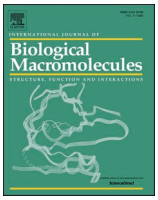
International Journal of Biological Macromolecules 267 (2024) 131446

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac



Development of a novel monoclonal antibody-based competitive ELISA for antibody detection against bovine leukemia virus

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ARTICLE INFO

Keywords:
Bovine leukemia virus
Competitive ELISA
Monoclonal antibody
p24 protein
Serological detection

ABSTRACT

Infection with bovine leukemia virus (BLV) leads to enzootic bovine leukosis, the most prevalent neoplastic disease in cattle. Due to the lack of commercially available vaccines, reliable eradication of the disease can be achieved through the testing and elimination of BLV antibody-positive animals. In this study, we developed a novel competitive ELISA (cELISA) to detect antibodies against BLV capsid protein p24. Recombinant p24 protein expressed by *Escherichia coli*, in combination with the monoclonal antibody 2G11 exhibiting exceptional performance, was used for the establishment of the cELISA. Receiver-operating characteristic curve analysis showed that the sensitivity and specificity of the assay were 98.85 % and 98.13 %, respectively. Furthermore, the established cELISA was specific for detecting BLV-specific antibodies, without cross-reactivity to antisera for six other bovine viruses. Significantly, experimental infection of cattle and sheep with BLV revealed that the cELISA accurately monitors seroconversion. In a performance evaluation, the established cELISA displayed a high agreement with Western blotting and the commercial BLV gp51 cELISA kit in the detection of 242 clinical samples, respectively. In conclusion, the novel p24 cELISA exhibited the potential to be a reliable and efficient diagnostic tool for BLV serological detection with a broad application prospect.

1. Introduction

Bovine leukemia virus (BLV) belongs to the *Deltaretrovirus* genus within the *Retroviridae* family and is closely related to human T-cell leukemia virus types 1 and 2 (HTLV-1 and -2) [1]. BLV is the causative agent of enzootic bovine leucosis (EBL) which is the most common neoplastic disease of cattle [2]. Cattle infected with BLV experience persistent infections throughout their lives, with around 30 % developing persistent lymphocytosis and approximately 5 % being susceptible to developing malignant B-cell lymphosarcoma tumors after long latency periods [3]. The clinical manifestations of BLV-infected cattle are diverse, primarily encompassing weight loss, reduced appetite, and decreased milk production [4]. In addition to the subclinical symptoms that can significantly affect cattle performance, BLV infection also causes direct financial losses resulting from the culling of cattle with malignant lymphomas and trade restrictions on live cattle, embryos and semen [5]. The World Organization for Animal Health (WOAH) has listed EBL as an economic disease that can cause drastic impacts on international trade.

BLV infection is widespread in cattle worldwide, with its prevalence varying from country to country [6]. Several European nations, along with Australia and New Zealand, have implemented eradication initiatives resulting in minimal BLV infection rates. However, BLV is still widely prevalent in North America, Africa, and Asia. It has been reported that BLV infects >40 % of dairy herds in United States [7,8], 84 % of dairy herds in Argentina [9] and 40.9 % of dairy herds in Japan [10]. In China, a meta-analysis of 35 epidemiological studies from 1985 to 2019 showed that the estimated pooled BLV prevalence was 10 %, and even >50 % in some areas [11]. In addition, several reports have surfaced indicating the potential presence of BLV in humans in recent

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https://doi.org/10.1016/j.ijbiomac.2024.131446
Received 3 February 2024; Received in revised form 17 March 2024; Accepted 5 April 2024
Available online 15 April 2024

decades, specifically in human breast tissue, lung cells, and blood cells [12–14]. Currently, serological tests and proviral DNA detection are commonly employed for diagnosing BLV infection. In regard to serological tests, the agar gel immunodiffusion (AGID) assay and enzyme-linked immunosorbent assay (ELISA) stand out as reference techniques recommended by the WOAH [15,16]. In contrast to AGID, ELISAs are more commonly utilized due to their increased sensitivity, capable of detecting infections in cattle with low antibody titers [17,18]. Antibodies that target the capsid protein p24 and the surface protein gp51 are generated shortly after infection, becoming detectable within 2 to 6 weeks, and persisting throughout the life of the host animal. Presently, commercially available ELISA kits for diagnosing BLV infection predominantly target antibodies against gp51, with limited availability of ELISA kits specifically designed for detecting p24 antibodies [19]. It has been reported that both the immunogenicity and concentration of p24 in virions and infected cells are higher than those of gp51 [20], and thus a p24-specific test could be especially useful as a confirmatory method when an eradication campaign reaches its final stage and false positive reactors in the gp51 tests are more likely to occur [9]. Moreover, in the context of potential vaccine applications, the p24 antibody serves as a crucial marker of response for the virus-like particle vaccine, assembled by the BLV *gag* gene, in immunized animals that test negative using gp51 ELISA. Additionally, when gp51-based vaccines are employed, p24-specific tests can effectively differentiate between vaccinated and naturally infected animals [21]. Significantly, the p24 protein has been widely employed as a surrogate marker for the diagnosis of various infections, including HIV-1, HTLV-1, HTLV-II, and STLV-I [6]. On the other hand, ELISA utilizing the virus p24 antigen has demonstrated the presence of IgG, IgM and IgA antibodies against BLV in humans [22]. This suggests that antibodies reactive with the BLV capsid antigen may serve as biomarkers for BLV exposure [23]. Consequently, there is a pressing need to develop sensitive and specific methods targeting p24 antibodies for the detection of BLV infection.

The aim of our study was to generate multiple mouse monoclonal antibodies (mAbs) targeting BLV p24 with exceptional efficacy, alongside the development of a robust mAb-based p24 competitive ELISA (cELISA). Subsequently, we assessed the sensitivity and specificity of this cELISA, validated its effectiveness using field serum samples, and compared its consistency with that of commercial ELISA and Western blotting. Our results underscore the vast potential of our method in detecting BLV infection in both cattle and sheep.

