IMMUNOCHROMATOGRAPHIC ASSAY FOR DIAGNOSIS OF BOVINE LEUKAEMIA VIRUS INFECTION IN COWS USING THE RECOMBINANT PROTEIN GP51

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Abstract. Infection with bovine leukaemia virus (BLV) is prevalent in herds in Kazakhstan. Current diagnostics of the BLV infection in Kazakhstan is based on the detection of antibodies against viral proteins p24 or gp51. Two methods are routinely used, enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID). Detection of the anti-gp51 antibodies has some advantage over targeting the anti-p24, because the anti-gp51 are present in higher titers and appear sooner after the onset of the disease. We describe an immunochromatographic (ICA) assay to detect the BLV infection in cows which utilizes recombinant protein gp51 as a capture antigen. Diagnostic characteristics of the ICA were determined in comparison with gold standard (AGID) using a panel of 310 cow sera. The ICA showed excellent diagnostic properties, such as high specificity (165/165, 100%) and sensitivity (141/145, 97.2%).

Keywords: enzootic bovine leukosis; gold nanoparticles; antigen gp51; immune chromatography; recombinant antigen

Introduction. Enzootic bovine leukosis is a contagious disease of cattle which is of high importance for herders because it leads to loss of productivity and ultimately necessitates euthanasia culling of cows. The disease is caused by infection with a retrovirus (bovine leukaemia virus, BLV), which belongs to the genus *Deltaretrovirus* of the *Retroviridae* family. The BLV infects lymphocytes, leading to their uncontrolled proliferation which in turn causes malfunction of lymphoid organs. BLV infection is most frequently found in dairy cows (Meas et al., 2002, Ravazzolo et al., 2007).

The BLV-associated leukosis is a persistent problem in many countries across the world. Disease results in considerable economic losses to farms (Burridge et al., 1981, Coulston et al., 1990, Zhao and Buehring, 2007) because of the decreased production of milk, export of meat (some countries prohibit importation of meat form the BLV-infected cows), costs of diagnostic and preventive measures, and the removal of infected livestock from production and breeding.

The BLV infection in all cases leads to the development of an immune response, including the appearance of antibodies against viral proteins. Considering the BLV biology, efficacious screening of livestock for this infection is possible with the use of serologic tests. Diagnostically important antibodies appear in the sera at 2^{nd}-8^{th} weeks post infection. The largest fraction of induced antibodies is targeted at the components of BLV virions: gp51 (glycoprotein exposed on the outer surface of BVL virions and on cytoplasmic membranes of the infected cells) and p24 (capsid protein forming the RNA-protein complex within the virion) (Troiano et al., 2013).

First serologic assays for the BLV infection utilized viral antigens which were produced in FLK cell cultures (sheep embryonic kidney). The FLK is a cell line chronically infected with the BLV (Van Der Maaten et al., 1975). In the last ten years, expression systems were developed to produce the recombinant gp51 in bacteria (*Escherichia coli*) (Ban et al., 1992, Siakkou et al., 1990, Ulrich et al., 1990) or yeast (*Saccharomyces cerevisiae*) (Legrain et al., 1989). This antigen was also produced in mammalian cell cultures with use of viral vectors such as cowpox virus (Kumar et al., 1990, Portetelle et al., 1991) or baculovirus (De Guiseppe et al., 2004, Kabeya et al., 1996, Russo et al., 1998).

Santos et al. (2012) used a phage display to select peptides which were termed as “peptide mimetic” of the epitopes of the BLV antigens. Selected peptides were used in a serologic assay. Aminoacid sequences of the peptide mimetics revealed homology to fragments within the sequences of BLV proteins p24, gp52 and gp30: three peptides D10, G5 and A9 were peptide mimetics of the p24, and one peptide D4 was homologous to a known linear epitope in the gp51.

