or have a poor bioavailability in plant derived-products.

Therefore, a low beef intake is recommended to avoid the risk of cancer, obesity and the metabolic syndrome [7]. Moreover, beef as a protein-rich and carbohydrate-low product is characterized by a low glycemic index, which is assumed to reduce chances to develop obesity, diabetes and cancer [7]. Taken together, beef contains several essential nutrients, which are beneficial for human health and development **(Table 2)**.

Table 2. Composition of several beef cuts (Pereira and Vicente [8]).

Beef cut	Energy value (kcal)	Protein (g)	Fat (g)	Saturated fat (g)	Vitamin B12 (mcg)	Na (mg)	P (mg)	Fe (mg)	Zn (mg)
Beef, steak cuts, raw	122	20.9	4.3	1.8	2	60	169	1.4	3.6
Beef, loin, raw	114	21	3.3	1.4	2	60	145	1.5	3.6
Beef, calf, loin, raw	148	19.9	7.6	3.2	1.2	24	195	0.9	3

To keep up with the global population growth, the dairy and beef industry need to face an increased production. As the world's resources are limited, the Food and Agriculture Organization of the United Nations estimates 70 % of the additional food supply must be safeguarded by developing and applying efficiency-enhancing technologies [9]. To meet the demands of the 21st century, individual cows must produce milk and beef more efficiently [10]. The rapid progress in genetics and management in the dairy and beef industry throughout the world has created a new era in which a smaller number of dairy and beef cows meets the growing demand for products derived from cattle [10]. Nevertheless, reproductive efficiency in cows is decreasing worldwide [11]. The importance of an optimized reproductive biology in cattle is based on its central role in the provision of high-quality protein for human nutrition (no pregnancy, no beef or milk) [12]. Consequently, to reach an optimal reproductive efficiency, the reproductive decline needs to be reversed by collective efforts from farmers, veterinarians, and researchers.

THE LINK BETWEEN PRODUCTION AND REPRODUCTION

Increased production in dairy cows has too often been linked with a concomitant decrease in reproductive capacity [13] and [14]. Dairy production systems that use cows that have been highly selected for milk production in recent decades have suffered

a decline in cow fertility [11]. Countries and regions that operate via diverse production systems from year round calving herds mostly found in Europe and North America, to grass based seasonal calving herds in Ireland, New Zealand and Australia are affected [10], [15], [16] and [17]. Consequently, one of the greatest challenges of reproductive biologists, pathologists, nutritionists and geneticists is to gain a better understanding of the underlying biology of the dairy cow that contributes to decreased fertility and develop strategies to reverse this negative trend. Fertility is a multi-factorial trait and its deterioration is caused by a network of genetic, environmental and managerial factors, and their complex interactions make it difficult to determine the exact reason for the fast decline [11].

Genetic correlations between milk yield and reproductive measures in dairy cows are unfavourable [18]. Dairy selection objectives have centred on milk production, with little attention being given to traits such as health and fertility [19]. Therefore, a negative genetic trend in fertility traits resulted in a phenotypic trend indicating a decline of ~ 1 % per year in pregnancy rates to first service [20]. However, in recent years, the emphasis within selection indices for Holstein-Friesians has shifted from predominantly production to functional nonproduction traits associated with improved health and fertility [21]. There is now evidence that the phenotypic historical decline in fertility has reached a nadir and has begun to improve [22] and [23]; however, future studies are needed to confirm this trend and to determine potential alleviating factors [19]. Inappropriate management of high yielding dairy cows may significantly contribute to poor fertility rather than direct genetic effects [24]. Understanding genotype by environment interactions is crucial in determining the best health and management practices to achieve high levels of productive and reproductive efficiency. In addition to augmented milk production, increases in herd size, changes in housing conditions and more in do-it-yourself activities have all contributed to an increased difficulty to manage the high producing dairy cow to also achieve optimal fertility [25].

During the period which extends from 2 weeks pre-calving to about 4 weeks post-calving, dairy cows experience the stress of parturition, the commencement of lactation, the increased demand for energy and protein to meet milk production all in combination with reduced feed intake which is generally inadequate to meet her maintenance and production requirements [11]. Deficient nutrition in this period may

lead to metabolic disorders, body condition score loss and consequently a pronounced negative energy balance triggering reduced immune function (cows more prone to disease). Hence, cows that suffer from metabolic disorders in the peri-parturient period are more likely to suffer from an increased incidence of mastitis, lameness and endometritis [26] all of which contribute to reduced reproductive efficiency.

Cattle are remarkable amongst mammals because the uterus almost always becomes contaminated with bacteria during and immediately after parturition [27]. Uterine contamination at parturition or in the following days is unavoidable and normal with 80–100 % of the animals having bacteria in the uterine lumen in the first 2 weeks postpartum [28] **(Figure 1)**. Over a similar time span, the endometrium initially sloughs and then regenerates, particularly over the maternal caruncles, where the maternal and foetal tissues have interfaced. Similarly, during parturition, the physical barriers of the cervix, vagina and vulva are compromised providing an opportunity for bacteria to ascend the genital tract from the environment and elicit uterine infection [27] and [29]. Many cows successfully deal with this bacterial contamination; however, at least 20 % of cows are unable to resolve the contamination and develop metritis within 21 days postpartum [12]. However, even cows that are treated successfully for clinical (endo)metritis have conception rates that are approximately 20 % lower than unaffected animals and an extra 3 % of animals remain infertile and are finally culled [30] **(Figure 1)**.

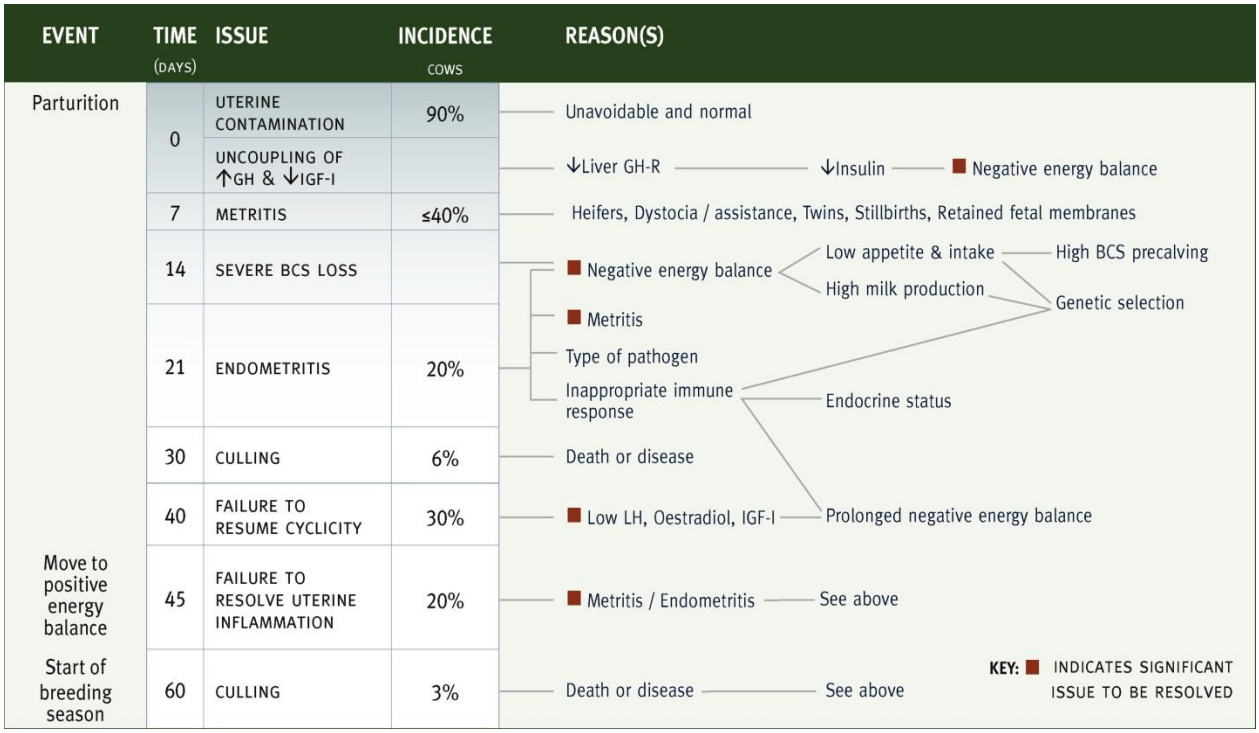

Figure 1. Schematic presentation of the reasons for the major problems contributing to low fertility in dairy cows in the postpartum period (Walsh et al. [11]).

An inflammatory milieu in the uterus decreases sperm motility, oocyte maturation, corpus luteum function and embryonic quality [31]. All of these aspects together impair successful reproduction at several crucial stages, with the final consequence of a decreased pregnancy rate [28] and [32]. In this aspect, subclinical endometritis is a highly prevalent uterine disease which courses asymptomatically, negatively affecting the reproductive outcome of the cow [33]. Subclinical endometritis is considered to be a postpartum disease, presumably associated with endometrial recovery after clinical endometritis, trauma or other non-microbial diseases [30]. Taken together, the marked changes in the physiological status during early lactation due to augmented milk production and the limitation in the management system have increased the prevalence of postpartum uterine disease in dairy cows. Our particular interest in postpartum uterine diseases is because it significantly contributes to the reproductive efficiency, and specifically in subclinical endometritis since it is suggested to be highly prevalent and its diagnosis is not fully established yet.

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POSTPARTUM UTERINE DISEASES IN DAIRY CATTLE

POSTPARTUM UTERINE DISEASE

Postpartum uterine disease is the leading cause of reproductive inefficiency in dairy cattle [1]. Amongst the mammals, dairy cattle farmed in intensive systems, commonly acquire microbial contamination of the uterus during parturition [2]. Almost all dairy cows (80 to 100 %) experience bacterial contamination of the uterus immediately after calving [3] and [4]. Due to this fact, as well as due to the substantially required repair of the endometrium following parturition, uterine inflammation is a normal and necessary component of the postpartum uterine involution [5]. However, in a proportion of the postpartum cows, the inflammation runs out of hands and leads to uterine disease [6]. The scope of the postpartum uterine disease complex includes retention of fetal membranes (RFM), clinical metritis (CM), clinical endometritis (CE) and/or purulent vaginal discharge (PVD), pyometra, and subclinical endometritis (SCE) **(Table 1)**.

Retention of fetal membranes

Retention of fetal membranes or retained placenta is the failure to expulse the placenta between 12 to 24 hours after parturition [7], [8] and [9]. If RFM occurs, the membranes are retained in the uterine lumen for on average 7 days [10], enhancing bacterial contamination and delaying uterine involution [11]. The incidence of RFM ranges from 4 to 12 % [7], with a median incidence rate of 8.6 % [12]. Predisposing factors are: twins, dystocia, stillborn calf, abnormal length of gestation, induced parturition, abortion, nutritional imbalance, fetotomy, cesarean section (non elective), increasing age, and seasonal effects [11] and [13]. Retained placenta has no direct impact on milk production, reproduction or culling if the condition not evolves to CM, CE, or SCE [14]. The increased risk for the previously mentioned diseases constitutes the main reason of the economic importance of RFM. A variety of methods has been used to treat RFM, nevertheless, the topic is still controversial. Manual removal, local antibiotics, and ecbolic drugs are commonly used treatments, although current evidence does not support their use [11], [15] and [16]. Intrauterine infusion of oxytetracycline is a common treatment among practitioners. The latter may reduce the incidence of subsequent fever [17], however, it has no effect on subsequent reproductive performance [18] and is associated with detectable milk residues which can persist up to 144 hours [19]. Moreover, the local antibiotic therapy (especially with tetracyclines)

Table 1. General overview of the main characteristics of the most common postpartum uterine diseases.

Postpartum uterine disease	Definition	Days after calving and reported incidence	Treatment
Retention of fetal membranes	Failure to expulse the placenta between 12 to 24 h after parturition	24 hours after parturition Incidence: 4 to 12%	Strong traction is discouraged Local antibiotics?
Metritis (puerperal and toxic)	Enlarged, atonic uterus Fetid, watery red-brown discharge Signs of systemic illness (fever >39.5°C, decreased milk yield, signs of toxemia)	Within 21 days after calving Usually at the end of the first week after calving Incidence: 5 to 15%	Local antibiotics? In case cows become febrile, systemic antibiotics for at least 3 consecutive days Supportive therapy if required
Clinical endometritis and/or purulent vaginal discharge	Local inflammation of the endometrium (clinical endometritis) Presence of purulent or mucopurulent material in the vagina (PVD) Absence of systemic symptoms (fever)	≥ 21 days after parturition Incidence: 20 to 30%	Intrauterine antibiotics (cephapirin) ≥ 26 after parturition Benefit of PGF2α is not clear
Pyometra	Presence of purulent material in the uterine lumen Corpus luteum present Cervix often closed	Depending on the days post calving after first ovulation Incidence: 1 to 2%	Two doses of PGF2α with an interval of 11 to 14 days between applications
Cytological endometritis	Abnormal presence of PMNs in endometrial cytology samples Absence of any clinical sign	Diagnosed between 21 to 64 days after calving 9 to 76% of incidence, most commonly between 25 to 35%	Intrauterine antibiotics (cephapirin) or PGF2α (both under discussion)

may prolong the RFM due to its ability to inhibit matrix metalloproteinases [16]. In case cows become febrile, systemic antibiotics (ceftiofur) appear to be beneficial in reducing disease and aiding in the return to normal reproductive function [13]. However, in many European countries, the systemic use of broad spectrum antibiotics is currently severely under pressure because of the potential association with the increase in antibiotic resistance.

Clinical metritis

Clinical metritis is characterized by an enlarged uterus and a watery red-brown fluid to viscous off-white purulent uterine discharge, which often has fetid odor, occurring

within 21 days postpartum [20]. The diagnosis of CM is made on the basis of clinical signs of fetid uterine discharge and/or systemic illness. The severity of the disease has been categorized according to the health signs of the animal in grade 1, 2 and 3 [3]. **Clinical metritis grade 1** is characterized by an abnormally enlarged uterus and uterine discharge without systemic signs of illness [20]. **Clinical metritis grade 2 (or puerperal metritis)** refers to animals that suffer from additional signs of systemic illness such decreased milk production, dullness, and fever > 39.5° C [20]. **Clinical metritis grade 3 (or toxic metritis)** includes animals with clinical signs of toxemia such as inappetence, cold extremities, depression, and/or collapse [3]. Risk factors for any degree of CM are often associated with RFM, dystocia, stillbirth or twins. Clinical metritis usually occurs at the end of the first week after calving, being less common after the second week postpartum [21] and [22]. The impact of metritis on milk production and reproduction is controversial [14]. When reported as detrimental, the impact on milk production is between 2 and 13 kg of milk per day during a period of 2 to 20 weeks [23], [24] and [25]. Giuliodori at al. [25] proposed that puerperal CM is associated with an impaired early pregnancy rate and an extended calving to conception interval, while other studies did not find a link between CM and a decline in reproductive capacity [14]. A common treatment for CM (puerperal/toxic) is the intrauterine infusion of antibiotics. However, the efficacy of a local antibiotic treatment is a controversial issue [21]. Nowadays, the use of systemic antibiotics (ceftiofur for 3 consecutive days) in cows with an abnormal vaginal discharge at days 4 to 6 after calving and a rectal temperature ≥ 39.5°C has gained popularity and is the most used treatment for puerperal/toxic metritis (CM grade 2 and 3), although this treatment has become controversial in terms of prudent use of antibiotics [21] and [26]. Recently, a randomized clinical trial studied the efficacy of the initial use of ketoprofen versus ceftiofur in cows with CM to reduce the usage of antibiotics drugs [27]. However, no beneficial effects were found when cows were only initially treated with ketoprofen, finally more doses of medical applications had to be expected [27].

Clinical endometritis and/or purulent vaginal discharge

Clinical endometritis is basically referring to a local inflammation of the endometrium, characterized by the presence of purulent or mucopurulent (> 50% pus) material in the vagina ≥ 21 days postpartum originating from the uterus, not

accompanied by systemic illness [20] and [28]. It affects around 20 % of dairy cows between 21 to 40 days postpartum [29]. Usually, CE is diagnosed by means of a vaginoscope, a gloved hand or the metri-check [30] and [31]. However, it has become clear that the presence of abnormal vaginal exudate may not precisely be related to endometrial inflammation. Endometritis requires endometrial cytology or biopsy (or ultrasound examination) for a convincing diagnosis [16]. The presence of vaginal exudate nowadays is referred to as 'Purulent vaginal discharge' (PVD). It is generally assumed that PVD is the result of endometritis, cervicitis/vaginitis or the combination of both [28] and [32] **(Figure 1)**. Prevalence of endometritis alone is around 13 %, cervicitis only 11 %, while 32 % of the cows suffer from both conditions [32]. The detrimental effects of endometritis and cervicitis/vaginitis on reproductive performance are additive [28]. In general, cows affected with PVD need on average 30 days more to become pregnant in comparison to unaffected cows [14], [28] and [33]. Currently, several controversial reports on the efficiency of treatment protocols for CE/PVD are available in the literature. However, two main approaches are commonly used: parenteral injections of prostaglandins (PGF2α) and intrauterine antibiotics. Prostaglandins are reported to be slightly beneficial [34] or inefficient [35]. Routine use of PGF2α after 30 days postpartum may be relevant, but there is lack of evidence to sustain its efficiency [14]. The use of an intra-uterine cephapirin application after 26 days postpartum has been proven to be useful for treating PVD [14], [36], [37] and [38].

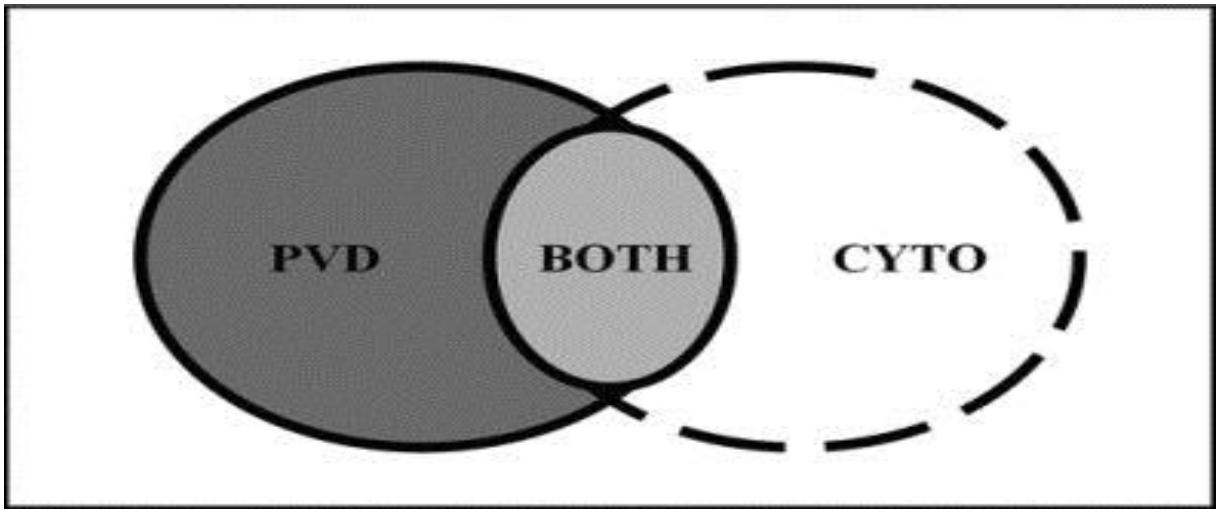

Figure 1. Cows could have 3 different uterine disease statuses: purulent vaginal discharge (PVD; cervicitis/vaginitis) only, subclinical endometritis (cytological endometritis, CYTO) only, or both PVD and CYTO (Dubuc et al. [28]).

Pyometra

Pyometra is defined as the accumulation of purulent or mucopurulent material in the uterine lumen provoking a distension of the uterus, accompanied by the presence of an active corpus luteum [20]. Often in pyometra, the cervix is functionally closed, although its lumen is not always completely occluded, and some purulent material may discharge through the cervix, vagina or vulva [20]. Based on a large field study [39], pyometra showed to affect approximately 1.2 % of the investigated cows, and the affection was related to problems during the postpartum period in most of the cases [40]. Generally, ovulation is delayed in cows with a pathologic uterine load [41], but in case cows do ovulate during an ongoing uterine infection, they may develop pyometra [16]. The diagnosis of pyometra can be done by rectal palpation and/or ultrasound, echography being the preferred and most accurate method to diagnose the disease. Treatment is based on the injection of two doses of PGF2α with an interval of 11 to 14 days between the applications, with a fair rate of healing [42], [43] and [44]. Prognosis after PGF2α treatment is generally favorable, with a first service conception rate of approximately 30 %, and an expected pregnancy rate of 80 % after three or four inseminations [44] and [45].

SUBCLINICAL ENDOMETRITIS

Subclinical endometritis is defined as the superficial inflammation of the endometrium, without visible clinical signs, but significantly impairing reproductive performances [20], [46], [47] and [48]. As SCE cannot be detected by simple visual inspection, complementary examinations are necessary for its diagnosis, being: histopathology, ultrasonography, leukocyte esterase strips (LES) and endometrial cytology.

Endometrial biopsy and histopathology

Structures observed in bovine endometrial biopsy samples include: 1) luminal epithelium; 2) endometrial glands arranged in two distinguishable layers (stratum compactum, superficially, and stratum spongiosum, more deeply); 3) stromal tissue; 4) lymphatics; 5) blood vessels; and 6) nerves. Definitely, histopathology is considered as the gold standard to diagnose endometritis in mares [49] and [50] **(Figure 2)**. In cows,

there currently are no peer reviewed papers available that comment on the effect of the histopathological findings of endometrial biopsies of clinically healthy cows on the reproductive capacity of the sampled animals. Moreover, in cattle, endometrial biopsy is rarely used since it is considered to be time-consuming, expensive and potentially detrimental for further fertility [20], [51], [52], [53] and [54]. Consequently, in the attempt to avoid the use of endometrial biopsy, it was compared with endometrial cytology in mares [55] and [56] and in cows [57] and [58]. However, only a fair/low agreement was found between both diagnostic methods. In this context, a more objective and deep study of the association between endometrial cytology and histopathology to explore the association between chronic and active alterations and by comparing the distribution of active inflammation (polymorphonuclear cells [PMNs]) at different levels (superficially and deeply) of the endometrium is warranted. Moreover, it would be very useful to develop a detailed scale of endometrial alterations that potentially have an effect on the reproductive performance of sampled animals and also to identify which specific endometrial alterations are associated with a substantial decrease in the fertility of cows.

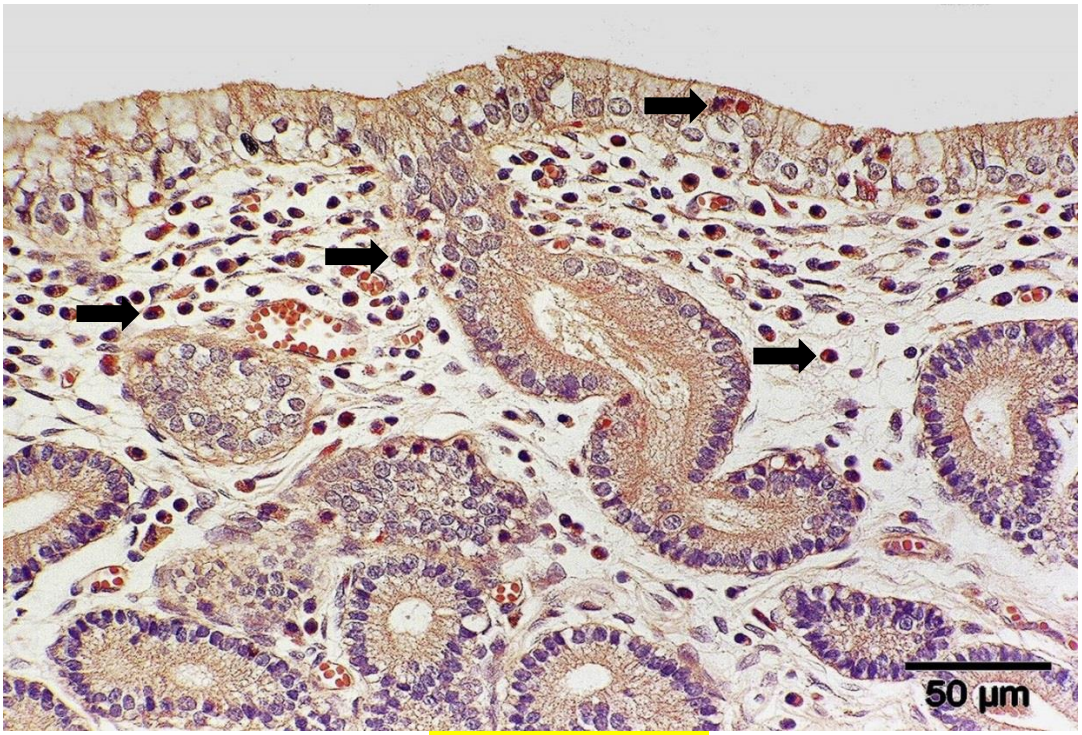

Figure 2. Histological evaluation (equine endometrium) of PMNs with naphtol-AS-D-chlorazetat-esterase staining. Polymorphonuclear cells appear bright red (Overbeck et al. [59]).

Subclinical endometritis and ultrasound

Nowadays, ultrasonography is considered to be part of the routine clinical examination of the reproductive tract of dairy cows [60]. Consequently, its potential to diagnose SCE has already been proven [1], [46] and [58]. The presence of fluid in the uterus between 20 to 47 days postpartum has been shown to be associated with a significant reduction in the relative pregnancy rate in comparison to cows that appeared “clean” at examination [46], [61] and [62]. However, the agreement between ultrasound and endometrial cytology is low, meaning that ultrasound and cytology measure two different representations of SCE. On one hand, the clearance mechanism of the uterus (luminal fluid) and on the other hand the cellular response of the inflamed uterus (PMNs in cytology slides) [1] and [46]. Another parameter used to diagnose SCE is the endometrial thickness measured by ultrasound. However, this technique is underused since it can easily be influenced by the location of the probe on the uterine horn. Consequently, ultrasonography is considered to be not accurate when it is not accompanied with endometrial cytology [1]. In general, ultrasound is an easy and fast method to diagnose SCE, nevertheless alone it is considered to be not precise enough to assess a final diagnose.

Leukocyte esterase colorimetric strips

The LES has been used for rapid diagnosis inflammation in fluids such as urine, pleural fluid, peritoneal fluid and cerebrospinal fluid [63]. The leukocyte esterase is a type of enzyme produced by neutrophils and is associated with infection . The LES has been used as an indirect method to detect inflammation due to its reaction with the diazoniun salt released indoxil which is oxidized, yielding a violet azo dye [64]; which intensity is related with the leukocyte counts. In order to create a “cow side” diagnostic method for SCE, LES were tested to assess its potential to diagnose SCE [63], [65] and [66]. Although results obtained by LES are positively correlated with endometrial cytology results [63] and [65], they initially were not correlated with the odds of pregnancy [63]. However, in a recent large study [66], a strong correlation was found between the LES results and the odds to become pregnant. Consequently, authors considered the LES as a valid alternative for on-farm SCE diagnosis. However, to fully

recommend the use of leukocyte esterase reagents as a final method to diagnose SCE, this method needs further refinement [65].

ENDOMETRIAL CYTOLOGY

Endometrial cytology is the most used technique to diagnose SCE in cattle in both field and research setups [28] and [67]. The measurement of the proportion of PMNs in cytology slides is the hallmark for SCE diagnosis, to the point that some authors refer bovine SCE to as "cytological endometritis" (CYTO) [67]. Consequently, starting from this point, SCE diagnosis by endometrial cytology will be referred as CYTO. Similarly to PVD, CYTO diagnostic criteria are established based on subsequent reproductive performances [14]. In general, in comparison to their negative counterparts, cows affected by CYTO experienced a detrimental effect regarding their reproductive capacity [28], [35] and [47] **(Figure 3)**. Although there is no impact of CYTO on milk production [14], its importance is mainly based on the increased time to pregnancy in positive cows [47] and the concomitant economic effect [14]. Assuming that each "extra" open day costs to the farmer approximately 2 € [68], and that a "CYTO positive" cow has on average an increased time to pregnancy of 25 d [28] and [47], CYTO associated costs reach up to 50 € per positive cow, plus the cost (material and service) of extra unsuccessful inseminations and eventual treatments. Moreover, CYTO has been shown to be a highly prevalent disease ranging on average from 20 to 30 % of examined postpartum cows [14], becoming one of the most important reproductive impairments in dairy cows studied in the last decade.

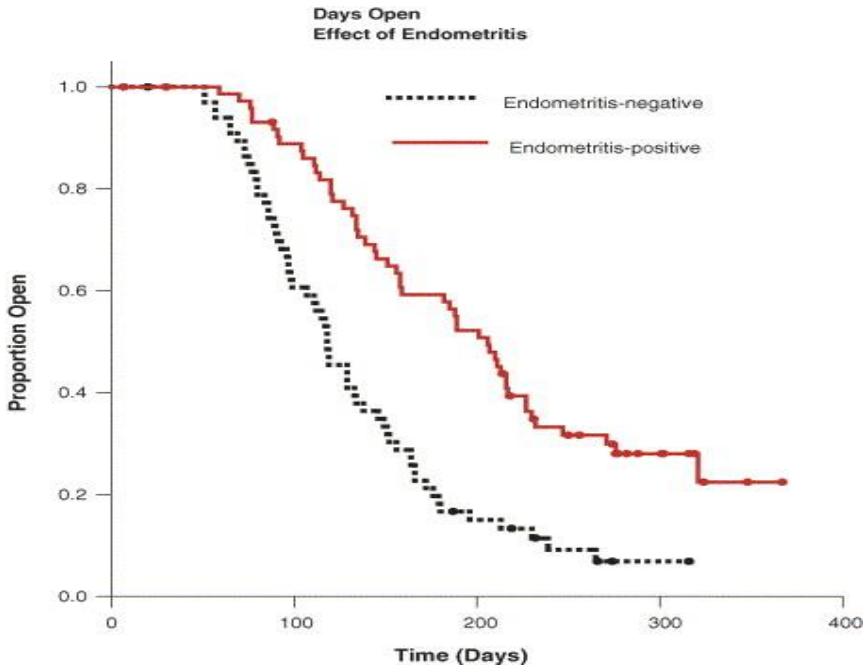

Figure 3. Kaplan-Meier survival curves for proportion of open cows by endometritis status (Gilbert et al. [47])

Endometrial cytology techniques in dairy cows: cytobrush and low volume lavage

Cytology is the science that evaluates the structure, chemistry and functionality of the cells [69]. Polymorphonuclear granulocytes represent the first and principal immunologic defense mechanism in the uterus [3], [4], [51] and [70]. Thus, an elevated number of PMNs in the uterine lumen indicates an inflammatory reaction of the endometrium (endometritis). Different techniques have been described to obtain endometrial samples for cytological examination in both mares and cows [1], [46], [47], [55], [71], [72] and [73]. In cows, cytobrush (CB) and low volume lavage (LVL) are the most used techniques to harvest endometrial surface scrapings. The CB consists of a small brush that is commonly used to sample cells from the vagina, cervix or endocervix for Papanicolaou testing in women [74]. As such, CB use was first reported in women to collect cervical samples to diagnose malign tumors [75]. In 2004 for the first time, the CB was adapted to harvest endometrial samples in cows [34]. The first described process to take endometrial samples by CB in cows was as follows: the CB was adapted onto a stainless steel road, placed in a stainless steel tube, and covered with a sanitary plastic sleeve. Then (under rectal guidance), by passing the instrument through the cervix (as an artificial insemination), the brush is released in the uterine lumen (corpus uteri) and with gentle rotation against the uterine wall, endometrial cytology samples

were obtained. Lastly, cytology slides were prepared by rolling the CB onto a clean glass slide.

The cytologic sampling technique "Low volume uterine flushing" was firstly described by Ball et al. [73] in mares, and subsequently modified by Gilbert et al. [47] and [76] for its use in cows. Briefly, an infusion pipette covered with a sanitary sleeve is manipulated through the cervix (as previously described for the CB sampling), and once in the uterine lumen 20 ml sterile physiologic solution is infused. After a gentle massage of the uterus (with the gloved hand in the rectum), liquid is recovered (at least 2 ml), and then transferred to a sterile plastic tube. Once at the laboratory facilities, samples are centrifuged (1000 rpm [179 g] for 7 min), supernatant excluded and the pellet smeared onto a clean microscope glass slide.

Before staining, both CB and LVL smears need to air-dry (or fixed with cyto-fixative). The staining method should be fast, easy to perform, and yield high-quality samples that allow accurate interpretation [77] and [78]. In most of the publications, a modified Wright-Giemsa staining (Diff Quick) is recommended to stain endometrial cytology slides [20] **(Figure 4)**. After staining, endometrial slides need to be microscopically evaluated (100 x and 400 x). Certainly, full evaluation is not only influenced by the sample itself, but also by the counting technique implemented to evaluate the samples [79]. The C-300 and C-100 methods (counting in total respectively 300 or 100 cells) are the methods of choice for the evaluation of the proportion of PMNs in endometrial samples to diagnose CYTO.

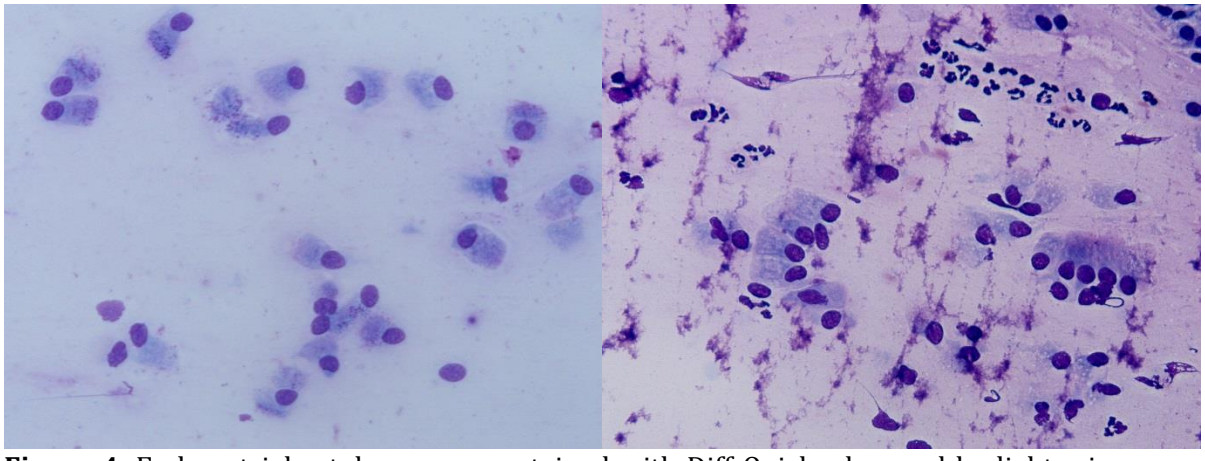

Figure 4. Endometrial cytology smears stained with Diff Quick, observed by light microscope (400 x).

