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QUARTERLY

Expression of bovine leukemia virus protein p24 in *Escherichia coli* and its use in the immunoblotting assay✪

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The *gag* gene encoded protein, p24 of bovine leukemia virus (BLV), was cloned and expressed as thioredoxin-6xHis-p24 protein in *Escherichia coli*. The bacterial cells carrying plasmid pT7THis-p24 expressed the protein of 38 kDa that was detected by immunoblotting analysis using anti-p24 monoclonal antibodies and sera from BLV infected cattle and sheep. The purified p24 fusion protein was shown to be sensitive and specific for detection of BLV antibodies in the infected cattle.

Enzootic bovine leukemia (EBL) is an infectious lymphoproliferative disease of cattle, caused by the retrovirus: bovine leukemia virus (BLV). Like other complex retroviruses the BLV genome contains the *gag, pol* and *env* structural genes and regulatory genes [1]. Most of the structural proteins of BLV are immunogenic but the naturally infected animals develop antibodies to *env*-encoded glycoproteins gp51 and gp30 as well as to *gag*-encoded proteins p24 and p15 [2]. The antibodies against p24 and gp51 are predominant [3]. BLV protein structure studied with monoclonal antibodies revealed the presence of three major conformational epitopes, F, G and H in the N-terminal region of gp51 and two epitopes in C-terminal region of p24 [4].

Since the presence of antibodies to BLV is a constant and early feature of BLV infection, serological examination of cattle sera is the best method for detection of infected animals. Most commonly used serological tests are the agar gel immunodiffusion (AGID) and the enzyme-linked immunosorbent assay (ELISA). However, variable results have been obtained by the two methods using the p24 or gp51 antigen. The disparities were often the result of differences in specificity

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Abbreviations: BLV, bovine leukemia virus; FLK, foetal lamb kidney; IPTG, isporopyl-1-thio–D-galactoside.

and sensitivity of the tests used. AGID is less sensitive and is not useful for detection of antibodies to p24 antigen. ELISA has been shown to detect both anti-gp51 and anti-p24 antibody with equal sensitivity but this method is prone to generate non-specific reactions. While in the ELISA the non-specific reactions are difficult to distinguish from specific ones, Western blot analysis allows precise resolution of the two reactions. So far limited studies have been performed to confirm the usefulness of the immunoblotting assay in the routine serological detection of BLV antibodies [5, 6]. Recently, the recombinant viral proteins have been found to be widely applicable in immunoassays for detection of specific antibodies. In particular, the use of the recombinant proteins synthesized in *E. coli* has been well documented in retroviral serology [7, 8].

In this study, the *gag* gene of BLV which encodes protein p24 was cloned and expressed as thioredoxin-6xHis-p24 fusion protein in *E. coli.* The recombinant p24 fusion protein was tested for sensitivity and specificity in detection of antibodies in sera of BLV-infected cattle by Western blotting assay.

MATERIALS AND METHODS

Plasmids and bacterial strains. Plasmid pAMP1 and *E. coli* strain DH5α were purchased from Life Technologies. The pT7THis expression vector is a derivative of pT7-7 vector (USB) which contains the sequence encoding the *E. coli* thioredoxin (Trx) gene and 6 histidine residues (6xHis). This sequence is under control of the T7 RNA polymerase promoter. pT7THis is modified by insertion of *E. coli* Arg-tRNA gene into *Bgl*II site to allow the expression of eukaryotic genes rich in AGG and/or AGA codons. The multiple cloning site at 3′-end of 6xHis sequence allows the insertion of a DNA fragment fused in phase with ATG start codon of the *trx* gene sequence.

