



## A diagnostic study of bovine endometritis integrating cytological, histopathological assessments, and immunohistochemical localizations of interleukins

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### Abstract

Endometritis significantly impairs cows' reproductive productivity, yet existing diagnostic practices lack the comprehensive accuracy required for precise diagnosis and pathological staging. This study aimed to evaluate and validate a combined diagnostic model integrating cytology, histopathology, and immunohistochemistry to diagnose and categorize the severity of endometritis precisely. Post-mortem endometrial samples (n=76) were collected from slaughtered cows and were subjected to endometrial cytobrush and biopsy sampling. Cytobrush smears were quantified for polymorphonuclear leukocyte (PMNL) and classified according to recognized thresholds: mild ( $\leq 5\%$ ), moderate ( $>5-15\%$ ), and high ( $>15\%$ ). The severity of endometrial inflammation was categorized into mild, acute, and chronic. Immunohistochemistry was utilized to determine the localization and expression patterns of interleukins (IL-8, IL-1 $\beta$ , and IL-10). The moderate threshold of PMNL count revealed a significantly higher rate (51.3%) than the mild and high groups, demonstrating a correlation with the histopathological assessment. Histopathological evaluation identified chronic inflammation as the most prevalent pathological lesion (47.4%). Receiver operating characteristic curve analysis supported the diagnostic efficacy of PMNL cut-off values, exhibiting high sensitivity and specificity for differentiating inflammation severity. Immunohistochemistry revealed differential localization and expression patterns of utilized inflammatory interleukins correlating with endometritis severity. Specifically, elevated expression intensity of IL-8 and IL-1 $\beta$  was consistently associated with severe inflammation, whereas a stronger expression of IL-10 predominated in chronic endometritis. In conclusion, the integration of cytobrush, histopathology, and immunohistochemistry significantly enhances the diagnostic accuracy and staging. The differential interleukin expression serves as a promising molecular biomarker, presenting novel insights into the complicated inflammatory dynamics underlying endometritis progression.

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### Introduction

In postpartum cows, the most critical event is uterine involution, which involves the reduction in uterine size, exclusion of microbial contaminants, and the re-

establishment of endometrial tissue for restoring uterine function and ovarian cyclic activity (1). However, due to diminished immune responses of postpartum cows, particularly those affected with dystocia or other related reproductive problems, they are not always able to eliminate

uterine pathogens promptly (2,3). This statement was approved by other works, where various bacterial species, including gram-positive/ negative and aerobic/ anaerobic pathogenic bacteria, were isolated from cows with postpartum endometritis (2,4). The prolonged existence of these pathogenic bacteria in the uterine cavity frequently causes various degrees of uterine inflammation ranging from undetectable subclinical endometritis to severe clinically distinguishable metritis (4,5). Endometritis is one of the most prevalent reproductive disorders that affects around 20% of post-partum cows, which refers to the inflammation of the endometrial superficial layer extending no deeper than the stratum spongiosum (6). Specifically, endometritis has a detrimental implication on cows' fertility due to the consequent endometrial pathological changes and the massive infiltration of polymorphonuclear leukocytes (PMNLs) in the uterine lumen (7,8). Endometritis causes economic losses resulting in extended open days, a decreased offspring rate, reduced milk production, costs of treatment, and repeated insemination due to pregnancy failure (9-11). Subsequently, due to decreased productivity, improper diagnostic procedures, and the increased costs, most of the affected cows are usually excluded and culled from the flock (10). For decades, various diagnostic approaches, such as vaginal inspection, the Metricheck device, ultrasonography, cytobrush, and histopathological assessment of uterine tissue, have been employed to evaluate the uterine health status and to guide therapeutic interventions (4,12). According to the endometrial histopathological lesions or the severity of PMNL infiltration, endometritis was classified into mild, acute, or chronic (8,13). These lesions might include desquamation of endometrial epithelium, glandular necrosis, atrophy, dilation, vascular congestion and hemorrhage, oedema, and various degrees of fibrosis (4,8,14). Another diagnostic approach manifested by molecular strategies has been tested and employed for the detection of endometrial inflammation. For instance, by comparison with an uninfected uterus, cows with endometritis expressed upregulated mRNA levels of several inflammatory interleukins (15,16). These interleukins are involved in immunological processes, cell adhesion, chemotaxis, and cellular death (17). Previous research indicates that immune cells, endometrial epithelial cells, endometriotic stromal cells, and blood platelets produce these interleukins (18). Previous studies in various species (e.g., cows, humans, and mares) specified that the elevated mRNA expression levels of interleukins in uterine endometrial tissue and/or blood are recognized as indicative and predictive markers of endometritis (19-21). For example, overexpression of IL-1 $\beta$  (a proinflammatory cytokine) and IL-8 (a chemokine) were linked with increased infiltration of PMNL and chemoattraction to strengthen phagocytic activities, respectively (22). Conversely, the reduced endometrial inflammatory response against bacterial infection was associated with upregulated uterine level of

anti-inflammatory interleukin manifested by IL-10 (23). Another recent study detailed a pro-fibrotic implication of IL-10 in the inflamed endometrial tissue compared with tissue from healthy endometrium (24). Moreover, the early activation of the anti-inflammatory response may disrupt regular tissue repair processes. Consequently, appropriate and consecutive expression of pro/ anti-inflammatory interleukins is critical for effective tissue healing and regeneration (22,25). Compared to non-infected endometrium, the mRNAs of both the pro/ anti-inflammatory cytokines were expressed with variable levels in endometrial tissues diagnosed with various degrees of inflammation (15,16).