Challenges faced when diagnosing CYTO in dairy cows

In spite of the vast application of CYTO, the high prevalence, and the impact of the disease on fertility (and profitability of the farm), multiple controversies about CYTO still remain. More specifically, the two main points of discussion are:

1) which cytologic technique should preferable be implemented to acquire samples, and

2) when exactly should samples be taken and which cut-off levels should be used to most accurately diagnose CYTO with regard to its negative impact on the subsequent pregnancy risk.

Cytologic technique implemented to acquire samples

Since CB and LVL are the most used cytologic techniques to harvest endometrial samples in cows, both techniques have already been compared regarding feasibility and reliability. However, in the studies where both techniques (CB and LVL) were compared, two critical points were overlooked: 1) both comparative samples were harvested in the same animal, and 2) the use of one single sample (when CB is used) to assess the inflammatory status of the complete endometrium was not evaluated. First, harvesting two samples in the same animal probably compromises the reliability of the second sample by causing endometrial irritation. Secondly, in cows, the description of the distribution of inflammation over the different regions of the endometrium has not been studied yet **(Figure 5)**.

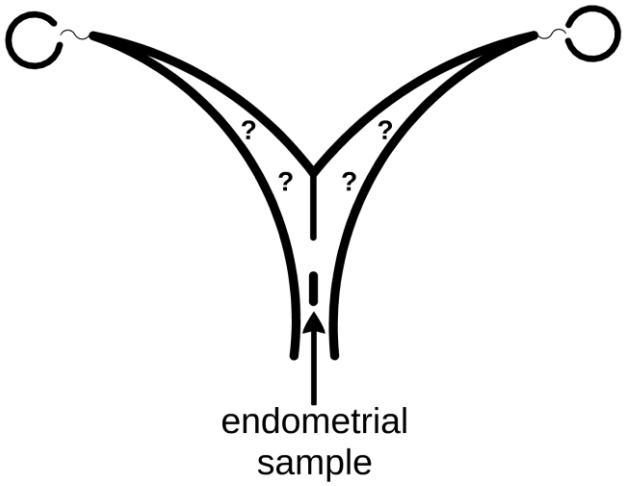

Figure 5. Image showing where endometrial samples are generally taken (CB). However, the representativeness of one single sample to visualize the inflammatory status of the complete endometrium is unknown.

Days in milk at the sampling moment and cutoff values used to define CYTO

Although CYTO is a major cause of subfertility in dairy cows, essential factors that could interfere with its diagnosis (days in milk at sampling and the PMN cut-off point) are completely unstandardized **(Table 2)**. It is clear that the choice of the cytological threshold to diagnose CYTO significantly influences its prevalence, especially since CYTO is closely associated with the involution and recovery of the endometrium after the parturition [80].

Table 2. Summary of studies diagnose CYTO, considering different time points postpartum and thresholds for percentage of PMN (%) (Sens and Heuwieser [81]).

Reference	Days in milk	PMN (%)	Prevalence (%)
Kasimanickam et al. (2004)	20–33	>18	45
	34–47	>10	41
Dubuc et al. (2010a)	35±3	≥6	13.5
	56±3	≥4	9.6
Plöntzke et al. (2010)	18–38	>5	38
	32–52	>5	19
McDougall et al. (2011)	29±2.4	≥9	29
	43±2.3	≥7	23

Cytological endometritis commonly is diagnosed within the voluntary waiting period (VWP), with few exceptions [82], [83] and [84]. The voluntary waiting period is a key management decision in which the herd manager designates a target number of days postpartum after which cows will be inseminated [85]. The interval from calving to first insemination provides time for uterine involution [85]. The VWP varies among farms and ranges between 30 to 90 days postpartum with a mean of 56±0.6 days [86]. The genital tract of the cow should have little evidence of the previous pregnancy by 42 days after calving [3]. However, in most of the CYTO studies, the sampling time overlaps with normal uterine involution [1], [46], [80], [87] and [88]. Moreover, around 40 % of cows [3] have dystocia, twins, RFM, (endo)metritis or/and or a marked negative energy that may extend the time for complete uterine recovery [89] and [90], and could interfere with the results of the cytologic sampling. The timing of the uterine involution processes varies among individual cows [80]. The presence of PMNs in the uterine lumen is a dynamic phenomenon [29], which makes it hard to predict the amount of PMNs that

will be present at the moment the animal is inseminated **(Figure 6)**. The timing of CYTO examination should allow the normal process of involution [20]. Therefore, it is of crucial importance to take cytologic samples at a standardized moment which should allow the use of a universal cut-off point and when normal uterine involution does no longer interfere with the cytologic results: after the VWP. Sampling during insemination may be ideal in this perspective, because it offers the opportunity to determine the uterine health status at the moment the animal is fertilized, allowing to study the effect of CYTO on the subsequent conception rate.

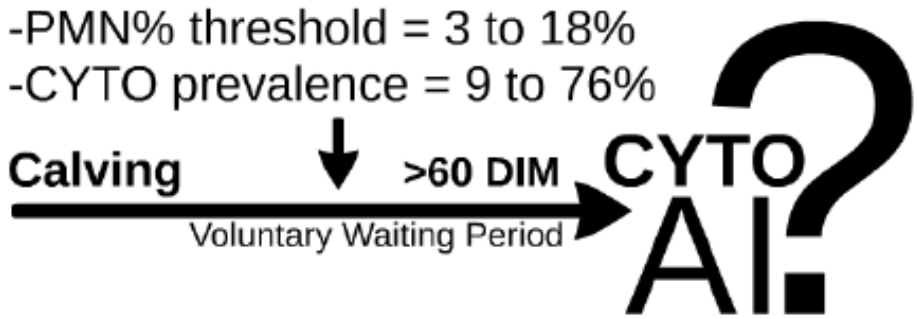

Figure 6. The prevalence of CYTO at the most critical, the peri-fertilization time, remains unknown.

THE LINK BETWEEN CYTO AND BACTERIOLOGY

It is fundamental to differentiate between bacterial contamination and bacterial infection. In the early postpartum period, the uterus is usually contaminated with a broad range of bacteria, not associated with clinical disease. On the other hand, bacterial infection implies adherence, penetration, and colonization of pathogenic organisms to the endometrial mucosa, and depending on the organism, the release of bacterial toxins [2] and [3]. The "most common" uterine pathogens associated with clinical uterine disease in dairy cattle are *Trueperella pyogenes, Escherichia coli, Fusobacterium necrophorum* and *Prevotella melaninogenicus* [41]. Nevertheless, it is important to mention that bacterial infection is not merely linked to the presence of pathogenic bacteria; the development of uterine disease depends as well on the immune response of the cow and on the bacterial load [3].

There are several papers which tried to associate the occurrence of CYTO with the presence of pathogenic bacteria in the uterus. Outcomes of these studies demonstrate that the agreement between pathogenic bacteria and CYTO was low [57], [80] and [83]. On the other hand, Sens and Heiwieser [81] confirmed the positive correlation between *Trueperella pyogenes* and *α-hemolytic streptococci* in the early postpartum (< 21 DIM) with higher odds of PMNs later on (> 21 DIM). Apparently, the association between pathogenic bacteria in the uterus and higher odds of CYTO is indirect (endometrial damage and delayed uterine recovery due to a primo-bacterial infection). In conclusion, an association has been demonstrated between CYTO prevalence and delayed uterine involution (i.e. previous uterine disease) [46], [89] and [89].

RISK FACTORS ASSOCIATED WITH CYTO IN DAIRY COWS

Cytological endometritis is presumably associated with endometrial recovery after CE, trauma or other non-microbial diseases [3]. Prevalence of CYTO widely ranges among farms (9 to 76 %) [47] and [89], suggesting that herd as well as individual risk factors are highly associated with the occurrence of the disease [89]. Risk factors associated with CYTO diagnosed during the VWP are mainly related to other postpartum uterine diseases such as retained placenta and acute metritis [46] and [88]. However, the metabolic status in the prepartum as well as in the early postpartum period was already identified as a risk factor for CYTO [89] and [90]. A thin body condition score as well as elevated hatoglopbin levels and hyperketonemia in the first week postpartum, all were associated with a higher risk to suffer from CYTO in dairy cows [90], but not to other postpartum diseases as CM, CE, and/or PVD. Consequently, some risk factors for CYTO are different than for other postpartum diseases, which supports the hypothesis that CYTO probably is a different manifestation of uterine disease. At least some authors mention CYTO rather to be a consequence of an immune suppression or a metabolic imbalance in the peripartum period [88].

CYTOLOGICAL ENDOMETRITIS IN DAIRY HEIFERS

Up until today, there is no evidence for presence (or absence) of CYTO in nulliparous dairy heifers. To the best of our knowledge, there currently are no peer reviewed papers

available that describe the presence of uterine diseases in nulliparous heifers. In this context, the congenital hymen persistence is a rare disease in nulliparous heifers [91]. In some cases, the complete blockage by the persistent hymen results in accumulation of uterine secretions and formation of mucometra, mucocervix, and/or mucovagina [92]. As a consequence of this mucus accumulation in the anterior vagina, contaminants or bacteria may invade the uterus causing endometrial infection. Lately, the bovine herpesvirus type 4 has gained importance due to its apparent association with reproductive disease in cattle [93]. This virus has an distinct tropism for endometrial cells, and it may have an association with CYTO in dairy cattle, including nulliparous heifers. In general, heifers should become rather easily pregnant after a first or second AI [94], however occasionally, some heifers remain non-pregnant after several AI's. As the genetic merit of some heifers could be considerably high, it might be important to study the inflammatory status of the endometrium in this group of animals and eventually the risk factors associated with non-pregnancy.

CONCLUSION

To resume, SCE is a highly prevalent disease that runs without clinical symptoms, but significantly impairs the fertility of dairy cows. Cytology is the preferred technique to diagnose SCE in both field and research setups, however, standardization of sampling is not fully established yet. Therefore, it is warranted to set up some research in the field in order to assess the clinical value of an endometrial cytology sample, to explore the distribution of inflammation within the uterine endometrium, and to study the CYTO prevalence and effect on fertility during AI. In order to maximize the reproductive efficiency in dairy farms, it is necessary also to study the risk factors associated with CYTO diagnosed at AI. Finally, although uterine pathologies are not highly prevalent in nulliparous dairy heifers, it is a weakly studied topic that merits further research.

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SCIENTIFIC AIMS

The general objective of this doctoral thesis was to research the cytologic diagnosis of subclinical endometritis (SCE) in dairy cattle. This doctoral thesis was split in two main parts in accordance to the specific objectives of each section. In the first section (chapter 4) we aimed to assess the clinical value of an endometrial cytology sample to diagnose SCE in dairy cows. To do so, specific aims of Chapter 4 were:

1. To compare cytology versus histopathology to evaluate subclinical endometritis in dairy cows (Chapter 4.1).

2. To assess the distribution of inflammation and the association between active and chronic alterations within the endometrium of dairy cows (Chapter 4.2).

Our goal in the second part of this doctoral thesis (Chapter 5) was to substantiate an innovative sampling technique to diagnose cytological endometritis (CYTO) during artificial insemination (AI). This in order to study the prevalence of CYTO and its effect on the pregnancy outcome of that insemination in nulliparous dairy heifers as well as in dairy cows. Furthermore, the risk factors associated with CYTO at AI in both types of animals were researched. So, the following aims were specified in Chapter 5:

1. To create a novel cytological sampling technique to diagnose cytological endometritis at artificial insemination in dairy cattle (Chapter 5.1).

2. To evaluate the prevalence and effect of cytological endometritis diagnosed at artificial insemination in dairy cows (Chapter 5.2).

3. To determine the risk factors associated with cytological endometritis diagnosed at artificial insemination in dairy cows (Chapter 5.3).

4. To assess the prevalence of cytological endometritis at the moment of insemination and its effects on the pregnancy outcome in nulliparous dairy heifers (Chapter 5.4).

Chapter 4.1

Comparison between cytology and histopathology to evaluate subclinical endometritis in dairy cows

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ABSTRACT

The aim of the present study was to compare endometrial cytology with histopathology to diagnose subclinical endometritis (SCE) in dairy cows. Endometrial cytology samples were collected from Holstein-Friesian cows (n = 32) just before slaughtering. Half of them were obtained by *in vivo* cytobrush (IV-CB), whereas the other half by *in vivo* low-volume lavage (IV-LVL). After slaughtering, reproductive tracts were collected, and the endometrium was sampled at eight locations. At each location, both an *ex vivo* cytobrush sample (EV-CB) and a tissue sample for histopathologic examination were taken. In the histopathology slides, polymorphonuclear (PMN) cell counts were differentiated as PMN cells in direct contact with the epithelial cells of the endometrium (PMN-EP), and PMN cells present in the deeper *stratum compactum* (PMN-SC). Summation of both countings was referred to as PMN-total. Pearson's correlation and Cohen's kappa coefficient were used to assess the correlation and agreement between both sampling methods (*in vivo* cytology [IV-CB and IV-LVL] with EV-CB and PMN-total). A Poisson mixed effect model was used to analyze the PMN cells' distribution. The prevalence of SCE was 18.75 % (n = 6/32) for *in vivo* cytology. The SCE prevalence based on EV-CB analyses and on the assessment of PMN-total was determined both at the sample (n = 256) as well as at the cow level (n = 32): EV-CB 25 % (n = 64/256) and 35.5 % (n = 12/32), and PMN-total 37.11 % (n = 95/256) and 59.38 % (n = 19/32). Correlation and agreement between IV-CB and EV-CB were r = 0.81 and k = 0.97, whereas between IV-CB and PMN-total r = 0.15 and k = 0.23, respectively. *In vivo* low-volume lavage correlation and agreement were r = 0.52 and k = 0.66 with EV-CB, and r = 0.45 and k = 0.44 with PMN-total. Moreover, correlation and agreement between EV-CB and PMN-total were r = 0.60 and k = 0.50, respectively. More PMN cells ($P < 0.05$) were detected in PMN-SC when compared to PMN-EP and EV-CB. A higher SCE prevalence was found using histopathology, rendering the latter as a more sensitive method to diagnose SCE in comparison to *in vivo* and *ex vivo* cytology. Although cytology had low and/or moderate sensitivity to diagnose SCE when compared with histopathology, its specificity is 100 %, implying that all cows that were indicated to suffer from SCE using *in vivo* cytology were confirmed to do so by histopathologic examination. There is an uneven distribution of PMN cells throughout the endometrium,

generally more PMN cells being found in the deeper *stratum compactum* than in contact with the superficial layers of the endometrium.

INTRODUCTION

Uterine inflammatory processes in dairy cows may persist for enduring time periods, triggering a detrimental effect on further reproductive capacity [1], [2] and [3]. Histologically, endometritis is defined as the presence of inflammatory cells in the uterine endometrium, with disruption (or not) of the epithelial layer [4]. When endometritis occurs without the presence of clinical signs, it is designated as "Subclinical Endometritis" (SCE). Subclinical endometritis refers to cows showing no clinical signs of endometritis but having an increased percentage of polymorphonuclear (PMN) cells in endometrial cytology being associated with reduced reproductive performance [5]. As SCE cannot be detected by simple visual inspection, complementary examinations are necessary for its diagnosis.

Cytology is the preferred technique to diagnose SCE in both field and research setups, mainly for reasons of simplicity and low cost [6] and [7]. Measuring the proportion of PMN cells in endometrial cytology is the hallmark of SCE diagnosis, which is therefore often also referred to as "cytological endometritis" [1], [5] and [8]. The controversy, however, lies in which cytologic technique is most reliable, cytobrush (CB) or low-volume lavage (LVL) [9]. Advantages and disadvantages have been described for both techniques [10]. Sampling with CB is easier, yields an *in situ* sample with less distorted cells [8], and provides results faster in comparison to LVL [8]. However, CB evaluates only a very small portion of the endometrium [7], [11] and [12], whereas LVL is considered to provide a more representative sample of the entire uterus [13], [14] and [15], yielding a higher chance to harvest PMN cells from a larger endometrial surface [16] and [17].

Although no diagnostic test can be considered 100 % accurate [18] and [19], histopathology is considered the gold standard to diagnose endometrial alterations, mainly because it allows to directly visualize both acute and chronic alterations in the epithelium and *stratum compactum* of the endometrium [14] and [20]. Unfortunately, at least in cows, biopsy sampling for histopathology is technically complicated and may be detrimental to subsequent fertility [19], [21], [22], [23] and [24]. Because biopsy sampling itself may affect fertility [21], [23] and [24], it is hard to objectively interpret the reproductive performance of sampled animals and identify which alterations should

be considered as critically interfering with fertility. To the best of our knowledge, there currently are no peer reviewed articles available that demonstrate a significant association between the results of a histopathologic examination of the bovine uterus and the reproductive capacity of the sampled animal. Consequently, endometrial cytology has become more common in the last 10 years [25], among other reasons, because of its possibility to predict the cows' further reproductive capacity even in cows that have calved for longer periods [3].

Thus, the main objective of the present study was to assess the accuracy and efficacy of endometrial *in vivo* cytology to diagnose SCE using *ex vivo* cytology and histopathology as the gold standard, and to find out which *in vivo* sampling technique (CB vs. LVL) renders more reliable results. Moreover, we aimed to assess the representativeness of *ex vivo* endometrial cytology (CB sampling) versus histopathology by comparing multiple samples taken in close proximity of each other evenly spread over the whole uterus.

MATERIALS AND METHODS

All experiments described in the present article were carried out with permission of the Ethical Committee of the Faculty of Veterinary Medicine of the Ghent University (EC 2013/174).

Animals and procedures

For the present study, 35 Holstein-Friesian cows from one single dairy farm were initially enrolled. The day before cows were planned to be slaughtered, the first author was informed about this decision by the herd manager. Main reasons for culling were reproductive failure and high somatic cell count. Additional reasons were lameness and aging. Just before slaughter, a complete reproductive examination was performed by an experienced veterinarian. This included vaginal examination by the gloved hand method [26] and transrectal reproductive ultrasonography (Tringa, Esaote-Pie Medical, Maastricht, The Netherlands). Cows presenting any type of purulent vaginal discharge were excluded from the study. Uterus and ovaries were evaluated by ultrasound for

presence (> 0.5 cm) of fluid in the uterine lumen [1] or presence of follicular or luteal cysts (fluid-filled anechoic structure > 2.5 cm in diameter) on either of the ovaries [27]. Presence of fluid (> 0.5 cm) in the uterine lumen, an ovarian cyst or severe lameness, were reasons to discard cows from the study.

In vivo sampling

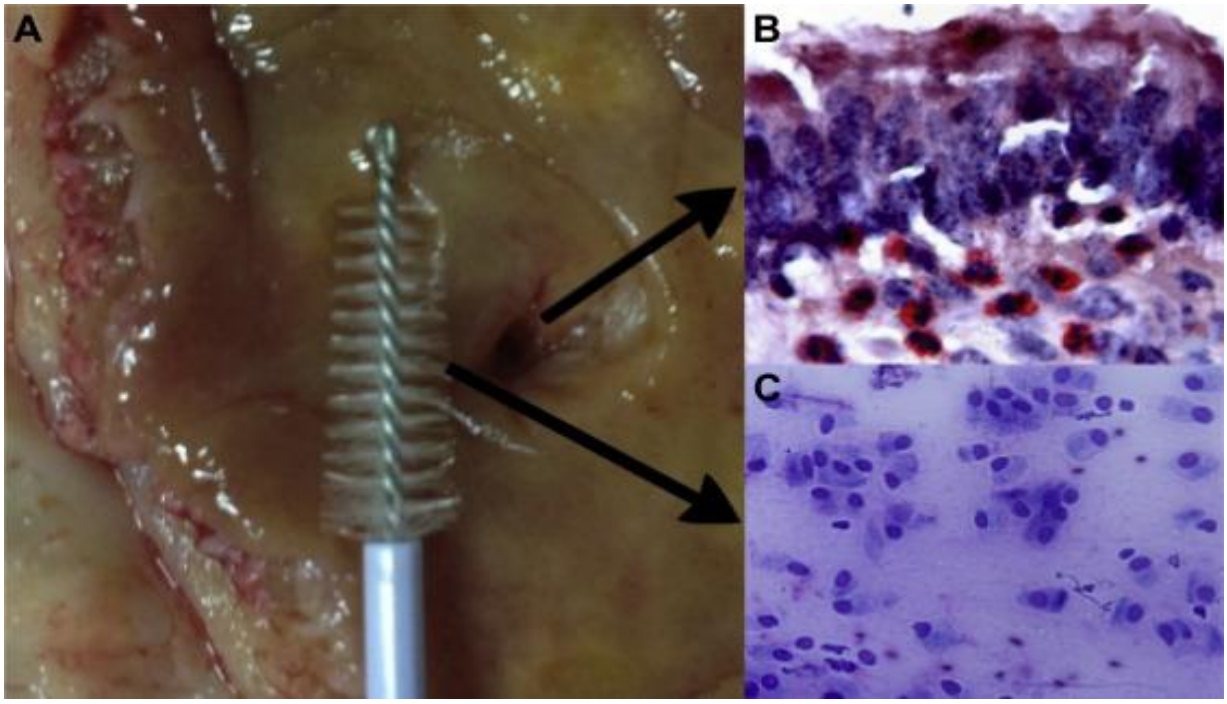
Endometrial cytology samples were taken based on the ear tag of the cow. *In vivo* CB (IV-CB) was performed in even-, whereas *in vivo* LVL (IV-LVL) in odd-numbered cows. Before sampling, the perineum of the cows was cleaned with fresh water and dried with a paper towel. For the IV-CB technique, a Cytobrush Plus GT (Cooper Surgical, Berlin, Germany) was adapted to a stainless steel stylette of an universal insemination gun (Agtech, Manhattan, KS, USA), by heating the tip of the stylette with a lighter and fitting it to the base of the handle of the CB. After this, the CB was introduced in a sterile 22″ long equine infusion pipette (Agtech). Then, to protect the infusion pipette from contamination by vaginal and cervical cells, it was covered with a 21″ long sanitary sheath (IMV, L'Aigle, France). Next, under rectal guidance of a gloved hand, the pipette was introduced into the vagina and manipulated through the cervix. Once in the entrance of the uterine lumen, the sanitary sheath was punctured with the tip of the pipette so that the CB was released. Then, it was rolled twice with some gentle pressure of the index finger through the rectum, sampling in this way the dorsal part of the uterine body. In the end, the CB was reintroduced in the pipette, and the device was carefully removed from the genital tract. Immediately after the IV-CB sampling, the bristles of the brush were gently rolled on a microscope slide (Marienfeld, Lauda-Königshofen, Germany), which was then air-dried and subsequently housed in a slide box.

For the IV-LVL, a sterile 22″-long equine infusion pipette (Agtech) was introduced into the reproductive tract of the cow as described for the IV-CB sampling. Once in the uterine lumen, a 60-ml syringe (Terumo, Binan, Laguna, Philippines) filled with 50 ml of sterile 0.9% saline solution (Eurovet, Heusden-Zolder, Belgium) was adapted to the infusion pipette. After infusion of the physiologic solution, an extra 10 ml of air was perfused to flush the residual liquid through the pipette. Next, the uterus was gently massaged for 10 seconds to equally distribute the liquid within the uterus. Finally, the

uterus was manipulated by the gloved hand in the rectum to recover the infused liquid by gravity in a 15-ml falcon tube. At least 5 mL of the infused fluid had to be recovered to consider the sampling as successful. After the IV-LVL collection, samples were kept in a refrigerated container and processed within 3 hours after sampling. Once at the laboratory facilities, IV-LVL samples were centrifuged at 700 × g for 5 minutes [9]. After discarding the supernatant, a drop of the pellet was placed on a microscope slide and gently smeared on another slide, spreading in this way the cellular content of the drop.

Ex vivo sampling

Within 2 to 3 hours after the *in vivo* sampling, cows were slaughtered in an European Union–accredited abattoir. Subsequently, their reproductive tracts were removed, identified with numbered tags, and placed in a refrigerated container. Reproductive tracts were transported to the laboratory facilities, and tissue samples were taken within 1 to 3 hours after slaughter. First, surgical scissors were used to make a sagittal section from the dorsal part of the external cervical os until the cranial end of the uterine body. Uterine horns were incised from the beginning of the bifurcation until the end of their tips, along the major curvature. Once the uterus was opened, samples were taken in eight predefined locations evenly spread over the entire uterus. For all uteri, the same sampling order was used: (1) tip of the left horn; (2) median part of the left horn; (3) bifurcation of the left horn; (4) left side of the uterine body; (5) right side of the uterine body; (6) bifurcation of the right horn; (7) median part of the right horn; and (8) tip of the right horn. Endometrial biopsies and cytology samples were taken with an 8.0-mm biopsy punch (Mediware, Wesel, Germany) and Cytobrush Plus GT (Cooper Surgical), respectively, **(Figure 1)**. Punch biopsies were rotated with gentle pressure in each location of the exposed endometrium to obtain an intact sample. Each endometrial biopsy sample was placed in a tube containing 4 % formaldehyde with proper identification of the cow and the location of sampling. *Ex vivo* cytobrush (EV-CB) samples were obtained by rotating a new CB just next to the site where the histopathology sample was taken. Then, collected cellular material was spread onto a clean microscope slide (Marienfeld, Lauda-Königshofen, Germany) accordingly identified.



(Figure 1). Image showing how the endometrial biopsies and cytology samples were taken next to each other with an 8.0-mm punch biopsy and a cytobrush, respectively. (B) Endometrial histopathology sample stained with Naphthol-AS-D-chloroacetate-esterase. (C) Endometrial cytology sample stained with Wright-Giemsa.

Staining and evaluation

Cytology slides (IV-CB, IV-LVL, and EV-CB) were stained with Wright-Giemsa **(Figure 1)** (Diff Quick, Fisher Diagnostics, Newark, DE, USA) and conventionally mounted with Eukitt (O.Kindler GmbH, Freiburg, Germany). The cytologic evaluation was made by light microscopy (Kyowa Optical, Tokyo, Japan) at magnification x 400. A total of 100 nucleated cells were counted in 10 random high-power fields (HPF; 10 cells per HPF), and PMN cells' ratio averaged (HPF-100 method) [28]. The threshold level for cytologic SCE was set at greater than or equal to three PMN cells/HPF [3].

Biopsies were routinely processed: embedded in paraffin wax, sectioned at 4-µm thickness, and stained with Naphthol-AS-D-chloroacetate-esterase, a histochemical technique considered as the gold standard to stain PMN cells **(Figure 1)** [29] and [30]. Biopsies were examined by a single observer using a conventional light microscope (Kyowa Optical, Tokyo, Japan) at magnification x 400. Five HPF were randomly selected, and PMN cells were counted and averaged. Polymorphonuclear cell counts were evaluated in two ways: PMN cells in direct contact (underneath or between) with the epithelial cells of the endometrium (PMN-EP; **Figure 2**), and PMN cells present in the

stratum compactum (PMN-SC), without direct contact with the epithelial layer **(Figure 2)**. Summation of both countings was referred to as the PMN-total. Because no SCE cutoff point for PMN counts in bovine histopathology is available in literature, we used a similar threshold level as applied for cytology (≥ 3 PMN cells in the average of five HPF). The same cutoff point was set either in PMN-EP, PMN-SC, and PMN-total.

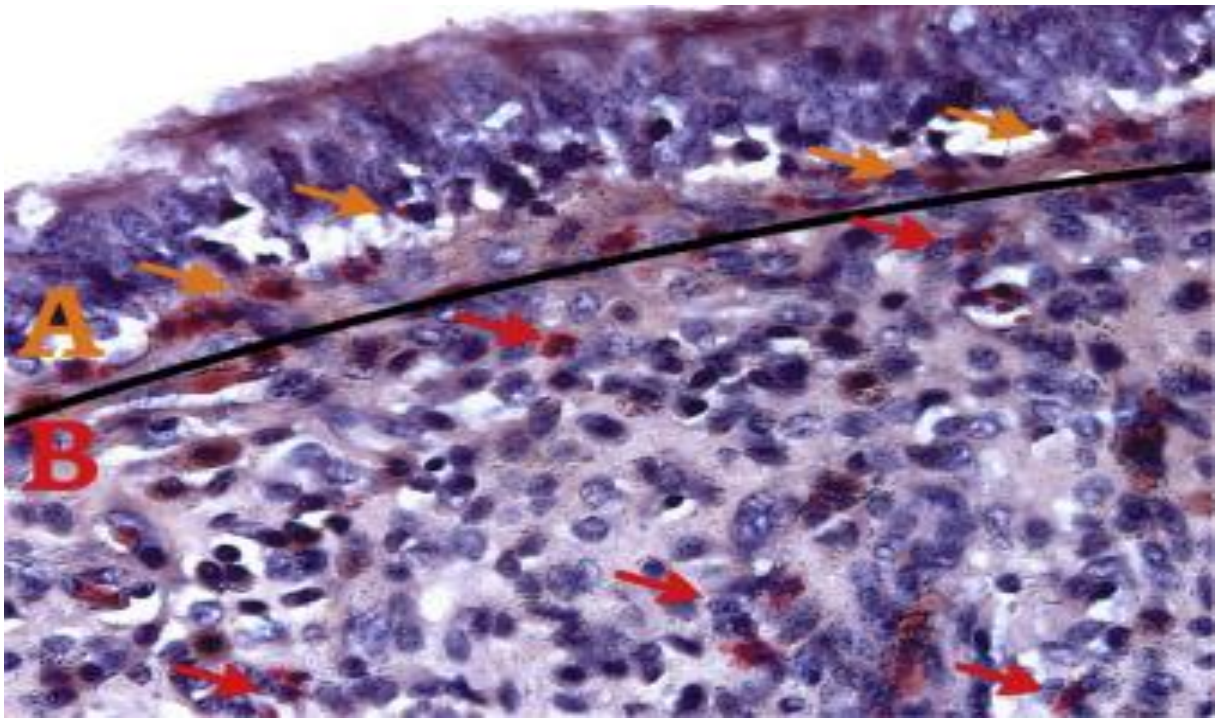


Figure 2. Image showing how histopathology samples were evaluated. A refers to the area of the figure in yellow (polymorphonuclear [PMN] cells present in direct contact with the epithelial cells of the endometrium (PMN-EP)). B refers to the area of the figure in red (PMNs present in the *stratum compactum* of the endometrium [PMN-SC] (PMN-EP + PMN-SC = PMN-total). The black line separates both areas.

Statistical analysis

Data of individual cows were exported to an excel spreadsheet file (Microsoft Corporation, Seattle, USA), both from the farm managing software and from the manually collected data. Statistical analyses were performed using R version 3.2.1, 2015 (R Inc., Boston, USA). Descriptive analyses were done using the function summary of the R coding system (package Base) to discover the prevalence of SCE both at the cow (n = 32) as well as at the individual sample (n = 256) level.

To compare PMN counts between different sampling methods (IV-CB and IV-LVL, EV-CB, and PMN-total), Pearson correlation coefficients were calculated for continuous variables using the function cor of the Hmisc package [31]. Cohen's kappa coefficient,

sensitivity (Se), and specificity (Sp) were calculated to assess the agreement among SCE positive and negative samples between the different sampling methods, using the function confusionMatrix of the package caret [32]. Correlation and agreement were considered as follows: 1 reflects total positive correlation, 0 no correlation, and −1 total negative correlation; less than 0.2 slight agreement, 0.2 to 0.4 fair agreement, 0.4 to 0.6 moderate agreement, 0.6 to 0.8 substantial agreement, and greater than 0.8 almost perfect agreement [33].

To compare the PMN counts between PMN-EP, PMN-SC, and EV-CB, a Poisson mixed effect model was used because the distribution of the data followed a log-normal pattern [34]. The model was built using the lem4 package [35], including a random intercept per cow. The Tukey test was used to compare differences between the estimates (cld function). The level of significance was set at $P < 0.05$ for all analyses. Results are expressed as back transformed least square means with standard error.

RESULTS

In the final evaluation, 32 cows (parity 2–6) were included, of which half were sampled by IV-CB and the other half by IV-LVL. Average days in milk at slaughtering were 315±173. The mean body condition score was 3.59±0.74 (1–5) [36].

The postmortem macroscopic examination of the reproductive tracts revealed that each cow presented at least one follicle greater than or equal to 0.8 cm and a CL greater than or equal to 2.0 cm on either of its ovaries. Because all cows had a CL in at least one of their ovaries at the moment of sampling, they all were considered to be in the luteal phase. We aimed to achieve a study with 80% power and 95% confidence interval. The sample size was calculated to assess the agreement between two methods expecting a SCE prevalence of 20%, an expected lower point of agreement between methods $k = 0.4$, and a clinically acceptable agreement $k = 0.7$. Therefore, 512 acceptable *ex vivo* sample slides were obtained: 256 EV-CB and 256 histology samples.

Prevalence of SCE in EV-CB and histology samples was evaluated in two ways: (1) at individual samples at each predefined location (n = 256), and (2) by considering the

whole uterus as affected by SCE if greater than or equal to 1 location (individual sample) was positively evaluated **(Table 1)**. For further evaluation of the IV-CB and IV-LVL, cows were divided in two equal groups according to the method of *in vivo* sampling (IV-CB vs. IV-LVL), to assess SCE prevalence in each of the groups **(Table 2)**.

Table 1. Polymorphonuclear counts and SCE prevalence: *in vivo* cytology, *ex vivo* cytobrush (*EV*-CB) and histopathology (PMN-total).

Variable	Sampling method		
	In vivo cytology	*EV*-CB	PMN-total
PMNs*	1.50±2.72	1.81±2.53	4.44±6.61
SCE prevalence**	18.75%	25%	37.11%
SCE prevalence*** (≥ 1 location)	18.75%	37.5%	59.38%

*PMNs mean and standard deviation.
**For individual locations (n=256 in *EV*-CB and PMN-total).
***The whole uterus considered SCE-positive if ≥1 location was considered positive (n=32).

Table 2. Polymorphonuclear counts and SCE prevalence: *in vivo* cytobrush (*IV*-CB), *in vivo* low volume lavage (*IV*-LVL), *ex vivo* cytobrush (*EV*-CB) and histopathology (PMN-total).

Variable	Sampling method					
	IV-CB	*EV*-CB_{CB}	PMN $total_{CB}$	*IV*-LVL	*EV*-CB_{LVL}	PMN $Total_{lVL}$
PMNs*	0.88±1.2	0.91±1.8	2.43±4.50	2.13±3.5	2.72±7.6	6.45±3.5
SCE prevalence**	12.5%	13.28%	26.56%	25%	36.71%	47.65%
SCE prevalence*** (≥ 1 location)	12.5%	18.75%	52.25%	25%	56.25%	62.5%

*PMNs mean and standard deviation.
**For individual locations (n=128 in *EV*-CB and PMN-total).
***The whole uterus considered SCE-positive if ≥1 location was considered positive (n=16).

Results of both *in vivo* sampling techniques were compared to each of the EV-CB and PMN-total samples at the eight predefined locations. Further comparison of IV-CB and IV-LVL techniques with EV-CB and PMN-total was also made by considering the whole uterus as being affected by SCE if at least one (≥ 1 location) of the eight EV-CB or PMN-total samples was positive. Correlation, agreement, sensitivity, and specificity between *in vivo* cytology techniques with EV-CB and PMN-total (as the golden standard) are shown in **Table 3**. Furthermore, Pearson correlation coefficient and kappa value agreement between EV-CB and PMN-total were calculated using PMN-total as the gold standard. Further comparison was also made between PMN-EP and PMN-SC versus EV-CB. Detailed information about these comparisons is depicted in **Table 4**.

