



Development of a new recombinant p24 ELISA system for diagnosis of bovine leukemia virus in serum and milk

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Abstract

Bovine leukemia virus (BLV) is a retrovirus that causes enzootic bovine leucosis. Here, we designed a p24 enzyme-linked immunosorbent assay (ELISA) to detect antibodies specific for BLV capsid protein p24 (encoded by the *gag* gene) in bovine serum samples. The *p24* gene was inserted into an *Escherichia coli* expression system, and recombinant proteins (GST-p24, p24, and His-p24) were purified. His-p24 was the most suitable antigen for using in the ELISA. The cut-off point was calculated from a receiver operating characteristic curve derived from a set of 582 field samples that previously tested positive or negative by BLV-CoCoMo-qPCR-2, which detects BLV provirus. The new p24 ELISA showed almost the same specificity and sensitivity as a commercial gp51 ELISA kit when used to test field serum samples, and allowed monitoring of p24 antibodies in raw milk and whey. Comparing the results for the p24 ELISA and gp51 ELISA revealed that p24 antibodies were detected earlier than gp51 antibodies in three out of eight calves experimentally infected with BLV, indicating improved detection without diminishing BLV serodiagnosis. Thus, the p24 ELISA is a robust and reliable assay for detecting BLV antibodies in serum or milk, making it a useful tool for large-scale BLV screening.

Introduction

Bovine leukemia virus (BLV) is the causative agent of enzootic bovine leucosis (EBL), a chronic lymphoproliferative neoplastic disease of cattle [1–3, 54]. The virus is closely

related to human T-cell leukemia viruses type 1 and 2 [3, 14]. BLV has spread worldwide [40–43, 45, 50]; indeed, BLV seropositivity is detected in 40.5% of dairy herds in Japan [33], 78% of dairy herds in Canada [34], and 79.5% of dairy herds in the USA [4].

BLV is a major animal health problem causing digestive disturbances, loss of appetite, weight loss, weakness or general debility and sometimes neurological manifestations [37], leading to huge economic losses worldwide [36, 38]. Previously, the economic losses were estimated only in terms of animal health, semen intended for export [28], and a low percentage of BLV-infected cattle that develop B-cell lymphoma [3]. However, a recent study reported that BLV-positive cows produce 218 kg less milk than BLV-negative healthy cows [38], indicating that even subclinical forms of the disease can cause economic losses by reducing yields [45, 55] and longevity [5, 11], and increasing morbidity due to weakened immune responses [14, 39]. Rhodes et al. reported estimated economic losses of \$412 per case of lymphosarcoma [45].

BLV comprises structural proteins encoded by the *gag* gene matrix protein p15, capsid protein p24 (the most abundant capsid protein), and nucleocapsid protein p12 [26, 52]. The envelope proteins (surface glycoprotein [gp51] and transmembrane

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protein [gp30]) are encoded by the *env* gene. Upon infection by BLV, cattle generate antibodies directed primarily against viral proteins p24 and gp51 [6].

BLV is diagnosed by the detection of viral antibodies and viral antigens [43]. During natural or iatrogenic transmission of BLV, one of the earliest indications of infection is onset of a humoral anti-viral response (at about 1–8 weeks post-inoculation) [25, 44]. The most commonly used serological tests are radioimmunoassay, the agar gel precipitation test (AGID), phytohemagglutinin (PHA) tests, and enzyme-linked immunosorbent assays (ELISAs) [18, 43]. The AGID and PHA methods can yield variable results due to different sensitivities and specificities. In addition, AGID is less sensitive and can miss infected cattle harboring a low antibody titer [9, 31] whereas ELISA is more sensitive and specific [47]. Most commercially available ELISAs are designed to detect antibodies specific for gp51 [18, 23]. For example, the gp51 antigen for the commercial ELISA kit used in Japan is purified from inactivated and solubilized virus obtained from persistent BLV-infected sheep fetal kidney cells using monoclonal antibody which makes gp51 production a rather difficult, expensive, and time-consuming process. A previous study indicated that both the immunogenicity and concentration of retroviral capsid proteins in virions and infected cells are higher than those of gp51 [44]. Furthermore, p24 antibodies present in most animals during the early period post-BLV infection [41]. A certain number of field infected cattle have p24 antibodies at high levels [22, 30], and tests targeting BLV p24 antigen test as well as gp51 antigen are approved by the World Organization for Animal Health (OIE) [37]. In addition, p24 antibody is an important marker of response for the virus-like particle (VLP) vaccine (VLP: assembled by BLV *gag* gene [19, 20]) in immunized animals that are negative using gp51 ELISA. The Gag p24 protein is also widely used as a surrogate marker for diagnosis of HIV-1, HTLV-1, HTLV-II, and STLV-I infection [12, 46, 48, 51].

