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Efficacy of endometrial cytology and ultrasonography for diagnosis of subclinical endometritis in postpartum Murrah buffaloes

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ABSTRACT

The present study was conducted to investigate the efficacy of trans-rectal ultrasonography and endometrial cytology to diagnose subclinical endometritis in postpartum Murrah buffaloes. Forty two buffaloes (6 normal, 6 clinical endometritic and 30 subclinical endometritic) were selected from college livestock farm and organised farms of Jabalpur. Trans-rectal ultrasonography, endometrial cytology and microbial assay were performed. Efficacy of trans-rectal ultrasonography and endometrial cytology for diagnosis of subclinical endometritis along with microbial assay was recorded as 69.23 and 87.17%, respectively. It is concluded that endometrial cytology is effective diagnostic technique for diagnosis of subclinical endometritis. Subclinical endometritis cases diagnosed as negative or doubtful by ultrasonography could be confirmed by endometrial cytology.

Key words: Endometrial cytology, Endometritis, Subclinical endometritis, Trans-rectal ultrasonography

Postpartum subclinical endometritis is defined as an endometrial inflammation occurring 21 days or more after parturition without any clinical signs whereas clinical endometritis is indicated by the presence of purulent/ mucopurulent cervico-vaginal mucus discharge. Postpartum endometritis has a negative effect on reproductive performance, causing an increase in the number of services per pregnancy and in the length of calving-conception interval. The prevalence of subclinical endometritis varies between 20 to 53% from 20 to 60 days postpartum (Gilbert *et al*. 2005).

A variety of Gram-positive and negative aerobes and anaerobes alone and in combinations have been isolated from infected uterus (Azawi 2010). Early and efficient diagnosis is essential for proper and effective treatment of condition. Although there are various methods for diagnosis of endometritis in buffaloes but the diagnosis still remains challenging due to lack of simple and effective diagnostic technique (Gilbert *et al*. 2005). Trans-rectal ultrasonography is a useful diagnostic tool in determining uterine size, echotexture and fluid accumulation in endometritis (Honparkhe *et al*. 2014).

Researches have also indicated that endometrial cytology might be useful and accurate procedure for detecting existence and severity of endometritis (Honparkhe *et al*. 2014). Keeping this in view, the present study was planned to study the efficacy of ultrasonography and endometrial cytology as diagnostic aids in subclinical endometritis in postpartum Murrah buffaloes.

MATERIALS AND METHODS

A total of 150 postpartum (28 to 45 days) apparently healthy Murrah buffalo cows with normal calving history and free from peripartum disorders maintained at college livestock farm and organized dairy farms of Jabalpur were selected for the study. After recording history, all the animals were subjected to gynaeco-clinical examination and Whiteside test. Whiteside test was performed by adding 1 ml of 5% sodium hydroxide solution to 1 ml cervical mucus. The mixture was heated upto boiling point. Development of yellow colour is indicative of subclinical endometritis. Among the 150 postpartum buffaloes screened, 6 each (normal and clinically endometritic buffaloes) were taken as reference animals while 30 subclinical endometritic buffaloes were randomly selected among their groups for studying the efficacy of ultrasonography and endometrial cytology as diagnostic aid for diagnosis of subclinical endometritis.

Transrectal ultrasonography: Transrectal ultrasonography was done using 5 MHz, linear array transducer and results were documented in the form of cervical diameter, presence of uterine fluid, uterine luminal diameter, endometrial thickness and ovarian structures

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(corpora lutea and follicle) etc. The cut off point for diagnosing subclinical endometritis with ultrasonography was endometrial thickness > 8 mm, uterine lumen diameter > 3 mm and presence of uterine fluid (Barlund *et al*. 2008).

Endometrial cytology by cytobrush technique: After proper restraining, backracking was done to evacuate the rectum. The perineal region and vulva were washed with antiseptic solution and disinfected with spirit swab. The vulvar lips were pulled apart and the modified cytobrush assembly (Madoz *et al*. 2014) specially fabricated for buffaloes (Fig. 1) was introduced in the vagina and endometrial sample were collected as per standard cytobrush technique procedure.

