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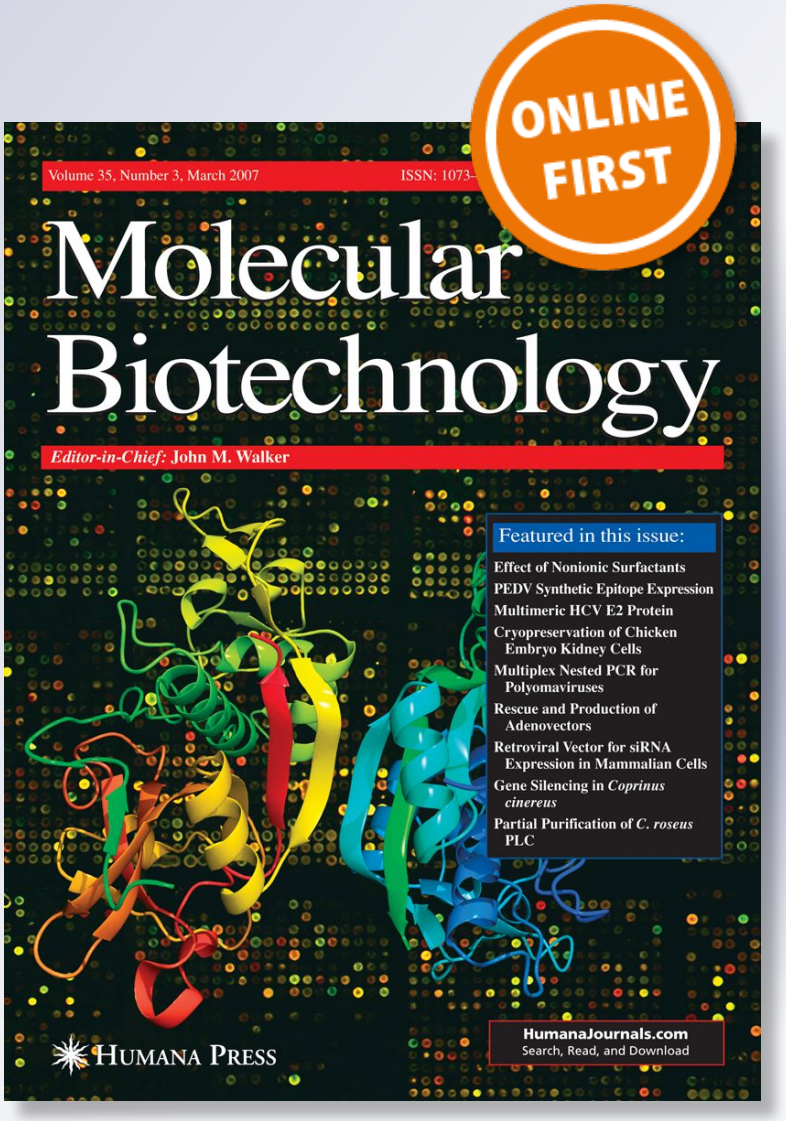

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RESEARCH

Expression of p24 gag Protein of Bovine Leukemia Virus in Insect Cells and Its Use in Immunodetection of the Disease

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Abstract Bovine leukemia is a common retroviral infection of cattle. The disease is characterized by a strong immunological response to several viral proteins, but the antibodies against p24 and gp51 are predominant. In this study, a recombinant baculovirus containing the gag gene *p24* was constructed and the protein, used as antigen, analyzed by western blot and an indirect in-house rp24-ELISA test. This allowed detecting the presence of antibodies for bovine leukemia virus in a panel of cattle sera. The authentication of the protein expands its potential use for different medical applications, from improved diagnosis of the disease to source of antigens to be included in a subunit vaccine.

Keywords Bovine leukemia virus · Immunodetection · Baculovirus system · Recombinant p24 protein · Immunoblotting assay

Introduction

Bovine leukemia virus (BLV), a B-cell lymphotropic retrovirus, causes a chronic lymphoproliferative neoplasic disease of cattle called enzootic bovine leukosis (EBL). Most of the infected animals remain clinically asymptomatic throughout their lives and act as carriers for the spread of the disease. Approximately, one third of BLV-infected cattle develop a persistent lymphocytosis, and only a minor fraction of these develop lymphosarcoma [1]. BLV infection is an "economic" disease. The main losses for producers arise from the impact on production and premature culling of the cows that show clinical signs. However, there may be an added factor to this economic loss that comes from the restricted market for BLV-infected animals. Many countries, especially those of the European Union, have a ban against cattle infected with BLV or any "live" product from these animals (i.e., semen and embryos) [2, 3].