Simard et al. (2000) compared four commercially available BLV enzyme-linked immunosorbent assay (ELISA) kits with the agar gel immunodiffusion (AGID) test. ELISA kits based on the gp5l protein gave an excellent correlation with the AGID test. Based on the dilution experiment, the gp5l ELISA kits were found to be more sensitive than the AGID test. Although the AGID has some advantages, such as low costs and use of simple equipment. Because of high sensitivity, ELISA is preferable to use with pool of sera. Initial testing of the pool of sera during screening of herds is a way to reduce costs of screening, especially in regions where BLV prevalence is low.

Modern time-saving diagnostic techniques such as the immune chromatographic assay (ICA) gain increasing

applications for rapid diagnostics of various infectious and noninfectious diseases. ICA is rapid and easy to use test, suitable for application in field conditions because it is an instrument-independent. In remote regions this is a crucial advantage over the instrument-dependent assays because no transportation of samples is needed. Furthermore, for some applications the sensitivity and specificity of ICA are not inferior to that of ELISA.

In this paper we describe the development and assessment of diagnostic properties of the ICA to diagnose BLV infection. Described ICA utilizes gold nanoparticles with adsorbed recombinant protein gp51 which is used to trap specific antibodies.

Materials and methods

Production of recombinant gp51. Sequence of the gp51 gene was obtained from public database: https://www.ncbi.nlm.nih.gov/protein/BAP81672.1. DNA fragment comprising the gene of interest, optimized for expression in *E. coli,* was synthesized *de novo* in a constructive PCR. The DNA fragment was cloned into expression vector pET32a (Invitrogen, Carlsbad, USA), which provides expression of a fusion protein. The fusion protein has *E. coli* thioredoxin on the N-terminus, gp51 on the C-terminus and His-tag for affinity chromatography in a linker region. All genetic engineering was performed using protocols published in Maniatis et al. (1982). The cloned gene was sequencing-verified (Sanger et al., 1977). Expression strain *E. coli* BL21(DE3) was transformed with the expression construct using procedure of Hanahan (1983). Immobilized metal affinity chromatography (IMAC) on columns HisTrap HP 1 ml (GE Healthcare, Sweden) was used to purify the recombinant protein gp51. Purification was performed in accordance with the manufacturer's instructions.

Serum samples and reference viral antigen. Samples of cattle sera (n=310) were obtained from the Republican Veterinary Laboratory (RVL, Astana, Kazakhstan). All sera were initially tested in the commercial AGID test ("Antigen", Almaty, Kazakhstan) according to the manufacturer's instructions. Negative control serum (NCS) and a positive control serum (PCS) containing antibodies to BLV antigens were used to test the ICA performance. The NCS and PCS are commercially available materials which are used in all veterinary laboratories in Kazakhstan as control samples in AGID. The NCS, PCS and BLV antigen produced in the FLK cell cultures were purchased from LLP "Antigen" (Almaty, Kazakhstan).

Western blot. IMAC-purified recombinant gp51 was separated in 12% SDS-PAGE. Upon electrophoresis, proteins were transferred to nitrocellulose membrane (Millipore). The membrane was blocked in a phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 1% bovine serum albumin (BSA). The membrane was incubated with an anti-His-tag mouse monoclonal antibody (Sigma-Aldrich, St. Louis, USA) as a primary antibody and then with a peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich, St. Louis, USA) as a secondary antibody. The immune complexes were detected by reaction with 4-chloro-1-naphthol and hydrogen peroxide.

Preparation of conjugate with colloidal gold. Colloid gold nanoparticles were produced using a hot chloroauric acid-sodium citrate method described in Byzova et al. (2010). Gold nanoparticles were used to produce conjugate with recombinant protein G (Sigma-Aldrich, St. Louis, USA), which binds to Fc fragment of IgG with high affinity. Before preparing the conjugate, pH of the colloid solution was adjusted to 6.0 by addition of 0.2 M K_2CO_3. Then the amount of protein G which provides saturation of the surfaces of gold nanoparticles was measured. For this purpose portions of 100 µl of the colloid solution were dispensed into wells of a 96-well ELISA plate. Aliquots (10 µl) of the progressive dilutions of protein G were added to wells and the plate was incubated for 10 min at room temperature. Upon that, aliquots (10 µl) of 10% NaCl were added to each well. Adsorption of the protein onto gold nanoparticles results in changes of color of the solution, from red to blue. Optic adsorption in wells was recorded at 510 nm and 550 nm using plate spectrophotometer.

