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New hematological key for bovine leukemia virus-infected Japanese Black cattle

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ABSTRACT. The European Community's (EC) Key, which is also called Bendixen's Key, is a well-established bovine leukemia virus (BLV) diagnostic method that classifies cattle according to the absolute lymphocyte count and age. The EC Key was originally designed for dairy cattle and is not necessarily suitable for Japanese Black (JB) beef cattle. This study revealed the lymphocyte counts in the BLV-free and -infected JB cattle were significantly lower than those in the Holstein cattle. Therefore, applying the EC Key to JB cattle could result in a large number of undetected BLV-infected cattle. Our proposed hematological key, which was designed for JB cattle, improves the detection of BLV-infected cattle by approximately 20%. We believe that this study could help promote BLV control.

KEY WORDS: BLV, EC Key, Japanese Black cattle, lymphocyte counts, proviral load

Bovine leukemia virus (BLV), a member of the *Retroviridae* family and *Deltaretrovirus* genus, is an etiological agent of fatal B-cell leukemia and malignant lymphoma in cattle. These diseases are collectively called enzootic bovine leukosis (EBL). A high BLV seroprevalence and an increase in the incidence of EBL have been problems in Japan [14, 15]. More than nine-tenths of BLV-infected cattle remain EBL free for life [4, 21]. However, cattle with EBL invariably die within months and are not approved for human consumption in Japan. BLV can cause lifelong infection, and no vaccines or therapeutic procedures are currently available for preventing BLV infection and EBL development. Therefore, preventing cattle from becoming infected is the only feasible measure to reduce the incidence of EBL. Although 70% of BLV-infected cattle remain asymptomatic carriers of the virus, approximately 30% of these cattle have continually increasing lymphocytes and develop persistent lymphocytosis (PL). Cattle with PL have a high BLV proviral load and become infectious sources for BLV-free cattle on farms [8, 19]. Therefore, performing lymphocyte counts in BLV-seropositive cattle is one way tool to evaluate the subclinical progression and for detecting major infectious sources [10, 16]. The European Community's (EC) Key, which is also called Bendixen's Key, is a hematological BLV diagnostic standard [2]. The EC Key classifies cattle as "normal", "suspect" and "lymphocytic" according to absolute lymphocyte count and age (Table 1). The Danish government recommends using this key to detect cattle with PL, and the virus has been almost eliminated throughout the country [1, 5]. Although real-time PCR has become a common quantification method for detecting the BLV proviral load, lymphocyte count gives veterinarians a great estimation for the diagnosis because automated cell counters have become popular in veterinary clinics [7, 9].

Japanese Black (JB) cattle are raised throughout Japan and produce high-quality beef. Although there are four Japanese beef breeds called Wagyu, JB is the most popular (approximately 97%) breed of Wagyu in Japan [13]. In our previous survey, only a few JB cattle had abnormal lymphocyte counts on BLV epidemic farms based on the EC Key. However, most JB cattle with normal numbers of lymphocytes had a high BLV proviral load. Therefore, we strongly suspected that these BLV-infected JB cattle might have significantly increased number of lymphocytes but was hardly detected. Because JB and Holstein (Hol) cattle are genetically distinct species, the disease susceptibility or hematological characteristics after BLV infection might differ [17, 22]. The purpose of this study was to evaluate the expansion of lymphocytes in BLV-infected JB cattle and to modify the EC Key for JB cattle.