Thus, the present work hypothesized that its relevant proteins might also be detectable and their expression might be associated with the severity of endometrial inflammation. This study aimed to validate a diagnostic and staging protocol of endometritis integrating localization of IL-8, IL-1 $\beta$ , and IL-10 proteins, endometrial cytobrush, and histopathological assessments of uterine biopsies.

## **Materials and methods**

### **Ethical approval**

This work was approved by the Institutional Animal Care and Use Committee (approval number: UM.VET.2024.031). Uterine samples were obtained from slaughtered animals and were handled following standard procedures.

### **Animals and tissue sampling**

The study utilized the reproductive tracts of crossbred slaughtered cows (n=137) obtained from local slaughterhouses in Mosul/ Iraq, from the period extended from September 2024 to April 2025. Genitalia that were identified as pregnant or diagnosed with any reproductive physiological disorders, such as cystic ovaries or inactive ovaries, were excluded. Furthermore, genitalia demonstrating significant inflammation characterized by conditions such as severe uterine congestion, adhesions, or distension with purulent or mucoid contents were also excluded from sampling. For consistency and elimination of the hormonal implication on the cytological assessment, only genitalia with complete uterine involution and functional ovaries at the proestrus period (1-4 days of estrus cycle) were selected for further processing. The ovarian physiological stage was determined by the presence of numerous growing follicles (15). Therefore, according to the mentioned criteria and the initial inspection, only 76 genitalia were selected for sampling, which were directly labelled and transferred to a local workplace for processing.

### **Endometrial smear and cytological examination**

To maintain consistency and ensure the collection of representative endometrial tissue that accurately reflects the overall health of the uterus, an incision measuring 10 cm was

made on the dorsal surface of the uterine body at the bifurcation area to expose the endometrium. Following a careful inspection of the lumen, a disposable plastic cytobrush (LOT: 20220730, India) was utilized to obtain endometrial smears. All smears were obtained from the uterine bifurcation region. The microscope slide was brushed with the obtained endometrial materials to perform a smear, and the smear was allowed to air-dry. According to the manufacturer, the smear was fixed for three minutes in methanol, followed by air drying. The dried smears were stained with the MGG stain kit (Atom Scientific RRSK27-100). Slides were immersed in the diluted May Grunwald stain (1:1 buffer) for 15 minutes. Following washing in buffer, slides were stained with the diluted Giemsa (1:9 buffer) for 10 minutes. Then, the stained slides were washed, air-dried, mounted in DPX (Loba Chemie, 03600), and cover-slipped. Slides were examined and imaged using a camera (OMAX, A35180U3, China) fitted on a light microscope (Motic BA210E), where high-power microscopy was employed for the cytological assessment.

For each stained slide, approximately 200 cells were counted, and the percentage of PMNL against the endometrial cells was estimated. According to a previous study, a threshold of <5% was considered negative/mild for cytological endometritis (26). Samples detected with greater than 5% demonstrated a considerable variability in the proportion of PMNL; therefore, classification was implemented with further subdivisions. Specifically, samples demonstrating a PMNL proportion between >5-15% were categorized as moderate, whereas those with a proportion of more than 15% were classified as high (15). To determine the efficiency of the cytological count on detecting the severity of endometritis, the results of the endometrial cytology were compared with the histological findings of each tested sample (13,27). In addition, receiver operating characteristic (ROC) curves were employed to estimate the optimal cut-off values, the sensitivity (specifying the correctly recognized cases with endometritis), and specificity (correctly diagnosing negative cases) of cytobrush in detecting the severity of endometritis (28).

### **Tissue sampling and staining**

Endometrial tissue samples were obtained from the uterine body at the intercaruncular space. These biopsies were preserved for three days in neutral buffered formalin (10%). Then, following a standard protocol, the formalin-fixed samples were embedded in paraffin blocks. A manual Histocut microtome (Reichert-Jung) was used for sectioning the paraffin-embedded tissues at a thickness of 5µm. For histopathological examination, the uterine sections were stained with Gill's hematoxylin II and aqueous eosin (1%) stain, following a standard protocol (29). The stained sections were utilized for the histopathological assessment to determine the severity of inflammation.

### **Classifying the severity of endometritis**

According to the histopathological lesions, the severity of endometrial inflammation was categorized into mild, acute, and chronic. In addition to infiltration of the inflammatory cells, the pathological lesions affecting the endometrial epithelium, uterine glands, blood vessels, and stroma were considered. Moreover, the standard inflammatory changes, e.g., the degree of endometrial hemorrhages, oedema, and the presence of fibrotic tissue, were also considered to determine the severity of inflammation (13,14,30).