2. Materials and methods

2.1. Recombinant p24 (rp24) expression and purification

The sequence of *p24* was optimized using GenSmartTM Codon Optimization software. The optimized *p24* gene was synthesized and inserted into the plasmid pCold-GST at the *Bam*HI and *Hin*dIII restriction sites. The resulting recombinant plasmid was then introduced into *Escherichia coli* (*E. coli*) strain Rosetta (DE3) and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After a 16-h induction at 16 °C, the bacterial cells were harvested, resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0), sonicated, and centrifuged at 12,000 ×*g* for 15 min at 4 °C. The supernatant, which contained rp24-GST, was then collected, and loaded onto Ni^{2+}-nitrilotriacetate affinity resin (Ni-NTA, Qiagen), followed by washing with a buffer consisting of 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl and 40 mM imidazole. The p24 protein was subsequently eluted using a buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl and 250 mM imidazole. The eluted protein was then dialyzed with 0.02 M PBS overnight at 4 °C. Following this, rp24-GST underwent tobacco etch virus protease (TEV) (Solarbio, China) digestion to remove the GST tag. The purity of the rp24 protein was confirmed using a commercial anti-mouse antibody against p24 (VMRD, USA).

2.2. Production of p24 mAbs

Mice were immunized with the rp24 protein every 14 days for a total of three immunizations and were then euthanized to collect splenocytes on day 3 following the final booster immunization. Subsequently, SP2/0 myeloma cells were fused with the splenocytes using polyethylene glycol (PEG) (Sigma, USA) and cultured in RPMI-1640 medium supplemented with 20 % fetal bovine serum and 1 × Hypoxanthine aminopterin thymidine (Sigma, USA). The cell suspension was dispensed into 96-well plates (Corning, USA) and cultured under 5 % CO_2 at 37 °C. The supernatants of the fused cells were screened using indirect ELISA (iELISA) with the rp24 as the coated antigen. Positive hybridomas in confluent wells were subcloned three times through limited dilution to obtain a single hybrid cell. Subsequently, the amplified hybridomas (2×10^6 cells) were administered intraperitoneally into BALB/c mice that had been pre-treated with liquid paraffin a week earlier. After 7 days, mouse ascites was collected, and the mAbs were purified using Protein G affinity chromatography beads (Genscript, China). The purified mAbs were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The subclass and light chain type of the mAbs were determined using a commercial Mouse Monoclonal Antibody Isotype Elisa Kit (Proteintech, China).

2.3. Stable expression of the p24 protein in HEK293T cells

The pLVX-IRES-Puro-p24-2HA-3FLAG construct was transfected into HEK293T cells for lentivirus packaging. The resultant lentivirus was utilized to infect HEK293T cells, followed by the addition of puromycin-containing medium after 2 days for selection, resulting in the establishment of a stable HEK293T cell line expressing p24.

2.4. Indirect immunofluorescence assay (IFA)

Prior to virus infection, cells (4×10^5/ml) were seeded in 96-well plates. The following cells were infected with specific viruses: Vero cells with lumpy skin disease virus (LSDV), MDBK cells with bovine viral diarrhea virus (BVDV) and infectious bovine rhinotracheitis virus (IBRV), HRT18G cells with bovine coronavirus (BCoV), MARC145 cells with bovine rotavirus (BRV), and BHK cells with akabane virus (AKAV). After infection, cells were fixed using 4 % paraformaldehyde for 10 min at room temperature and then permeabilized with 0.2 % Triton X-100. Following blocking with 1 % bovine serum albumin, mouse polyclonal antibodies against the corresponding viruses and mAb 2G11 were separately added and incubated for 1 h at 37 °C. Subsequently, the cells were washed with PBS buffer, and 1:1000 Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Invitrogen, USA) was applied. After three washes with PBS, the cells were observed using the EVOS imaging system (Life Technology, USA).

2.5. Indirect ELISA

The purified p24, diluted in carbonate buffer (CBS), was coated on 96-well plates (Corning, USA) at a concentration of 5 μg/ml (100 μl/well) and allowed to incubate overnight at 4 °C. Following this, the coated plate was washed thrice with PBST (0.05 % Tween in 0.01 M PBS) and then blocked with 5 % skimmed milk (BD, USA) in PBST for 1 h at 37 °C. After three additional washes with PBST, 100 μl of hybridoma supernatants were added to the wells and incubated for 1 h at 37 °C. Sera from p24 immunized mice and non-immunized mice were diluted to 1:1000 as positive and negative controls, respectively. Subsequent washes were followed by the addition of 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Merck, USA) diluted 1:5000 in PBST into the plates and then incubated for 1 h at 37 °C. Following additional washing, 100 μl of tetramethylbenzidine (TMB) (Beyotime, China) was added to each well. The plates were incubated at room temperature for 10 min, followed by the addition of 100 μl of 2 M

H_2SO_4 per well. The results were measured using a microplate reader (Bio Tek, USA) at an optical density of 450 nm (OD450).

2.6. Serum panel

A total of 247 serum samples were collected from 5 different farms in Heilongjiang Province (188) and Tianjin (59) between March 2020 and June 2023. One hundred and sixty of them were negative sera obtained from cattle farms that were free from BLV. The other 87 of them were positive sera collected from cattle farms occurring BLV infection. The negative sera and positive sera were used to determine the cut-off value of the p24 cELISA after being confirmed by PCR for the BLV-*pol* gene and Western blotting.

To evaluate the concordance between the developed cELISA, a commercial ELISA kit (ID Screen, France), and Western blotting, 242 clinical serum samples from 9 cattle farms in Heilongjiang Province and Xinjiang Uygur Autonomous Regions were collected in 2023. Among them, 179 sera were obtained from 6 cattle farms with an epidemic of BLV, while 63 sera were collected from 3 cattle farms without BLV infection.

