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Running head: Evaluation of bovine leukemia virus using stable flies

Abstract. The incidence of enzootic bovine leukosis (EBL), a type of B-cell lymphoma, is increasing in Japan. EBL is caused by bovine leukemia virus (BLV; *Retroviridae*, *Deltaretrovirus bovleu*) infection; EBL is diagnosed by detecting antibodies against BLV in milk and blood or BLV DNA in blood. We assessed the feasibility of using stable flies (*Stomoxys calcitrans*) as a sampling tool to assess BLV infection status in cattle herds. First, we collected blood from 3 cattle herds and, based on the measurement of BLV-PVL by quantitative real-time PCR (qPCR), identified 1) a BLV-free herd, 2) a herd with a low prevalence of BLV-infected cattle and low proviral load (PVL), and 3) a herd wherein half of the cattle were BLV-infected with low-to-high PVLs. Next, we collected stable flies from the 3 herds, extracted DNA from their blood meals, analyzed it for BLV DNA, and measured the BLV PVL. Cattle DNA and BLV DNA, but not other mammalian DNA, were successfully detected by digestion of the flies. Based on fly blood meal qPCR, we identified one herd as BLV-free and the other 2 herds as having <50% prevalence of BLV-infected cattle with low PVLs. Our fly results were not consistent with preliminary BLV-PVL measurements on cattle blood. Our pilot study indicated that, to assess the feasibility of a stable fly blood meal test as an alternative technique for evaluating BLV infection status in dairy and beef cattle, additional investigations involving more cattle herds and stable flies are needed.

Keywords: bovine leukemia virus; cattle; enzootic bovine leukosis, feasibility studies.

Enzootic bovine leukosis (EBL) is a B-cell lymphoma caused by infection with bovine leukemia virus (BLV; *Retroviridae*, *Deltaretrovirus bovleu*).[20] Among BLV-infected cattle, >50% remain healthy throughout their life; however, ~30% develop persistent lymphocytosis, and <5% develop EBL.[6] A nationwide serosurvey of BLV infection in Japan from 2009 to 2011 revealed that the seroprevalence of BLV infection was 28.7% in 9,722 beef breeding cattle and 40.7% in 11,113 dairy cattle.[18] The Ministry of Agriculture, Forestry and Fisheries, Japan, reported that the annual number of EBL cattle was 99 in 1998 and this increased gradually to 4,491 in 2023[13] (Suppl. Fig. 1).

There are no commercial vaccines or therapeutic drugs for BLV infection; thus, countermeasures against BLV infection and EBL development are urgently required. Most western European countries, Australia, and New Zealand have established eradication programs and control measures, which have resulted in negligible BLV infection rates.[11,20] However, in Japan, the high prevalence of BLV antibodies makes it impractical to cull all BLV-infected cattle. Currently, herd management for BLV is performed by detecting antibodies against BLV in milk and blood using ELISAs and/or BLV DNA in blood using PCR testing. High BLV-proviral load (PVL) levels in cattle blood constitute a risk factor for EBL progression[9]; however, milk does not contribute to the measurement of BLV-PVL in cattle, and milk tests are available only for the detection of antibodies against BLV for dairy cattle herds, but not for beef cattle herds. Consequently, blood collection is still used for the routine detection of antibodies and BLV DNA, and assessment of BLV-PVL.

Stomoxys calcitrans (*Diptera*: *Muscidae*), commonly referred to as a stable fly, is a blood-sucking ectoparasite that is globally considered an economically important pest for the livestock industry. Its painful bites disrupt the feeding behavior of livestock and cause direct harm through blood loss, tissue damage, and allergic reactions.[12,25] Moreover, stable flies are suspected to play a crucial role in the spread of infectious diseases owing to their potential as mechanical pathogen carriers, particularly in livestock.[1] Stable flies carry pathogens, such as bovine viral diarrhea virus,[4] lumpy skin disease virus,[14] and *Anaplasma marginale.*[14] In addition, BLV[3] and BLV genes[22] have been detected in stable flies that feed on BLV-infected cattle. We hypothesized that the BLV infection status of cattle herds would be reflected in the blood meals of stable flies. Furthermore, we aimed to determine the feasibility of using stable files as a sampling tool to assess BLV infection status in cattle herds without the need for blood collection.

First, we collected blood from cattle of 3 farms (A-C) in Gifu, Japan, which had ~20, 40, and 80 Holstein cattle, respectively. Hematologic tests, detection of serum antibodies against BLV via ELISA, and measurement of the BLV-PVL (copies/10^5 WBCs) using quantitative real-time PCR (qPCR) were performed by the Gifu Chuo Livestock Hygiene Service Center (Gifu, Japan), as described previously.[23] Studies havereported that higher activity of serum lactate dehydrogenase (LDH) and/or increased ratios of LDH isozymes 2 and 3 are diagnostic biomarkers for EBL.[7,10] Therefore, we had the serum LDH activity and ratio of LDH isozymes analyzed by a clinical testing company (SRL, Tokyo, Japan). Although the percentage of LDH 2+3 from 2 of 33 cattle in 2023 on farm B and 2 of 78 cattle in 2023 on farm C increased 50% or more, these 4 cattle did not develop to EBL during our study period. BLV infection of cattle on each farm was also confirmed using nested PCR detection of the pX[19] or envelope regions[5] of BLV in the blood (GoTaq hot start green master mix; Promega; Suppl. Table 1).

We detected no BLV-infected cattle on farm A among the 13 cattle tested in 2021 and 18 cattle in 2023 (Fig. 1; Suppl. Table 2). The prevalence of BLV-positive cattle on farm B was 9 of 36 (25%) in 2022 and 6 of 36 (17%) in 2023; on farm C, the prevalence was 51 of 74

(69%) in 2022 and 39 of 78 (50%) in 2023 (Fig. 1A; Suppl. Tables 3,4). On farm B, most of the BLV-positive cattle were in the lower PVL category (< 25,000 copies/10^5 WBCs), according to a classification described previously,[9] and the cattle with the highest PVL were included in the second PVL category (25,000–50,000 copies/10^5 WBCs). On farm C, most BLV-positive cattle were in the first PVL category (< 25,000 copies/10^5 WBCs), but some were in the 4 other PVL categories, including the highest PVL category (≥ 100,000 copies/10^5 WBCs). Based on the PCR results, each cattle herd was characterized as follows: farm A was BLV-free, farm B had a low prevalence of BLV-infected cattle and low PVL, and on farm C, >50% of the cattle were BLV-infected with low-to-high PVLs (Fig. 1B).

Next, we captured stable flies (Table 1) on the bodies of the cattle and inside the barns of each farm using a butterfly net. To avoid viral contamination, new butterfly nets were used for each farm and sampling period. The flies were transferred to our laboratory on the same day, and precautions were taken to prevent secondary viral pollution during sample delivery or preparation. All flies collected in plastic bags were killed by placing them in a −80°C freezer, followed by subsequent storage at -30°C. Captured flies were pooled and placed into 15-mL tubes, and the body surfaces of the flies were rinsed with 2 mL of PBS by gently rotating the tube for 10 min without crushing or releasing their bodily fluids. Then, the flies were crushed using sterile cotton swabs. The crushed liquid was filtered using 1.0- and 0.45-µm pore filters (Merck Millipore), and the filtrate was centrifuged at 20,400 × *g* for 1 h at 25°C. Total DNA was extracted from 200 µL of the lower layer after centrifugation (DNeasy blood & tissue kit; Qiagen), according to the manufacturer's instructions.

To determine the origin of the stable fly blood meals, a multiplex PCR assay was performed, as described previously.[17] Briefly, the mitochondrial DNA (mtDNA) copy number of each extracted DNA sample was quantified using a universal primer set that amplified a conserved region of the 16S rRNA gene in vertebrates (SmartCycler II system; Cepheid). Multiplex PCR was performed (Multiplex PCR assay kit v.2; Takara Bio) in a 25-µL reaction mixture containing 5,000 copies of sample DNA in an iCycler (Bio-Rad; Suppl. Table 5). Multiplex PCR products were analyzed (3500xL Genetic Analyzer with a 36-cm array and POP-4 polymer; Thermo Fisher), and the results were analyzed with GeneMapper ID-X Software v.1.4 (Thermo Fisher) with a peak amplitude threshold of 175 RFU and customized panel and bin sets. The origin of the blood meal in all DNA samples was successfully determined (Table 1; Suppl. Table 6); for all farms, we tested for 21 other mammalian species, and only cattle DNA was detected in the stable fly blood meals. The distance between the capture location and the target animal intended for evaluation is crucial when using stable fly blood meals. Studies have shown that when stable flies are captured near locations with different types of livestock[21] or in zoos,[15] genes from various animal species are detected in their blood meal. We had captured stable flies inside barns extremely close to the cattle, leading to the detection of only cattle genes in their blood meals.

