A non-invasive diagnostic test for subclinical endometritis in buffaloes

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ABSTRACT

This study was conducted to evaluate the potential of uterine lavage sample optical density (ULSOD) test at the time of estrus for diagnosis of subclinical endometritis (SCE). Buffaloes (86) at the time of estrus having >5% polymorphonuclear (PMN) cells in endometrial cytosmears were designated as positive (21) and buffaloes with ≤5% PMN cell as negative (65) for SCE. Presence of E. coli, A. pyogenes and F. necrophorum in the uterus was detected based upon PCR amplification of genes related to bacteria specific virulence factors (finH, fimA and lktA genes, respectively). Pathogenic bacteria were isolated from 76.2% buffaloes with SCE as compared to 39.4% buffaloes without SCE. E. coli (finH) and F. necrophorum (lktA) represented the major bacteriological risk factor for occurrence of SCE. The optical density of uterine lavage was measured at 352, 500, 620, 790 and 960 nm wavelengths. ULSOD620 was selected as reference wavelength because it presented the greatest area under curve (0.80). The recommended threshold for the receiver operator curve was 0.029 with a sensitivity and specificity of 85.7 and 73.8%, respectively. In the current study, the level of agreement (kappa) of ULSOD620 with cytobrush cytology was moderate (0.49) and the diagnostic accuracy was good (76.7%). Buffaloes with ≤0.029 ULSOD620 at the time of estrus had significantly lower conception rate at corresponding AI as compared to buffaloes with >0.029 ULSOD620. It is suggested that ULSOD620 measurement could be used as alternative to endometrial cytology and can be a tool to predict the outcome of artificial insemination in buffaloes.

Key words: Buffalo, Estrus, Optical density, Subclinical endometritis, Uterine lavage

Subclinical endometritis (SCE) is the superficial inflammation of endometrium without visible clinical signs, but significantly impairing reproductive performance (Kasimanickam et al. 2004, Gilbert et al. 2005). Studies have demonstrated the presence of SCE to be a key factor in pregnancy rate, with an increase in days open ranging from 25 days (Dubuc 2011) to 30 days (Madoz et al. 2013). The extra days without conception (Kasimanickam et al. 2004, Gilbert et al. 2005, Barlund et al. 2008), extra artificial inseminations (Gilbert et al. 2005, Barlund et al. 2008), and subsequent culling result in economic losses.

In cows and buffalo, several tests have been developed to evaluate the inflammatory processes of the endometrium, viz. histopathology (Pascottini et al. 2016b), ultrasonography (Kasimanickam et al. 2004, Meira et al. 2012), leukocyte esterase test (Couto et al. 2013, Denis-Robichaudand Dubuc 2015), endometrial cytology (Kasimanickam et al. 2004, Singh et al. 2018), cytology of small volume uterine lavage (Gilbert et al. 2005, Dar et al. 2015), bacteriology (Bariński et al. 2012, Pothmann et al. 2015), optical density of uterine lavage (Machado et al. 2012) etc. Cytological assessment using cytobrush technique is the reference method for diagnosis of subclinical endometritis, owing to ease of collection of samples, quality of the samples, rapid results, and repeatability of the test (Wagener et al. 2017). On the basis of endometrial cytology, the incidence of SCE in buffaloes had been reported to be between 23% (Gahlot et al. 2016) and 26% (Bajaj et al. 2016); however, higher incidence of subclinical endometritis 41.7% based on uterine lavage cytology (Dar et al. 2015) had also been recorded. Uterine lavage sample optical density (ULSOD) test was recently developed as a non-invasive test for diagnosis of SCE. Machado et al. (2012) reported good accuracy (100% sensitivity, 82.3% specificity) for dichotomized ULSOD compared with endometrial cytology. The technique of measuring ULSOD is simple, does not require special training and cannot be influenced by the researcher. However, this test has not yet been standardized in buffaloes, especially during estrus.