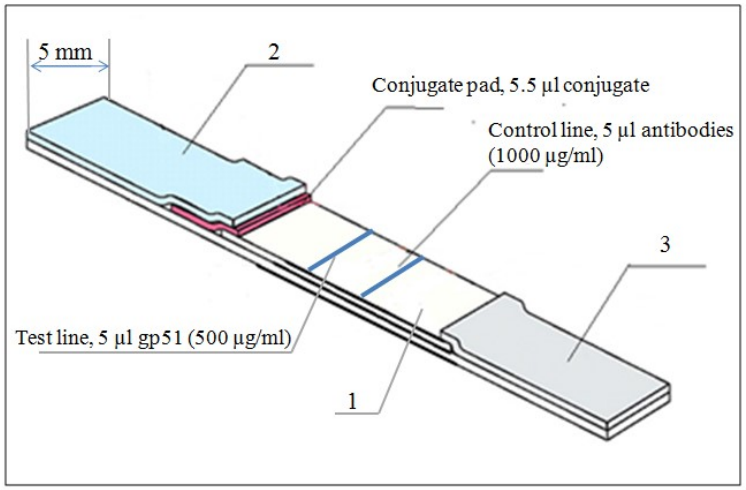
Preparation of large amounts of the conjugate was done as follows. A portion (2 µl) of solution of the protein G (8 µg/ml) was mixed with 10 ml of colloidal gold (pH 6.0). Mixture was incubated for 10 min at room temperature, and then 0.4 ml of 1% Polyethylene glycol (PEG) was added to achieve final concentration of the PEG 0.04%. Upon incubation for 30 min at room temperature the mixture was centrifuged for 45 min at 60000 g. Supernatant was removed and the pellet was resuspended in 1.5 ml of PBS containing 0.04% PEG as stabilizer. The conjugate was stored at 4°C. Before utilization for the preparation of immunochromatographic strips, the conjugate was diluted 1/10 in a PBS containing 0.02% PEG.

Production of immunochromatographic strips. Methods published by Byzova et al. (2010) were used to produce nitrocellulose membranes covered with reagents and to assemble multi-membrane strips (Fig.1).

Conjugate of colloidal gold with protein G was loaded onto conjugate pad (made from glass fiber matt) using the automatic dispenser BioDot XYZ3050. The conjugate was applied in amount 11 µl per 1 cm of length of the fiber matt. In the test system, a solution of the recombinant gp51 (500 µg/ml or different concentration if indicated) was used to form the test line. To form a control line, a solution of anti-bovine IgG antibody (Sigma-Aldrich, St. Louis, USA) (1000 µg/ml) was used. All solutions were applied with 10 µl per 1 cm of membrane width. The resulting glass fiber and membranes were air dried at 20-22°C for not less than 20 hours. The absorbent pad, the nitrocellulose membrane, the conjugate pad and the sample tube were collected in a single strip and placed in a plastic casing. The final strip tests for ICA are 65 mm long and 3 mm wide.

Performing ICA. Samples (100 µl) of cattle sera were diluted in sample dilution buffer (900 µl of PBS+0.05% Tween 20 (PBST) per sample). Sample pads of the ICA strips were submerged in the diluted sera for

2.5-3 min, and then the strips were placed on a horizontal surface. Following incubation for 20 min at room temperature, result was considered positive if the red bands were present at both test line and control line. Result was negative if one red band appeared at only the control line. If no clearly visible red bands developed on strip or the only band appeared at the test line but not in the control line, the result was considered invalid.