Recently, recombinant proteins have become widely used in immunoassays for detecting antibodies [13, 32]. Therefore, we expressed the BLV p24 protein in *Escherichia coli* (*E. coli*) and used the purified product as an antigen to develop a p24 ELISA to detect anti-BLV antibodies. This fast, simple, specific, and low cost p24 ELISA assay is a robust alternative to the currently available commercial kit used to screen for BLV diagnosis, and will be useful for future vaccine development such as VLP or a subunit p24 immunogen.

Materials and methods

Animals, serum, genomic DNA, milk, and whey

BLV-negative three Holstein calves, two Jersey calves, and three Japanese Black calves were inoculated with white

blood cells containing more than 10^6 proviral copies of BLV (isolated from naturally infected cattle). Serum samples were obtained at weekly intervals over a period of 12 weeks post-inoculation.

Blood samples (in EDTA) were also collected from 582 cattle from seven farms in four provinces in Japan. Serum was obtained (tube containing clot activator and serum separator gel), and genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega Corporation, Tokyo, Japan). BLV-positive serum which was designated as EBL serum was isolated from cattle that develop B cell lymphoma and used as a positive control for all tests. Raw milk was obtained from 194 cows that were diagnosed for BLV infection by ELISA using gp51 antibodies and p24 antibodies for testing serum samples: 63 were BLV-negative and 131 were BLV-positive. Milk was centrifuged at 20400 \times g for 30 min to obtain whey. All samples were stocked at -20°C until use.

Construction of the p24 plasmid

The *p24* gene was amplified from an infectious molecular clone, pBLV-IF [16], using primer set p24-EcoRI-F, 5'-AAGAATTCCTTGCCAATCATATCTGAAGGG-3' and p24-XhoI-R, 5'-TTCTCGAGTCAGAGAACTGCAGGCTGTTTACC-3'. The *EcoRI* and *XhoI* sites are underlined. The 648 bp PCR product was subcloned into the *EcoRI* and *XhoI* sites of the entry vector pGEX-6p-3 (GE Healthcare, Uppsala, Sweden) and designated glutathione S-transferase (GST)-p24/pGEX-6p-3. To yield His-p24/pGEX-6p-3, the GST tag of GST-p24/pGEX-6p-3 was replaced with a 6 \times histidine (6-His) tag using primer set His-p24-F, 5'-ATGCATCATCACCATCACCCTCGGATCTGGAAGTTCTG-3' and His-p24-R, 5'-GTGGTGATGGTGATGATGCATGAA TACTGTTTCCTGTGT-3 (the 6-His tag is indicated in double underlined). All of the plasmids were confirmed by sequencing.

Expression and purification of recombinant proteins

GST-p24/pGEX-6p-3 and His-p24/pGEX-6p-3 were transformed into *E. coli* BL21-CodonPlus[®] (DE3)-RIPL competent cells (Agilent Technologies, Tokyo, Japan), and expression of recombinant proteins were induced by addition of 1mM isopropyl- β -d-thiogalactoside at 25°C with agitation. The expressed GST-p24 protein was harvested using Glutathione Sepharose[™] 4 Fast Flow (GE Healthcare Bio-Sciences, Uppsala, Sweden), according to the manufacturer's instructions, and concentrated using a centrifugal filter column (Amicon[®] Ultra-15; Merck Millipore Ltd., Darmstadt, Germany). The GST-p24 was digested with a precision protease to release the GST tag. The His-p24 protein was purified using a His GraviTrap column (GE Healthcare Life Sciences), according

to the manufacturer's instructions. The concentration of each recombinant protein was measured using a bicinchoninic acid protein kit (ThermoFisher Science, Waltham, MA).

Detection of recombinant proteins

Purified recombinant GST-p24, p24, His-p24, and GST proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels, which were then stained with Coomassie brilliant blue (CBB). The proteins were transferred to a membrane and blocked with 5% skim milk in phosphate-buffered saline (PBS)-T buffer at room temperature. After washing three times, the membrane was incubated overnight at 4°C with an anti-BLV p24 monoclonal antibody (Mab) (BLV-3; VMRD, Pullman, WA), an anti-GST polyclonal antibody (Pab) (GE Healthcare Life Sciences), or an anti-His tag Mab [HRP] (GenScript, Piscataway Township, NJ), followed by either peroxidase-conjugated affinity-pure goat anti-mouse IgG (Jackson ImmunoResearch Inc., West Grove, PA) or horseradish peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology, Texas, TX).