The main disadvantages of cytology based methods are observer bias and the vast variety of cut-off values to differentiate affected versus unaffected animals taking into account the days after calving samples are taken. Furthermore, in buffalo, the time period for sampling and cut-offs values have not been standardized. Endometrial sampling especially during estrus could be ideal in the
perspective that it offers the opportunity to determine the uterine health status at the time when the animal is fertile and thus allow studying the effect of SCE on the subsequent conception rate (Pascottini et al. 2016a, Singh et al. 2018). Estrus could also provide a standardized time for the use of a universal cut-off point without interference of normal uterine involution process, thus further increasing sensitivity and specificity of the diagnostic test. Hence, the aim of present study was to evaluate the potential of ULSOD at the time of estrus for the diagnosis of subclinical endometritis in buffaloes.

MATERIALS AND METHODS

Pluriparous buffaloes (86) detected in standing estrus were subjected to endometrial cytobrush sampling (Barlund et al. 2008) and uterine lavage followed by artificial insemination 4 to 6 h later. Each animal was subjected to two cytobrush sampling, once each for endometrial cytology and DNA extraction for detection of uterine bacteria. The threshold values for percentage of PMN cells in endometrial cytology for diagnosis of uterine inflammation (subclinical endometritis) using universal PMN cell cut-off (Gilbert et al. 2005, Singh et al. 2018). Accordingly buffaloes at estrus with >5% in endometrial cytosmears were considered as positive for subclinical endometritis.

Endometrial cytology: The first cytobrush was rolled on a clean grease free microscope slide and the slides were dried, fixed with methanol and stained with Giemsa stain. Slides were examined using light microscopy and 300 cells per slide were counted to record the % of PMN cell.

Detection of uterine bacteria: The second cytobrush was immediately immersed in 2 ml phosphate buffered saline (PBS) in a 15 ml centrifuge tube and transported at 4°C in an icebox until it was processed in the laboratory. The extraction of DNA from the cytobrush washing was carried through Cetyltrimethyl ammonium bromide (CTAB) method (Atashpaz et al. 2010). The extracted DNA was subjected to quantitative and qualitative assessment using recommended protocols. Finally, DNA extracted from uterine cytobrush samples was used for PCR amplification of fimH gene of E. coli, fimA gene of A. pyogenes and lktA gene of F. necrophorum using published primers (Table 1). The primer sequences were synthesized by Genaxy India Pvt. Ltd. The amplicons generated were resolved on agarose (1.2%) and images were recorded using gel-doc system.

Uterine lavage sample optical density (ULSOD) test

Uterine flushing was performed by slowly infusing 20 ml of sterile normal saline solution (NSS) in to the uterus with the help of a sterile 50 ml syringe, while holding the catheter per-rectally. After a brief gap of 10–15 sec, uterine horn was massaged gently and a minimum of 5 ml of infused fluid was sucked out with the same syringe. The obtained flush sample was immediately transported to the laboratory. Once inside laboratory, uterine lavage samples were diluted with hypertonic saline solution (10%) in a 1:1 proportion. The diluted samples were incubated at room temperature for 30 min and then centrifuged at 4°C for 30 min at 3000 g. The supernatant was collected and an aliquot of 2 ml from each sample was taken in a cuvette. The optical density was measured at the following wavelengths (352, 500, 620, 790 and 960 nm) in double beam spectrophotometer.

Artificial insemination: All the buffaloes were inseminated with good quality frozen thawed semen 4–6 h after cytobrush sampling and uterine lavage (twice as per AM/PM schedule) and were confirmed for pregnancy by ultrasonography at day 40 post-insemination.

