



## NOTE

Virology

# Intrauterine infection with bovine leukemia virus in pregnant dam with high viral load

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**ABSTRACT.** Enzootic bovine leukemia is caused by the bovine leukemia virus (BLV). BLV is transmitted vertically or horizontally through the transfer of infected cells via direct contact, through milk, insect bites and contaminated iatrogenic procedures. However, we lacked direct evidence of intrauterine infection. The purpose of this study was to confirm intrauterine BLV infection in two pregnant dams with high viral load by cesarean delivery. BLV was detected in cord and placental blood, and the BLV in the newborns showed 100% nucleotide identity with the BLV-*env* sequence from the dams. Notably, a newborn was seropositive for BLV but had no colostral antibodies. In this study, we presented a direct evidence of intrauterine BLV transmission in pregnant dam with a high proviral load. These results could aid the development of BLV control measures targeting viral load.

**KEY WORDS:** bovine leukemia virus, high proviral load, intrauterine infection, vertical transmission

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Bovine leukemia virus (BLV), which belongs to the family *Retroviridae* and genus *Deltaretrovirus*, causes enzootic bovine leukosis (EBL) and is genetically closely related to human T-cell leukemia virus-1 (HTLV-1) [13]. Most BLV-infected cattle remain subclinical and are referred to as aleukemic, but approximately 30% develop persistent lymphocytosis. Additionally, 1–5% of BLV-infected cattle develop fatal lymphoma or lymphosarcoma [8]. BLV is highly endemic in many countries, including Japan. Several studies have reported that the seroprevalence and number of EBL cattle are increasing in Japan because there is no effective treatment or vaccine for BLV infection [9, 10]. Previously, we reported that BLV-infected cattle with persistent lymphocytosis or EBL and high proviral load are considered major sources of both horizontal and vertical BLV transmission [7, 11]. Furthermore, maternal proviral load is closely correlated with the frequency of vertical transmission to calves [7]. Thus, BLV-infected cattle with high proviral loads present a high risk for BLV transmission. However, no direct evidence of intrauterine BLV infection has been confirmed in infected cattle. Thus, the purpose of this study was to confirm intrauterine BLV infection in two pregnant dams with high viral load by cesarean delivery.

In this study, to confirm the vertical transmission of BLV, six newborns from infected-pregnant cattle (Holstein breed) were investigated. Four natural delivery newborns from the dams (Pr. S1808, Pr. M1635 and Pr. M10221) were tested BLV-infection before colostrum administration. A dam, Pr.H1453, was clinically and histopathologically diagnosed as EBL. Cesarean section was aseptically conducted in two dams, Pr. H368 and Pr.H1453, with a high proviral load at Faculty of Veterinary Medicine, Hokkaido University to directly confirm the intrauterine BLV infection. A newborn was taken on day 277 of the pregnancy in Pr. H368. The other one was taken on day 190 of the pregnancy in Pr.H1453, and venous blood was collected immediately from the newborns. Maternal samples, amniotic fluid, and peripheral, placental, and cord blood were also collected during delivery, and purified genomic DNA was obtained for the detection of BLV with PCR analyses. BLV infection was confirmed with nested PCR targeting the LTR to detect provirus [6] and with a commercial enzyme-linked immunosorbent assay kit (ELISA; JNC Inc., Tokyo, Japan) to detect anti-BLV antibody. The proviral load was further confirmed by real-time PCR using a Cycleave PCR BLV detection kit

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**Table 1.** Detection of bovine leukemia virus and antibody in the infected dams and newborns

Dam ID	Pr. H368	Pr. H1453	Pr. S1808		Pr. M1635	Pr. M10221
Age (year)	3	4	4		3	3
BLV diagnosis of dam						
PCRs	+	+	+		+	+
ELISA (S/P value)	+(1.492)	+(3.055)	+(1.570)		+(1.264)	+(1.378)
Provirus load (copies/50 ng DNA)	3,775	4,868	10,215		2.7	9.9
Lymphoma	–	+	–		–	–
No. of lymphocytes (/μl)	15,900	88,200	22,000		6,300	4,200
Disease stage	PL	EBL	PL		AL	AL
Obstetric delivery	Cesarean section (day 277)	Cesarean section (day 190)	Natural delivery		Natural delivery	Natural delivery
No. of newborn	1	1	2 (Twins)		1	1
Sex of newborn	Male	Female	Female	Female	Male	Female
Colostrum administration	No	No	No	No	No	No
BLV diagnosis of newborn						
Nested-PCR	+	+	+	+	–	–
ELISA (S/P value)	+(1.222)	N.D	+(1.082)	+(0.885)	–	–
Western blotting	+	N.D	N.D	N.D	N.D	N.D

PCRs: nested PCR and real-time PCR, +: positive, –: negative, AL: aleukemia stage, PL: persistent lymphocytosis stage, EBL: enzootic bovine leukemia, N.D: not demonstrated.