2.7. Development of the cELISA

To assess the competitive ability of the mAbs, we employed five positive sera and five negative sera. Briefly, the 96-well ELISA plates were coated with the purified rp24 at 4 °C overnight. After being washed thrice with PBST, the plates were blocked with 5 % skimmed milk (BD, USA) in PBST for 1 h at 37 °C. Testing mixtures containing 50 μl testing serum samples and 50 μl mAb were added in the 96-well plates and incubated at 37 °C for 60 min and followed by three-times washing. Then we added 100 μl of HRP-conjugated goat anti-mouse IgG (Merck, USA) diluted 1:5000 in PBST into the plates and then incubated for 1 h at 37 °C. Finally, after being washed again, TMB was added for color reaction, and 2 M H_2SO_4 was added to stop the reaction. The results were measured using a microplate reader (Bio Tek, USA) at OD450, and the percent inhibition (PI) was calculated using the following formula:

$$\mathrm{PI}\ (\%) = [(\text{mean OD of negative control} - \text{OD of sample}) / \text{mean OD of negative control}] \times 100\ (\%).$$

To optimize the cELISA process, a range of different concentrations of p24 proteins from 0.3125 μg/ml to 40 μg/ml, were utilized for coating. Additionally, the dilution ratios of mAbs of p24 were varied between $1{:}2^8$ and $1{:}2^{14}$. The final optimal conditions were determined to achieve approximate OD450 values of 1.0 with iELISA through checkerboard titration. Following this, three BLV antibody-negative and positive sera, with dilutions ranging from 1:1 to 1:64, were tested for cELISA. The optimal serum dilution was set to obtain the smallest ratio of OD450 values between the positive and negative sera (P/N). The incubation time of the testing mixtures or HRP-conjugated goat anti-mouse IgG (Merck, USA) was set at intervals of 30, 45, and 60 min, respectively. After the addition of TMB, the colorimetric reaction time was set at 10 and 15 min. The two optimal reaction conditions were selected based on the smallest ratio of P/N. Ultimately, the procedure that enabled the highest level of differentiation between the positive and negative reference sera samples was determined.

2.8. Validation of the p24 cELISA

Twenty-four serum samples against various bovine viruses, including BVDV, BRV, IBRV, BCoV, AKAV, and LSDV, were collected from infected or vaccinate cattle to assess the specificity of the p24 cELISA. The repeatability of the cELISA was gauged by examining eight serum samples, comprising four negative and four positives for BLV. The coefficient of variation (CV), computed from the OD values as (standard deviation SD/mean) × 100 %, was employed to measure the consistency. The intra-assay CV was determined by assessing each serum on three plates, while the inter-assay CV was calculated from three measurements of each serum.

2.9. Infection of animals with BLV

Four 3-month-old female sheep and three 4-month-old female cattle, which were confirmed as BLV-free animals by PCR and ELISA, were selected for infection with the cell lysates from transfecting with a plasmid that contains the entire BLV proviral genome (pBLV344) as described previously [24]. In preparation for transfection, 9×10^5 HEK293T cells were seeded in 20 ml of complete growth medium in a 15 cm culture dish. The transfection mixture, consisting of 60 μl PEI (1 μg/μl), 20 μg pBLV344 provirus, and 1 ml OPTI-MEM, was incubated for 15 min at room temperature and then added dropwise to each dish. After 48 h in the incubator, the transfected cells were harvested with trypsin-EDTA and centrifuged at 1100 rpm for 5 min. The resulting cell pellets were resuspended in 1 ml of PBS, frozen at −80 °C overnight, and subsequently thawed to yield cell lysates. Each sheep or cattle was administered with a total volume of 1 ml lysate, with 500 μl injected into the jugular vein and 500 μl intracutaneously. Following the injection, blood samples were collected from all infected individuals twice monthly.

2.10. Statistical analysis

Receiver operator characteristic (ROC) curve analysis was conducted using 87 sera from the BLV-infected animals and 160 sera from uninfected animals to determine the cut-off value, sensitivity, and specificity of the p24 cELISA. Statistical analysis and data visualization were carried out using GraphPad Prism software (version 8.0; GraphPad Software, Inc., La Jolla, CA).

The degree of agreement (kappa value) between the cELISA and Western blotting, as well as a commercial ELISA kit, was calculated using Microsoft Excel. A higher kappa value indicates better consistency between the two methods; a kappa value between 0 and 0.40 suggests poor consistency, while a kappa value over 0.40 indicates high consistency between the methods.

3. Results

3.1. Characterization and purification of rp24 protein

The codon-optimized BLV *p24* gene was integrated into the pCold-GST vector, embellished with a 6 × His tag and GST tag at the N-terminal, and then introduced into *E. coli* Rosetta (DE3). The recombinant p24-GST (rp24-GST) were expressed in a soluble form in *E. coli*, which was then purified via a Ni^{2+} NTA affinity column. Following purification, the protein was subjected to digestion by TEV enzyme to yield the rp24 protein without the GST tag. As expected, the SDS-PAGE analysis demonstrated that predominant bands of rp24 were at approximately 24 kDa (Fig. 1A). Additionally, Western blotting analysis showed that a single protein band of rp24 was recognized by the p24 commercial mouse mAb (Fig. 1B).

3.2. Generation of mAbs against BLV p24

MAbs against the p24 of BLV were prepared using hybridoma technology. Ultimately, three clones that produced antibodies specifically targeting the p24 protein were obtained, and were designated as 3E6, 2G11, and 2A7. The isotypes of mAbs 3E6 and 2A7 were identified as IgG1 with the kappa light chain, while the isotype of mAb 2G11 was classified as IgG1 with the lambda light chain (Table 1). These mAbs were purified from the ascites fluid of mice injected with hybridoma cells and subsequently assessed using SDS-PAGE. The heavy and light chains of the mAbs were observed at approximately 53 kDa and 24 kDa,

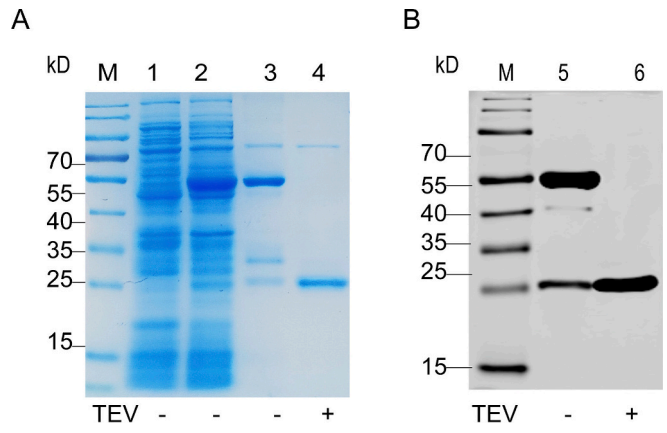

Fig. 1. Analysis of rp24 protein. (A) SDS-PAGE analysis of rp24 protein; M, Protein marker, lane 1, whole cell lysates of uninduced bacteria; lane 2, Supernatants of bacterial cell lysates induced by 0.5 mM IPTG; lane 3, purified rp24-GST, lane 4, rp24 protein after TEV protease cleavage (B) Western blotting analysis of the purified rp24 protein with the commercial p24 mAb. lane 5, purified rp24 protein with GST tag; lane 6, rp24 protein after TEV protease cleavage.