Statistical analysis: The statistical analysis was carried out using SPSS Statistics 16.0 (SPSS, Chicago, USA). Mean values of PMN cell count, optical density, etc were compared using independent sample T-test. p-value of less than 0.05 was considered as statistically significant. Association of presence of bacterial virulence factors and subclinical endometritis was analysed using Chi-square test. Different wavelengths were compared using receiver operator characteristic (ROC) curve analysis and the wavelength having highest area under curve (AUC) was selected. For the selected wavelength, the optimum cut-off was taken as a point at which the Youden’s index (Se + Sp – 1) was maximum (Habibzadeh et al. 2016). Furthermore, the diagnostic potential of selected cut-off value was compared against a gold standard test (PMN cell %) by calculating sensitivity, specificity, diagnostic accuracy, and Kappa statistic (κ) using standard formulae. Kappa statistic (κ) measures the agreement of test with gold standard. κ was calculated using crosstab function of SPSS Statistics 16.0 (SPSS, Chicago, USA). Value of ≤0 indicate no agreement, 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement.

RESULTS AND DISCUSSION

Prevalence of subclinical endometritis using cytobrush

Table 1. Primers used for PCR amplification

| Target gene | Primer sequence (5’–3’) | Tm (°C) | Product size (bp) | Reference |
|-------------|-------------------------|--------|------------------|-----------|
| E. coli (fimH) | Forward-TGCAGAAACGGATAAAGCCGTGG Reverse-GCAGTCACCTGGCCCTCGGTA | 63 | 508 | Moreno et al. (2005) |
| A. pyogenes (fimA) | Forward-CACTACGCTACCATATCAACAAG Reverse-GCTGTAATCCGCTTTGGTCTGT | 59 | 605 | Silva et al. (2009) |
| F. necrophorum (lktA) | Forward-AATCGGAGTAGTATGGTTTCG Reverse-TTTGGTAACTGCCACTGC | 60 | 401 | Zhou et al. (2009) |
cytology: A prevalence rate of 24.4% was recorded for SCE based on the endometrial cytobrush cytogram (>5% PMN cell count); accordingly 21 out of total 86 buffaloes were declared as positive for SCE at estrus. Buffaloes diagnosed positive for SCE had mean PMN % of 6.14 (95% CI: 5.30–6.98) and buffaloes negative for SCE had mean PMN% of 2.09 (95% CI: 1.79–2.39) at the time of estrus.

Association between uterine bacterial virulence factors and subclinical endometritis: E. coli (fimH) and F. necrophorum (lktA) represented the major bacteriological risk factor for occurrence of subclinical endometritis (Table 2).

Table 2. Association between the presence of E. coli in the uterus at the time of estrus and uterine inflammation in buffalo

| Bacteria (VF)       | % Incidence | χ² (sig.) |
|---------------------|-------------|-----------|
| E. coli (fimH)      | 52.4        | 24.6      |
| A. pyogenes (fimA)  | 38.1        | 21.5      |
| F. necrophorum (lktA) | 42.8        | 7.7       |

*Continuity correction was used when expected value of any cell was less than 5.

It is well known that bacterial invasion of the uterus induces an inflammatory response in endometrium (Sheldon et al. 2006). The uterine pathogenic bacteria act synergistically to enhance the endometrial inflammation (Singh et al. 2008). E. coli infection affects the phenotype and function of polymorphonuclear cells, damages the uterus, favours the absorption of endotoxin and supports the co-infection by A. pyogenes (Azawi et al. 2008). A. pyogenes expresses a cholesterol dependent cytotoxoinylosis (PLO) which drills a pore in cell membrane, leading to osmotic death of the cell and increasing the susceptibility of the endometrium to E. coli (Singh et al. 2008). F. necrophorum, on the other hand, actively invade uterine tissues and produces a leucocidal toxin that inhibits phagocytosis (Tan et al. 1994).