### **Immunohistochemical staining**

Immunohistochemical staining was used to evaluate the localization pattern of the inflammatory interleukins in uterine sections that were either identified with mild, acute, or chronic endometritis. Purchased Interleukins (IL) 8, 10, and IL-1β (Elabscience: E-AB-33423, E-AB-70235, and E-AB-40530, respectively) and used them at dilution rates of 1:200, 1:400, and 1:400, respectively. According to the manufacturer, for dewaxing, the utilized endometrial sections were processed in xylene (2x5 minutes), followed by rehydration steps in declining concentrations of ethanol (100%, 95%, and 70%; 3 minutes each). To expose the protein epitopes (antigen retrieval), the rehydrated sections were treated with 0.01M citrate buffer (pH 6.0). Sections were heated in a microwave for four intervals of five minutes, followed by a double-wash in phosphate-buffered saline (pH 7.4), then sections were incubated in blocking serum for 20 minutes at 24°C. Subsequently, the sections were incubated overnight at 4°C with the predefined diluted primary antibodies. For the negative control, sections were treated with an equivalent concentration of non-immune rabbit IgG. At the end of the incubation, sections were washed in PBS and exposed to a goat anti-rabbit secondary antibody (Elabscience, E-AB-1003, 1:500) for half an hour at room temperature. Following double washes in PBS, the slides were incubated in diaminobenzidine substrate until the brown color was established. Then, sections were washed in PBS, counterstained with hematoxylin, rinsed with tap water, and dehydrated in ethanol. The stained sections were cleared in xylene before being mounted and cover-slipped.

### **Statistical analysis**

One-way ANOVA with Tukey's Multiple Comparisons Test was used to detect the statistical variation among the mean proportion values of the counted PMNL (31). The Chi-squared test was utilized to determine statistical differences between the rates of categorized cytobrush and histopathological groups. All statistics were performed using GraphPad Prism 8, where the probabilities at  $P \leq 0.05$  were considered significantly varied.

## Results

### Endometrial cytobrush

The stained endometrial smears were employed to determine the efficiency of the PMNL count for the initial detection of endometritis and its linkage with the severity of endometrial inflammation. Figure 1 shows endometrial smears of four different endometrial stained sections demonstrating the presence/ absence of inflammatory cells among the endometrial cells. The majority of the determined inflammatory cells were PMNL; however, mononuclear inflammatory cells were also evident.

Based on the selected thresholds, the assessed endometrial smears revealed that the moderate threshold (>5-15) was the most frequently detected, occurring in 51.3% of cases, followed by the high and mild thresholds (34.2% and 14.5%, respectively). The Chi-Square test demonstrated that the rate of the moderate threshold appeared significantly higher than the mild and high thresholds ( $P \leq 0.01$  and  $P \leq 0.05$ , respectively); additionally, the rate of the high threshold also revealed a significant variation ( $P \leq 0.05$ ) compared to the mild group. Furthermore, One-way ANOVA analysis showed that the mean PMNL proportion of the category high (>15) was substantially higher ( $P < 0.01$ ) than the mean proportions of PMNL of the other two threshold groups. Similarly, the mean proportion

in cows with the moderate proportion appeared statistically higher ( $P \leq 0.05$ ) than the means of the mild groups (Table 1).

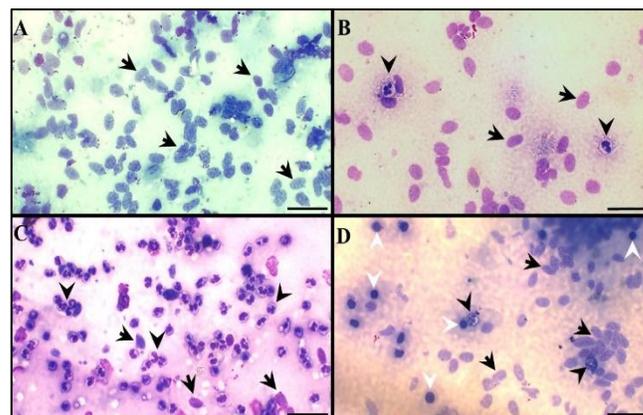


Figure 1: Endometrial cytobrush-stained smears. The stained smears revealed either the absence of inflammatory cells among the epithelial and stroma cells (A, tailed arrows), a mild proportion of PMNL (B, head arrows), a massive quantity of PMNL (C), and the presence of both PMNL and mononuclear cells (D, white arrows). Smears were stained with MGG stain kit™ and imaged under high power microscopy (40x), scale bar 50µm.

Table 1: Proportion of PMNL in the endometrial cytobrush

Categories	Samples (n)	%	Mean PMNL (Proportion)	Std Dev
Mild ( $\leq 5$ )	11	14.5 <sup>c</sup>	2.4 <sup>c</sup>	1.6
Moderate (>5-15)	39	51.3 <sup>a**</sup>	10.5 <sup>b*</sup>	2.5
High (>15)	26	34.2 <sup>b*</sup>	29.8 <sup>a**</sup>	12.2
Total	76	100		

Cows with the moderate range of PMNL count were significantly higher ( $P < 0.01$ ) than cows with the high and mild rates. However, the mean proportion of the PMNL count greater than 15% (High) demonstrated a statistical variation ( $P < 0.01$ ) compared to the other two groups. Different letters in the same column represent statistical difference (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ ).