Like the genomes of other complex retroviruses, the BLV genome contains the gag, pol and env structural genes and regulatory genes [4]. One of the earliest indications of infection is the onset of a humoral antiviral response at about 2–6 weeks post-inoculation. Antibodies recognize the epitopes from the env-encoded glycoproteins gp51 and gp30 as well as those from the gag-encoded proteins p24 and p15. They also recognize regulatory proteins such as Tax and Rex. While in experimentally infected animals anti-gp51 antibodies seem to appear earlier and at consistently higher titers than the p24 antibodies [5, 6], a certain number of naturally infected cattle develop an antibody response to p24 at a higher level [7–10]. Because the presence of antibodies to BLV is a constant and early feature of BLV infection, serological examination of cattle is the best method to control and detect infected animals. According to OIE, the serological tests most commonly used to detect BLV antibodies are agar gel immune diffusion (AGID) and the enzyme-linked immunosorbent assay (ELISA). A potential reason to apply a p24-specific

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test in addition to the commonly used gp51 test during an EBL advanced eradication campaign is that some cattle may develop a very important antibody response to p24, as mentioned earlier, and may not be detected during routine testing.

Recently, recombinant viral proteins have been found to be widely applicable in immunoassays for detection of specific antibodies. Different heterologous expression systems are used to produce recombinant proteins and, within these, the baculovirus expression system is one of the most popular and efficient. Baculoviruses are large (30–60 × 250–300 nm), rod-shaped, double-stranded DNA (80–180 kbp) viruses that are highly specific and capable of replication only in arthropod hosts [11]. This is why baculoviruses were first studied as biological control agents to protect crops and forests [12]. The *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) is the most studied baculovirus at the molecular level and most expression vectors are based on this virus [13]. There are many advantages of using the baculovirus expression system, such as its high expression levels and post-translational modifications that allow the expressed heterologous proteins to be correctly folded and biologically active [14, 15].

The use of baculovirus expression systems for the synthesis of p24 by recombinant baculovirus in insect cells has not yet been described. In this study, a recombinant baculovirus containing a cDNA encoding the *p24* gene of BLV was constructed and used to infect insect cells. The protein produced was tested for its antigenicity to obtain a recombinant p24 protein able to be used in diagnostic tests to detect the presence of anti-p24 antibodies in BLV-infected animals.

Materials and Methods

Cells and Viruses

BLV-infected FLK cells (BLV–FLK) from the National Veterinary Laboratory, Copenhagen, Denmark were cultured in Eagle's minimum essential medium (Sigma-Aldrich) containing 10 % fetal bovine serum (FBS). *Spodoptera frugiperda* (Sf21) insect cells were cultured in TC-100 Insect Medium (Sigma-Aldrich) containing 10 % of FBS. Linear DNA from the BaculoDirect™ C-Term Transfection kit (Invitrogen) derived from AcMNPV DNA was used to build the recombinant baculovirus. This allows transferring the gene of interest from the entry plasmid to the baculovirus DNA directly in vitro without the need of recombination in bacterial cells, using specific recombination sites from the bacteriophage lambda. The presence of the *Herpes simplex* virus thymidine kinase gene (*HSV1tk*) and the *lacZ* gene located between the two recombination sites allows inhibiting the replication of non-recombinant baculovirus in the presence of ganciclovir (Sigma-Aldrich) and determining viral purity using β-galactosidase staining [16, 17]. The expressed protein is placed in fusion with a tag containing hexahistidine and the V5 epitope sequence, a 14 amino acid peptide derived from the proteins P and V of the simian paramyxovirus SV5, to allow detecting and purifying the recombinant fusion protein [18].

Amplification of the *p24* Gene and Cloning

Genomic DNA was extracted from BLV–FLK cells by a commercial kit (Wizard® Plus Minipreps DNA Purification System, Promega) and then used as a template for the PCR technique. The *p24* gene was amplified by PCR using the specific oligonucleotides (5′-CACCATGCCAATCA TATCTGAA-3′) and (5′-GAGAAGTGCAGGCTGT-3′) located upstream and downstream of the *p24* gene, respectively. The oligonucleotides were designed according to the nucleotide sequence of the BLV genome bank (GenBank ID: K02120.1). An expected 642-bp PCR fragment corresponding to the *p24* gene was amplified and a *CACC* sequence was introduced in the forward primer for directional cloning into the entry vector (pENTR™/D-TOPO®—BaculoDirect™ GST Gateway®, Invitrogen), following the manufacturer's instructions.