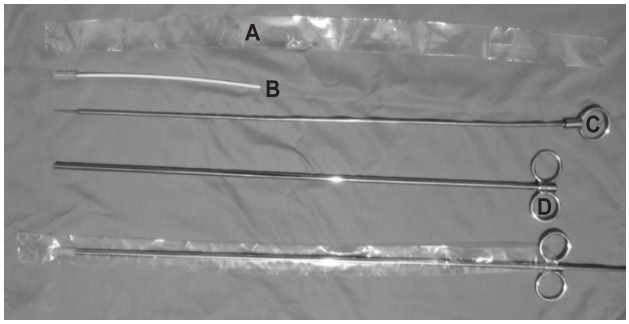


Fig. 1. Cytobrush assembly parts - sanitary sheath (A), cytobrush (B), inner stylette (C) and outer catheter (D).

After sample collection, cytobrush was rolled over on clean microscopic glass slide, air dried and stained with modified Wright Giemsa stain solution for 2 min on staining rack. The stain was then diluted with equal volume of triple glass distilled water and kept for 5 min. The slide was then washed with triple glass distilled water and air dried. The slides were then screened for the presence of endometrial cells and polymorphonuclear cells (PMN cells). Three hundred cells were counted under the microscope at 400× and 1000× and per cent PMN cells were calculated. This data was used to classify the health status of uterus along with nature of discharge (clear, purulent or mucopurrulent) as clinical or subclinical endometritis or normal (without inflammation or healthy).The threshold cut off values for diagnosis of subclinical endometritis by endometrial cytology were >18% PMNs between day 20–33 days postpartum and >10% PMNs between day 34–47 days postpartum as described by Kasimanickan *et al*. (2004).

Microbial assay: Uterine samples were aseptically collected for microbial assay by low volume lavage technique. Autoclaved Brain Heart infusion (BHI) broth tubes were incubated for 6–8 h using nichrome loops. It was then gently streaked on BHI, Muller-Hinton (MH) and Eosin Methylene Blue (EMB) agar medium in petri dishes and incubated for 48 h at 37°C (Quinn *et al*. 1999a). Isolation and identification of bacteria were based on the morphology, cultural characters and biochemical tests as described by Quinn *et al*. (1999b). All the isolates were characterized morphologically using Gram staining (Quinn *et al*. 1999b). Total viable bacterial count was calculated using spread plate technique as per the method described by Sarkar *et al*. (1996) with minor modifications.

Statistical analysis: The data was analysed statistically by analysis of variance (ANOVA). The means were compared using Duncan's new multiple range test (DNMRT) described by Snedecor and Cocharn (1994).

RESULTS AND DISCUSSION

On the basis of characteristics of cervico-vaginal mucus, Whiteside test, per-rectal examination, trans-rectal ultrasonography and endometrial cytology; 50.67% (76/150) buffaloes were found to be suffering from endometritis. The incidence of subclinical and clinical endometritis was recorded as 26.00 (39/150) and 24.67% (37/150), respectively. The incidence of subclinical endometritis has been reported to vary between 20–90% during first 2–3 months postpartum (Cheong *et al*. 2011). However, clinical endometritis has been encountered in 21 to 35% cattle revealing mucopurulent discharges (Plontzke *et al*. 2011). In the present study, the incidence of clinical and subclinical endometritis was also within the range of aforesaid studies.

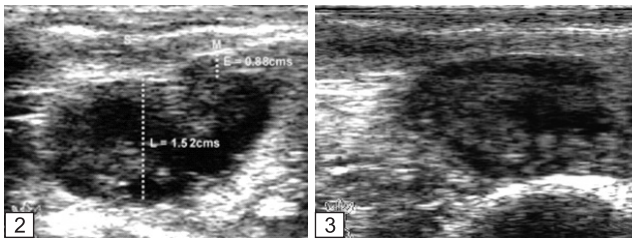
Trans-rectal ultrasonography examination revealed that cervical diameter did not vary significantly (P>0.05) in normal, clinical and subclinical endometritic buffaloes (Table 1).