In present study, presence of A. pyogenes (fimA) was not significantly associated with subclinical endometritis. Previous studies had also reported that A. pyogenes is risk factor for more severe forms of endometritis (Prunner et al. 2014, Dar et al. 2015). The lack of association could be attributed to clearance of A. pyogenes infection in postpartum animals by the time of first estrus or due the presence of virulent factors other than fimA such as plo, cbpA, fimG, nanH and nanP (Santos et al. 2010, Bicalho et al. 2012). Interestingly, Madoz et al. (2014) also showed that the likelihood of isolating A. pyogenes increased by only 0.6% with increasing percentage points of PMN. In present study, no pathogenic bacteria were isolated from 5 out of 21 buffaloes with subclinical endometritis. Uterine inflammation detected in the absence of bacteria had also been reported (McDouggall et al. 2011, Barański et al. 2012), where not all cows with detectable uterine inflammation (PMN %) were culture-positive for bacteria. The failure to isolate any of the above bacteria in buffaloes with SCE may be attributed to presence of bacteria of other genera as reported previously (Singh et al. 2018).

Association between optical density of uterine lavage and SCE: The ROC analysis was performed to evaluate the accuracy of optical density of uterine lavage samples measured at several different wavelengths (Table 3). The ULSOD620 was selected as reference wavelength for detection of subclinical endometritis at estrus because it presented the greatest AUC among all wavelengths evaluated. The optimum cut-off was the optical density value having maximum Youden’s index (Sensitivity + Specificity – 1) (Habibzadeh et al. 2016). Therefore, OD value >0.029 at 620 nm, with sensitivity and specificity of 85.7% and 73.8%, respectively, was selected as optimum cut-off value.

Table 3. Area under curve (AUC) for OD values of uterine lavage samples collected on first postpartum estrus at different wavelengths obtained by ROC analysis

| Wavelength | AUC  | SE  | Sig. |
|------------|------|-----|------|
| 352 nm     | 0.47 | 0.08| 0.65 |
| 500 nm     | 0.57 | 0.08| 0.36 |
| 620 nm*    | 0.80 | 0.06| 0.01 |
| 790 nm     | 0.65 | 0.06| 0.03 |
| 960 nm     | 0.63 | 0.07| 0.08 |

*Cut-off value = 0.029 (sensitivity, 85.7%; specificity, 73.8%).

Prevalence of subclinical endometritis using ULSOD620: Based on the derived ULSOD620 cut-off (>0.029), 35 out of total 86 buffaloes were declared as positive for SCE at estrus; accordingly a prevalence rate of 40.7% was recorded. Buffaloes diagnosed with SCE by ULSOD620 had mean OD of 0.039 (95% CI: 0.034–0.044) and buffaloes without subclinical endometritis had mean OD of 0.022 (95% CI: 0.020–0.024).

Endometrial cytology is the most commonly used technique to diagnose SCE in cattle in both field and research setups (Dubuc et al. 2010, de Boer et al. 2014). 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 (de Boer et al. 2014). The data presented suggested that the dichotomized ULSOD620 could be used to identify buffaloes with SCE with high sensitivity (85.7%) and specificity (73.8%) (Table 4). It is believed that higher ULSOD620 could be a result of presence of bacteria in uterine lavage samples, as well as exudation of protein and inflammatory cells within the uterine lumen as a result of endometritis (Cheong et al. 2012, Machado et al. 2012). In the present study, percentage of neutrophils in uterine lavage samples varied significantly (P<0.001) with respect to dichotomized ULSOD620. The PMN % was 4.5±0.4 in buffaloes with ULSOD620 >0.029 as compared to 2.1±0.2 in buffaloes with ULSOD620 ≤0.029.

