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ORIGINAL ARTICLE

Development of a luminescence syncytium induction assay (LuSIA) for easily detecting and quantitatively measuring bovine leukemia virus infection

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Abstract

Bovine leukemia virus (BLV) causes enzootic bovine leukosis and is closely related to the human T cell leukemia virus. Since BLV infection mostly occurs *via* cell-to-cell transmission, BLV infectivity is generally measured by culturing BLV-infected cells with reporter cells that form syncytia upon BLV infection. However, this method is time-consuming and requires skill. To visualize the infectivity of BLV, we developed a new assay called the luminescence syncytium induction assay (LuSIA) that is based on a new reporter cell line designated CC81-BLU3G. CC81-BLU3G is stably transfected with pBLU3-EGFP, which contains the BLV long terminal repeat U3 region linked to the enhanced-green fluorescence protein (EGFP) gene. CC81-BLU3G expresses the EGFP in response to BLV Tax expression specifically, and forms fluorescing syncytia when transfected with an infectious BLV plasmid or when cultured with BLV-infected cells. Compared to the conventional assay, LuSIA was more specific and detected cattle samples with low proviral loads. The fluorescing syncytia was easily detected by eye and automated scanning and LuSIA counts correlated strongly with the proviral load of infected cattle ($R^2 = 0.8942$).

Introduction

Bovine leukemia virus (BLV) can be taxonomically classified within the family *Retroviridae* genus *Deltaretrovirus* and is an oncogenic virus closely related to the human T cell leukemia virus type 1 (HTLV-1). BLV infection causes enzootic bovine leukemia, which is the most common neoplastic disease of cattle [1]. After infection, the BLV genomic DNA integrates into the host genome as a provirus and remains in the infected host throughout its lifetime. Furthermore, it has recently been reported that BLV infection decreases milk production and cow longevity without onset of leukosis [2]. For this reason, BLV infects cattle worldwide and causes serious problems for the cattle industry.

BLV is transmitted *via* cell-containing fluids such as blood and milk. BLV mainly infects $CD5^+$ IgM^+ B cells. However, it is also known to infect $CD5^-$ IgM^+ B cells, $CD2^+$, $CD3^+$, $CD4^+$, $CD8^+$, and γ/δ T cells, monocytes, and granulocytes [1, 3–9]. BLV is transmitted most efficiently by cell-to-cell contact. Cell-free virus transmission can occur, but this is less efficient [1]. The natural BLV host is cattle and water buffalo but it can also be experimentally transmitted to sheep, goats, and alpaca. It can also infect cultured cells from humans, monkeys, cattle, dogs, goats, sheep, and bats [10].

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The infectivity of BLV is typically measured by using the syncytium induction assay (SIA). In this assay, infected peripheral blood mononuclear cells (PBMCs) or cell lines are cocultured with a reporter cell line such as CC81, F81 and CRFK (a family of immortalized feline kidney cell lines), and fetal lamb kidney cells (FLK) for several days [11–16]. When these reporter cells are infected with BLV, they fuse with each other, thereby forming large multinuclear syncytia. However, SIA has some drawbacks: the culture duration is long, and skill is needed to count the syncytia. Moreover, this method is unsuitable for screening because the counting must be performed visually.

Two studies have reported improved BLV infectivity assays. One, the immunoperoxidase infectivity assay, measures the levels of a major BLV antigen [17]. Generally, measurement of human immunodeficiency virus (HIV) infectivity is based on established reporter cell lines such as TZM-bl and MAGIC-5 cells that are stably transfected with a plasmid that contains a reporter gene with the long terminal repeat (LTR) of HIV on its upper promoter region. The reporter gene is expressed when HIV replicates [18–20]. Jewell and Mansky reported a similar method for measuring BLV infectivity using reporter cells, which has the enhanced-green fluorescence protein (EGFP) reporter with a BLV LTR full-length promoter, and assessed their signal intensity by flowcytometry [21]. However, neither method has become the mainstream method for measuring BLV infectivity.

These reports suggest that plasmids bearing the BLV LTR may be useful for generating reporter lines that can more sensitively quantitate BLV infectivity than reporter lines used in conventional SIA. This is supported by the fact that the BLV LTR bears many transcriptional regulator-binding sites and is responsible for virus replication and integration [22–29]. The BLV LTR consists of three regions, namely, the U3, R, and U5 regions. The U3 region bears three Tax-responsive elements (TxRE) that are recognized by the BLV protein Tax, which is the main regulator of BLV replication. In particular, the binding of Tax to TxRE-2 is reported to predominantly regulate BLV viral replication [26]. Moreover, the binding of Tax to BLV TxRE is mediated by a cAMP response element-binding protein (CREB) [30]. The possibility that plasmids bearing BLV LTR could be used to generate sensitive reporter lines for BLV infection is supported by two studies that generated reporter plasmids containing the BLV LTR U3 region promoter to assay BLV replication [13, 31].

Here, we established several novel reporter cell lines, derived from CC81, CRFK and CHO-K1 cells, to measure BLV infectivity. Subsequently, we selected the best line that is most responsive to BLV infection and grows sustainably. This line was designated CC81-BLU3G and was generated by stably transfecting CC81 cells with a pBLU3-EGFP reporter plasmid containing the BLV LTR-U3 region promoter and the gene encoding EGFP. When cultured with BLV-infected cells, CC81-BLU3G cells form syncytia and express EGFP as a result of Tax-induced transactivation of BLV LTR-U3. The CC81-BLU3G line was used to establish the Luminescence Syncytium Induction Assay (LuSIA). We found that LuSIA measured BLV infection more easily, sensitively, and quantitatively than the conventional SIA. LuSIA may be useful as a high-throughput screening test for measuring BLV infection in large numbers of samples, for testing candidate BLV vaccines, and for identifying compounds that inhibit BLV transmission.

Materials and methods

Cell culture

The following cell lines were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's Medium (DMEM) (Thermo Fisher, Waltham, MA) with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO): FLK-BLV cells, which are persistently infected with BLV, 293T cells, which are human embryonic kidney cells that express the large T antigen of simian virus 40, CC81 cells, which are cat cells transformed by mouse sarcoma virus, CRFK cells, which are cat kidney cells, Tb1-Lu cells, which are bat lung cells, and their transfectants, namely, CC81-BLU3G and CRFK-BLU3G. The Chinese hamster ovary CHO-K1 cell line and its transfectant CHO-BLU3G were cultured at 37 °C with 5% CO_2 in F-12 Nutrient Mixture (Thermo Fisher) with 10% FBS.