We captured 140 stable flies in 2021 and 112 in 2023 on farm A, 52 in 2022 and 71 in 2023 on farm B, and 38 in 2022 and 27 in 2023 on farm C (Table 1). To verify the feasibility of using stable flies for the detection of cattle and BLV DNA, blood meal DNA was extracted from pooled flies, as described above, or from a single fly. For single-fly samples, 1 mL of PBS was added to the fly before crushing with sterile toothpicks. After removing the fly body, the blood color of the crushed liquid was confirmed (Suppl. Table 7). Preliminary tests examined for BoLA-DRA, the bovine internal control DNA, in blood meal DNA extracted from pooled flies (1, 5, or 10 flies) captured at farm A. Using qPCR analysis, both pooled and single samples had detectable levels of BoLA-DRA in the blood meals, indicating that they could be used for BLV DNA detection. BoLA-DRA was detected in DNA extracted from crushed liquid samples with visible blood color, but not from colorless samples (Suppl. Table 7), indicating successful extraction of cattle DNA only from visibly colored blood meal samples. In preliminary experiments, pooled samples containing both visible and colorless blood tended to contain low amounts of BoLA-DRA (Suppl. Table 7). BoLA-DRA was detected ~8.8-fold more often in single samples with visible blood color than in pooled samples (Suppl. Fig. 2). Therefore, only blood meal liquid samples with visible blood color were selected, and their extracted DNA was used to detect the origin of the blood meal source and to measure BLV-PVL. As a result, of 140 flies captured in 2021 and 112 flies in 2023 on farm A, 50 flies in 5 pools (10 flies per pool) and 5 flies were tested, respectively (Table 1). For farm B, of 52 flies captured in 2022 and 71 flies in 2023, all 52 flies in 5 pools (10-12 flies per pool) and 21 flies were tested (Table 1). For farm C, of 38 flies captured in 2022 and 27 flies in 2023, all 38 flies in 5 pools (4-10 flies per pool), and 19 of 27 flies were tested (Table 1).

Blood color was confirmed in all 15 pooled samples (5 pooled samples for each farm). For single samples, 5 flies with deep blood color among the 112 flies captured at farm A were used for PCR and qPCR. Blood color was confirmed in 21 of the 71 flies at farm B and 19 of the 27 flies at farm C, and these samples were selected for PCR. BLV-PVL was measured using a 5-μL template DNA sample from the fly blood meal via qPCR (StepOne Plus analytical thermal cycler; Applied Biosystems), according to the manufacturer's instructions. The reaction mixture contained 10 μL of Thunderbird Probe qPCR Mix (Toyobo), 0.3 μL of CoCoMo-BLV Primer/Probe (Nippon Gene), 5 μL of a template DNA sample, and PCR-grade water to make the volume up to 20 μL. PVL was calculated using the following formula: (number of BLV LTR copies/number of BoLA-DRA copies) × 10^5 WBCs. The data were analyzed for significance using the Mann–Whitney *U* test and Kruskal–Wallis *H*-test ($p \leq 0.05$). All statistical analyses were performed using EZR software (v.1.64).[8]

The prevalence of BLV DNA in blood meals, as determined using PCR, varied for each farm (Table 1). The BLV-positive blood meals in farms A, B, and C were 0 of 5, 2 of 5, and 2 of 5 for pooled samples, respectively, and 0 of 5, 6 of 21, and 1 of 19 for single samples, respectively (Fig. 2A). For farm C, the BLV-positive cattle were 51 of 74 cattle (69%) in 2022 and 39 of 78 cattle (50%) in 2023, as determined by PCR using cattle blood (Fig. 1A), whereas that of BLV-positive blood meals was low at 2 of 5 pooled samples and 1 of 19 single samples. Moreover, based on the results of the BLV-PVL blood meal tests, each herd was characterized as follows: farm A was a BLV-free herd, and farms B and C were herds with a prevalence of less than half of BLV-infected cattle, 6 of 21 and 1 of 19 single samples in farms B and C (Fig. 2A), respectively, and low PVL (Fig. 2B), which was inconsistent with the BLV infection statuses determined using the cattle blood tests. This discrepancy in the BLV infection status in cattle herds between cattle blood tests and blood meal tests could be caused by DNA degradation in flies.

Comparing the detected amounts of BoLA-DRA among farms, farm A had significantly higher amounts, ~8.5- and 14.3-fold, than farms B and C, respectively (Suppl. Fig. 3). It has been difficult to discern the stage of the digestive cycle when using blood meals from field-captured flies.[21] Farm A was on the university campus; farms B and C were 15 and 35 km distant, respectively. These results indicate that the delayed processing for farms B and C may have led to DNA degradation in the blood meals via fly digestion. In addition, stable flies have been reported to fly 29 km in 24 h.[2] Others[24] indicated that 50% of adult stable flies dispersed beyond 1.6 km from their natal sites on farms, suggesting that stable flies could fly to neighboring farms. However, it is considered that, once stable flies find hosts, most tend to remain close to the hosts for several days.[2,24] Moreover, phylogenetic analysis of BLV using 1,823 cattle from 117 farms in 2 adjacent districts demonstrated that genetically distinct BLV strains were spread on each farm.[16] Therefore, horizontal transmission of BLV between neighboring farms by stable flies that fly between farms should occur only rarely. Further study is needed to determine whether blood meals from captured flies are derived only from cattle on the sampled farm.

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Declaration of conflicting interests

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Supplemental material

Supplemental materials for this article are available online.

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Table 1. Blood origin, nested-PCR, and qPCR analyses of stable fly blood meals.

Farm	Sample	No. of stable flies in a pool	Blood origin	Nested-PCR for BLV	qPCR		
					BoLA-DRA	BLV-LTR	BLV-PVL
					(Copies/100 ng DNA)		(Copies/10^5 WBCs)
A	2021 Sept., 50 of 140 flies were tested in 5 pools (10 flies per pool)						
	Pools						
	1	10	Cattle	-	54.6	ND	ND
	2	10	Cattle	-	143	ND	ND
	3	10	Cattle	-	273	ND	ND
	4	10	Cattle	-	787	ND	ND
	5	10	Cattle	-	150	ND	ND
	2023 Sept., 5 of 112 flies were tested						
	1	1	NT	-	685	ND	ND
	2	1	NT	-	1,140	ND	ND
	3	1	NT	-	1,210	ND	ND
	4	1	NT	-	2,230	ND	ND

	5	1	NT	-	582	ND	ND
B	2022 Sept., 52 flies were tested in 5 pools						
	Pools						
	1	12	Cattle	+	4.8	0.3	6,670
	2	10	Cattle	-	11.1	ND	ND
	3	10	Cattle	-	15.3	ND	ND
	4	10	Cattle	+	48.8	3.5	7,240
	5	10	Cattle	-	9.7	ND	ND
	2023 Oct., 21 of 71 flies were tested						
	1	1	NT	+	431	18.9	4,380
	2	1	NT	+	282	28.9	10,200
	3	1	NT	+	776	253	32,700
	4	1	NT	+	111	20.3	18,300
	5	1	NT	+	116	25.4	21,900
	6	1	NT	+	447	140	31,400
	7	1	NT	-	76.1	ND	ND
	8	1	NT	-	14.0	ND	ND