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Table 1. European Community Key (EC Key) and hematological key designed for Japanese Black cattle in this study (JB Key)

Age in years	EC Key [2]			JB Key					
	Lymphocyte counts (/μl)			Lymphocyte counts (/μl)			White blood cell counts (/μl)		
	Normal	Suspect	Lymphocytic	Normal	Suspect	Lymphocytic	Normal	Suspect	Lymphocytic
0–1	<10,000	10,000–12,000	>12,000	<7,000	7,000–8,000	>8,000	<12,500	12,500–13,500	>13,500
1–2	<9,000	9,000–11,000	>11,000	<5,500	5,500–6,500	>6,500	<11,000	11,000–12,000	>12,000
2–3	<7,500	7,500–9,500	>9,500	<4,500	4,500–6,000	>6,000	<10,000	10,000–11,500	>11,500
3–4	<6,500	6,500–8,500	>8,500						
>4	<5,000	5,000–7,000	>7,000	<4,000	4,000–5,500	>5,500	<9,000	9,000–10,500	>10,500

Table 2. Comparision of white blood cell and lymphocyte counts of BLV-seronegative and -seropostive Holstein and Japanease Black cattle

BLV-infection	Age in years	WBC (×10^2/μl)			Lymphocyte (×10^2/μl)		
		Holstein (*n*)	Japanese Black (*n*)	*P* value	Holstein (*n*)	Japanese Black (*n*)	*P* value
Seronegative	0–1	111.3 ± 9.06 (30)	96.55 ± 2.61 (93)	0.22	53.47 ± 3.31 (30)	47.59 ± 1.48 (93)	0.12
	1–2	99.71 ± 4.50 (28)	86.38 ± 2.01 (71)*	0.01	49.82 ± 2.59 (28)	41.45 ± 1.22 (71)*	<0.01
	2–3	96.93 ± 2.64 (59)	77.28 ± 2.94 (40)*	<0.01	46.73 ± 1.72 (59)	35.08 ± 1.30 (40)*	<0.01
	3–4	75.05 ± 3.35 (38)	71.54 ± 2.58 (35)	0.48	32.32 ± 1.36 (38)	31.80 ± 1.18 (35)	0.96
	>4	71.00 ± 2.94 (58)	63.86 ± 1.43 (125)*	0.01	32.69 ± 2.51 (58)	25.99 ± 0.70 (125)*	<0.01
	All	88.36 ± 2.12 (213)	78.89 ± 1.21 (364)*	<0.01	41.69 ± 1.19 (213)	36.14 ± 0.70 (364)*	<0.01
Seropositive	0–1	119.9 ± 10.78 (26)	93.74 ± 3.66 (42)*	0.01	61.46 ± 7.85 (26)	49.50 ± 2.77 (42)	0.26
	1–2	128.6 ± 11.2 (7)	99.79 ± 6.07 (29)*	0.02	82.86 ± 8.27 (7)	58.86 ± 4.68 (29)*	<0.01
	2–3	127.7 ± 7.44 (41)	108.5 ± 6.43 (32)*	0.03	72.54 ± 6.08 (41)	61.56 ± 5.36 (32)	0.18
	3–4	123.6 ± 8.56 (57)	88.13 ± 4.14 (47)*	<0.01	64.72 ± 7.23 (57)	46.38 ± 2.85 (47)	0.32
	>4	118.0 ± 4.90 (148)	81.16 ± 2.29 (387)*	<0.01	64.19 ± 7.23 (148)	41.43 ± 1.75 (387)*	<0.01
	All	121.0 ± 3.46 (279)	85.39 ± 1.81 (537)*	<0.01	65.74 ± 2.60 (279)	44.63 ± 1.39 (537)*	<0.01

Asterisk shows blood counts of JB were significantly lower than that of Holstein.