Isolation of PBMCs, extraction of genomic DNA, and quantification of BLV proviral DNA

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) from breeding cows in the Institute of Livestock and Grassland Science, NARO or the School of Veterinary Medicine of Azabu University. The PBMCs were isolated by Percoll gradient centrifugation as described by Miyasaka and Trnka [32]. Genomic DNA was extracted from the whole bloods by using the Wizard Genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions. Proviral DNA was quantified by using the BLV-CoCoMo-qPCR-2 method, as described previously [33–35].

Construction of plasmids

To construct a reporter plasmid that expresses EGFP when it encounters the Tax protein of BLV, the LTR-U3 region from the BLV infectious molecular clone pBLV-IF [13] was amplified by polymerase chain reaction (PCR) by

using KOD-plus (TOYOBO, Osaka, Japan) and the specific primers AAACATATGTGTATGAAAGATCATGCC GACCTAGG and AAGAGAGCTCAGGACCGAGAG. The underlined sequences in the two primers are restriction enzyme-specific sites for *Nde*I and *Sac*I, respectively. The PCR product was purified by using a FastGene Gel/PCR Extraction kit (Nippon Genetics, Tokyo, Japan). It was then digested by *Nde*I and *Sac*I and the digested fragment was inserted into the *PshB*I and *Sac*I sites of the pEGFP-N1 plasmid by compatible cohesive ends and sticky end ligation. This plasmid contains a neomycin-resistance gene and encodes a red-shifted variant of wild-type GFP that exhibits brighter fluorescence than the wild-type GFP [36]. Ligation was achieved by using Ligation high ver.2 (TOYOBO). XL-10 competent cells transformed with the ligated plasmid (Agilent, Santa Clara, CA) were propagated overnight at 37 °C with kanamycin. The inserted clone was selected by colony-direct PCR by using GoTaq Green Master Mix (Promega) and directional specific primer sets: GTAAACCAG ACAGAGACGTCAGCTGCC and GGCCGTTTACGTCGC CGTCC. The constructed plasmid was verified by nucleotide sequencing using the primers described above. The resulting plasmid was designated pBLU3-EGFP.

To construct pME18neo/BLV Tax-FLAG, which encodes FLAG-tagged BLV Tax, the *tax* gene from the pBLV-IF template was amplified by using the AACTCGAGGCC ACCATGGCAAGTGTTGTTGGTTGGGGGCC and AAA AAAGCGGCCGCTCACTTGTCGTCATCGTCTTTGTA GTCAAAAAGGCGGGAGAGCC primers. The underlined sequences in these forward and reverse primers correspond to the restriction sites for *Xho*I (TOYOBO) and *Not*I (TOYOBO), respectively. The amplification yielded the *tax* gene with the FLAG sequence at its 3′ end. The PCR products were then introduced into the *Xho*I and *Not*I sites of the pME18neo plasmid by using Ligation high Ver. 2 according to the manufacturer's instructions. Transformation and purification were performed as described above. The construct was verified by nucleotide sequencing using the sequencing primers AACTCGAGGCCACCATGGCAAGTGTTGTTG GTTGGGGGCC, AAAAAAGCGGCCGCTCACTTGTCG TCATCGTCTTTGTAGTCAAAAAGGCGGGAGAGCC, TTTTATTTCAGGTCCCGGATCC, and GGAATTAAT TCGAGCTCGGT.

Transient cotransfection of 293T cells with plasmids

293T cells seeded at 5×10^4 cells/well in a 24-well plate were incubated overnight at 37 °C with 5% CO_2. The cells were then transfected with 0.2 μg of pBLU3-EGFP together with 0.5 μg of pME18neo/BLV Tax-FLAG (or the pME-18neo control) or 0.5 μg pBLV-IF (or the pKSII control) by using the FuGENE HD reagent (Promega) according to the manufacturer's instructions. After 48 h, the culture medium was removed and the cells were fixed with 3.6% formaldehyde/Phosphate buffered saline (PBS) with 10 μg/mL of Hoechst 33342 (Sigma-Aldrich). Fluorescence was observed by FV-1000D fluorescence microscopy (Olympus, Tokyo, Japan).

Establishment of reporter cells that are stably transfected with pBLU3-EGFP

CHO-K1, CRFK, Tb1-Lu, and CC81 cells seeded at 3×10^5 cells/well in 6-well plates were incubated overnight at 37 °C with 5% CO_2. The cells were transfected with 1.25 μg pBLU3-EGFP by using the FuGENE HD reagent or the Lipofectamine 3000 reagent (Thermo Fisher) according to the manufacturer's instructions. After 48 h, the cells were cultured in fresh medium in the presence of 500 μg/mL G418 (Roche, Basel, Switzerland) and passaged for several weeks to select antibiotic-resistant clones. Stable transfectants were cloned by limiting dilution culture in 96-well plates and cultured until the clones had expanded sufficiently. When multiple clone cells from one parental cell line were detected, the clone that responded best to transfection with 0.5 μg pME18neo/BLV Tax-FLAG was chosen. Unfortunately, the cloning of Tb1-Lu transfectants could not be obtained due to their poor growth during limiting dilution. The reporter cell clones that exhibited high EGFP expression with low backgrounds were designated CHO-BLU3G, CRFK-BLU3G, and CC81-BLU3G.

Transient transfection of reporter cells with plasmids

The CHO-BLU3G, CRFK-BLU3G, and CC81-BLU3G reporter cells were seeded at 5×10^4 cells/well in 24-well plates and incubated overnight at 37 °C with 5% CO_2. They were then transfected with 0.5 μg of pME18neo/BLV Tax-FLAG (or the pME18neo control) or with 0.5 μg pBLV-IF (or the pKSII control) by using the FuGENE HD reagent. Three days post transfection, they were fixed by using 3.6% formaldehyde/PBS with 10 μg/mL of Hoechst 33342. Fluorescence was observed using FV-1000D fluorescence microscopy.

Development of the LuSIA

To detect the best reporter cell for LuSIA, the candidate reporter cells were cocultured with FLK-BLV cells. Thus, the CHO-BLU3G, CRFK-BLU3G, and CC81-BLU3G reporter cells were seeded at 2×10^5 cells/dish in 6-cm dishes and incubated overnight at 37 °C with 5% CO_2. After removing the culture medium, FLK-BLV cells were added at 6×10^4 cells/dish in the 6-cm dishes. The LuSIA coculture medium was 10% FBS/DMEM supplemented with 1

× non-essential amino acid (Thermo), 5 × penicillin-streptomycin-Glutamine (Thermo), 2.5 μg/mL Amphotericin B solution (Sigma-Aldrich), 2-mercaptoethanol (Sigma-Aldrich), and 4 μg/mL of polybrane (TOYOBO). After three days of coculture, the cells were washed with PBS and fixed by using 3.6% formaldehyde/PBS with 10 μg/mL of Hoechst 33342. Fluorescence was observed by FV-1000D fluorescence microscopy. As a control for this experiment, the reporter cell lines were transfected with 2.8 μg pBLV-IF and cultured for two days.

