Purification of *Escherichia coli*-expressed HIS-tagged Maedi-Visna p25 core antigen by Ni^{2+}-chelate affinity chromatography

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ABSTRACT: In this study, recombinant histidine tagged p25 capsid protein of Maedi-Visna virus was developed. Part of the viral genome coding p25 protein was positioned downstream and in frame with a metal binding domain in pRSET-B vector. Recombinant protein was expressed in *E. coli* cells and soluble fraction of the protein was subsequently purified by Ni^{2+}-chelate affinity chromatography. Purified protein was then used as antigen in an indirect ELISA test. One hundred fifty ovine serum samples were screened for antibodies to p25 protein of the virus. Immunoblot with whole virus antigen was used as a gold standard. The total number of positive results in the ELISA was 38 (25.33%). Immunoblot failed to confirm a positive result in 2 (1.33%) of them and these results were therefore considered to be false positive. The number of true positive results in the ELISA was thus 36 (24%). All immunoblot positive samples were also positive by ELISA test. In conclusion, recombinant His-tagged capsid protein showed very high sensitivity and specificity in detecting antibodies to Maedi-Visna virus.

Keywords: lentivirus; recombinant antigen; ELISA; immunoblot

INTRODUCTION

Ovine lentiviruses (OvLV) represented by Maedi-Visna virus (MVV) cause slowly progressing disease characterized by interstitial pneumonia, encephalitis, indurative mastitis and non-suppurative arthritis (Gudnadottir, 1974). In The Czech Republic, Maedi-Visna virus (MVV) has been proved by serological examination (Literák *et al*., 1995), isolated in 1997 (Celer *et al*., 1997) and recently analyzed by phylogenetic analysis (Celer *et al*., 2000).

Several serological methods for detecting antibodies to Maedi-Visna virus have been described, e.g. complement fixation (Torfason, 1992), indirect fluorescence and virus neutralization (Gudnadottir, 1990), agar gel immunodiffusion test (Winward *et al*., 1979) and different indirect ELISAs combined with immunoblot (Houwers and Nauta, 1989; Zanoni *et al*., 1994; Celer *et al*., 1998). Indirect ELISA test based on whole virus antigen is commercially available.

The major capsid protein p25 is considered as one of the immunodominant antigens in lentivirus infection (Houwers and Nauta, 1989). It is the first antigen recognized by the immune system of infected animals and the production of p25 specific antibodies persists for a long time. For this reason, the MVV p25 in its native or recombinant form has been extensively used in the first and second generation of serological tests for MVV diagnosis (Zanoni *et al*., 1991; Boshoff *et al*., 1997).

For Maedi-Visna diagnosis, the most widely used test in the Czech Republic is the AGID test (Celer, 1993a). It is easy to carry out and highly specific. Programs to eradicate the disease, however, require seropositive animals to be diagnosed in the very early stages of infection and this requirement can only be met by more sensitive immunoenzymatic tests. The purpose of our study was to develop a suitable antigen based on recombinant p25 protein of MVV for indirect ELISA test.

MATERIAL AND METHODS

Tissue culture and virus strain

A strain of ovine lentivirus K1514 was used for whole virus antigen production. Sheep choroid plexus (SCP) cells from tissue explants of an ovine lentivirus-free newborn lamb were used for virus cultivation. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 300 mg/l L-glutamine and 40 mg/l gentamycin. Antigen for SDS-PAGE was concentrated and purified as described previously (Celer *et al*., 1993b).

Supported by the Grant Agency of Ministry of Education, Youth and Sports of the Czech Republic (Grant No. 161700001).

Plasmid construction

For cloning of the p25 gene a subgenomic fragment was PCR-amplified from viral DNA. Primer pairs were derived from published sequences of Maedi-Visna virus (Sonigo *et al*., 1985). Primers were designed in such a way that restriction enzyme sites BamHI (underlined) were introduced at the 5′- and 3′-ends of the fragment, respectively (Primer 917: TAGGGA**GGATCC**TCCTATTGTG AATTTGCA; Primer 1556: GCAT**GGATCC**AAATCCTT CGGATCCCACAT).

For expression, the p25 gene was cloned into the multiple cloning site of pRSET-B plasmid vector, under control of strong bacteriophage T7 transcription and translation signals. DNA insert was positioned downstream and in frame with a sequence that encodes an N-terminal fusion peptide – a series of six histidine residues that function as a metal binding domain in the translated protein. For the protein expression, recombinant plasmid was then transformed into BL21 (DE3) pLysS *E. coli* cells.

Expression of p25 protein in *E. coli*

A fresh overnight culture of *E. coli* in BL21 (DE3) pLysS carrying recombinant plasmid was diluted 1 : 100 in 2 L of fresh LB-medium in the presence of 100 mg/ml ampicillin and grown to OD_{600} 0.6 at 37°C at a shaking speed of 270 rpm. The expression of recombinant p25 protein was induced by adding isopropyl-β-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM for three hours at 30°C. Cells were then pelleted at 4 000g at 4°C for 20 min. The sediment was either used directly or stored at –70°C until use.