Our results support the use of ULSOD620 for the diagnosis of subclinical endometritis in buffalo at the time of estrus. In current study, 40.7% of the buffaloes were...
diagnosed as having endometritis using ULSOD_{620} versus 24.4% with cytobrush cytology and the level of agreement between the two tests was moderate (k=0.49). The diagnostic accuracy of ULSOD_{620} for prediction of SCE was good (76.7%). On the other hand, the diagnostic accuracy of bacteriology for prediction of SCE and the level of agreement with cytobrush cytology was fair (63.9/7% and kappa=0.27, respectively) (Table 4). ULSOD test is rapid, inexpensive, has the ability to arrive at universal cut-off value, and may not be detrimental for future fertility of animal (Machado et al., 2012). The results indicate that the ULSOD_{620} can be effectively used for diagnosis of SCE at the time of estrus.

**Conception rate**: Presence of subclinical endometritis at the time of estrus (>5% PMN) significantly (P<0.05) decreased the conception rate at the corresponding AI. Similarly, buffaloes with ULSOD_{620} of >0.029 at the time of estrus had significantly (P<0.05) lower conception rate at the corresponding AI as compared to buffaloes with ULSOD_{620} of ≤0.029 (Table 5).

Table 5. Relation between presence of dichotomized endometrial cytology and ULSOD_{620} at estrus and conception rate in buffaloes.

| Diagnostic test | Threshold | No. of female | No. of conception |
|-----------------|-----------|---------------|-------------------|
|                 | ≥5% PMN   | ≤5% PMN       | (%)               |
| Endometrial     | ≥5% PMN   | 21            | 1                 | 4.8 | 5.82 |
|                  | ≤5% PMN   | 65            | 20                | 30.8 (P<0.05) |
| ULSOD at ≥0.029 | 620 nm    | 35            | 3                | 8.5  | 6.99 |
|                 | ≤0.029    | 51            | 18                | 35.3 (P<0.05) |

In conclusion, it is suggested that ULSOD_{620} measurement could be used as alternative to endometrial cytology for the diagnosis of SCE in estrual buffaloes. The findings of present study also suggest that ULSOD_{620} is associated with fertility and buffaloes with ULSOD_{620} >0.029 at the time of estrus had lowered conception rates compared to buffaloes with ULSOD_{620} ≤0.029.

**REFERENCES**

Atashpaz S, Khani S, Barzagari A, Barar J, Vahed S Z, Azerbajiani R and Omidi Y. 2010. A robust universal method for extraction of genomic DNA from bacterial species. *Microbiology* 79: 538-42.

Azawi O. 2008. Postpartum uterine infection in cattle. *Animal Reproduction Science* 105: 187-208.

Bajaj N K, Shukla P, Agrawal R G, Agrawal S and Honparkhe M. 2016. Subclinical endometritis in postpartum buffaloes: an emerging threat. *Journal of Animal Research* 6(5): 819.

Barański W, Podhalicz-Dzięgielewska M, Zduńczyk S and Janowski T. 2012. The diagnosis and prevalence of subclinical endometritis in cows evaluated by different cytologic thresholds. *Theriogenology* 78(9): 139-47.

Barlund C S, Carruthers T D, Waldner C L and Palmer C W. 2008. Subclinical endometritis in postpartum buffaloes: an emerging threat. *Journal of Animal Research* 6(5): 819.

Bicalho M L S, Machado V S, Oikonomou G, Gilbert R O and Bicalho R C. 2012. Association between virulence factors of *Escherichia coli*, *Fusobacterium necrophorum*, and *Arcanobacterium pyogenes* and uterine diseases of dairy cows. *Veterinary Microbiology* 157(1–2): 125–31.

Cerri R L A, Rutigliano H M, Lima F S, Araújo D B and Santos J E P. 2009. Effect of source of supplemental selenium on uterine health and embryo quality in high-producing dairy cows. *Theriogenology* 71: 1127–37.

Cheong S H, Nydam D V, Galvao K N, Crosier B M, Ricci A, Caixeta L S, Sper R B, Fraga M and Gilbert R O. 2012. Use of reagent test strips for diagnosis of endometritis in dairy cows. *Theriogenology* 77(5): 858–64.