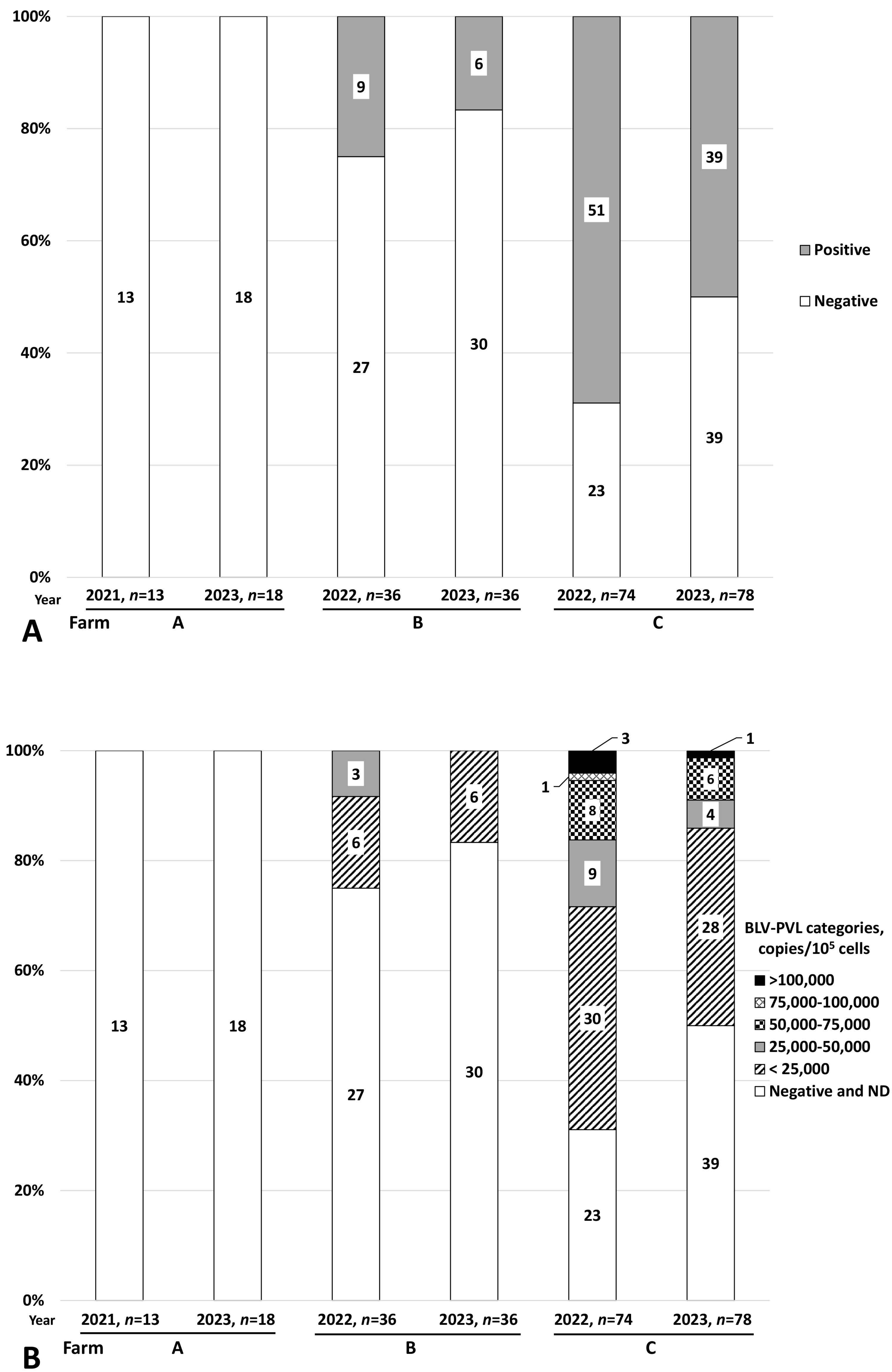
	9	1	NT	-	74.8	ND	ND
	10	1	NT	-	12.5	ND	ND
	11	1	NT	-	58.5	ND	ND
	12	1	NT	-	252	ND	ND
	13 to 21	9	NT	-	NT	NT	NT
C	2022 Oct., 38 flies were tested in 5 pools						
	Pools						
	1	10	Cattle	-	5.3	ND	ND
	2	8	Cattle	+	32.4	4.8	14,700
	3	6	Cattle	+	44.3	7.2	16,200
	4	4	Cattle	-	12.2	ND	ND
	5	10	Cattle	-	5.3	ND	ND
	2023 Oct., 19 of 27 flies were tested						
	1	1	NT	+	1,190	79.8	6,720
	2	1	NT	-	121	ND	ND
	3	1	NT	-	505	ND	ND
	4	1	NT	-	391	ND	ND

5	1	NT	-	1.6	ND	ND
6	1	NT	-	262	ND	ND
7 to 19	1	NT	-	NT	NT	NT

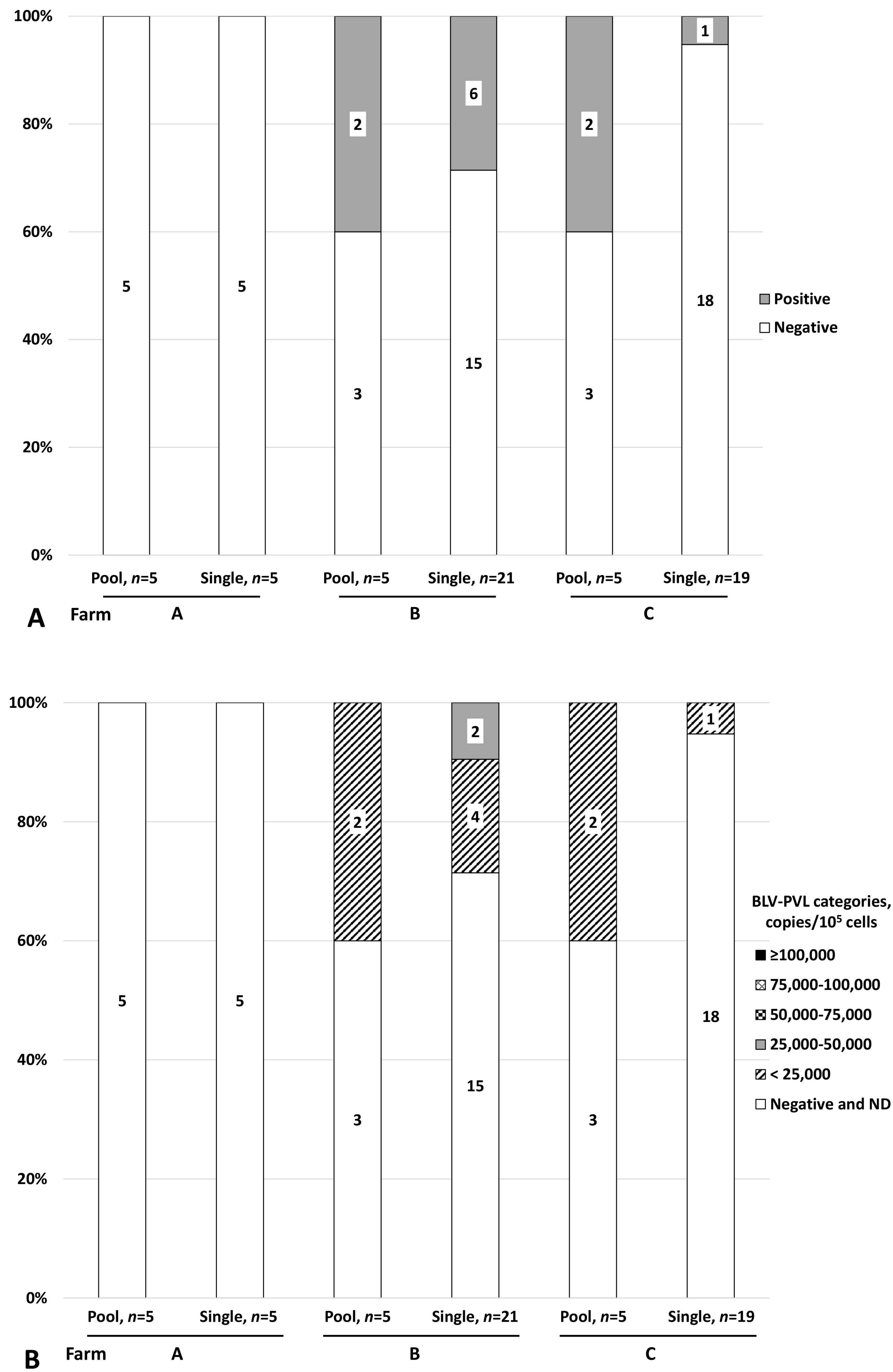
BLV = bovine leukemia virus; + = positive; - = negative; ND = not detected; NT = not tested; PVL = proviral load.