Table 1
Identification of isotypes of p24 mAbs.

	Monoclonal Antibodies		
	3E6	2G11	2A7
Ig subclass	IgG1	IgG1	IgG1
Light chain type	Kappa	Lambda	Kappa

respectively (Fig. 2A).

Furthermore, we conducted an iELISA to assess the superior binding ability of the anti-rp24 mAbs. The three mAbs were set to a concentration of 1 mg/ml, which was then diluted at a 2-fold ratio. The reactions of the serially diluted mAbs with rp24 revealed that 2G11 exhibited the highest titer and demonstrated exceptional immunoreactivity against BLV p24 (Fig. 2B).

Five positive sera and five negative sera were employed in the cELISA to evaluate competitive binding capability of the three mAbs with rp24. The results indicated that all five positive BLV sera effectively interfered with mAb 2G11 by >70 %, with a mean PI value of 80 %, while the five negative sera exhibited a PI value <20 % (Fig. 2C). For the other two mAbs, the mean PI value of all five positive BLV sera were 60 %. Thus, mAb 2G11 displayed the most effective competitive activity among the three mAbs.

To confirm the specificity of the mAb 2G11, IFA assay was performed utilizing six bovine viruses (BVDV, BRV, IBRV, BCoV, AKAV, and LSDV) and HEK293T-p24. Each of the virus-infected cells captured the corresponding mouse polyclonal antibody, while none of them reacted with the healthy mouse antibody. MAb 2G11 specifically interacted with HEK293T-p24 cells but did not exhibit reactivity towards cells infected with any other viruses (Fig. 2D), demonstrating excellent specificity towards BLV p24. Consequently, 2G11 was selected as the detection antibody for subsequent cELISA development.

3.3. Development of cELISA based on mAb 2G11

Following the checkerboard titration results, the optimal dilution of mAb 2G11 was determined to be $1{:}2^{12}$, and the most suitable coating concentration of p24 protein was 2.5 μg/ml (Supplementary Data Table S1). Correspondingly, the optimal dilution of sera was ascertained to be 1:2 (Supplementary Data Table S2). A preferential incubation time of 45 min for both serum and mAb, as well as the secondary antibody, was determined through a checkerboard assay. Similarly, a colorimetric reaction time of 10 min was determined (Supplementary Data Table S3). Finally, the best reaction conditions for the p24 cELISA were determined (Table 2), we performed the detection process for further evaluation (Fig. 3).

A set of 160 negative sera and 87 positive sera was utilized to determine the cut-off value, sensitivity, and specificity of the p24 cELISA. Thereafter, the percent of inhibition values for each sample were calculated, and subsequently delineated in an interactive dot plot diagram (Fig. 4A). Additionally, a ROC analysis was performed to determine of the cut-off value under appropriate diagnostic sensitivity and specificity of the assay (Fig. 4B). According to the ROC analysis, the area under the curve (AUC) was determined to be 0.9992 (95 % confidence interval: 0.9979 to 1.00). Furthermore, when the cut-off value was set at 44.45 % for the developed cELISA, the diagnostic sensitivity and specificity were established at 98.85 % (95 % confidence interval: 0.9377 to 0.9949) and 98.13 % (95 % confidence interval: 0.9463 to 0.9949), respectively. Hence, the cut-off value of PI was conclusively set at 44.45 %. Consequently, serum samples with a PI of <44.45 % were deemed negative, whereas those with a ≥ 44.45 % were categorized as positive. Notably, out of the 160 negative sera analyzed, 3 were erroneously identified as false positive with PI values of 46.05 %, 47.00 %, and 49.99 %, while only 1 out of the 87 positive sera fell into the category of false negative, with a PI value of 40.43 %.

3.4. Specificity and sensitivity of the p24 cELISA

To confirm the specificity of the cELISA, the positive sera specifically against six bovine viruses including BVDV, BRV, IBRV, BCoV, AKAV, and LSDV, together with BLV positive sera, were measured. The PI values of the six bovine virus sera were below 44.45 %, whereas the PI values of BLV positive sera were over 44.45 %. These results indicated that the cELISA exclusively detected antibodies against BLV but did not recognize antibodies against other bovine viruses in the sera (Fig. 5).

Furthermore, to evaluate the sensitivity of the developed cELISA, we examined the serum antibody dynamics in experimentally infected animals. Initially, the sera of four sheep, taken at different timepoints post-infection, were assayed using the p24 cELISA and a commercial gp51 cELISA kit. As shown in Fig. 6A, all infected sheep seroconversions were firstly detected by the commercial cELISA kit at the fourth week post-infection. In the p24 cELISA detection, two of the four sheep, S3 and S4, were seroconverted at the fourth week, while the other two sheep, S1 and S2, were seroconverted at the second week (Fig. 6B), earlier than the time detected by the commercial test. Furthermore, we compared the two methods in detecting antibody dynamics in cattle, the primary target animal of BLV infection naturally. Both the p24 cELISA and commercial gp51 cELISA kit exhibited similar dynamics of the BLV-specific immune response in the 3 infected cattle. All of them displayed a positive BLV-specific antibody response at the fourth week post-infection. Hence, these data validated that the p24 cELISA have excellent sensitivity for detecting serum dynamics in infected animals.

3.5. Repeatability and reproducibility of the p24 cELISA

Four BLV positive and four BLV negative sera were measured using developed the p24 cELISA on one plate on one run or on three plates in three separate runs to assess the cELISA's repeatability and reproducibility. As shown in Table 3. The intra-assay CV of the PI ranged from 0.54 % to 4.73 %, and the inter-assay CV of the PI ranged from 0.87 % to 6.84 %, indicating remarkable repeatability and reproducibility of the p24 cELISA.

