

## Comparison of bacterial endotoxin lipopolysaccharide concentrations in the blood, ovarian follicular fluid and uterine fluid: a clinical case of bovine metritis

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**ABSTRACT.** We investigated the concentration of the bacterial endotoxin lipopolysaccharide (LPS) in the blood, ovarian follicular fluid and uterine fluid of a clinical case of bovine metritis. A 2-year-old lactating Holstein cow exhibited continuous fever  $>39.5^{\circ}\text{C}$  for more than 2 weeks after normal calving. The cow produced a fetid, watery, red-brown uterine discharge from the vagina and was diagnosed with metritis. The LPS concentrations in plasma and uterine fluid were 0.94 and 6.34 endotoxin units (EU)/ml, respectively. One of seven follicles showed an extremely high level of LPS (12.40 EU/ml) compared to the other follicles (0.62–0.97 EU/ml). These results might suggest the presence of high concentration of LPS in follicles in cows with postpartum metritis.

**KEY WORDS:** dairy cow, follicle, lipopolysaccharide, metritis

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In postpartum dairy cows, uterine bacterial infection commonly occurs and causes infertility by disrupting uterine and ovarian function. Uterine inflammatory diseases, such as metritis and endometritis, cause enormous economic losses related to compromised reproductive performance; conception rates are approximately 20% lower in cows with endometritis, the median calving to conception interval is 30 days longer, and 3% more animals are culled owing to failure to conceive [1, 5]. *Escherichia coli* (*E. coli*) is among the main types of bacteria causing uterine inflammation, and the tissue pathology is largely associated with the bacterial endotoxin lipopolysaccharide (LPS) [11]. LPS has been detected in plasma, uterine fluid [7] and follicular fluid [4] of cows with endometritis, suggesting that LPS derived from uterine inflammation translocates to the ovary and affects follicular function. However, the occurrence of LPS absorption from the uterus into the bloodstream or its translocation into the follicle remains controversial [2, 4, 7]. There has been no report comparing the LPS concentration in different organs of the same animal, which makes it difficult to evaluate the possible translocation of uterine LPS to the bloodstream or follicles. In the present study, we evaluated the LPS concentration of blood, follicular fluid and uterine fluid of a clinical case of bovine metritis.

A 2-year-old lactating Holstein cow exhibited anorexia

and continuous fever  $>39.5^{\circ}\text{C}$  for more than 2 weeks after normal calving. Both intrauterine and intravenous administrations of tetracycline were performed every day during the first week postpartum, and then, intramuscular injection of ceftiofur sodium was used during the second week postpartum. The cow showed a fetid, watery, red-brown uterine discharge from the vagina. Transrectal ultrasonography of the uterus using an ultrasonic scanner (HS-101V; Honda Electronics Co., Ltd., Toyohashi, Japan) equipped with a 5-MHz linear-array transducer (HLV-155; Honda Electronics Co., Ltd.) revealed an enlarged uterus and accumulation of echogenic fluid in the uterine horns and the uterine body. The cow did not show any clinical signs of mastitis, such as udder swelling or increased somatic cell count. Based on these findings, the cow was diagnosed as metritis. On day 23 postpartum, the cow was euthanized according to ethical and animal welfare requirements under the guidelines of the Care and Use of Agriculture Animals of Obihiro University (No. 25–25), and a post-mortem examination was carried out.

At necropsy, the right uterine horn was enlarged compared to the left uterine horn (right uterine horn; length of 24.6 cm and width of 6.0 cm, left uterine horn; length of 18.0 cm and width of 2.4 cm), and the mucosa of both uterine horns was congested. For histopathological examination, uterine tissues were fixed in 10% formalin and embedded in paraffin wax. Four- $\mu\text{m}$ -thick sections were deparaffinized and stained using hematoxylin and eosin. Histologically, all layers of the uterine wall of both uterine horns showed prominent infiltration of lymphocytes, plasma cells, macrophages and neutrophils (Fig. 1). Fibrosis with atrophy and depletion of endometrial glands were observed in the endometria of both uterine horns. Infiltration of neutrophils and fibrosis were more prominent in the right uterine horn compared to