LuSIA with FLK-BLV cells

LuSIA was conducted by coincubating 5×10^4 CC81-BLU3G cells/well with the indicated concentrations of FLK-BLV cells in LuSIA coculture medium in a 12-well plate for three days. The culture medium was then exchanged with fresh medium and the cells were cultured for an additional 1 day. The cells were washed with PBS and fixed by using 3.6% formaldehyde/PBS with 10 μg/mL of Hoechst 33342. Fluorescence was observed using FV-1000D fluorescence microscopy.

Conventional SIA with FLK-BLV cells

SIA was conducted by coincubating 5×10^4 cells/well CC81 with the indicated concentrations of FLK-BLV with 10% FBS/DMEM in a 12-well plate at 37 °C with 5% CO_2 for three days. The culture medium was then exchanged with fresh medium and the cells were cultured for an additional day. Thereafter, the cells were washed with PBS and fixed with 3.6% formaldehyde/PBS with 10 μg/mL of Hoechst 33342. The CC81 cells were stained with May-Grunwald solution (Merck, Darmstadt, Germany) and the Gimsa solution (Sigma-Aldrich) as described previously [37]. The stained syncytia was counted by FV-1000D fluorescence microscopy.

LuSIA with BLV-infected PBMCs from cattle

CC81-BLU3G cells (2×10^5 or 2×10^4 cells/well) were cocultured in LuSIA coculture medium with the indicated concentrations of PBMCs in 12-well plates for three days. The culture medium was then replaced with fresh medium and the cells were cultured for an additional two to three days. Fluorescence was observed by FV-1000D fluorescence microscopy each day. In the quantitative analysis, the cells were washed with PBS and fixed with 3.6% formaldehyde/PBS with 10 μg/mL of Hoechst 33342 on two days after the change of medium. Subsequently, the fluorescent syncytium were automatically scanned in 9 fields-of-view from each well by EVOS2 fluorescence microscopy with a 4 x objective; counting was performed by HCS Studio Cell Analysis software (Thermo fisher). Fluorescent syncytium were recognized by EGFP expression and gated by their area size and intensity.

Results

Construction of the novel pBLU3-EGFP reporter plasmid

To measure BLV infection specifically, we constructed a new reporter plasmid that bears the BLV LTR-U3 region and expresses EGFP when it is exposed to the BLV transactivating protein Tax. As shown in Fig. 1, we first cloned the TxRE-containing LTR-U3 region from the infectious BLV molecular clone called pBLV-IF [13] by PCR amplification. Thereafter, we replaced the cytomegalovirus promoter region of the EGFP expression vector pEGFP-N1 with the LTR-U3 region of BLV.

Response of the novel reporter plasmid pBLU3-EGFP to Tax expression

To determine whether the pBLU3-EGFP plasmid expresses EGFP when it is exposed to Tax, we first transiently transfected 293T cells with both the pBLU3-EGFP plasmid and the Tax-expressing plasmid pME18neo/BLV Tax-FLAG. As a control, the pME18neo was transfected instead of pME-18neo/BLV Tax-FLAG. EGFP expression only occurred when the pBLU3-EGFP-transfected cells were cotransfected with the Tax expression vector (Fig. 2A).

The infectious BLV clone pBLV-IF can induce cellular fusion in 293T cells [38]. This cytopathic effect is due to the BLV Envelope protein [13]. Therefore, we next transfected 293T cells with both the pBLU3-EGFP plasmid and pBLV-IF and examined syncytium formation. This combination had a remarkable effect (Fig. 2B). In the case of the 293T cells cotransfecting pBLU3-EGFP and the Tax-expressing plasmid, strong fluorescence is emitted by one cell (Fig. 2A, lower panel). By contrast, many cells exhibited strong fluorescence that formed huge syncytia when 293T cells were cotransfected with pBLU3-EGFP and the pBLV-IF plasmid (Fig. 2B, lower panel). In the cotransfection of pBLU3-EGFP with the pME-18neo and pKSII, only a small amount of fluorescence was emitted and there was no syncytia formation (Fig. 2A and B, upper panel).

Establishment of stable reporter cell lines and their response to Tax expression

Several cell lines, including CC81, CRFK and Tb1-Lu [10], HeLa, NHI3T3 and FLK [13, 16], have been shown to form syncytia when they are infected with BLV. Since