3.6. Clinical performance assessing of the p24 cELISA

A total of 242 clinical serum samples were collected and subjected to BLV testing using the developed cELISA, the commercial ELISA kit, and

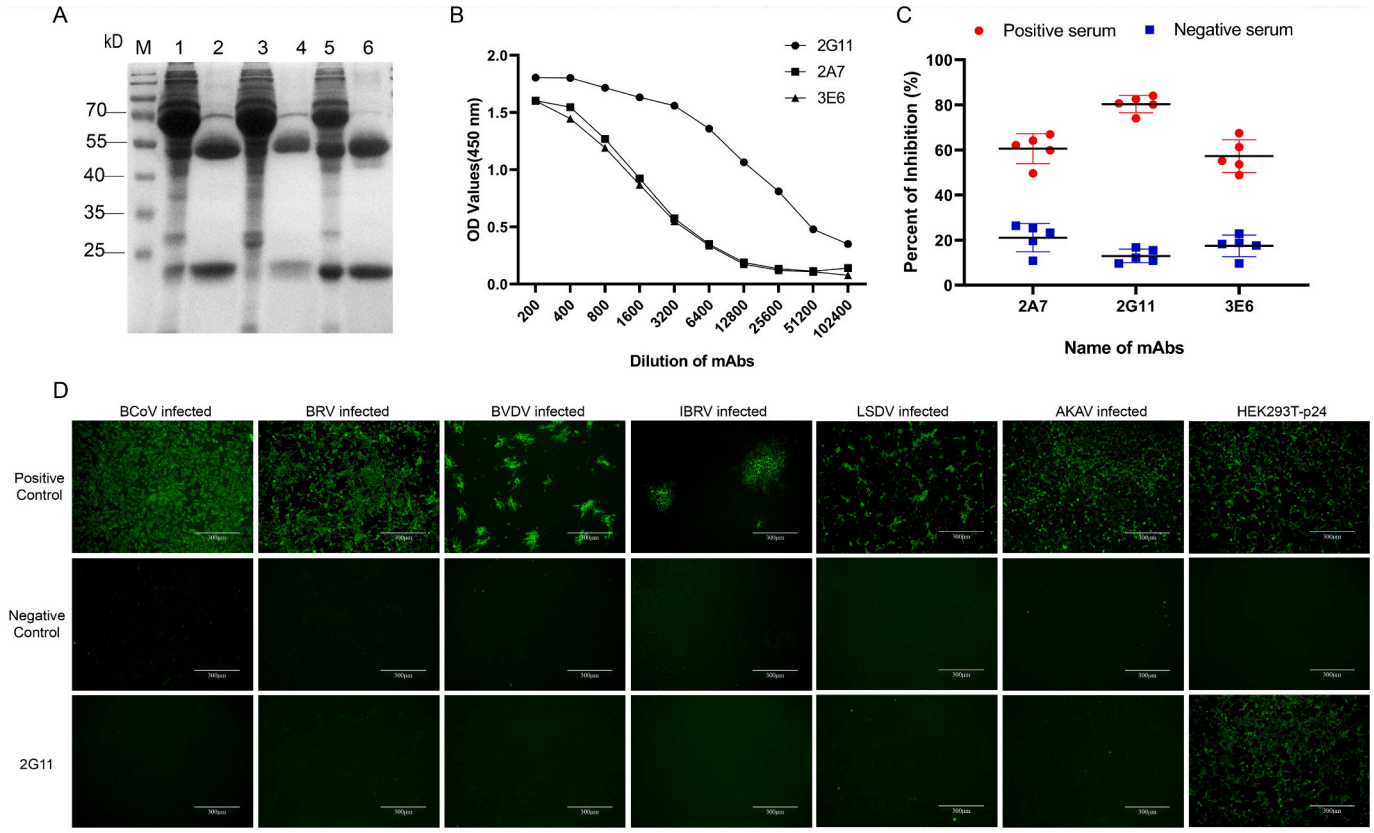

Fig. 2. Characterization of mAbs 3E6, 2G11, and 2A7. (A) Purification of mAbs 3E6, 2G11, and 2A7. The purified mAbs were analyzed by SDS-PAGE. M, protein marker; lane1, mAb 2G11 pre-purification; lane 2, mAb 2G11 post-purification; lane 3, mAb 3E6 pre-purification; lane 4, mAb 3E6 post-purification; lane 5, mAb 2A7 pre-purification; lane 6, mAb 2A7 post-purification. (B) Comparison of antibody reactivity to rp24 by iELISA. (C) Determination of the ability of mAbs to distinguish positive sera and negative sera by cELISA. Each symbol shape denotes the identity inhibition of serum for respective mAb. Five positive BLV sera (Red) and five negative sera (Blue) were tested, and the average PI of positive and negative sera was recorded for each mAb. (D) Determination the specificity of mAb responses to different viruses by IFA. Infected cells with BCoV, BRV, BVDV, IBRV, LSDV, and AKAV, and a stable HEK293T cell line expressing p24 were immunostained with mAb 2G11, while uninfected cells were used as negative controls. Scale, 300 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Optimized conditions of the P24 cELISA.

Optimized dilutions and reaction conditions	P24 cELISA
Coating condition	0.25 μg/well in CBS 4 °C, 12 h
Blocking condition	5 % skimmed milk 37 °C, 1 h
Serum samples and mAb 2G11	1:2 and $1:2^{12}$, respectively 37 °C, 45 min
The HRP-conjugated secondary antibodies	1:5000 37 °C, 45 min
Chromogenic substrate	100 μl RT, 10 min

Western blotting. The coincidence rates of the developed cELISA, compared with the commercial ELISA kit and Western blotting, were 94.63 % (229/242) and 97.52 % (236/242), respectively (Table 4). The statistical analysis showed that the developed cELISA had a high level of consistency with the Western blotting (Kappa = 0.95) and the commercial ELISA kit (Kappa = 0.88) (Table 4). No significant differences were found between cELISA and either the Western blotting or the commercial ELISA kit (all Kappa values were > 0.4). Thus, these results demonstrated a high level of concordance between our p24 cELISA, Western blotting, and the commercial ELISA kit, indicating that the p24 cELISA exhibited remarkable application potential for clinical detection.

4. Discussion

Given the widespread prevalence of BLV on dairy farms in China and the absence of commercially available vaccines for viral prevention [25,26], it is imperative to segregate infected cattle from healthy individuals through systematic detection methods to effectively control the spread of the virus within the herd. Seropositivity stands out as the most reliable indicator of BLV infection, since BLV-infected animals display a persistent immune response [27], marked by the production of high-titer antibodies against p24 and gp51 [18]. Although most commercial BLV serology methods primarily focus on testing antibodies against gp51 [19], the presence of antibodies against p24 is widely acknowledged as a crucial diagnostic marker for BLV infection [28,29]. Thus, we established a cELISA to detect antibodies against p24.