Cloning the* gag *gene in the expression vector. The preparation of plasmid DNA, DNA restriction, agarose gel electrophoresis, cloning and transformation of *E. coli* were carried out according to Sambrook *et al.* [9]. The *gag* gene (678 bp) encoding the protein p24 was amplified using forward primer 5′-CCAATCATATCTGAAGGGAA-3′ and reverse primer 5′-CAGAAGTGCAGGCTGTTTCA-3′ complementary to the BLV provirus sequence previously published [10]. In the 5'-ends of the primers, the extensions of dUMP residues and *Eco*RI and *Bam*HI recognition sites were added to the forward and reverse primers, respectively, for further cloning and subcloning purposes. DNA isolated from FLK cells infected with BLV (kindly provided by Dr J. Miller, NADC, Ames, IA) was used as the template. The amplified fragment was cloned into pAMP1 vector using the CloneAmp System (Life Technologies) according to manufacturer's instructions. The sequence of the insert was confirmed by the di-deoxy termination sequencing (Sanger). The recombinant plasmid pAMP1 containing the p24 encoding sequence was digested with *Eco*RI and *Bam*HI and the insert was ligated into the pT7THis expression vector digested with the same enzymes and transformed to *E. coli* strain B BL21 (DE3). The resulting plasmid pT7THis-p24 which, under the control of the T7 promotor, expressed thioredoxin and a short His tag fused to the N-terminus of full-length p24, was isolated. The p24 expression was regulated by *lac* repressor produced by *lacI* gene in *E. coli* host cells.

Isolation and detection of fusion protein. The *E. coli* BL21 (DE3) carrying plasmid pT7THis-p24 was grown at 30°C in LB medium [9] containing 100 μg ml^{-1} of ampicillin. When the culture had reached an A_{600} of 0.6 the expression of p24 was induced by addition of 1 mM IPTG. After 16 h of induction the cells were collected, sonicated and proteins were extracted with loading buffer (125 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate, 9% glycerol, 0.7 M 2-mercaptoethanol, 0.002% bromophenol blue). The soluble fraction of cell proteins was subjected to SDS/PAGE analysis [11]. The p24 fusion protein was purified by metal affinity chromatography on a Chelating Sepharose Fast Flow column (Pharmacia). The 6xHis-tagged protein was eluted with linear gradient of imidazole (0–0.25 M). In the immunoassay, the purified fusion protein p24 was transferred to

nitrocellulose membrane (Hybond-C, Amersham) by electroblotting and incubated on the filters with monoclonal antibodies anti-p24 of BLV (BW5C6H11 clone, CER, Belgium) or with bovine and sheep sera from BLV-infected and BLV-negative animals. The immunoassays were carried out as described previously [12].

Western blot analysis of sera from BLV infected cattle. Twenty two serum samples derived from cattle of different clinical status (lymphosarcoma, persistent lymphocytosis, and aleukaemic cattle showing no clinical symptoms) were tested by immunoblotting with the purified recombinant p24 fusion protein or, with culture supernatant- derived semipurified BLV virions [12] as test antigen. The results were compared to those obtained in the commercial ELISA (Synbiotics, France) and AGID (Dr Bommeli, Switzerland).

RESULTS

A 642 bp fragment of the *gag* gene containing the entire encoding sequence of the 24 kDa protein of BLV was amplified and inserted into vector pAMP1 by using a ligation-independent cloning system. The plasmids isolated from single white colonies of Amp^r transformants were screened for the correct insert by restriction analysis using *Eco*RI and *Bam*HI enzymes. Sequence analysis of the inserted fragment in the recombinant plasmid pAMP1.2 revealed 100% homology with the corresponding *gag* gene sequence of BLV (GenBank Accession No. M10987). The *Eco*RI/*Bam*HI fragment containing the *gag* gene encoding protein p24 was then inserted into the expression plasmid pT7THis. The single colonies of Amp^r transformants of *E. coli* BL21-DE3 were screened for the presence of the recombinant plasmids using restriction analysis, and the plasmid designated pT7THis-p24 containing the *Eco*RI/*Bam*HI insert of 654 bp was identified. The *E. coli* clone containing pT7THis-p24 expressed the fusion protein thioredoxin-6xHis-p24 following IPTG induction. The presence of this protein in the cell lysate was demonstrated by SDS/PAGE analysis (Fig. 1A, lane 1). After purification by metal-chelate affinity chromatography, the fusion protein migrated consistently as a single band with the molecular mass of 38 kDa (Fig. 1A, lane 3). The molecular mass of p24 was increased to 38 kDa with the 14 kDa thioredoxin-6xHis moiety at the N-terminus. The purified fusion protein was repeatly obtained at 55 mg/L of the *E. coli* culture.