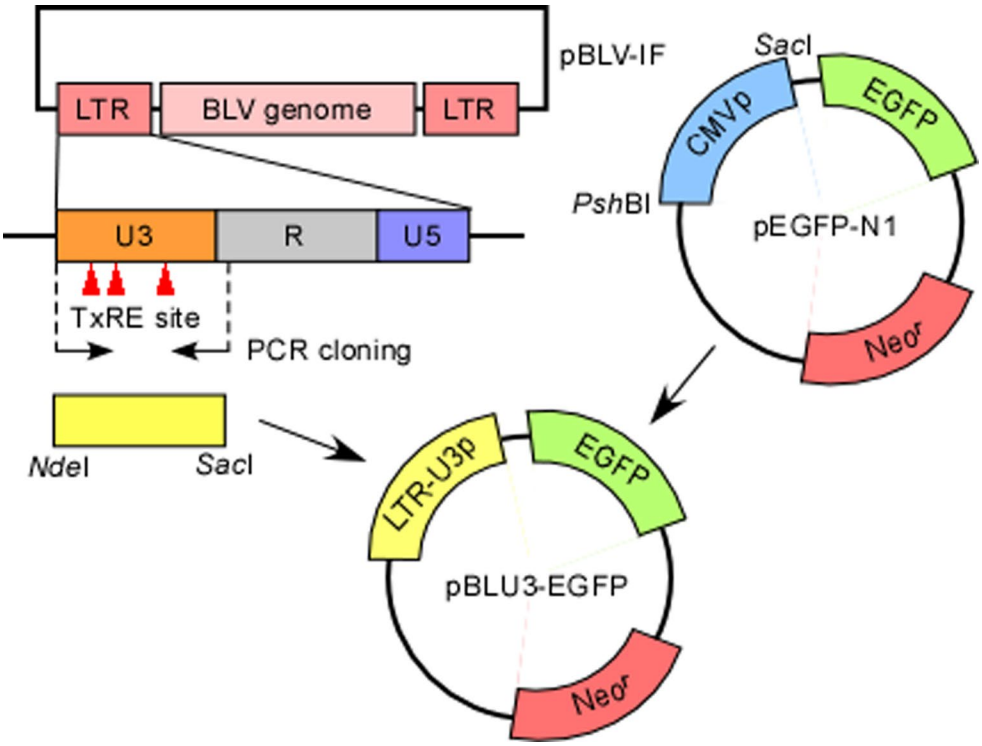


Fig. 1 Construction of the pBLU3-EGFP plasmid. The long terminal repeat (LTR)-U3 region of BLV, which bears three Tax-responsive elements (TxREs; indicated by the red triangles), was cloned by PCR from the infectious BLV molecular clone pBLV-IF. The PCR added the restriction enzyme sites *Nde*I and *Sac*I. The amplified PCR product and the EGFP-expressing vector pEGFP-N1 were digested by these restriction enzymes. The cytomegalovirus promoter (CMVp) region of pEGFP-N1 was replaced by the BLV LTR-U3 region. LTR-U3p, BLV LTR U3 promoter; CMVp, cytomegalovirus promoter; Neo^r, neomycin-resistance gene

it would be useful to have a reporter cell line that both fluoresces and forms syncytia upon BLV infection, we assessed whether CC81, CRFK, or Tb1-Lu would be suitable for establishing a BLV-specific reporter cell line. Thus, they were transfected with the pBLU3-EGFP plasmid. We also transfected CHO-K1 cells with the plasmid because this cell line has unknown susceptibility to BLV infection. The transfected cell lines were selected with 500 µg/mL G418. The first selected cells were cloned by using the limiting dilution method. Thereafter, the cloned cell lines were transiently transfected with 0.5 µg of pME18neo/BLV Tax-FLAG. The clones that exhibited low background signals and high response signals were selected.

Stable transfectants that expressed EGFP in the presence of Tax were generated from all cell lines except Tbl-Lu. These transfectants were designated CC81-BLU3G, CRFK-BLU3G, and CHO-BLU3G. These clones expressed EGFP when they were transfected with a Tax expression vector but not the pME18neo control vector (typical results are shown in Fig. 3; data not shown for CRFK-BLU3G). Notably, CC81-BLU3G and CRFK-BLU3G also formed fluorescing syncytia when transfected with the infectious BLV molecular clone pBLV-IF: this was not observed when the cells were transfected with the control pKSII vector (Fig. 3, lower panel, and data not shown for CRFK-BLU3G). Unexpectedly, CHO-BLU3G exhibited EGFP expression but no syncytium formation when it was transfected with pBLV-IF (Fig. 3, upper panel).

Development of the LuSIA

Cell-free infection with BLV is believed to be very inefficient, probably due to the instability of BLV virions [39–41]. Consequently, to efficiently infect cells, they must come into direct contact with BLV-infected cells. Therefore, we determined whether the stably transfected cell lines could be used to measure BLV infection by culturing them with the FLK cell line that is productively infected with BLV (FLK-BLV). As a positive control, the lines were transfected with pBLV-IF. CC81-BLU3G and CRFK-BLU3G formed fluorescing syncytia when cocultured with FLK-BLV (Fig. 4 and data not shown). In contrast, when CHO-BLU3G was cocultured with FLK-BLV, there was no EGFP expression or syncytium formation. However, as has already been shown in Fig. 3, the pBLV-IF-transfected CHO-BLU3G did express EGFP (Fig. 4). This indicates that although CHO-BLU3G can synthesize and express detectable BLV protein after transfection with pBLV-IF, it cannot be infected with BLV.

Of the two stably transfected cell lines that expressed EGFP and formed syncytia when they underwent cell-to-cell BLV transmission, CC81-BLU3G exhibited rapid growth and the best response to BLV infection. Consequently, CC81-BLU3G was used to establish the novel LuSIA assay.