Development of a new recombinant p24 protein ELISA system

To determine the optimal concentrations of GST-p24, p24, and His-p24 required for immobilization, recombinant proteins were serially diluted 2-fold from 40 to 0.156 µg/ml in 0.1 M NaHCO₃ buffer (pH 9.0) and coated onto multiwell plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) overnight at 4°C. The coated plates were washed with PBS-T buffer and applied with the anti-BLV p24 Mab (BLV-3) or with serum from BLV-free cattle or cattle experimentally infected with BLV (samples were diluted in sample dilution buffer [7.20 mM Na₂HPO₄ 12H₂O, 2.82 mM NaH₂PO₄ 2H₂O, 150.24 mM NaCl, 1% tween 20, and 5% skim milk]) and incubated with antigens for 1 h at 37°C. The plates were then washed and incubated at 37°C for 30 min with HRP-conjugated goat anti-mouse IgG (Amersham Bioscience, Piscataway, NJ) or peroxidase-conjugated goat anti-bovine IgG (Jackson ImmunoResearch Inc.). Finally, 3,3',5,5'-tetramethylbenzidine (TMB) substrate was applied to the plates and the reaction was stopped by addition of 1 N H₂SO₄. Optical density (OD) values were read, and the status of a sample that are evaluated by the sample to positive ratio (S/P ratio) was calculated as follows:

$$\frac{S}{P} = \frac{\text{Sample(antigen - no antigen) - Blank}}{\text{Positive control(antigen - no antigen) - Blank}}$$

The cut-off point for the S/P value was calculated from receiver operating characteristics (ROC) curves.

To analyze the sensitivity and specificity of the assay, a series of dilutions of serum E4 (the European Community's

reference serum), EBL serum (isolation from BLV-infected cattle with B cell lymphoma) and BLV-negative serum were evaluated by p24 ELISA.

In addition, the collected 582 serum samples, serum from eight experimentally infected calves and 194 milk/whey samples were analyzed.

Measurement of BLV proviral load

BLV proviral load (PVL) in all the DNA samples (the collected 582 samples and 8 experimentally infected calves) was measured using the BLV-CoCoMo-qPCR-2 method as described previously [17, 29, 35, 39, 40, 49, 56].

Measurement of gp51 antibodies

Anti-BLV gp51 antibodies were measured using an anti-BLV antibody ELISA kit (JNC, Tokyo, Japan), according to the manufacturer's instructions.

Results

Preparation of recombinant proteins

As shown in Fig. 1, the 648 bp fragment of *p24* gene encoding the BLV Gag capsid protein p24 was inserted into pGEX-6p-3 with an N-terminal GST or His tag. The calculated (using Compute pI/Mw tool) molecular weights of GST-p24, p24, GST, and His-p24 were 50, 24, 26, and 25 kDa, respectively; a single band with the correct size for each recombinant protein was detected by CBB staining (Fig. 1A, bottom). Western blotting with an anti-p24 Mab, an anti-GST Pab, and an anti-His Mab detected p24 (GST-p24, p24, and His-p24), GST-p24 and GST, and His-p24, respectively (Fig. 1A, bottom). This indicated that the target proteins were expressed and produced correctly.

Development of a new p24 ELISA assay

As shown in Fig. 1B, p24, GST-p24, and His-p24 reacted specifically with the anti-BLV p24 Mab and with BLV-positive serum. An anti-BLV p24 Mab reacted strongly with antigens, while the optimal concentrations of p24, GST-p24, and His-p24 for immobilization on the ELISA plate were 5, 2.5, and 2.5 µg/ml, respectively. Likewise, each peak reaction value was obtained for BLV-positive bovine serum when the concentrations of p24, GST-p24, and His-p24 were 10, 5, and 5 µg/ml, respectively. However, GST-p24 reacted non-specifically with BLV-negative serum when used at a concentration > 10 µg/ml. In contrast, p24 and His-p24 showed specific reactions at high concentrations. Furthermore, when