### Histopathological findings

The histopathological examination of the H&E-stained sections revealed diverse inflammatory changes in the endometrium. Therefore, according to the severity of these changes, the endometrial inflammation was categorized into mild, acute, and chronic. The mild endometritis demonstrates an intact surface epithelium with a moderate infiltration of PMNL in the subepithelial stroma and blood vessels. Only several endometrial glands were detected atrophic, and blood vessels revealed a modest congestion. Sections with acute endometritis revealed degenerative epithelial changes and desquamation, massive infiltration of inflammatory cells, mainly the PMNL, and numerous mononuclear cells, in the stroma and blood vessels. Hyperplasia of the functional layer, glandular dilation and atrophy, stromal oedema, thickening of the vascular walls, and subepithelial hemorrhages. The chronic inflammation

was mainly distinguished by the presence of glandular islands, peri-glandular fibrosis, glandular atrophy and necrosis, and infiltration of inflammatory cells (particularly the plasma cells). Additionally, vascular thickening and massive oedema were evident (Figures 2 and 3).

### Histopathological assessment and its association with the cytobrush count

The data revealed a significant association between the severity of histopathological lesions and the indicated ranges of PMNL count. The majority of cytological smears that were detected with a mild PMNL count ( $\leq 5$ ) displayed mild endometrial inflammatory changes (72.7%), which appeared statistically varied ( $P \leq 0.01$ ) compared to chronic endometrial changes (27.3%); Interestingly, the relevant histopathological section of cows detected with less than 5% PMNL count do not demonstrate acute changes. As the

PMNL count increased to the moderate range (>5-15), there was a marked reduction in mild cases (7.7%) alongside a notable rise ( $P \leq 0.01$ ) in both acute (33.3%) and chronic (59%) endometrial lesions. In cases with high PMNL counts (>15), although the percentage of acute cases revealed the highest score (61.5%), statistics failed to indicate a significant variation against chronic cases (38.5%). However, the high category does not specify any case with mild histopathological changes. These findings suggest a linkage between the elevated PMNL counts and the severity of histopathological changes. The Chi-square test indicated that chronic inflammation exhibited a significantly higher prevalence rate (47.4%,  $P \leq 0.01$ ) compared to both acute and mild endometritis (Table 2).

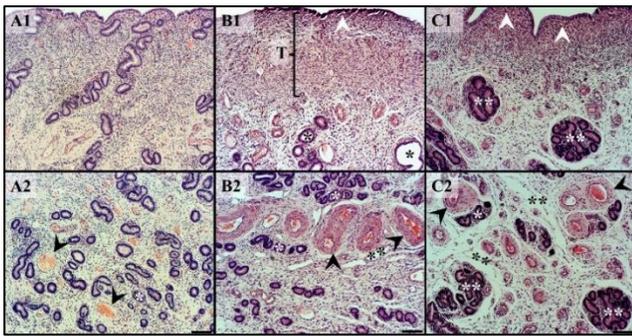


Figure 2: Low-power microscopy of the various forms of endometritis. The left panel (A1-A2) reveals mild endometritis, the middle panel (B1-B2) demonstrates the acute endometritis, and the right panel (C1-C2) is for the chronic inflammation. T: Hyperplasia of the functional layer, black star: glandular dilation, white stars: glandular atrophy, two black stars: edema, black arrow: thickening of the

vascular walls, white arrow: subepithelial hemorrhage, two white stars: glandular islands, peri-glandular fibrosis, glandular atrophy and necrosis, two black stars: edema. Sections were stained with H and E, where the upper panel (A1-C1) represents the functional layer, while the lower one (A2-C2) represents the basal region. Scale bar 200µm.

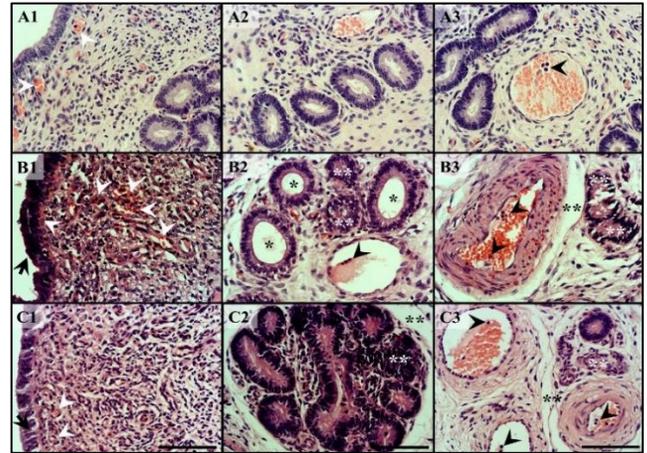


Figure 3: High-power microscopy of the various forms of endometritis. The mild endometritis (A1-A3) reveals minor lesions in the endometrial compartments. However, the acute (B1-B3) and chronic inflammation (C1-C2) demonstrated more severe histopathological changes. Black tailed arrows: degenerative changes of epithelial cells, white arrows: subepithelial hemorrhage, black head arrows: vascular PMNL, black star: glandular dilation, two white stars: glandular atrophy and degeneration, Panel C2: glandular islands, two black stars: oedema. Sections were stained with H&E, scale bar 200µm.

Table 2: Classification of endometritis integrating the histopathological and cytobrush assessments

Cytobrush	Samples (n)	Histopathological assessment		
		Mild (%)	Acute (%)	Chronic (%)
Mild ( $\leq 5$ )	11	8 (72.7) <sup>a**</sup>	0	3 (27.3) <sup>b</sup>
Moderate (>5-15)	39	3 (7.7) <sup>c</sup>	13 (33.3) <sup>b**</sup>	23 (59) <sup>a**</sup>
High (>15)	26	0	16 (61.5)	10 (38.5)
Total	76	11 (14.5) <sup>c</sup>	29 (38.1) <sup>b**</sup>	36 (47.4) <sup>a**</sup>

Data were analyzed with the chi-squared test, where different letters in the same row are statistically varied (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ ).

