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BIOTECHNOLOGY

Endometrial Cytology to Diagnose Subclinical Endometritis in Cows

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ABSTRACT

Endometrial cytology is a method commonly used in gynaecological practice for the diagnosis of reproductive disorders in cattle. This is due to the unusual structure of the cervix, which make reaching the uterus difficult. Proper preparation and evaluation of cytological smears depend not only on the experience of the personal performing the examination but also on the selection of an appropriate method for obtaining material from the uterus. Cytological examination is performed in cows mainly in order to exclude or confirm subclinical endometritis when ultrasonography reveals only slight changes or no irregularities. Subclinical endometritis (SE) is defined as inflammation of the endometrium in the absence of any signs of clinical endometritis such as purulent vaginal discharge and is associated with delayed uterine involution. SCE is also known as cytological endometritis on the basis of "an elevated ratio of polymorphonuclear cells (PMN) in endometrial cytology samples. Endometritis is indicated 21-33 d after parturition if leukocytes exceed 18% and 34-47 d after parturition if the percentage exceeds 10%.

Highlights

- Endometrial cytology is important for diagnosis of subclinical endometritis because absence of clinical signs.
- Subclinical Endometritis has a negative effect on reproductive performance as it increases services per conception, the calving to first service interval and the calving to conception interval, reduces the risk of pregnancy and decreases the conception rate.

Keywords: Endometrial cytology, Subclinical endometritis, Leukocytes, Smears, Cytological endometritis

Endometrial cytology is a method commonly used in gynaecological practice for the diagnosis of reproductive disorders in cattle. This is due to the unusual structure of the cervix, which can make to reach the uterus difficult. Proper preparation and evaluation of cytological smears depend not only on the experience of the personnel performing the examination, but also on the selection of an appropriate method for obtaining material from the uterus (Prieto *et al*. 2012). Cytological examination is performed in cows mainly in order to exclude or confirm subclinical endometritis when ultrasonography reveals only slight changes or no irregularities (Hassing *et al.* 2010).

Subclinical endometritis (SE) is defined as inflammation of the endometrium in the absence of any signs of clinical endometritis such as purulent vaginal discharge and is associated with delayed uterine involution (Sheldon *et al.* 2006).

SCE is also known as cytological endometritis on the basis of "an elevated ratio of polymorphonuclear cells (PMN) in endometrial cytology samples.

Endometritis is indicated 21-33 d after parturition if leukocytes exceed 18% and 34-47 d after parturition if the percentage exceeds 10%. The cytological examination in such cases is limited to calculating in various ways the percentage of cells characteristic for inflammatory infiltration, i.e. leukocytes, mainly segmented neutrophils and lymphocytes. The evaluation involves counting 100-150 of all cells visible in the smear and determining the percentage of leukocytes among them, or counting all cells visible in 10 fields of view and determining the percentage of leukocytes (Baranski *et al.* 2013). It seems interesting to create a cytological model of the endometrium representative of the cyclical and pathological changes that take place on the ovaries, which alter the appearance and functioning of the endometrium by producing the hormones associated with these changes (Brodzki *et al.* 2014).

Subclinical Endometritis has a negative effect on reproductive performance as it increases services per conception, the calving to first service interval and the calving to conception interval, reduces the risk of pregnancy and decreases the conception rate. This results in major economic losses (Kasmanickman *et al*. 2004).

Endometrial and inflammatory cells may be collected by a guarded cotton swab, uterine biopsy, uterine lavage, or cytobrush techniques to evaluate endometrial cytology, especially as an aid in the diagnosis of subclinical-endometritis. A technique that yields well-preserved cells representative of a large uterine surface area without causing harm to the reproductive tract is required for consistent and reliable cytological results.

UTERINE DEFENSE MECHANISM

The postpartum uterus in dairy cow is succeptible to multiple bacterial pathogens and succeptibility to infection appears to be associated with periparturient immunosuppression and energy status. The uterine immune response to microbes leads to an influx of neutrophils from the peripheral circulation into the endometrium and uterine lumen (Dhaliwal *et al*. 2001 and Zerbe *et al*. 2003).

Neutrophils play an important role as they provide the first line of cellular defense against bacterial colonization within the uterus. Peripheral blood PMN functions of periparturient dairy cows are impaired relative to non-parturient cattle. The impaired PMN function around the time of parturition is associated with nutrient deficiencies that occur prior to parturition (Hammon *et al*. 2006).