Purification of p25 protein

The cell pellet was resuspended in 180 ml of phosphate lysis (20 mM sodium phosphate, 500 mM sodium chloride, 0.2% Trition-X-100, 1 mg/ml lysozyme, 10 mg/ml DNAse 1 and 10 mg/ml RNAse A, pH 7.8) by stirring in an ice-cold water bath. The cells were lysed on ice by ten cycles of sonication at 20 s each with 3 min intervals to avoid heating of the material. Cell debris was removed by centrifugation at 10 000g at 4°C for 30 min. Recombinant p25 protein was separated from contaminating proteins by Ni^{2+} chelate affinity chromatography. The column with 5 ml of Ni-NTA agarose (Qiagen, Hilden, Germany) was equilibrated with phosphate buffer pH 7.8 (20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8). The column was loaded at a flow rate of 1 ml/min for 1 h and washed with 10 volumes of washing phosphate buffer pH 6.0 (20 mM sodium phosphate, 500 mM sodium chloride, pH 6.0) and 10 volumes of the washing buffer pH 5.5. Bound protein was eluted with elution buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 4.0) in a total volume of 40 ml. The eluate was collected in 1ml fractions. Aliquots of the fractions were run on 12% SDS-PAGE and stained with Coomassie Brilliant Blue to analyze their purity. The concentration of recombinant protein was determined spectrophotometrically (OD_{280}). Fractions containing the pure protein were pooled and stored (concentration of pooled protein was 1mg/ml).

ELISA

Polystyrene 96-well microtitre plates were coated with 100 ml of viral antigen in PBS (pH 7.2) over night. Ovine sera tested in duplicate were diluted 1 : 100 and 100 ml per well were added. For the detection of the specific binding of antibodies, 100 ml of anti sheep and goat IgG horseradish peroxidase conjugate (Sigma, St. Louis, Missouri) were added per well.

Finally, 100 ml of 1mM ABTS [2,2-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] (Sigma, St. Louis, Missouri) dissolved in 500 mM citrate buffer pH 4 with 15 mM H_2O_2 was added. Optical absorbance was measured at 405 nm.

A test serum was considered to be positive if the mean of the test serum absorbance was higher than the mean absorbance of the weak positive serum.

Negative control sera were obtained from 20 adult sheep and 20 lambs that were tested negative for ovine lentivirus antibodies by the immunoblot and the AGID tests; these sera were used to determine the cut-off value of the weak positive serum (mean absorbance of negative control sera plus 3 times their standard deviation).

SDS-PAGE and Western blotting

The antigen was mixed with a Laemmli sample buffer and heated to 95°C for 2 min. This material was separated by electrophoresis on a 12% resolving and 4% stacking polyacrylamide gel. The composition of the electrophoresis buffer was 0.025 M Tris, 0.192 M glycine, 0.1% SDS. The gel was run at a constant voltage (200 V) at room temperature. Proteins were then blotted onto a nitrocellulose membrane at 110 V for 1 hour. The composition of the blotting buffer was 0.025 M Tris, 0.192 M glycine, 0.01% SDS and 20% ethanol. After transfer, the nitrocellulose membrane was dried, cut into strips and stored at 4°C.

Immunostaining

The nitrocellulose strips were blocked with 1% nonfat dry milk in Tris buffered saline with Tween-20 (TBST;

0.025 M Tris, 0.9% NaCl, 0.1% Tween-20, pH 8.0) for 1 hour.

Each strip was then incubated with Maedi-Visna virus positive ovine serum diluted 1/100 in TBST (3 hours); bound antibody was detected by incubation with anti sheep and goat IgG conjugated with alkaline phosphatase for 2 hours. The reaction was then made visible with NBT/BCIP (Promega Corporation, Madison, USA). Unequivocal reactivity with capsid and matrix protein was considered to be a positive result.

Statistical analysis

An agreement between the test with recombinant p25 and immunoblot was evaluated using the kappa statistic. Samples, which scored positive in the immunoblot with whole virus (WV) antigen, were considered to be "true positive".

RESULTS

Nucleotide sequences 917–1 556 (Sonigo *et al*., 1985) of the K1514 MVV strain were amplified incorporating BamHI sites at both ends. Plasmid with cloned fragment was designated pRSET/p25. This plasmid construction expressed protein with molecular weight of 30 kDa after induction. The highly purified recombinant protein migrated as a single band in the SDS-PAGE gel (Figure 1).