Figure 1. Bovine leukemia virus (BLV) infection status in cattle herds on farms A-C using cattle blood tests. Percentages and absolute numbers of **A)** BLV infection detected in cattle blood, and **B)** BLV-proviral load (PVL). BLV-PVL categories were classified according to a previous study.[8]

Figure 2. Detection of bovine leukemia virus (BLV) DNA and the measurement of BLV-proviral load (PVL) in the blood meals of flies. Percentages and absolute numbers of **A)** BLV DNA, and **B)** BLV-PVL detected in stable fly blood meals. The BLV-PVL categories were classified based on a previous study.[8]



100%
80%
60%
40%
20%
0%
13
18
9
27
6
30
51
23
39
39
Positive
Negative
Year
2021, n=13
2023, n=18
2022, n=36
2023, n=36
2022, n=74
2023, n=78
Farm
A
B
C
A
100%
80%
60%
40%
20%
0%
13
18
3
6
27
6
30
3
1
8
9
30
23
1
6
4
28
39
BLV-PVL categories, copies/10^5 cells
>100,000
75,000-100,000
50,000-75,000
25,000-50,000
< 25,000
Negative and ND
Year
2021, n=13
2023, n=18
2022, n=36
2023, n=36
2022, n=74
2023, n=78
Farm
A
B
C
B


100%
80%
60%
40%
20%
0%
5
5
2
3
6
15
2
3
1
18
Positive
Negative
Pool, n=5
Single, n=5
Pool, n=5
Single, n=21
Pool, n=5
Single, n=19
A
Farm
A
B
C
100%
80%
60%
40%
20%
0%
5
5
2
3
2
4
15
2
3
1
18
BLV-PVL categories, copies/10^5 cells
≥100,000
75,000-100,000
50,000-75,000
25,000-50,000
< 25,000
Negative and ND
Pool, n=5
Single, n=5
Pool, n=5
Single, n=21
Pool, n=5
Single, n=19
B
Farm
A
B
C

Supplemental Table 1. Primers and conditions used in nested PCR for the detection of pX and envelope regions of bovine leukemia virus.

Target	Primers		Sequence (5'-3')	PCR conditions				References
				Denaturation	Annealing	Extension	Cycles	
pX	1st	AF	CAGACACCAGGGGAGCCATA	94ºC, 45 s	62ºC, 30 s	72ºC, 30 s	25	19
		BR	CTGCTAGCAACCAATTCGGA					
	2nd	CF	AGCCATACGTTATCTCTCCA	94ºC, 45 s	62 ºC, 30 s	72ºC, 30 s	25	
		DR	CAGGTTAGCGTAGGGTCATG					
envelope	1st	5032F	TCTGTGCCAAGTCTCCCAGATA	95ºC, 30 s	62ºC, 30 s	72ºC, 60 s	40	5
		5608R	AACAACAACCTCTGGGAAGGGT					
	2nd	5099F	CCCACAAGGGCGGCGCCGGTTT	95ºC, 30 s	70ºC, 30 s	72ºC, 60 s	40	
		5521R	GCGAGGCCGGGTCCAGAGCTGG					

Supplemental Table 2. Assessment of bovine leukemia virus infection and the clinical status of cattle on farm A.

Cattle	Age, mo	ELISA* antibody	Nested PCR†	WBC, ×10^9/L	Lymphocyte, ×10^9/L	LDH‡, μkat/L	LDH isozymes§, %					
							1	2	3	2+3	4	5
2021 June (n = 13)												
1	41	-	-	5.4	28	22	64	20	11	31	4	1
2	66	-	-	4.8	20	21	68	18	10	27	3	1
3	93	-	-	5.4	28	22	66	18	10	29	4	2
4	57	-	-	5.4	27	20	73	15	7	22	2	3
5	34	-	-	4.8	21	22	70	17	9	27	2	2
6	56	-	-	10.4	61	16	65	20	11	31	3	2
7	33	-	-	9.1	44	18	62	22	12	33	4	1
8	26	-	-	8.2	43	25	67	19	9	29	3	2
9	31	-	-	6.1	32	22	71	16	7	23	4	2
10	78	-	-	7.1	35	21	69	18	8	26	2	3
11	81	-	-	6.0	31	20	61	22	12	34	4	2
12	46	-	-	8.6	42	20	67	19	10	29	3	1
13	17	-	-	7.4	41	22	62	21	11	32	4	2
2023 November (n = 18)												
1	49	-	-	7.4	29	15	49	28	15	43	5	3
2	49	-	-	7.2	31	15	50	27	14	41	5	4
3	29	-	-	5.5	23	17	48	28	16	44	5	3
4	18	-	-	7.6	43	17	50	27	13	40	6	4
5	82	-	-	5.7	20	17	48	27	15	42	6	4
6	26	-	-	6.6	23	16	50	26	15	41	5	4
7	34	-	-	5.3	27	17	47	27	16	43	6	4
8	58	-	-	7.9	26	14	48	28	16	44	5	3
9	109	-	-	6.1	21	13	46	28	16	44	6	4
10	49	-	-	5.4	29	21	45	27	15	42	6	7
11	21	-	-	6.4	35	18	48	29	15	44	5	3
12	44	-	-	3.0	13	21	38	27	21	48	8	6
13	9	-	-	11.7	65	19	36	25	14	39	8	17
14	37	-	-	6.3	33	14	50	29	14	43	4	3
15	40	-	-	6.0	30	13	52	28	14	42	4	2
16	4	-	-	9.2	57	15	42	31	17	48	6	4
17	8	-	-	12.0	72	15	41	31	18	49	6	4
18	4	-	-	13.4	63	20	49	28	15	43	5	3

LDH = lactate dehydrogenase, - = negative.

* Using anti-bovine leukemia virus (BLV) antibody ELISA kit (JNC, Tokyo, Japan).

† Using primers for the envelope or pX region of BLV.

‡ Using an auto analyzer JCS-BM6050 (JEOL) and an enzymatic method (L-Type Wako LD IF or L-Type Wako J, Fujifilm Wako Pure Chemical).

§ Using a Hydrasys 2 Scan (Sebia) and Hydragel 7 ISO-LDH (Sebia).

Supplemental Table 3. Assessment of bovine leukemia virus infection and the clinical status of cattle on farm B.

Cattle	Age, mo	ELISA[*] antibody	Nested PCR[†]	BLV-PVL[‡], copies/ 10^5 WBCs	WBC, $\times 10^9$/L	Lymphocyte, $\times 10^9$/L	LDH[§], μkat/L	LDH isozymes[¶], %					
								1	2	3	2+3	4	5
2022 May (n = 36)													
1	23	+	+	332	14.4	8.9	20	57	25	14	39	4	1
2	26	-	-	NT	10.6	3.9	23	66	20	12	31	2	1
3	27	-	-	NT	6.4	3.9	20	49	24	20	44	5	2
4	26	-	-	NT	18.3	4.1	28	69	17	10	28	3	1
5	77	-	-	NT	9.6	2.5	20	60	21	12	33	4	3
6	28	-	-	NT	21.3	4.2	32	69	17	11	27	3	1
7	61	-	-	NT	8.5	2.7	21	57	21	14	35	5	3
8	33	-	-	NT	10.0	3.9	26	70	15	8	23	5	3
9	31	-	-	NT	10.0	2.5	23	64	16	13	30	5	2
10	69	-	-	NT	7.7	3.9	24	63	17	10	27	5	5
11	24	-	-	NT	12.0	7.4	29	49	18	14	32	9	11
12	22	-	-	NT	11.2	5.2	25	61	21	12	33	4	2
13	22	+	+	14,238	13.1	6.6	23	56	22	14	36	5	4
14	21	-	-	NT	9.3	5.3	20	58	23	13	35	4	3
15	33	-	-	NT	7.2	3.6	23	62	17	12	30	5	4
16	39	-	-	NT	10.1	3.8	23	64	15	11	26	5	5
17	46	-	-	NT	9.6	3.9	21	62	18	13	30	5	3
18	44	-	-	NT	8.0	4.8	19	65	15	11	26	5	4
19	67	-	-	NT	7.5	2.3	26	66	18	9	28	4	3
20	37	-	-	NT	8.7	4.9	21	62	18	10	28	6	5
21	45	-	-	NT	7.9	4.4	22	58	22	13	35	5	2
22	45	-	-	NT	7.8	3.2	21	56	22	13	35	6	4
23	76	-	-	NT	7.6	3.1	24	20	5	18	23	18	4
24	27	-	-	NT	12.2	4.8	22	65	18	10	28	4	3
25	86	-	-	NT	6.1	2.1	19	57	17	14	30	9	4
26	47	-	-	NT	8.8	3.6	22	60	21	13	33	4	3
27	32	-	-	NT	10.6	4.6	27	75	10	9	19	4	3
28	48	-	-	NT	9.0	3.2	24	75	12	8	20	3	2
29	32	-	-	NT	11.5	3.8	23	66	14	13	26	5	3
30	71	NT	+	102	8.8	1.8	19	72	11	9	20	5	4
31	80	NT	+	6,010	8.6	4.7	22	63	12	11	22	7	8
32	66	NT	+	17,357	10.6	3.6	18	66	13	11	24	5	5
33	109	NT	+	46,458	16.5	9.7	21	69	17	10	27	3	2
34	42	NT	+	41,186	18.3	10.2	21	69	15	9	24	4	3
35	46	NT	+	36,185	15.8	6.8	25	76	13	7	20	2	2