Effective defense against reproductive tract invasion by environmental organisms is mediated by anatomical and functional barriers as well as nonspecific and specific immune responses (Dhaliwal *et al*. 2001: Azawi 2010). The uterine defense mechanisms against contaminant microorganisms are maintained in several ways: anatomically, by the simple or pseudo stratified columnar epithelium covering the endometrium; chemically, by mucus secretions from the endometrial glands; immunologically, through the action of polymorphonuclear inflammatory cells and humoral antibodies.

It is generally accepted that the cyclical pattern of steroid hormone concentration, characteristic for different stages of the oestrous cycle regulates the potential pathogenicity of micro-organisms that contaminate the uterus postpartum. For example, the endometrium is more susceptible to infection under progesterone than oestrogen dominance. Cattle are resistant to uterine infections when progesterone concentrations are basal and they are susceptible when progesterone concentrations are increased (Lewis 2003).

ENDOMETRIAL CYTOLOGY (SAMPLE COLLECTION METHOD)

Different method have been used for harvesting luecocytes viz., direct swab (Azawi *et al*. 2007 and Yavari *et al*. 2009), uterine lavage /aspiration (Azawi *et al*. 2008; Chapwanya *et al*. 2009 and Ramesh Babu 2010) and cytobrush technique (Barlund *et al*. 2008; Moscuzza *et al*. 2015 and Pothman *et al*. 2015).

1. Cytobrush method

Endometrial cytology has been used as a diagnostic tool in horses, but Kasimanickam *et al*. (2004) and Barlund *et al.* (2008) used a modified cytobrush to collect endometrial cytology samples in cows. The cytobrush is cut to 1 cm in length, threaded onto a solid stainless steel rod, 65 cm in length and 4 mm in diameter and placed in a stainless steel tube, 50 cm in length and 5-6 mm in diameter, to pass it through the cervix. The instrument is placed in a sanitary plastic sleeve to protect it from vaginal contamination. The vulva is cleaned with paper

towels and the lubricated instrument is passed through the vagina to the external os of the cervix. The plastic sleeve is perforated and the stainless steel sheath and extension is manipulated through the cervix and into the body of the uterus where the cytobrush is turned clockwise approximately ¼ turn to obtain endometrial cells from the uterus. The cytobrush is retracted into the stainless steel tube prior to removal from the uterus. The cytobrush is rolled onto a clean glass slide and allowed to air-dry on farm. Slides are stained with modified Wright Giemsa stain and evaluated using 400x magnification for differential cell count (endometrial, PMNs and squamous cells). The cytobrush technique has been used to evaluate the relationship between PMNs and conception.

2. Cytotape method

Cytotape consist of a 1.5-cm piece of paper tape roll on the top of a standard AI catheter, covered with a double guard sheet. All the CT sheets were prepared in advance to be ready to use at the farm. Briefly, the AI gun is introduced into the vagina under rectal guidance manipulation through the cervix. Once it reach the uterine lumen (corpus uteri), the tip of the catheter is released from the double guard sheet and then it is rolled twice on the dorsal wall of the uterine body with a gentle pressure of the index finger through the rectum and removed from the cow's genital tract (Pascottini *et al*. 2016).

3. Aspiration

After thorough clinical examination at estrus phase, a fluid (2-3 ml approximately) is aspirated from uterine lumen using sterile uterine catheter connected to a 20 ml disposable syringe and transferred into sterile tubes, then transported to laboratory aseptically and carefully at 4°C for the determination of percentage of polymorphonuclear cells. The fluid is smeared on the slide and stained by Papanicolaou and Giemsa stains (Kasimanickam *et al*. 2005 and Yavari *et al*. 2009).

4. Lavage technique

The uterine body is lavaged by infusing 60 ml of 0.9% sodium chloride solution into the uterine body with a 60-mL syringe attached to a 52-cm disposable plastic infusion rod. The uterus is massaged and then recovers the fluid by aspiration. Uterine lavage samples are brought to the laboratory within 2 h and centrifuged at 1000 rpm for 15 min. After discarding the supernatant, cytological smear is prepared from the sediment. A drop of sediment is streaked on to a clean microscopic slide and air dried (Kasimanickam *et al*. 2005). Smear is stained with hematoxillin and eosin stain and examined under oil an immersion lens of a microscope (Singh and Sulochana 1996; Nibret Moges 2015).