Table 3. Pearson correlation coefficients *r*, kappa values for agreement (*k)*, and sensitivity (Se) and specificity (Sp) of *in vivo* cytobrush and low volume lavage (*IV*-CB and *IV*-LVL) with *ex vivo* cytobrush (EV-CB) and histopathology (PMN-total) as references.

Technique	Individual sample level (n= 128)				Cow level (n= 16)*		
	Correlation	Agreement	Se	Sp	Agreement	Se	Sp
IV-CB/ *EV*-CB	0.8	0.9	94.1	100	0.7	Se=66.6	Sp=100
IV-CB/ PMN-total	0.1	0.2	56.2	77.6	0.2	Se=22.2	Sp=100
IV-LVL/ *EV*-CB	0.5	0.6	93.7	82.2	0.4	Se=44.4	Sp=100
IV-LVL/ PMN-total	0.4	0.4	47.5	95.5	0.3	Se=40	Sp=100

*The whole uterus considered SCE-positive if ≥1 location was considered positive.

Table 4. Pearson correlation coefficients (*r)*, kappa values for agreement (*k)*, and sensitivity (Se) and specificity (Sp) of *EV*-CB (*ex vivo* cytobrush) with histopathology [total numbers of the PMNs counted (PMN-total)], PMN-EP (PMN epithelium), and PMN-*SC* (PMN *stratum compactum*) as references.

Method	*Ex Vivo* Cytology (*EV*-CB)			
	Correlation	Agreement	Sensitivity	Specificity
PMN-total	*r*=0.6	*k*=0.5	Se=81.2	Sp=77.6
PMN-EP	*r*=0.6	*k*=0.6	Se=69	Sp=91.8
PMN-*SC*	*r*=0.5	*k*=0.5	Se=66.6	Sp=87.7

*Total of samples in each method n=256.

Significantly (P < 0.05), higher PMN counts were found in the deep *stratum compactum* (PMN-SC), in comparison to the superficial endometrium (PMN-EP), and the *ex vivo* cytology (EV-CB) samples. However, no differences were found in the number of PMN cells between EV-CB and PMN-EP (P > 0.05; **Figure 3**).

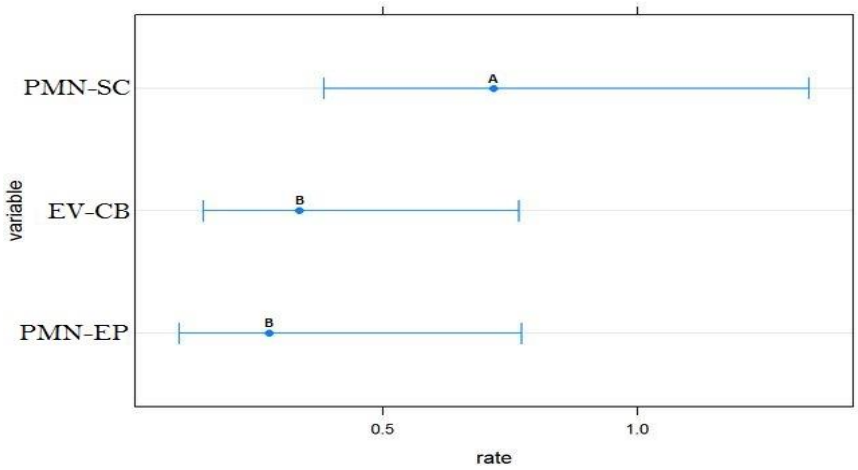


Figure 3. Line graph illustrating the PMN means and standard deviations between PMN *stratum compactum* (PMN-SC), *ex vivo* Cytobrush (*EV*-CB), and PMN Epithelium (PMN-EP). Significant differences (*P* < 0.05) were found between PMN-SC with *EV*-CB and PMN-EP.

DISCUSSION

In the present study, we compared two currently popular techniques (CB and LVL) to diagnose SCE in dairy cows with histopathological examination of multiple samples evenly spread over the entire endometrium. All harvested samples were carefully controlled and designated to be of high quality and were acceptable for further analysis. Mean neutrophil counts based on histopathological examination (PMN-total) were higher than those in both the *ex vivo* and *in vivo* cytology samples (**Tables 1** and **2**). All the cows diagnosed SCE-positive by *in vivo* cytology (IV-CB or IV-LVL), were also diagnosed positive with EV-CB and histopathology (PMN-total). Almost all (11/12; 91.67 %) cows diagnosed positive by EV-CB were also positively evaluated by histopathology. Prevalence of SCE diagnosed via PMN-total was 59.38 % (19/32), which was higher than via EV-CB 37.5 % (12/32) and *in vivo* cytology 18.75 % (6/32). *In vivo* cytology detected only 50 % and 31.58 % of the cows that were positively diagnosed via EV-CB and PMN-total, respectively. Moreover, IV-CB detected 66.66% of cows, which were positive by EV-CBCB, but only 22.22 % of those which were positive via HP-total^{CB}. On the other hand, IV-LVL detected 44.44 % of the cows, which were diagnosed positive via EV-CBLVL, and 40% of those diagnosed positive via PMN-total^{LVL}. The fact that more positive samples were found by EV-CB and histopathology may be due to: (1) when multiple samples are assessed within the same uterus, the chance is higher to collect more PMN cells and hence to have more positive samples, and (2) *in vivo* samples are "blind" samples, meaning that the exact location of the CB, including the pressure applied when rotating the brush, or the proper distribution of the physiologic solution (applying LVL) throughout the uterus is uncertain. This "blind effect" is absent for both *ex vivo* techniques because the exact sampling location could carefully be assessed. Nevertheless, a higher SCE prevalence was found using histopathology in comparison to EV-CB. Obviously, biopsies are in situ specimens in which all cellular components are clearly visualized throughout the whole thickness of the *stratum compactum*, whereas cytology only reflects the superficial layer of the tissue. These results suggest that standard cytology methods generally underestimate inflammatory reactions of the endometrium in dairy cows.

Because there are many speculations about the preferred sampling technique (low-volume lavage [1] vs. cytobrush [5]) for *in vivo* cytology, both techniques have already

been compared in terms of feasibility and reliability in both mares [10], [17] and [37] and cows [8] and [38]. In these studies, comparative samples were harvested in the same animal. Because the act of taking a sample may affect the result of the next sampling by causing endometrial irritation [8] and [10], we considered it indispensable to use only one *in vivo* sampling method per cow. In the present study, both techniques provided samples of similar cytological quality and cellularity; however, CB was experienced as a quicker and relatively easier technique to perform. When PMN counts were compared between *in vivo* and *ex vivo* cytology, EV-CB results were highly correlated with IV-CB results, but only moderately correlated with results of IV-LVL. The kappa value assessed the agreement between IV-CB and EV-CB as very high, whereas it was only moderate between IV-LVL and EV-CB. When a single positive sample at greater than or equal to 1 location was used to consider the cow to suffer from SCE, EV-CB also highly agreed with IV-CB whereas only fairly with IV-LVL. However, when IV-CB and IV-LVL were compared with PMN-total as the gold standard, results changed for the IV-LVL technique. The correlation between PMN counts in histopathology and IV-CB was weak but remained moderate with IV-LVL. Agreement between histopathology and IV-CB was also weak, but still moderate with IV-LVL, both at the sample and the cow level. This switch of agreement and correlation of the *in vivo* cytology sampling technique when compared first to EV-CB and then with PMN-total may have a logical explanation. In comparison with the CB technique, LVL has more chances to collect higher amounts of granulocytes because a greater surface of the endometrium is sampled, allowing more PMN cells lying loose in the endometrial lumen to be collected. Cytobrush samples (IV-CB and EV-CB) only harvest a small portion of the endometrium, and loose PMN cells are therefore only locally collected.

Generally, more PMN cells were visualized in the histopathology samples in comparison to the *in vivo* samples. However, granulocytes harvested after *in vivo* sampling do not originate from the same area as those identified in the histopathology samples. Low-volume lavage PMN cells are mainly harvested from the uterine lumen and the superficial endometrium, whereas PMN cells in the histopathology samples were mainly localized in the *stratum compactum*. Consequently, the higher correlation and agreement between IV-LVL and PMN-total in comparison to between IV-CB and

PMN-total, is probably because of the higher capacity of the LVL technique to concentrate a higher amount of PMN cells.

The sensitivity of both *in vivo* cytology techniques was only moderate (sample level) in comparison to histopathology, meaning that IV-CB and IV-LVL only identify approximately half of the samples evaluated positive by histopathology. The Sp of both *in vivo* techniques was however 100 %, implying that all of the samples evaluated positive by IV-CB and IV-LVL were confirmed by histopathology. At the cow level, the Se of IV-CB was low whereas the Se of IV-LVL was moderate, confirming the higher correlation and agreement between IV-LVL and histopathology. Sensitivity and Sp of both *in vivo* techniques are relatively high when compared to EV-CB (sample level). However, at the cow level, the Se is higher for IV-CB than for IV-LVL using EV-CB as a reference. To conclude, both *in vivo* sampling techniques (CB and LVL) are relatively easy to perform but have a low sensitivity to identify inflammatory reactions of the endometrium.

Neutrophils are considered as the first line of defense against endometrial infection and are rapidly recruited from the peripheral blood circulation to the site of injury [22], [39], [40], [41] and [42]. In case of endometritis, neutrophilic dominance area includes the endometrial epithelium (and lumen), and the *stratum compactum* [4]. However, endometrial sampling by cytology limits harvesting of epithelial cells segregated in the uterine lumen, the epithelial layer of the endometrium, and the cellular components of the superficial portion of the *stratum compactum*; and eventually PMN cells present in all these locations. Uterine histopathology, on the other hand, allows examination of the endometrial epithelium and the complete *stratum compactum*. Interestingly, we found an uneven distribution of PMN cells within the uterine endometrium, with significantly more neutrophils present in the deep *stratum compactum* (PMN-SC) than adjacent to the uterine epithelium (PMN-EP). As could be expected, PMN counts in PMN-EP and EV-CB were similar, confirming cytology to render a superficial sampling of the uterine endometrium. This finding is also demonstrated by a high correlation and agreement between EV-CB and PMN-EP, whereas correlation and agreement between EV-CB and PMN-SC were only moderate. Hence, cytology can be considered as an accurate method to evaluate the presence of PMN cells in the superficial endometrium, but when PMN

cells are located in deeper layers, cytology significantly loses sensitivity to diagnose inflammatory reactions of the uterine wall.

CONCLUSION

In comparison to histopathology, cytology has a lower sensitivity to diagnose inflammatory reactions of the endometrium in dairy cows. Although both *in vivo* sampling techniques, CB, and LVL are used to diagnose SCE, their diagnosis is based on essentially different approaches. The CB collects cells from a very limited area of the superficial endometrium, whereas the LVL technique collects cells from a larger surface of the superficial endometrium, including cells that are segregated in the uterine lumen. Polymorphonuclear cells have an uneven distribution throughout the endometrium; more PMN cells were found in the deep *stratum compactum* than adjacent to the endometrial epithelium. Although the results of *in vivo* cytology have earlier been shown to be associated with the reproductive capacity of the sampled cows, more research is needed to evaluate whether inflammatory cells present in the deeper endometrial layers significantly interfere with the fertility outcome.

Interesting questions in this context that warrant further research are: (1) are the PMN cells segregated in the uterine lumen still physiologically active and therefore able to interfere with further fertility; (2) why do some cows keep on suffering from high amounts of intrauterine PMN cells even long after the postpartum period; and (3) what is the trigger for the continuous attraction of PMN cells toward the endometrium in at least some cows that have calved for very long periods.

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Chapter 4.2

Distribution of inflammation and association between active and chronic alterations within the endometrium of dairy cows

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ABSTRACT

Objectives of the present study were twofold: 1) to assess the association between polymorphonuclear (PMN) counts and chronic alterations within the bovine endometrium, and 2) to determine the distribution of inflammation throughout the endometrium of clinically healthy dairy cows. Holstein-Friesian cows (n= 32) from a single dairy farm were selected for this experiment. Before slaughtering, a complete reproductive examination was performed to discard any type of clinical disease. After slaughtering, reproductive tracts were collected, and the endometrium was sampled at 8 pre-defined locations. At each location, endometrial biopsies (EB) and cytology (CY) samples were harvested. Histopathology samples were stained with haematoxylin-eosin (EB-HE) and Naphtol-AS-D-chloracetate-esterase (EB-Naphtol) while CY samples were stained with Wright-Giemsa. In the EB-HE samples, parameters assessed were: epithelium height, mononuclear cells infiltration, lymphocytic aggregates, periglandular fibrosis, angiosclerosis, and hemorrhage. In EB-Naphthol and CY slides, PMNs counts were evaluated. Binomial logistic regression was used to assess the association between the number of PMNs present in both the EB-Naphthol and CY samples and alterations identified in the EB-HE samples; and to analyze the distribution of the histopathologic alterations (EB-HE). A Poisson mixed effect model was used to analyze the distribution of PMNs within the endometrium. A significant positive association was found between the PMN counts and the mononuclear cells infiltration. The presence of erythrocytes was associated with higher odds to detect PMNs in the *stratum compactum*. Significantly higher infiltration of PMNs and mononuclear cells were detected in the uterine body and the right horn region. Concluding, CY is a technique that allows the evaluation of PMN counts and therefore only evaluates active inflammation. A complete assessment of endometrial health can only be obtained using EB. To optimize the sensitivity to diagnose endometrial inflammation in cows, adjacencies of the corpus uteri should be considered as the preferred region to harvest samples.

INTRODUCTION

Subclinical endometritis (SCE) is a major cause of subfertility in cows causing important economic losses to dairy farmers [1]. This disease refers to cows showing no clinical signs of endometritis but having an increased percentage of polymorphonuclear cells (PMNs) in cytology (CY) that is associated with reduced reproductive performance [2]. As SCE cannot be detected by simple visual inspection, complementary examinations need to be implemented for its diagnosis [3]. Endometrial CY obtained by means of cytobrush (CB) [2] or low volume lavage (LVL) [4] are the most commonly used techniques to diagnose SCE in dairy cows [5] and [6]. The proportion of PMNs detected is the only parameter taken into account when SCE is evaluated by means of CY. Endometrial biopsy (EB), however, is known to allow the evaluation of a wider range of histopathologic alterations to assess uterine health [7], albeit there are to the best of our knowledge currently no peer reviewed papers available that were able to demonstrate that the presence of these alterations is significantly associated with an impairment of the reproductive capacity.

Histological examination of the endometrium allows to reveal the presence of a variety of cellular components such as lymphocytes, plasma cells, mast cells, macrophages, and leucocytes [8] and [9]; or chronic changes like periglandular fibrosis, angiosclerosis, endometrial gland atrophy or lymphoid aggregates [10] and [11]. After recovering from active endometritis (PMNs infiltration), chronic alterations may persist [10], while being unnoticeable when only applying cytological analyzes. In cows, EB is rarely used to diagnose SCE or other uterine alterations because it is considered to be time-consuming, expensive and potentially detrimental to future fertility [10], [12], [13], [14] and [15].

When endometrial samples are taken (either by CY or EB), they should be representative for the health status of the complete uterus [16]. However, sampling by CB or EB represents a section of only 1-2cm^2 of the entire endometrium. Generally, these endometrial samples are harvested in the adjacencies of the corpus uteri [17]. In mares, the representativeness of a single EB sample is still a matter of serious debate [16], [18], [19], [20], [21] and [22], while in cows, the representativeness of samples taken in the vicinity of the corpus uteri has not been studied yet. Therefore, aims of the

present study were twofold: 1) to assess the association between PMN counts and chronic alterations at 8 pre-defined locations of the endometrium; and 2) to determine the distribution of chronic and acute inflammation throughout the endometrium by sampling at 8 locations evenly spread over the entire uterus.

MATERIALS AND METHODS

This experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine of the Ghent University (EC 2013/174). The present study was performed jointly with a trial in which we aimed to assess the accuracy and efficacy of endometrial cytology to diagnose SCE in dairy cows. A total of 32 healthy Holstein-Friesian cows which were designated by the farmer to be culled, were clinically examined before slaughtering. A thorough clinical examination was performed in order to exclude cows suffering from purulent vaginal discharge [23], uterine content (> 0.5 cm) [2], follicular or luteal cysts (> 2.5 cm in diameter) [24], or any other disease which could interfere with the diagnosis of SCE. All the 32 selected cows were from one single dairy farm located in the Flemish region of Belgium. Cows were free-stall housed, milked twice daily and fed with a total mixed ration supplemented with protein and minerals according to their requirements. The body condition score (BCS; 1-5) [25] was assessed at the time of examination. Individual cow's data such as days in milk (DIM) at slaughtering and parity were as well recorded in a work file at the moment of sampling.

Sample collection

Cows were slaughtered in an EU-accredited abattoir. Then, reproductive tracts were collected, identified, and placed in a refrigerated container for transportation (4 to 8 °C; no more than 3 hours of transportation). Estrous cycle stage of each cow was evaluated by visual inspection of the ovaries [26]. At the laboratory facilities, samples were taken at 8 pre-defined locations evenly spread over the opened uterus. Locations were as follows: (1) tip of the left horn, (2) median part of the left horn, (3) basis of the left horn, (4) left side of the uterine body, (5) right side of the uterine body, (6) basis of the right horn, (7) median part of the right horn, and (8) tip of the right horn **(Figure 1)**. Locations 1, 2 and 3 corresponded to the left horn region; 4 and 5 to the corpus uteri

region; and locations 6, 7 and 8 to the right horn region. At each location, EB and CY (CB) samples were taken with an 8.0 mm biopsy punch (Mediware, Wesel, Germany) and a Cytobrush Plus GT® (Cooper Surgical, Berlin, Germany), respectively. Both samples (EB and CB) were taken next to each other. After sampling, EB samples were placed in 4 % formaldehyde. Cellular material collected by the CB was gently rolled on a clean microscope slide (Marienfeld, Lauda-Königshofen, Germany), accordingly identified, air-dried, and placed in a microscope slide box.

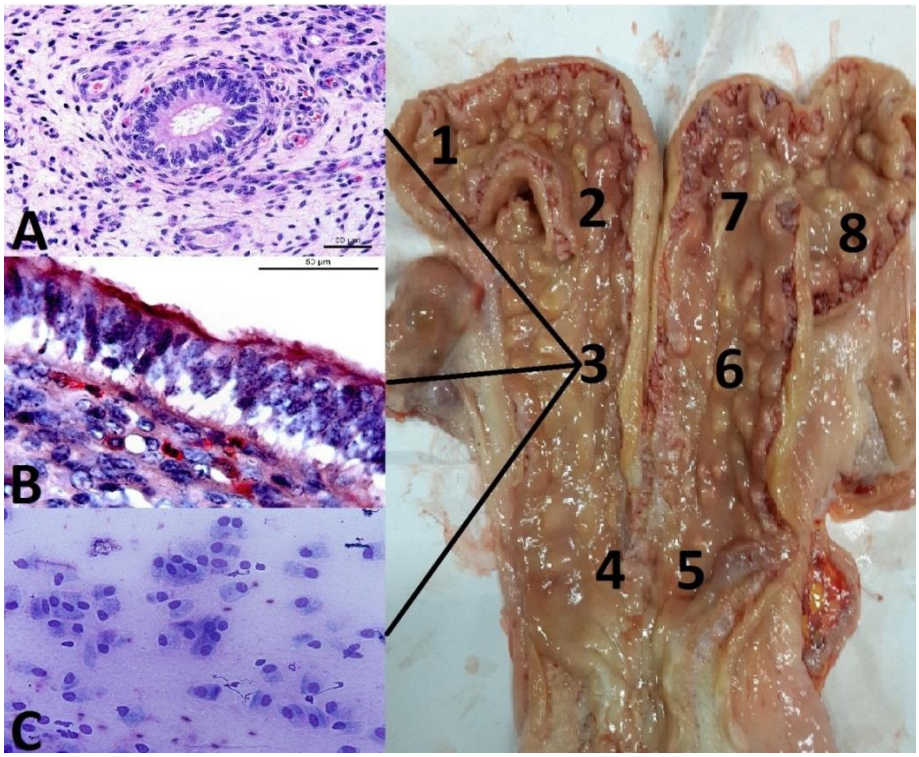

Figure 1. Schematic illustration of an opened uterus including the 8 predefined locations of sampling. At each of these locations, 3 samples were obtained: A) Endometrial Biopsy Haematoxylin and Eosin (EB-HE), B) Endometrial Biopsy Napthol (EB-Napthol), and C) Cytology by Cytobrush (CY-CB).

Histopathological staining and evaluation

Endometrial biopsies were embedded in paraffin wax, sectioned at 4 µm thickness and stained with the conventional histopathological staining haematoxylin and eosin (EB-HE) (**Figure 1**, A), and with Naphtol-AS-D-chloracetate-esterase (EB-Naphtol) [16] and [27] (**Figure 1**, B). Each biopsy was stained by both staining methods and examined by a single observer using a conventional light microscope (Kyowa Optical, Tokyo, Japan) at 100 x and 400 x magnifications. For the EB-HE staining, parameters considered were: epithelium height, mononuclear cell infiltration, lymphocytic

aggregates, periglandular fibrosis, angiosclerosis and haemorrhage [7], [28], [29] and [30] **(Table 1)**. All these parameters were evaluated and averaged in five high power fields (HPF) at 400 x and categorized as: flattened, cuboidal or columnar (epithelium); normal, mild, moderate or severe (mononuclear cell infiltration, lymphocytic aggregate, periglandular fibrosis and angiosclerosis); and absent or present (haemorrhage) [7], [28], [29], [30] and [31]. For the EB-naphthol staining, 5 HPF were randomly selected, and PMNs present in the *stratum compactum* were counted and averaged (400 x).

Table 1. Descriptive statistics of the endometrial biopsy haematoxylin and eosin (EB-HE) samples

Variable	Category	Diagnosis	Dichotomization	Odds ratio and CI	
				EB-Naphtol	CY-CB
Epithelium height	Columnar (control)*	181	181 (absent)	0.99 (0.93, 1.03)	1.07 (0.96, 1.19)
	Cuboidal	70	75 (present)		
	Flattened	5			
Mononuclear cell infiltration	Normal (control)*	89	89 (absent)	1.06** (1.01, 1.13)	1.08** (1.02, 1.16)
	Mild (21 to 40 cells/HPF)	149			
	Moderate (41 to 70 cells/HPF)	18	167 (present)		
	Severe (> 70 cells/HPF)	0			
Lymphocytic aggregate	Absent (control)*	200	200 (absent)	0.96 (0.89, 1.02)	0.98 (0.84, 1.11)
	Mild (3 aggregates/HPF)	54			
	Moderate (4 to 5 aggregated/HPF)	2	56 (present)		
	Severe (> 5 aggregates HPF)	0			
Periglandular fibrosis	Absent (control)*	104	104 (absent)	1.02 (0.97, 1.07)	0.92 (0.77, 1.06)
	Mild (1 to 3 layers/HPF)	133			
	Moderate (4 to 5 layers/HPF)	19	152 (present)		
	Severe (> 5 layers/HPF)	0			
Angiosclerosis	Absent (control)*	121	121 (absent)	0.99 (0.94, 1.04)	0.93 (0.83, 1.03)
	Mild (1 to 3 layers/HPF)	120			
	Moderate (4 to 5 layers/HPF)	15	135 (present)		
	Severe (> 5 layers/HPF)	0			
Hemorrhage	Absent (control)*	237	237 (absent)	1.17** (1.09, 1.26)	0.97 (0.81, 1.13)
	Present	19	19 (present)		

Odds ratios of the binomial logistic regression: endometrial biopsy naphthol (EB-naphthol) and cytology by cytobrush (CY-CB) samples. Below the odds ratio values, between brackets are the 95% confidence intervals (CI). Slide evaluations were made at 100× and 400× magnifications.
a The odds ratio of the control groups is 1.00.
b Odds ratios between PMN counts and indicators of the alteration are significantly different ($p \leq .05$).

Cytological staining and evaluation

Cytobrush slides (CY-CB) were conventionally stained with Wright-Giemsa (Diff Quick, Fisher Diagnostics, Newark, DE, USA) (**Figure 1**, C) and mounted with Eukitt® (O. Kindler GmbH, Freiburg, Germany). The cytologic evaluation was made under light microscopy (Kyowa Optical, Tokyo, Japan) at 400 x magnification. One hundred nucleated cells in 10 random HPF (10 cells per HPF) were evaluated and PMN counts averaged [32].

Statistical analyses

For the statistical analyses, manually collected data were transferred to an Excel spreadsheet file (Microsoft Corporation, Seattle, USA), and then exported to R version 3.3.1, 2015 (R Inc., Boston, USA).

A binomial logistic regression model was built in order to analyze the association between PMN counts in both EB-Napthol and CY-CB samples, with each alteration identified in EB-HE. The glm function of the package stats was used to build models where each evaluated parameter (EB-HE) was dichotomized as present (cuboidal or flattened; mild, moderate or severe; present) or control (columnar; normal; absent). Odds ratios (OR) were calculated with their 95 % confidence interval (CI) and P-values. Outcomes were interpreted as OR = 1: PMN counts do not affect the odds of having the considered alteration; OR > 1: higher PMN counts are positively associated with the odds of having the considered alteration, and OR < 1: higher PMN counts are negatively associated with the odds of having the considered alterations [33].

To examine the distribution of PMNs within the uterus, a Poisson mixed effect model was used since the distribution of the data followed a log-normal pattern [34]. Using the function glmer (package lem4) [35], models were built to compare the PMN counts between locations and regions of the endometrium, including a random intercept per cow. In order to examine the distribution of alterations (among the 8 pre-defined locations) identified in the EB-HE samples, a binomial logistic regression model was built using the function glm of the package stats. For both models, the Tukey test was used to compare the differences between the estimates per location and region (cld function). The level of significance was set at $P < 0.05$ for all analyses.

RESULTS

Parity of the selected cows ranged from 2 to 6, and the average days DIM at inclusion was 315±173. The mean BCS was 3.59±0.74 (1-5) [25]. In total, 768 samples were obtained: 256 EB-HE, 256 EB-Napthol, and 256 CY-CB; representing the 8 predefined locations evenly spread over each of the 32 uteri. This number of samples was achieved in order to perform a study with 80% power and 95% confidence interval. The sample size was computed expecting a lower point of agreement between methods k = 0.4 and a clinically acceptable agreement k = 0.7. The expected SCE prevalence was set at 20%. None of the sampled cows showed symptoms of estrus during clinical examination. The post-mortem macroscopic examination of the reproductive tracts revealed that each cow presented at least 1 follicle ≥ 0.8 cm and a CL ≥ 2.0 cm on either of its ovaries [26]. Since all cows bore a CL in either of their ovaries at the moment of sampling, they were all considered to be in the luteal phase [36].

Associations between the number of PMNs detected in the EB-Napthol and CY-CB samples and each of the alterations identified in the EB-HE samples, are shown in **Table 1**. A positive association ($P < 0.05$) was found between the number of PMNs found in the EB-Napthol and CY-CB samples and the infiltration of mononuclear cells in EB-HP, with OR 1.06 (CI 1.01, 1.13) and OR 1.08 (CI 1.02, 1.16), respectively. Higher PMN counts in EB-Napthol samples were furthermore positively associated ($P < 0.05$) with the presence of hemorrhages in the corresponding EB-HE samples, with an OR 1.17 (CI 1.09, 1.26) **(Table 1)**.

The distribution of PMNs sorted by uterine location (1 to 8), and uterine region (left horn, corpus uteri, and right horn) is described in **Table 2**. Differences ($P < 0.05$) concerning the number of PMNs were found between the different locations, with higher PMN counts in locations 4 and 6. When PMN counts were sorted by uterine region, more PMNs were found in the corpus uteri and the right horn in comparison to the left horn ($P < 0.05$). A heat map of the PMN distribution throughout the 8 pre-defined locations in the EB-Napthol and CY-CB samples is shown in **Figure 2**. For the parameters evaluated in the EB-HE samples, an uneven distribution was found for the infiltration of mononuclear cells ($P < 0.05$). Higher odds to detect mononuclear cells were found at locations 4, 5, 6 and 7. When evaluating by uterine region, higher

amounts of mononuclear cells ($P < 0.05$) were found in the corpus uteri and right horn in comparison to the left horn region. For the remaining parameters, no significant differences were found.

Table 2. Distribution of PMNs sorted by location and region of the uterus [Endometrial Biopsy Napthol (EB-Napthol), and Cytology by Cytobrush (CY-CB)].

Location	CY-CB*	EB-Napthol*	Region	CY-CB*	EB-Napthol*
1(a)	1.44±2.61	2.40±4.52			
2(a)	1.78±3.36	3.44±5.48	Left horn(a)	1.41±2.48	3.63±5.91
3(ab)	1.84±3.11	5.03±7.28			
4(b)	2.38±3.55	6.47±8.73	Corpus uteri(b)	2.31±3.53	5.45±7.6
5(ab)	2.25±3.57	4.44±6.25			
6(b)	1.94±2.80	5.38±6.91			
7(ab)	1.47±2.44	4.28±6.33	Right horn(b)	1.60±2.55	4.58±6.52
8(ab)	1.41±2.45	4.06±6.43			

CY-CB and EB-naphthol values are expressed as polymorphonuclear mean with their respective standard deviation. Values with different superscript (a, b) are different ($p < 0.05$).

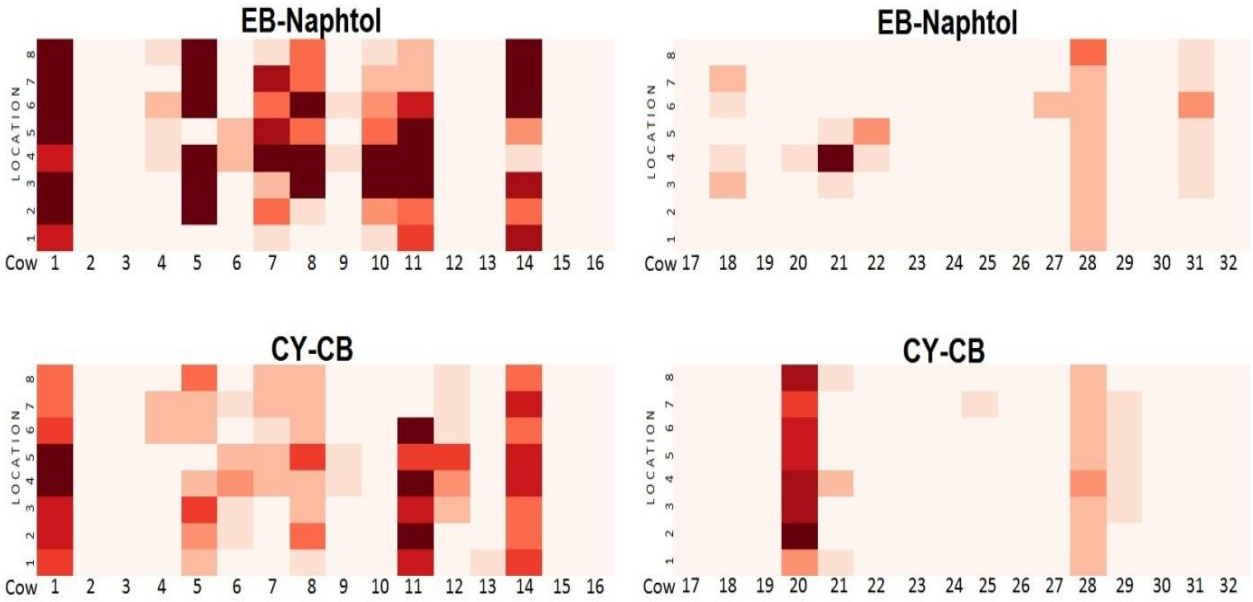

Figure 2. Heat map showing in a red spectrum the PMN counts (darker red refers to higher PMN count) sorted by location (1–8). Endometrial biopsy naphthol (EB-naphthol) and cytology by cytobrush (CY-CB)

DISCUSSION

There is little information about the histopathological interpretation of EB in cows. Since EB sampling in cattle has been mentioned to be detrimental to further fertility [13], [14] and [15], it is underused to examine routinely subfertile cows. Thus,

nowadays CY is the most popular method to diagnose SCE in dairy cows [5], even in cows that have calved for longer time periods as for example repeat breeders [37]. However, when only evaluating CY samples, chronic endometrial alterations may go unnoticed since only a few mononuclear cells and eosinophils are sporadically visualized in CB slides [38]. Consequently, in the present paper, we aimed to assess the association between the number of PMNs detected in EB-Napthol and CY-CB samples with chronic alterations such as reduced epithelium height, monocyte infiltration, lymphocytic aggregates, periglandular fibrosis, and angiosclerosis as identified in EB-HE samples. Also, the presence of red blood cells (hemorrhage) in the *stratum compactum* was evaluated in the EB-HE samples. To simplify the associations, and since no severe chronic alterations were found in the EB-HE samples, we dichotomized the output of each respective alteration to be absent (control) or present **(Table1)**.

Although the odds ratios were relatively low (but the confidence interval was over 1), a significantly positive association was found between the number of PMNs detected in both the EB-Napthol and CY-CB samples and the infiltration of mononuclear cells as evaluated by EB-HE. An initial infiltration of granulocytes, attracts new PMNs by secreting pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor alpha (TNF-α) in the injured tissue [39], [40], [41] and [42]. If this active inflammation persists and continues to stimulate the influx of mononuclear cells, degenerative changes may occur (chronic inflammation and fibrosis) by long-lasting irritation of the endometrium [10], [43] and [44]. The latter may explain the positive association between the number of PMNs and the infiltration of mononuclear cells in the endometrium. However, no further significant associations were found with the presence of other chronic alterations. Probably, the persistent infiltration of PMNs and mononuclear cells took not long enough to produce concomitant degenerative changes in the endometrium. Furthermore, while degenerative changes in the endometrium (endometrosis) have been proven to impair fertility in ageing mares [43], [44], [45] and [46], little is known about the effect of "bovine endometrosis" on the reproductive capacity of the affected animals. Therefore, more research about the effect of endometrosis on fertility in dairy cows is warranted.

A significantly positive association was found between the number of PMNs counted in the EB-Naphtol samples and the detection of hemorrhages in the concomitant

histopathological samples (EB-HE). This finding was previously mentioned by Pascottini et al. [47], who suggested that bloody samples harvested by CB may imply the concomitant presence of PMNs, in this way interfering with SCE diagnosis. Perhaps these PMNs are hemorrhage-derived, which also explains the positive correlation between the number of PMNs and erythrocytes as found in the EB-HE samples. Furthermore, hemorrhages observed in the EB-HE samples were mainly allocated in the deep *stratum compactum*, as was the majority of PMNs found in the EB-HE slides. The latter may explain the lack of association between the number of PMNs found in CY-CB samples and the presence of hemorrhages in EB-HE slides since CY samples typically merely visualize the uppermost layers of the endometrium. In resume, endometrial cytology based on evaluating the number of PMNs is a diagnostic tool useful to diagnose mainly active endometritis, while a complete and more accurate assessment of endometrial health can only be obtained by means of EB.