Construction of the Baculovirus Entry Vector

The 642-bp DNA fragment corresponding to the *p24* gene, obtained by PCR, was separated by electrophoresis in an agarose gel (0.8 %) and purified from the gel using a commercial kit (QIAquick Gel Extraction Kit, Qiagen), according to the manufacturer's instructions. The amplification product was re-amplified in electrocompetent *Escherichia coli* cells (One Shot® TOP10F′, Invitrogen). Clones containing the *p24* gene were amplified in Luria–Bertani (LB) broth and plasmids were purified with a commercial kit (QIAprep® Spin Miniprep Kit, Qiagen). To confirm the insertion in frame and the absence of mutations in the *p24* gene, plasmids were sequenced by the Nucleic Acid Exploration Facility, University of Wyoming, Wy, USA, using M13 forward and reverse primers. The sequencing results were analyzed by comparing with databases and using the basic local alignment search tool (BLAST) software.

Construction of the Recombinant Baculovirus

To obtain the recombinant baculovirus, the reaction was performed for 18 h at room temperature in a microcentrifuge tube containing 100 ng (2 µl) of the purified entry

vector, 4 μl of 5× reaction Buffer and 4 μl of enzyme mix (Gateway LR Clonase Plus Enzyme Mix, Invitrogen). After incubation, 2 μl of proteinase K solution (Invitrogen) was added to the reaction and incubated for 10 min at 37 °C. Lipid-mediated transfection of the Sf21 cells was performed with Cellfectin reagent (Invitrogen) in six-well plates. Each well was seeded with 1.5×10^6 Sf21 cells and cells were allowed to attach for 1 h at room temperature. The transfection mixture was prepared with 10 μl of recombination reaction (LR), 6 μl of Cellfectin reagent and 200 μl of unsupplemented Grace's Insect Medium (Invitrogen), and incubated at room temperature for 45 min. The medium was removed from each well and wells were carefully rinsed with fresh medium. Then, 800 μl of unsupplemented Grace's Insect Medium was added to the transfection mixture and dropped onto the cells. The plate was incubated at 26 °C for 5 h. After incubation, the transfection mixture was removed and 2 ml of complete growth medium with 10 % FBS, antibiotics and 100-μM ganciclovir (Sigma-Aldrich) were added to each well. The plate was incubated at 27 °C for 72 h in a moisturized box. When the first signs of infection appeared, the cell culture medium containing virus was harvested and purified by plaque assay. Isolated recombinant viruses were propagated and the supernatant was designated as P1 viral stock. To prepare a high-titer viral stock, 500 μl of the P1 viral stock was used to infect 1.5×10^6 Sf21 cells in 1.5 ml of complete growth medium with antibiotics and 100-μM ganciclovir (Sigma-Aldrich). The plate was incubated at 27 °C for 72 h in a moisturized box. When the first signs of infection appeared, the cell culture medium containing virus was harvested and designated as P2 viral stock. PCR was used to confirm the presence and orientation of the *p24* gene in the recombinant baculovirus.

Protein Concentration Assay

Protein concentration was determined by thin layer chromatogram scanning and the Bradford total protein content assay using the Bio-Rad Protein Assay kit (Bio-Rad) with bovine serum albumin (BSA) as the standard.

Recombinant Protein Expression Analysis and Antigenicity Assay by Western Blot

Sf21 cells were infected with the recombinant baculoviruses at a multiplicity of infection (MOI) of 1 and incubated at 27 °C. At 3 days post-infection, cells were harvested, lysed by the buffer sample and then analyzed in duplicate in a discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 12 % according to the target protein molecular weight. The gel was stained with Coomassie blue and other gel was transferred to a nitrocellulose membrane (Sigma-Aldrich) by the standard electric transfer protocol for western blot analysis. Briefly, the membrane was blocked at room temperature with PBS buffer (0.025 % Tween-20) containing 4 % skim milk and then incubated at 37 °C for 1 h in a 1:100 dilution international reference anti-BLV serum sample E4 (Veterinary Laboratories Agency, New Haw, Adelestope Surrey, UK) as a positive control, and also a panel of 22 representative sera covering the biological variability of cattle sera: 16 from BLV naturally infected cows and 5 from non-infected cows. The panel included a commercial anti-p24 monoclonal antibody (VMRD Inc.). To perform the analysis, all the cow sera were diluted 1:100 and the monoclonal antibody was diluted 1:500.