5. Low volume lavage technique

The cytologic sampling technique "Low volume uterine flushing" was firstly described by Ball *et al.* (1988) in mares and subsequently modified by Gilbert *et al.* (1998) for its use in cows. Sterile 0.9% sodium chloride solution (20ml) is infused into the uterus using a sterile, plastic infusion pipette. The vulva and perineum is cleaned, and the pipette is inserted into the vagina. The pipette is then manipulated through the cervix and the saline solution is infused into the uterus. 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 for 7 min), supernatant excluded and the pellet smeared onto a clean microscope glass slide. The slides are then stained by the May Grunwald-Giemsa technique and examined by light microscopy (magnication x400) to perform the differential cell count of 200 cells by two independent technicians (Galvao *et al.* 2011; Saut *et al.* 2013).

6. Guarded Swabs

Guarded swab method is essentially the same as cytobrush technique. The only difference is on solid stainless steel rod cotton swabs are placed instead of cytobrush. The double-guarded culture swab is guided manually through the vulva, vagina, and cervix and advanced into the uterus. The swab is extended and gently rolled for approximately 15 seconds. The swab is retracted through the guard and rolled onto a pair of microscope glass slides. The slide is air-dried, fixed in alcohol and stained (i.e., Diff-Quick stain). Examination of the stained cytological specimen is done under a microscope (Williams *et al.* 2005 and Kenide *et al.* 2016).

STAINING METHODS

Different staining methods have been used for endometrial cytology viz., Giemsa staining (Nibret Moges 2015 and Salah *et al*. 2017) May-Grunwald Giemsa staining (Saut *et al.* 2013 and Kenide *et al*. 2016) Diff-Quik staining (Pascottini *et al.* 2016) Papanicolau staining (Samatha *et al*. 2013) and Leishman staining (Suman Kumar G. 2010).

1. Giemsa Staining

Contents of the staining reagents:

Methanol	73%
Glycerol	26%
Giemsa's Azur-Eosin-Methylene blue	0.6%

Procedure

- Prepare the mucus smear on the surface of clean microscopic glass slide.
- Fix the smear in absolute methyl alcohol for 5 minute.
- Drain off the alcohol and allow the slide to dry.
- Flood the diluted stain on the smear for 20 minutes.
- Finally wash with distilled water.
- Wipe the back of the slide to remove excess stain and allow to dry in an upright position.

Smear slide is evaluated by light microscope. At least 100 to 200 cells are counted at 20 microscopic fields (×900). The counted cells are epithelial cells, large vacuolated epithelial cells, neutrophil and lymphocyte (Ahmadi *et al.* 2006).

2. May-Grunwald Giemsa Staining

Contents of the staining reagents:

May-Grunwald solution –	0.2%
Methanol —	99%
May-Grunwald eosin-methylene blue —	0.2%

Procedure

- Prepare the smear from endometrial sample on the clean glass slide.
- Fresh air-dried smears are immersed in a jar of absolute methyl alcohol for 3 minute.
- Transfer to a jar containing May-Grunwald stain diluted with 1 or 2 parts of buffered distilled water and leave for 3 to 15 minute.
- Without washing, transfer the slide to Giemsa stain diluted with 9 parts of buffered distilled water and leave for 7 to 30 minute.
- Finally washed in distilled water and air dry the smear.

3. Wright-Giemsa Staining (Diff-Quik staining)

Procedure

- Cover the dried endometrial fluid smear completely with Wright-Giemsa stain and allow standing for 1 to 3 minute.
- Add an equal amount of buffered distilled water or neutral water.
- Allow the mixture to stand for 3 to 5 minute. A metallic scum should appear green.
- Float off the metallic scum with a stream of water from a wash bottle or from the tap.
- Wipe the stain from the back of the slide.
- Stand the slide on end and wave gently in the air.
- Examine the slide on an oil immersion lense.

4. Papanicolau Staining

Principles of PAP Stain

- Haematoxilyn (violet): A basic stain with chemical affinity for acid substances (i.e. nuclei of cells, filled with DNA).
- EA50 (light blue): An acid stain that reacts with the cytoplasm of less mature squamous (exocervical) cell (basal, parabasal and intermediate cell) and with glandular (endocervical) cell.
- OG6 (orange): An acid stain that reacts with superficial squamous cell, filled with keratin.
- The haematoxylin nuclear stain demonstrates chromatinic patterns of normal and abnormal cells.
- The counterstains, Orange-G and E.A. (eosin-azure) have a high alcoholic concentration which provides cytoplasmic transparency. This enables clear visualization through areas of overlapping cells, mucus and debris.