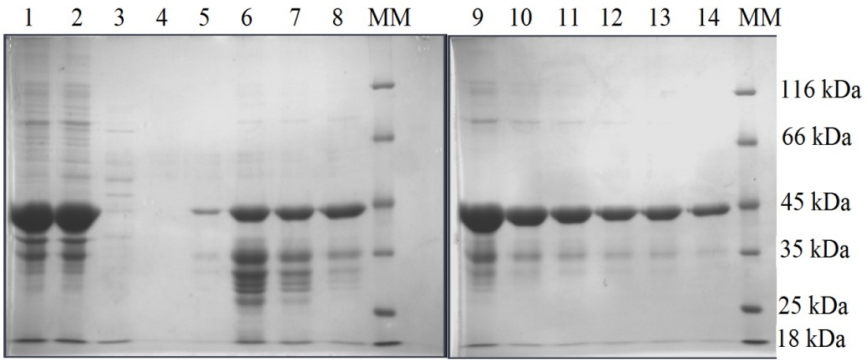
1 – nitrocellulose membrane; 2 – sample pad; 3 – absorbent pad

Figure 1. **Components of the immunochromatographic strip**

ELISA. To perform indirect ELISA, the recombinant antigen gp51 was immobilized in wells of 96-well plate. For this purpose, aliquots (100 µl) of solution of the recombinant gp51 with concentration 10 µg/ml were dispensed into wells and the plates were incubated for 12 hrs at 4°C. After washing and blocking in 1% BSA in PBST, samples of cattle serum (diluted 1:10 in PBST) were placed into the wells. Upon 1 hr incubation the wells were thoroughly washed. Solution of conjugate of anti-bovine IgG with horseradish peroxidase (diluted 1:10000 in PBST) was dispensed into wells and the plate was incubated at 37°C for 1 hr. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added to wells. Upon development of color, the plate was scanned on the plate spectrophotometer.

Results

Codon-optimized gene gp51 was synthesized from synthetic oligonucleotides and cloned into an expression plasmid pET32a. The obtained construct pET-gp51 was transformed into *E. coli* strain BL21(DE3) to produce the expression strain BL21(DE3)/pET-gp51. Several clones of the producing strain were used to check for the expression of the recombinant protein gp51. In these experiments, optimal conditions of the expression of the recombinant protein were determined. The highest levels of expression of the recombinant gp51 were observed during incubation of culture of the producing strain at room temperature in presence of the isopropyl β-D-1-thiogalactopyranoside at 0.2 mM and incubation time 6 hrs. Under these conditions, a product with an apparent molecular mass 44 kDa accumulated in bacterial cells, in a soluble fraction (Fig. 2). We estimate that at the end of incubation the recombinant protein amounted to 80% by weight of the total protein in the soluble fraction. IMAC was used to purify the recombinant gp51 (Fig. 2).

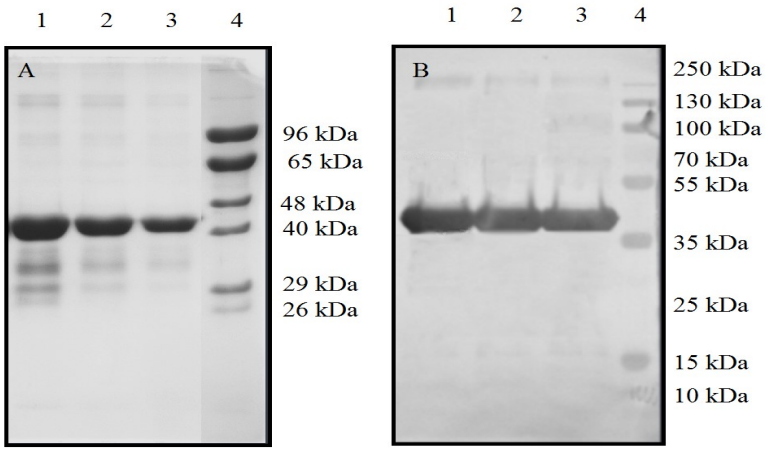


Lanes: 1-2, insoluble fraction of lysed bacterial cells; 3, flowthrough after passing the HisTrap HP column; 4, column wash (20 mM imidazole); 5, sample eluted into 50 mM imidazole; 6-8, three fractions collected during elution into 100 mM imidazole; 9-14, consecutive fractions eluted into 500 mM imidazole; MM marker of molecular weights. Molecular masses of the marker's bands are indicated