36	32	NT	+	3	7.5	3.0	24	71	15	10	24	4	1
2023 June (n = 33)													
1	35	NT	+	8,434	12.8	5.7	17	44	29	17	46	6	4
2	36	NT	+	22,164	21.9	13.9	20	46	28	16	44	6	4
3	84	NT	-	16	6.2	2.3	15	48	27	15	42	6	4
4	40	+	-	69	11.8	3.7	17	46	29	17	46	5	3
5	33	+	+	2,972	8.6	4.6	19	50	27	15	42	5	3
6	40	-	-	NT	8.8	4.5	23	45	33	16	49	4	2
7	39	+	+	6,893	13.3	7.8	15	45	29	17	46	6	3
8	35	-	-	NT	10.1	5.0	20	45	30	16	46	6	3
9	37	-	-	NT	7.7	3.2	20	49	28	15	43	5	3
10	44	-	-	NT	5.7	1.7	19	44	28	17	45	7	4
11	74	-	-	NT	6.4	2.9	18	41	28	18	46	8	5
12	27	-	NT	NT	4.8	1.5	25	50	28	13	41	5	4
13	90	-	-	NT	8.3	3.6	16	44	28	16	44	7	5
14	29	-	-	NT	8.6	4.7	20	52	23	14	37	7	4
15	22	-	-	NT	8.4	3.6	16	43	31	17	48	6	3
16	24	-	-	NT	12.0	5.4	19	43	32	18	50	5	2
17	45	-	-	NT	2.1	1.0	16	41	30	18	48	7	4
18	61	-	-	NT	8.9	4.5	15	50	29	15	44	4	2
19	45	-	-	NT	5.7	2.5	18	45	29	16	45	6	4
20	28	-	-	NT	7.9	2.3	18	40	27	20	47	9	4
21	99	-	-	NT	10.4	6.0	17	43	29	17	46	7	4
22	32	-	-	NT	7.0	4.1	18	46	27	15	42	7	5
23	30	-	-	NT	6.8	2.4	19	41	30	18	48	6	5
24	58	-	-	NT	7.2	3.7	16	49	27	14	41	6	4
25	34	-	-	NT	6.6	3.2	20	47	28	16	44	5	4
26	50	-	-	NT	7.9	4.3	18	49	28	15	43	5	3
27	39	-	-	NT	10.0	5.2	18	43	28	16	44	8	5
28	57	-	-	NT	10.8	5.4	18	47	29	15	44	6	3
29	28	-	-	NT	6.0	2.8	16	49	26	15	41	7	3
30	52	-	-	NT	3.8	1.5	15	44	28	17	45	7	4
31	32	-	-	NT	8.2	3.7	18	48	26	15	41	6	5
32	27	-	-	NT	7.4	3.6	18	53	27	14	41	4	2
33	21	-	-	NT	6.8	3.1	22	42	32	18	50	5	3

BLV = bovine leukemia virus, LDH = lactate dehydrogenase, NT = not tested, PVL = proviral load, + = positive, - = negative.

* Using anti-BLV antibody ELISA kit (JNC, Tokyo, Japan).

† Using primers for the envelope or pX region of BLV.

‡ Using a CoCoMo-BLV primer/probe (A803, Riken Genesis).

§ Using an auto analyzer JCS-BM6050 (JEOL) and an enzymatic method (L-Type Wako LD IF or L-Type Wako J, Fujifilm Wako Pure Chemical).

¦ Using a Hydrasys 2 Scan (Sebia) and Hydragel 7 ISO-LDH (Sebia).

Supplemental Table 4. Assessment of bovine leukemia virus infection and the clinical status of cattle on farm C.

Cattle	Age, mo	ELISA[*] antibody	Nested PCR[†]	BLV-PVL[‡], copies/ 10^5 WBCs	WBC, $\times 10^9$/L	Lymphocyte, $\times 10^9$/L	LDH[§], µkat/L	LDH isozymes[‖], %					
								1	2	3	2+3	4	5
2022 April (n = 74)													
1	35	+	+	2	10.6	3.7	21	54	21	14	35	7	5
2	49	-	-	NT	9.2	4.1	18	52	20	16	36	6	6
3	49	-	-	NT	9.5	4.9	20	58	20	13	33	5	4
4	39	-	-	NT	10.4	5.8	24	58	20	13	33	6	4
5	39	+	+	6,821	9.3	5.3	22	51	23	14	37	7	5
6	48	+	+	ND	5.2	2.8	23	61	21	12	33	4	3
7	70	-	-	NT	6.1	2.0	21	54	22	14	35	7	4
8	67	NT	+	61,116	18.4	13.3	21	62	21	11	33	4	2
9	61	NT	-	ND	7.0	2.5	19	58	20	13	32	6	4
10	51	+	+	24,114	10.1	5.0	27	67	17	9	26	4	3
11	25	-	-	NT	7.7	4.1	24	60	20	13	33	4	3
12	74	-	-	NT	12.7	3.3	28	38	18	18	36	12	15
13	32	-	-	NT	8.4	4.0	20	61	19	11	30	5	3
14	43	+	+	10,403	7.2	4.3	23	51	20	12	32	6	11
15	131	NT	+	29,977	12.5	7.2	16	51	25	14	38	6	4
16	100	+	+	1,367	11.9	3.1	20	56	23	13	35	5	3
17	109	NT	+	1,063	6.5	2.2	19	47	20	22	42	6	6
18	100	-	-	NT	4.0	1.6	17	59	20	12	32	6	4
19	110	NT	+	353	5.9	2.6	18	50	19	19	39	8	4
20	46	+	+	807	9.4	3.3	22	64	20	11	30	3	2
21	44	+	+	368	11.5	4.0	20	59	21	12	33	5	3
22	44	+	+	11,467	14.2	3.9	39	31	11	7	18	7	4
23	40	+	+	25,524	11.2	5.2	17	48	24	17	41	6	5
24	87	-	-	NT	7.2	2.4	21	58	23	13	37	4	1
25	42	+	+	8,751	12.4	2.3	20	51	23	14	37	7	5
26	50	+	-	ND	4.4	3.0	24	59	20	11	31	6	5
27	32	-	-	NT	7.8	3.1	22	56	22	14	36	5	3
28	33	-	-	NT	7.8	3.2	22	53	24	15	39	5	3
29	64	+	+	2,402	5.3	2.9	20	57	21	14	35	5	3
30	121	NT	+	121,600	7.8	5.0	20	61	18	11	29	5	5
31	84	+	+	0	9.6	3.1	17	61	20	12	32	4	3
32	78	+	+	20,045	7.3	4.1	25	65	21	10	31	3	1
33	66	-	-	NT	4.8	2.3	20	54	23	14	37	6	4
34	39	-	-	NT	11.7	5.7	18	58	22	15	36	5	2

35	62	+	+	384	8.7	4.7	24	63	22	12	33	3	1
36	39	+	+	51,781	14.8	8.9	21	54	23	12	35	4	7
37	63	-	-	NT	7.0	3.0	18	65	19	11	30	3	2
38	21	+	+	34,742	10.2	3.9	19	62	18	12	31	5	2
39	39	+	+	3,062	7.9	2.5	18	68	11	12	23	6	4
40	42	-	-	NT	20.4	2.9	18	72	12	10	23	3	2
41	117	NT	+	1,407	6.7	2.5	16	77	10	7	17	3	3
42	112	NT	+	41,123	9.3	3.9	22	70	16	9	25	3	1
43	66	NT	+	56,204	27.1	17.9	25	76	11	8	19	3	2
44	57	-	-	NT	8.5	3.7	24	64	14	11	25	5	6
45	98	-	-	NT	6.2	2.3	20	66	10	11	21	7	5
46	44	+	+	53,633	16.4	6.4	20	75	9	9	17	5	4
47	26	-	-	NT	9.0	4.2	18	75	10	9	19	4	2
48	37	+	+	68,140	15.2	10.9	22	73	12	9	21	4	2
49	40	+	+	4,383	9.0	4.4	16	80	9	7	16	2	2
50	26	-	-	NT	7.7	4.7	25	81	6	6	13	3	3
51	60	+	+	72,825	17.0	3.8	20	69	15	9	24	4	3
52	65	+	+	142	10.2	2.5	19	62	15	14	29	7	2
53	126	+	+	32,805	10.6	5.6	25	47	28	16	44	5	3
54	44	+	+	35,298	20.5	9.2	24	69	12	7	19	4	9
55	38	-	-	NT	9.4	3.7	21	70	14	10	24	4	3
56	88	NT	+	103,534	27.7	22.2	22	74	14	9	22	2	2
57	91	NT	+	297	18.3	2.9	25	74	13	8	20	3	2
58	103	NT	+	65,468	14.0	10.4	20	67	16	11	27	4	3
59	151	NT	+	76,287	16.3	6.9	19	68	9	12	21	7	5
60	72	+	+	25,154	18.3	6.7	19	77	9	9	18	3	2
61	29	-	-	NT	8.8	4.1	22	78	6	8	14	4	4
62	93	NT	+	51,893	8.8	4.8	17	78	4	9	12	5	5
63	67	+	+	65	12.2	4.2	22	74	6	12	18	5	3
64	65	+	+	8,294	10.3	4.1	18	77	10	10	20	3	1
65	52	+	+	113,639	24.4	12.6	20	74	11	9	19	4	3
66	40	+	+	266	3.7	1.9	25	74	11	8	18	3	5
67	42	+	+	5,495	26.3	3.5	21	73	12	9	21	4	2
68	32	-	-	NT	10.0	4.6	22	65	11	12	22	7	6
69	65	NT	+	45,748	8.9	5.2	18	67	14	10	24	5	4
70	31	+	+	2,476	30.1	4.8	18	69	12	11	23	5	4
71	42	-	-	NT	16.0	2.6	20	73	9	9	18	5	5
72	41	+	+	303	10.1	4.5	19	75	8	9	16	5	4
73	52	+	+	1,037	13.7	2.8	23	73	10	9	19	5	3
74	44	+	+	42,515	17.1	3.3	18	70	11	10	21	5	5
2023 August (n = 78)													