After 1 h, the membrane was washed and subsequently treated with horseradish peroxidase (HRP)-conjugated goat anti-bovine IgG antibody and rabbit anti-mouse HRP-IgG conjugated antibody (Sigma-Aldrich) at a 1:5,000 dilution for 1 h at 37 °C. Immunoreactive bands were detected by standard techniques for HRP enzyme.

Standardization of the In-House Indirect rp24-ELISA Procedure

The optimal dilutions of the recombinant p24 antigen and serum were determined by a checker board titration test with positive and negative BLV sera. The antigen was coated in 96-well ELISA plates (Nunc Immuno Plate, PolySorb, InterMed, Denmark) ranging from 10 to 0.25 μg/ml diluted in 0.05 mol/l carbonate buffer (22.4 mmol/l $NaHCO_3$, 11.9 mmol/l Na_2CO_3, pH 9.6). Both reference positive and negative sera diluted serially from 6.25 to 200 were used and tested to determine the optimal serum dilution. The dilutions that gave the maximum difference in absorbance at 405 nm between positive and negative sera were selected to test the sera panel. The working dilution of goat anti-bovine HRP-IgG (Sigma-Aldrich), the reaction temperature, time and other conditions were also optimized.

In-House rp24-ELISA Procedure

After optimization, 100 μl of 2.5 μg/ml rp24 protein in 0.05 mol/l carbonate buffer (pH 9.6) was coated onto the wells of an ELISA plate, and the plate was incubated at 37 °C for 2 h. The antigen-coated plate was washed three times with PBST (0.01 mol/l phosphate-buffered saline, pH 7.4, 0.05 % Tween 20) and blocked with PBST containing 1 % (W/V) skimmed milk at 37 °C for 2 h. After washing, 100 μl of the 1/100 diluted sera panel was added, and then incubated at 37 °C for 2 h. After washing, 100 μl of goat anti-bovine HRP-IgG antibodies (1:2,500) was added and incubated at 37 °C for 1 h. The plates were washed three times and then incubated with 100 μl of

30 mg of 2,2′-azino-bis-(3-benzthiazoline-6-sulfonic acid) (ABTS) (KPL, Inc.) substrate in 100-mM phosphate–citrate buffer pH 4.0 and 3 µl of 30 % H_2O_2 for 15 min at room temperature. The optical density (OD) of each well was read at 405 nm using a microplate reader (Bio-Rad). Duplicate positive and negative control sera were included in the plate.

The sera panel mentioned before was tested by our in-house rp24 ELISA, in addition to AGID and indirect p24 ELISA developed by Gutierrez et al. [19] (data not shown).

The negative serum samples as determined by western blot, AGID and p24 ELISA [19] tests were used to determine the cut-off value. All sera were subjected to the in-house rp24-ELISA three times to abate the deviation. The cut-off value was defined as the mean OD values of all negative sera plus three standard deviations between them (mean + 3 SD).

Results

Generation of the Baculovirus Entry Vector and the Recombinant Baculovirus

After cloning the PCR product (Fig. 1) to the entry vector and transformation of competent cells, eight clones were isolated and screened with a specific PCR using the primers mentioned in the Materials and Methods section (Fig. 2). Only four clones were transformed with the plasmid containing the *p24* gene of BLV in the correct orientation. The four plasmids were designated p24/1, p24/2, p24/3 and p24/4. The two entry vectors p24/2 and p24/3 were

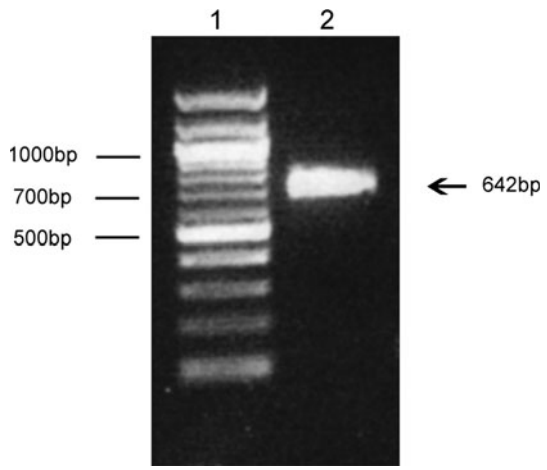

Fig. 1 DNA amplification of the *p24* gene. Agarose gel (0.8 %) electrophoresis showing the amplification of the BLV *p24* gene by PCR. *Lane 1*: molecular marker and *lane 2*: amplification product of approximately 642 bp

