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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 insemination 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 (*fimH*, *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* (*fimH*) 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. $ULSOD_{620}$ 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 $ULSOD_{620}$ with cytobrush cytology was moderate (0.49) and the diagnostic accuracy was good (76.7%). Buffaloes with ≤0.029 $ULSOD_{620}$ at the time of estrus had significantly lower conception rate at corresponding AI as compared to buffaloes with ≤0.029 $ULSOD_{620}$. It is suggested that $ULSOD_{620}$ 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 (Barañski *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

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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 4ºC 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 messaged 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 (ºC)	Product size (bp)	Reference
E. coli (*fimH*)	Forward-TGCAGAACGGATAAGCCGTGG Reverse-GCAGTCACCTGCCCTCCGGTA	63	508	Moreno *et al*. (2005)
A. pyogenes (*fimA*)	Forward-CACTACGCTCACCATTCACAAG Reverse-GCTGTAATCCGCTTTGTCTGTG	59	605	Silva *et al*. (2009)
F. necrophorum (*lktA*)	Forward-AATCGGAGTAGTAGGTTCTG Reverse-TTTGGTAACTGCCACTGC	60	401	Zhou *et al*. (2009)

cytology: A prevalence rate of 24.4% was recorded for SCE based on the endometrial cytobrush cytology (>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		χ^2 (sig.)
	SCEP (21)	SCEN (65)	
E. coli (*fimH*)	52.4	24.6	5.7 (0.02)
A. pyogenes (*fimA*)	38.1	21.5	2.3 (0.13)
F. necrophorum (*lktA*)	42.8	7.7	11.9* (0.001)

*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 cytotoxinpyolysin (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 invades 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*. (2104) 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 (McDougall *et al*. 2011, Barañski *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 $ULSOD_{620}$ 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 $ULSOD_{620}$: Based on the derived $ULSOD_{620}$ 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 $ULSOD_{620}$ 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 $ULSOD_{620}$ could be used to identify buffaloes with SCE with high sensitivity (85.7%) and specificity (73.8%) (Table 4). It is believed that higher $ULSOD_{620}$ 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 $ULSOD_{620}$. The PMN % was 4.5±0.4 in buffaloes with $ULSOD_{620}$ of >0.029 as compared to 2.1±0.2 in buffaloes with $ULSOD_{620}$ of ≤0.029.

Our results support the use of $ULSOD_{620}$ for the diagnosis of subclinical endometritis in buffalo at the time of estrus. In current study, 40.7% of the buffaloes were

Table 4. Diagnostic potential of ULSOD at 620 nm (cut-off = >0.029) for detection of subclinical endometritis in buffaloes (gold standard: >5% PMN at first postpartum estrus)

Diagnostic test		Gold standard		Sensitivity (%)	Specificity (%)	Diagnostic accuracy (%)	kappa statistic (κ)
		≥5% PMN	<5% PMN				
$ULSOD_{620}$	>0.029	18	17	85.7	73.8	76.7	0.49
	≤0.029	3	48				
Bacteria	Present	16	26	76.2	60.0	63.9	0.27
	Absent	5	39				

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 buffalo

Diagnostic test	Threshold	No. of animal	No. of animal conceived	Conception rate (%)	χ^2 (sig.)
Endometrial cytology	>5% PMN	21	1	4.8	5.82
	≤5% PMN	65	20	30.8	(P<0.05)
ULSOD at 620 nm	>0.029	35	3	8.5	6.99
	≤0.029	51	18	35.3	(P<0.05)

In recent years, several publications had reported a negative impact of SCE in dairy cattle on subsequent reproductive performance (Gilbert *et al*. 2005, Barlund *et al*. 2008, Kaufmann *et al*. 2009, McDougall *et al*. 2011). However, similar studies on buffalo are lacking in literature. Kaufmann *et al*. (2009) reported a significant reduction in first service conception rate in cows with high PMN count (15% PMN) as compared to medium PMN count (0–15% PMN) (29.6% vs 57.6%). The exact mechanism by which subclinical endometritis decreased conception is unclear. Soto *et al*. (2003) suggested that mediators of the inflammatory cascade, including cytokines can impair early embryo development and might be part of the mechanism by which fertility is depressed in cows suffering from inflammatory diseases in early lactation. Inflammation in the endometrium has been shown to reduce fertilization in postpartum dairy cows (Cerri *et al*. 2009). Cows diagnosed with subclinical endometritis have altered endometrial and embryonic gene expression that might be the reason for reduced fertility (Hoelker *et al*. 2012); the same explanation could be true for the reduced fertility in subclinical endometritic buffaloes of the present study.

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.

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