For many years, EB sampling has been regarded as an integral part of breeding soundness evaluation in mares, mainly to diagnose endometritis [48]. Consequently, multiple studies have examined the representativeness of a single EB to evaluate the health of the entire uterus [16], [18], [19], [20], [21] and [22], although often reporting controversial results. To the best of our knowledge, this study is the first that describes the distribution of inflammation over different locations throughout the entire uterus of clinically healthy cows.

Results of the present study confirm the uneven distribution of inflammation over the 8 pre-defined locations as graphically evidenced by the heat map **(Figure 2)**. Significantly more PMNs were found at the corpus uteri and nearby the bifurcation of the right horn. It seems logical to find higher odds for the detection of PMNs in the corpus uteri since this is the location most proximal to the cervix, the latter being the evacuation site of uterine debris towards the external environment. The fact that in cattle more pregnancies are allocated in the right than in the left uterine horn [49] and [50] and the concomitant mechanical effect of the fetus during parturition may furthermore explain why we detected greater PMN counts at the bifurcation of the right horn. Correspondingly with the higher PMN counts detected in the corpus uteri and the right horn, the odds to detect mononuclear cells in these regions were also higher. The latter finding re-confirms the hypothesis that mononuclear cells are chemo-attracted by

pro-inflammatory factors mainly secreted by PMNs [39], [40], [41] and [42]. Consequently, the most representative region for CB and EB sampling in dairy cows is the adjacencies of the corpus uteri.

CONCLUSION

A significantly positive association was found between higher PMN counts and the infiltration of mononuclear cells. Higher PMN counts are associated with higher odds to detect erythrocytes in the *stratum compactum* of the endometrium. Since more PMNs and mononuclear cells were found in the adjacencies of the corpus uteri, sampling in this region may enhance the sensitivity to diagnose SCE in dairy cows.

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Chapter 5.1

Cytotape: a novel technique to diagnose cytological endometritis in dairy cows

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Pascottini OB, Dini P, Hostens M, Ducatelle R, Opsomer G. A novel cytologic sampling technique to diagnose subclinical endometritis and comparison of staining methods for endometrial cytology samples in dairy cows. Theriogenology. 2015;84:1438-46.

ABSTRACT

The present article describes a study of the diagnosis of subclinical endometritis in dairy cows having two principal aims: first, to validate a novel technique for taking endometrial cytology samples to diagnose subclinical endometritis in dairy cows. Second, to compare the percentage of polymorphonuclear cells (PMNs) in cytology samples stained with Diff Quick versus a specific staining method for PMNs, naphthol-AS-D-chloroacetate-esterase (CIAE). In the first experiment, Holstein-Friesian cows (n = 204) were used to take two cytology samples at the same time using the conventional cytobrush (CB) and the new cytotape (CT). Both devices were assembled within the same catheter allowing sampling at the same time, and approximately at the same location. Cytotape consisted of a 1.5-cm piece of paper tape rolled on the top of an insemination catheter covered with a double guard sheet. Parameters used to evaluate both methods were: PMNs percentage, total cellularity, quality of the smears, and red blood cell contamination. The concordance correlation coefficient analysis was used to assess agreement between continuous and Pearson chi-square tests for categorical variables. Agreement between the percentage of PMNs in both methods was good ρ = 0.84 (0.79, 0.87) with a minor standard error of 2 %. Both methods yielded similar total cellularity (P = 0.62). Cytotape yielded better quality smears with more intact cells (P < 0.01) while samples that were taken by CB were more likely to be bloody (P < 0.01). Hence, CT and CB methods yielded smears with a similar PMNs percentage and a total number of cells, but CT provided smears with higher quality and significantly less blood contamination. For the second experiment, 114 duplicate cytology slides were stained using both Diff Quick and CIAE. Agreement between PMNs percentage in both staining techniques was good ρc = 0.84 (0.78, 0.89) with a standard error of only 2 %. Hence, Diff Quick was confirmed as an easy, fast, and high-quality staining technique, which can be routinely used to stain endometrial cytology samples satisfactorily.

INTRODUCTION

Subclinical endometritis (SCE) is one of the most important reproductive impairments in dairy cows studied in the last decade. It can be defined as the superficial inflammation of the endometrium (no deeper than the stratum spongiosum) [1], without visible clinical signs, but significantly affecting reproductive performances [2] and [3]. Cytology is considered the best technique to diagnose SCE due to its feasibility and fair reliability [2] and [3]. Consequently, a practical definition for SCE was established by consensus in 2006, stating that SCE is diagnosed when in endometrial cytology, samples taken between 21 and 33 days postpartum (DPP) greater than 18 % of harvested cells are identified as polymorphonuclear cells (PMNs), or greater than 10 % of the cells are PMNs when samples are taken at 34 to 47 DPP, in the absence of clinical endometritis [4]. Although endometrial cytology is actually considered the best technique to diagnose SCE [5], the main problem lies in the vast variety of cutoff values to differentiate affected versus unaffected cows taking into account the days after calving samples are taken [6] and [7].

Cytology samples to diagnose SCE are mainly obtained either by cytobrush (CB) [2] or low-volume uterine lavage (LVL) [3], either of them providing similar results [8] and [9]. In equine medicine, sampling by cotton swab (CS) is also considered a valid technique to diagnose endometritis [10]. However, there seems not to be an ideal cytology technique; each method has specific advantages and disadvantages [11]. Yet, CB is recommended as the technique of choice because of its feasibility, safety, and reasonably high-quality samples [8] and [9]. The ideal cytology technique should represent an equilibrium between practicability and the possibility to yield highly accurate results. A harmless technique yielding a high number of well-preserved cells is indispensable for reliable cytologic results [12].

Subclinical endometritis commonly is diagnosed during the voluntary waiting period before insemination, usually from 21 to 64 DPP [2], [3], [8] and [13]. However, diagnosing SCE at that time has two major disadvantages: (1) often, sampling interferes with routine management at the dairy farm (provoking extra handling of animals and hence extra labor); and (2) the percentage of PMNs present in the uterine lumen is a dynamic phenomenon [14], which makes it hard to predict the amount of PMNs that

will be actually present at the moment the animal is inseminated, creating parameters for SCE diagnosis relatively erratic. Consequently, the prevalence of SCE fluctuates widely among different studies, mainly because of the large variation in both the DPP when samples are taken and the cutoff value applied to define an animal as SCE positive [7], [15] and [16]. Therefore, it is imperious to take cytology samples at a standard moment that is both more convenient in relation to the general herd management and allows the use of a universal PMNs threshold. Sampling during artificial insemination (AI) may be an ideal proposal in this perspective because it does not require extra manipulation of the animals and offers opportunities to determine the uterine health status at the moment the animal is fertilized. Therefore, aim of the present study was to compare and validate an innovative technique to take endometrial cytology samples using the generally accepted CB as the gold standard.

After cytologic sampling, smears need to be air-dried, stained, and microscopically evaluated. The staining method should be fast, easy to perform under practical circumstances, and yield high-quality samples allowing accurate interpretation [17] and [18]. According to the literature, in more than 90 % of the studies, a modified Wright-Giemsa staining, such as Diff Quik, Tincion 15, Haema Quick, Hemacolor, is used [3], [19], [20] and [21]. Because these stainings are fast and easy to perform, they are well accepted and widely used, although they have not been compared with a gold standard to evaluate more objectively the PMNs-to-epithelial cells ratio. Naphthol-AS-D-chloroacetate-esterase (CIAE) is an enzyme histochemical method in which PMNs appear bright red after staining and is therefore regarded as the preferable staining to identify and count PMNs [22] and [23]. Because a relatively small number of PMNs is needed to consider a cow positive for SCE, it is essential to have a good quality staining not to miss any PMN and hence sub-evaluate the number of cows suffering from SCE.

Hence, the following were the aims of the present study:

−To compare and validate an innovative technique to take endometrial cytology samples using the generally accepted CB as the gold standard;

–To compare the PMNs percentage in twin endometrial cytology samples stained with the widely applied Diff-Quik versus the CIAE, a histochemical preparation considered as the gold standard to stain PMNs.

MATERIALS AND METHODS

All experiments mentioned in the present study were carried out with permission of the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University (EC 2013/174).

Study design

We based a sample size calculation in order to achieve a study with $\alpha = 0.05$, 80% power, and an expected correlation $\rho c = 0.2$. Consequently, the study population consisted of 204 Holstein-Friesian cows, 140 from one commercial dairy herd, and 64 that were selected at the slaughterhouse before slaughtering. Cows chosen at the slaughterhouse were of unknown origin and health status and thus no data concerning health and previous reproductive status were available. Cows from the commercial farm were between 31 and 37 DPP at the moment of sampling **(Figure 1)**.

Examinations and sampling

Only cows with a body condition score greater than 2 (1–5) [24] were enrolled in the study. Vaginal examination was performed by the gloved hand method [25]. The presence of purulent vaginal discharge (PVD) was not used as an exclusion criterion. Vaginal discharge (VD) was classified as: clear mucus (VD-0), mucus with pus flecks (VD-1), mucopurulent discharge (VD-2), and purulent (or fetid) discharge (VD-3) [26]. A transrectal reproductive ultrasound (Tringa, Esaote-Pie Medical, Maastricht, the Netherlands) examination (uterus and ovaries) was performed before sampling. Reproductive ultrasound findings were classified as presence (> 0.5 cm) or absence of fluid in the uterine lumen [2] and presence (Corpus luteum (CL), > 2 cm) or absence of corpus luteum [27] at the moment of examination.

A special device was developed to take two endometrial cytology samples at the same time. A human use Cytobrush Plus GT (Cooper Surgical, Berlin, Germany) was adapted to a stainless steel stylet of an universal insemination gun (Agtech, Manhattan, KS, USA), by heating the top of the stylet with a lighter and fitting it to the base of the handle of the CB. Then, the stylet-CB was introduced in a 22″-long equine infusion pipette individually wrapped (Agtech, Manhattan, KS, USA). Next, a 1.5-cm clean piece of paper tape (Tesa 4322; Hamburg, Germany) was rolled on the top of the equine infusion pipette. To protect the equine infusion pipette (tape and CB) from contact with both the vaginal and cervical wall during sampling, the pipette was covered with a 12″-long Sani-Shield Rod (Agtech, Manhattan, KS, USA).

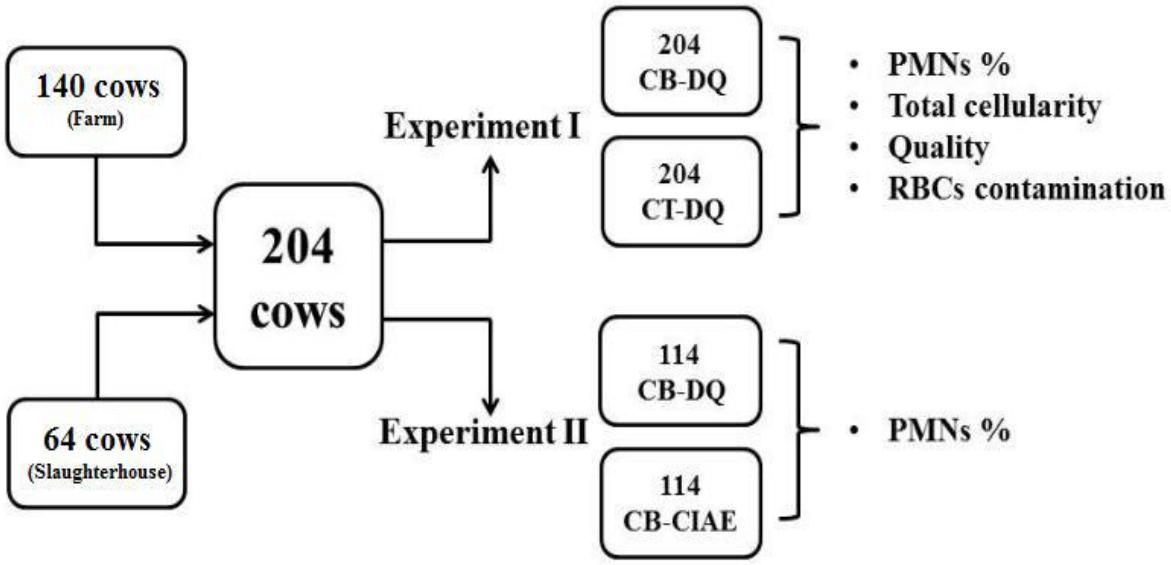

Figure 1. Brief overview of the study protocol for both experiments. CB-DQ, CB Diff-Quik; CT-DQ, CT Diff-Quik; PMN, polymorphonuclear cell.

Once the sampling pipette was armed, the perineal region of the cow was thoroughly cleaned with fresh water and dried with paper towel. Next, under rectal guidance, the pipette was introduced into the vagina and manipulated through the cervix. Once in the lumen of the uterine body, the top of the pipette (with the paper tape on it) was released from the Sani-Shield Rod. Then, it was rolled on the dorsal wall of the uterine body with a gentle pressure of the index finger through the rectum. After the pipette-paper tape (cytotape or CT) was rotated twice, the CB was released from the pipette **(Figure 2)** and also rolled twice in the dorsal part of the uterine body (just next to the place where CT had been rolled). Once the CB was rolled twice, it was retracted into the

pipette, and then the pipette was covered again with the Sani-Shield Rod to prevent contamination with cervical and vaginal cells. Finally, the device was carefully removed from the reproductive tract.

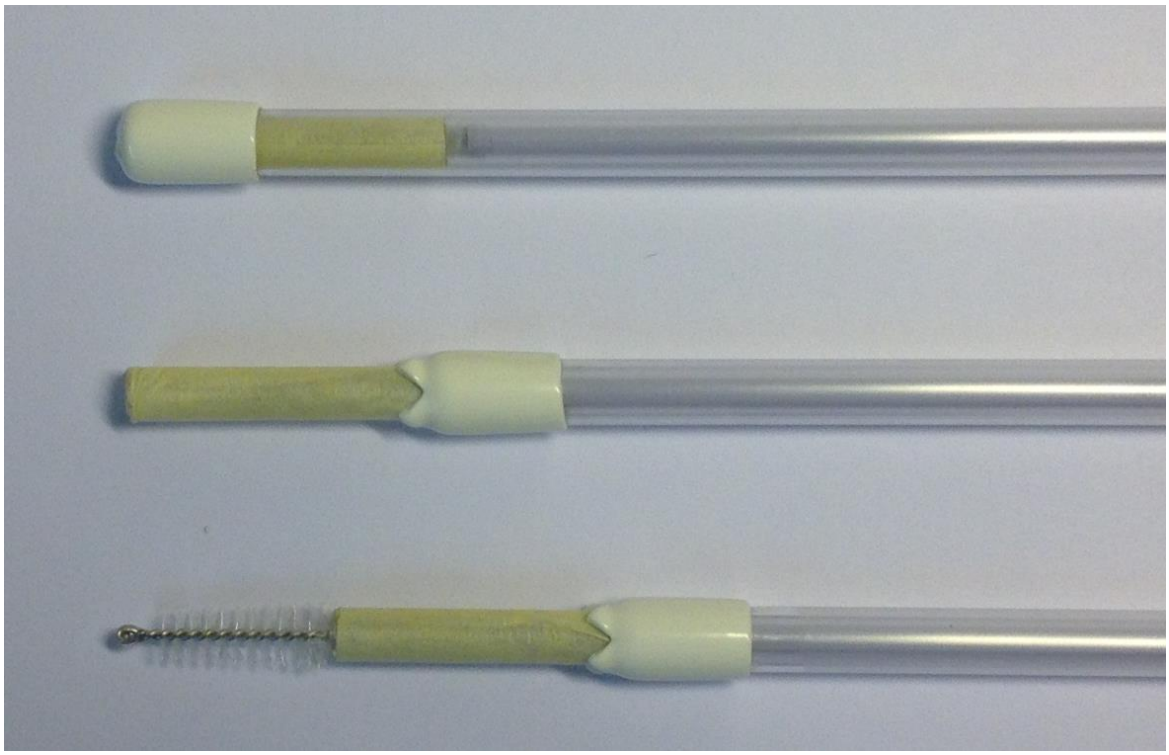

Figure 2. Image showing how the cytology samples were taken (from top to bottom). First, once the cytology device was manipulated through the cervix and reached the uterine lumen, the cytotape (CT) was released from the Sani-Shield Rod. After rotating twice, the cytobrush was released and rotated at approximately the same location where the CT sample had been taken. In this way, two cytology samples were taken at the same time.

Preparation and staining of the slides

Slides for cytologic examination were prepared at the farm and slaughterhouse immediately after sampling. First, the CT samples were gently rolled onto a clean microscope glass slide (Marienfeld, Lauda-Königshofen, Germany) to spread the collected cellular material. After that, two microscope slides were prepared by rolling half of the CB circumference on one slide (first duplicate) and the other half on another slide (second duplicate), in this way, obtaining a suitable and equally distributed quantity of cellular material on both slides. Before staining, smears were air-dried. In total, 408 slides including 204 from CT and 204 from the CB first duplicate stained with Diff-Quik (Fisher Diagnostics, Newark, DE, USA), according to the manufacturer's instructions. A subset (n = 114) of the second duplicates of the CB samples was randomly selected to be stained with the CIAE method, for the second experiment. In

the end, 522 slides were stained, 204 CT Diff Quick (CT-DQ), 204 CB Diff Quick (CB-DQ), and 114 CB CIAE (CB-CIAE) **(Figure 1)**.

For the CIAE staining, two stock solutions were prepared: a substrate solution and a hexasodium solution. For the substrate solution, 3.58 mg of naphthol-AS-D-chloroacetate (Sigma, ref. no. N0758, St. Louis, USA) was diluted in 0.9 mL of dimethyl sulfoxide (Sigma, ref. no. D5879, St. Louis, USA) and 0.1 ml Triton X-100 (Sigma, ref. no. X100, St. Louis, USA), becoming a light yellow solution. For preparing the hexasodium solution, first, a sodium nitrite solution 1 mol/l was prepared by diluting 345 mg of sodium nitrite (Carl-Roth, ref. no. 8604.1, Karlsruhe, Germany) in 5 mL of distilled water. Once the sodium nitrite solution was prepared, pararosaniline hydrochloride (Sigma, ref. no. P3750, St. Louis, USA) was diluted in 3 ml of 1 mol/l HCl (Chem-lab, ref. no. CL05.0311.1000, Zedelgem, Belgium), acquiring a dark brown color. In the end, 0.5 ml of nitrite solution was added to the pararosaniline-HCl solution, turning this into a light brown color. Both the substrate and the hexasodium solution had to rest for 5 minutes before use to reach stabilization. To prepare the final solution, 1 ml of substrate solution and 0.5 ml of hexasodium solution were added to 100 ml of a phosphate-buffered saline solution (pH = 7.2), on a vortex, until a light pink color appeared. Next, slides were incubated for 90 minutes at 37 °C in the CIAE solution. After incubation, slides were rinsed for 2 minutes with tap water and 5 minutes with distilled water. For the counterstaining, slides were submerged in a hemaluin Gill staining solution for 7 minutes. Finally, they were rinsed with tap water for 10 minutes and distilled water for 5 minutes.

Once dry, cover slips (Marienfeld, Lauda-Königshofen, Germany) were mounted on the stained glass slides using Eukitt (O.Kindler GmbH, Freiburg, Germany) as the mounting medium.

Cytologic evaluation

All slides (CB-DQ, CT-DQ, and CB-CIAE) were evaluated by light microscopy (Kyowa Optical, Tokyo, Japan) using x 100 and x 400 magnifications. For each slide, a total of 300 cells were counted by one observer, and the PMN-epithelial cell ratio was assessed [20]. In the CT-DQ and CB-DQ slides also, the total cellularity, quality of the harvested cells, and the background content were assessed.

Total cellularity, quality, and red blood cell (RBC) contamination were assessed after evaluating 10 high power fields at x 100 magnification [10], [11] and [28] and results averaged. To evaluate total cellularity, the number of cells was estimated and classified as no cells, low (< 50 cells), moderate (50–100 cells), and high (> 100 cells). Quality was evaluated by estimating the percentage of intact cells leading to a categorization in three different groups: very good (> 75 % intact cells), good (50 %–75 % intact cells), or bad (< 50 % intact cells). Finally, the RBC contamination was evaluated by assessing the quantity of erythrocytes and subsequent classification as no RBCs, low (disperse erythrocytes), moderate (high number of erythrocytes), and high RBCs (strong background of erythrocytes).

Statistical analysis

Comparison between CB and CT was made for PMNs %, total cellularity, quality, and RBC contamination. Data from all sampled cows were exported from data capture forms to an Excel (Microsoft Corporation, Seattle, USA) spreadsheet file. Statistical analyses were carried out using SAS software (SAS Institute Inc., Cary, USA). First, descriptive statistical analyses were executed using PROC FREQ and PROC MEANS. To assess agreement among continuous variables (PMNs %) between diagnostic methods, CT versus CB as gold standard, the concordance correlation coefficient (CCC) test was performed using the SAS macro reported by Crawford et al. [29]. Briefly, for CCC interpretation, values are between −1 and 1, in which −1 means complete disagreement, 0 translates to an independent situation, and 1 indicates a perfect agreement [30]. The CCCs are reported with a 95 % confidence interval. Enhanced Bland-Altman plots were created to visualize agreement between methods. Moreover, in order to assess the Kappa value between CT, CB, and both staining methods; PMNs' outcomes in slides were categorized as ≥1%, ≥5%, a ≥10%. Comparisons and further agreements between categorical variables were made using contingency tables, and Kappa and Pearson chi-square tests. The level of significance was set at P value less than 0.05 [31]. To compare the agreement of PMNs % between Diff Quick versus CIAE staining, the SAS macro CCC test was used, and an enhanced Bland-Altman plot was made to picture the agreement between both staining techniques.

RESULTS

Two hundred four Holstein-Friesian cows were included in the present study. In total, 522 slides were analyzed all of which were readable and acceptable for further analysis. In cows sampled at the slaughterhouse (n = 64), 10 cows (15.6 %) presented PVD at the moment of examination: VD-0, 54 of 64 (84.4 %); VD-1, 8 of 64 (12.5 %); VD-2, 2 of 64 (3.1 %); and VD-3, 0 of 64 (0 %). Eight of the 64 cows (12.5 %) showed uterine content, and 41 (64 %) had a CL (> 2 cm) on at least one of the ovaries. From the cows sampled at the farm (n = 140), 95 of 140 (67.8 %) showed VD-0, 28 of 140 (20 %) VD-1, 17 of 140 (12.1 %) VD-2, whereas no cows were found with VD-3. Sixteen of the 140 cows (11.4 %) had uterine content (> 0.5 cm), whereas 77 of them (55 %) presented a CL (> 2 cm) at the moment of examination. In general, from the 204 cows, 149 showed no VD (73 %, no PVD), 24 (17.7 %) presented uterine content (> 0.5 cm) and 118 (57.8 %) presented a CL (> 2 cm).

The CCC between CT-DQ and CB-DQ concerning the percentage of PMNs is summarized in **Table 1**. However, three outliers were noticed in the enhanced Bland-Altman Plot **(Figure 3)**. Re-checking the data revealed that the strong differences between CT-DQ and CB-DQ PMNs % in these three samples were probably due to contamination during cervical manipulation, as these three cows suffered from PVD. Omitting these three outliers and re-performing the CCC test showed an agreement between the two sampling techniques of ρc = 0.93 (0.91, 0.94) and a standard error (SE) of 1 %. The variance components such as subject variance, random error variance, and method effect values were analyzed to be 2.7 %, 0.1 %, and 0.01 %, respectively. The Kappa values between CT-DQ and CB-DQ at PMNs ≥1%, ≥5% and ≥10% were k = 0.77, k = 0.74, and k = 0.76, respectively.

Table 1. Concordance correlation coefficient (CCC) values achieved between both sampling techniques and staining methods.

Technique	Concordance correlation coefficient				
	CCC	SE CCC	Subject	Random error	Method effect
Brush/tape (n = 204)	0.84	0.02	0.02	0.004	0.0003
Diff Quik/CIAE (n = 114)	0.84	0.02	0.04	0.006	0.0008

Also included: standard error (SE) and variance components.

Table 2. Quality, total cellularity, and RBCs contamination in samples taken by cytobrush (CB) and cytotape (CT).

Technique	Quality			Cellularity			RBCs' contamination				Total
	Bad	Good	Vgood	Low	Moderate	High	None	Low	Moderate	High	
Brush	0	49	155	29	155	20	26	101	64	13	204
Tape	0	28	176	27	162	15	59	112	33	0	
P-value		0.0079			0.6249				<0.0001		

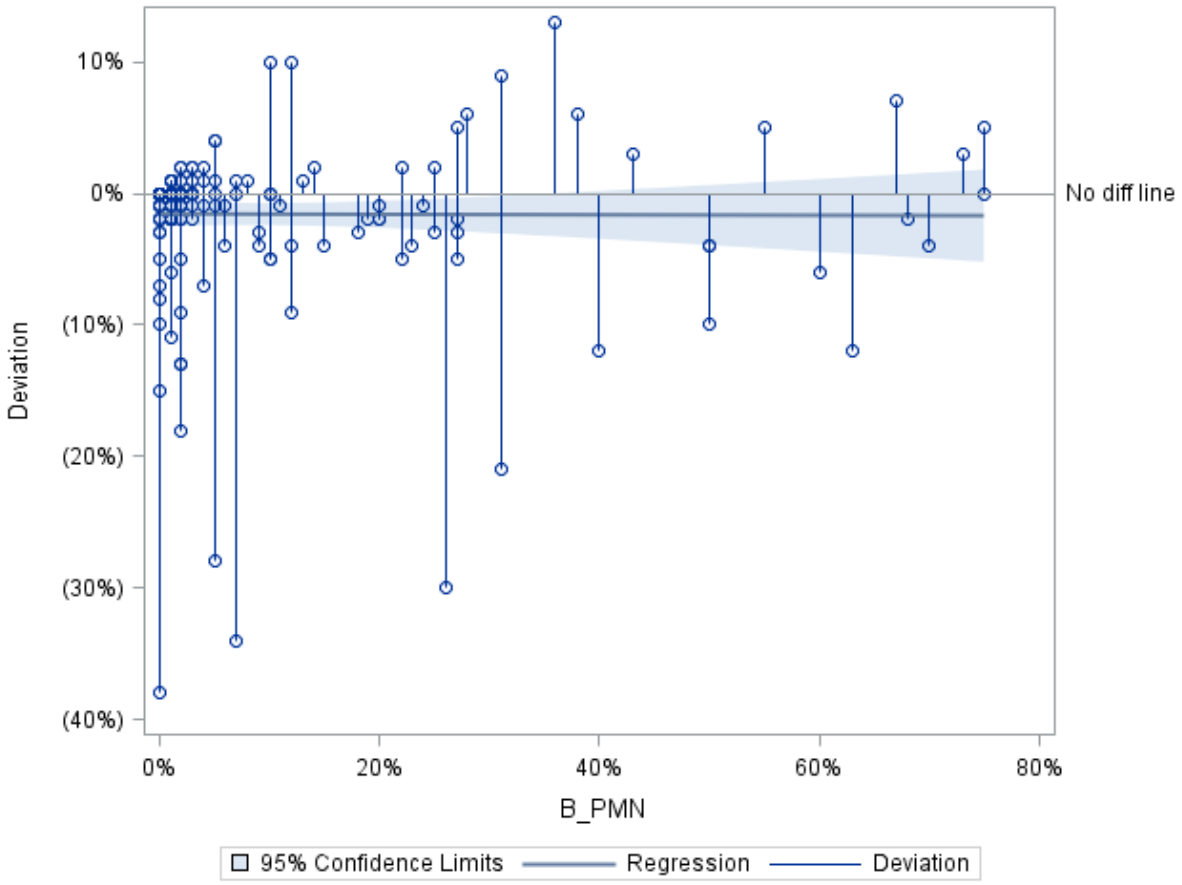

Figure 3. Enhanced Bland-Altman plot illustrating the agreement in polymorphonuclear cell (PMN) % between the cytotape (CT) and cytobrush (CB) sampling methods. In red, three outliers are notable. These correspond to a strong difference between CT and CB PMNs %.

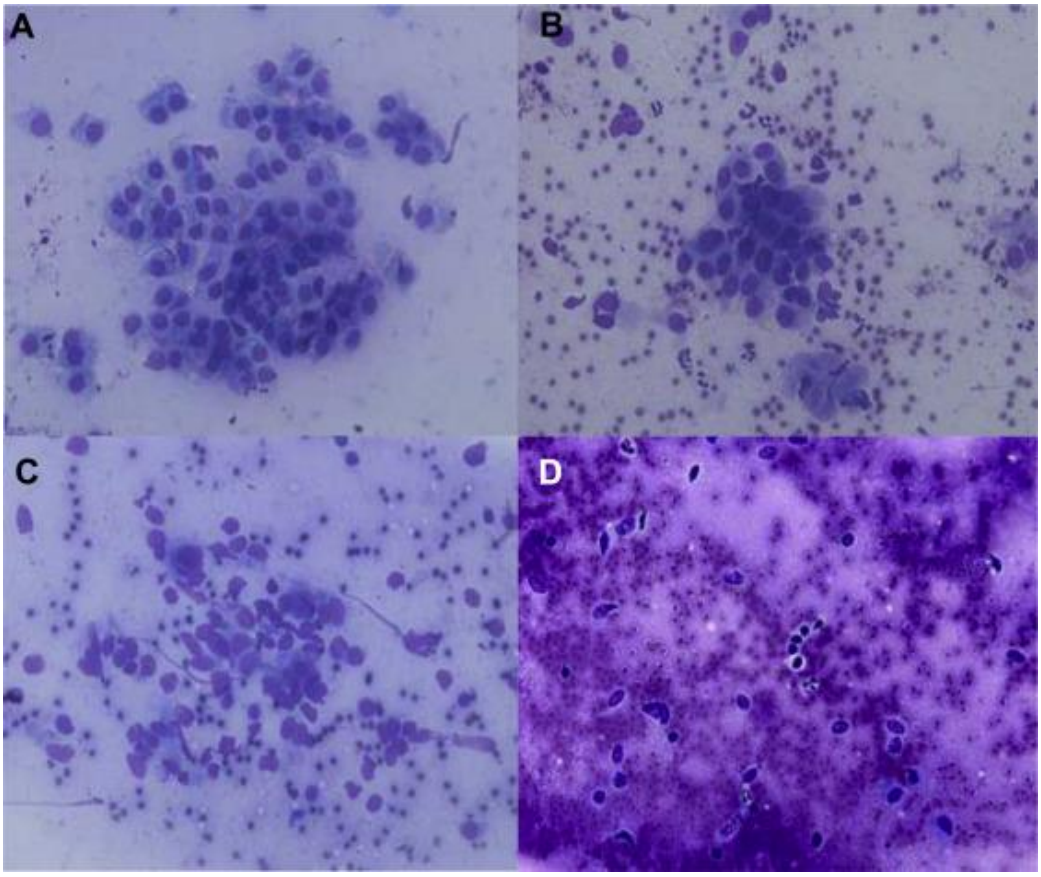

Figure 4. Cytology smears obtained by cytotape and cytobrush, stained by Diff Quick, observed by light microscope (x 400). (A) Cytotape sample, (B) Cytobrush sample with moderate RBCs contamination. (C) Cytobrush sample with fragmented cells and moderate RBC contamination. (D) Cytobrush sample with high RBC contamination

Separately, CCCs were calculated based on the presence and/or absence of a CL (>2 cm) and in cows suffering from PVD at the moment of sampling. The CCC in cows with CL was ρc = 0.85 (0.79, 0.89), whereas in cows bearing no CL on either of the ovaries, it was ρc = 0.82 (0.73, 0.87). In PVD positive cows, the CCC was ρc = 0.81 (0.70, 0.88), whereas in PVD negative cows, it was ρc = 0.83 (0.77, 0.87).

Table 3. Total number of samples achieved and stained by each technique

Sample	Polymorphonuclear (PMN) %				
	N	Minimum (%)	Maximum (%)	Mean (%)	Std. dev. (%)
Tape (Diff Quick)	204	0	75	11	18
Brush (Diff Quick)	204	0	75	8	14
Brush CIAE	114	0	85	15	16

Minimum, maximum, mean, and standard deviation of the PMNs % obtained by each sampling technique and staining method.

For the second experiment, the CCC value between the CB-DQ and CB-CIAE staining was ρc = 0.84 (0.78, 0.89) with an SE of 2 %. Variance components related to the subject

was 4 %, being much greater than the random error 0.6 % and method effect 0.08 % **(Table 1)**. Moreover, the Kappa values between CT-DQ and CB-CIAE at PMNs ≥1% was k= 0.5, at PMN's ≥5% was k = 0.79, and at ≥10% it was k = 0.94.

DISCUSSION

The principal aim of the present study was to validate a new diagnostic technique to take endometrial cytology samples to diagnose SCE using the CB as the gold standard. The secondary goal was to compare the percentage of PMNs in twin cytology samples stained with Diff Quick versus CIAE.

The high prevalence, its relatively difficult diagnosis and the economic impact of the condition, all make SCE one of the most important and challenging diseases in the modern dairy industry. Despite its undeniable importance, standardization of SCE diagnosis by cytology has not been fully established yet. Although it is well accepted that the best method to diagnose SCE is by measuring the proportion of PMNs in cytology samples [4], controversy remains because of the wide range of cutoff values used to define SCE [20], DPP at the sampling moment [16] and cytology technique implemented to acquire samples [8], [9], [10], [11] and [32]. To standardize SCE diagnosis, we developed an innovative device that allows to use the AI gun to take cytology samples. The latter would facilitate significantly sampling during insemination, which offers further advantages in terms of labor efficiency and knowledge of uterine health at the moment of fertilization. The concept of taking samples during AI has three main advantages in comparison to the more conventional sampling techniques: the standardization of the moment of sampling, the usage of ordinary materials, and avoiding "extra" handling of animals to obtain the samples. Furthermore, sampling at the time of insemination allows for a more direct examination of the effect of the counts of PMNs in the uterus on the success of pregnancy.

Central parameter used to evaluate the agreement between the here-presented innovative technique and the well-accepted CB was PMNs %. To obtain a wide distribution of PMNs % in the cytology smears, samples were harvested from cows in both early postpartum (31–37 DPP) and presumably late postpartum. Indeed, PMNs %

ranged from 0 % to 75 % for both techniques, with a mean of 11 % for CT and 8 % for CB **(Table 3)**. Agreement between both techniques concerning the PMNs % was good with a small SE. Interestingly, the variance components analysis found that the subject variance was significantly higher than the random error and the method effect. The latter implies that it is more likely to have a higher difference in PMNs % between samples when different cows are evaluated than that the disagreement is related to differences in reading the slides or to a method effect (CT or CB) related to the appreciation of the slides by the observer. The presence of PVD did not influence the CCC result. In both situations (PVD negative or positive), the CCC was at least "good". While inspecting the enhanced Bland-Altman plot, three outliers were noticed presumably because the CT was contaminated during cervical manipulation in PVD positive cows. The agreement became "high" when these three samples were omitted. Therefore, we can conclude that presence of PVD does not affect the agreement between CT and CB, but a practical recommendation would be to use a sanitary sheet [33] in cows which are not in heat (closed cervix) during the sampling. In 57.8 % of the sampled cows, a CL (>2 cm) was detected by ultrasonography, whereas the other 42.2 % were in anestrus or around the time of ovulation (no CL or CL < 2 cm). The CCC between CT and CB were furthermore "good" both in cows bearing and not bearing an active CL. In resume, CT is a sampling technique that achieves cytology samples with a similar number of PMNs than CB and can be used at any stage of the estrous cycle.