For further effective validation of cytobrush on detecting the severity of endometritis, the sensitivity and specificity were estimated using the ROC curve. The optimal cut-off threshold points were selected to exploit the stability between sensitivity and specificity. In mild endometritis, the cut-off points of less than 2.8 revealed a sensitivity of 100%, indicating that all cases with mild endometritis were correctly determined by the test. Additionally, the calculated specificity of the test was 98%, specifying that 98% of the

cases were accurately diagnosed as negative for mild inflammation. Regarding the acute inflammation, the best cut-off point was established at less than 8.8, yielding a sensitivity of 100% and a specificity of 74%. For the chronic endometritis, the best sensitivity at the obtained cut-off point (<1.5) was 79% with a specificity of 96%. This outcome underscores the reliability of the endometrial cytobrush not only for the initial diagnosis of endometritis but also for determining the severity of inflammation (Figure 4).

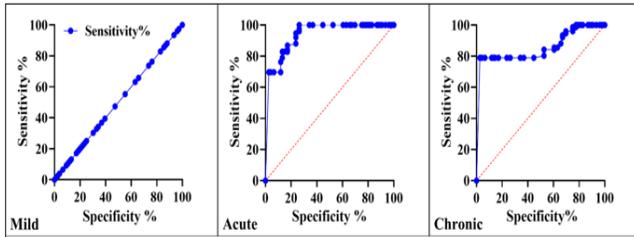


Figure 4: The sensitivity and specificity of the cytobrush for detecting the severity of endometritis. ROC curves were utilized to determine the optimal cut-off points for mild, acute, and chronic inflammation, where sensitivity and specificity were estimated for each category.

**Localization of interleukin proteins**

Immunohistochemical staining was utilized to assess the expression level of IL-8, IL-10, and IL-1 $\beta$  proteins in endometrial tissues diagnosed with varying severity of endometritis. To ensure the reliability, specificity, and interpretability of the interleukin antibodies utilized in the staining procedures, negative controls were incorporated into each staining trial. The uterine sections subjected to negative control staining exhibited no discernible staining in any of the endometrial compartments (Figure 5).

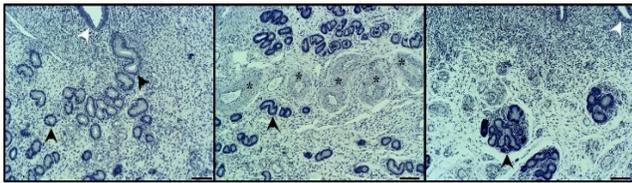


Figure 5: Negative control for the immunohistochemistry stain. Endometrial sections were incubated with non-immune rabbit IgG in three staining runs for IL-8, IL-10, and IL-1 $\beta$ , respectively. All sections revealed no stain in any endometrial structures, including epithelium (white arrows), glands (black arrows), or blood vessels (starred), scale bars 200 $\mu$ m.

In mild endometritis, IL-8 exhibited weak staining intensity within the vascular endothelium and glandular epithelial cells. In contrast, acute endometritis displayed a marked, strong staining pattern for IL-8, particularly in the surface epithelium, subepithelial stromal cells, vascular endothelium, and glandular epithelium. By comparison with the acute inflammation, while the detection pattern of IL-8 remained consistent, its staining intensity was notably diminished in the chronic form (Figure 6).

In mild endometritis, IL-1 $\beta$  was weakly expressed in the surface epithelial cells and was undetectable in blood vessels or uterine glands. However, in acute cases, prominent staining for IL-1 $\beta$  was observed in the glandular epithelium and the cytoplasm of mononuclear immune cells (mainly

macrophages) within the subepithelial and stroma. In cases of chronic inflammation, IL-1 $\beta$  demonstrated strong expression in both the surface epithelium and macrophages, though the staining intensity within the glandular epithelium was reduced. In all categories, the expression of IL-1 $\beta$  in blood vessels was detected as negative to a very weak intensity (Figure 7).

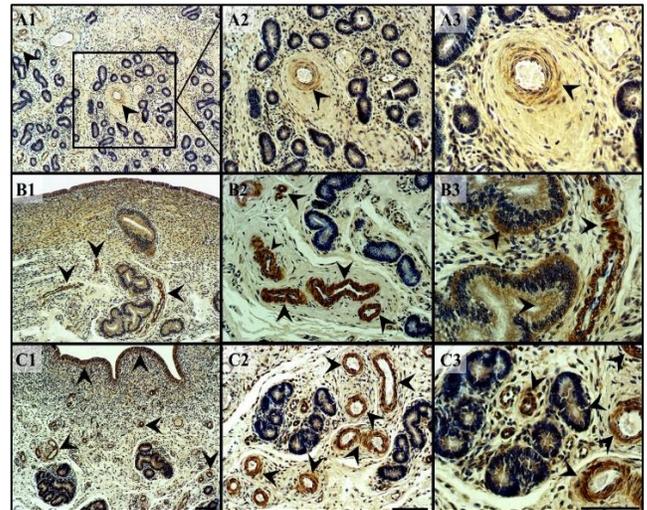


Figure 6: Expression of IL-8 in various forms of endometritis. In the mild endometritis (A1-A3), IL-8 was expressed with a weak intensity in the vascular endothelium and some glandular epithelium. In the acute form (B1-B3), strong localization of IL-8 was prominent in the surface and subepithelial cells, vascular endothelium, and glandular epithelium. In chronic form (C1-C3), the localization of IL-8 was similar to the acute form, but with less staining intensity. Black head arrows represent the positive localization of IL-8, scale bar 200 $\mu$ m (labels 1-3: magnifications 10x, 20x, and 40x, respectively).