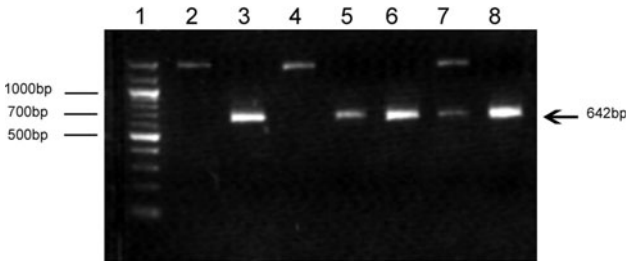

Fig. 2 DNA amplification of the *p24* gene in the baculovirus entry vector. Agarose gel (0.8 %) electrophoresis of the amplification by PCR of the BLV *p24* gene inserted in the baculovirus entry vector, showing a unique fragment with the expected molecular size of 642 bp. *Lane 1*: molecular marker; *lanes 2, 3, 4, 5, 6, 7 and 8*: clones 1–7, respectively. Clones 2, 4, 5 and 7 contained the *p24* gene in the correct orientation

sequenced and both constructs contained the *p24* gene in frame. Sequencing showed two substitutions that differed from the published sequence (GenBank ID: EF600696.1), in position 535 and 640 of the *p24* gene in both plasmids, resulting in an amino acid change from arginine to lysine and leucine to valine in positions 179 and 214 of the protein, respectively. The entry vector p24/3 was chosen to set up the recombination reaction.

Three days after cell transfection, as indicated in the Materials and Methods section, clear signs of cell infection (i.e., cessation of cell growth, increase in cell diameter and detachment of cells from the monolayer) were observed. The supernatant was collected and a plaque assay was performed to isolate individual plaques. Two possible recombinant viruses were propagated, the supernatant collected and Sf21 cells re-infected. After staining with 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-Gal) (Sigma-Aldrich), all the cells used to produce the P2 viral stock were blue, showing that there were no more non-recombinant baculovirus in the viral stock. PCR controls of the presence and orientation of the *p24* gene in baculovirus DNA were all positive (data not shown).

Detection of the Recombinant Protein Expression and Purification

Insect cell extracts infected with the p24 recombinant baculovirus (p24RBV) were used in an SDS-PAGE gel to detect the p24 protein. When compared with uninfected cells, infected cells presented a protein band of around 24 kDa (Fig. 3). The 24-kDa recombinant protein produced had the expected size for the recombinant protein. Analysis by western blot showed the presence of the p24 protein with a molecular weight of 24 kDa. The size of the recombinant protein approximates to the predicted molecular weight of the p24 fusion protein containing the C-terminal tag.

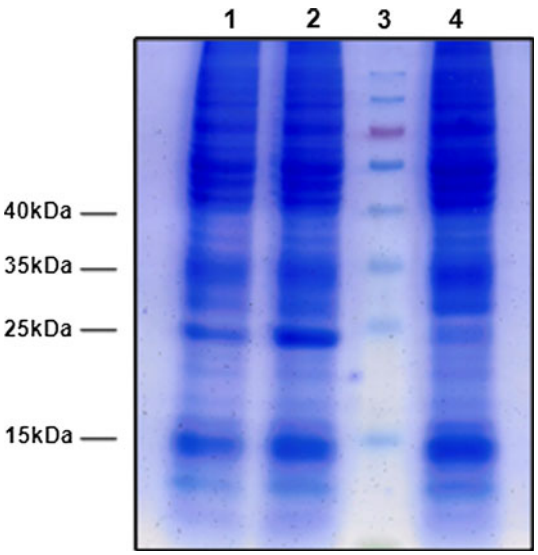

Fig. 3 SDS-PAGE gel of Sf21 cells non-infected and infected with the p24RBV stained with Coomassie blue. *Lanes 1 and 2*: cells infected with the p24RBV; *lane 3*: prestained molecular weight marker; and *lane 4*: mock-infected cells