- There are four main steps in the staining procedure: (1) Fixation (2) Nuclear staining (3) Cytoplasmic staining (4) Clearing

Staining steps

1. 95% Ethanol (fixation) 2 minutes
2. Distilled water 2 minutes
3. Harris Haematoxylin 1 minutes
4. Tap water 5 minutes
5. 95% Ethanol 15 second
6. OG 6 2 minutes
7. 95% Ethanol 15 second (twice)
8. EA 50 5 minutes
9. 95% Ethanol 15 second
10. Absolute ethanol 30 second (twice)
11. Xilene or Bioclear 2 minutes (twice)

Results

Nuclei should appear – blue/black cytoplasm (non-keratinised squmous cells) – blue/green keratinised cells – pink/orange

5. Leishman Staining

Staining steps

- Prepare the smear on the slide.
- Flood the smear with undiluted stock Leishman's stain and leave for 1 to 2 minute to fix.
- Dilute the stain on the smear with double the volume of buffered distilled water and stains for 5 to 15 minute.
- Blow the mixture or rock the slide gently to aid mixing.
- Wash with distilled water until the film has a pinkish tinge (0.5 to 2 min.)
- Wipe the back of the slide to remove excess stain and allow drying in an upright position.

The evaluation involves counting 100-150 of all cells visible in the smear and determining the percentage of leukocytes among them, or counting all cells visible in 10 fields of view and determining the percentage of leukocytes.

Table 1: Interpretation Of Endometrial Cytology

References	Days in milk (DIM)	PMN (%)
LeBlanc *et al.* (2002)	21-33	>18
Kasmanickman *et al.* (2004) Sheldon *et al.* (2006) Brodzki *et al*. (2014)	34-47	>10
Barlund *et al*. (2008)	28-41	>8
Salah *et al.* (2017)		
Plontzke *et al*. (2010) Egbert *et al.* (2016)	18-38	>5
Dubuc *et al*. (2010) Singh *et al*. (2016)	35±3	>6
	56±3	>4
McDougall *et al*. (2011)	30-45	>9
Madoz *et al.* (2013)	21-33	>8
	34-47	>6
	48-62	>4

Overall conclusion is that Subclinical endometritis is indicated 21-33 d after parturition if PMNs exceed 18% and 48-62 d after parturition if the percentage exceeds 4%.

OTHER DIAGNOSTIC METHODS

1. Endometrial biopsy

Biopsy sample is collected from caudal one-third portion of uterine horns by Albuchin's uterine biopsy catheter. The endometrial biopsy is immediately placed into a bottle containing 10% formal saline solution and stored at 4°C until preparation for sectioning, which included dehydration, clearing, embedding, sectioning, and staining, performed according to the methods described by Luna (1968). Microscopic examination of endometrial biopsy samples of endometritis reveal changes in endometrium like disruption of surface epithelium, leucocytic infiltration, varying degrees of glandular degeneration and periglandular fibrosis (Bajaj 2002). The drawbacks with this method is that it is time consuming, expensive, invasive, has detrimental effect on future fertility and is seldom useful on the spot for planning therapeutic regimen (Sheldon *et al*. 2006).

2. Leukocyte esterase colorimetric strips (LES)

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; which intensity is related with the leukocyte counts. It is a "cow side" diagnostic method for diagnosis of SCE. The results obtained by LES are positively correlated with endometrial cytology results (Pascottini *et al*. 2016).

3. White side test

W.H. Whiteside (1939) first used this test for diagnosis of mastitis. In this test, cervical mucus is collected aseptically from suspected animals and is boiled with equal amount of 5 per cent sodium hydroxide. The test is considered positive if the color turns yellow. This test is based on correlation between the number of leucocytes present in the mucus and intensity of yellow color (Pateria and Rawal 1990).

Kumar (2010) reported that the white side test had a significant positive correlation with pH, bacterial load in CVM and bacterial load in uterine flushings.

3. Per-rectal palpation

Though trans-rectal palpation of uterus is the most commonly practiced diagnostic method but it is not an accurate method for diagnosis of all type of endometritis (Oral *et al*. 2009). However, a holistic and systematic approach will be more helpful in providing information for such a multi factorial disease.

4. Vaginoscopy

Vaginoscopy is considered as a more accurate method than rectal palpation for diagnosis of uterine infections. Observation of vaginal discharges are useful for the diagnosis of clinical endometritis but not for sub-clinical endometritis as discharges may not always be collected due to closure of os, although these animals may harbour infection in uterus. A single vaginoscopic examination therefore lacks accuracy and hence not a true indicator of absence of uterine inflammation (Oral *et al*. 2009).