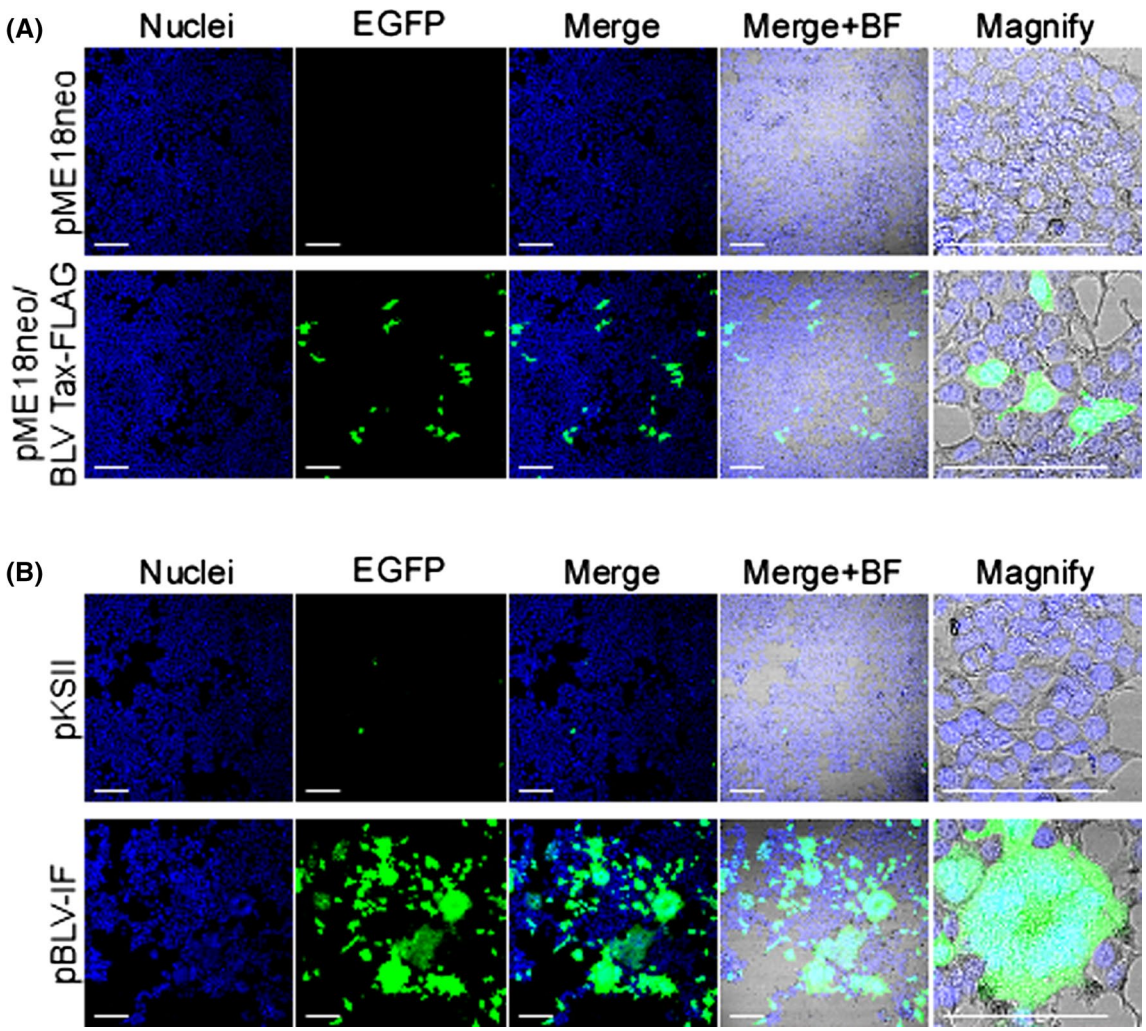


Fig. 2 Transient coexpression of pBLU3-EGFP with a Tax-expressing plasmid or an infectious BLV clone in 293T cells. 293T cells were cotransfected with pBLU3-EGFP and (A) pME18neo/BLV Tax-FLAG (or the pME18neo control) or (B) pBLV-IF (or the pKSII control). After two days of culture, the cells were fixed with 3.6% formaldehyde/PBS with Hoechst 33342. The scale bars (white bars) signify 100 μm. The images are representative of three independent experiments. EGFP, enhanced-green fluorescent protein; BF, bright field

Comparison of LuSIA with conventional SIA

Conventionally, BLV infection is assessed by using CC81 or CRFK cells as reporter cells in the SIA [11, 12]. We compared SIA and LuSIA in terms of their ability to generate syncytia after BLV infection by respectively culturing CC81 and CC81-BLU3G with various concentrations of FLK-BLV cells (1, 10, 50, 100, 250, 1000, and 6000 cells/well) for four days. Thereafter, the syncytia were counted by using FV-1000D fluorescence microscopy (Fig. 5). In SIA, the CC81 cells exhibited good syncytium formation when they were cocultured with FLK-BLV (upper panels of Fig. 5A, and Fig. 5B). However, the CC81 cells that were not cultured with FLK-BLV also exhibited some syncytia-like formation (blue triangles in the upper left panel). By contrast, in LuSIA, CC81-BLU3G only expressed EGFP and exhibited syncytium formation when they were cocultured with FLK-BLV (lower panels in Fig. 5A, and Fig. 5B).

Depending on the FLK-BLV cell concentration, the syncytium number obtained by LuSIA ranged from 4.67 ± 3.51 to 2294.33 ± 390.02 counts per well. In SIA, the equivalent numbers for SIA ranged from 4.33 ± 3.51 to 2702.00 ± 213.55 counts per well (Fig. 5B). Regression analysis showed a strong positive correlation between the LuSIA and SIA syncytia counts ($R^2 = 0.9975$) (Fig. 5C). Significantly, LuSIA was more sensitive than SIA because it had a lower nonspecific background: that meant it could still detect BLV-induced syncytia when low concentrations of FLK-BLV were used (Fig. 5B).

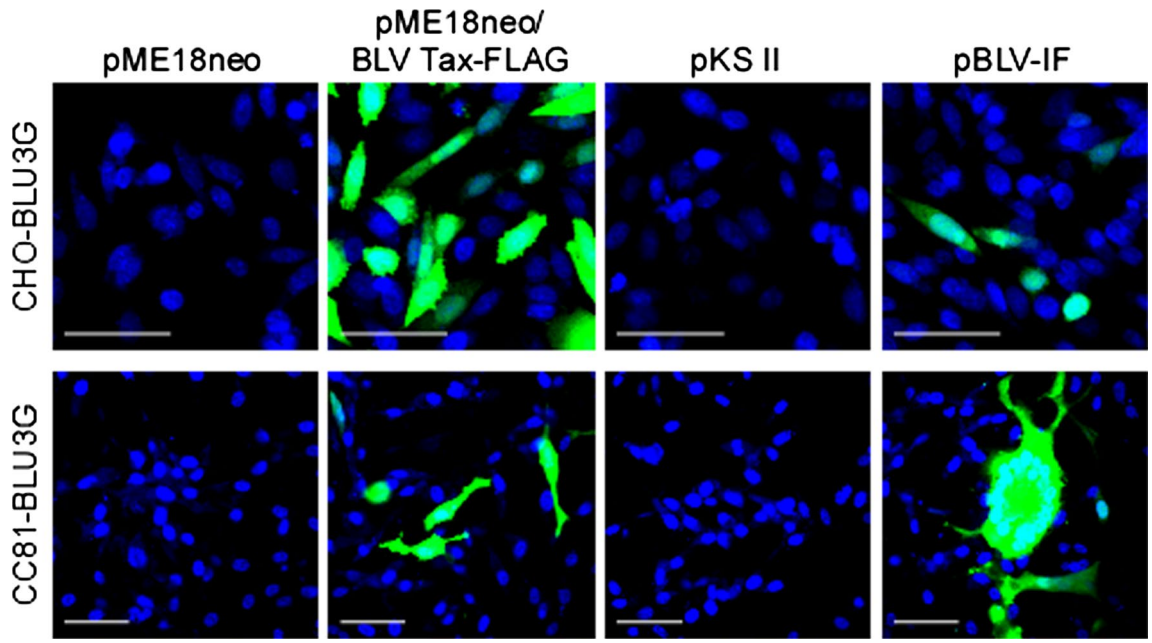


Fig. 3 Transient transfection of reporter cell lines with a Tax-expressing plasmid or the infectious BLV molecular clone. The reporter cells CHO-BLU3G and CC81-BLU3G were transfected with pME18neo/BLV Tax-FLAG (or pME18neo as a control) or pBLV-IF (or pKSII as a control). After three days of culture, the cells were fixed with 3.6% formaldehyde/PBS and Hoechst 33342. The scale bars (white bars) signify 50 µm. The images are representative of three independent experiments