In cases of mild endometritis, IL-10 exhibited a moderate expression in the surface epithelium, with a stronger expression in the vascular endothelium. In the acute form, a moderate expression of IL-10 was predominantly observed in the endothelium, while the surface epithelium only demonstrated numerous patches of staining. In chronic endometritis, a similar localization pattern of IL-10 was noted; however, a stronger intensity of staining was obtained relative to the mild and acute inflammation. In all categorized endometrial inflammations, the endometrial glands and immune cells were detected as negative for IL-10 (Figure 8).

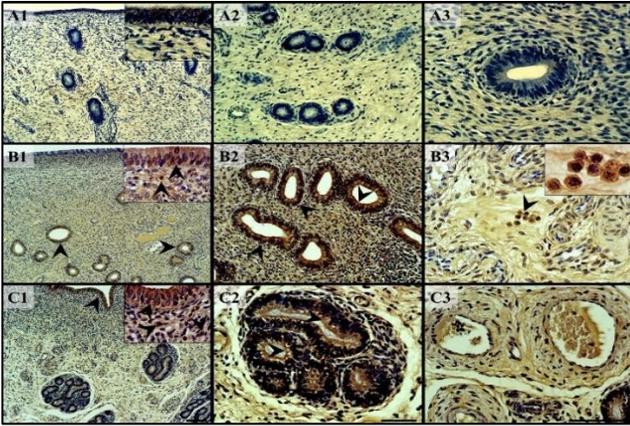


Figure 7: Expression of IL-1 $\beta$  in various forms of endometritis. In the mild endometritis (A1-A3), IL-1 $\beta$  was only expressed in the surface epithelium. In the acute form (B1-B3), a strong expression of IL-1 $\beta$  was revealed in the surface and glandular epithelium and in the cytoplasm of the mononuclear immune cells, mainly macrophages (B1 and B3, including boxed images 100x). A similar expression pattern was represented in the chronic form (C1-C3), but the intensity in the glandular epithelium was reduced. Black head arrows represent the positive localization of IL-1 $\beta$ , scale bar 200 $\mu$ m (labels 1-3 in each row: magnifications 10x, 20x, and 40x, respectively).

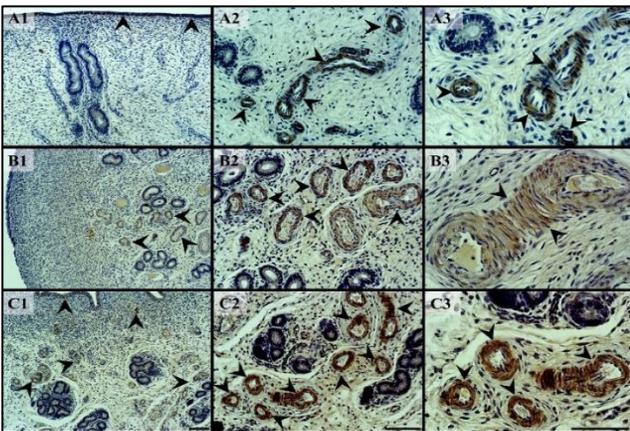


Figure 8: Localization of IL-10 in various forms of endometritis. In mild endometritis (A1-A3), IL-10 was expressed in the surface epithelium and vascular endothelium. In the acute form (B1-B3), a weakened expression of IL-10 was revealed in the surface and vascular endothelium. Compared to the acute, both the surface epithelium and vascular endothelium were detected with higher intensity in chronic endometritis (C1-C3). In all stained sections, the uterine glands failed to express staining for IL-10. Black head arrows represent the positive localization of IL-10, scale bar 200 $\mu$ m (labels 1-3: 10x, 20x, and 40x, respectively).

## Discussion

Endometritis, an inflammation of the uterine endometrial lining, is an important reproductive condition that adversely affects bovine reproductive efficiency, leading to decreased fertility, delayed conception, and substantial economic losses due to extended calving intervals and increased culling rates (9,10,32). For decades, various diagnostic procedures have been used for the clinical and/or laboratory detection of endometritis; however, a high proportion of cows have been abandoned from herds and culled due to uterine infections, reviewed (11,33,34). Therefore, the present study was designed to test the integrative application and potential association of the endometrial cytology with histopathological assessment as a rapid clinical procedure. Additionally, based on a previous study, numerous interleukin levels were found to be increased in response to endometrial infection, suggesting a critical role for these interleukins in the inflammatory process (1). Consequently, the present study hypothesized that proteins of the candidates' inflammatory interleukins might be detectable in the endometrium and their expression might be linked to the severity of the inflammation.