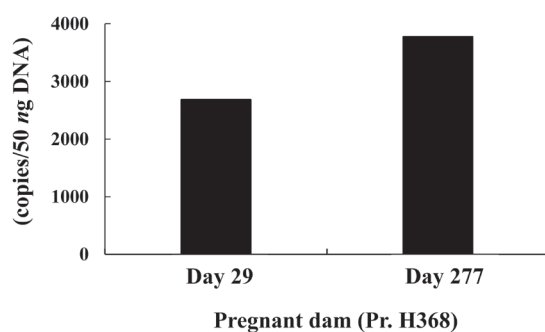
(Takara Bio, Otsu, Japan) according to the manufacturer's instructions. For confirmation of viral transmission to the newborns, the genotypes of the detected BLV from the dam and newborn were further identified via nested PCR targeting *env* gene (444 bp) and sequencing as previously described [2]. In addition to ELISA, western blotting using the recombinant BLV-*env* immunoglobulin (BLV-*env*-Ig) protein [6] was used to confirm the presence of anti-BLV antibody in serum. Four newborns from BLV-infected dams via natural delivery were also tested for BLV infection and presence of anti-BLV antibody. All animal experiments were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan) and all protocols were approved by the Institutional Animal Care and Use Committee of Hokkaido University, Japan (approval no. 11-0059 and 17-0024). Informed consent was obtained from clinical veterinarians and farmers before sample collection.

In a previous study, we reported that maternal proviral load increases the risk of vertical BLV transmission [7]. Indeed, BLV was detected in calves delivered via natural delivery from a dam (Pr. S1808) with a high proviral load, whereas it was not detected in calves from dams (Pr. M1635 and Pr. M10221) with a low proviral load (Table 1). The proviral load in peripheral blood derived from pregnant dam Pr. H368 was 2,685 copies/50 ng DNA at early pregnancy and 3,775 copies/50 ng DNA at cesarean section. The other pregnant dam with EBL, Pr. H1453, had 4,868 copies at cesarean section. These values confirmed the high risk of vertical BLV transmission (Fig. 1A, Table 1) [7]. As expected, BLV was detected in the both of newborns delivered via cesarean section (Fig. 1B, Table 1), and the detected BLV-*env* gene sequences were completely identical between dams and newborns (data not shown). The BLV was also detected in placental and cord blood from the dam (Fig. 1B), and among the maternal samples, the proviral load in the placenta was highest (Fig. 1C). Notably, the anti-BLV antibody was detected in the newborn without colostrum antibodies from the dam (Fig. 1D, Table 1). To confirm whether this result reflected a fetal immune response to the infection, we confirmed the presence of the antibody in newborns with vertical BLV infection or uninfected newborns from BLV-infected dams. The presence of anti-BLV antibody in natural delivery newborns from infected dams was tested with ELISA using sera without colostrum antibodies. Consistent with intrauterine transmission, the antibody was detected in newborns with vertically transmitted BLV, whereas uninfected newborns from infected dams with low proviral loads were seronegative (Table 1).

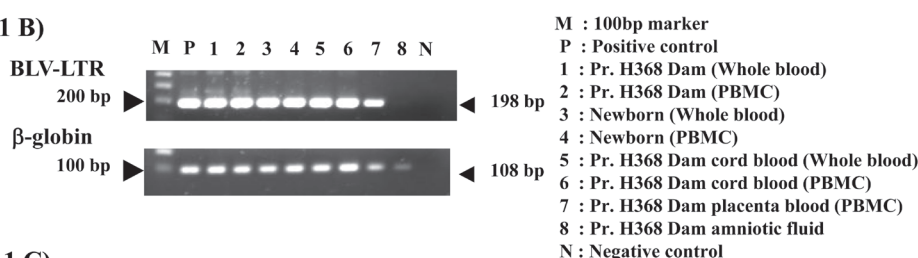
To date, several studies have indicated that colostrum intake could be a risk for vertical BLV transmission [5, 7]. In addition, suspected clinical cases of vertical BLV transmission via intrauterine infection have been reported [7]. However, no studies have reported direct evidence of intrauterine BLV infection in a BLV-infected dam. Thus, we confirmed intrauterine infection in a BLV-infected newborn delivered via cesarean delivery in a dam with a high viral load. This report is first to provide direct evidence of intrauterine infection in BLV-infected dam. There are two possible routes of BLV transmission via intrauterine infection: transmission of BLV-infected cells via cord blood and oral viral acquisition via the swallowing of amniotic fluid containing BLV-infected cells or cell-free virus particles. In this study, BLV provirus was detected in cord and placental blood but not in amniotic fluid. HTLV-1, which is genetically closely related to BLV, is mainly transmitted via cell–cell contact, including vertical transmission [1]. Similar to the findings in the present study, a previous study indicated that HTLV-1 was detected in the epithelium of the placenta [4]. BLV has also been detected in endothelial cells [12]. Thus, cord blood and placenta might be the routes of vertical BLV transmission.