Couto G B, Vaillancourt D H and Lefebvre R C. 2013. Comparison of a leukocyte esterase test with endometrial cytology for diagnosis of subclinical endometritis in postpartum dairy cows. *Theriogenology* 79(1): 103–07.
Jan M H, Kumar H, Chaudhary R K, Palanivelu M and Narayanan K. 2015. Association of Escherichia coli and Fusobacterium necrophorum with subclinical endometritis in postpartum Murrah buffalo. Indian Journal of Veterinary Pathology 39(4): 311–15.

de Boer M W, LeBlanc S J, Dubuc J, Meier S, Heuwieser W and Arsl S. 2014. Systematic review of diagnostic tests for reproductive-tract infection and inflammation in dairy cows. Journal of Dairy Science 97: 3983–99.

Denis-Robichaud J and Dubuc J. 2015. Determination of optimal diagnostic criteria for purulent vaginal discharge and cytological endometritis in dairy cows. Journal of Dairy Science 8: 6848–55.

Dubuc J, Duffield T F, Leslie K E, Walton J S and LeBlanc S J. 2010. Definitions and diagnosis of postpartum endometritis in dairy cows. Journal of Dairy Science 93: 5225–33.

Dubuc J. 2011. Postpartum uterus diseases: prevalence, impacts, and treatments. WCDSAdvances in Dairy Technology 23: 255–67.

Gahlot S C, Kumar S, Kumaresan A, Chand S, Baithalu R K, Lathika S, Pathbandha T K, Lathwal S S and Mohanty T K. 2016. Efficiency of uterine fluid cytology in the diagnosis of subclinical endometritis in the water buffalo (Bubalis bubalis). Reproduction in Domestic Animals 52(3): 513–16.

Gilbert R O, Shin S T, Guard C L, Erb H N and Fradjbat M. 2005. Prevalence of endometritis and its effects on reproductive performance of dairy cows. Theriogenology 64(9): 1879–88.

Habibzadeh F, Habibzadeh P and Yadollahie M. 2016. On determining the most appropriate test cut-off value: the case of tests with continuous results. Biochemiamedica 26(3): 297–307.

Hoelker M, Salilew-Wondim D, Drillisch M, Christine G, Ghanem Habibzadeh F, Habibzadeh P and Yadollahie M. 2016. On determining the most appropriate test cut-off value: the case of tests with continuous results. Biochemiamedica 26(3): 297–307.

Hoelker M, Salilew-Wondim D, Drillisch M, Christine G, Ghanem

Kassimanickam R, Duffield T F, Foster R A, Gartley C J, Leslie K E, Walton J S and Johnson W H. 2004. Endometrial cytology and ultrasonography for the detection of subclinical endometritis in postpartum dairy cows. Theriogenology 62: 9–23.

Kaufmann T B, Drillisch M, Tenhagen B A, Forderung D and Heuwieser W. 2009. Prevalence of bovine subclinical endometritis 4 h after insemination and its effects on first service conception rate. Theriogenology 71(2): 385–91.

Machado V S, Knauer W A, Bicalho M L S, Oikonomou G, Gilbert R O and Bicalho R C. 2012. A novel diagnostic technique to determine uterine health of Holstein cows at 35 days postpartum. Journal of Dairy Science 95(3): 1349–57.

Madoz L V, Giuliodori M J, Jaureguiberri M, Plöntzke J, Drillisch M and De la Sota R L. 2013. The relationship between endometrial cytology during estrous cycle and cutoff points for the diagnosis of subclinical endometritis in grazing dairy cows. Journal of Dairy Science 96(7): 4333–39.

Madoz L V, Giuliodori M J, Migliorisi A L, Jaureguiberri M and De la Sota R L. 2014. Endometrial cytology, biopsy, and bacteriology for the diagnosis of subclinical endometritis in grazing dairy cows. Journal of Dairy Science 97(1): 195–201.