The mean endometrial thickness (Table 1) was significantly higher (P<0.05) in clinical and subclinical

Table 1. Trans-rectal ultrasonographic findings in normal, clinical and subclinical endometritic postpartum buffaloes

Group	Trans-rectal ultrasonography findings		
	Cervical diameter (cm)	Endometrial thickness (cm)	Uterine lumen diameter (cm)
Normal (n=6)	1.94±0.18	0.38^{b}±0.09	0.41^{c}±0.07
Clinical endometritis (n=6)	2.04±0.17	0.88^{a}±0.03	0.99^{a}±0.06
Subclinical endometritis (n=30)	2.24±0.11	0.82^{a}±0.02	0.77^{b}±0.02

The means with the same superscript within the column did not differ significantly (P>0.05)



Figs 2–3. **2.** Transverse view of uterine horn showing dilated uterine lumen (L) containing marked amount of anechoic fluid with hyper/hypoechoic content and tortuous hypoechoic endometrium (E) in clinical endometritic postpartum buffalo **3.** Transverse view of uterine horn showing dilated anechoic uterine lumen (L) and thickened, wavy hypoechoic endometrium (E) in subclinical endometritic postpartum buffalo

endometritic buffaloes as compared to normal buffaloes.The difference was non-significant (P>0.05) between clinical and subclinical endometritic buffaloes. Similar findings were also observed for uterine lumen diameter. However, there was significant difference (P<0.05) for uterine lumen diameter between clinical and subclinical endometritic buffaloes (Figs 2 and 3).

The clearance of uterine fluid depends on the reabsorption process of the endometrium and the phagocytic clearance of the cellular debris. During the normal process of involution, the uterine lumen which is enlarged in size retracts to its nearly original size as the involution process progresses. Therefore, any accumulation of fluid in the uterus, whether sterile or infected, and its volume directly affects the endometrial thickness and inflammatory changes due to infections. This reflects in the variation in endometrial thickness and uterine lumen diameter as compared to cervical diameter. This could be the possible reason for non-significant change in the cervical diameter in normal, clinical and subclinical endometritic buffaloes. The significant variation (P<0.05) in endometrial thickness between normal, clinical and subclinical endometritic buffaloes reflects the volume of fluid, inflammatory response of the uterus resulting in accumulation of fluid and endometrial oedema (Figs 2 and 3). It was observed that the uterine lumen diameter also varied significantly (P<0.05) between clinical and subclinical endometritic buffaloes. This may be due to the fact that the clinical endometritic buffaloes were diagnosed as open endometritic buffaloes that have every chance of an infected uterus increasing the volume of uterine fluid. This is also correlated with the findings that the clinically endometritic buffaloes harboured higher bacterial load as compared to the subclinically endometritic buffaloes.

Findings in the present study for ultrasonographic diagnosis of subclinical endometritis are consistent with ultrasonographic findings of Purohit *et al*. (2013). Ultrasonographic findings such as increased amount of fluid accumulation in the uterine lumen (Kasimanickam *et al*. 2005), increase in uterine horn and cervical diameter and echotextural changes are associated with bacterial growth and delayed uterine involution after calving (Mateus *et al*. 2002), offer immediate diagnosis. Significant variation was not observed in between mean cervical diameter in cows with an inflamed (both cellular density and inflammatory status) and healthy uterus (Polat *et al*. 2015).

Polymorphonuclear (PMN) cells are the predominant inflammatory cell types found in intrauterine fluid accumulations and determination of the relative proportion of PMN has been shown to be predictors of reproductive performance in the postpartum cows (Kasimanickam *et al.* 2005). Endometrial cytology by cytobrush in the present study revealed significant difference (P<0.05) in PMN cell percentage in normal, clinical and subclinical endometritic buffaloes (Table 2). It is obvious from the results of present study that there was a dramatic increase in the percentage of PMN cells in clinically and subclinically endometritic buffaloes as compared to normal buffaloes.