Secondary parameters evaluated between CT and CB were total cellularity, quality, and RBC contamination **(Figure 4)**. Cellularity is a critical element in cytologic specimens and is closely correlated with the threshold used to define inflammation [9] and [34]. In the present investigation, both methods showed a similar total cellularity. In previous publications in which endometrial cytology methods were compared, CB yielded significantly more cells than LVL or CS [11], [32] and [35]. It is clear that in LVL, cells can be diluted [9]. The most probable reason why CT yielded a similar cellularity as CB is because the paper tape (on the top of the catheter) does not have absorbent properties as the CS, and most of the cellular material collected was adhered to the glass slide when the tape was rolled on it. In contrast with cellularity, both quality and RBC contamination were found to be significantly better for CT in comparison to CB. More intact cells were found in CT slides, resulting in significantly more CT samples being

categorized in the category “very good”. In human medicine, the phenomenon of cellular distortion and fragmentation was described when the CS was used [10] and [11]. This distortion and fragmentation are mainly related to the firm adherence of cells to the cotton fibers and the consequent pressure needed to take the samples and to roll them onto the glass slide [28] and [35]. The presumable reason why the CT slides yielded a better quality in comparison to the CB slides is the rigidity of the brush bristles and the fragmentation of cells when the brush is rolled on the microscope slide [11]. Also, the endometrial material is not strongly adhered to the CT; consequently, cells are easily detached from the tape and scattered on the glass slides resulting in a small percentage of fragmented-distorted cells.

Remarkably, when samples are taken by CB, there is a significantly higher risk to have a bloody smear in comparison to CT sampling. Presence of RBCs in endometrial cytology samples was already described earlier, demonstrating higher numbers of RBCs in LVL than in CB [9] (in cows) and more bloody samples in CB versus CS [28], in mares. We found a robust difference represented by around 35 % of CB samples with a high or moderate contamination of RBCs **(Figure 5)**. Rigid fibers of the CB might be responsible for high amount of RBCs in the samples [11], and this blood contamination should not be regarded as a negligible observation. Most of SCE publications lacked control groups when CB or LVL was performed. Only in one study, a control group was implemented [19]. Regarding the high amount of bloody samples when using the CB, one might argue damaging the uterine epithelium and the concomitant production of lesions in the endometrium, which opportunistic bacteria might use as an entrance gateway to cause a secondary infection. Additionally, bloody contamination of the samples implies the presence of 1 % or 2 % of PMNs [36]; hence, when a low threshold of PMNs is used to diagnose SCE [37] and [38], these PMNs could interfere with the diagnosis.

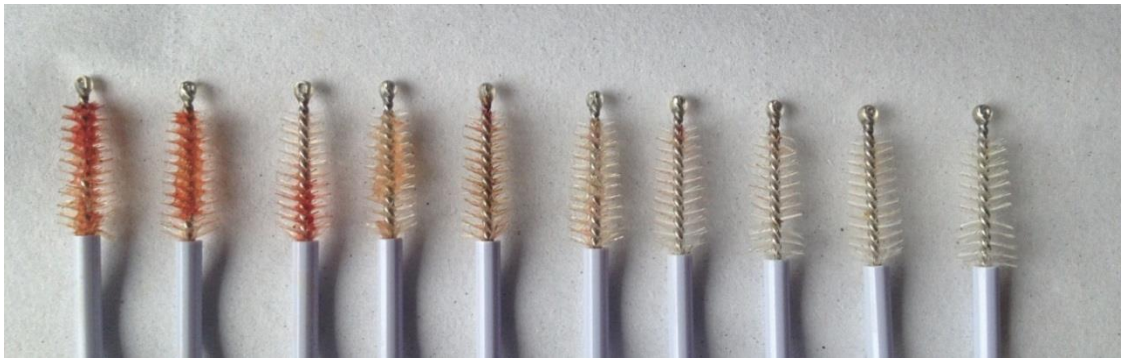

Figure 5. Cytobrush with different amounts of blood contamination after endometrial sampling.

It is mandatory to develop a cow-side diagnostic test to diagnose SCE with fair accuracy and at a low cost, under field conditions [39]. Both, CB and LVL are acceptable cytology techniques to diagnose SCE in dairy cows, but the principal reason why CB is preferred by practitioners is due to its practicability and cytologic quality [8] and [9]. The main advantage of LVL is that samples are taken from a larger endometrial surface and tend to be more representative for the health of the complete endometrium [10], [12], [28], [40] and [41]. However, more studies are needed to prove this hypothesis. Major disadvantage of LVL is related to the difficulty to recover fluid after the infusion (17 % of the cases), damage due to manipulation during sampling, irritation that flushing liquid can cause to the endometrium and a higher percentage of distorted cells, in comparison to CB smears [8], [9], [10], [11], [12], [32], [42], [43] and [44]. Main CB disadvantage is the requirement of specialized equipment [9]. Conversely, CT is not a 100 % cow-side diagnostic test because after sampling, slides need to be stained and analyzed under a microscope. However, CT is one step forward to an easier cytologic SCE diagnosis because it does not require special material (paper tape and double guard sheet), and it can be used with the AI gun achieving high-quality endometrial samples. Consequently, CT offers similar characteristics as the CB but requires less specialized material and can be used during AI.

A high-quality staining method is mandatory to yield an objective and accurate evaluation of the PMN-to-epithelial cell ratio in endometrial cytology samples. Modified Wright-Giemsa (Diff Quick) is currently the most widely used staining to evaluate endometrial cytology slides from dairy cows. To assess the capacity of Diff-Quik to stain PMNs, it was compared with an enzyme histochemical staining in which PMNs appear bright red after staining (CIAE; **Figure 6**), and which is therefore often used as the gold standard for identification and counting of these cells [22] and [23]. Because the CIAE staining method requires several preparatory steps including a time-consuming incubation, it is considered time demanding and relatively expensive. Therefore, this technique is not recommended for routine use under field circumstances. Concordance correlation coefficient between Diff Quick and CIAE PMNs' % was good. Nevertheless, when the number of PMNs increases, the divergence between both staining techniques is also growing. Major differences between CIAE and Diff Quick are only considerable

when very high amounts of PMNs have to be evaluated, and the threshold for SCE is by far exceeded **(Figure 7)**.

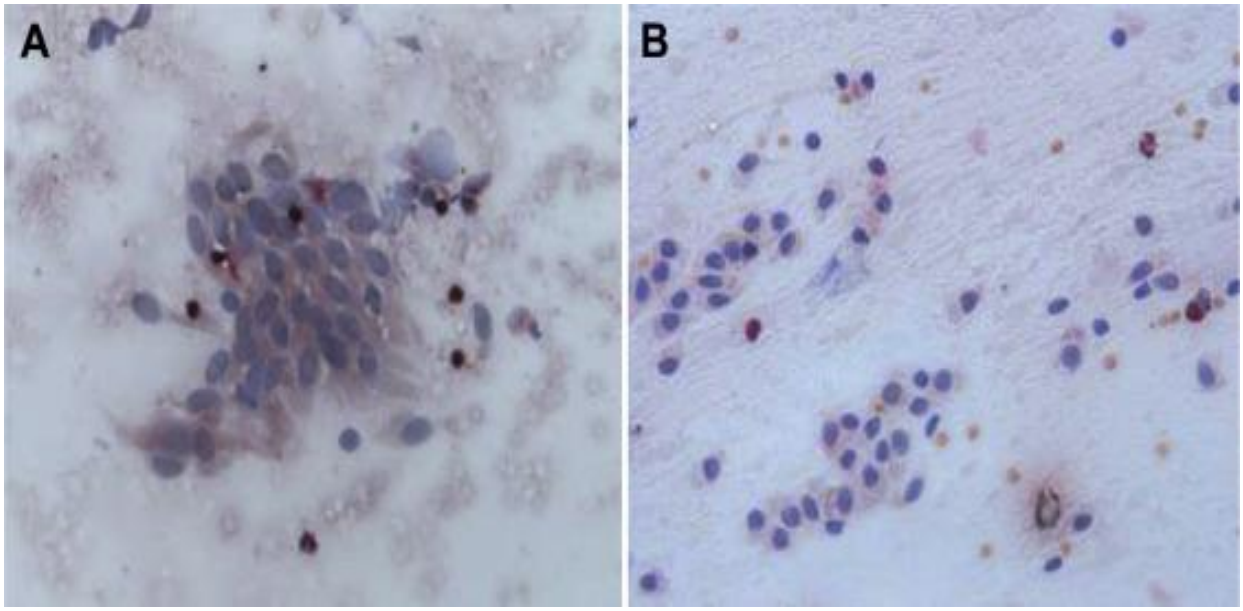

Figure 6. Cytology smears stained by CIAE, observed by light microscope (x 400). (A and B) Polymorphonuclear cells appearing in bright red.

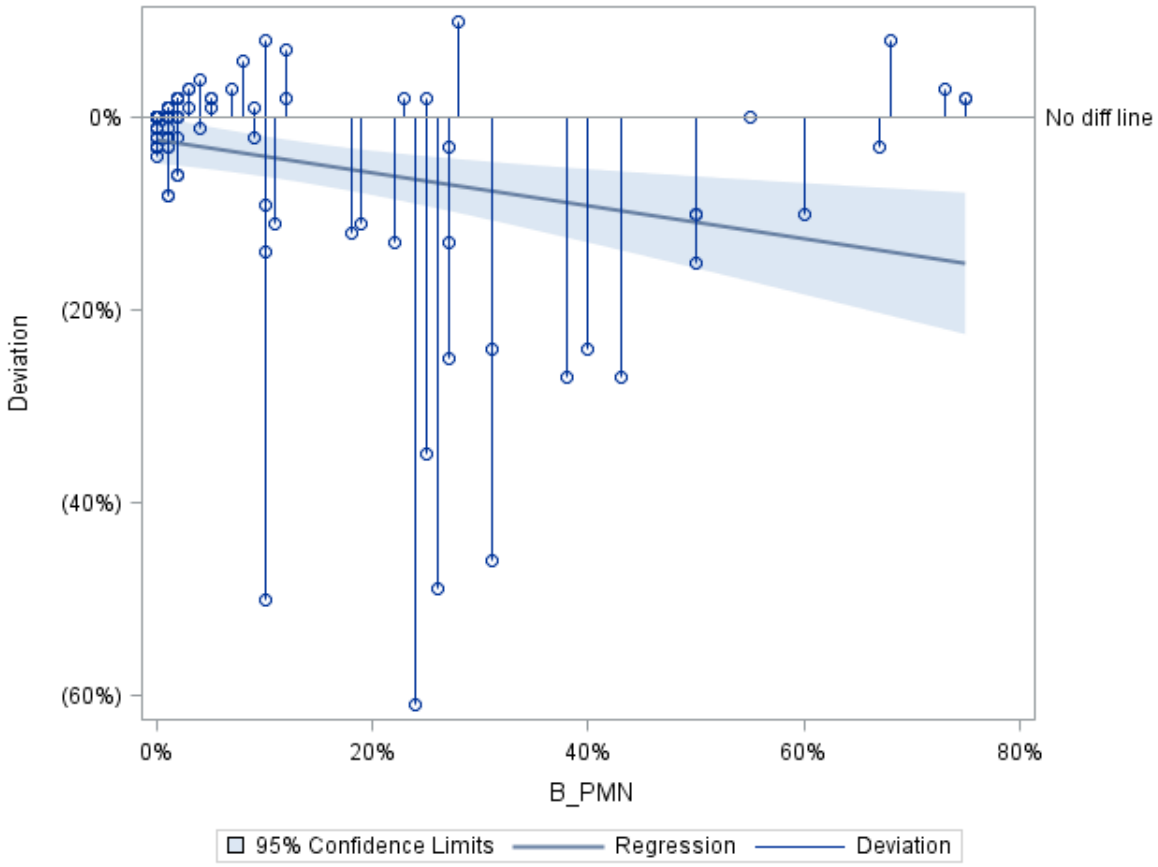

Figure 7. Enhanced Bland-Altman plot illustrating the agreement between the Diff Quick and CIAE staining. PMN, polymorphonuclear cell.

CONCLUSIONS

In summary, taking endometrial cytology samples with CB and CT yields similar results regarding parameters like PMNs % and total cellularity. However, techniques significantly differ in quality parameters and RBCs contamination in favor of the CT. When samples are taken by CT, less distorted–fragmented cells and a significantly lower contamination with RBCs were reported. On top of these advantages, the CT offers a technique that can be applied at the moment of insemination, by adhering the tape on the insemination pipette. The latter might allow field studies on a large scale to find a correlation between the number of PMNs in the uterine lumen and the conception result. Furthermore, this will allow to determine a straightforward cutoff value at a standardized moment (i.e., during insemination) above which the number of PMNs is associated with a reduced conception rate. Finally, modified Wright-Giemsa (Diff Quick) is a fast, easy, and high-quality technique to stain endometrial cytology samples.

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Chapter 5.2

Cytological endometritis at artificial insemination in dairy cows: prevalence and effect on pregnancy outcome

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ABSTRACT

The present paper reports a field study in dairy cows aiming: 1) to consolidate the cytotape (CT) as a valid technique to diagnose cytological endometritis (CYTO) during artificial insemination (AI); 2) to establish a cut-off point concerning the polymorphonuclear cells (PMNs) proportion to diagnose CYTO at AI; 3) to assess the prevalence of CYTO at AI; and 4) to evaluate the effect of CYTO on the pregnancy outcome of that AI. The investigation was performed using 1,625 AI-CT samples harvested from 873 Holstein-Friesian cows from 18 dairy farms in the Flemish region of Belgium. The CT device consisted in adapting a 1.5 cm piece of paper tape on the top of a conventional AI catheter covered with a double guard sheet, allowing to take an endometrial cytology sample when performing an AI. A receiving operator characteristic curve was built to assess the threshold level above which the PMN% significantly affects the AI success. Multilevel generalized mixed effect models were built to identify factors affecting the pregnancy outcome of the AI under investigation. Only seven samples (0,4 %) harvested in five cows were discarded because of low quality parameters. Cut-off point for CYTO at AI was set at ≥ 1 % PMN (Sensitivity = 33.8 % - Specificity = 88.6 %). Prevalence of CYTO at AI was 27.8 %. The conception rate for CYTO positive samples was 32.7 % while it was 47 % for CYTO negative samples. A CYTO negative AI had 1.8 [Odds ratio (OR)] more chances to become pregnant than a CYTO positive one. Other factors identified as detrimental for the pregnancy outcome were: BCS ≤ 1.5 (OR = 0.6), relative 305 days milk yield (OR = 0.9), dystocia (OR = 0.3), parity ≥ 2 (OR= 0.7) and warm months of the year. In conclusion, CT is a consolidated technique to diagnose CYTO at AI, PMN 1 % is the threshold level to diagnose CYTO at AI, around one-quarter of the uteri that are inseminated suffer from CYTO, affected uteri having a significantly lower chance to become pregnant from that insemination.

INTRODUCTION

A high reproductive performance is a decisive factor for production and hence profitability in modern dairy herds [1] and [2]. To assess an adequate reproductive efficiency, the first insemination conception rate must be as high as possible. However, multiple factors such as semen quality, insemination technique, heat detection efficiency, delayed ovulation, and uterine diseases like purulent vaginal discharge and/or subclinical endometritis (SCE) are known to significantly affect the success of an artificial insemination (AI) [3] and [4].

It is well accepted that the "most convenient" method to diagnose SCE is by measuring the polymorphonuclear (PMN)/epithelial cell ratio in endometrial cytology samples (CYTO) [5]. However, standardization of SCE diagnosis by means of CYTO has not been fully established yet [6]. In different publications, the time relative to calving the samples were taken varied from 21 to 64 days in milk (DIM) [7], [8], [9] and [10]. Concomitantly, PMN threshold levels to diagnose CYTO ranged from 3 to 18 % [11] and [12], resulting in a wide variation (9 to 76 %) in reported CYTO prevalence [13]. Consequently, comparing results between studies is almost unfeasible. However, a novel technique "Cytotape" (CT), was developed in order to take endometrial samples at the moment of AI [6]. This simple technique achieved high cytology standards when compared with the cytobrush, its main advantage being the possibility to sample cows during AI, by using ordinary material. Sampling during AI may have three significant benefits: 1) standardization of the moment of sampling, and assessment of the uterine health status at the most critical point: the moment of insemination; 2) allowing the use of a universal PMN cut-off point, since the moment of sampling is standardized; and 3) no extra manipulation of the animal is required, since CYTO sampling and AI are performed simultaneously.

The timing of SCE (CYTO) examination should allow to take into consideration the proper process of uterine involution, which is based on an inflammatory reaction [5]. However, an inflammatory status at inappropriate stages of the reproductive cycle, inflicts damage on gametes and zygotes, impairing the pregnancy outcome [14]. Polymorphonuclear cells represent the first defense line and the principal cell type recruited during uterine inflammation [15] and [16]. The presence of PMNs in the

uterine lumen is the result of an inflammatory cascade that originated by the activation of immune receptors leading to a pro-inflammatory state characterized by the secretion of inflammatory cytokines and chemokines [14], [17], [18], [19] and [20]. This pro-inflammatory milieu interferes with fertility by creating suboptimal conditions for sperm cell transportation and storage, oocyte maturation and ovulation, fertilization, zygote development, implantation and embryonic and fetal growth [14], rising the amount of sub-fertile animals in dairy farms.

Thus, aims of the present study were to: 1) consolidate the CT as a valid technique to diagnose CYTO during AI, 2) define the PMN % cut-off point above which the conception result of the AI is significantly decreased, 3) establish the prevalence of CYTO at AI in dairy cows, and 4) evaluate the effect of CYTO at AI on the conception rate.

MATERIALS AND METHODS

Study design

This prospective observational cohort study was conducted from July 2014 to March 2015. In order to achieve a study with 80 % power and a 95 % confidence interval (CI) (Dohoo et al., 2009), the sample size was calculated to identify a difference of 10% in conception rate between diseased (30 %) and non-diseased (40 %) cows, with an expected CYTO prevalence of 30 %. Based on this calculation, a total of 1,625 AIs were performed in 873 Holstein-Friesian cows from 18 dairy farms. All participating dairy herds were located in the Flemish region of Belgium and were using free stalls for housing. Herd size ranged from 24 to 176 with an average of 76±38 cows per herd. Cows were fed a total mixed ration according to their production level and were milked twice daily. Farm level inclusion criteria were: the willingness of the farmer to cooperate, and the availability of a data record-keeping software for fertility and milk production parameters. All included herds participated in the official milk recording system in which cows are sampled every four to six weeks to assess daily milk yield, fat, protein, lactose, urea and somatic cell count (SCC) level. Cow-level inclusion criteria were: healthy Holstein-Friesian cows presenting estrus and offered for insemination. Cows were sampled more than once if they did not conceive at the first AI-sample and

were offered for a next AI. Body condition score (BCS; 1-5) was evaluated just before the AI-sample in all cows [21].

Sampling procedure

Cows were inseminated based on the AM-PM rule [22], generally after spontaneous heat expression. All inseminations were done after the voluntary waiting period (VWP), which was set at 60 DIM. One experienced veterinarian from the company Cattle Improvement Co-operative (CRV-Belgium) performed all AIs and simultaneously acquired the endometrial cytology samples using the CT. Cytotape consisted of a 1,5 cm piece of paper tape (Tesa 4322, Hamburg, Germany) rolled on the top of a standard AI's catheter, covered with a double guard sheet (Sani-Shield Rod®; Agtech, Manhattan, KS, USA) [6]. All the CT sheets were prepared in advance, so once at the farm, they were ready to use. Briefly, the AI gun was introduced into the vagina and under rectal guidance manipulated through the cervix. Once in the uterine lumen (corpus uteri), the tip of the catheter was released from the double guard sheet. Then, it was rolled twice on the dorsal wall of the uterine body [23] with a gentle pressure of the index finger through the rectum. At the end, prior to the removal of the AI gun from the genital tract, the AI was performed, the catheter was covered with the double guard sheet, and removed from the cow's genital tract.

Preparation, staining of the slides and microscopic evaluation

Slides were prepared at the farm immediately following the AI-sampling. The top of the CT was gently rolled on a clean microscope glass (Marienfeld, Lauda-Königshofen, Germany) procuring to spread the cellular material homogeneously over the entire slide. Next, after proper identification, slides were air dried and housed in a slide box. Approximately every two weeks, boxes were delivered to the laboratory facilities for staining and evaluation. Stainings were done using Diff Quick (Fisher Diagnostics, Newark, DE, USA) according to the instructions of the manufacturer. Mounting media (Eukitt®; O.Kindler GmbH, Freiburg, Germany) was applied on the slides with a Pasteur pipette and then covered with coverslips (Marienfeld, Lauda-Königshofen, Germany). Samples were conventionally evaluated by light microscopy (Kyowa Optical, Tokyo, Japan). Neutrophils and epithelial cells were assessed, and their ratio was calculated at 100 x and 400 x magnifications **(Figure 1)** after counting 300 nucleated cells [24]. Also,

total cellularity and quality of the samples were assessed in 10 high power fields. Total cellularity and quality were respectively classified as low (< 50 cells), moderate (50-100 cells), and high cellularity (> 100 cells); bad (< 50 % intact cells), good (50-75 % intact cells), and very good quality (> 75 % intact cells).

Conception results were assigned as follows: an insemination was considered successful when pregnancy was confirmed by rectal palpation at least 45 d post-AI. Inseminations were defined as not successful when they were followed by another insemination or when the animals were diagnosed empty by rectal palpation at least 45 days post-AI.

Statistical analyses

Data exploration and descriptive statistics. Individual cow data were exported both from the computerized record system of the herd as well as from the manually collected data sheets to a Microsoft Excel (Microsoft Corporation, Seattle, USA) work file. Prime data exploration was done with the PivotTables function (Microsoft Excel), and then re-organized and classified accordingly. All statistical analyses were done using R version 3.3.0, 2016 (R Inc., Boston, USA). The function summary of the R code system (package Base) was used for the descriptive statistical analyses.

Factors affecting AI success including PMN% as an indicator of uterine health. Multilevel generalized mixed effect models were built to determine the association between the pregnancy outcome of the AI and independent variables. The responsive variable for the AI success was binomial, with cows classified as being pregnant or not pregnant. The function glmer of the package lme4 [25] was used to run the models. First, univariable models were constructed and factors that were associated with the AI success ($P < 0.2$) were identified. Next, Pearson and Spearman's correlation coefficients were calculated among the significant independent variables to avoid multicollinearity in the next steps. If two independent variables had a correlation coefficient ≥ 0.55, only the one with the highest statistical significance or the most biologically relevant variable was selected for further analysis. Finally, multivariable models were built by manual stepwise backward elimination. Only risk factors and first order interactions with P-values < 0.05 were retained in the final model. The AI-CT sample number nested within cow, nested within farm (three level model) was chosen as random effect to correct for

the fact that some cows were sampled more than once. The fixed effects tested were: ease of calving at previous parturition (eutocia, dystocia [traction, caesarean section, twins]), previous performance of AI (no, yes), previous harvesting of a CT sample (no, yes), PMN% at AI, BCS (1-5), DIM at AI, month of the AI, bull sire, parity (primiparous, multiparous) and the milk production measures which were obtained during the official milk recording closest to the AI [milk yield (Kg), protein (g/l), fat (g/l), lactose (g/l), urea (mg/l) and SCC (x 1000/ml)]. The SCC was expressed as the log to normalize the data. Moreover, the relative 305 days milk yield (Kg) was offered to the models. These values were expressed as the proportional 305 d yield as compared to the average of the farm blocked by parity [26] and [27]. Body condition score and DIM at AI were categorized into lean (< 1.5), normal (2-3.5) and fat (> 3.5), and 61 to 81, 82 to 102, 103 to 123, 124 to 144, and ≥ 145 DIM, respectively. All results are expressed as Odds Ratio (OR) with their respective 95 % CI.

Construction of the ROC curve to assess the PMN cut-off point for CYTO diagnosis. A receiver operator characteristics curve (ROC curve; package pROC [28]) and the area under the curve (AUC, package cvAUC [29]) were constructed to find the optimal cut-off point value revealing the greatest summation of sensitivity and specificity of the PMN percentage in the endometrial cytology sample significantly affecting the pregnancy outcome. The multivariate generalized mixed effect model identifying the factors being associated with the AI success as described above, was used to compute predictions of the pregnancy outcome, at the individual sample level. The prediction results were interpreted as continuous variables ranging from 0 to 1. To dichotomize the predictions, a dummy variable was created using a threshold level of 0.5 (prediction 0.5 = 50 % chance to become pregnant). All the values > 0.5 were considered as positive predictors for pregnancy, and all variables ≤ 0.5 were considered as negative predictors for pregnancy. To construct the ROC curve, the newly developed dummy variable representing the individual conception result was used as the classifier and the PMN percentage of the sample-AI as the predictor.

Prevalence and effect of CYTO on the pregnancy outcome. After establishing the cut-off point for CYTO diagnosis, the prevalence of CYTO at the insemination and farm level was assessed by the function summary of the R code system (package Base). Finally, CYTO diagnosis was introduced as a categorical fixed factor, first in the univariate

model and then in the multivariate generalized mixed effect model to evaluate its effect on the AI success.

RESULTS

Descriptive statistics

A total 1,625 AI-samples from 873 cows in 18 dairy farms were harvested. Ninety-nine point six percent (n = 1,618) of these samples were considered readable and thus acceptable for further analysis. Seven samples were rejected (five cows) because of none or scant cells (< 300 cells). Of the readable samples, 82.9 % (n = 1,342) revealed moderate cellularity **(Figure 1 A)**, 12.1 % (n = 196) high cellularity and the remaining 4.9 % (n = 80) a low number of cells. The greatest majority (93 %, n = 1,505) of the slides displayed a high number of intact cells (> 80 %) and were therefore classified as very good regarding quality **(Figure 1 B)**. Only 7 % (n = 113) were of moderate quality, while no samples were considered to have bad quality. The average PMN % per slide was 1.5±5. Initially, 873 cows were enrolled but 27 cows were excluded due to unavailability of fertility data (culled or sold). Thus, 1,578 AI-samples from 846 cows had satisfactory cytology and reproductive follow-up data. Milk production information was available from 797 cows (1,508 samples). Cows with no milk production data were recorded as missing values and therefore excluded from the risk factor analysis. The average individual 305 days milk yield and the milk yield closest to the AI time were 9,210±1,680 and 31.6±7.6 Kg, respectively. Average protein, fat, lactose, urea and SCC in milk were 34.8±3.6 g/l, 41.8±6.7 g/l, 46.1±1.9 g/l, 231.7±7 mg/l, and 179.5±514 (x 1000/ml), respectively. The mean number of AIs per cow was 2.2±1.5 while an average of 1.8±1.2 AI-samples were obtained per cow. Average DIM at the AI-sample was 122±54. Overall conception rate at the sample level was 43 % (n= 679).

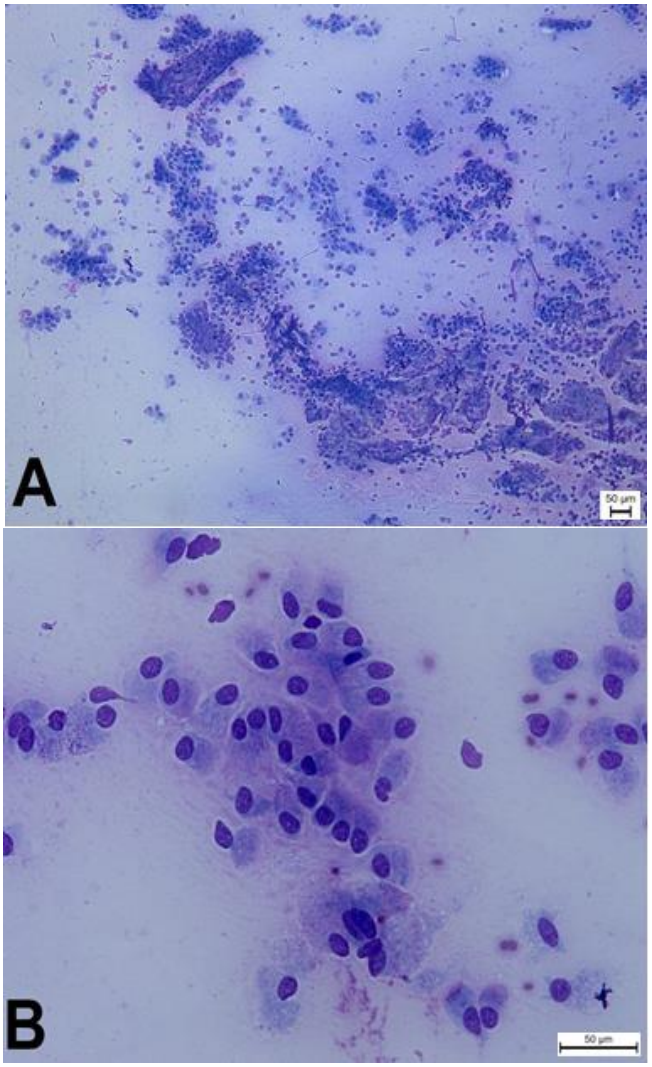

Figure 1. Microscopic evaluation of endometrial cytology samples harvested by Cytotape during AI. A) 100 x. B) 400 x.

Factors affecting AI success including PMN% as an indicator of uterine health

Results of the univariable analysis are summarized in **Table 1**. Individual risk factors ($P < 0.2$) in the univariable analysis were dystocia, BCS, parity, the month of the AI, performance of a previous AI, the PMN % at AI, individual milk production closest to the AI-sample, and the proportional 305 days milk yield. Manual stepwise backward regression of the individual risk factors ($P < 0.05$) and their significant first degree interactions resulted in the final multivariable model, which included dystocia, BCS, lactation, the month of the AI, performance of a previous AI, the PMN % at AI, proportional 305 days milk yield and the interactions BCS*dystocia and previous AI*parity **(Table 2)**. The performance of a previous AI was not significant in the multivariable analysis, still it was retained in the final model since its interaction with parity was significant ($P < 0.05$).

Table 1. Results of the univariable models of factors affecting the AI success in dairy cows.

Variables	Odds ratio	95% CI	*P*-value
Last calving[1]	-	-	0.04[a]
Eutocia	Reference	-	-
Dystocia	0.58	0.35-0.97	0.04[a]
Previous CT sampling[1]	-	-	0.51
No	Reference	-	-
Yes	1.07	0.88-1.31	0.51
Previous AI[1]	-	-	0.13[b]
No	Reference	-	-
Yes	0.85	0.69-1.04	0.13[b]
BCS[1]	-	-	0.12[b]
Normal	Reference	-	-
Fat	1.02	0.79-1.31	0.85
Lean	0.77	0.59-1.01	0.05[a]
BULL[1]	-	-	0.31
Month of AI[1]	-	-	0.003[a]
Parity[1]	-	-	0.14[b]
Primiparous	Reference	-	-
Multiparous	0.84	0.68-1.05	0.14[b]
DIM[1]	-	-	0.56
61-81	Reference	-	-
82-102	1.11	0.89-1.41	0.41
103-123	0.86	0.61-1.24	0.71
124-144	0.98	0.68-1.41	0.91
≥145	1.03	0.79-1.33	0.82
Rmilk 305,%[2]	0.99	0.98-0.99	0.01[a]
Daily milk production[2]	0.99	0.97-1.01	0.18[b]
Milk fat[2]	1.08	0.93-1.26	0.31
Milk protein[2]	0.98	0.74-1.31	0.92
Milk lactose[2]	1.05	0.63-1.79	0.83
Milk urea[2]	0.99	0.98-1.01	0.58
Milk SCClog[2]	0.89	0.75-1.08	0.15[b]
PMN,%[2]	0.92	0.89-0.95	0.0001[a]
CYTO[1]	-	-	0.0001[a]
Positive	Reference	-	-
Negative	1.85	1.46-2.35	0.0001[a]

[a] and [b] risk factors with $P < 0.05$ and < 0.2, respectively; CI confidence interval, AI artificial insemination, CT cytotape, BCS body condition score, DIM days in milk, Rmilk 305 relative milk production in 305 days blocked by farm and parity, SCC somatic cell count, PMN polymorphonuclear cells, CYTO cytological endometritis.
[1]Categorical variables; [2]Continous variables.

Table 2. Results of the multivariable mixed effects analysis on parameters affecting the AI success in dairy cows.

Variables		Odds Ratio	95% CI	*P*-value
BCS[1]	Normal	Reference	-	
	Fat	0.97	0.68-1.27	0.22
	Lean	0.64	0.44-0.85	0.03[a]
Previous AI	No	Reference	-	
	Yes	0.83	0.56-1.23	0.36
Last calving[1]	Eutocia	-	-	-
	Dystocia	0.34	0.16-0.74	0.006[a]
Parity[1]	Primiparous	Reference	-	-
	Multiparous	0.65	0.45-0.93	0.02[a]
Rmilk 305[2]	%	0.99	0.98-0.99	0.02[a]
CYTO[1]	Positive	Reference	-	-
	Negative	1.76	1.37-2.25	0.0001[a]
Month of the AI[1]	January	Reference	-	-
	July	0.64	0.35-1.17	0.15
	August	0.58	0.38-0.89	0.01[a]
	September	0.57	0.38-0.86	0.007[a]
	October	0.86	0.57-1.28	0.45
	November	1.17	0.71-1.93	0.51
	December	0.76	0.51-1.12	0.16
	February	0.75	0.49-1.16	0.21
	March	1.04	0.68-1.61	0.86
BCS*Last calving[1]	Fat*Dystocia	0.19	0.05-0.79	0.02[a]
	Lean*Dystocia	0.29	0.07-1.15	0.07
Previous AI*Parity	No*Primiparous	1.68	1.05-2.68	0.03[a]

[a] Factors significantly affecting the AI success; CI confidence interval, AI artificial insemination, BCS body condition score, Rmilk 305 relative milk production in 305 days blocked by farm and parity, CYTO cytological endometritis.
[1]Categorical variables; [2]Continous variables.

Construction of the ROC curve to assess the PMN cut-off point for CYTO diagnosis

Based on the outcome of the multivariable model, a ROC curve to reveal the PMN % cut-off point was constructed **(Figure 2)**. The ROC curve revealed that the cut-off point for CYTO at AI was ≥ 1 % PMN. The area under the ROC curve was 0.62 (CI; 0.6-0.64). Sensitivity and specificity for predicting pregnancy using this threshold level were 33.8 % and 88.6 %, respectively **(Table 3)**. Then, after dichotomization of the PMN % (CYTO positive = PMN ≥ 1 %, CYTO negative = PMN < 1), CYTO was introduced first in an univariable model **(Table 1)**, and since its effect was highly significant (P = 0.0001), it was included in the final multivariable model **(Table 2)**. The prevalence of CYTO using PMN ≥ 1 % as the cut-off point in the readable samples was 27.8 % (n = 450). Conception rate in CYTO positive samples (n = 443) was 32.7 % (n = 145) while it was 47 % (n = 534) in the negative specimens (n= 1,135) (P < 0.01). Cytological

endometritis prevalence per farm ranged from 10.7 to 39.7 % with an average of 28.1 %.