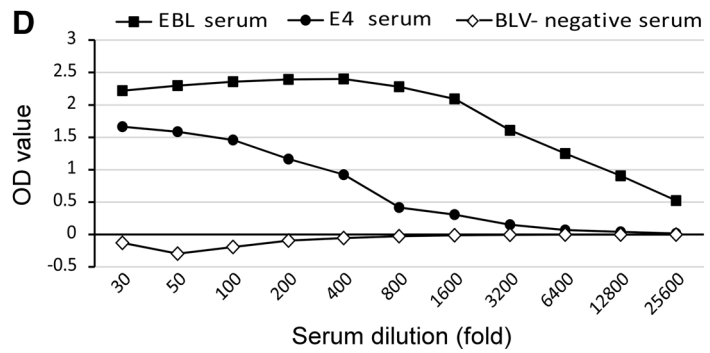
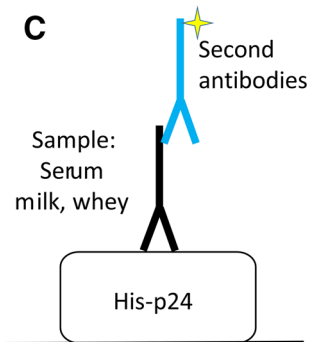
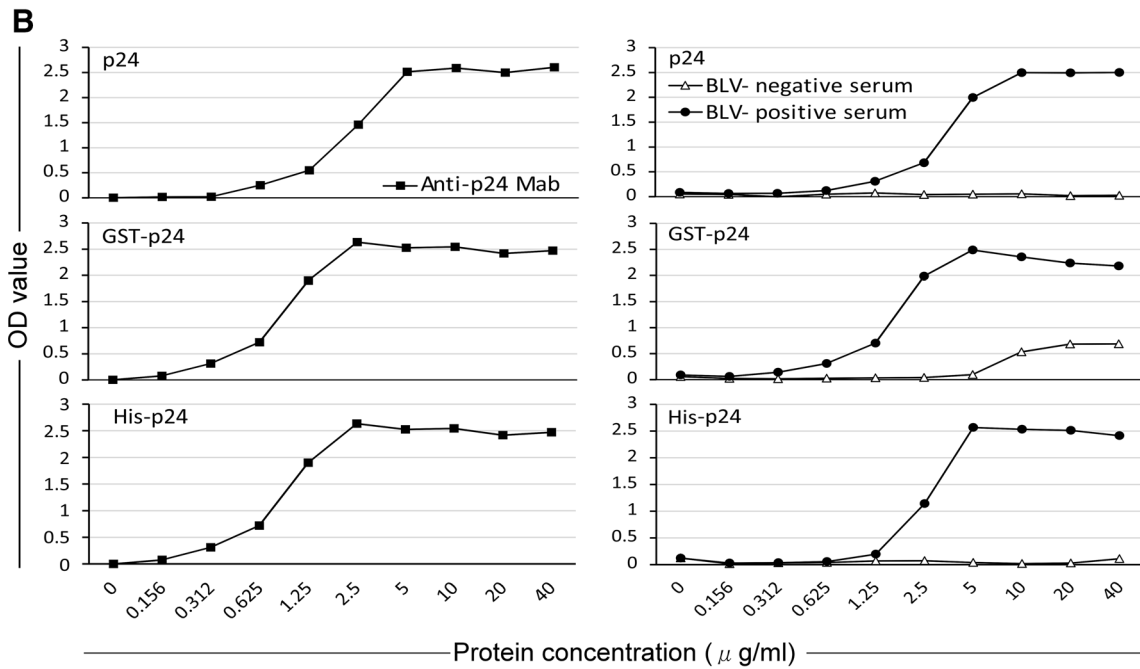
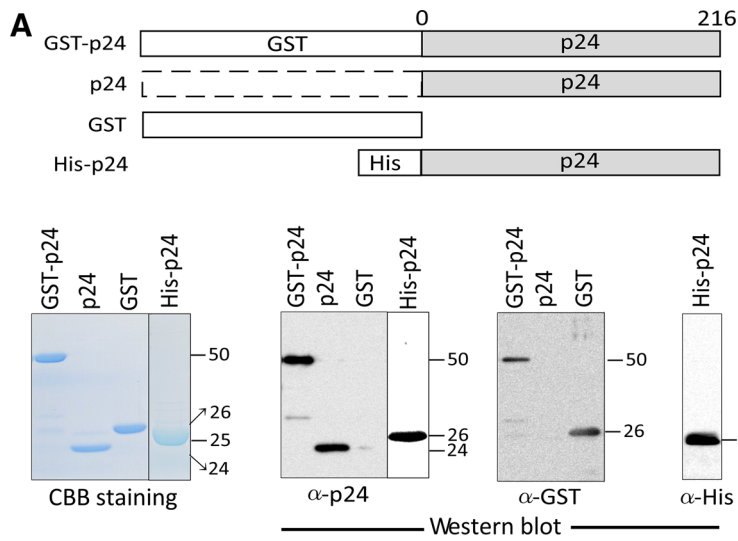