Table 2. Endometrial cytology in normal, clinical and subclinical endometritic postpartum buffaloes

Group	Endometrial cytology				
	PMN*	Endometrial cell*	Fibroblast*	RBC*	PMN (%)
Normal (n=6)	$13.00^{c}\pm$ 5.56	$286.67^{a}\pm$ 5.70	$0.00^{c}\pm$ 0.00	0.33± 0.33	$4.34^{c}\pm$ 1.85
Clinical endo-metritis (n=6)	$106.67^{a}\pm$ 10.30	$164.50^{c}\pm$ 10.98	$12.33^{a}\pm$ 1.61	0.00± 0.00	$35.56^{a}\pm$ 3.43
Subclinical endo-metritis (n=30)	$65.13^{b}\pm$ 1.36	$233.47^{b}\pm$ 1.57	$0.20^{b}\pm$ 0.09	1.93± 0.61	$21.17^{b}\pm$ 0.45

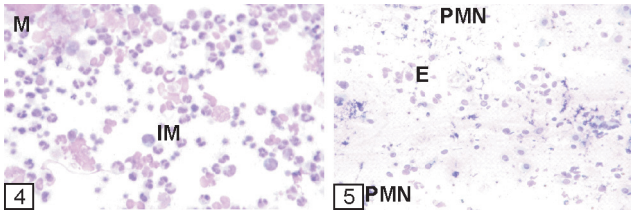
The means with the same superscript within the column did not differ significantly (P>0.05). *Numbers per 300 cells counted.

However, the PMN percentage in subclinically endometritic buffaloes was lower than in the clinically endometritic buffaloes (Figs 4 and 5). This clearly reflects that in endometritis whether it is infected or moderately infected, there is an influx of PMN cells in the uterine lumen indicating an inflammatory process. Ghasemi (2011) also reported that >18% PMNs on endometrial cytobrush cytology was the lowest percentage of PMN associated with an elevation of inflammatory cytokines (IL-6, IL-8, TNF-α).

The cut off threshold of PMN percentages by cytobrush technique in the present study are higher than what has been reported earlier where the most appropriate threshold was >8% PMNs for defining endometritis-positive disease status in cows sampled between 28 to 41 (Barlund *et al*. 2008) and 25 (Dourey *et al*. 2011) days postpartum using 150–270 day pregnancy status as the outcome, respectively.

Kasimanickam *et al*. (2004) reported that a threshold of >18% PMNs was most appropriate for cows examined between 20–33 DIM and, that >10% PMNs should be used for cows examined between day 34 and 47 DIM. Although the DIM ranged between 28 and 41 in the present study, the mean was 34.26±0.73 DIM which supports for a higher threshold to differentiate between PMN influx associated with bacterial infection rather than the PMN influx associated with the normal uterine involution process.

Bacterial contamination should be actually cleared off by day 28 for normal postpartum recovery. But if the bacterial load is present and animal comes in early oestrus then after oestrus, the luteal phase is dominated by progesterone hormone which has immunosuppressive action resulting in increase in bacterial load. In the present study, total viable bacterial count in normal group buffaloes differed significantly (P<0.05) with clinical endometritis group buffaloes (Table 3). However, difference between subclinical endometritis group and normal group buffaloes and also between subclinical and clinical endometritis



Figs 4–5. **4.** Endometrial smear from clinical endometritic buffalo (32 days postpartum) showing abundant amount of mature (M) and immature (IM) polymorphonuclear cells. Modified Wright Giemsa stain (×400) **5.** Endometrial smear from subclinical endometritic buffalo (32 days postpartum) showing Polymorphonuclear cells (PMN) and endometrial cells (E). Modified Wright Giemsa stain (×400).

buffaloes did not differ significantly (P>0.05).

Uterine lavage samples obtained from buffaloes diagnosed as normal were sterile (Table 4). All the 6 uterine lavage samples screened for bacterial isolates were found to be positive in clinical endometritis group. Out of these 6 samples that were found to be positive for bacterial isolates, 7 isolates were obtained; 2 (28.57%) were of *E. coli* and 2 (28.57%) were *Streptococcus* followed by 1 (14.28%) of *Citrobacter*, 1 (14.28%) of *Proteus* and 1 (14.28%) was *Enterococcus* species (Table 4).

Table 3. Total viable bacterial count in normal, clinical and subclinical endometritic postpartum buffaloes

Group	Total viable bacterial count (10^6 CFU/ml)
Normal (n=6)	$0.00^{b}\pm0.00$
Clinical endometritis (n=6)	$0.425^{a}\pm0.134$
Subclinical endometritis (n=30)	$0.186^{ab}\pm0.047$

The means with the same superscript did not differ significantly (P>0.05).