Table 3. Cytological endometritis (CYTO) prevalence, pregnancy outcome, sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), and significance level (P-value [Pearson chi-squared]) of PMN% thresholds at different levels of endometrial cytology samples (n= 1,578) acquired in dairy cows (n= 797) simultaneously with the AI.

Threshold (PMN%)	CYTO prevalence (%)	Pregnancy outcome[1] (%)	Se (%)	Sp (%)	PPV (%)	NPV (%)	P-value
≥ 1%	27.8	32.7	33.8	88.6	89.2	32.4	< 0.01
≥ 2%	19.9	28.7	25.3	95.2	93.6	31.3	< 0.01
≥ 3%	14.6	26.9	19.1	98.8	97.7	30.4	< 0.01
≥ 4%	9.3	22.5	12.7	99.8	99.3	29.1	< 0.01
≥ 5%	7.4	17.8	10	100	100	28.6	< 0.01

[1]pregancy outcome of the AI-CYTO sample.

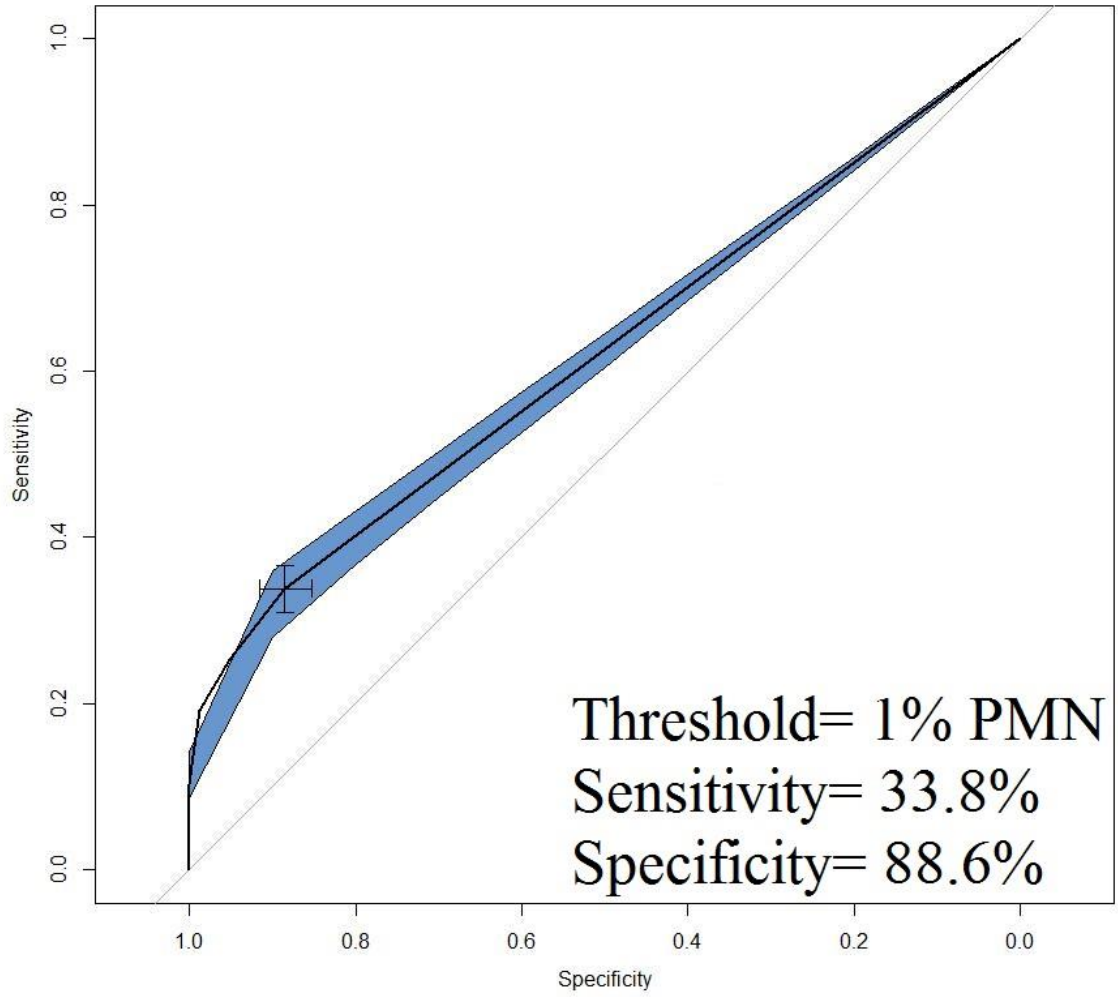

Figure 2. Receiving operating characteristic curve showing the cytological endometritis (CYTO) cut-off point at AI (highest point for sensitivity and specificity [+]).

DISCUSSION

Descriptive statistics

The finding that more than 99 % of the samples harvested by CT were adequate for microscopic evaluation, indicates that it is a consolidated technique to diagnose CYTO at AI. Moreover, the outcome of the conception rate in CT sampled cows during insemination (43 %) was identical in comparison to recent analogous studies in the Flemish and Dutch regions [26], [27] and [30]. The latter demonstrates that CT is a technique which allows to explore the uterine health status at the moment of insemination without detrimental effect on the AI pregnancy outcome. Currently, uterine cytology samples are mostly taken for scientific purposes during the VWP [13], while only a very limited number of farms are routinely using cytology to diagnose SCE. The lack of feasibility and the "extra" labor are main reasons to not routinely evaluate uterine health by cytology in commercial dairy farms. Consequently, CYTO is considered to only have a secondary role when inadequate pregnancy results are evaluated. Although, the CT technique is not a 100 % cow side diagnostic test, it is one step forward towards an easier CYTO diagnosis. In this context, the use of the leukocyte esterase (LE) colorimetric test could be an on farm alternative for CYTO [31]. However, the LE diagnosis for SCE should be preceded by a cytobrush [32] or low volume lavage [33] sampling, which is still done during the VWP and involves "extra" labor.

Factors affecting AI success

Many publications extensively described risk factors affecting the AI success in dairy cows [30], [34], [35], [36], [37] and [38], each of them generally focusing on distinct and specific factors. In the present study, we aimed to assess the effect of uterine health at the moment of insemination on the AI pregnancy outcome. Fertility is however, a multifactorial trait, the final pregnancy outcome being dependent on numerous influencing factors. Consequently, a multivariable analysis was performed to identify factors that are significantly associated with the pregnancy result. In this model, a low BCS, dystocia, insemination during the warmer months of the year, a high PMN % during AI, an elevated proportional 305 days yield, parity, and the interaction between a high BCS (≥ 3.5) and dystocia were found to have a detrimental effect on the AI success. In previous studies, the exacerbated fall of BCS following a dystocic parturition was

already demonstrated to negatively affect the reproductive performance of a cow mainly due to a strong negative energy balance [39] and [40]. On the other hand, a well-balanced nutritional status as reflected by an adequate BSC, is generally correlated with a better reproductive performance [41]. It is well known that heat stress is associated with an impaired quality of the oocyte and embryo development and hence also with a higher risk of early embryonic loss [42]. Although in the present study the sampling procedure did not go through all months of the year, fertility was significantly lower for inseminations that were performed in warm months such as August and September. Furthermore, we also found a significantly lower chance of pregnancy in cows yielding more milk than their peers in the herd. Probably, the metabolic stress of producing more milk than cows under the same condition (same management and parity) could interfere with the fertility outcome [35]. Moreover, as described by Lucy [43], in response to genetic selection for milk production, the reproductive physiology of the cow has changed to longer intervals to first ovulation, higher incidence of anestrus, abnormal luteal phases, and greater embryonic loss, resulting in a decline of the reproductive efficiency. In concordance with Inchaisri et al. [27], we found a higher conception rate in first parity animals in comparison to ≥ 2 parity cows. The latter especially at the first service (no previous AI*primiparous). Contrary to Inchaisri et al. [27] however, the probability of a successful AI did not vary with DIM. None of the measured milk quality parameters (protein, fat, lactose, urea, and SCC) nor their interactions were found to have a significant effect on the AI success.

Cut-off point for CYTO diagnosis, CYTO prevalence at the moment of insemination and its effect on pregnancy outcome

The cut-off point for CYTO at AI was set at ≥ 1 % PMN. However, the sensitivity and specificity of this threshold level was relatively low (33.8 %) and high (88.6 %), respectively. Additionally, the high positive predictive value (89.2 %) and the low negative predictive value (32.4 %) **(Table 3)** indicate that a CYTO positive cow at insemination is very likely to not become pregnant from that insemination. On the other hand, a CYTO negative sample at insemination is not a good predictor of the pregnancy outcome of that insemination. The latter seems obvious since as previously mentioned, multiple factors affect the conception rate, not only the absence of CYTO. In studies in which CYTO was evaluated in relation with repeat breeding and hence samples were

also taken late after calving, different cut-off points for CYTO diagnosis have been implemented [12] and [44]. In the study of Salasel et al. [12], results of a ROC curve analysis revealed a PMN level of 3 % as the threshold level for CYTO. In the other study [44], the threshold level was arbitrarily set at 5 % PMN. The cut-off level of PMN 3% is relatively close to our finding, however, Salasel et al. [12] performed a low volume lavage technique to acquire the endometrial cytology samples. By using this technique, a larger surface of the endometrium is sampled, yielding a higher chance to collect more PMNs [9], [45], [46] and [47], which may have revealed the slightly higher cut-off level in comparison to our study.

Approximately one quarter of the uteri that were inseminated suffered from CYTO, positive uteri having a significantly lower chance to become pregnant from that insemination. Consequently, we suggest the moment of AI to be convenient to sample for CYTO diagnosis, since a sample harvested at that time may better reflect the health of the endometrium and hence its receptivity to a young embryo. However, further studies to compare sampling during the postpartum period with sampling at the moment of insemination are necessary to confirm this finding. The wide variation in both DIM as well as in the applied PMN % threshold level at sampling, may explain the results of studies in which no association was found between CYTO and subsequent fertility [48]. Furthermore, in our experience, sampling during estrus while inseminating the cow, allows to take advance of the fact that the cervix is open. Reaching the uterine body is relatively easy at that moment, while the act of sampling did not impede to inseminate the cow. The latter furthermore minimizes the risk of traumatization of the genital tract which is a real concern when samples are taken when the cervix is closed .

Sampling during AI may however be controversial since according to some authors, high estrogen concentrations might provoke a physiological PMN-infiltration in the endometrium [49] and [50], which might give rise to false positive CYTO results. However, in the present study, in > 72 % of all readable samples that had been harvested during AI, no single PMN was found. The latter is in agreement with Madoz et al. [51] who also concluded, based on cytology sampling by cytobrush, that no PMN-infiltration could be shown when samples are taken during estrus. On the other hand, a

post-breeding inflammatory response following AI was illustrated in cows [52] as it is widely known to occur in mares [53] and [54].

Acquiring cytology samples during AI creates furthermore innovative perspectives in CYTO diagnosis and treatment. For example, the examination by CT during first insemination allows to explore uterine health without any "extra labor cost". If the breeding is not successful and the cow is diagnosed CYTO positive, it will be possible to set up a more targeted treatment to increase the odds of pregnancy at a subsequent AI. The use of this new tool could furthermore be interesting when inseminating and simultaneously sampling repeat breeder cows aiming to more accurately diagnose the underlying reason of the repeat breeding. However, it is important to mention that there currently is no consensus concerning an effective CYTO treatment. Some authors demonstrated the benefits of an intra-uterine cephapirin treatment [55] and [56] while others did not [57]. Recently, we have demonstrated the possibility to reduce the amount of PMNs present in the uterine lumen by applying an uterine lavage with 500-600 ml sterile saline solution [58]. However, further field studies are necessary to confirm this as a potential treatment for repeat breeder cows suffering from CYTO. Contradictory results have been put forward regarding the application of non-steroidal anti-inflammatory drugs (NSAIDs) following AI [59], [60], [61] and [62]. However, a treatment with NSAIDs specifically targeted for cows suffering from CYTO diagnosed at AI, could represent a valid strategy to improve the pregnancy outcome of that AI. The wide variation in CYTO prevalence among different herds, furthermore suggests that management factors significantly influence the risk for CYTO [7] and [63]. The latter invites to identify the risk factors associated with CYTO during AI.

CONCLUSIONS

Cytotape is a consolidated technique to diagnose CYTO at AI. The PMN cut-off point to diagnose CYTO during AI is ≥ 1 %. Approximately one-quarter of inseminated uteri of modern dairy cows suffer from CYTO. Conception rate is significantly lower in CYTO positive inseminations. The odds to become pregnant for a cow that is diagnosed CYTO negative at the moment of insemination are 1.8 times higher than for a CYTO positive animal. Innovative strategies regarding CYTO diagnosis and treatment can be foreseen

when cytology samples are harvested simultaneously with AI. Further research is needed to investigate the risk factors that are significantly associated with CYTO diagnosed during AI to reduce the prevalence of the disease.

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Chapter 5.3

Risk factors associated with cytological endometritis diagnosed at artificial insemination in dairy cows

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ABSTRACT

In this study, we aimed to determine risk factors associated with cytological endometritis (CYTO) diagnosed at artificial insemination (AI) in dairy cows. The CYTO risk factors were evaluated based on 1.625 AI-CYTO samples obtained from 873 Holstein-Friesian cows from in total 18 dairy herds in Flanders (Belgium). The endometrial cytology samples were obtained using the cytotape technique, which consisted in adapting a 1.5 cm piece of paper tape to a standardly loaded AI catheter, covered with a double guard sheet. The polymorphonuclear cells' (PMNs) cut-off point for CYTO at AI was set at ≥ 1 %. We constructed multilevel generalized mixed effect models in order to identify the risk factors associated with the presence of CYTO at AI. The CYTO prevalence at AI was 27.8 % at the animal level, while the within-herd level prevalence ranged from 10.7 to 39.7 %, with an average of 28.1 %. Risk factors associated with the occurrence of CYTO were parity ≥ 2 [odds ratio (OR) = 1.8], days in milk (DIM) at AI ≥ 124 (OR = 0.4), and warm moths of the year [July (OR = 2.9), August (OR = 2.3), and September (OR = 1.4)]. In conclusion, the present study supports that multiparous cows and cows that are inseminated in the summer months have a higher risk to suffer from CYTO at insemination, while the risk for CYTO is lower when the insemination is taking place at ≥ 124 DIM.

INTRODUCTION

Dairy cows farmed in intensive systems commonly need to deal with a high microbial contamination during and immediately after parturition [1]. If this bacterial contamination evolves to uterine infection, a proportion of the postpartum cows will eventually develop uterine disease [2]. Within the postpartum uterine disease complex, subclinical endometritis plays an extraordinary role, being: highly prevalent, asymptomatic, and with a negative effect on subsequent fertility [3] and [4]. Although endometrial cytology (cytobrush [5] or low volume lavage [6]) are well-accepted techniques to diagnose cytological endometritis (CYTO) during the voluntary waiting period (VWP) [7], it may not reflect the uterine health status at the time of fertilization and subsequent implantation. Consequently, an innovative technique (cytotape [CT]) was recently developed to take endometrial samples during artificial insemination (AI) in order to evaluate the prevalence and effect of endometrial inflammation at the time of mating [8].

Prevalence of CYTO widely ranges among farms (9 to 76 %) [6] and [9], suggesting that herd as well as individual risk factors are highly associated with the occurrence of this disease [9]. Risk factors for CYTO occurring during the voluntary waiting period, have been identified as retained placenta, negative energy balance, and acute metritis [5], [9] and [10]. However, to the best of our knowledge, no study exists about the risk factors that are significantly associated with CYTO at AI. A high CYTO prevalence causes considerable economic losses to dairy farmers due to inadequate fertility results. Consequently, identifying risk factors associated with CYTO at AI, may allow to reduce the prevalence of this disease and the concomitant costs by relatively simple management interventions. Thus, in the present study, we aimed to determine the risk factors associated with CYTO diagnosed at AI in dairy cows.

MATERIALS AND METHODS

A convenience sampling protocol of dairy farms in the Flemish region of Belgium based on the availability of a computerized record system and the farmers' willingness to collaborate, was implemented for this prospective cross-sectional cohort study. First,

a sample size calculation was done to assess the minimal number of inseminations to be included in order to perform a study with 80 % power and a 95 % confidence interval (CI) [11]. This sample size was designed based on an expected CYTO prevalence of 30 % and an anticipated difference of 10 % in the conception rate between affected (30 %) versus unaffected (40 %) cows. This sample size calculation revealed that minimally 1.498 inseminations should be included in the study. Finally, from July 2014 to March 2015, a total of 1.625 AIs in 873 Holstein-Frisian cows from 18 herds were included. Herd size ranged from 24 to 176 with an average of 76 cows per herd, all cows being housed in free-stall barns with cubicles. Cows were milked twice daily and fed with a partially mixed ration and additive concentrates according to their milk production level. All included farms participated in the official milk recording system in which milk samples were analyzed every four to six weeks in order to assess milk yield as well as the fat, protein, lactose, urea and somatic cell count (SCC) level in milk. Cows presenting standing estrus (visual observation), and that were identified by the farmer to be inseminated, were enrolled in the trial. Cows showing signs of abnormal vaginal discharge were not included. Cows that not conceived at the first AI-sample were eventually sampled more than once. The body condition score (BCS; 1-5) [12] was assessed at AI.

All herds implemented exclusively AI for breeding the cows. All inseminations were done after the VWP [which was set at 60 days in milk (DIM)] by one experienced veterinarian from the company Cattle Improvement Co-operative (CRV-Belgium), generally after spontaneous heat expression (based on the a.m./p.m. rule [13]). Endometrial cytology samples were taken during AI using the CT technique [14]. Briefly, the AI sheath (IMV, L'Aigle, France) was prepared in advance by rolling a 1,5 cm piece of paper tape (Tesa 4322, Hamburg, Germany) on the top of the AI pipette and then covered with a 12-inch-long Sani-Shield Rod® (Agtech, Manhattan, KS, USA). At the farm, after conventionally loading the universal insemination gun (Agtech, Manhattan, KS, USA) with a frozen-thawed semen straw (0.5 or 0.25 ml), it was mounted with the CT-AI sheet. Once ready, the CT-AI gun was carefully introduced in the genital tract of the cow and manipulated through the cervix under rectal guidance. In the uterine lumen, the top of the CT-AI gun was released from the Sani-Shield Rod®. Then, with some gentle pressure of the index finger through the rectum, the cytology sample was

taken by rotating the top of the AI catheter on the dorsal wall of the corpus uteri. Next, after injection of the semen, the catheter was covered with the Sani-Shield Rod® and carefully removed from the genital tract of the cow. Finally, the cellular material on the top of the paper tape was uniformly spread (rolled) on a clean microscope slide (Marienfeld, Lauda-Königshofen, Germany), air-dried, and housed in slide boxes. At the laboratory facilities, slides were stained with Diff Quick (Fisher Diagnostics, Newark, DE, USA), and once dry mounted with Eukitt mounting media (O.Kindler GmbH, Freiburg, Germany). The cytologic evaluation was made by light microscopy (Kyowa Optical, Tokyo, Japan) at 400 x. The polymorphonuclear cells proportion (PMNs %) was assessed by counting 300 cells (epithelial and PMNs) [15]. The threshold level for CYTO was set at ≥1 % PMNs.

All statistical analyses were performed using R version 3.3.0, 2016 (R Inc., Boston, USA). Individual cow data such as CYTO (positive, negative), previous calving ease [eutocia, dystocia (traction, caesarean section, twins)], performance of a previous AI (no, yes), body condition score (BCS) at AI (1-5) [12], DIM at AI, month in which the AI was performed, parity (primiparous, multiparous) and milk yield measurements that were obtained during the official milk recording closest to the AI [milk yield (Kg), protein (g/l), fat (g/l), lactose (g/l), urea (mg/l) and SCC in milk (x 1000/ml)], were exported from the data record-keeping software of the farm into a Microsoft Excel spreadsheet file (Microsoft Corporation, Seattle, USA), and the R working environment. First, descriptive statistics were computed using the package Base of the R code system. Then, in order to determine the risk factors being significantly associated with CYTO (positive, negative), we constructed multilevel generalized mixed effects models (function glmer, package lme4 [16]). The CT sample number nested within cow, nested within herd were included as a random effect. The binomial CYTO condition (positive, negative) was set as the responsive variable. All information collected at individual cow level (previously mentioned above) was tested as fixed effects (explanatory variables). The BCS was categorized as lean (< 1.5), normal (2-3.5) and fat (> 3.5). The DIM at AI was categorized as follows: 61 to 81, 82 to 102, 103 to 123, 124 to 144, and ≥ 145 DIM [17] and [18]. The SCC in milk was expressed as SCC-log in order to normalize the data distribution. Moreover, the proportional 305 days milk yield was calculated and offered to the models. The proportional 305 days milk yield consisted in the 305 days milk yield

of each cow blocked by parity and by farm [17]. All fixed factors showing P < 0.2 in the univariable models, and which were not highly inter-correlated (r < 55), were offered to the multivariable analysis by backward stepwise elimination. The final model of the multivariable analysis consisted of fixed effects (and first degree interactions) with P-value < 0.05. Results are expressed as odds ratios with their respective confidence interval [11].

RESULTS

Initially, a total 1.625 AI-samples from 873 cows in 18 dairy farms were included. However, 27 cows (47 AI-samples) were excluded due to the unavailability of their fertility outcome (culled or sold; n = 40) or bad quality of cytologic samples (zero or scant cells; n = 7). Finally, 1.578 AI-samples from 846 cows had satisfactory cytology and reproductive follow-up data. Of the 1.578 AI-samples, 537 (34 %) were harvested in heifers and 1.041 (66 %) in multiparous cows. Milk production information was available from 797 cows (1.508 samples). Milk production information with no data available were recorded as 'missing value'.

The average DIM at the moment of AI-sampling was 122±54, while the mean BCS was 2.78±0.65. The mean number of AIs per cow was 2.2±1.5 while each cow was AI-sampled 1.8±1.2 times on average. The overall conception rate at the sample level (n= 1.578) was 43 % (n = 679). The individual milk yield closest to the AI was 31.62±7.58 kg and the individual 305 days milk yield was on average 9.210±1.680 kg. Average protein, fat, lactose, urea and SCC in milk were 34.8±3.6 g/l, 41.8±6.7 g/l, 46.1±1.9 g/l, 231,7±70 mg/l, and 179.53±513.9 (x 1000/ml), respectively. The CYTO prevalence at AI was 27.8 %. At the herd level, the CYTO prevalence was 28.1 % ranging from 10.7 to 39.7 %.

Results of the univariable analyses are depicted in **Table 1**. Risk factors (P < 0.2) offered to the multivariable model were: performance of a previous AI, the month in which the AI was performed, parity, DIM at AI, and fat and protein in milk (g/l; measurement closest to the AI). After manual backward stepwise elimination of the individual risk factors and their respective first degree interactions (P < 0.05), a final model was obtained **(Table 2)**. Risk factors positively associated with CYTO at AI were:

warm months of the year (July, August and September) and parity (≥ 2), while a significantly negative association with CYTO at AI was found when cows were sampled after 123 DIM.

Table 1. Results of the univariable model of risk factors associated with cytological endometritis diagnosed at AI in dairy cows.

Variable	Odds ratio	95% CI	*P*-value
Last calving[1]	-	-	0.84
Eutocia	Reference	-	-
Distocia	1.1	0.62-1.81	0.84
Previous AI[1]	-	-	0.02[a]
No	Reference	-	-
Yes	1.3	1.1-1.62	0.02[a]
BCS[1]	-	-	0.21
Normal	Reference	-	-
Fat	0.8	0.68-1.05	0.24
Lean	1.2	0.98-1.35	0.33
Month of AI[1]	-	-	0.0001[a]
Lactation[1]	-	-	0.17[b]
1	Reference	-	-
≥2	1.9	0.95-1.48	0.17[b]
DIM[1]	-	-	0.15[b]
61-81	Reference	-	-
82-102	0.9	0.68-1.05	0.31
103-123	0.7	0.56-0.87	0.08[b]
124-144	0.7	0.59-0.82	0.07[b]
≥145	0.7	0.66-0.91	0.07[b]
Rmilk 305, %[2]	1.0	0.81-1.25	0.38
Daily milk production[2]	1.0	0.81-1.24	0.53
Milk fat[2]	0.8	0.64-0.99	0.008[a]
Milk protein[2]	0.7	0.55-0.85	0.02[a]
Milk lactose[2]	0.9	0.72-1.12	0.71
Milk urea[2]	0.9	0.81-1.25	0.89
Milk SCClog[2]	1.0	0.83-1.29	0.71

[a] and [b] risk factors with $P < 0.05$ and < 0.2, respectively; CI confidence interval, AI artificial insemination, BCS body condition score, DIM days in milk, Rmilk 305 relative milk production in 305 days blocked by farm and parity, SCC somatic cell count.
[1]Categorical variable; [2]Continuous variable.

Table 2. Results of the multivariable mixed effects analysis of risk factors associated with cytological endometritis (CYTO) in dairy cows.

Variable		Odds ratio	95% CI	*P*-value
Month of the AI	January	-	Reference	-
	July	2.9	1.65-5.27	0.0002[a]
	August	2.3	1.39-3.87	0.001[a]
	September	1.4	0.22-0.69	0.001[a]
	October	0.7	0.43-1.07	0.09
	November	1.1	0.72-1.69	0.64
	December	1.1	0.77-1.79	0.46
	February	0.4	0.91-2.17	0.13
	March	0.7	0.44-1.15	0.16
DIM	61-81	-	Reference	-
	82-102	1.3	0.71-2.52	0.77
	103-123	0.5	0.23-1.05	0.06
	124-144	0.4	0.14-0.99	0.05[a]
	≥145	0.4	0.22-0.82	0.01[a]
Lactation	1	-	Reference	-
	≥2	1.8	1.17-3.01	0.008[a]

*Factors significantly affecting the CYTO occurrence, CI confidence interval, AI artificial insemination, DIM days in milk

[1]Categorical variable; [2]Continuous variable.

DISCUSSION

This is, to the best of our knowledge, the first study that evaluated risk factors associated with CYTO at the most critical point, i.e. at the moment of AI in dairy cows. Postpartum uterine inflammation is a dynamic phenomenon starting immediately after calving and in many cases continuing till the end of the VWP. Earlier we have shown that in 27 % of the animals that are inseminated, CYTO is still present and is associated with lower pregnancy results.

Selection of potential risk factors to be examined, was based on biological relevance and data from the literature. One of the risk factors that we tested was DIM. To do so, we categorized the DIM at AI-CT sampling in five levels based on 21 day-intervals (a standard estrus cycle), starting from the end of the VWP (60 DIM) till ≥ 145 DIM. Although we did not find an association of CYTO occurrence from 60 till 123 DIM, uteri sampled after 123 DIM had a significantly lower chance to suffer from CYTO at AI. Interestingly, cows already showed a trend ($P > 0.06$) to be CYTO negative after 100 DIM. The moment of sampling for CYTO examination should take into account the duration of the uterine involution process [4], which is mainly fulfilled after 60 DIM. Yet,

in some cows, inflammation may persist even after 60 DIM. Literature supports that a previous uterine infection [9] or/and severe negative energy balance [19] and [20], delay the process of normal uterine involution [21], [22] and [23] and is associated with CYTO prevalence. In the present study, we did not have credible records of postpartum metritis or endometritis occurrence, and we did not measure negative energy balance indicators, reason why we did not include them in the risk factors analysis. However, independently of a previous postpartum uterine disease or a severe negative energy balance, we have demonstrated that most of the cows completed the uterine clearance (CYTO) starting from 100 to 120 DIM.

There is a general consensus nowadays that heat stress is a major contributing factor for low fertility in lactating dairy cows [24]. Multiple aspects contribute herein, among which the most important are the decreased expression of overt estrous symptoms and the reduced feed intake [25] and [26]. Although the present study did not include all the months of the year (July 2014 to March 2015), our results show that cows are more prone to suffer from CYTO during summer (July, August and September), suggesting that heat stress in lactating dairy cows is positively associated with CYTO diagnosed at AI. This is the first study to propose this hypothesis. Heat stress affects the reproductive performance of dairy cows both directly and indirectly [25]. Indirectly, mainly mediated through its deleterious effect on dry matter intake, finally prolonging the negative energy balance [25], [26] and [27]. The negative energy balance has shown to impair neutrophil function [23] and [28]. The PMNs represent the principal cell type recruited during uterine infection [29], and its dysfunctionality during the period of negative energy balance (accentuated by heat stress) may explain the increased prevalence of CYTO during the warm months of the year.

The effect of parity on the postpartum uterine disease complex is far from clear [30]. Gilbert et al. [6] found no significant effect of parity on CYTO prevalence. However, some authors [9] and [31] have found that primiparous cows had a higher risk to suffer from CYTO prevalence (in comparison to multiparous cows). They often refer to the fact that the initiation of milk production generates a greater metabolic stress in primiparous cows [21], which consequently causes the latter to be at a higher risk to suffer from CYTO. Other publications however indicated that the incidence of postpartum uterine diseases grows with increasing parity [32] and [33]. Our results

similarly show that multiparous cows have higher chances to suffer from CYTO in comparison to primiparous cows. Recently, Baez et al. [34] demonstrated that there is a negative association between the size of the uterus and the reproductive outcome, particularly in multiparous cows. Nevertheless, the biology behind the effect of uterine size on fertility is unknown. Although interpretations of results should be made with caution, we suggest that the greater size of the uterus in multiparous cows may interfere with complete uterine clearance after parturition, resulting in a higher CYTO prevalence at AI (persistent inflammation).

A low BCS at parturition has been described as a risk factor for CYTO [10]. Our study failed to demonstrate the BCS measured at AI to be a risk factor for CYTO. Cheong et al. [9] identified that primiparous cows with higher milk yield were at a higher risk for CYTO in comparison to multiparous cows. The high milk production is directly related to the metabolic stress (negative energy balance) in cows under the same condition (management and parity). However, we did not find an association between CYTO diagnosed at AI with an elevated milk production. In concordance with other similar publications [9] and [10], dystocia was not associated with CYTO occurrence. Probably, this may be due to the relatively extended period between the delivery of the cow and the AI-CT sampling (> 60 days). No milk related parameters were found to have a significant association with CYTO diagnosed at AI

CONCLUSIONS

Cows inseminated after 123 DIM had significantly lower chances to be CYTO positive. During summer, the CYTO prevalence diagnosed at AI increased significantly. Multiparous cows are 1.8 times more at risk to suffer from CYTO in comparison to primiparous ones. Although some risk factors may be controlled by relatively simple management decisions [cooling systems in well-designed barns accompanied with strategic feeding (high concentrate and low fiber) during the hot months of the year)], CYTO occurrence will still be inevitable. Therefore, more research regarding the development of an efficient cow side treatment that not involves an increase in the usage of antibiotics, is mandatory.

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Chapter 5.4

Prevalence of cytological endometritis and effect on pregnancy outcomes at the time of insemination in nulliparous dairy heifers

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ABSTRACT

The objectives of the present study were to assess the prevalence of cytological endometritis (CYTO) at the time of artificial insemination (AI) and its effect on pregnancy outcomes in nulliparous dairy heifers. In total, 512 endometrial cytology samples were taken during AI from 351 nulliparous Holstein-Friesian heifers using cytotape (a 1.5-cm piece of paper tape rolled on the top of an AI catheter covered with a double guard sheet). After sampling, the top of the AI catheter was gently rolled onto a glass slide, air-dried, and stained using Diff Quick (Fisher Diagnostics, Newark, DE). For each slide, 300 nucleated cells were counted, and the polymorphonuclear cell ratio (% PMN) was assessed at 400 x magnification. We constructed a receiver operating characteristic curve to find the cutoff point at which sensitivity and specificity (% PMN) affected pregnancy outcomes. The receiver operating characteristic curve revealed that the threshold level for diagnosing CYTO in nulliparous dairy heifers was 1% PMN. An insemination was considered successful when pregnancy was confirmed by rectal palpation at least 45 days post-AI. Heifers were considered not pregnant when they received a subsequent insemination or were diagnosed empty by rectal palpation. We built multilevel generalized mixed-effect models to test factors affecting pregnancy outcomes and the occurrence of CYTO at AI. We excluded 16 samples harvested from 12 heifers due to poor sample quality or unavailability of reproductive data. Of the 496 AI samples, the prevalence of CYTO at AI was 7.86 % (n = 39). The conception rate was 62.8 % (n = 287) in CYTO-negative samples (n = 457) and 38.46 % (n = 15) in CYTO-positive samples. Risk factors for non-pregnancy were a previous AI (odds ratio 2.96; 95 % confidence interval: 1.21–7.26) and the interaction between CYTO and previous AI. The only risk factor identified as being associated with the occurrence of CYTO was a previous AI (odds ratio 4.7; 95 % confidence interval: 2.15–10.34). The performance of unsuccessful inseminations significantly affects reproductive outcomes in subsequent AI and may lead to CYTO in nulliparous dairy heifers.

INTRODUCTION

Ensuring the efficiency of AI involves paying attention to many factors, among them uterine health. It is well known that an adverse uterine environment provokes breakdown of uterine homeostasis, significantly decreasing the reproductive performance of the cow [1] and [2]. Subclinical endometritis is a highly prevalent but asymptomatic uterine disease that can impair a cow's reproductive capacity [3]. In the field, subclinical endometritis is diagnosed primarily by measuring the proportion of inflammatory cells in a cytology sample taken from the uterus and is therefore often referred to as “cytological endometritis” (CYTO) [1] and [4]. Cytological endometritis can be diagnosed using the cytobrush [5] and [6], low-volume lavage [3] and [6], or cytotape [7]. The main advantages of cytotape are its versatility (enabling sampling at AI or during the luteal phase) and its high-quality samples [7].

Subclinical endometritis is considered a postpartum disease, presumably associated with endometrial recovery after clinical endometritis, trauma, or other nonmicrobial diseases [8]. However, CYTO samples have not, to our knowledge, been collected in nulliparous heifers to diagnose the condition in this group of animals. Although nulliparous heifers have not been exposed to risk factors for CYTO, such as retained placenta, acute metritis, or severe negative energy balance[5], [9] and [10], to date there is no evidence of the presence (or absence) of CYTO in these animals. The objectives of the present paper were to assess the prevalence of CYTO and its effect on pregnancy outcomes in nulliparous dairy heifers. We also identified risk factors for the occurrence of CYTO and non-pregnancy following AI.

MATERIALS AND METHODS

Experimental Design

A total of 512 AI in Holstein-Friesian nulliparous heifers (n = 351) from 18 commercial dairy herds were included in this prospective observational cohort study, conducted from July 2014 to March 2015 in the Flemish region of Belgium. Participating herds were required to use a computerized record system for herd management. The

number of heifers in the herds ranged from 10 to 96. All heifers were housed in freestall barns and had access to pasture during summer time. Heat was detected based on visual observations. Heifers that were showing signs of standing heat and were clinically healthy (no abnormal vaginal discharge) were included in the study at the time of AI. Heifers that required multiple inseminations before pregnancy occurred were sampled more than once.