Fig. 1 Development of the new p24 ELISA system. (A) Schematic showing the BLV recombinant fusion proteins: Glutathione S-Transferase (GST)-p24, p24, GST (left upper), and His-p24 (right upper). The p24 protein is shown in gray and comprises amino acids 1–216. Proteins of the correct size are visible on coomassie brilliant blue (CBB) stained gels and on western blots detected using antibodies specific for p24, GST, and His (bottom). (B) Optimization of p24, GST-p24, and His-p24 concentrations used to coat the plate wells. Recombinant proteins were serially diluted 2-fold and coated. Plates were incubated with either an anti-p24 Mab (left) or BLV-negative/positive cattle serum (right) prior to measurement of OD values. (C) Schematic representation of the new recombinant p24 ELISA system. Purified His-p24 protein was immobilized on plate. Serum samples were added to response and the antigen-antibody complex was detected using horseradish peroxidase (HRP)-conjugated anti-bovine IgG. After washing, the wells were incubated with substrate solution and the reaction was stopped with H₂SO₄ prior to reading of optical density. (D) Analysis of sensitivity and specificity of p24 ELISA. The European Community's reference serum E4, EBL serum (isolation from BLV-infected cattle with B cell lymphoma) and BLV negative serum were diluted into a series to detect p24 antibodies by p24 ELISA

used at low concentrations (2.5–5 µg/ml), His-p24 reacted more strongly with BLV-positive bovine serum than p24.

His-p24 was therefore chosen as the diagnostic antigen for the p24 ELISA. The optimal concentration for immobilization was 5 µg/ml. The recombinant p24 ELISA assay is illustrated in Fig. 1C. Furthermore, the data obtained with a series of dilutions of E4 and EBL serum showed that the this p24 ELISA has high sensitivity and specificity (Fig. 1D): E4 was reacted at 1:12800 and EBL serum showed antibody activity of >1:26000. By contrast, control negative serum showed no reaction with His-p24.

Determining the cut-off point for the p24 ELISA system

The PVL in 582 cattle was measured using BLV-CoCoMo-qPCR-2. The results showed that 295 samples were positive and the remaining 287 samples were negative. To determine the cut-off point for the S/P value, ROC curves were constructed to evaluate the potential of antibodies to discriminate between animals with a detectable or undetectable PVL. ROC curve analysis yielded an area under the curve (AUC) of 0.905, which enables meaningful interpretation of p24 antibody levels in BLV-positive and -negative cattle. From this, the cut-off for the S/P value of the new p24 ELISA was calculated as 0.40 (Fig. 2).

Comparison of diagnostic results obtained by the p24 ELISA and other detection methods

The new p24 ELISA was used to measure anti-BLV antibody levels in 582 samples collected from cattle in the field. First, we compared the specificity and sensitivity of the new p24 ELISA with that of BLV-CoCoMo-qPCR-2 for the diagnosis

of BLV infection (Table 1). The results showed that 249/582 cattle (42.8%) were positive for both PVL and anti-p24 antibodies and that 255/582 (43.8%) cattle were negative with both PVL and anti-p24 antibodies. Overall, 295 cattle were positive for PVL, but 46 of them were negative with anti-p24 antibodies (sensitivity, 84.4%; 95% CI, 79.8–88.4%). Similarly, 287 cattle were negative for PVL, but 32 of them were positive with anti-p24 antibodies (specificity, 88.9%; 95% CI, 84.6–92.2%).

Next, we compared the specificity and sensitivity of the new p24 ELISA with those of a commercial gp51 ELISA kit. As shown in Table 2, 266/582 (45.7%) cattle were positive for both anti-p24 and anti-gp51 antibodies, and 260/582 (44.7%) were negative for both anti-p24 and anti-gp51 antibodies. Overall, 307 cattle were positive with anti-gp51 antibodies, but 41 of these were negative with anti-p24 antibodies (sensitivity of 86.6%; 95% CI, 86.6–90.2%). Similarly, 275 cattle were negative for with anti-gp51 antibodies, but 15 of these were positive with anti-p24 antibodies (specificity, 94.5%; 95% CI, 91.2–96.9%). Taken together, the data suggest that the new p24 ELISA has high sensitivity and high specificity when compared with BLV-CoCoMo-qPCR-2 and the gp51 ELISA kit.