In this study, the *E. coli* expression system was chosen for expressing the p24 protein due to its cost-effectiveness, enabling rapid and economical antigen production. Moreover, to minimize the interference from tagged proteins, TEV protease was used to remove the GST tag (Fig. 1). The performance of the cELISA greatly relied on the properties of the mAb used. Here, we screened 3 distinct mAbs, of which 2G11 exhibited a higher reaction titer against the p24 protein compared to the other two mAbs, 3E6 and 2A7. (Fig. 2B). Importantly, BLV-positive serum demonstrated a stronger capability to compete with mAb 2G11 for binding to the p24 protein when compared to the other two mAbs (Fig. 2C). This suggests that mAb 2G11 likely represents the dominant antibody against p24 in BLV serum, and that the epitope it recognized most likely located within the dominant antigenic region of the p24

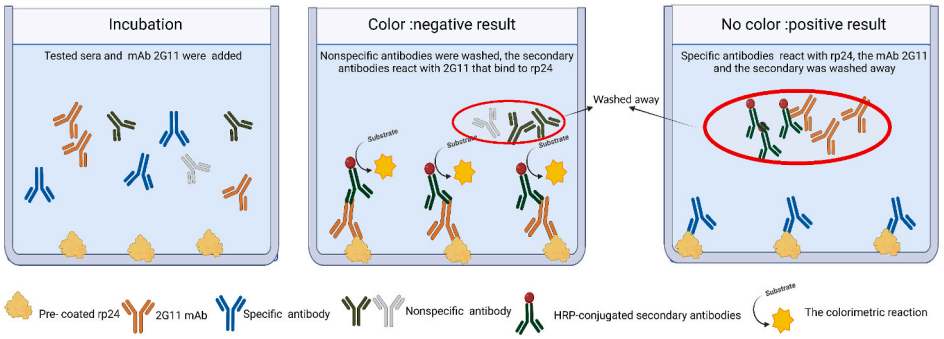

Fig. 3. Schematic diagram of the p24 cELISA. The specific antibody in the sample has the same binding site as the antibody conjugate. Through step-by-step incubation and washing, only one antibody can occupy the binding site. Little or no color is visible in the positive samples. Conversely, negative samples have a darker color.

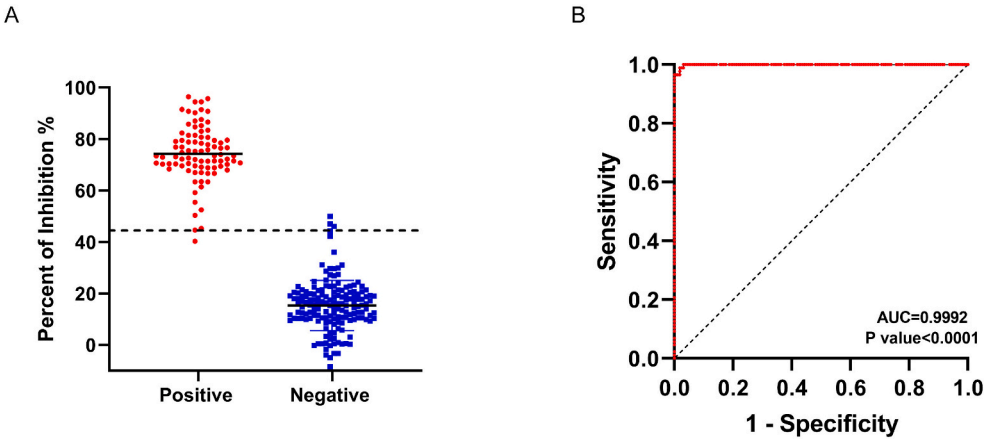

Fig. 4. ROC analysis for the p24 cELISA. The assay was conducted using BLV-negative sera (n = 160) and BLV-positive sera (n = 87). (A) Interactive dot plot diagram displaying the PI values of sera while the cut-off value was set to 44.45 %. (B) ROC analysis of cELISA results while the AUC of the test was 0.9992.

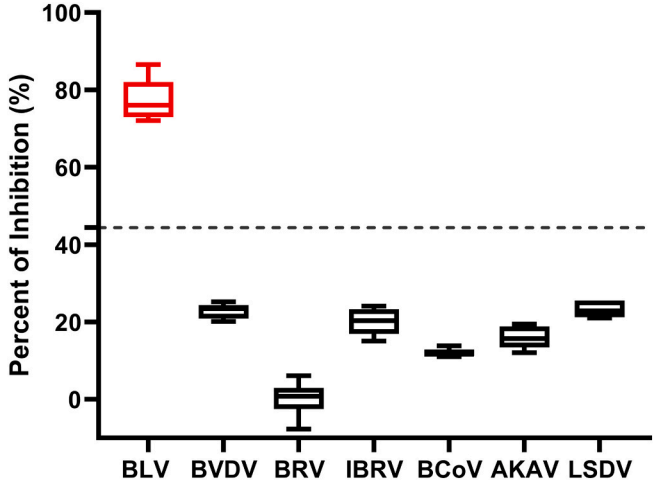

Fig. 5. Specificity of the p24 cELISA. Evaluation of the cELISA for detecting antibodies against seven bovine viruses, including BVDV, BRV, IBRV, BCoV, AKAV, LSDV, and BLV. The horizontal dotted line represents the cut-off value.

protein. Furthermore, 2G11 exhibited no cross-reactivity with other prevailing bovine viruses and specifically targeted the p24 protein of BLV (Fig. 2D). It not only reacted with HEK293T cells expressing the p24 protein in immunofluorescence assay (Fig. 2D), but also exhibited reactivity with BLV-persistently infected cells BL3.1 (ATCC CRL-2306) [30], with a distinct band at 24 kDa in Western blotting (Supplementary Fig. S1). The specificity of the mAb is crucial to ensure that the developed cELISA specifically detects BLV antiserum (Fig. 5). In conclusion, both the coated antigen and the mAb used in this study were suitably selected for the establishment of the cELISA.