1	34	-	+	NT	9.0	3.7	17	51	27	14	41	5	3
2	83	+	+	62,368	14.7	9.7	17	48	28	15	43	6	3
3	37	+	+	16,699	11.1	4.9	15	47	28	15	43	6	4
4	29	-	-	NT	9.8	5.3	17	46	28	15	43	7	4
5	48	-	-	NT	7.3	2.7	20	52	25	14	39	6	4
6	33	-	-	NT	8.1	3.1	20	51	26	14	40	6	3
7	67	+	+	67,262	25.1	20.0	16	47	29	15	44	6	3
8	48	-	-	NT	14.4	8.0	15	45	28	16	44	7	4
9	41	-	-	NT	7.7	3.0	18	52	26	14	40	5	3
10	104	+	+	3,458	9.9	2.7	14	47	28	15	43	6	4
11	31	+	+	ND	11.2	4.3	15	44	29	16	45	7	4
12	34	-	-	NT	11.1	5.6	18	51	26	14	40	6	3
13	65	-	-	NT	5.2	2.9	14	48	27	15	42	6	4
14	60	+	+	8,020	8.3	4.2	13	45	27	15	42	8	5
15	24	-	-	NT	9.0	4.3	14	43	29	16	45	8	4
16	103	-	-	NT	6.6	3.7	20	50	28	14	42	5	3
17	39	-	-	NT	8.2	4.2	15	47	29	15	44	6	3
18	71	-	-	NT	6.1	2.2	17	44	26	16	42	7	7
19	56	+	+	218	11.8	6.6	15	47	28	15	43	6	4
20	29	-	-	NT	6.9	3.2	17	45	27	15	42	7	5
21	61	+	+	25,003	10.4	6.2	15	48	27	14	41	6	5
22	32	-	-	NT	5.0	1.9	21	51	25	14	39	6	4
23	30	-	-	NT	7.7	4.3	13	46	29	16	45	6	3
24	78	+	+	358	5.2	2.1	14	48	27	14	41	7	4
25	52	-	-	NT	9.4	4.6	17	46	28	16	44	6	4
26	45	+	+	66	6.1	2.6	17	52	24	14	38	6	4
27	66	+	-	9	3.9	1.2	19	46	25	15	40	8	6
28	77	+	+	ND	5.1	2.7	15	47	27	14	41	7	5
29	49	-	-	NT	4.4	1.5	15	45	28	15	43	7	5
30	80	+	+	3,235	7.6	4.8	15	47	26	15	41	7	5
31	125	+	+	ND	5.2	2.3	12	45	27	17	44	7	4
32	48	-	-	NT	4.8	2.4	13	47	27	15	42	7	4
33	65	-	-	NT	7.0	2.9	14	50	27	15	42	5	3
34	55	+	+	1,561	6.5	1.9	17	42	25	17	42	8	8
35	86	-	-	NT	4.9	1.9	14	44	29	16	45	7	4
36	82	-	-	NT	6.3	2.6	14	44	28	18	46	7	3
37	41	+	+	18,777	11.6	6.7	19	40	28	17	45	7	8
38	79	-	-	NT	4.4	1.2	14	45	26	15	41	6	8
39	47	+	+	3,213	7.4	3.7	11	45	29	17	46	6	3
40	81	+	+	60,403	10.4	6.9	21	40	31	19	50	7	3
41	55	-	-	NT	7.5	2.4	13	46	28	17	45	6	3

42	24	-	-	NT	12.0	2.7	14	46	29	14	43	6	5
43	58	+	+	4,520	7.9	3.3	15	42	27	18	45	8	5
44	25	-	-	NT	9.3	4.4	15	42	31	18	49	6	3
45	56	+	-	ND	1.4	0.7	16	46	28	15	43	6	5
46	56	+	+	65,694	11.5	6.8	15	37	25	17	42	9	12
47	114	-	-	NT	5.7	1.1	15	46	26	16	42	7	5
48	62	+	+	891	7.8	3.6	14	46	27	15	42	7	5
49	128	+	+	18,512	10.0	5.9	22	38	31	21	52	7	3
50	56	+	+	251	3.8	1.7	15	46	25	14	39	8	7
51	58	-	-	NT	6.6	2.9	15	45	28	16	44	7	4
52	45	+	+	4,560	1.8	0.9	19	50	26	13	39	6	5
53	53	+	+	40,400	12.0	8.7	18	44	30	17	47	6	3
54	46	+	+	39,596	13.1	8.8	16	49	26	14	40	7	4
55	60	+	+	60,763	14.7	10.1	22	35	20	14	34	10	21
56	42	-	-	NT	7.7	3.7	15	47	27	15	42	7	4
57	33	-	-	NT	8.2	5.2	21	49	27	14	41	6	4
58	25	-	-	NT	8.0	3.9	16	43	30	16	46	7	4
59	57	+	+	706	6.9	2.8	13	50	26	14	40	6	4
60	58	-	-	NT	7.6	3.4	14	50	26	14	40	6	4
61	54	-	+	NT	8.8	3.3	15	47	28	16	44	6	3
62	55	+	+	70,098	15.2	9.4	33	29	19	16	35	11	25
63	88	+	+	17,667	11.6	6.2	12	48	27	14	41	7	4
64	36	-	-	NT	10.1	4.3	16	47	26	15	41	6	6
65	55	+	+	2,016	9.4	4.5	19	41	24	15	39	8	12
66	27	-	-	NT	6.1	2.8	16	49	29	15	44	5	2
67	28	-	-	NT	10.1	5.5	19	49	27	15	42	6	3
68	69	+	+	124,524	22.9	16.5	16	52	27	13	40	5	3
69	32	-	+	NT	13.1	7.2	20	50	26	14	40	6	4
70	31	+	+	5,425	9.6	4.2	20	47	29	15	44	6	3
71	58	+	+	12,773	8.6	4.0	16	47	27	15	42	6	5
72	30	-	-	NT	6.9	4.5	17	50	28	14	42	5	3
73	35	-	+	NT	9.2	4.4	18	49	27	14	41	6	4
74	64	+	-	ND	5.3	2.4	19	54	24	13	37	6	3
75	31	+	+	13,147	15.8	9.3	17	46	28	15	43	7	4
76	42	-	-	NT	9.9	4.6	14	45	28	16	44	7	4
77	31	+	-	23	13.4	5.4	20	50	26	15	41	6	3
78	60	+	+	45,365	13.9	8.8	14	46	29	16	45	6	3

BLV = bovine leukemia virus, LDH = lactate dehydrogenase, NT = not tested, PVL = proviral load, + = positive, - = negative.

* Using anti-BLV antibody ELISA kit (JNC).

† Using primers for the envelope or pX region of BLV.

‡ Using a CoCoMo-BLV Primer/Probe (A803, Riken Genesis).

§ Using an auto analyzer JCS-BM6050 (JEOL) and an enzymatic method (L-Type Wako LD IF or L-Type Wako J, Fujifilm Wako Pure Chemical).

¦ Using a Hydrasys 2 Scan (Sebia) and Hydragel 7 ISO-LDH (Sebia).