Table 3 summarizes the results obtained using the three different methods. Overall, 249 (42.8%) cattle were positive for provirus, gp51 antibodies, and p24 antibodies, and 242 (41.6%) cattle were negative for provirus, gp51 antibodies, and p24 antibodies. By contrast, 18 cattle were PVL-positive only, 15 were p24 antibody-positive only, and 13 were gp51 antibody-positive only. Of 17 cattle that were negative for PVL, all had detectable p24 and gp51 antibodies. In addition, 56 samples gave different results in the p24 and gp51 ELISAs, including 28 cattle that were PVL- and gp51-positive, 13 samples that were gp51-positive and PVL-negative, and 15 that were p24-positive and PVL-negative. This suggests that production of anti-BLV antibodies may vary from cow to cow. According to our data, 277 (93.9%) of 295 cattle which tested positive for BLV provirus, as determined by BLV CoCoMo-qPCR-2, were found with at least one type of antibodies, and 277 (85.5%) of 324 cattle which tested positive for antibody had provirus. Combined together, the data suggests that naturally infected cattle produce anti-p24 and anti-gp51 antibodies, but it is unclear which is generated first.

Determination of the period post-infection during which p24 and gp51 antibodies are induced

To determine the time course of anti-p24 and anti-gp51 antibody production, we measured their concentrations in eight calves experimentally infected with BLV. Serum samples were diluted 50-fold and tested using the p24 ELISA and the gp51 ELISA kit. As shown in Table 4, three Holstein, two Jersey,

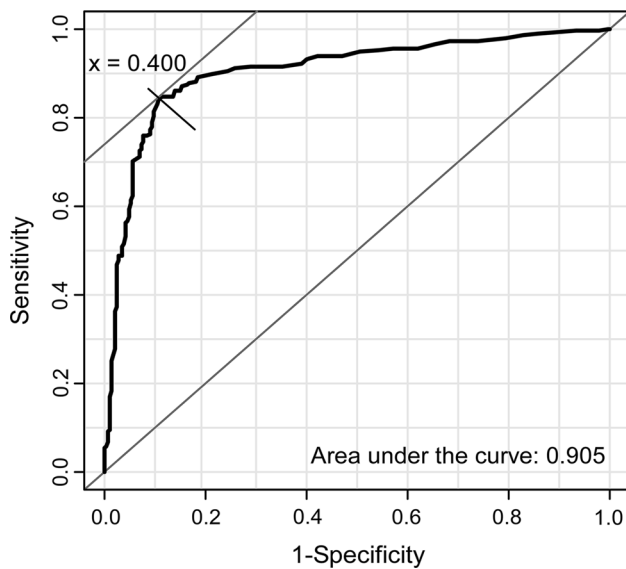


Fig. 2 A receiver operating characteristics (ROC) curve was used to calculate the cut-off point for the S/P value of the new p24 ELISA using serum from 582 cattle that were either positive or negative for BLV (as determined by BLV-CoCoMo-qPCR-2). The cut-off point for the S/P value was 0.400, while the area under the curve was 0.905

Table 1 Testing of 582 samples using the new p24 ELISA and BLV-CoCoMo-qPCR-2

		BLV-CoCoMo-qPCR-2		Total
		Positive	Negative	
p24 ELISA	Positive	249	32	281
	Negative	46	255	301
Total		295	287	582
		Sensitivity (%)	Specificity (%)	
		(95% CI)	(95% CI)	
		84.4	88.9	
		(79.8–88.4)	(84.6–92.2)	

CI, confidence interval

Table 2 Testing of 582 samples using the new p24 ELISA and the gp51 ELISA kit

		gp51 ELISA		Total
		Positive	Negative	
p24 ELISA	Positive	266	15	281
	Negative	41	260	301
Total		307	275	582
		Sensitivity (%)	Specificity (%)	
		(95% CI)	(95% CI)	
		86.6	94.5	
		(82.3–90.2)	(91.2–96.9)	