Figure 2. **SDS-PAGE of samples collected during purification of the recombinant gp51 protein expressed in *E. coli***

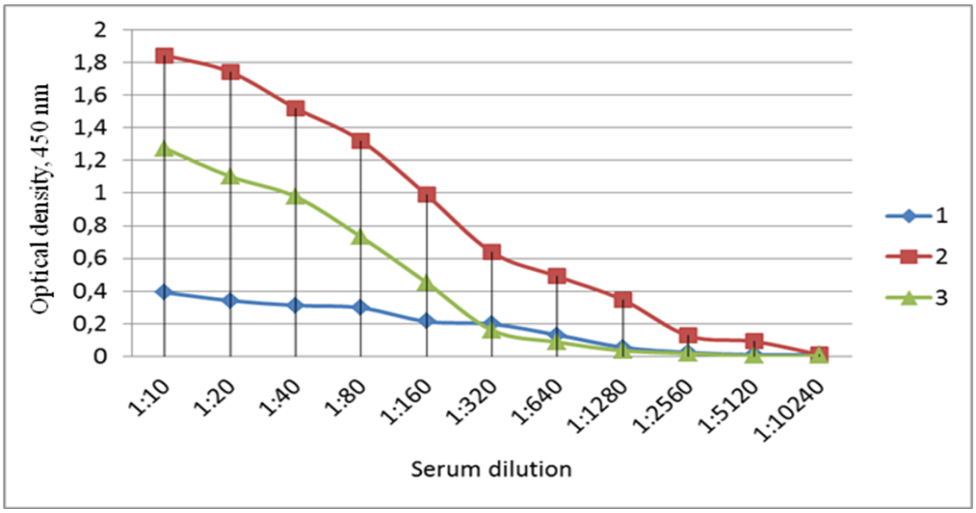
In the western blot a product with an apparent molecular mass 45 kDa reacts with the anti-His-tag monoclonal antibody. This band corresponds to the expected molecular mass of the recombinant protein gp51 (Fig. 3).

Diagnostic value of the recombinant gp51 antigen was tested in ELISA using positive and negative control sera (Fig. 4).



Panel A, SDS-PAGE of the recombinant antigen gp51. Panel B, western blot with the anti-6X His tag monoclonal antibody. Lines 1-3, samples of protein gp51; line 4, marker of molecular weights. Molecular masses of the marker's bands are indicated

Figure 3. **SDS-PAGE and western blot of the purified recombinant antigen gp51**



Line 1, negative control serum; line 2, positive control serum; line 3, serum from the BLV-infected cow

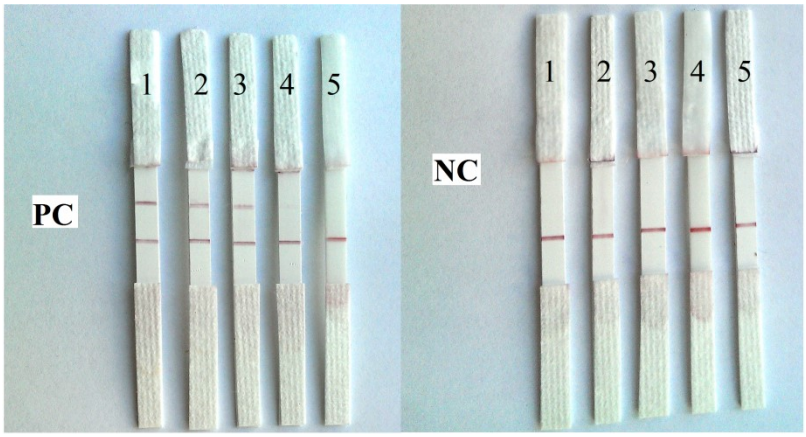
Figure 4. **Use of the recombinant gp51 antigen in ELISA**

The control positive serum (Fig. 4, line 2) reacted with the gp51 antigen in dilution 1:1280. In a range of dilutions 1:10-1:160, signal of the positive control serum significantly exceeded that of the negative control serum (Fig. 4, line 1). One serum from cow with confirmed BLV infection (Fig. 4, line 3) reacted with the gp51 in dilution 1:320.