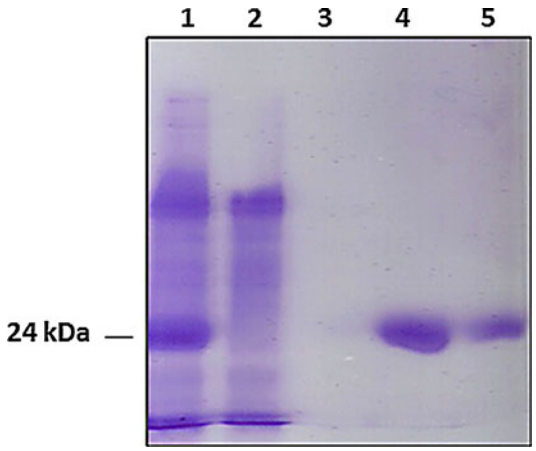

Fig. 5 SDS-PAGE gel purification of p24 recombinant protein from Sf21 cells p24RBV infected. *Lane 1*: supernatant of Sf21 infected with the p24RBV after lysis and centrifugation; *lane 2*: flow-through of immobilized-metal (Ni2+) affinity chromatography (IMAC); *lane 3*: flow-through of the last wash of the column; and *lanes 4 and 5*: first and second eluates from IMAC

Analysis of the cell lysate showed that the recombinant protein is expressed in infected Sf21 cells (Fig. 4). The presence of 2 % FBS in the culture medium increased the production of the recombinant p24 protein.

The purification of the p24 recombinant protein from Sf21 cells by a simple one-step Ni2+ affinity purification procedure is shown in Fig. 5. The concentration of the purified protein was determined with a commercial kit (Micro BC Assay protein Quantitation Kit, Uptima-Interchim), and a total of 0.2 mg of purified recombinant protein was obtained from 5×10^7 Sf21 cells. The p24 recombinant protein tested by western blot analysis against the sera panel mentioned in Materials and Methods (Fig. 6) accurately differentiated positive from negative samples. The results obtained by western blot analysis showed 100 % consistency with the results obtained by AGID and indirect p24 ELISA [19] demonstrating that the antigenic response of the p24 recombinant protein is equivalent to that of the antigens used in the AGID and ELISA assays.

Based on the result of the western blot, we developed a rapid, sensitive and specific in-house rp24 ELISA using purified recombinant p24 protein as antigen.

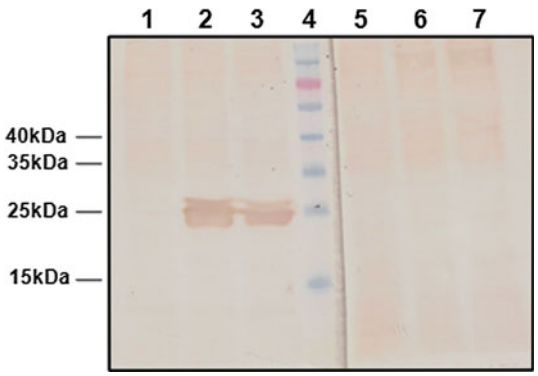

Fig. 4 Western blot analysis of recombinant p24 protein of BLV in Sf21. Proteins were separated by SDS-PAGE and analyzed by immunoblotting. *Lane 1*: mock-infected cell lysate; *lanes 2 and 3*: cell lysate infected with the p24RBV; *lane 4*: prestained molecular weight marker; *lanes 5 and 6*: p24RBV-infected cell lysate; and *lane 7*: mock-infected cell lysate. Samples 1–3 were analyzed using international reference anti-BLV serum and samples 5–7 were analyzed using negative control serum as primary antibodies

In-House rp24-ELISA Procedure

The recombinant p24 protein was tested for its suitability as a diagnostic antigen in the indirect rp24 ELISA. The checker board titration tests indicated that the OD value that gave the maximum difference between positive and negative sera (P/N value of 6.530) was when the final concentration of coating antigen was 2.5 μg/well and the serum dilution was 1:100. An optimal dilution at 1:2,500 for the goat anti-bovine HRP-IgG was determined. All the experiments were performed in triplicate.

In the optimized test conditions as described earlier, the negative serum samples were used to decide the cut-off value, which revealed a mean OD value of 0.768 with a standard deviation (SD) of 0.070. Therefore, the suitable cut-off value to decide the status of serum samples in the rp24-ELISA was set as 0.978 (mean + 3 SD). The serum sample with an absorbance ≥0.978 was scored as BLV-antibody-positive; otherwise, it was determined to be BLV-antibody-negative (Table 1).