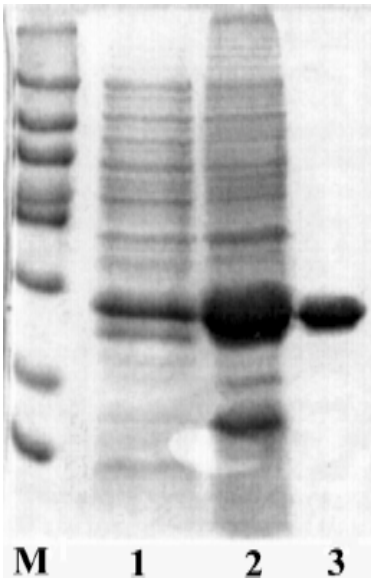

Figure 1. Purification of recombinant p25 protein of Maedi-Visna virus

Lane 1 is *E. coli* fusion protein lysate prior to induction; lane 2 represents protein lysate following induction; lane 3 was loaded with affinity purified recombinant p25 protein; lane M contains molecular size marker (Promega)

Recombinant protein was isolated from *E. coli* cells by sonication in lysis buffer. Soluble p25 protein remained in the supernatant. The preparations were applied to a Ni-NTA column allowing the binding of the histidine tagged proteins via the N-terminal histidine residues. Then, the protein was eluted from the column by acidic pH (4.0). The total amount of soluble recombinant p25 protein was 40 mg/l of induced culture. About the same amount of the protein remained in insoluble form and was lost during the purification.

A total of 150 ovine serum specimens were analyzed for antibodies to ovine lentivirus. The sera were tested by ELISA based on recombinant p25 antigen and by immunoblot based on whole virus antigen. The results of whole virus test were considered as a gold standard. The comparison of the two tests is summarized in Table 1.

Table 1. Comparison of immunoblot and recombinant p25 ELISA test

Immunoblot	ELISA positive	ELISA negative	ELISA total
positive	36	0	36
negative	2	112	114
total	38	112	150

As expected, the largest group of 148 (98.66%) sera reacted identically in both tests.

The total number of positive results in the ELISA was 38 (25.33%). Immunoblot failed to confirm a positive result in 2 (1.33%) of them and those results were therefore considered to be false positive. The number of true positive results in the ELISA was thus 36 (24%). All immunoblot positive samples were also positive by ELISA test.

The agreement between the two tests beyond chance was excellent with a kappa value of 0.96.

DISCUSSION

Increasing the efficiency of Maedi-Visna eradication programs requires very sensitive immunoenzymatic tests e.g. ELISAs preferably combined with immunoblot. The antigens used in these tests are based on whole virus particles or on recombinant viral proteins. The main advantage of recombinant proteins is their relative low cost and simplicity of purification.

The most widely used recombinant systems for this purpose are fusion proteins tagged with glutathione S-transferase (GST) (Zanoni *et al*., 1991; Rosati *et al*., 1994). Unfortunately some animals have antibodies reacting with the GST part of the fusion protein and thus, all examined sera have to be checked not only against recombinant antigen, but also against GST alone.

This drawback of GST fusion proteins led us to use the polyhistidine tagged expression system. The polyhistidine is poorly immunogenic and generally does not affect secretion, compartmentalization or folding of the fusion protein within the bacterial cell (Kaslow and Shiloach, 1994). Such protein produced in sufficient amount would be an ideal candidate to be used as an antigen in immuno-enzymatic tests.

Many target proteins are expressed in both soluble and insoluble forms. Culture conditions can have a dramatic effect on solubility of a given recombinant protein. In general, conditions that decrease protein synthesis, such as low rate of protein synthesis or low induction temperature, tend to increase the percentage of target protein found in soluble form. Although the insoluble fraction is easily purified following solubilization, the soluble form retains proper folding of the protein and is thus more suitable as antigen in any serological test. Despite the relatively high production of our protein, a significant fraction was expressed in soluble form. We found that after lysis of *E. coli* cells only about half of the p25 protein could not be recovered from the supernatant but was present in the insoluble fraction.

Recombinant capsid p25 protein of the virus was used as antigen in an indirect ELISA test. The reason to use the capsid proteins in our testing scheme is based on previous observation that most OvLV infected sheep react against this viral protein (Kwang *et al*., 1996). Infected animals develop a strong immune response against the capsid protein early in infection.

Our testing scheme compared the sensitivity of serological tests with recombinant and whole virus proteins as antigens. The immunoblot analysis of the whole virus antigen demonstrated mainly the presence of the p25 capsid protein and p16 matrix protein. The reactivity with p25 protein was consistently found in all examined sera but reactivity with matrix protein was evident only with strong positive sera. According to the composition of whole virus antigen, very high concordance of WV immunoblot and recombinant p25 antigen based ELISA is not surprising. Two samples were found to be positive by ELISA test and negative by immunoblot. Both samples showed repeatedly weak reactivity in ELISA test and no reactivity with viral proteins in WV immunoblot. This result may be explained by trace amount of contaminating *E. coli* proteins in recombinant antigen preparation.

In conclusion, recombinant His-tagged capsid protein showed sufficient sensitivity and specificity in detecting antibodies to OvLV.

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Received: 01–02–06
Accepted after corrections: 01–03–22

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