CI, confidence interval

Table 3 Detection of BLV in 582 samples using three different methods

Provirus ^a	gp51 Abs ^b	p24 Abs ^c	Head
+ ^d	+	+	249
+	+	-	28
+	-	+	0
+	-	-	18
-	+	+	17
-	+	-	13
-	-	+	15
-	-	-	242

^aThe proviral load (PVL) was measured by BLV-CoCoMo-qPCR-2

^bAnti-gp51 antibodies were measured using a gp51 ELISA kit

^cAnti-p24 antibodies were measured using the new p24 ELISA

^d+: BLV-positive; -: BLV-negative

and three Japanese Black cattle (all BLV-negative and aged 5–12 months) were inoculated with 10^6 – 10^7 proviral copies (sufficient to induce infection and antibody production). The time of BLV inoculation was defined as week 0. Serum samples were collected weekly up until week 12. In three calves (N2, KB57, and 587), antibodies specific for p24 were detected earlier than those specific for gp51. In three calves (KB64, KB19, and KB55), anti-p24 and anti-gp51 antibodies were detected simultaneously. Finally, anti-gp51 antibodies were detected earlier than p24 antibodies in two calves (N1 and KB17). Taken together, these results suggest that the BLV p24 protein is a useful marker for diagnosis of BLV infection, since it seems that in many cases p24 antibodies are detectable earlier than gp51 antibodies.

Detection of p24 antibodies in milk and whey

Finally, we asked whether the new p24 ELISA could detect antibodies in raw milk and whey (Table 5). The results showed that, of 131 cows with a positive BLV result in blood, 106 showed a positive result in raw milk and whey samples (specificity, 80.9% [95% CI, 73.1–87.3%]). From 63 cattle with BLV-negative blood samples, 58 raw milk samples were negative (sensitivity, 92.1% [95% CI, 82.4–97.4%]). From 43 cattle with BLV-negative blood samples, 37 whey samples were negative (sensitivity, 86.0% [95% CI, 72.1–94.7%]). The gp24 ELISA thus allows a rapid and specific detection of anti-BLV antibodies in milk and whey samples that are easier to obtain in the field than blood samples.

Table 4 Detection of anti-p24 and -gp51 antibodies (Abs) in eight cattle experimentally infected with BLV using new p24 ELISA and gp51 ELISA

Cattle no.	KB55		KB57		KB64		KB19		KB17		N1		N2		587	
Breed	Holstein		Holstein		Holstein		Jersey		Jersey		Japanese Black		Japanese Black		Japanese Black	
Sex	♂		♂		♂		♂		♂		♀		♀		♂	
Age (months)	5		5		9		9		9		5		5		12	
Number of proviral copies inoculated	1.0×10 ⁷		1.0×10 ⁷		0.5×10 ⁷		1.2×10 ⁷		1.2×10 ⁷		4.0×10 ⁷		4.0×10 ⁷		0.5×10 ⁷	
Abs against BLV	p24	gp51	gp51	p24	gp51	p24	gp51	p24	gp51	p24	gp51	p24	gp51	p24	gp51	p24
Time after BLV inoculation (weeks)	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	2	+	+	—	+	—	—	—	—	—	—	—	—	—	—	—
	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+: Positive for anti-BLV Abs

-: Negative for anti-BLV Abs

NT: Not tested

Table 5 Sensitivity and specificity of the new p24 ELISA for detecting BLV in raw milk (194) and whey (174) samples

		Blood		Total
		Positive	Negative	
Raw milk	Positive	106	5	111
	Negative	25	58	83
Total		131	63	194
		Sensitivity (%)	Specificity (%)	
		(95% CI)	(95% CI)	
		80.9	92.1	
		(73.1-87.3)	(82.4-97.4)	
		Blood		Total
		Positive	Negative	
Whey	Positive	106	6	112
	Negative	25	37	62
Total		131	43	174
		Sensitivity (%)	Specificity (%)	
		(95% CI)	(95% CI)	
		80.9	86.0	
		(73.1-87.3)	(72.1-94.7)	

CI, confidence interval

Discussion

Here, we showed that (1) of the three examined GST-p24, p24 and His-p24 reagents, the recombinant His-p24 protein was the most suitable one for developing an ELISA system for the rapid and early diagnosis of BLV infection. Larsen et al. have also previously reported that His-p24 gave a very clear reaction with the serum of 16 infected animals [24]. (2) the p24 ELISA showed almost similar sensitivity and specificity for BLV as the commercially available gp51 ELISA kit (although the production of BLV antibodies may vary in individual cattle); (3) p24 antibodies were detected earlier post-infection than gp51 antibodies in three out of eight experimentally infected animals; (4) the p24 ELISA allows monitoring of anti-p24 antibody levels in raw milk and whey. In addition, Gutierrez et al. have also demonstrated that the His-p24-ELISA is a precise assay with good reproducibility [15]. The new p24 ELISA assay has therefore considerable potential for wide scale use.