McDougall S, Hussein H, Aberdeen D, Buckle K, Roche J, Burke J, Mitchell M and Meier S. 2011. Relationships between cytology, bacteriology and vaginal discharge scores and reproductive performance in dairy cattle. Theriogenology 76: 229–40.

Meira E B S, Henriques L C S, Sá L R M and Gregory L. 2012. Comparison of ultrasonography and histopathology for the diagnosis of endometritis in Holstein-Friesian cows. Journal of Dairy Science 95(12): 6969–73.

Moreno E, Planells I, Prats G, Planes A M, Moreno G and Andreu A. 2005. Comparative study of Escherichia coli virulence determinants in strains causing urinary tract bacteremia versus strains causing pylonephritis and other sources of bacteremia. Diagnostic Microbiology and Infectious Diseases 53: 93–99.

Pascottini O B, Hostens M, Dini P, Van Eetvelde M, Vercauteren P and Opsomer G. 2016a. Prevalence of cytological endometritis and effect on pregnancy outcomes at the time of insemination in nulliparous dairy heifers. Journal of Dairy Science 99(11): 9051–56.

Pascottini O B, Hostens M, Dini P, Vandepitte J, Ducatelle R and Opsomer G. 2016b. Comparison between cytology and histopathology to evaluate subclinical endometritis in dairy cows. Theriogenology 86(6): 1550–56.

Pothmann H, Prunner I, Wagener K, Jaureguiberri M, de la Sota R L, Erber R, Aurich C, Ehling-Schulz M and Drillisch M. 2015. The prevalence of subclinical endometritis and intrauterine infections in repeat breeder cows. Theriogenology 83(8): 1249–53.

Prunner I, Pothmann H, Wagener K, Giuliodori M, Huber J, Ehling-Schulz M and Drillisch M. 2014. Dynamics of bacteriologic and cytologic changes in the uterus of postpartum dairy cows. Theriogenology 82(9): 1316–22.

Santos M A, Caxeta L S, Machado V S, Rauf A K, Gilbert R O and Bicalho R C. 2010. Antimicrobial resistance and presence of virulence factor genes in Arcanobacterium pyogenes isolated from the uterus of postpartum dairy cows. Veterinary Microbiology 145: 84–89.

Sheldon I M, Lewis G S, LeBlanc S and Gilbert R O. 2006. Defining postpartum uterine disease in cattle. Theriogenology 65: 1516–30.

Silva E, Leitao S, Tenreiro T, Pomba C, Nunes T, Lopes de Costa L and Mateus L. 2009. Genomic and phenotypic characterization of Escherichia coli isolates recovered from the uterus of puerperal dairy cows. Journal of Dairy Science 92: 6000–10.

Singh H, Brar P S, Arora A K, Dhindsa S S and Honparkhe M. 2018. Bacterial presence and fertility in subclinical endometritis buffaloes at oestrus. Indian Journal of Animal Sciences 88(4): 415–19.

Singh J, Murray R D, Mselha G and Woldehiwet Z. 2008. The pathomechanism of subclinical endometritis in dairy cows. Theriogenology 515–19.

Soto P, Natzke P R and Hansen P J. 2003. Actions of tumor necrosis factor-α on oocyte maturation and embryonic development in cattle. American Journal of Reproductive Immunology 50: 380–88.

Tan Z L, Nagaraja T G, Chengappa M M and Smith J S. 1994. Biological and biochemical characterization of Fusobacterium necrophorum leukotoxin. American Journal of Veterinary Research 55: 515–19.

Wagner K, Gabler C and Drillisch M. 2017. A review of the ongoing discussion about definition, diagnosis and pathomechanism of subclinical endometritis in dairy cows. Theriogenology 94: 21–30.

Zhou H, Bennett G and Hickford J G. 2009. Variation in Fusobacterium necrophorum strains present on the hooves of footrot infected sheep, goats and cattle. Veterinary Microbiology 135: 636–67.