In HTLV-1 infection, breast milk intake is considered the main route of vertical transmission in human. Although a few reports indicate that HTLV-1 is transmitted within the uterus [3, 14, 16], the risk of intrauterine infection is low in humans, unlike BLV transmission in cattle. This difference might be explained by the viral load during pregnancy. In HTLV-1 infection, high proviral load develops over a long period, and most pregnancies occur in young women who have low viral loads if even they are carriers.

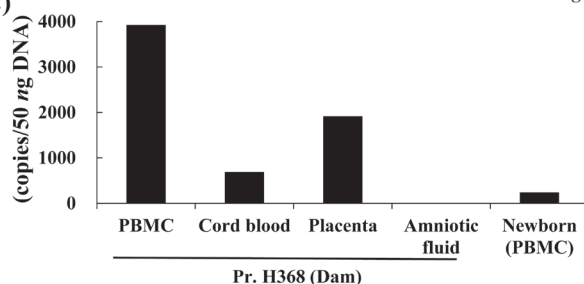
(Fig. 1 A)



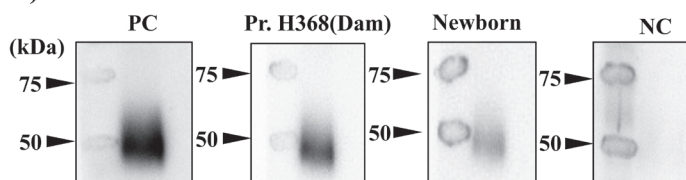
(Fig. 1 B)



(Fig. 1 C)



(Fig. 1 D)



**Fig. 1.** Direct evidence of intrauterine bovine leukemia virus (BLV) infection in a pregnant dam with high proviral load. (a) Proviral load in BLV-infected pregnant dam Pr. H368 in early and late pregnancy. (b) Nested PCR detection of BLV in the newborn of Pr. H368. Lane 1: Pr. H368 dam (whole blood); lane 2: Pr. H368 dam (peripheral blood mononuclear cells [PBMCs]); lane 3: newborn (whole blood); lane 4: newborn (PBMC); lane 5: Pr. H368 dam cord blood (whole blood); lane 6: Pr. H368 dam cord blood (PBMCs); lane 7: Pr. H368 dam placental blood (PBMCs); lane 8: Pr. H368 dam amniotic fluid; P: positive control; N: negative control; M: 100-bp marker. (c) Proviral loads in maternal samples and newborn. (d) Detection of BLV antibody in Pr. H368 dam and newborn with western blotting using recombinant BLV-env protein.

On the contrary, the latency period between BLV infection and the development of high proviral load is shorter, although the lifespan of cattle differ.

Notably, the BLV antibody was detected in intrauterine-infected newborns, whereas uninfected newborns from infected dams were negative for BLV antibody. In cattle, maternal antibodies do not transition from dam to offspring through the placenta [15]. Instead, the bovine fetus generally obtains immunocompetence at approximately 3–4 months' gestation [15]. Thus, intrauterine infection might be acquired from mid-pregnancy onward. However, pregnant dam Pr. H368 already had a high proviral load in early pregnancy. It remains unknown why the newborn did not show immunological tolerance similar to calves infected with bovine viral diarrhea virus in early pregnancy. Further studies are needed to elucidate the mechanism of BLV transmission.

In conclusion, the results of this study present the first direct evidence of intrauterine BLV infection. In Japan, many BLV-

infected pregnant adult cattle have high proviral loads, which indicates that BLV infection occurs in young animals, as has been observed in the field. Thus, the prevention of BLV infection in young generations is crucial in decreasing vertical transmission.

CONFLICT OF INTEREST. The authors declare that they have no competing interests.

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