Blood samples were collected from 901 JB and 492 Hol cattle between April 2016 and June 2017 by local clinical veterinarians during their BLV diagnostic assessments. The numbers of white blood cells (WBC) and lymphocytes were assessed using an automated veterinary hematology analyzer (MEK-6550 Celltac α, Nihon Kohden, Tokyo, Japan). All blood samples were kept refrigerated, and the blood counts were conducted within three days of each blood sampling. After performing the blood counts, the blood samples were centrifuged (1,500 × g, 5 min, 4°C), and 500 μl of plasma were dispensed into 96 deep-well plates. The separated plasma was diagnosed using BLV gp51 antibody detection enzyme-linked immunosorbent assay (ELISA) (JNC, Tokyo, Japan) according to the manufacturer's instructions. Genomic DNA was extracted from all BLV-seropositive blood using a Wizard Genomic DNA Purification kit (Promega, Fitchburg, MA, U.S.A.) according to the manufacturer's instructions. The DNA concentration and purity were determined using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.), and the DNA was diluted to 20 *n*g of genomic DNA/μl. Proviral load quantification was performed using a LightCycler 96 System (Roche Diagnostics, Indianapolis, IN, U.S.A.). The amplification was performed in a reaction mixture containing 5 μl of 2x Cycleave PCR Reaction Mix (TaKaRa Bio, Kusatsu, Japan), 0.2 μl of Probe/Primer Mix for BLV (TaKaRa Bio), 0.6 μl of a template DNA sample and PCR-grade water to increase the volume to 10 μl. To determine the proviral loads, calibration curves were generated from the measured concentration of the dilution series of the positive control containing a portion of the BLV *tax* gene. Each amplification procedure was performed in duplicate and expressed as the number of proviral copies per 50 *n*g of genomic DNA. Comparisons of the blood cell counts and proviral loads were evaluated by a normality test (D'Agostino-Pearson omnibus test) and a Mann Whitney test using GraphPad Prism 6 (GraphPad Software, San Diego, CA, U.S.A.). $P<0.05$ was considered statistically significant in this study.

The BLV-free and -infected JB cattle (n=364 and 537) had a significantly lower number of WBC and lymphocytes than those of Hol cattle (n=213 and 279), respectively (Table 2). The blood counts were significantly different between the BLV-free Hol and JB cattle in all age groups, except for the 0- to 1- and 3- to 4-year-old groups. According to our previous study, BLV-infected pregnant cows with more than 2,000 copies/50 *n*g of proviral load transmitted the virus to their neonates at a significantly higher rate than cows with less than 2,000 copies/50 *n*g [11]. In another study, we showed that BLV-infected cattle with less than 500 copies/50 *n*g were less likely to transmit the virus horizontally to BLV-free cattle (H. Mekata, unpublished data). Therefore, we classified the BLV-infected cattle into three groups as follows: "very high copies" (more than 2,000 BLV proviral copies/50 *n*g), "high copies" (500–2,000 copies/50 *n*g) and "low copies" (less than 500 copies/50 *n*g). Then, we analyzed the correlation between the BLV proviral load and lymphocyte counts classified by the EC Key (Table 3). In the Hol cattle, 20.5 and 53.2% of the "very high copies" cattle were classified as "normal" and "lymphocytic", respectively. However, 44.0% and only 28.6% of the "very high copies" JB cattle were classified as "normal" and "lymphocytic", respectively. Applying the EC Key to the lymphocyte count

Table 3. Correlation between BLV proviral load and lymphocyte counts classified by EC Key and hematological key for Japanese Black cattle (JB Key)

Classification by BLV proviral load	Classification by EC key (% (*n*))						Classification by JB Key (% (*n*))		
	Holstein cows			Japanese Black cattle			Japanese Black cattle		
	Normal	Suspect	Lymphocytic	Normal	Suspect	Lymphocytic	Normal	Suspect	Lymphocytic
Very high copies[a)]	20.5 (22)	26.1 (28)	53.2 (57)	44.0 (66)	27.3 (41)	28.6 (43)	18.0 (27)	32.0 (48)	50.0 (75)
High copies[b)]	75.3 (49)	12.3 (8)	12.3 (8)	89.4 (110)	10.5 (13)	0 (0)	60.1 (74)	32.5 (40)	7.3 (9)
Low copies[c)]	81.3 (87)	14.9 (16)	3.7 (4)	98.4 (260)	1.1 (3)	0.3 (1)	91.6 (242)	8.0 (21)	0.3 (1)
Seronegative	95.7 (204)	3.2 (7)	0.9 (2)	100 (364)	0 (0)	0 (0)	92.5 (337)	5.5 (20)	1.9 (7)

a) Very high copies: BLV-infected cattle with proviral loads of more than 2,000 copies/50 *n*g, b) High copies: BLV-infected cattle with proviral loads of 500–2,000 copies/50 *n*g, c) Low copies: BLV-infected cattle with proviral loads of less than 500 copies/50 *n*g.