Supplemental Table 5. Primers and conditions used in PCR for 16S rRNA gene and multiplex PCR for origin identification.

Target	Primers (species)	Sequence (5'-3')	PCR conditions	Cycles	References
16S rRNA	Forward	TACGACCTCGATGTTGGATCA	95ºC, 5 s 60ºC, 20 s	40	17
	Reverse	AGATAGAAACCGACCTGGATT			
Multiplex PCR	CYTB_UniF	GACCAATGATATGAAAAATCATCGTTGT	94ºC, 30 s 58ºC, 30 s 72ºC, 30 s	27	17
	CYTB_CattleR	GGCTGGAAGGTCGATGAATGTA			
	CYTB_RabbitR	GTGAAAATTTGAATTATAAGGCACAG			
	CYTB_HumanR	ATAGTCCTGTGGTGATTTGGAGGATC			
	CYTB_SheepR	TGCTAGGAATAGGTCTGTTGGAATC			
	CYTB_PigR	GTCTGATGTGTAATGTATTGCTAAGAAC			
	CYTB_HorseR	ACGGATGAGAAGGCAGTTGTC			
	CYTB_GoatR	CGACAAATGTGAGTTACAGAGGGA			
	CYTB_CatR	TGATTCAGCCATAATTAACGTCG			
	CYTB_CamelR	GTAGGAGCCGTAGTAAAGCCCA			
	CYTB_SikaR	GCTGTGGCTATAACTGTAAATAGGACA			
	DL_UniF	CACCATCAGCACCCAAAGCT			
	DL_UniR	ATGGGCCCGGAGCGAGAAGAG			
	DL_Bird_UniF	TCGTGCATACATTTATATTCCACATA			
	DL_Bird_UniR	GTGTACGATTAATAAATCCATCTGGTAC			
	DL_Bird_UniR2	GTGGACGATCAATAAATCCATCTGATAC			

Supplemental Table 6. Vertebrate mtDNA concentration and the origin identification in stable fly blood meals.

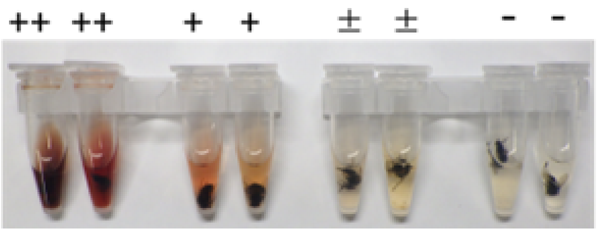
Farm	Sample number	No. of stable flies in a pool	DNA, ng/μL	A260/280	Vertebrate mtDNA, copies/μL	Origin
A	1	10	438	2.15	1,670,800	Cattle
	2	10	326	2.10	907,500	Cattle
	3	10	255	2.12	914,300	Cattle
	4	10	406	2.13	1,465,100	Cattle
	5	10	357	2.14	1,409,900	Cattle
B	6	12	365	2.15	152,300	Cattle
	7	10	514	2.13	396,100	Cattle
	8	10	288	2.13	68,000	Cattle
	9	10	161	2.10	275,500	Cattle
	10	10	266	2.11	115,300	Cattle
C	11	10	213	2.12	130,400	Cattle
	12	8	168	2.11	30,060	Cattle
	13	6	166	2.18	26,870	Cattle
	14	4	23	2.09	5,532	Cattle
	15	10	239	2.13	145,300	Cattle

Supplemental Table 7. Detection of bovine BoLA-DRA gene in stable fly blood meals.

Sample number	No.of stable flies in a pool	Blood color intensity*	qPCR†	
			BoLA-DRA	
			Ct	Copies/100 ng DNA
1	1	++	26.0	4,250
2	1	++	29.1	562
3	1	++	25.5	6,040
4	1	+	30.9	178
5	1	+	30.0	329
6	1	±	33.3	37
7	1	±	ND	ND
8	1	±	39.2	1
9	1	-	ND	ND
10	1	-	ND	ND
11	1	-	ND	ND
12	1	-	ND	ND
13	1	-	ND	ND
14	5	-, -, -, -, -	ND	ND
15	5	-, -, -, -, -	ND	ND
16	5	+, +, +, +, +	27.7	1,470
17	5	+, +, +, +, +	28.4	932
18	10	+, +, +, +, +, -, -, -, -, -	30.4	247
19	10	+, +, +, +, +, -, -, -, -, -	30.9	179

ND = not detected.

* Blood color intensity. Individuals before pooling in sample nos. 14-19.



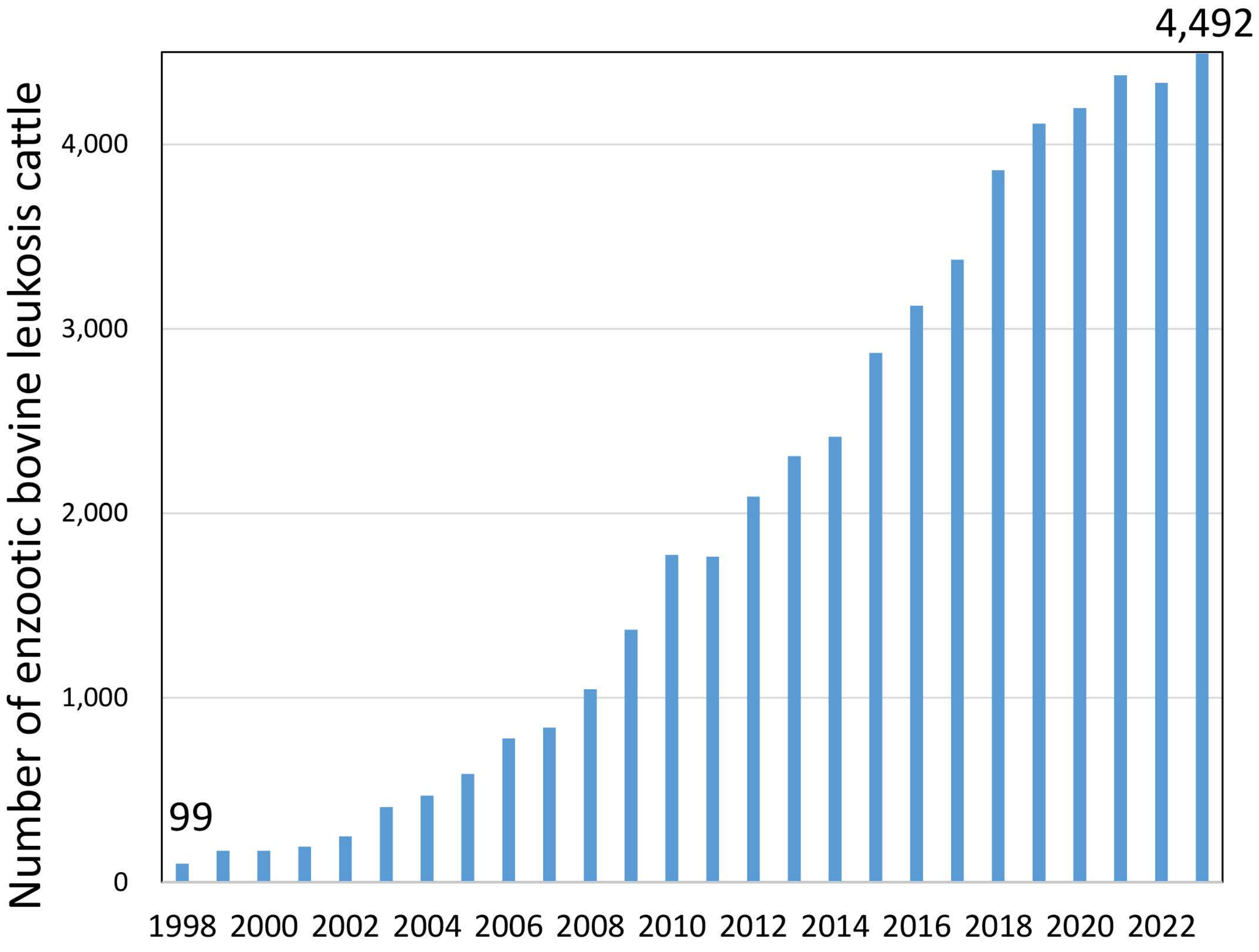
† Using a CoCoMo-BLV Primer/Probe (A803, Riken Genesis).