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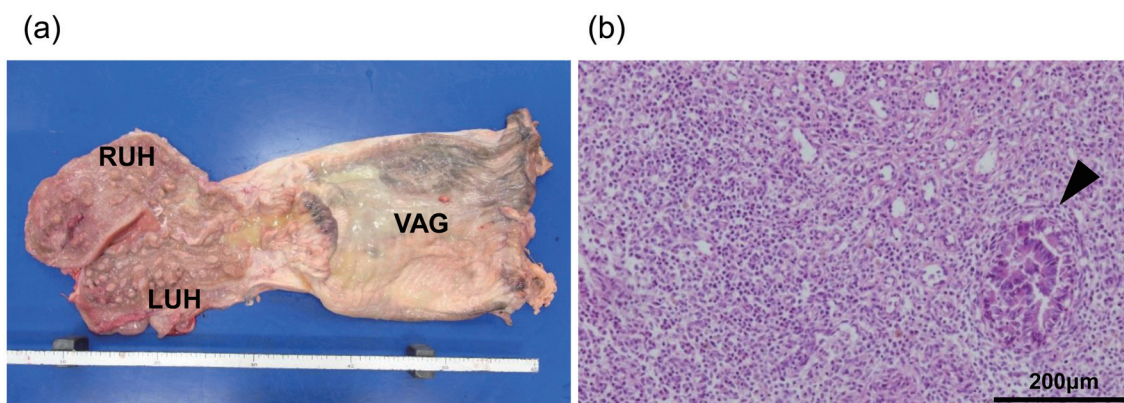


Fig. 1. (a) The right uterine horn was enlarged compared to the left uterine horn (right uterine horn; length of 24.6 cm and width of 6.0 cm, left uterine horn; length of 18.0 cm and width of 2.4 cm), and the mucosa of both uterine horns was congested. RUH: right uterine horn, LUH: left uterine horn, VAG: vagina. (b) The uterine endometrium of the right uterine horn shows prominent infiltration of neutrophils, lymphocytes, macrophages and plasma cells. Fibrosis with atrophy and depletion of endometrial glands (arrowhead) were observed. Hematoxylin and eosin staining. Scale bar=200  $\mu$ m.

Table 1. Concentrations of lipopolysaccharide (LPS) in plasma, uterine fluid and follicular fluid of a clinical case of bovine metritis

	Follicle position <sup>a)</sup>	Follicle diameter (mm)	E/P ratio <sup>b)</sup>	LPS (EU/ml)
Plasma	-	-	-	0.94
Uterine fluid	-	-	-	6.34
Follicle 1	LO	10.5	0.26	0.97
Follicle 2	LO	7.9	0.05	12.40
Follicle 3	LO	7.9	0.03	0.62
Follicle 4	RO	12.1	0.32	0.87
Follicle 5	RO	8.9	0.06	0.70
Follicle 6	RO	6.3	0.02	0.71
Follicle 7	RO	7.2	0.01	0.64

a) Follicle position: LO; Left ovary, RO; Right ovary. b) The ratio of estradiol and progesterone concentrations in the follicular fluid.

the left uterine horn. A uterine swab was obtained from each uterine horn using a sterile swab (Eiken Chemical Co., Ltd., Obihiro, Japan), and the swab was cultured aerobically and anaerobically at a commercial laboratory (Daiichi Kishimoto Clinical Laboratory, Inc., Obihiro, Japan). An aerobic gram-positive bacterium,  $\beta$ -streptococcus group C, was detected from the right uterine horn.

Intrauterine fluid was aseptically collected from the right uterine horn at the necropsy, and aliquots of fluid were centrifuged at  $600 \times g$  at  $4^\circ\text{C}$  for 30 min. Before the necropsy, blood sample was collected from the coccygeal vein into heparinized tubes and centrifuged at  $1,750 \times g$  at  $4^\circ\text{C}$  for 15 min. Follicular fluid from medium to large follicles ( $>6$  mm in diameter,  $n=7$ ) was aspirated using a syringe with a 22-gauge needle and centrifuged at  $1,500 \times g$  at  $4^\circ\text{C}$  for 1 min. Follicle diameter was determined from the weight of follicular fluid, as previously described by Murasawa *et al.* [9]. Obtained supernatants of uterine fluid, blood and follicular fluid were stored frozen at  $-30^\circ\text{C}$  in pyrogen-free siliconized glass tubes until the LPS assay. The LPS concentrations in the uterine fluid, plasma and follicular fluid

were measured using the QCL-1000 Chromogenic Limulus Amebocyte Lysate Endpoint Assay Kit (Lonza Walkersville, Inc., Walkersville, MD, U.S.A.) as previously described [6]. A standard curve for the endotoxin assay was prepared using endotoxin-free water with concentrations of 0.1, 0.25, 0.5 and 1 endotoxin units (EU)/ml (1 EU=0.1 ng LPS). The internal recovery rate, as determined using positively spiked samples, was  $>80\%$ , and the limit of detection was 0.1 EU/ml. Table 1 presents the LPS concentration in plasma, follicular fluid and uterine fluid. The LPS concentrations of plasma and uterine fluid were 0.94 EU/ml and 6.34 EU/ml, respectively. Follicular fluid LPS concentration ranged from 0.62 EU/ml to 12.40 EU/ml (Table 1).