in JB cattle could miss many "very high copies" cattle and classify these cattle as "normal". Therefore, we proposed a modified hematological key optimized to blood counts in JB cattle, named JB Key, as shown in Table 1. The cutoff values of lymphocyte and WBC counts in JB Key were defined by 40–60 and 90–95% of percentile value of blood counts in BLV-infected cattle with "very high copies" and BLV-seronegative cattle, respectively. In the lymphocyte counts of the JB Key, 50.0% of the BLV-infected JB cattle with "very high copies" were classified as "lymphocytic". Regarding the sensitivity, the proportion of classified as "lymphocytic" from among "very high copies", was improved 21.4, 91.6 and 92.5% of the "low copies" and seronegative JB cattle were classified as "normal", respectively. Although using WBC counts was less accurate than using lymphocyte counts, 38.6 and 40.0% of the "very high copies" JB cattle were classified as "normal" or "lymphocytic", respectively (Supplemental table1).

The number of immune cells, particularly during childhood, is significantly different among human races [3]. Although little is known about immune cell differences among cattle species, the number of WBCs and B cells in JB cattle was reported to be lower than that in Hol cattle [6, 18]. However, the studies did not consider the status of BLV infection or proviral loads, which strongly associate with the blood counts. In this study, the numbers of lymphocytes and WBCs in BLV-seronegative JB cattle were 10–15% lower than those in Hol cattle. Moreover, the lymphocyte counts in the BLV-infected JB cattle were significantly lower than those in the Hol cattle. However, whether the lower lymphocyte counts associated with BLV infection affect the pathology of BLV infection in JB cattle remains unknown. To the best of our knowledge, no comparisons of the possibility of developing EBL in BLV-infected JB and Hol cattle have been performed. In this study, the average and median viral loads were higher in the Hol cattle (Average=1,936; Median=1,218) than in the JB cattle (Average=1,369, Median=518) (P<0.01). However, further studies are required to confirm that the lower copy number was correlated with the lower lymphocyte count in the BLV-infected JB cattle. The EC Key classifies cattle into groups in 12-month intervals because the total number of lymphocytes decreases with age [20]. Although the number of WBCs and lymphocytes in BLV-free JB cattle also decreases with age, there was no significant difference between 2- to 3- and 3- to 4-year-old cattle (P=0.20 and 0.12). Therefore, we classified the age group in the JB Key as 2- to 4-year-old cattle. The number of lymphocytes in newborn calves has been shown to fluctuate during the first 3 months of life [12]. Therefore, further classification in 12-month-old cattle and avoiding the use of the hematological key until 3 months of age might lead to further improvements. The older JB cattle were classified as the same age group in JB Key, as like in the EC key. In our data, more than 50% (18/34) of cattle with "very high copies" over 8 years of age were classified in "lymphocytic" in JB Key. Therefore, the older JB cattle could be correctly classified based on this key.

This study reveals that applying the EC Key to BLV-infected JB cattle could result in many BLV-infected cattle with very high proviral loads being undetected. Therefore, we propose a new hematological key for JB cattle, named JB Key. The JB Key improves the sensitivity by 21.4% and maintains the specificity, the proportion of classified as "normal" from among BLV-seronegtive, of the diagnosis at more than 90%. In veterinary clinics, a combination of BLV antibody testing and measurements of blood counts remain simple, useful tools for BLV diagnosis. We believe that this study could help to reduce the oversight of a high BLV proviral load in JB cattle and could improve BLV control in endemic areas.

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