Conjugate of the protein G with colloidal gold was produced. Optimal concentration of the protein G in a mixture with gold nanoparticles was determined as the one which produces maximum saturation. Corresponding graph was built by spectrophotometric measurements of mixtures of the protein G with gold nanoparticles at 580 nm. The maximum saturation occurred at a concentration of the protein G 5 µg/ml. Further increase in protein concentration did not lead to increase in adsorption. Based on these results, we used protein G at a final concentration 8 µg/ml, to produce the conjugate which was subsequently used to produce ICA strips. The conjugate was loaded on a glass wool membrane (RT-R5, MDI, India) in amount 7 µl of conjugate per strip.

The concentration of antigen applied to the membrane plays a significant role during the immunochromatographic process. To determine the optimal amount of protein, recombinant gp51 was used at different concentrations: 500 µg/ml, 250 µg/ml, 125 µg/ml, 60 µg/ml, 30 µg/ml and 15 µg/ml. Produced strips were used to test reactions with the positive and negative

reference sera. With both reference sera we observed development of the control line on all strips independently of the amount of the applied antigen. Test lines appeared on strips immersed in the positive reference serum (Fig. 5).

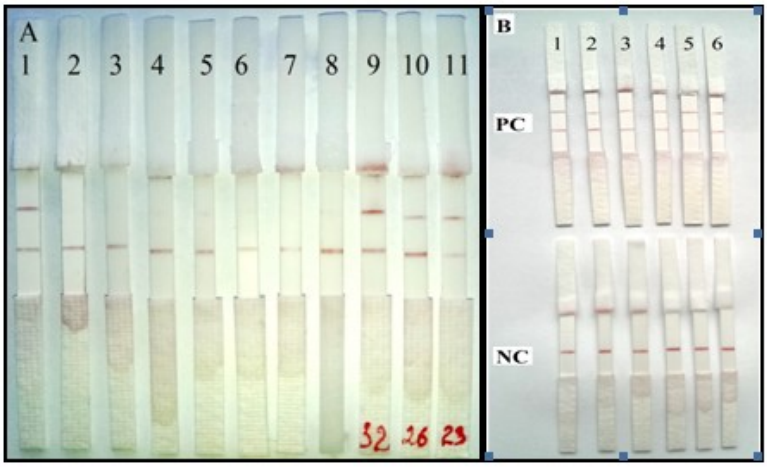


Panel PC, ICA testing of the positive reference serum. Panel NC, ICA with the negative reference serum. Numbers 1-5 indicate strips to which varying amounts of the gp51 were applied during production of the strips. Concentrations of the recombinant gp51 were as follows: 1, 500 µg/ml; 2, 250 µg/ml; 3, 125 µg/ml; 4, 60 µg/ml; 5, 30 µg/ml

Figure 5. **Influence of the amount of the diagnostic antigen (the recombinant gp51) on performance of the ICA**

Not all amounts of the antigen were equally effective to detect specific antibodies. Test line was evident on the strips with gp51 applied at concentrations 500 µg/ml, 250 µg/ml and 125 µg/ml. Lower concentrations of the gp51 did not result in appearance of the test line. Testing of negative reference serum worked as expected: only control lines appeared on all strips. Based on the obtained results the recombinant gp51 antigen concentration of 500 µg/ml was chosen for use in the ICA.

To determine sensitivity of the ICA strips we used a panel of samples which included the reference positive serum and a number of AGID-positive cow sera. All samples in this panel were positive in ICA. To test for possible unspecific reactivity of the produced ICA strips with unspecific antibodies in cattle sera, we tested the strips with a panel of samples including the reference negative serum and AGID-negative sera from cows with non-BLV infections (brucellosis, foot-and-mouth disease) (Fig. 6A). We also included in the panel AGID-negative sera from clinically healthy cows. These samples were negative in the ICA. ICA strips gave positive reaction with the positive reference serum in dilutions up to 1:1600 (Fig. 6B).