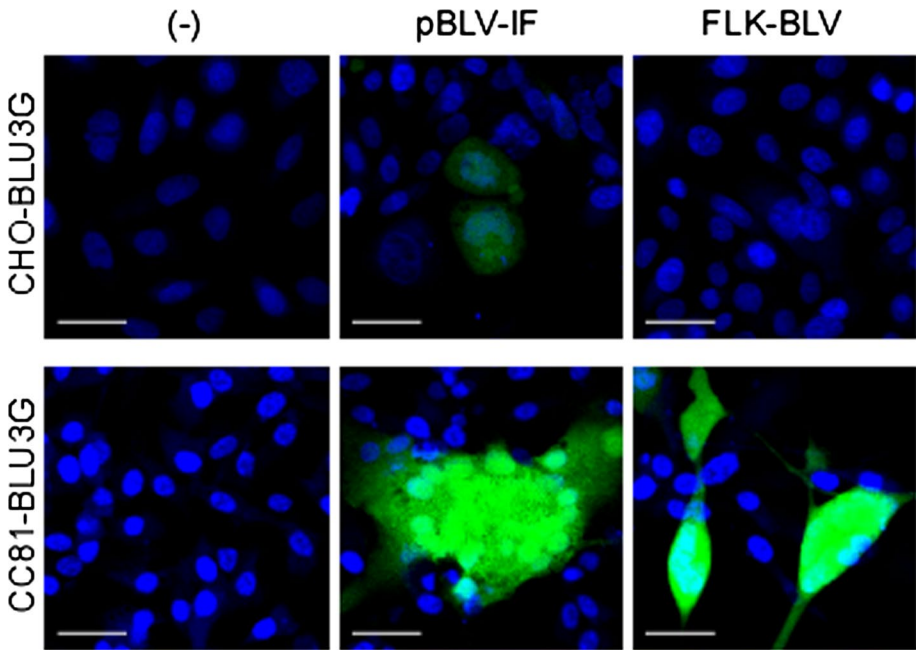
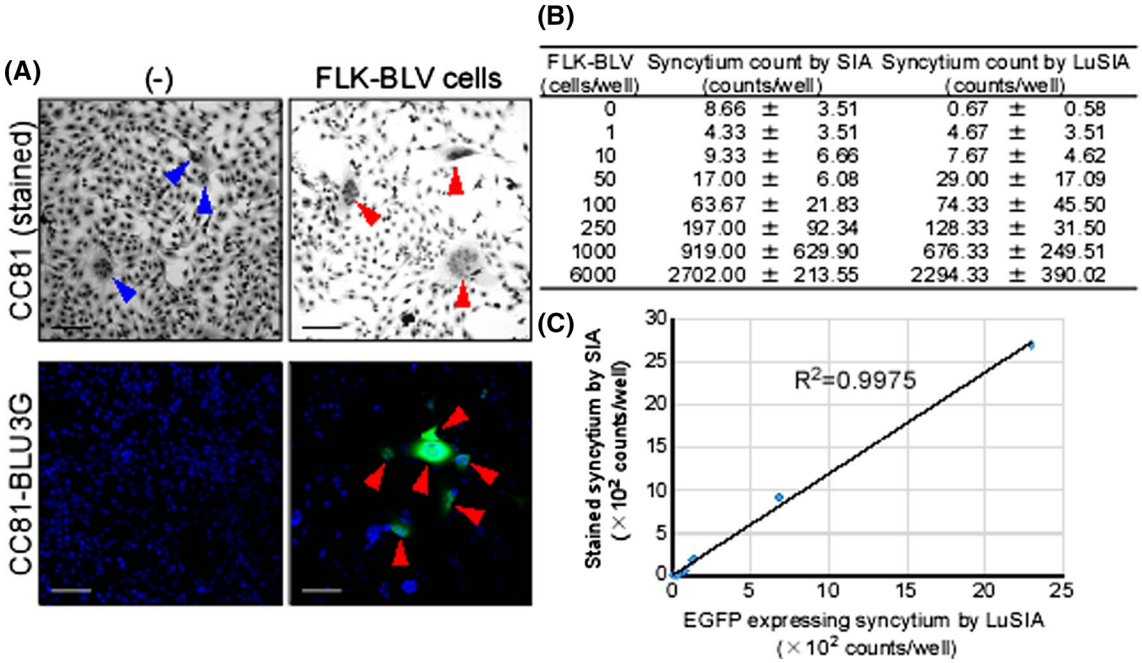


Fig. 4 Coculture of BLU3G reporter cells with the BLV-infected cell line FLK-BLV. CC81-BLU3G and CHO-BLU3G cells were cultured overnight and then cocultured with FLK-BLV for three days. Alternatively, they were transfected with pBLV-IF and cultured for two days. The cells were fixed with 3.6% formaldehyde/PBS and Hoechst 33342. The scale bars (white bars) signify 30 µm. The images are representative of three independent experiments

LuSIA with PBMCs from BLV-infected cattle

Jimba *et al.*[37] reported that the proviral loads in the PBMCs from BLV-infected cattle (as calculated by using BLV-CoCoMo-qPCR, which can measure the proviral load of known and novel BLV variants) correlate with the syncytium counts when the PBMCs are cocultured with CC81 cells in SIA. To determine whether LuSIA is useful for assessing the BLV infectivity of PBMCs from BLV-infected cattle, we first subjected PBMC samples from infected and uninfected cattle to BLV-CoCoMo-qPCR2 and a qualitative LuSIA. PBMCs from four BLV-infected cattle (cows #2–5) and one uninfected cow (#1) were used. The proviral loads of infected cows #2, 3, 4, and 5 were 40.5, 740.5, 4196, and 50887 copies/10^5 cells,



(B)

FLK-BLV (cells/well)	Syncytium count by SIA (counts/well)	Syncytium count by LuSIA (counts/well)
0	8.66 ± 3.51	0.67 ± 0.58
1	4.33 ± 3.51	4.67 ± 3.51
10	9.33 ± 6.66	7.67 ± 4.62
50	17.00 ± 6.08	29.00 ± 17.09
100	63.67 ± 21.83	74.33 ± 45.50
250	197.00 ± 92.34	128.33 ± 31.50
1000	919.00 ± 629.90	676.33 ± 249.51
6000	2702.00 ± 213.55	2294.33 ± 390.02