The uterine cytobrush is a minimally invasive method for evaluating endometrial cellularity, particularly focusing on the proportion of PMNL as biomarkers for uterine inflammation (35). In previous studies, for detecting endometritis, various thresholds of PMNL count were utilized as diagnostic values, ranging from > 5% (27) to >18% (35,36). In the current study, due to a wide range of PMNL counts being estimated and to link the obtained values with the severity of inflammation, the data were categorized into three varied diagnostic thresholds. The statistical analysis confirmed that higher PMNL levels were significantly associated with increased severity of inflammation. For instance, the increased PMNL proportions are strongly associated with severe histological lesions, such as epithelial desquamation, glandular degeneration, and stromal edema. It has been suggested that infiltration of PMNL into the uterine lining not only represents an essential immune mechanism but also serves as a marker of the existence of inflammation (8,35). These findings strengthen the use of cytobrush as an essential diagnostic tool for endometrial inflammation, capable of guiding treatment decisions even in the absence of clinical signs (37,38). However, since deeper endometrial stroma may have higher PMNL proportions than superficial epithelium, there are concerns about possible false negative results (8,39). The current study confirms earlier findings that the most reliable method for detecting mild endometritis is still histopathology. This is demonstrated by the 11 samples that detected with mild or chronic histopathological lesions (72.7% and 27.3% of cases, respectively) despite having less than 5% polymorphonuclear leukocytes (PMNLs) detected by cytobrush. The observed inconsistencies between

cytobrush results and histopathological evaluations, especially in mild cases, could be attributed to either the lower PMNL counts that are indicative of chronic inflammation or the superficial nature of cytobrush sampling, which might not accurately reflect deeper tissue conditions (8,35). For consistency, the present study adhered to conventional pathological categorization of endometrial inflammation (mild, acute, or chronic), which was primarily guided by precise tissue alteration and damage. The reported histopathological lesions affecting endometrial epithelium, glands, vessels, stroma, and cellular infiltration align with previous topic-relevant studies in cows (4,14,30). Interestingly, the chronic form exhibits a unique feature manifested by glandular nests, periglandular fibrosis, and plasma cell infiltration, suggesting an alteration from an innate to adaptive immune response (40,41). These histopathological lesions align with the findings of previous topic-relevant studies in cows (4,30) and buffaloes (14).

A substantial association was recognized between the cytological assessment (PMNL count) and the severity of endometrial lesions. Precisely, the PMNL count of 5% or less was correlated to mild pathological changes; however, groups displaying moderate to high PMNL proportions exhibited acute or chronic endometritis. These results highlight the predictive precision of cytological assessment for consequent histopathological examination. These outcomes are similar to observations reported in other works, including cows (39,42), buffaloes (13), and mares (43). Such correlations enhance understanding of disease progression and strengthen the use of integrated cytological and histological diagnostic approaches. In this study, chronic endometritis was the most prevalent endometrial inflammation (47.4%), aligning with previous research (44.7%) (13). This high prevalence may be due to untreated or inadequately treated mild cases progressing to chronic forms.

Interestingly, ROC curve analysis validated the diagnostic utility of cytological PMNL thresholds. While previous studies reported variable sensitivity (17-86%) and specificity (42-94%) for the endometrial cytobrush in subclinical endometritis (11,39). Although acute inflammation showed lower specificity (74%), 100% sensitivity ensured the detection of nearly all true cases. For chronic cases, 79% sensitivity and 96% specificity indicated strong classification accuracy. These metrics validate PMNL thresholds and highlight cytobrush cytology's potential to reduce diagnostic uncertainty, especially when combined with histopathology (4,39,42), bacteriology (39), or even ultrasonography (35). Given that elevated genomic inflammatory interleukins are linked to endometritis (16,19,23) and play a role in modulating local immunity and inflammation (43,44). Therefore, this study utilized immunohistochemical localization of IL-8, IL-1 $\beta$ , and IL-10 to investigate their expression patterns with inflammation severity.

The immunoreactivity of IL-8 exhibited a strong expression in sections diagnosed with acute inflammation and a declined intensity in chronic cases, consistent with its role in early immune recruitment (mild endometritis). It has been suggested that bacterial infection induces an inflammatory response manifested by upregulation of inflammatory interleukins. These inflammatory mediators, including pro-inflammatory interleukins (e.g., TNF- $\alpha$ , IL-1 $\beta$ , and IL-6) and chemokines (e.g., IL-8), prompt the leukocytes' extravasation and chemoattraction of neutrophils and monocytes, and encourage phagocytosis of bacteria and degenerated cells (16,41). Thus, the higher proportion of PMNL in the acute form might be a consequence of the elevated protein expression of IL8. In buffaloes, a similar correlation between the increased PMNL count and the upregulated levels of endometrial *IL-8* mRNA was documented and indicated to use the expression of IL-8 as a marker for severe endometritis (45).