The concentrations of estradiol (E2) and progesterone (P4) in follicular fluid were measured by enzyme immunoassay as previously described [8]. Standard curve ranges were 2–2,000  $\mu\text{g/ml}$  for E2 and 50–50,000  $\mu\text{g/ml}$  for P4. Follicular fluid was diluted with assay buffer when E2 and P4 concentrations were too high for the respective standard curve ranges. The intra- and inter-assay coefficients of variation averaged 7.7% and 4.9% for E2, and 6.5% and 8.7% for

P4, respectively. The ratio of E2 and P4 concentrations (E/P ratio) in follicular fluid is shown in Table 1.

This is the first report to document the comparison of LPS concentrations in plasma, follicular fluid and uterine fluid of a clinical case of bovine metritis. The LPS concentrations in ovarian follicular fluid was similar to that in plasma and was lower than that in uterine fluid (Table 1). Compared to previous studies [4, 7, 12], the LPS concentrations in follicular fluid and uterine fluid detected in the present case were much lower, although the plasma LPS concentration was similar to that reported by Mateus *et al.* [7] and Williams *et al.* [12]. This discrepancy among studies might be due to the condition of the animals; the severity of uterine inflammation in the present case might be rather mild compared to that of cows assessed in previous studies.

Interestingly, one of seven follicles showed an extremely high level of LPS (12.40 EU/ml) compared to other follicles (0.62 to 0.97 EU/ml). No distinctive feature in the follicular size, appearance, vascularization or location was noted among the seven follicles. The mechanism by which LPS accumulates in the follicular fluid is not known. Although we currently have no clear explanation for this specific accumulation of LPS in a single follicle, these results suggest that some specific follicles might accumulate LPS selectively, passively or by chance. One potential candidate molecule that could be involved in the accumulation of LPS is LPS-binding protein (LBP). LBP is an acute-phase protein produced in the liver, which circulates in the bloodstream where it recognizes and forms a high-affinity complex with LPS [10]. In humans, LBP is constitutively present in serum and increases by 10- to 50-fold during acute-phase inflammation [13]. Thus, it might be possible that LBP produced by the acute uterine inflammation binds to LPS, which would then contribute to accumulation of LPS in the follicular fluid.

In general, the E/P ratio can be used to determine the follicular activity in cattle, and follicles are classified as E2-active (E/P ratio  $\geq 1$ ) or E2-inactive (E/P ratio  $< 1$ ) [3]. We recently reported that follicles with a follicular fluid LPS concentration greater than 0.5 EU/ml were classified as E2-inactive, with suppressed E2 production [6]. Similarly, in the present case, all follicles with a follicular fluid LPS concentration greater than 0.5 EU/ml were classified as E2-inactive, although the E/P ratio of individual follicles did not correlate with follicular LPS concentration (Table 1. Pearson's correlation coefficient =  $-0.17$ ,  $P=0.73$ ). These findings indicate that follicular steroid production was suppressed in all follicles of the present case, which is consistent with previous studies.

LPS is derived from the cell membranes of gram-negative bacteria, such as *E. coli*; however, gram-negative bacteria were not detected from the uterus of the present case. In general, *E. coli* dominates the uterus in the first few days after parturition, and *E. coli* numbers decrease within 2 weeks postpartum due to the physiological involution process [12]. Thus, it is likely that the cow used in the present study was in a state of spontaneous recovery from the early postpartum uterine inflammation, and any gram-negative bacteria that had initially contaminated the uterine rumen had already

been eliminated. Indeed, histopathological findings of the endometrium indicated that the uterine condition was consistent with a transition period to chronic inflammation. In our previous study, we observed that the follicular LPS concentration was not associated with the severity of uterine inflammation (unpublished data). Perhaps, this inconsistency between the uterine condition and follicular LPS concentration indicates the possibility of long-term accumulation of LPS in follicular fluid after the uterine inflammation has resolved.

In summary, various concentrations of LPS were detected in the plasma, follicular fluid and uterine fluid of a cow with postpartum metritis. Although the concentration of LPS in ovarian follicular fluid was almost the same as that in plasma and was lower than that in uterine fluid, one follicle showed an extremely high concentration of LPS. Further studies using more clinical cases of uterine inflammation are required to examine the possible accumulation of LPS in follicular fluid.

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