Fig. 5 Correlation between the syncytium induction assay (SIA) and luminescence syncytium induction assay (LuSIA) syncytium counts. CC81 and CC81-BLU3G cells were used in the SIA and LuSIA, respectively. (A) CC81 and CC81-BLU3G were cocultured with 1000 FLK-BLV per well for three days. The culture medium was then removed, exchanged with fresh medium, and the cells were cultured for an additional day. Thereafter, the cells were fixed with 3.6% formaldehyde/PBS with Hoechst 33342. In SIA, the CC81 cells were stained with the May-Grunwald and Gimsa solutions. The stained and fluorescing syncytia in SIA and LuSIA, respectively were observed by FV-1000D fluorescence microscopy. The scale bars (white and black bars) signify 100 μm. The syncytia that formed during coculture with FLK-BLV are indicated by the red triangles. The syncytia-like formations that arose in the absence of FLK-BLV are indicated by the blue triangles. The images are representative of three independent experiments. (B) CC81 and CC81-BLU3G cells were cultured with 0, 1, 10, 50, 100, 250, 1000, and 6000 FLK-BLV cells for three days. The culture medium was then removed, exchanged with fresh medium, and the cells were cultured for an additional day. The stained CC81 syncytia in the SIA and the fluorescing CC81-BLU3G syncytia in LuSIA were counted by FV-1000D fluorescence microscopy. The data from three independent experiments are expressed as mean ± SD. (C) The correlation between the stained syncytia counts in SIA and the EGFP-expressing syncytia counts in LuSIA

respectively. The proviral load of the uninfected cow was 0 copy/10^5 cells (Fig. 6A). When the CC81-BLU3G cells were cocultured with the PBMCs, syncytia with EGFP fluorescence were observed after three (cow #5) or four (cows #2, 3, and 4) days of culture (Fig. 6B). Thus, LuSIA detected PBMCs that had very low BLV copy numbers. By contrast, no syncytia with EGFP were detected in PBMCs collected from the uninfected cow (#1).

We then subjected the PBMCs of eight BLV-infected cattle and two uninfected cattle to BLV-CoCoMo-qPCR2 and quantitative LuSIA. The LuSIA-measured counts of EGFP-expressing syncytia correlated strongly with the provirus copy counts [correlation coefficient (R^2) = 0.8942] (Fig. 6C).

Discussion

In the present study, we constructed a novel BLV infectivity assay called LuSIA that measures the formation of EGFP-expressing syncytia when the CC81-BLU3G reporter cell is cocultured with BLV-infected cells. This method has the advantage of higher sensitivity, specificity, and quantification, when compared to traditional methods. The first characteristic feature of the LuSIA assay is based on the newly constructed reporter plasmid pBLU3-EGFP. This plasmid bears the BLV LTR-U3 promoter region linked to the reporter gene *egfp* (Fig. 1). Transiently transfected 293T cells expressed EGFP in response to BLV-Tax expression (Figs. 2 and 3). This is a novel approach given that previous approaches either generated Tax response reporter plasmids that bore the BLV LTR-U3 region promoter or created BLV reporter cells that were stably transfected with a reporter plasmid bearing the full-length LTR promoter [13, 21, 31]. This is significant because the Tax-induced replication of BLV is regulated by BLV LTR-U3 [42] whereas the BLV LTR U5 region has an interferon regulatory factor-binding site that drives the Tax-independent replication of BLV [43]. Thus, it is likely that a reporter plasmid that bears the LTR-U3 promoter only (*i.e.*, not the whole LTR) will yield a sensitive and BLV-specific method that measures BLV infection. The second characteristic feature of LuSIA is based on

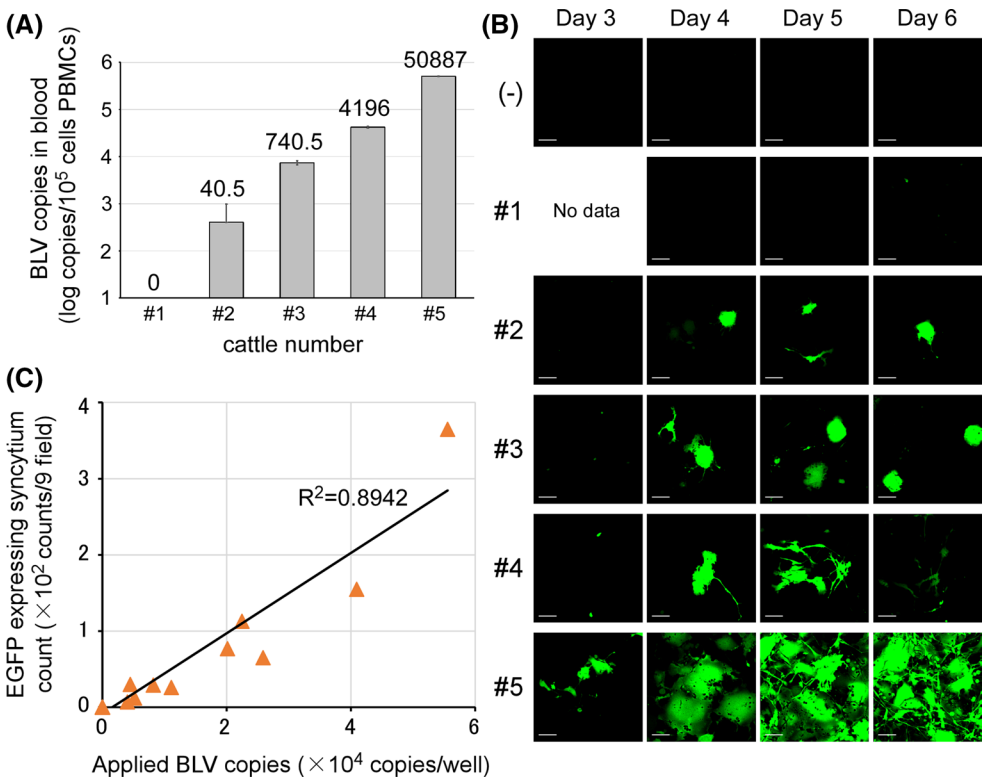


Fig. 6 Luminescence syncytium induction assay (LuSIA) with peripheral blood mononuclear cells (PBMCs) from BLV-infected and uninfected cattle. (A and B) PBMCs were collected from one uninfected cow (#1) and four BLV-infected cattle (#2–5). (A) Their provirus loads were measured by using the BLV-CoCoMo-qPCR-2 method. The average ± standard deviation from duplicate experiments are shown. (B) Qualitative LuSIA with the PBMCs. CC81-BLU3G cells (2×10^5 cells) were cocultured with 2×10^5 PBMCs in 12-well plates for three days. The culture medium was then replaced with fresh medium. During another three days of culture, the fluorescing syncytia were counted daily by FV-1000D fluorescence microscopy. The scale bars (white bars) signify 100 µm. (C) PBMCs from eight BLV-infected and two uninfected cattle were subjected to BLV-CoCoMo-qPCR-2 and quantitative LuSIA was performed. Thus, CC81-BLU3G (5×10^4 cells) and PBMCs (2×10^4 or 1×10^5 cells) were cocultured for three days. Thereafter, the culture medium was exchanged with fresh medium. After two more days of culture, the fluorescing syncytia were automatically scanned by EVOS2 fluorescence microscopy and counted by HCS studio software. The correlation between provirus copy number and fluorescing syncytia numbers is shown

the novel reporter cell line, CC81-BLU3G, that expresses EGFP and forms syncytia in response to BLV infection. CC81-BLU3G was generated by stably transfecting CC81 cells with a pBLU3-EGFP reporter plasmid, thereby allowing BLV infection induced syncytia to be easily detected and distinguished from Tax only expressed single cells. Collectively, these two characteristic features led us to speculate that CC81-BLU3G might be useful for measuring BLV infection.