One experienced inseminator from the Cattle Improvement Co-operative (CRV, Belgium) performed all inseminations. The farmer informed the inseminator when a heifer was detected in estrus, and inseminations were performed using the a.m./p.m. rule [11]. Endometrial cytology samples were obtained at the same time as AI using the newly developed cytotape [7]. Briefly, cytotape consists of a 1.5-cm piece of paper tape (Tesa 4322, Hamburg, Germany) rolled on top of a loaded insemination catheter (Agtech, Manhattan, KS), and covered with a 12-inch-long Sani-Shield rod (Agtech). The cytological sampling and AI procedure was as follows. First, the heifer's vulva was cleaned with a paper towel. Then, the AI catheter (with the cytotape on top) was manipulated through the cervix and, once in the uterine lumen, the catheter was released from the Sani-Shield rod. Next, with some gentle pressure of the index finger through the rectum, the top of the catheter was rotated twice on the dorsal wall of the corpus uteri. Finally, after injecting the semen into the lumen of the uterine body, the AI catheter was covered with the Sani-Shield rod and carefully removed from the genital tract. Microscope glass slides (Marienfeld, Lauda-Königshofen, Germany) were prepared on the farm by rolling the top of the AI catheter on the readable area of the glass slide, homogeneously spreading the collected cellular material. Finally, smears were air-dried and housed in a slide box for storage and transportation.

Slide boxes were delivered every 2 weeks to the laboratory facilities. All slides were stained with Diff Quick (Fisher Diagnostics, Newark, DE), and once the slides were dry, Eukitt mounting medium (O. Kindler GmbH, Freiburg, Germany) was used to protect the specimens and hold the coverslips on the slides. Microscopic evaluation was done at 100 x and 400 x magnifications (Kyowa Optical, Tokyo, Japan) by a single experienced reader. In total, 300 nucleated cells were counted, and the polymorphonuclear cell ratio (% PMN) was evaluated [12]. The total cellularity and quality of the samples were assessed in 10 high-power fields at 100 x [7] and [13]. Samples were classified as low

cellularity (< 50 cells), moderate cellularity (50–100 cells), and high cellularity (> 100 cells); and as poor quality (< 50 % intact cells), good quality (50–75 % intact cells), and very good quality (> 75 % intact cells).

An insemination was considered successful when pregnancy was confirmed by rectal palpation at least 45 days post-AI. Inseminations were considered unsuccessful when they were followed by another insemination or when the animals were diagnosed as not pregnant by rectal palpation at least 45 days post-AI.

Statistical Analyses

Individual heifer data were taken from the data capture forms collected by the inseminator, and from the computerized record system at each farm and exported to Excel (Microsoft Corp., Redmond, WA). Statistical analyses were performed using R version 3.3.0 (R Inc., Boston, MA), considering the AI sample as the unit of interest.

Before the start of the study, a sample-size calculation was conducted to identify the difference in conception rate between diseased and non-diseased animals, with a 95 % CI and 80 % power [14]. However, because neither the prevalence of CYTO nor the pregnancy risk in affected nulliparous heifers was available, we extrapolated variables that had been described in cows. We used a CYTO prevalence of 30 % [15] and considered the conception rate in healthy heifers to be 67 % [16], versus 40.2 % in diseased animals. Cows with CYTO had lower odds [odds ratio (OR): 0.6] of becoming pregnant than healthy cows [9].

For the data set assessment, descriptive statistical analyses were conducted using the summary function of the R coding system (package base). A receiver operating characteristic (ROC) curve (package pROC; [17]) was constructed to assess the cutoff point where the higher summation of sensitivity and specificity (% PMN) negatively affects pregnancy outcomes. To build the ROC curve, pregnancy outcomes for each AI was set as the response variable and the endometrial cytology result (% PMN) as the predictor. Based on the cutoff point of the ROC curve, we calculated Cohen's kappa coefficient to assess intra-observer agreement in a random subsample of 100 endometrial cytology slides (package caret, function confusionmatrix;[18]).

We built multilevel generalized mixed-effect models to test which explanatory variables significantly affected the presence of CYTO at AI and pregnancy outcomes. We used the function glmer (family = binomial) of the package lme4 [19] to construct the models. The fixed effects (explanatory variables) included in the model to evaluate the response variable of pregnancy outcomes were as follows: presence or absence of CYTO; whether the heifer had been previously inseminated or not; whether the heifer had been previously sampled using cytotape or not; bull identification; month of AI; and age (in months) of the heifer (continuous variable). Risk factors tested for CYTO were as follows: whether the heifer was previously inseminated or not; month of AI; and age (in months) of the heifer (continuous variable). All effects with P-values < 0.2 (univariate) and their interactions were included in the final models, and the ultimate model was computed using backward stepwise elimination. For all models, we used the identification of the heifer nested within herd as a random effect. We used this random effect combination because of its better fit compared to other random effects (lowest Akaike and Bayesian information value [20] and [21]). In the final models, statistical significance was $P < 0.05$ [14].

RESULTS

Results of the sample-size calculation revealed that a minimum of 496 animals should be sampled. In the end, 512 samples were collected in the present study, of which 99.61 % (n = 510) were considered readable and accepted for further analysis. Samples that were not considered readable generally lacked cells on the slide. Of the readable samples, 85.69 % (n = 437) displayed moderate cellularity, 11.37 % (n = 58) had high cellularity, and 2.94 % (n = 15) had low cellularity. Most of the readable samples (98.24 %; n = 501) were of very good quality. Of the rest, 1.76 % (n = 9) were of moderate quality and no samples were of poor quality.

Initially, 351 nulliparous heifers were included in the study. However, 12 heifers (14 samples) were excluded from statistical analysis due to the unavailability of pregnancy results (heifers were culled or sold). The final data set was based on 496 AI samples (339 heifers). The mean number of AI per heifer was 1.72±1.06, and on average

1.45±0.78 cytotape samples were obtained per heifer. The overall conception rate was 60.89 % (302 pregnancies from 496 AI samples).

The ROC curve revealed that the threshold level for diagnosing CYTO in nulliparous dairy heifers was 1 % PMN. The sensitivity and specificity for predicting pregnancy at this cutoff were 0.12 and 0.95, respectively. The area under the curve was 0.54 (95 % CI: 0.51–0.56). The mean % PMN in the cytology samples was 0.32±1.5, ranging from 0 to 18 % PMN. Intra-observer agreement for the cytologic evaluation was 0.93 (95 % CI: 0.86–1). Based on the 496 AI samples with acceptable cytology results and reproductive follow-up data, the prevalence of CYTO at AI was 7.86 % (n = 39). The conception rate for CYTO-negative uteruses (n = 457) was 62.8 % (n = 287); for CYTO-positive uteruses (n = 39), it was 38.46 % (n = 15).

More descriptive statistics and interactive data exploration of the analyzed variables can be found at https://public.tableau.com/profile/bovianalytics#!/vizhome/OB-Heifers/Published.

Factors affecting AI success are summarized in **Table 1** and **Table 2**. In the univariate model, bull identification, previous cytotape sampling, age (in months) of the heifers, and month of AI were not associated with pregnancy outcomes at $P < 0.2$ **(Table 1)** and were not offered to the multivariate model. In the final multivariate model, previous AI (OR 2.96; 95 % CI: 1.21–7.26) and the interaction CYTO × previous AI had a negative effect on pregnancy outcomes **(Table 2)**. The performance of a previous AI had a significant effect on the prevalence of CYTO (OR 4.7; 95 % CI: 2.15–10.34; $P < 0.0001$). Other factors, such as age in months and month of AI were not significantly associated with CYTO prevalence.

Table 1. Univariate model of factors affecting AI success in nulliparous dairy heifers, using heifer nested in farm as a random effect

Factors affecting the pregnancy outcome			
Variable	Odds ratio	Confidence interval (95%)	*P*-value
CYTO			
Negative	Referent	-	-
Positive	0.37	0.18-0.72	0.0042[a]
Bulls	-	-	0.22[b]
Previous AI			
No	Referent	-	-
Yes	0.76	0.53-1.11	0.16[a]
Previous cytotape sample			
No	Referent	-	-
Yes	0.93	0.62-1.37	0.76
Age in months	1.082	0.98-1.19	0.75
Month of AI	-	-	0.34[b]

Odds ratio (OR), > 1 (<1) is positively (negatively) associated with odds of pregnancy.
CYTO cytological endometritis.
Variables offered to the multivariate model.
Overall *P*-value.

Table 2. Multivariate mixed effects analysis of factors affecting AI success in nulliparous dairy heifers, using heifer nested in farm as a random effect and all possible cytological endometritis (CYTO) × previous AI interactions

Factors affecting pregnancy outcome			
Variable	Odds ratio	Confidence interval (95%)	*P*-value
CYTO negative	1.55	0.63-3.78	0.33
No previous AI	2.96	1.21-7.26	0.01*
CYTO x Previous AI	-	-	0.02*

CYTO x Previous AI (multiple comparisons)							
CYTO	AI		CYTO	AI	Odds ratio	Confidence interval (95%)	*P*-value
No	No	vs.	No	Yes	1.06	0.72-1.58	0.75
No	No	vs.	Yes	No	0.55	0.12-2.66	0.46
No	No	vs.	Yes	Yes	4.6	1.98-10.7	0.0004*
No	Yes	vs.	Yes	No	0.52	0.11-2.53	0.42
No	Yes	vs.	Yes	Yes	4.32	1.83-10.19	0.0009*
Yes	No	vs.	Yes	Yes	8.26	1.45-47.27	0.02*

Odds ratio (OR), > 1 (<1) is positively (negatively) associated with odds of pregnancy.
*variable significantly affecting the pregnancy outcome ($P < 0.05$).

DISCUSSION

Subclinical endometritis has been considered a postpartum uterine disease [8]. To the best of our knowledge, this is the first study to evaluate the presence of polymorphonuclear cells in nulliparous heifers from endometrial cytology samples taken at AI, as well as the effect of subclinical endometritis on pregnancy outcomes.

In a previous study, Kaufmann et al. [22] evaluated the effect of CYTO diagnosed 4 h after AI (cytobrush) in Holstein-Friesian cows and found no association between pregnancy outcome and % PMN. It is likely that a postbreeding inflammatory reaction explained the influx of PMN and may have interfered with the cytology results, as has been shown in mares [23] and [24]. Also, high estrogen concentrations during heat may provoke the infiltration of PMN into the endometrium [25] and [26], causing false-positive diagnoses of CYTO. Nevertheless, a more recent experiment [27] suggested that the physiological infiltration of PMN was not related to the stage of the estrous cycle. In the present study, samples were harvested during AI, and most of the samples (> 92 %) showed no PMN. The outcomes of the present study support the finding that the results of sampling during insemination are not affected by physiologic PMN infiltration during estrus or by any other physiologic inflammatory reaction of the endometrium, at least in nulliparous dairy heifers. Sampling during AI (open cervix) was fast, easy, and noninvasive (absence of erythrocytes in the slides), and led to high-quality samples (99 % of readable samples were classified as very good). As well, we proved that sampling during AI is justifiable, because the conception results were in a comparable range to those in similar studies [16] and [28].

We collected a relatively high number of AI samples in the present study (n = 512), but because of the small number of CYTO-positive uteri (7.86 %, n = 39), the results should be interpreted with caution. However, this number of samples was enough to prove the effect of CYTO on conception rate (CYTO-negative 62.8 % vs. CYTO-positive 38.4 %) in nulliparous dairy heifers. As described by Gilbert [29], an inflammatory milieu in the uterus decreases sperm motility, oocyte maturation, corpus luteum function, and embryonic quality. Taken together, these factors impair successful reproduction at multiple crucial stages, and a decreased conception rate is the final consequence. However, in the final model identifying factors that affected pregnancy

outcomes of the insemination, only previous insemination and the interaction of previous AI × CYTO were significantly associated. Moreover, the prevalence of CYTO and the occurrence of a previous AI had a high positive correlation. When we made multiple comparisons in the previous AI × CYTO interaction **(Table 2)**, we found a significantly lower conception rate in previously inseminated heifers with CYTO compared with heifers that only had CYTO or only had a previous insemination. As previously mentioned, only 39 samples (7.86 %) were positive for CYTO. From the 39 CYTO-positive samples, 30 (70 %) were taken from heifers that had undergone a previous unsuccessful AI.

Risk factors for CYTO in dairy cows have been well described by several authors [5], [9] and [10]. In nulliparous dairy heifers, however, there is no information about this uterine problem or its associated risk factors. However, the results of the risk factor analysis indicating CYTO as a potential risk factor for non-pregnancy should be interpreted with caution due to the low prevalence of CYTO. More studies including a higher number of heifers are needed to confirm our conclusions with more power. The prevalence of CYTO in heifers inseminated more than once was 16.13 % (n = 30), but in heifers who were not previously inseminated, it was only 3.32 % (n = 9). This finding strongly suggests that CYTO in nulliparous heifers may originate from a previous unsuccessful insemination. It is likely that CYTO can be induced by introducing contaminants during insemination, by the inflammatory response provoked by the inseminated material (semen, diluter, contaminants), or by inseminating heifers that are not truly in heat. Inadequate heat detection, periovulatory hormonal disturbance, or early embryonic loss could all be underlying reasons why insemination is unsuccessful [30], [31] and [32]. It is generally accepted that steroid hormones affect the uterine immune response [33]; progesterone suppresses the immune response [34] and [35] but it is more difficult to establish infections while estrogens are dominant [36]. When animals are not in heat (metestrus or early diestrus) when they are inseminated, accidental contaminants or the semen itself may trigger an iatrogenic inflammatory reaction. Practitioners must take special care in sterile manipulation when loading the AI pipette to avoid introduction of contaminating material when it is manipulated through the vulva-vagina-cervix into the uterine lumen, and to inseminate only heifers with an open cervix (estrus). Also, sanitary sheets are highly recommended to avoid

iatrogenic contamination of the uterus [37] during AI, particularly in dairy heifers. However, it is unknown why some heifers were positive for CYTO without having experienced a prior insemination. Contaminants (bacteria, viruses, or both) could have invaded the uterus via an opened cervix during a previous estrus, or the endometrium may have been infected via the peripheral blood by bacteria (e.g., Histophilus somni) or a virus that has a distinct tropism for endometrial cells such as the bovine herpesvirus type 4 virus [38] and [39].

Heat stress has been previously noted to have a major effect on fertility, mainly in dairy cows [40] and [41]. Donovan et al. [28] demonstrated that heifers inseminated in the summer were less likely to conceive at first insemination. However, in the present study, the month in which the AI took place did not affect pregnancy outcome. Unfortunately, samples were acquired only from July 2014 to March 2015, so not all months of the year were included. Consequently, “season” could not be introduced in the model, only month of sampling, although it was not significantly associated with the prevalence of CYTO, or with pregnancy outcomes. In accordance with the results of previous studies [28] and [42], bull identification and age of the heifer at AI were not associated with pregnancy outcomes. Furthermore, harvesting a cytotape sample during a prior insemination was not significantly associated with the conception rate, confirming that cytotape sampling has no detrimental effect on subsequent fertility.

CONCLUSIONS

In the present study, approximately 8 % of uteri sampled from nulliparous heifers at the time of insemination had CYTO. Positive samples were associated with lower conception rates compared to their negative counterparts. Previous unsuccessful insemination was associated with lower rates of pregnancy and a higher risk of CYTO. The latter might be regarded as the underlying reason for the impaired fertility results. More field studies that include higher numbers of animals are needed to confirm these results with more power.

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GENERAL DISCUSSION

At the very beginning of this PhD project, we performed an in-depth revision of the literature, to precisely acknowledge what is known and what is necessary to know about the topic. It became clear that there was limited information about the real value of an endometrial cytology sample to diagnose subclinical endometritis (SCE) as well as about the inflammatory status of the endometrium at the moment of artificial insemination (AI) in dairy cattle.

Subclinical endometritis is generally defined as an inflammation of the endometrium (no deeper than the *stratum compactum*) occurring without the presence clinical signs, but with a significantly deleterious effect on reproductive performances [1], [2] and [3]. Subclinical endometritis can be diagnosed by different methods such as histopathology, ultrasound, leukocyte esterase colorimetric strips and endometrial cytology [4] and [5]. However, as SCE is mainly diagnosed by cytology, it is often referred to as 'cytological endometritis' (CYTO). In the present section, when SCE is only referring to the cytological analysis of endometrial samples, it will be referred to as 'CYTO.'

The most frequently used techniques to diagnose CYTO are low volume lavage (LVL) and cytobrush (CB). However, the gold standard to evaluate endometrial alterations is the histopathological evaluation of endometrial biopsies. Endometrial biopsy sampling is a standard procedure in mares, but not in cows, since the same biopsy sampling in these animals is invasive enough to affect the future fertility outcome. Consequently, in the first part of the project we aimed to compare LVL versus CB using histopathology as the gold standard in order to diagnose SCE in dairy cows. Also, we took both, a biopsy and a cytology samples at eight pre-defined locations evenly spread throughout the uterus to study the potential impact of the distribution of inflammation within the bovine endometrium on the diagnostic outcome. The only way to assess multiple high-quality samples from a single uterus was using post-mortem uteruses. Therefore, to make the comparisons between endometrial cytology and histopathology, we used a model simulating the uterus in the "breeding period" (cows generally in late lactation).

Cytological endometritis is mainly diagnosed during the voluntary waiting period (VWP) with the aim to associate its presence with the future reproductive performance of the cow. In this respect, we hypothesized that uterine inflammation is a dynamic process from the postpartum period till the time of breeding. Taking cytology samples

during the VWP may not reflect the uterine health status at the time of fertilization and subsequent implantation. Therefore, in this project, we developed a novel technique to diagnose CYTO during AI, which allowed us to describe the prevalence, effect and risk factors associated with CYTO diagnosed at AI in both dairy cows and nulliparous heifers.

Here, in the general discussion, we aim to interpret all the observations achieved in this PhD project and use all the knowledge acquired throughout these years in order to describe the "Practical approach of subclinical endometritis in dairy cattle."

NOVEL INSIGHTS IN SUBCLINICAL ENDOMETRITIS DIAGNOSIS IN DAIRY COWS

Subclinical endometritis plays a critical role in the modern dairy industry. It is highly prevalent, asymptomatic, and with a profound detrimental effect on the reproductive performance [3] and [4]. Subclinical endometritis is indeed one of the most important reproductive impairments in dairy cows studied in the last decade. Endometrial cytology is the most commonly used technique to diagnose SCE (CYTO), mainly for reasons of low cost and simplicity [6] and [7]. Two main techniques are generally accepted to diagnose CYTO in dairy cows: CB [1] and LVL [2]. Although most of the authors describe the CB technique as technically easy to perform and often leading to high-quality smears [1], [8] and [9], samples represent only 1-2 cm^2 of the entire endometrium. The LVL technique on the other hand, is technically harder to perform in comparison to CB, since it requires more manipulation of the genital tract and a post-sampling centrifugation step [1] and [4]. Furthermore, it is considered as a more accurate technique since samples are thought to be more representative for the entire uterus (harvest cells from a larger endometrial surface) [10], [11], [12] and [13]. Therefore, the following questions can be raised: which cytology technique is superior? Are the sample outcomes representative for uterine health? Are both methods essentially diagnosing the same uterine problem?

In bovine and equine literature, histopathology is considered the gold standard to diagnose endometrial alterations because it allows to directly visualize both acute and chronic changes of the endometrium [14] and [15] **(Figure 1)**. In mares, for many years

endometrial biopsy sampling has been regarded as an integral part of the breeding soundness evaluation [16] and [17]. Also, in mares, the representativeness of one single biopsy sample to evaluate the health of the entire uterus is a matter of debate, due to controversial results obtained by different authors [8], [18], [19], [20] and [21]. Unfortunately, in cows, biopsy samples for endometrial histopathology are detrimental to subsequent fertility [22], [23], [24] and [25]. Therefore, they cannot be used routinely to diagnose endometrial alterations. In the present thesis, the currently most commonly used cytology techniques (CB and LVL, *in vivo*) were compared with multiple post-mortem biopsy and cytology (CB) samples, in order to assess the value of CYTO diagnosis using histopathology as the golden standard.

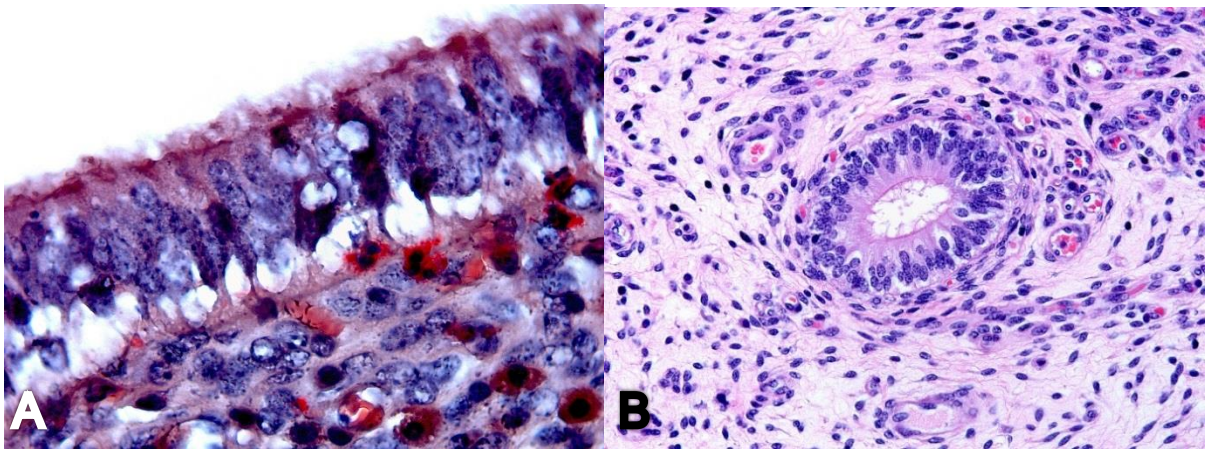

Figure 1. A) Polymorphonuclear cells stained in bright red in endometrial histopathology sample stained with Naphtol-AS-D-chloroacetate-esterase. B) Mild periglandular fibrosis in a endometrial histopathology sample stained with haematoxylin and eosin.

Cytobrush and LVL lavage techniques are essentially based on different approaches. Our results show that neither cytological technique is better than the other. Each technique has particular advantages and disadvantages. For example, when the uterine cavity is flushed with the liquid infused during LVL sampling, it harvests cells and debris present in the uterine lumen, and probably the superficial layer of the endometrial epithelium. Then, during centrifugation of the recovered liquid, the collected cellular material and debris are concentrated and subsequently spread on a microscope slide. Microscopic evaluation of these slides revealed a high content of debris (background) and higher values of polymorphonuclear cells (PMNs) in comparison to CB sampling. The CB technique on the other hand only samples a small portion of the endometrium, and loose PMNs are therefore only locally collected. These appreciations are in line with published literature where both CB and LVL samples were harvested in the same animal

and compared with each other [4], [8], [9] and [26]. In these papers, it was found that the PMN concentration was always higher in LVL samples (in comparison to CB). Moreover, the LVL technique may only evaluate a previous (probably already resolved) inflammatory status of the endometrium, since this technique mainly harvests segregated luminal cells that already migrated through the endometrium. However, what exactly triggers the PMNs' persistence long after calving in the uterine lumen, and how they may interfere with the fertility of the cow remains unclear **(Figure 2)**.

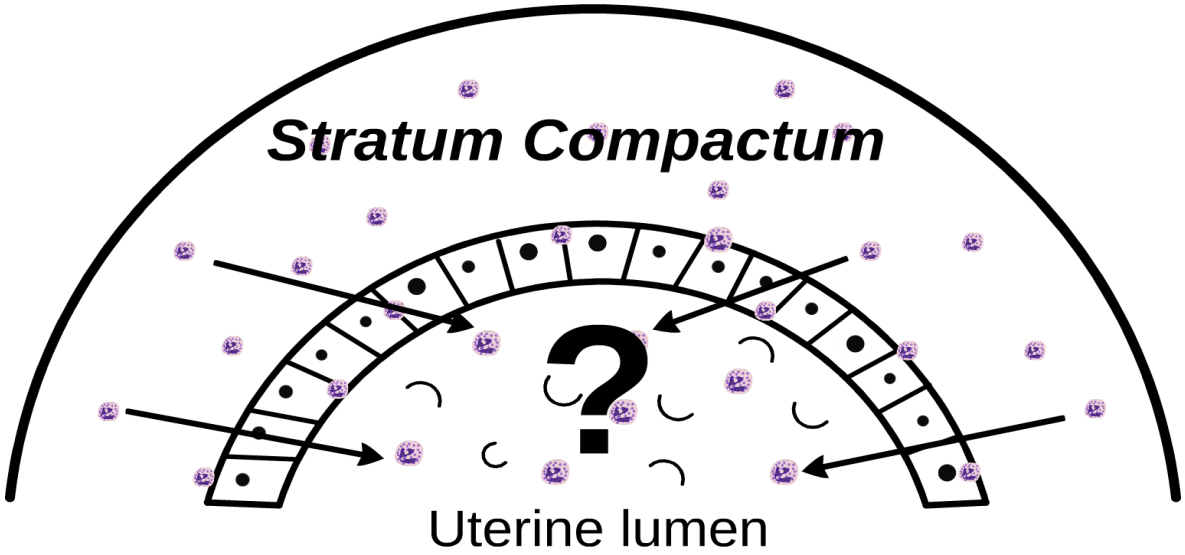

Figure 2. Illustration of the PMN migration from the *stratum compactum* toward the uterine lumen. However, it is unclear whether luminal PMNs are still physiologically active and how they interfere with the fertility of the cows.

Earlier, a decreased PMN function in cows suffering from uterine health disorders, was already demonstrated [27]. The killing ability of PMNs could be evaluated using two indices: the myeloperoxidase activity and the cytochrome c reduction assay. When the phagosome fuses with primary granules of the neutrophil, myeloperoxidase is released. Myeloperoxidase catalyzes the reaction between hydrogen peroxide and chloride anions to form hypochlorite. Hypochlorite reacts with tyrosine and other residues of bacterial proteins to kill bacteria. On the other hand, the cytochrome c reduction measures the amount of superoxide anions produced by the PMNs during the oxidative metabolic burst associated with phagocytosis of pre-opsonized zymosan. In this context, more research regarding the PMN killing ability in cows with subclinical endometritis is warranted.

How much information is obtained from an endometrial cytology sample? This is a crucial question which practitioners should take into account when they diagnose CYTO in the field. In this context, the value of CB samples has been criticized since they may not represent the inflammatory status of the complete uterus. Cytobrush generally harvests cells from the adjacencies of the corpus uteri in dairy cows [7]. Therefore we investigated the distribution of inflammation within the uterine endometrium, and the representativeness of one single CB sample harvested in the uterine body. Based on our results, we suggest that the neighborhood of the uterine body is the most sensitive region to detect endometrial inflammation. The uterine body is the link between the uterine milieu and the external environment. Independently of the location of the gravid horn (right or left), during birth, the fetus necessarily has to pass through this region. The corpus uteri is like a bottleneck, where the risk of damage (and consequent inflammation) is higher. Thus, to diagnose CYTO in dairy cows more accurately, we recommend to harvest CB samples from the corpus uteri **(Figure 3)**. Moreover, using histopathology, we found an uneven distribution of PMNs between the superficial versus deep *stratum compactum*, with significantly more granulocytes present in the deep *stratum compactum*. No differences were however found between PMN counts in cytology samples (CB) versus the number of PMNs in the superficial endometrium of histopathology samples. This finding allows us to confirm the hypothesis that endometrial cytology only visualizes the superficial endometrium. Still, when no PMNs are found in CB smears, extra PMNs may be allocated in the deep *stratum compactum*. However, the significance of PMNs present in the deep *stratum compactum* with respect to further reproductive capacity is still unknown. More research is needed to evaluate whether inflammatory cells present in this area interfere with the fertility outcome.

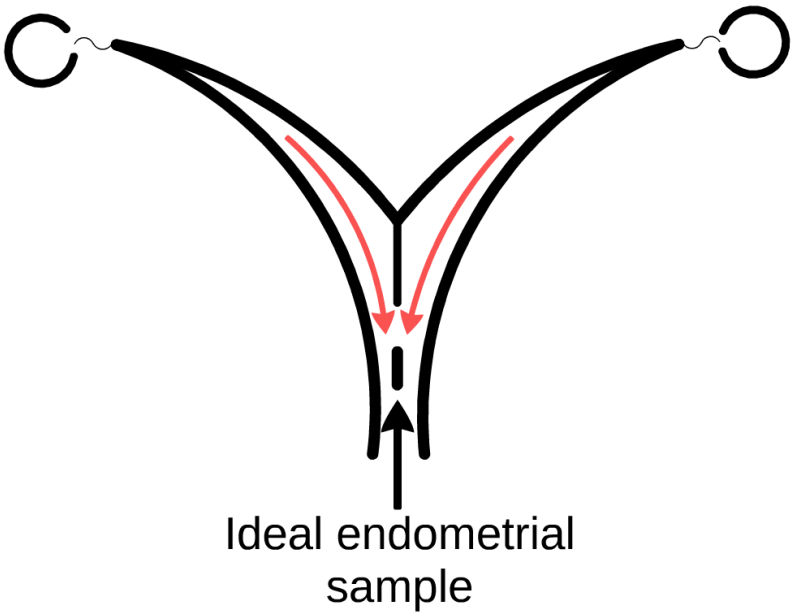

Figure 3. Acquiring endometrial samples in the *corpus uteri* region enhances the sensitivity to diagnose CYTO in dairy cows since higher PMN counts were found in this region.

We also hypothesized that cytology methods might underestimate the health status of the endometrium of dairy cows. The CYTO diagnosis is based on the mere visualization of the PMNs' proportion. On the other hand, histological examination of the endometrium may also reveal chronic changes such as periglandular fibrosis, angiosclerosis, endometrial gland atrophy or lymphoid aggregates [3] and [17]. Indeed, after recovering from active endometritis (PMN infiltration), chronic alterations may persist [3], being left unnoticed when only applying cytological analysis. Since our experiments did not find associations between PMNs and the chronic changes, we conclude that the CYTO outcome only represents the acute inflammatory status of the superficial endometrium. A definitive conclusion about the uterine health can only be reached by an endometrial biopsy. However, very little is known about chronic bovine endometrosis and its consequences for the reproductive capacity of the cow.

A NOVEL CYTOLOGICAL SAMPLING TECHNIQUE TO DIAGNOSE CYTOLOGICAL ENDOMETRITIS IN DAIRY COWS

In an attempt to facilitate CYTO diagnosis in dairy cows, in the second part of this PhD project, we aimed to create a device to harvest endometrial cytology samples

during AI in order to reveal the inflammatory status of the uterus at peri-fertilization time. Currently, CYTO diagnosis is far from being fully standardized. The PMN cut-off values reported in the literature to differentiate CYTO positive versus negative cows widely vary among authors (from 3 to 18 %) [7] and [28]. The latter is closely linked with the time point (days in milk (DIM); from 21 to 64) that samples are taken [1], [2], [4] and [27]. Consequently, extreme differences in CYTO prevalence have been reported among studies, between 9 to 76 % [29]. The percentage of PMNs present in the uterine lumen is a dynamic phenomenon during the postpartum period [30], so taking cytology samples during the VWP may not be representative for the amount of PMNs that will be present at breeding time.

Kaufmann et al. [31], were the first to harvest cytology samples at the peri-fertilization time, 4 hours after AI. In the studies of the present thesis, cytology samples were taken "simultaneously" with AI, in order to accurately evaluate the effect of CYTO on fertility. In the case of sampling 4 hours after AI, the injected seminal material could provoke a post-breeding inflammatory reaction, interfering with the cytology results [32] and [33]. Moreover, from a practical point of view, it would be tedious and probably more traumatizing to go through the cervix twice in a short period of time. Consequently, by simply rolling a 1,5 cm piece of paper tape on the tip of a standardly loaded AI catheter, covered with a double guard sheet, we developed the "Cytotape". By using the Cytotape technique, the post-breeding inflammatory reaction provoked by semen is avoided. Moreover, the use of endometrial cytology probably would become more popular among practitioners since the Cytotape device only demands cheap and ordinary material. However, in order to recommend the Cytotape as a valid tool to harvest samples for cytologic examination, several field studies were performed by our research group.

To assess the sampling capacity of the Cytotape, it was compared with the CB as gold standard **(Figure 4)**, taking samples in a random stage of the estrous cycle. Both samples (Cytotape and CB) were taken in the same animal, at the same time and approximately at the same location. The principal parameter used to evaluate the correlation between both techniques was the PMN %, while also cellularity, quality, and red blood cell contamination were assessed for both techniques. In comparison to the CB technique, cytologic analysis following sampling by Cytotape yielded similar PMN

counts. Furthermore, the total cellularity as assessed by both methods was similar. The quality of the smears, however, was better in the Cytotape versus the CB slides (**Figure 4, A** and **B**). Cellularity and quality are keystone parameters to evaluate cytology slides properly. An accurate evaluation of the PMN/epithelial cell ratio is only possible with a high number and a clear differentiation of the cellular components of the smears. Moreover, Cytotape samples revealed a lower erythrocyte contamination in comparison to the CB slides. The red blood cell contamination should not be regarded as a negligible factor because its excessive presence may indicate endometrial damage through sampling, and may also imply the presence of blood derived PMNs which could interfere with CYTO diagnosis (false positive samples). Taking together, the Cytotape clearly surpassed standards among sampling techniques to diagnose CYTO in dairy cows.

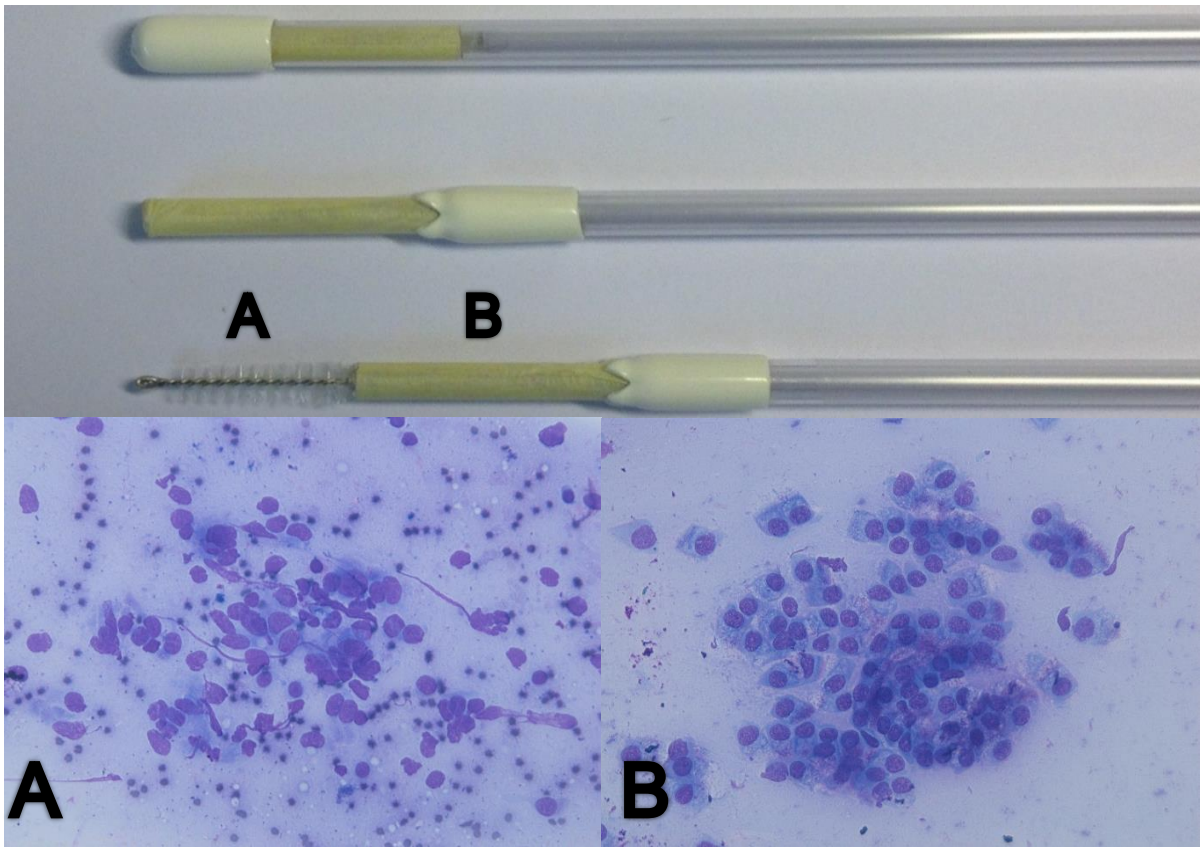

Figure 4. Image showing how both (CB and CT) cytology samples were taken at the same time, **A)** Cytobrush sample with fragmented cells and moderate red blood cells contamination. **B)** Cytotape sample exhibiting good quality (intact) endometrial cells.