We detected BLV antibodies and PVL in a large number of samples collected from field cattle. Most PVL-positive samples were also positive for anti-p24 and anti-gp51 antibodies. When cattle are infected with BLV, they generate antibodies specific for the viral p24 and gp51 proteins. Our data showed that infected cattle generate antibodies to both of these proteins. In addition, 17 PVL-negative cattle had detectable anti-p24 and anti-gp51 antibodies, indicating the presence of a recent infection, although not enough provirus was produced to be detectable by

BLV-CoCoMo-qPCR-2). In contrast, 18 cattle were only PVL positive only. This result is supported by the finding of Kelly et al. [21] that using PCR allows for the detection of the provirus as early as 7 days after infection, whereas antibodies can only be detected 3 weeks post infection. Interestingly, only 15 and 13 cattle were positive for either anti-p24 or anti-gp51 antibodies, respectively. It remains unclear which type of antibody appears first in BLV-infected animals because this may depend on the individual animal. Because most field cattle initially have a low PVL, the humoral immune responses are weak. In three of the eight cattle with BLV experimental infection, anti-p24 antibodies were detected earlier than anti-gp51 antibodies although both antibodies were detected at the same time in three other cattle. Radke et al. showed that p24 antibodies were present in most BLV-infected animals very early [44]. In addition, it is possible that retroviral capsid proteins are highly immunogenic and are present in both virions and infected cells at concentrations higher than those of gp51 [44]. However, in two cattle we detected anti-gp51 antibodies earlier than anti-p24 antibodies. The gp51 protein localizes at the surface of the viral particle and since it contains the recognition site for receptors required for virus entry, it is considered to be the natural target for neutralizing antibodies, it may generate a strong immune response in infected cattle [10, 27, 53]. Collectively, these data suggest that a significant proportion of infected cattle may generate anti-p24 antibodies earlier than anti-gp51 antibodies, indicating that the p24 ELISA may improve BLV serodiagnosis and be useful for suppressing virus spread.

Many assays for BLV diagnosis require collection of blood from target cattle [43]. However, collecting milk is less invasive and saves both time and labor. In addition, many false-positive results are obtained for calves below 6 months of age due to ingestion of colostrum, suggesting that anti-BLV antibodies are present in milk. Therefore, we used the p24 ELISA to test samples of raw milk and whey. We detected p24 antibodies in both milk and whey from cattle that had shown positive results with serum. The p24 ELISA is thus a cost-effective method for detecting BLV antibodies in a non-invasive manner.

The gp51 protein is important for the induction of virus neutralizing antibodies, because it contains three conformational epitopes in the N-terminal region of gp51 [7, 8] which is the reason we tried to express the entire gp51 in *E. coli*. However, purifying the entire gp51 was difficult (data not shown) and the purified protein was not recognized by serum from BLV-infected cattle, presumably because major epitopes are not accessible in recombinant gp51 protein. In contrast, p24 protein has two major epitopes present in the C terminal region [7, 8]. Furthermore, the recombinant p24 protein expressed in *E. coli* reacts specifically with p24

antibodies in serum from BLV infected field serum and with BLV p24 monoclonal antibody making it is a good antigen for BLV detection.

In conclusion, the new p24 ELISA based on a recombinant protein produced on an inexpensive *E coli* platform is a highly sensitive and specific method for detecting antibodies in serum from both experimentally and naturally infected animals. The assay may soon become available commercially for detecting anti-BLV p24 antibodies in milk and whey and could be valuable for the improved diagnosis of the disease as well as for studying antigens for developing a subunit vaccine or a VLP vaccine.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflicts of interest.

Animal handling and research ethics All animals were handled by veterinarians from the Animal Research Center in Hokkaido, and RIKEN, Japan in strict accordance with good animal practice following the guidelines of Hokkaido Research Organization and RIKEN. The study was approved by the Animal Ethical Committee and the Animal Care and Use Committee of the Animal Research Center, Hokkaido Research Organization (approval number 1302) and RIKEN Animal Experiments Committee (approval number H29-2-104).

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