We found that when the reporter cells were cultured without FLK-BLV, some nonspecific small syncytia-like structures were formed with CC81 but not with CC81-BLU3G (Figs. 5A and B). The EGFP-expressing syncytium counts in LuSIA correlated strongly with the syncytium counts in SIA ($R^2 = 0.9975$), when the reporter cells were cocultured with 0–6000 FLK-BLV cells per well (Fig. 5B and C). However, LuSIA was superior to SIA for detecting BLV-specific EGFP-expressing syncytia when smaller FLK-BLV cell concentrations were used. This was due to the higher nonspecific background levels of syncytium formation in SIA. These observations suggest that LuSIA detects BLV infection as effectively as SIA, and is in fact more sensitive than SIA for low infected-cell concentrations. Furthermore, the conventional SIA method is used to assess the infection of other viruses, such as bovine immunodeficiency virus and bovine foamy virus. In contrast, LuSIA specifically responds to BLV-Tax, to detect BLV specific infection. CC81-BLU3G cells were passaged over twenty times (over about two months) and stocks were maintained by freezing. Their reactivity to BLV infection did not change following passaging or freezing.

In the present study, we also established other cell lines that were stably transfected with pBLU3-EGFP, namely, CHO-BLU3G (based on CHO-K1) and CRFK-BLU3G. While the CRFK-BLU3G cells also responded to Tax expression, BLV expression, and BLV infection, they were

less sensitive than CC81-BLU3G cells (data not shown). Finally, while the CHO-BLU3G clone expressed EGFP when transfected with the Tax-expressing pME18neo/BLV Tax-FLAG plasmid (like CC81-BLU3G), they exhibited EGFP expression without syncytium formation (unlike CC81-BLU3G) when they were transfected with pBLV-IF (Fig. 3). Furthermore, when CHO-BLU3G was cocultured with FLK-BLV, neither EGFP expression nor syncytium formation was observed (unlike the CC81-BLU3G cells, which exhibited both) (Fig. 4). Hence, although CHO-BLU3G synthesized and expressed detectable BLV proteins after transfection with pBLV-IF, it could not be infected with BLV. Thus, CHO-BLU3G was not suitable for the assay. This was expected because we could not detect specific syncytium formation when we cocultured the untransfected CHO-K1 parental line with FLK-BLV cells (data not shown). This observation may reflect the fact that CHO-K1 cells secrete a factor that blocks infections with retroviruses [44]. It is also possible that CHO-K1 cells lack the, as yet unidentified, plasma membrane receptor(s) through which BLV infects its target cells. Additional studies are needed to determine which of these mechanisms renders CHO-K1 cells resistant to BLV infection.

We confirmed that LuSIA could measure BLV infection of PBMCs collected from naturally infected cattle. Our initial qualitative assay showed that fluorescing syncytia formed even when the PBMCs had a very low proviral load (40.5 copies/10^5 cells, as shown by BLV-CoCoMo-qPCR2) (Fig. 6A and B). Our subsequent quantitative LuSIA assay with PBMCs from eight BLV-infected and two uninfected cattle, whose provirus loads ranged from 0 to 55,546 copies/10^5 cells, showed a strong correlation between fluorescing syncytium counts and the provirus copy number (Fig. 6C). Since the proviral load, as measured by the BLV-CoCoMo-qPCR method, correlates closely with BLV infection [37], our result confirms that LuSIA accurately measures BLV infection. Here, we show the results of analysis performed using automated scanning and quantitative systems. This demonstrated that the automated LuSIA could analyze samples and count syncytia far faster and more easily than conventional counting by eye. Using LuSIA, we may be able to develop a high-throughput screening system. We therefore have adopted automated scanning and analysis, based on the fluorescence microscopy system applied in LuSIA. Previously, an improved infection assay based on the full-length LTR reporter system was reported and used for analyzing EGFP expression by flow cytometry [21]. However, we think that flow cytometric detection is not suitable for our reporter cell assay. This is because flow cytometry requires cellular resuspension and is unable to detect syncytia formation. The fluorescence signal intensity of BLV infected cells does not reflect infection directly, but reflects the expression of BLV Tax and its activity in infected cells. It forsakes information on envelope integrity and efficiency and does not reflect precise infectivity. Furthermore, in the early stages of syncytia formation, infected cells decrease their overall fluorescence intensity, which is caused by EGFP flow into newly infect cells. For this reason, infection cannot be measured by the expression of EGFP alone, and requires the specific detection of syncytium formation.

There are two problems in the present study: (i) We used samples collected from only one farm and one breed, the Holstein. To develop LuSIA as a diagnostic tool, we need to test multitude samples collected from other farms and other breeds. (ii) Although the LuSIA method successfully shortens the duration for detecting BLV-specific syncytia as compared with the conventional SIA method, it still requires a long-time of cultivation (at least three days) because the constitutive steps in BLV infection are also required, i.e. virus-cell attachment, viral fusion, reverse transcription, proviral integration, viral transcription, processing of viral transcripts and nuclear export, assembly of new virions. In addition, induction of syncytia formation by expression of Envelope may take at least three days. Therefore, to develop a truly "rapid" infection quantification assay, we need to further improve our reporter cells or plan a new strategy for mimicking these physiological processes, such as proviral integration, viral transcription and the processing of viral transcripts, etc.

LuSIA is an easy and fast method for detection and a highly sensitive method for quantitating BLV infection. These advantages mean that LuSIA may be useful for high-throughput screening of many samples or for long-duration follow-up surveys. It may also be useful for detecting BLV neutralizing antibodies, validating candidate BLV vaccines, and identifying chemical compounds that could be used to treat BLV-infected cattle. Thus, LuSIA may be highly useful for suppressing the horizontal spread of BLV.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interests to declare.

Ethics statement All animal experiments were conducted in accordance with the Guidelines for Laboratory Animal Welfare and Animal Experiment Control that were set out by the Institute of Livestock and Grassland Science, NARO (permit numbers: 1711B082) and by the School of Veterinary Medicine of Azabu University (permit numbers: 161121-2).

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