Panel A, numbers 1-11 correspond to the following sera: 1, positive reference serum; 2, negative reference serum; 3-6, sera from cows with brucellosis; 7-8, sera from cows with food-and-mouth disease; 9-11, sera from the BLV-infected cows. Panel B: part PC, ICA with the positive reference serum; part NC, ICA with the negative reference serum. Numbers 1-6 indicate various dilutions of the positive or negative reference sera (1, 1:100; 2, 1:200; 3, 1:400; 4, 1:800; 5, 1:1000; 6, 1:1600)

Figure 6. **Determination of the specificity and sensitivity of the ICA**

Diagnostic characteristics of the ICA were compared with the gold standard (AGID) on a sufficiently large panel of cattle sera (n=310). This comparison revealed high specificity of the ICA (165/165, 100%) and sensitivity (141/145, 97.2%) (Table 1).

Table 1. **Sensitivity and specificity of the ICA in comparison with golden standard (AGID)**

AGID	ICA (gp51-based)		
	Positive	Negative	Total
Positive	141	4	145
Negative	-	165	165
Total	141	169	310

Discussion

A variety of ICA has been developed for the detection of various infectious diseases in animals (Yang S et al., 2010, Nguyen T et al., 2015, Li X et al., 2015). The ICA for the BLV infection is supposed to have advantages over routinely used AGID test and even more advanced tests like ELISA. Such advantages include simplified testing procedure, rapidity of obtaining of results, absence of the need in complex equipment and good diagnostic performance comparable to that of the AGID (DaPeng et al., 2007).

To have competitive cost on the market, the diagnostic test must be produced from relatively cheap components. Among the most valuable components in ICA is the BLV antigen which is used to capture specific antibodies. Cheap production of the BLV antigen was achieved with the use of recombinant DNA technology. Production of the recombinant antigens for diagnostics possesses attractive features such as the purity and high concentration of antigen in the resulting product, and easy scale-up of the production.

Components of the BLV virion - glycoprotein gp51 and capsid protein p24 - are commonly used in diagnostic assays to capture specific antibodies because these viral proteins elicit high titers of antibodies during the BLV infection. Naturally, titers of antibodies against the gp51 are even higher than against the p24 and thus use of the gp51 may provide greater sensitivity (De Giuseppe et al., 2004).

In this paper we describe successful utilization of the recombinant gp51 produced in *E. coli* for the development of the ICA. The purified protein successfully worked as the specific antigen in the western blot and ELISA demonstrating that the bacterially-expressed gp51 reacts with at least a fraction of antibodies raised against the BLV glycoprotein. Similar results were obtained by Ban et al. (1992) in an investigation to map the gp51 antigenic determinants with use of the bacterially-expressed gp51 as one of the antigens. In this study authors described three conformational epitopes located in the N-terminal part of the gp51 molecule which immune reactivity depends on the glycosylation. Also, the gp51 molecule has a number of linear epitopes in the non-glycosylated C-terminal part. Antibodies against these epitopes seem to neutralize the virus and these antibodies are present in animals vaccinated with whole-virion vaccines.

Based on the obtained recombinant gp51 antigen, ICA was developed for the rapid detection of BLV infection. This test showed high diagnostic parameters and can be used to provide quality data. The successful use of test strips for the rapid diagnosis of BLV infection in situ provides invaluable information for the development of timely preventive measures.

Conclusions

The bacterially expressed gp51 was used to capture serum antibodies in the ICA. ICA is a particularly convenient test for field diagnostics because it is quick (~20 min) and unlike current routine assays, ICA does not require transportation of samples to laboratory. A panel of cow sera was tested by ICA and AGID for comparison of diagnostic performance. In this comparison, AGID was used as a gold standard, because in Kazakhstan it is the official test for the diagnosis of enzootic bovine leukosis. Sensitivity of the ICA is 97.2%, specificity - 100%.

Acknowledgments

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