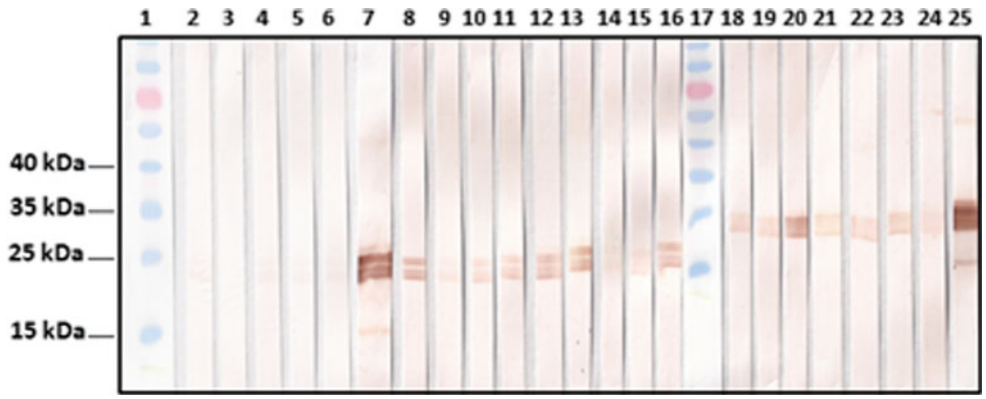

Fig. 6 Characterization of the p24 recombinant protein of BLV expressed in insect cells. The western blot analysis was carried out with a panel of representative cow sera. *Lanes 1 and 17*: prestained molecular weight marker; *lanes 2–6*: 1–5 sera samples from non-infected cows; *lane 7*: international reference anti-BLV serum; *lanes 8–16* and *18–24*: 6–14 and 15–21 sera samples from positive naturally infected animals, respectively; and *lane 25*: anti-p24 monoclonal antibody

Table 1 In-house rp24 ELISA assay in optical density (OD_{405nm}). Results of the sera panel

Sera panel	OD_{405nm}	Sera results
1	0.7912	−
2	0.7031	−
3	0.7720	−
4	0.8743	−
5	0.7942	−
6	1.7241	+
7	0.9897	+
8	1.0472	+
9	0.9901	+
10	1.4943	+
11	1.7474	+
12	1.7135	+
13	0.9886	+
14	0.9928	+
15	1.2052	+
16	1.3411	+
17	2.0135	+
18	1.4164	+
19	1.2923	+
20	1.3335	+
21	0.9930	+

Agreement Assessment for Western Blot, AGID, p24 ELISA [19] and In-House rp24 ELISA

The in-house rp24-ELISA showed 100 % of concordance when compared with the western blot, the AGID test and the indirect p24 ELISA [19]. All the assays were tested with the sera panel.

The current in-house rp24 ELISA detected the specific BLV antibodies present in all the positive samples, including the low-reacting samples by western blot technique, indicating a sensitivity equivalent to that of the AGID test and the indirect p24 ELISA [19]. All the negative control sera showed a baseline response, providing no evidence of non-specific binding in this assay.

Discussion

In Argentina, BLV infection is endemic with about 84 % of the dairy herds and 34 % of dairy cattle infected [20]. BLV-infected animals mount a persistent immune response, characterized by high-titer antibodies directed to the envelope glycoprotein gp51 together with the p24 major structural protein [21, 22]. Therefore, seropositivity is one of the best indicators of BLV infection.

In this study, the p24 recombinant protein was chosen because, certain number of naturally infected cattle have been found to develop high level of antibody response to p24 [1, 7, 8], in addition to gp51 protein antibody response.

The expression of proteins from different viruses, such as bovine immunodeficiency virus [23] and bovine respiratory syncytial virus [24], has been already described. In the last decade, BLV gag proteins p24 have been expressed in heterologous expression systems such as *Escherichia coli* [25–28] or *Saccharomyces cerevisiae* [29]. In a direct comparison study for recombinant protein production in the *E. coli*, *Pichia pastoris*, *Saccharomyces cerevisiae* and *Spodoptera frugiperda* systems, Christopher et al. [30] reported that baculovirus-mediated expression in Sf21 cells is the most efficient system capable of producing large amounts of recombinant protein.