Out of 30 samples from subclinical endometritic buffaloes, 25 (83.33%) samples were found positive for bacterial isolates. Among these 25 positive samples, 26 bacterial isolates were isolated; *E. coli* was highly prevalent 8 (30.76%) followed by *Staphylococcus* 7 (26.92%), *Streptococcus* 3 (11.53%), *Proteus* 3 (11.53%), *Acinetobacter* 3 (11.53%) and *Bacillus* species 1 (07.69%) (Table 4).

The prevalence of *E. coli* as observed in the present study was in accordance with the findings of Udhayavel *et al*. (2013) and Biswal *et al*. (2014). Costa *et al.* (2010) isolated *E. coli*, *Pseudomonas* spp., *Klebsiella* spp. and *Proteus* spp. in more numbers from uterine washings of cows suffering from metritis.

Any diagnostic test for endometritis will have <100% sensitivity when measured against reproductive performance, because there are numerous unaccountable reasons due to which cows fail to become pregnant, resulting in false negative results (Kasimanickam *et al*. 2004). Moreover, the spontaneous resolution of uterine disease may lead to false positive results for any test performed during the early postpartum period.

Table 4. Bacterial isolates from normal, clinical and subclinical endometritic buffaloes

Group	Bacterial isolation		Positive bacterial isolates			Types of bacterial isolates							
	-ve sample	+ve sample	Single type	Mixed type	Total	*Streptococcus* spp.	*Bacilli* spp.	*Pseudomonas* spp.	*E. coli*	*Staphylococcus* spp.	*Acinetobacter* spp.	*Citrobacter* spp.	*Proteus* spp.	*Enterococcus* spp.
Normal (n=6)	6 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	0	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Clinical endometritis (n=6)	0 (0.00)	6 (100.00)	5 (80.00)	1 (20.00)	7	0 (0.00)	0 (0.00)	0 (0.00)	2 (28.57)	2 (28.57)	0 (0.00)	1 (14.28)	1 (14.28)	1(14.28)
Subclinical endometritis (n=30)	5 (16.67)	25 (83.33)	24 (96.00)	1 (4.00)	26	3 (11.53)	2(7.69)	3 (11.53)	8 (30.76)	7 (26.92)	3 (11.53)	0 (0.00)	0 (0.00)	0 (0.00)
Overall (All Groups) (n=42)	11 (26.19)	31 (73.80)	29 (93.54)	2 (6.45)	33	3 (9.09)	2 (6.06)	3 (9.09)	10 (30.03)	9 (27.27)	3 (9.09)	1 (3.03)	1 (3.03)	1 (3.03)

Figures in paranthesis indicate percentage.

The efficacy of endometrial cytology is dependent on the thresholds of PMN per cent with respect to days of sampling postpartum. Therefore, various research workers have used different thresholds (>18 and >10% PMN, Kasimanickam *et al*. 2004; >5% PMN, Gilbert *et al*. 2005 and >8% PMN, Barlund *et al*. 2008) to define subclinical endometritis. Consequently, lower PMN per cent cut off may increase number of false positive cases.

As per Barlund *et al*. (2008), cows appearing apparently normal may either have impaired uterine clearance (good volume of fluid in lumen) or those with increased inflammatory response (lower or no fluid volume in lumen). Animals with increased inflammatory response may be without infection or infection might be cleared off by their natural defence mechanism on time (cytologic endometritis), while animals with impaired uterine clearance may harbour bacterial load and infection (infective endometritis). Therefore, if endometrial cytology with higher PMN per cent cut off threshold with respect to day postpartum is coupled with microbial assay, it will definitely aid in detecting infective versus cytologic endometritis more accurately.

In the light of above statements and the results obtained in present study, it is clear that when the animals were examined with endometrial cytology alone between days 28 to 33, 34 to 47 days or irrespective of days postpartum; all the 23 (100%), 16 (100%) and 39 (100%) animals examined were found to be positive (Table 5). But when animals were examined for endometrial cytology combined with microbial assay between days 28 to 33 postpartum; out of 23 animals, 20 (86.69%) were found to be positive and between day 34 to 47 postpartum, 14 (87.50%) out of 16 animals and irrespective of days postpartum, out of 39 animals, 34 (87.17%) animals were found to be positive (Table 5). These findings prove that sensitivity of endometrial cytology to detect false positive cases increased when microbial assay was combined with endometrial cytology.