During the development of the p24 cELISA, several key operational steps were optimized. Firstly, the optimal concentration of antigen and mAb was determined to minimize the usage of raw materials and reduce costs without affecting the optimal reactivity between the antigen and antibody. Next, the dilution fold of the serum sample was investigated using the minimum P/N value as the criterion. This optimization aimed to achieve the optimal sensitivity and specificity of the cELISA. By determining the appropriate dilution fold, the assay could accurately detect the presence of the p24 antibody in the serum. Additionally, the reaction time of the cELISA was optimized to ensure the assay could be completed in the shortest possible time. Through these optimizations, the established p24 cELISA became cost-effective, efficient, and convenient, providing a solid foundation for further development of commercial kits.

In the evaluation of the specificity of the cELISA, positive sera against six common bovine viruses circulating in China were selected. These viruses included BVDV, IBRV, BCoV, BRV, AKAV, and LSDV. Among these viruses, IBRV is commonly detected in dairy cows in China and affects the respiratory tract and reproductive system. BCoV and BRV are notable pathogens causing neonatal calf diarrhea. BVDV primarily causes immunosuppression and damage to the respiratory and digestive systems in cattle [31,32]. AKAV and LSDV are important arbovirus pathogens transmitted by vectors [33–35]. We found that no antigen was shared between these viruses with BLV via bioinformatic analysis. Furthermore, the sera against the six tested viruses were negative in the

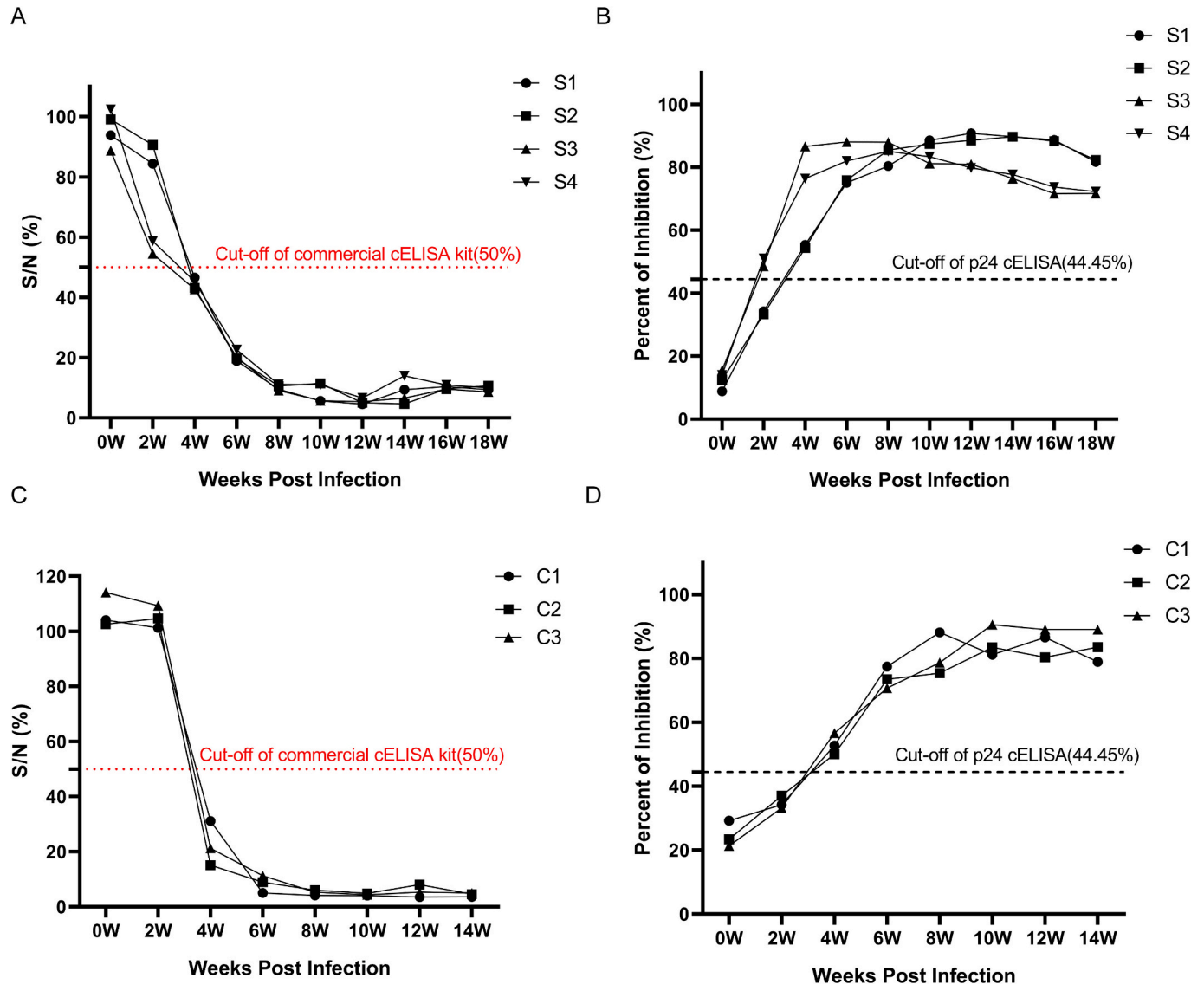

Fig. 6. Detection of the antibody dynamics to BLV using the p24 cELISA and a commercial cELISA kit. (A) Sera from experimentally infected sheep were tested for the presence of antibodies to BLV using the commercial cELISA. The red horizontal dotted line represents the cut-off value. (B) Sera from experimentally infected sheep were tested for the presence of antibodies to BLV using the p24 cELISA. The black horizontal dotted line represents the cut-off value. (C) Sera from experimentally infected cattle were tested for the presence of antibodies to BLV using the commercial cELISA. The red horizontal dotted line represents the cut-off value. (D) Sera from experimentally infected cattle were tested for the presence of antibodies to BLV using the p24 cELISA. The black horizontal dotted line represents the cut-off value. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Repeatability of the p24 cELISA.