In this study, *p24* gene from BLV–FLK (GenBank ID: EF600696.1) inserted in the baculovirus entry vector was subjected to sequence analysis and showed 99.7 % homology with the BLV strains from Japan (GenBank ID:

K02120.1) and USA-Maryland (GenBank ID: M10987.1), and 98 % of homology with those from Argentina (GenBank ID: AF257515.1) and Australia (GenBank ID: D00647.1). Sequence analysis of the *p24* gene in the vector and comparison of other known sequences of the *gag* gene available in GenBank indicates the success in cloning the gene into the vector. The p24 protein is highly conserved in worldwide BLV strains and shares common stable epitopes that can cross-react [31]. In fact, in this study, a change was shown in the in-house amplified and expressed p24. It is very likely that the changes occurred as a consequence of the continuous passages of the FLK cell line in the laboratory. However, the substitutions from arginine to lysine and leucine to valine are substitutions to a similar amino acid. Therefore, the bonding properties of the amino acids are nearly identical and should not cause any issues regarding the function or structure of the protein, as demonstrated by western blot and ELISA analysis. However, characterization of the p24 nucleotide and amino acid sequence of natural variants should be carried out to ensure that the circulating variants are not variable enough to produce antibodies that do not match with the recombinant p24.

In this report, Sf21 cells infected with p24RBV expressed p24 recombinant protein. The predicted molecular weight of the recombinant protein, including the C-terminal tag, was determined to be 24 kDa. The size of the recombinant protein was determined by SDS-PAGE and the antigenicity by western blot and ELISA.

The infected cell extracts analyzed by western blot were positively immunostained with the international reference polyclonal antibody raised against BLV. Purified recombinant p24 protein was used as a diagnostic reagent in western blot and ELISA analysis. The results obtained from the sera panel reflected the accuracy of the antigen to detect positive and negative reactors when compared with the results obtained from the other two well-known tests: AGID and p24 ELISA [19]. The fact that the whole sera panel recognized the p24 recombinant protein confirms that this protein was antigenically similar to the intact virus. In addition, no cross-reaction was observed with BLV negative sera that may have contained antibodies against many pathogenic and non-pathogenic viruses. Our western blot and ELISA analysis results showed a complete correlation with the AGID and p24 ELISA test results, suggesting that the recombinant p24 protein exhibits the antigenic epitopes and conformation necessary for specific antigen–antibody recognition.

The preliminary results of the use of recombinant p24 protein as antigen for diagnosis and the validation approach of the in-house rp24 ELISA are very promising and ensure the suitability of the assay as a standard laboratory tool, including verification by other tests such as AGID and indirect p24 ELISA [19]. The performance of our in-house rp24 ELISA will be improved with further development and validation of the technique, including a larger number of serum samples.

Because cross-reactivity with different bovine retroviruses during serological tests is possible [32], the use of recombinant proteins of the virus, or even of smaller parts of it, may diminish this kind of problem. This could also reduce costs, because nowadays, the use of the whole virus as antigen for diagnosis is quite expensive.

The western blot immunoassay and the in-house rp24 ELISA described in this publication could help to confirm BLV infections by seroconversion detection and could be used in further studies towards the development of an immunodiagnostic test for BLV, to obtain new epidemiologic data about EBL.

Concerning the binding antibodies of gp51 protein, there is disagreement between the results achieved with the conventional serological tests and the western blot assays [33, 34]. However, the antibody reaction against the gag-related protein p24 is very clear, even when just after infection the reaction against gp51 may be weaker or absent [21]. While in naturally infected animals, anti-p24 antibodies were found less frequently and at lower titers than anti-gp51, their detection by western blot analysis was consistent, despite the use of crude BLV antigen containing both p24 and gp51 [35, 36].

The recombinant p24 protein produced and the potential use in indirect ELISA assay could be especially useful in the eradication campaign of the disease. Furthermore, the potential development of an ELISA containing both the gp51 and p24 proteins as antigens could allow detecting BLV-infected animals, minimizing the number of false-negative results.

When p24 recombinant protein was used as an antigen in the immunodiagnostic test, we did not observe non-specific reaction to components of the cell culture medium or other viruses that might be present in the producer cell line. As shown here, there was only one band reactive with any of the sera.

The use of recombinant p24 protein could be extended in the development of a safer vaccine which does not include the active virus at all. Furthermore, the detection of anti-p24 antibodies could potentially discriminate between vaccinated and naturally infected animals, if BLV envelope glycoproteins were used as immunogens [10, 37].

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