Trans-rectal ultrasonography has been used as a diagnostic aid for diagnosis of subclinical endometritis in number of studies. However, it is challenging to distinguish the pathological changes in the endometrium of postpartum cows with subclinical endometritis. Criteria for diagnosis of subclinical endometritis in postpartum buffaloes like endometrial thickness and uterine lumen diameter (fluid in lumen) only measures endometrial oedema and inflammatory fluid volume. It does not give an idea about quality of fluid whether sterile and inflammatory or infective. Fluid volume in uterine lumen is also influenced by oestrus phase. Mateus *et al*. (2002) in their study have reported an association of bacterial growth and impaired uterine involution with ultrasonographically detectable accumulation of intrauterine fluid and increased endometrial thickness and echotextural changes. Therefore, trans-rectal ultrasonography remains a good practical tool for on-farm diagnosis but with less efficiency. Trans-rectal ultrasonography when combined with microbial assay and/or endometrial cytology will help in differentiating between inflammatory oedema/fluid and infection.

In the present study, when the animals were examined for endometrial thickness and presence of fluid in uterine lumen by trans-rectal ultrasonography alone between days 28 to 33 postpartum; out of 23 animals, 20 (86.95%) animals were found to be positive and between day 34 to 47 postpartum, out of 16 animals, 12 (72.72%) animals were found positive (Table 5). When microbial assay in addition to ultrasonography was considered between days 28 to 33 postpartum; out of 23 animals, 17 (73.90%) animals were found to be positive and between day 34 to 47 postpartum, 10 (62.50%) out of 16 animals and irrespective of days postpartum, out of 39 animals, 34 (87.17%) animals were found to be positive (Table 5). This signifies that sensitivity to rule out false positive cases was increased when trans-rectal ultrasonography was coupled with microbial assay.

Endometrial cytology (EC) and trans-rectal ultrasonography (USG) techniques measure two different causative factors. EC measures the cellular response and USG measures the clearance mechanism of uterus (Kasimanickam *et al*. 2004). Endometrial cytology is more efficient technique than ultrasonography but still not practicable to perform under field conditions. Therefore, the best diagnostic aid for subclinical endometritic animals with precision is to use combination of USG and EC. Animals diagnosed as positive by both endometrial cytology and trans-rectal ultrasonography positive were found to be associated with more severe detrimental effect on pregnancy (60%) than either positive by EC or USG alone when

Table 5. Efficacy of trans-rectal ultrasonography and endometrial cytology for diagnosis of subclinical endometritis with respect to days of examination postpartum

Day of sampling	Buffaloes positive	Buffaloes diagnosed positive by							
		Endometrial cytology				Trans-rectal ultrasonography			
		Without microbial assay		With microbial assay		Without microbial assay		With microbial assay	
		No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
28-33 day postpartum	23	23	100.00	20	86.69	20	86.95	17	73.90
34-47 day postpartum	16	16	100.00	14	87.50	12	72.72	10	62.50
Irrespective of day postpartum	39	39	100.00	34	87.17	32	82.05	27	69.23

sampling was done minimum 14 days apart (Kasimanickam *et al*. 2004). To differentiate the sterile or infected uterine fluid, microbial assay may be coupled with endometrial cytology and trans-rectal ultrasonography for more precise diagnosis of subclinical endometritis.

The aim of the study was to investigate the efficacy of trans-rectal ultrasonography and endometrial cytology to diagnose subclinical endometritis in postpartum Murrah buffaloes. Forty two buffaloes were selected for the study. Trans-rectal ultrasonography, endometrial cytology and microbial assay were performed. Efficacy of trans-rectal ultrasonography and endometrial cytology for diagnosis of subclinical endometritis along without and with microbial assay was recorded as 69.23 and 87.17% respectively. Endometrial cytology by cytobrush technique was found to be reliable and effective diagnostic technique for diagnosis of subclinical endometritis and proved to be better when coupled with microbial assay. Subclinical endometritis cases diagnosed as negative or doubtful by ultrasonography could be confirmed by endometrial cytology.

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