The localization of IL-1 $\beta$  in the endometrial tissue is consistent with another previous study (36). The highest expression of IL-1 $\beta$  (a proinflammatory interleukin and an indicator of macrophage activation) was demonstrated in the acute stage and declined in chronic stages; its expression in the mild form was limited only to the surface epithelium. The strong expression of IL-1 $\beta$  in the acute inflammation might indicate a sustained pro-inflammatory activity. A previous study reported that IL-1 $\beta$  is produced and secreted by diverse types of immune and non-immune cells in response to inflammatory signals, which in turn upregulate the immune responses (46). These data are indicated in this study, where a strong intensity of staining was evident in the macrophages. Interestingly, other studies specified that to resolve acute inflammations, IL-1 $\beta$  employs effective pyrogenic action, which is essential for the effective initiation of the innate and adaptive immune responses (47). It has been indicated that pro-inflammatory interleukins enhance the immune status of the infected endometrium by encouraging the local cellular production of antibacterial peptides and infiltration of PMNL; for instance, by comparison with healthy cows, the expression levels of *IL-1 $\beta$*  and *IL-8* were dramatically increased (3 and 2.5-fold, respectively) in cows with endometritis (19,22). Correspondingly, in this study, the increased immunoreactivity of IL-1 $\beta$  and IL-8 in severe endometritis is consistent with the outcome of cytobrush, suggesting the validity of these two interleukins as markers for diagnosing severe endometritis.

IL-10, an anti-inflammatory interleukin, was moderately expressed in mild cases but markedly increased in chronic inflammation; Conversely, its expression largely decreased in acute inflammation. In the mild form, such an expression pattern might be essential to maintain the endometrial tissue with a balanced status against the detrimental effects of proinflammatory interleukins (25). In the acute form, the lowest expression of IL-10 might be essential to sustain the

inflammatory process against infection (23). However, the strong and continued expression of IL-10 in chronic endometritis is essential to promote fibrous tissue formation, immunosuppression, cellular proliferation, and tissue repair (24,41). Therefore, in contrast with IL-8 and IL-1 $\beta$ , the increased endometrial expression of IL-10 might be utilized as a marker of chronic endometritis.

## Conclusion

The diagnosis and staging of cows' endometritis were accurately and effectively determined by the utilized multimodal diagnostic approach (cellular, pathological, and interleukin expression). Therefore, the observed correlations between these parameters strengthen the rationale for employing this integrated diagnostics strategy in veterinary practices, eventually enhancing therapeutic decision-making.

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## Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication and/or funding of this manuscript.

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## دراسة تشخيصية لالتهاب بطانة الرحم في الأبقار تدمج التقييمات الخلوية والنسجية المرضية وتحديد التوضع النسيجي المناعي للأنترليوكينات

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### الخلاصة

يؤثر التهاب بطانة الرحم بشكل كبير على الكفاءة التناسلية في الأبقار، ومع ذلك فإن الأساليب التشخيصية الحالية غالباً ما تقتصر إلى الدقة اللازمة للتشخيص والتصنيف المرضي. تهدف هذه الدراسة إلى تقييم والتحقق من كفاءة نموذج تشخيصي يجمع بين علم الخلايا وعلم الأمراض النسيجي والكيمياء النسيجية المناعية لتشخيص وتصنيف شدة التهاب بطانة الرحم في الأبقار. جمعت ٧٦ عينة لبطانة الرحم من الأبقار المذبوحة خلال فترة ما قبل الشبق، وخضعت هذه العينات لاستحصا المسحات الخلوية والخزاع من بطانة الرحم. تم إجراء التقييم الكمي لكريات الدم البيضاء متعددة أشكال النوى في المسحات المصبوغة وتصنيفها وفقاً لعتبات محددة: البسيطة ( $\geq 5\%$ )، معتدلة ( $< 15\%$ )، وعالية ( $< 5\%$ ). وفقاً للتغيرات النسيجية المرضية في بطانة الرحم، صنفت شدة التهاب بطانة الرحم إلى خفيفة وحادة ومزمنة. فضلاً عن ذلك، صبغت مقاطع بطانة الرحم باستخدام الكيمياء النسيجية المناعية لتحديد تموضع وأنماط التعبير للأنترليوكينات الالتهابية. أظهرت العتبة المعتدلة لعد الخلايا متعددة أشكال النوى نسبة معنوية أعلى من المجموعتين البسيطة والعالية، مما يدل على وجود ارتباط إيجابي مع شدة الالتهاب النسيجي. أظهر التقييم النسيجي المرضي أن الالتهاب المزمن

هذه الدراسة أن دمج احتساب عدد الخلايا الالتهابية المتعددة النوى والتقييم النسيجي المرضي مع التلوين الكيميائي النسيجي المناعي يُحسّن دقة التشخيص وتحديد المرحلة المرضية لالتهاب بطانة الرحم. إن اختلاف التوضع وأنماط التعبير للإنترلوكينات المستعملة يعد بمثابة مؤشرات حيوية جزيئية واعدة تُقدّم رؤى جديدة حول الديناميكية الالتهابية المُعقدة والكامنة لتطور التهاب بطانة الرحم.

كان الأفة المرضية الأكثر انتشارًا. أكد تحليل منحنى الخصائص التشغيلية للمستقبل فعالية تشخيصية لقيم القطع للخلايا المتعددة النوى، حيث أظهرت حساسية ونوعية عالية لتمييز شدة الالتهاب. أظهر التصيغ الكيميائي النسيجي المناعي اختلاف في التوضع وأنماط التعبير للإنترلوكينات المستعملة ارتباطا بشدة التهاب بطانة الرحم. بشكل خاص، اقترنت شدة التعبير للإنترلوكينات الثامن والأول بيتا بالالتهاب الشديد، بينما لوحظ تعبير شديد للإنترلوكين العاشر في الحالات المزمنة. تُظهر