5. Trans-rectal ultrasonography

Trans-rectal ultrasonography is a useful diagnostic tool in determining uterine size, echotexture and fluid accumulation in endometritis (Honparkhe *et al*. 2007). Monitoring endometrial echotexture alterations, especially homogeneity and contrast changed dependency on the cellular density and inflammation status may be potential diagnostic markers for sub-clinical endometritis in cows (Polat *et al.* 2015). Ultrasonography though a practical cow-side test, but when used alone is not specific enough to accurately diagnose subclinical endometritis in animals (Barlund *et al.* 2008 and Bajaj *et al.* 2015).

Purohit *et al*. (2013) reported that subclinical endometritis is a frequent cause of endometritis in cows and can be diagnosed by ultrasonographic appearance of uterine luminal fluid and /or increased endometrial thickness.

Polat *et al*. (2015) studied echotexture of endometrium by ultrasonography in postpartum cows with sub-clinical endometritis and concluded that monitoring endometrial echotexture alterations, especially homogeneity & contrast changed dependency on the cellular density and inflammation status may be potential diagnostic markers for sub-clinical endometritis in cows.

6. Acute phase protein levels in endometritis

Acute phase proteins (APP) refer to a group of hepatic glycoproteins which are stimulated by inflammatory mediators and respond to initial reaction to infection, inflammation or trauma in animals (Marinkovic *et al*., 1989). The function of APP is to promote immunoglobulin production and tissue repair, preventing further injury and recycling useful molecules and debris (Kent, 1992).

Ruminants are significantly different to other species in their acute phase response in that haptoglobin (Hp) is the major acute phase protein. The other APPs in cattle are serum amyloid A (SAA) and α_1 acid glycoprotein (AGP). In healthy cattle the serum haptoglobin concentration is <20mg/L but can increase upto >2g/L in 2 days of infection. In cattle, haptoglobin concentration is effective in the diagnosis and prognosis of mastitis, peritonitis, pneumonia and endometritis (Murata *et al*., 2004 and Petersen *et al*., 2004).

COMPARISON OF DIFFERENT METHODS USED FOR DIAGNOSIS OF ENDOMETRITIS

- Kasimanickam *et al*. (2005) compared the cytobrush and uterine lavage techniques to

evaluate endometrial cytology and concluded that the cytobrush technique is a consistent and reliable method for obtaining endometrial samples for cytologic examination from postpartum dairy cattle. The uterine lavage technique required more time to collect samples and 17% failure in attempts to recover lavage fluid, increased distorsion of cells was also observed.

- Barlund *et al*. (2008) compared the efficacy of vaginoscopy, ultrasonography and endometrial cytology by uterine lavage and by cytobrush technique for diagnosis of postpartum endometritis and concluded that cytobrush technique is the most precise technique for diagnosis of endometritis.
- Madoz *et al*. (2014) compared endometrial cytology, biopsy and bacteriology for the diagnosis of sub-clinical endometritis and reported that endometrial cytology is more reliable method than uterine biopsy for diagnosis of sub-clinical endometritis as the uterine biopsy outputs are influenced by the estrous cycle whereas there is no significant change in estrous cycle in superficial endometrial layer.
- Moscuzza *et al*. (2015) compared endometrial cytology with biopsy and quoted that endometrial cytology is a non invasive technique with high specificity and repeatability for the diagnosis of sub-clinical endometritis postpartum in dystocic beef heifers.

CONCLUSION

The cytobrush technique can provide sufficient uterine cells to perform both cytology and gene expression analysis with a single sample. The technique is non-invasive, and the samples obtained using a cytobrush are representative of endometrial cells which are directly involved in the uterine immune defense system.

The uterine lavage technique as compare to cytotape required more time to collect samples, failure in attempts to recover lavage fluid, increased distorsion of cells and it may cause irritation to the endometrium.

Cotton swabs are more likely to distort cells as compare to Cytobrush and Cytotape.

Endometrial cytology samples with Cytobrush and Cytotape yields similar results regarding parameters like PMNs % and total cellularity. However, techniques significantly differ in quality parameters and RBCs contamination in favor of the Cytotape. When samples are taken by cytotape, less distorted–fragmented cells and a significantly lower contamination with RBCs are reported.

Overall conclusion is that cytotape method for sample collection is better than cytobrush and lavage technique for diagnosis of subclinical endometritis.

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