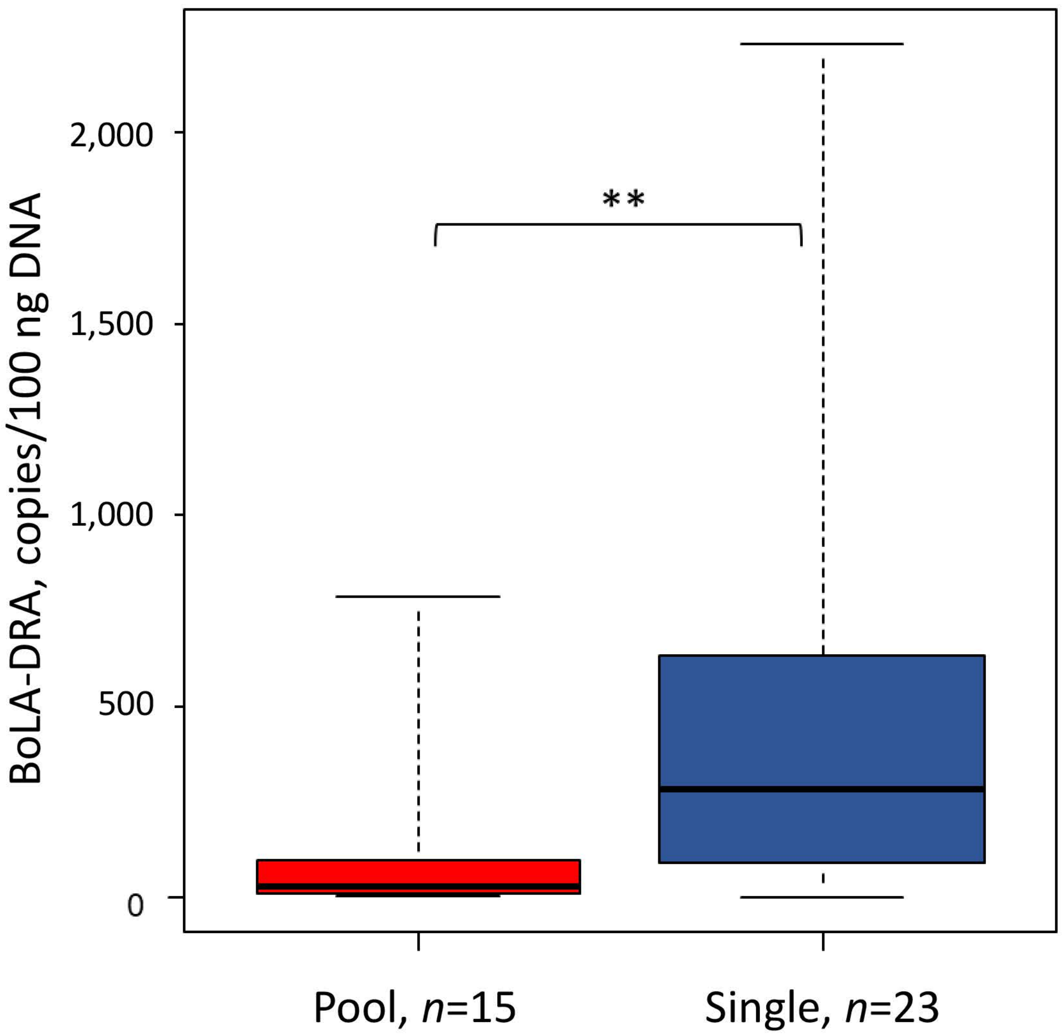
Supplemental Figure 1. Number of enzootic bovine leukosis cattle in Japan. Data from the Surveillance of Infectious Diseases, the Ministry of Agriculture, Forestry and Fisheries, Japan. (in Japanese) Accessed on April 18, 2024.

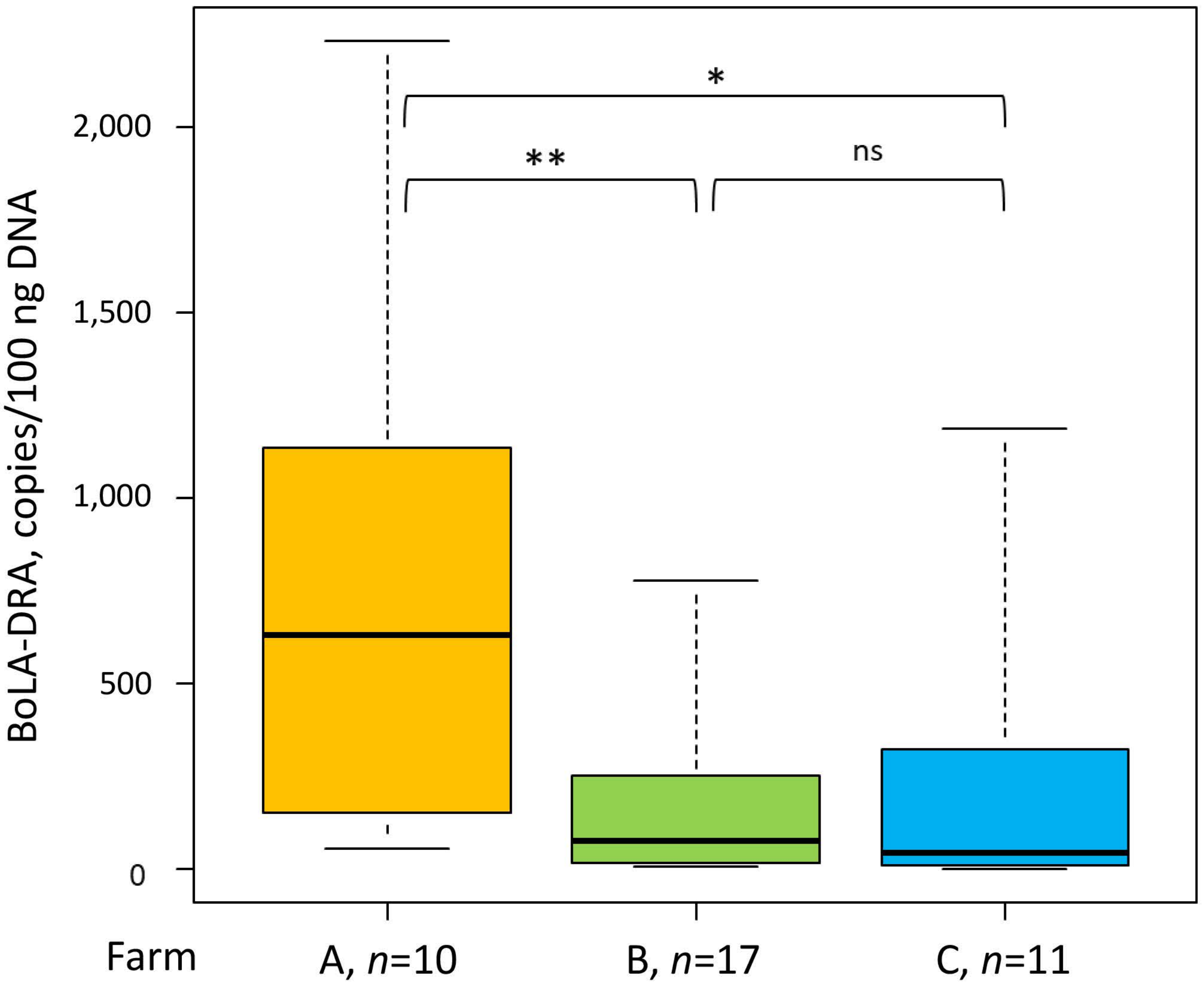
https://www.maff.go.jp/j/syouan/douei/kansi_densen/kansi_densen.html.

Supplemental Figure 2. Comparison of BoLA-DRA detection amounts in stable fly blood meals. Whiskers show minimum and maximum values, boxes represent 25%–75% data ranges, and horizontal lines within boxes are medians. The statistical significance was calculated by Mann–Whitney *U* test (**$p < 0.01$).

Supplemental Figure 3. Comparison of the BoLA-DRA detection amounts in stable fly blood meals among farms. Whiskers show minimum and maximum values, boxes represent 25%–75% data ranges, and horizontal lines within boxes are medians. The statistical significance was calculated by the Kruskal–Wallis *H*-test (* = p <0.05; ** = p <0.01). ns = not significant.


4,492
99
Number of enzootic bovine leukosis cattle
0
1,000
2,000
3,000
4,000
1998
2000
2002
2004
2006
2008
2010
2012
2014
2016
2018
2020
2022


BoLA-DRA, copies/100 ng DNA
2,000
1,500
1,000
500
0
**
Pool, n=15
Single, n=23



BoLA-DRA, copies/100 ng DNA
2,000
1,500
1,000
500
0
*
**
ns
Farm
A, n=10
B, n=17
C, n=11