Samples	Repeatability (intra-assay)			Reproducibility (inter-assay)		
	Mean PI (%)	SD	CV (%)	Mean PI (%)	SD	CV (%)
1	81.62	1.00	1.22	83.00	2.20	2.65
2	74.29	1.59	2.14	75.57	2.91	3.85
3	69.57	1.21	1.74	69.71	0.61	0.87
4	91.32	0.50	0.54	91.48	1.16	1.27
5	31.94	1.12	3.50	32.11	1.72	5.37
6	14.34	0.32	2.20	15.22	0.83	5.47
7	11.07	0.52	4.73	13.25	0.46	3.50
8	22.42	0.52	2.34	20.17	1.38	6.84

BLV P24 cELISA, with the PI value significantly lower than the cut-off value. This indicated that the cELISA exhibited remarkable specificity without cross-reactivity with the antisera of other common bovine viruses. Based on these considerations and the specificity evaluation results, our established p24 cELISA shows promise as a specific and reliable tool for detecting BLV antibody and can contribute to the study and control of BLV infections.

BLV naturally infects cattle, zebu and water buffalo, and experimentally infects other animals, such as sheep [36,37]. Compared with iELISA, cELISA is more convenient for the detection of BLV antibodies across various animals, because it eliminates the necessity to alter secondary antibodies. Here, we conducted BLV infections in both sheep and cattle, collecting serum samples at key time points to monitor antibody dynamics and assess the sensitivity of the p24 cELISA. In the experimental infected sheep (Fig. 6A, B), the established p24 cELISA demonstrated a sensitive detection capability for the humoral immune response induced by BLV infection, and even two out of four sheep exhibited seroconversion earlier than those detected by commercial gp51 cELISA kit. This discrepancy attributed to variations in sensitivity between the two detection methods, or it might be that p24 has better immunogenicity in sheep than that of gp51, as a previous study had

Table 4
Comparison of the p24 cELISA with Commercial ELISA kit and Western blotting.

Serum no.	cELISA	Commercial ELISA kit		Agreement (%)	Kappa value	Western blotting		Agreement (%)	Kappa value
		Positive	Negative			Positive	Negative		
82	Positive	75	7	94.63	0.88	79	3	97.52	0.95
160	Negative	6	154			3	157		

demonstrated that retroviral capsid proteins are highly immunogenic and more abundant than envelope glycoproteins in virions and infected cells [38]. Sheep serve as a crucial animal model for investigating the pathogenesis of BLV and vaccine development, and thus the developed p24 cELISA provides an effective tool for monitoring the antibody response to BLV infection and vaccination in sheep. In the bovine infection experiment, both the p24 cELISA and the commercial cELISA kit detected seroconversion at the fourth week post-infection (Fig. 6C, D) which was later than the second week reported by the previous study on p24 iELISA [29]. One reason might be that the characteristics of the two assays are different, iELISA is generally more sensitive, while cELISA is more specific. Additionally, variations in the breed and age of the inoculated animals, along with individual differences, could contribute to divergent induced immune responses [24]. Furthermore, another likely reason is that the inoculum used in the two infection tests is different, resulting in different efficiency of BLV infection [39,40]. Bai et al. inoculated BLV-negative cattle with leukocytes containing $>10^6$ copies of BLV provirus isolated from BLV-infected cattle, which is close to naturally occurring blood-borne infections and may induce a faster humoral immune response. In contrast, We inoculated cattle with the lysates from the cells transfected with BLV infectious clones pBLV344 [41]. Although the BLV reverse genetic system has been successfully applied to the study of BLV pathogenicity, the viral dose inoculated into animals is affected by the amount of transfected plasmid and the efficiency of the transfected plasmid assembling into viruses in cells. As a result, our infected cattle have a later seroconversion than infected cattle that are directly inoculated with leukocytes. In summary, the cELISA we established performs well in detecting the antibody dynamics of both infected cattle and sheep, showing a wide range of application.

Since there is no gold standard method for serological detection of BLV infection, the comparison of the p24 cELISA with a commercial gp51 cELISA kit and Western blotting was conducted in assessing the performance and accuracy of the p24 cELISA. A total of 242 serum samples are randomly collected from different farms in the northern regions of China, ensuring a broad representation of BLV infection in clinical settings. This diverse sample collection helps in capturing the variability of BLV prevalence and infection patterns in China, enhancing the generalizability and applicability of our assay. It was noted that the p24 cELISA demonstrated a slightly higher level of agreement with Western blotting (97.52 %, 236/242) compared to the commercial gp51 cELISA kit (94.63 %, 229/242) (Table 4). This result is not unexpected, as the p24 cELISA and the commercial kit detect antibodies against p24 and gp51, respectively. Given the variation in expression levels of these two proteins at different stages of infection and their differing immunogenicity towards the host, it suggests that neither method can completely exclude the possibility of false results. Therefore, in diagnostic and epidemiological investigations, combining both methods will yield more accurate results. In addition to its application in animals, p24 cELISA may also serve as a complementary method for studying the prevalence of BLV in the human body, complementing the detection of proviral DNA.

In conclusion, we successfully generated p24 mAbs against BLV and developed a cELISA for detecting antibodies against BLV using the mAb 2G11. The established cELISA exhibited a high degree of specificity, sensitivity, and reproducibility. In clinical testing, the cELISA showed high agreement with commercial ELISA kit and Western blotting. All data indicated that the p24 cELISA can serve as a complementary tool to the commercial gp51 ELISA kit in the detection of BLV antibodies. Therefore, the cELISA developed in this study is a valuable, simple, and reliable tool for serodiagnosis or surveillance of BLV, and may contribute to the prevention and control of BLV.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.131446.

Ethics statement

All animal experiments in this study received approval from the Animal Ethics Committee of Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences, under approval number 220401-01.

CRediT authorship contribution statement

Jing Wang: Writing – original draft, Investigation. **Chao Sun:** Investigation. **Zhe Hu:** Visualization, Resources, Conceptualization. **Fang Wang:** Resources. **Jitao Chang:** Writing – review & editing, Resources. **Ming Gao:** Methodology. **Dandan Ye:** Methodology. **Qi Jia:** Methodology. **Hui Zou:** Methodology. **Luc Willems:** Supervision. **Zhigang Jiang:** Writing – original draft, Project administration, Methodology, Investigation, Conceptualization. **Xin Yin:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that there is a patent related to this work (application no. 202410204796.6).

Data availability

No data was used for the research described in the article.

Acknowledgments

We thank Prof. Mingchun Gao and Prof. Jianfa Wang for generously providing a portion of the serum samples. This study was supported by the National Key Research and Development Program of China (grant no. 2022YFE0140700).

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