PREVALENCE, EFFECTS, AND RISK FACTORS ASSOCIATED WITH CYTOLOGICAL ENDOMETRITIS DIAGNOSED AT ARTIFICIAL INSEMINATION IN DAIRY COWS

Evaluating the success of an AI implicates paying attention to multiple factors, among others uterine health status. However, in practice, endometrial analysis to evaluate CYTO is not routinely done in dairy cows. Currently, endometrial cytology samples are mostly taken during the VWP for scientific purposes [29]. On commercial dairy farms, CYTO is rarely assessed mainly due to the "extra" management efforts to separate and catch the cows, and the unavailability of specialized materials to harvest the samples (practical and logistical reasons). The Cytotape technique was developed in order to facilitate the "on farm" CYTO diagnosis. Consequently, sampling during AI may have three significant benefits: (1) standard sampling moment, (2) standard PMN % threshold level, and (3) no extra manipulation of cows is required. In order to validate the technique to harvest samples during AI, to create a PMN % cut-off level at AI time, and to set the CYTO prevalence and its effect on fertility, a large field study was performed.

At the first step of the "Cytotape research," samples had been harvested in a random stage of the estrous cycle and were compared with CB as the gold standard. The next step was to validate the sampling capacity of the Cytotape when sampling during AI. Consistently, more than 99% of the samples harvested during AI were adequate for microscopic evaluation, most of them with a high number of intact cells. Moreover, collecting endometrial cytology samples during estrus showed to have three extra major advantages: (1) sampling with an open cervix was experienced to be fast, easy, and non-invasive; (2) risk for iatrogenic infection is less since estrogens are dominant at the time of sampling [34]; and (3) probably samples acquired during estrus are more representative, since during estrus, the contractility of the myometrium is enhanced and cellular components of the superficial endometrium may be better mixed in a matrix of mucus. We hypothesized that this mixed mucus material may be more representative for the entire uterus, similarly to the infused liquid during LVL. However, it may be arguable that sampling during estrus might lead to false-positive results since some authors reported a higher PMN infiltration during estrogenic dominance (histopathology) [35] and [36]. In line with our results (high number of samples with no

PMNs), Madoz et al. [37] suggested that the physiological infiltration of PMNs was not related to the stage of the estrous cycle, at least in studies based on cytologic samples.

A challenging objective for the present studies was to establish a universal PMN % threshold to diagnose CYTO during AI. To fulfill this goal, we included all the co-variants that significantly affected fertility in the receiver operator characteristics (ROC) curve as the classifier. Using this statistical approach, for the first time other variables significantly affecting the reproductive outcome were involved in the assessment of a PMN % cut-off point. In our field studies, of all factors that significantly affected the fertility outcome at AI, high PMN counts was the most significant. In agreement with this, a low PMN % threshold level for CYTO at AI was obtained in the ROC curve (1 % PMN). This cut-off point revealed a high positive predictive value and a low negative predictive value. Concomitantly, we can assume that a CYTO positive cow diagnosed at AI is very unlikely to become pregnant from that AI. On the other hand, a CYTO negative sample at AI is not a good predictor of the pregnancy outcome of that insemination. The latter seems obvious since multiple factors are affecting the AI success, not only the absence of CYTO.

The 1% PMN cut-off point revealed that the CYTO prevalence at AI was relatively high (27.8 %) **(Table 1)**. More than one-quarter of modern dairy cows that are inseminated, suffer from CYTO. Cytological endometritis negative uteri diagnosed at AI had 1.8 more chances to become pregnant from that AI than CYTO positive uteri. In other words, the conception rate in CYTO negative samples was 47 % while it was 32.7 % in CYTO positive samples **(Figure 5)**. As described by Gilbert [38], an inflammatory milieu during fertilization time significantly impairs the conception rate by creating a suboptimal condition for sperm cell transport and storage, oocyte maturation and ovulation, fertilization, zygote development, implantation and embryonic growth. This negative association between CYTO and fertility is well known, and has been described by several authors around the world [7] and [39]. However, this is the first study that demonstrated the presence of CYTO at AI and confirmed its deleterious effect on the subsequent insemination outcome. All other studies focus on diagnosing CYTO during the VWP, overlapping with the proper time of uterine involution, and sometimes leading to inconclusive results of the CYTO effect on fertility [40].

Table 1. Summary of CYTO studies from around the world, considering different time points postpartum, thresholds for percentage of polymorphonuclear cells (PMNs) and respective prevalence; including the new CYTO threshold level and prevalence at AI (adapted from Sens and Heuwieser [39]).

Reference	Country	Days in milk	PMN (%)	Prevalence (%)
Kasimanickam et al. (2004)	USA	20-33	>18	45
		34-47	>10	41
Dubuc et al. (2010)	Canada	35±3	≥6	13.5
		56±3	≥4	9.6
Plöntzke et al. (2010)	Argentina	18-38	>5	38
		32-52	>5	19
McDougall et al. (2011)	New Zealand	29±2.4	≥9	29
		43±2.3	≥7	23
Pascottini et al. (2016)	Belgium	>60	≥1	27.8

Although taking endometrial cytology samples while inseminationg could be considered as "too late", it nevertheless, creates new perspectives for CYTO treatment. Moreover, routine harvesting of Cytotape samples during the first insemination would allow exploring uterine health without "any extra labor cost." In case the breeding is not successful and the cow is diagnosed CYTO positive, it may be possible to set up a targeted treatment to increase the pregnancy outcome in subsequent insemination attempts. Also, taking Cytotape samples in repeat breeder cows may reveal the underlying reason for their problem to get in calf. However, it is important to mention that to date, there is no consensus about an effective treatment for CYTO positive cows. Two treatments are currently most discussed, prostaglandin F2-alfa and intrauterine antibiotics [41], [42], [43], [44] and [45]. The use of prostaglandins during diestrus results in luteolysis and subsequently estrus, which has been suggested to enhance local immunity by removal of the immunosuppressive effect of progesterone [46] and [47]. Furthermore, prostaglandins are claimed to possess an ecbolic effect by which they effectuate the elimination of bacteria and debris [44]. However, only one study showed

a significant benefit of prostaglandin treatment on CYTO prevalence [42], while other studies did not [43] and [44]. Although several authors unsuccessfully tried to correlate the presence of CYTO with bacterial infection [29], [39], [48] and [49], recently Denis-Robichaud and Dubuc [41] successfully increased the pregnancy outcome of CYTO positive cows treated with cephapirin in the postpartum period. In a recent paper, Dini et al. [50] demonstrated the possibility to reduce the amount of PMNs from the uterine lumen by applying an uterine lavage with saline solution. However, further studies are necessary to confirm this as a potential treatment for CYTO.

The wide variation of CYTO prevalence among herds [2] and [51] strongly suggests that management factors significantly affect CYTO occurrence **(Figure 6)**. Previous studies already identified risk factors such as retained placenta, an accentuated negative energy balance, and acute metritis [1], [51] and [52]. In all these studies, CYTO was evaluated in the postpartum period; no study exists about the risk factors associated with CYTO diagnosed during AI.

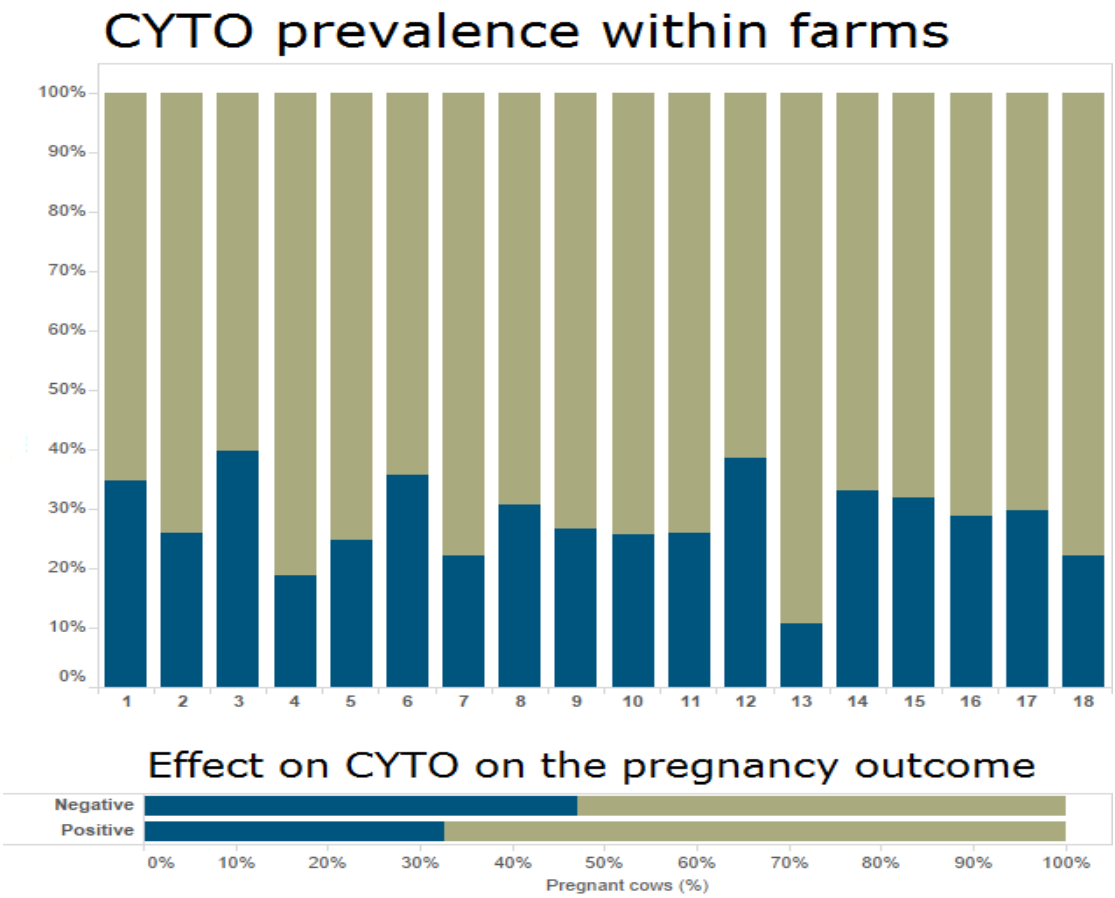

Figure 5. Bar charts showing the prevalence of CYTO within farms, and the effect of CYTO diagnosed at AI on the insemination outcome in dairy cows.

One interesting risk factor identified in our study was the effect of DIM at the AI Cytotape sampling time on the CYTO prevalence. In the late postpartum, after 123 DIM sampled uteri had significantly lower chances to suffer CYTO, but there was not a positive association between CYTO and uteri sampled between 60 to 123 DIM. This finding demonstrates that most of the cows are 'clean' at the end of the VWP, but the final uterine clearance is only long time after parturition. Another novel finding of factors associated with CYTO at the breeding time was heat stress. Cows sampled during summer (July, August, and September), were more prone to suffer from CYTO during AI than those sampled in other months of the year. To the best of our knowledge, this is the first study suggesting a contributing effect of heat stress on CYTO. The CYTO prevalence is maybe increased during summer because of the indirect influence of the accentuated negative energy balance in high yielding cows during this season (due to alteration decrease in the dry matter intake) [53], [54] and [55]. A severe negative energy balance, among many other things, may impair neutrophilic function [28] and [56]. The PMNs' dysfunctionality may lead to persistence of inflammation, and therefore more PMNs are continuously attracted to the endometrium of the cow (vicious circle).

In contrast to other studies [31] and [51], we found that multiparous cows had more chances to suffer from CYTO than primiparous cows. Authors attribute this to the greater metabolic stress primiparous cows have to overcome when establishing milk production in comparison to the multiparous ones. On the other hand, Baez et al. [57] recently demonstrated that there is a negative association between the size of the uterus and the reproductive outcome. Logically, uterine size grows concomitantly with increasing parity. Although the biology behind the effect of uterine size on fertility is unknown, we suggest that a large uterus encounters more problems to complete full clearance after parturition. Consequently, in a large pendulous uterus, we speculate a higher CYTO prevalence.

PREVALENCE, CONSEQUENCES AND RISK FACTORS ASSOCIATED WITH CYTOLOGICAL ENDOMETRITIS DIAGNOSED AT ARTIFICIAL INSEMINATION IN NULLIPAROUS DAIRY HEIFERS

Cytological endometritis is generally considered part of the postpartum uterine disease complex, presumably associated with endometrial recovery after clinical endometritis, trauma or (non) microbial diseases [58]. Since nulliparous heifers are generally expected to be unexposed to CYTO risk factors (parturition and negative energy balance), up to the present day there was no evidence for presence (or absence) of CYTO in these animals. In one of the studies of the present PhD thesis, we evaluated the uterine inflammatory status of nulliparous dairy heifers during AI. To do so, we first defined the PMN % cut-off point, after which we were able to describe the prevalence and risk factors associated with CYTO in this group of animals.

The CYTO cut-off point at AI in nulliparous dairy heifers was exactly the same as it is in cows, 1 % PMN (based on a standard ROC curve). Hence, the minimal presence of PMNs in the uterus at AI seems to affect the fertility success not only in dairy cows but also in nulliparous heifers. As expected, the prevalence of CYTO in nulliparous heifers was however low (7.9 %). The latter can probably be attributed to the fact that these animals did not experience a prior parturition and concomitant damage and bacterial contamination. The performance of an unsuccessful previous AI and the interaction previous of AI x CYTO had a deleterious effect on the conception rate **(Figure 6)**. In that regard, from the CYTO positive samples, 70 % were harvested from heifers that had undergone a previous unsuccessful AI. The latter suggests that the presence of CYTO in nulliparous heifers is associated with an unsuccessful previous AI. Further analysis showed that a heifer that was previously unsuccessfully inseminated had 4.7 more chances to suffer from CYTO than heifers which had not been previously inseminated. The CYTO prevalence in nulliparous heifers that had already been inseminated was 16.1% versus 3.3% in not previously inseminated counterparts. Probably, CYTO in nulliparous heifers is induced by the introduction of contaminants while inseminating the animal, by the inflammatory reaction produced by the semen (or diluter), or by inseminating heifers that are not perfectly in heat. The latter could be blamed to an inadequate heat detection or a hormone imbalance [59], [60] and [61]. It is well accepted that during progesterone dominance, the immunological response of the

uterus is suppressed [46] and [62] whereas it is more difficult to establish an infection in an estrogenic environment [34]. Consequently, practitioners should take special care while inseminating nulliparous heifers, in order to avoid inseminating heifers not being in heat. Furthermore, also the sterile manipulation of the AI gun before and during AI requires special attention. In this respect, the use of sanitary sheaths might be highly recommendable while inseminating nulliparous heifers [63]. It is unclear why heifers that were not previously inseminated were diagnosed as CYTO positive. Probably contaminants could have invaded the uterus via an opened cervix during a previous estrus, or the uterus may have been infected via the peripheral circulation as has been suggested for the bovine herpes virus type 4 [64].

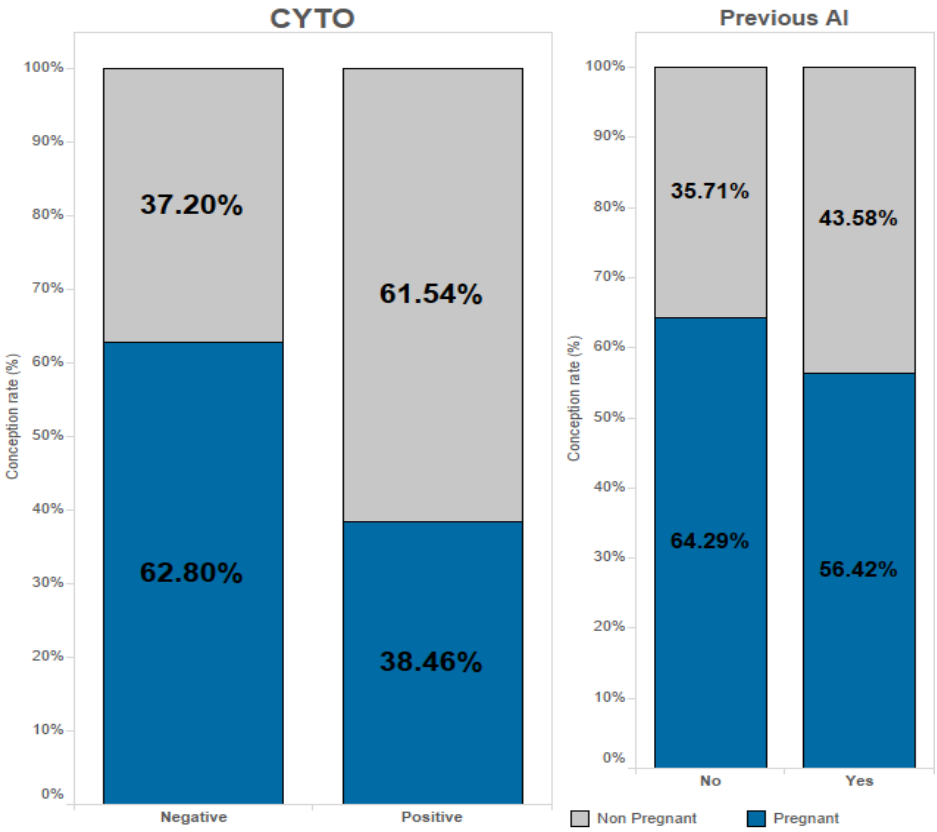

Figure 6. Bar charts representing the effect of CYTO diagnosed at AI, and the effect of a previous unsuccessful AI on the insemination outcome of nulliparous dairy heifers.

CONCLUSIVE REMARKS AND TARGETS FOR FURTHER RESEARCH

In the present thesis, we were able to answer some specific questions about SCE diagnosis in dairy cows, such as revealing the value of a cytologic sample in comparison to an endometrial biopsy and where in the uterus to ideally sample cows for CYTO

diagnosis. Also, a novel cytologic technique to diagnose CYTO simultaneously with AI was developed, and in a large field study, the prevalence, risk factors, and consequences of CYTO diagnosed at AI in both cows and nulliparous dairy heifers were investigated. Although we did answer some important questions, the following points need further clarification regarding the SCE phenomenon in dairy cows:

- Although we discovered that CB and LVL samples are based on fundamentally different approaches, some interesting questions that warrant further research are: (1) are the PMN cells segregated in the uterine lumen still physiologically active and therefore able to interfere with further fertility; (2) why do some cows keep on suffering from high numbers of intrauterine PMN cells even long after the postpartum period; (3) what is the trigger for the continuous attraction of PMN cells towards the endometrium in at least some cows a long time after calving.
- We compared cytology versus histopathology samples to examine the endometrium. However, we did not harvest biopsy samples in living cows in order to research the effect of biopsy sampling on further fertility. Biopsy sampling in live cows followed by histopathology is further highly warranted to research the effect of some specific alterations on the fertility of dairy cows.
- We created a simple and "easy to use" device in order to diagnose CYTO in dairy cows and heifers. Nevertheless, after sampling slides need to be stained and evaluated under a light microscope. It is imperative to develop a reliable "cow side" diagnostic test for SCE. In this context, the leucocyte esterase colorimetric test could be an alternative. However, it should be preceded by a CB or LVL sampling, which is still done during the VWP (extra labor). The leukocyte esterase strip needs to be adapted to the AI gun in order to prove its efficacy to diagnose SCE during AI.
- Currently, there is no effective treatment for CYTO. Field studies need to be done regarding the use of preferably non-antibiotic based treatments for the disease. In this context, a therapeutic flushing with saline solution and/or the utilization of non-steroidal anti-inflammatory drugs specifically targeted for cows suffering from CYTO, could represent a valid strategy to improve the pregnancy outcome of affected cows.

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SUMMARY

Postpartum uterine diseases are a common phenomenon in dairy cows. They are considered as a leading cause of reproductive inefficiency, increasing the number of open days and the culling rate, leading to a profound effect on the profitability of dairy farms. Criteria to diagnose postpartum uterine diseases are fundamentally based on clinical symptoms. However, subclinical endometritis (SCE), a highly prevalent postpartum uterine disease, courses without the presence of clinical symptoms.

Endometrial cytology is per definition the technique to use to diagnose SCE. Consequently, it is often referred to as 'cytological endometritis' (CYTO). However, its diagnosis has been hampered by the lack of universally accepted criteria. The primary practical approach of this doctoral thesis consisted of elucidating the clinical value of an endometrial cytology sample **(Chapter 4)**. Moreover, we attempted to standardize the CYTO diagnosis by the implementation of a novel sampling technique allowing to harvest samples during artificial insemination (AI) using a standardized threshold for the number of polymorphonuclear cells (PMNs) **(Chapter 5)**.

In **Chapter 4.1**, we compared cytology versus histopathology in order to assess the real value of a cytology sample for the evaluation of endometrial health in cattle. Firstly, we compared the most popular cytologic techniques, cytobrush and low volume lavage, using histopathology as the golden standard. We concluded that both techniques are based on inherently different approaches. Low volume lavage harvests cells mainly localized in the uterine lumen, while sampling by cytobrush leads to harvesting cells present in the superficial *stratum compactum*. Also, we discovered that more PMNs are localized in the deep in comparison to the superficial *stratum compactum*. However, similar PMN counts were found when comparing the locally collected cytobrush samples with the superficial PMNs visualized in the histopathology samples.

Chapter 4.2 describes the distribution of inflammation within the bovine endometrium. We found that more PMNs were allocated in the *corpus uteri* and right horn region in comparison to the left horn. Consequently, we recommend harvesting endometrial cytology samples from the *corpus uteri* region of dairy cows. Interestingly, we found a positive correlation between the number of PMNs and mononuclear cells, while no association was found between PMN numbers and the presence of chronic alterations in the histopathology samples. Thus, we concluded that endometrial

cytology only assesses active inflammation, while if the complete health of the uterus needs to be evaluated, histopathology remains the best option.

In **Chapter 5.1** we described the Cytotape, a novel device to harvest samples for cytology to diagnose CYTO, based on fixing a short piece of paper tape on the tip of an insemination gun. First, we validated the Cytotape using the cytobrush as the gold standard, sampling animals in a random stage of the estrous cycle. We evaluated the PMNs proportion, total cellularity, quality and the red blood cell contamination outcomes of both techniques. All samples were taken simultaneously in the same cow and approximately at the same uterine location. The PMNs proportion, as well as the total cellularity were similar between cytotape and cytobrush slides. However, cytotape samples were of a better quality and were less contaminated by red blood cells. Consequently, we concluded that cytotape is a valid technique to diagnose CYTO in dairy cows.

Chapter 5.2 describes an extensive field study in which we aimed to: set a standardized PMN threshold to diagnose CYTO at AI, establish the CYTO prevalence at AI, and evaluate the effect of CYTO diagnosed at AI on the pregnancy outcome of that AI. To detect the PMN threshold, we took into account other co-variables that were detected to affect the outcome of an AI. Finally, the PMN threshold level for cytology samples achieved during AI was found to be 1 %. Stunningly, approximately one-quarter of modern dairy cows suffered from CYTO during AI and CYTO-negative uteri at AI had 1.8 more chances to conceive in comparison to CYTO-positive ones. Then, in **Chapter 5.3** we studied the risk factors associated with CYTO diagnosed at AI in dairy cows. In this study, we discovered that dairy cows that are inseminated later than 123 days postpartum, had significantly lower odds to suffer from CYTO. Moreover, multiparous cows had more chances to have CYTO at AI in comparison to primiparous cows. Another, rather novel finding in this study was the positive association between heat stress and CYTO. Uterine samples harvested in July, August, and September had more chances to be CYTO positive in comparison to uteri sampled in other months of the year.

In the last chapter of this doctoral thesis (**Chapter 5.4**), we harvested cytology samples from uteri of nulliparous dairy heifers. To the best of our knowledge, this is the

first extensive field study to report about CYTO in nulliparous heifers. Also in these animals, it became clear that a PMN threshold of 1 % should be applied to diagnose this uterine aberration. Doing so, we found the prevalence of CYTO at AI to be 7.86 %. The conception rate associated with positive samples was 38.46 %, while in CYTO negative samples it was 62.8 %. Risk factors negatively associated with odds for pregnancy were a previous AI and the interaction previous AI x CYTO. Moreover, this novel study allowed us to describe for the first time CYTO as an iatrogenic disease, since its presence in nulliparous dairy heifers was associated with an unsuccessful previous AI. Hence, we speculate that when animals are not in heat during AI, accidental contaminants or maybe even the semen itself may trigger an inflammatory reaction of the endometrium of dairy heifers.

In conclusion, the findings of the present PhD thesis highly contribute to the discussion regarding the sampling methodology (technique, PMN threshold level, standardized moment to harvest samples) in order to accurately diagnose CYTO in dairy cattle. Furthermore, we herewith introduce the use of the Cytotape which allows sampling in an easy and much better standardized way in practice, and this both in cows as well as in nulliparous heifers. We are convinced that the results of the present work will facilitate and hence encourage practitioners to routinely sample animals that have problems to get in calf. We are both happy and proud that by this work our research team was able to contribute to this important topic, this was our *"Practical approach of subclinical endometritis in dairy cattle".*

SAMENVATTING

Baarmoederafwijkingen komen vaak voor bij melkkoeien. Zij worden momenteel beschouwd als het belangrijkste fertiliteitsprobleem vooral omdat ze vrijwel altijd gepaard gaan met een duidelijke verlenging van de tussenkalftijd en een toename van het aantal dieren dat te vroeg moet worden opgeruimd. Derhalve veroorzaken deze problemen een grote economische verliespost op melkveebedrijven. De diagnose van de verschillende vormen van baarmoederproblemen gebeurt in de praktijk op basis van klinische symptomen. Subklinische endometritis, volgens vele auteurs momenteel de meest voorkomende baarmoederafwijking bij melkvee, verloopt echter zonder dere symptomen.

Per definitie gebeurt de diagnose van subklinische endometritis op basis van de uitslag van een cytologisch onderzoek van het endometrium. Vandaar ook dat vaak de terminologie 'cytologische endometritis' wordt gebruikt om naar dere aandoening te verwijzen. Tot op heden is er echter nog heel wat discussie omtrent de diagnostische criteria. Het primaire doel van dit doctoraatsonderzoek was dan ook om meer duidelijkheid te brengen in het belang van het cytologisch onderzoek bij de diagnose van subklinische endometritis in de praktijk (**Hoofdstuk 4**). Daarnaast werd een innovatieve staalnametechniek voorgesteld die toelaat om endometriumstalen te nemen tijdens het insemineren, wat een veel betere standaardisatie toelaat en bovendien de mogelijkheid biedt om zicht te krijgen op de gezondheid van het endometrium op het meest aangewezen moment, namelijk op het moment van de bevruchting (**Hoofdstuk 5**).

In hoofdstuk **4.1** werd het resultaat van een cytologisch onderzoek van het endometrium vergeleken met dat van een histopathologisch onderzoek dat als goudstandaard werd gehanteerd. In dit onderzoek werden de twee momenteel populairste staalnametechnieken, namelijk de cytobrush en de low volume lavage met elkaar vergeleken. De verschillen tussen deze twee technieken zijn zonder meer duidelijk: met behulp van de low volume lavage worden de losliggende cellen aanwezig in het uteruslumen geoogst en dit over de volledige lengte van de uterus, terwijl met de cytobrush het oppervlakkige deel van het *stratum compactum* van het endometrium wordt bemonsterd en dit slechts op een heel beperkte plaats. Verder leerden de resultaten van dit onderzoek ons dat er zich in de dieper gelegen delen van het *stratum compactum* vaak meer polymorphonucleaire cellen (PMNs) bevinden in vergelijking

met de meer oppervlakkig gelegen delen. Het aantal PMNs dat via histopathologisch onderzoek werd gevonden in deze meer oppervlakkig gelegen gebieden, was echter identiek aan het aantal PMNs dat via cytobrush werd gedetecteerd.

In hoofdstuk **4.2** werd de distributie van de endometriale inflammatie doorheen de uterus onderzocht. Geconcludeerd werd dat PMNs vooral kunnen teruggevonden worden ter hoogte van het *corpus uteri* en in de omgeving van de rechter uterushoorn. Op basis hiervan kon de waarde van staalname ter hoogte van het uteruslichaam, zoals op dit moment reeds algemeen gebeurt, worden bevestigd. Interessant was ook de positieve correlatie die werd gevonden tussen het aantal PMNs en het aantal mononucleaire cellen, terwijl er geen associatie werd gevonden tussen het aantal PMNs en het al dan niet aanwezig zijn van chronische endometriumafwijkingen. We concludeerden dan ook dat cytologie enkel kan worden aangewend voor het diagnosticeren van een actieve inflammatie, terwijl histopathologie de meest aangewezen techniek blijft voor het bepalen van de algehele gezondheidstoestand van het endometrium.

In hoofdstuk **5.1** werd het gebruik van de Cytotape beschreven. Het betreft een innovatieve doch zeer eenvoudige methodiek om stalen te nemen voor cytologisch onderzoek van het endometrium, gebaseerd op het aanbrengen van een klein stukje papier plakband op een inseminatiepipet. Vooreerst werden de resultaten van de staalname via Cytotape vergeleken met die verkregen via de standaard cytobrush methode en dit bij dieren die zich in een verschillend stadium van de oestrische cyclus bevonden. Cytotape en cytobrush stalen werden bij één en hetzelfde dier en op vrijwel exact dezelfde lokalisatie in de uterus genomen. In het onderzoek werden het totaal aantal geoogste cellen, het proportionele aandeel van PMNs, de kwaliteit van de geoogste cellen en de contaminatie met rode bloedcellen bepaald. Uit het onderzoek bleek dat de Cytotapestalen van betere kwaliteit en minder gecontamineerd met rode bloedcellen waren. Geconcludeerd werd dan ook dat de Cytotape kan ingezet worden om op een gevalideerde manier stalen voor cytologisch onderzoek van het endometrium te nemen.

In hoofdstuk **5.2** worden de resultaten gerapporteerd van een uitgebreide veldstudie waarin werd getracht om:

-de afkapwaarde van het PMN % tijdens KI, waarboven de kans op drachtig worden significant kleiner is, te detecteren;

-de prevalentie van CYTO op het moment van KI te bepalen;

-de invloed van het PMN % tijdens KI op het drachtig worden, te kwantificeren.

Voor zover ons bekend, werd hierbij voor het eerst een afkapwaarde bepaald rekening houdend met andere co-variabelen die een invloed hebben op het drachtig worden. Een PMN% van 1 bleek uiteindelijk de beste afkapwaarde te zijn waarboven de kans op drachtigheid werd verminderd. Verder bleek in totaal ongeveer een kwart van de koeien die werden geïnsemineerd aan CYTO te lijden, terwijl CYTO-negatieve uteri 1,8 keer meer kansen hadden om drachtig te worden in vergelijking met CYTO-positieve. Daaropvolgend werd in hoofdstuk **5.3** een onderzoek gedaan naar de risicofactoren die significant geassocieerd zijn met de aanwezigheid van CYTO op het moment van de KI. Uit dit onderzoek bleken koeien die geïnsemineerd werden later dan 123 dagen na afkalven, een lagere kans op CYTO te hebben. In vergelijking met vaarzen, hadden multipare koeien dan weer meer risico op CYTO. Een andere, eerder innovatieve vondst was dat koeien die geïnsemineerd werden in de warmere maanden (juli, augustus en september) een hoger CYTO-risico bleken te hebben. De resultaten suggereren dan ook een mogelijks positief effect van heat stress op het ontwikkelen van CYTO.

In het laatste hoofdstuk (**5.4**) van dit doctoraat wordt een onderzoek bij pinken beschreven. Voor zover ons bekend, is dit het eerste veldonderzoek waarbij tijdens KI stalen voor cytologisch onderzoek van het endometrium werden genomen bij jonge, nog niet gekalfde vaarzen. Vooreerst werd ook hier de afkapwaarde voor het PMN% bepaald, wat eveneens 1 bleek te zijn. Uiteindelijk werd een CYTO-prevalentie van 7,86% gevonden bij deze dieren. Het drachtigheidspercentage bij de positieve stalen bleek 38,46 te zijn, in vergelijking met 62,80 in geval het staal negatief was. Factoren die zorgden voor een lagere kans op drachtig worden bij deze jonge dieren waren: het feit dat ze eerder waren geïnsemineerd en de interactie tussen een eerdere inseminatie en

de aanwezigheid van CYTO. De bekomen resultaten suggereren dat CYTO bij pinken mogelijks aanzien kan worden als een iatrogene aandoening, aangezien het probleem vooral werd gedetecteerd bij dieren die voordien reeds waren geïnsemineerd. Het feit dat dieren mogelijks niet goed tochtig waren op het moment van KI met daarbovenop het inbrengen van vreemd materiaal in de uterus, kunnen er mogelijks hebben voor gezorgd dat er een inflammatoire reactie van het endometrium is ontstaan.

CURRICULUM VITAE

Osvaldo Bogado Pascottini was born on February 25th 1986, in Asuncion, Paraguay. He finalized his secondary studies in the Colegio Cristo Rey in 2004, in Asuncion. In 2005 he continued his academic formation at the Facultad de Ciencias Veterinarias of the Universidad Nacional de Asuncion, where he obtained his DVM diploma in 2011.

In 2012 Osvaldo achieved a post graduate study in 'Biotechnology of Reproduction in Ruminants' at the Institute of Bovine Reproduction of Cordoba, Argentina. Between 2012 and 2013 he worked as a bovine practitioner in the Paraguayan beef industry, performing mainly reproduction related activities in cows. In 2013 he successfully obtained a grant from the Erasmus Mundus (Mundus Lindo) project for a full doctoral training program at the Faculty of Veterinary Medicine of the Ghent University (Belgium).

Osvaldo is first author and co-author of several articles published in international peer-reviewed journals. His experimental work has been presented during various European and international congresses.

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