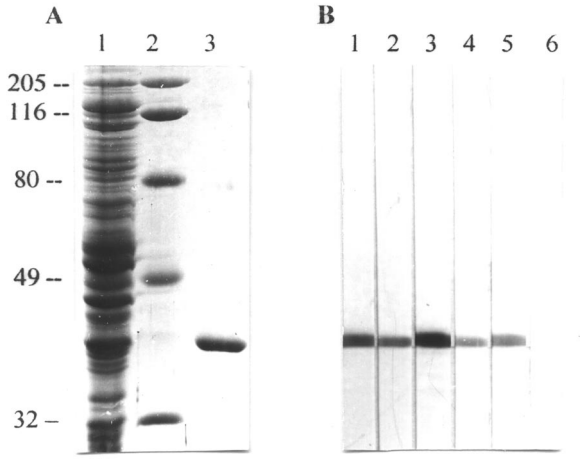

Figure 1. Characterization of BLV recombinant fusion protein p24.

(A) The extract of *E. coli* BL21 pT7THis-p24 total proteins induced with IPTG (lane 1); fusion protein Trx-6xHis-p24 purified by metal affinity chromatography (lane 3) separated by 10% SDS/PAGE as described by Laemmli [11] and stained with Coomassie blue. Positions of molecular size markers (lane 2) are given (in kDa) to the left of the figures (B) The purified fusion protein p24 separated by SDS/PAGE, transferred to Hybond C filter and incubated with: anti-p24 monoclonal antibody diluted 1:2000 (lane 1); BLV-infected cattle sera diluted 1:100 (lanes 2–4), BLV-infected sheep serum diluted 1:200 (lane 5) and BLV-negative cattle serum diluted 1:100 (lane 6). The colorimetric reaction was developed after incubation with peroxidase-labelled anti-species IgG according to the procedure described earlier [13].

To analyze BLV-specific immunoreactivity of the p24 fusion protein we performed Western blot analysis (Fig. 1B). Anti-p24 monoclonal antibodies (Fig. 1B, lane 1), bovine sera from BLV infected cattle (Fig. 1B, lanes 2–4) and serum from sheep experimentally infected with BLV (Fig. 1B,

lane 5) specifically and efficiently recognized the 38 kDa fusion protein. The intensity of the reactions varied between the antibodies used in the assay, with the monoclonal antibody and sheep serum being the most reactive, while the cattle sera were somewhat less reactive. No reaction was observed between the p24 fusion protein and serum from non-infected cattle (Fig. 1B, lane 6). The immunoreactivity of fusion protein p24 was highly specific, as demonstrated by the absence of cross-reaction of BLV-positive sera with thioredoxin moiety expressed in *E. coli* (not shown).

After evaluation of the BLV specific nature of p24 fusion protein, we determined whether the *gag* encoded protein could be used for the detection of anti-BLV p24 antibodies in sera from infected animals. Twenty two bovine serum samples were tested for the presence of BLV antibodies by ELISA and AGID. The results were compared to those obtained from Western blot analyses based on the recombinant protein p24 as antigen. Both ELISA and AGID detected only anti-gp51 antibodies. When the sera were tested by AGID, 18/22 were positive and 4 were negative. The ELISA tests detected anti-p24 antibodies in 20/22 with two doubtfull reactions. The latter two sera were also negative by the AGID assay. When the immunoblotting analysis was used, specific antibodies were found in all sera tested. All sera from clinically and haematologically positive cattle were found to be positive in all tests; inconsistent results were found only for sera from serologically positive animals.

In the next experiment we tested whether the immunoblotting assay with recombinant fusion protein p24 could be used as a confirmatory test for both AGID and ELISA. Four sera that were negative by AGID and gave inconsistent results by ELISA were tested with the blotted antigen p24, and all were found positive (Fig. 2, lanes 5–8). When these sera were tested by immunoblotting with the semi-purified virus from FLK-BLV cell culture supernatant as an antigen the antibodies anti-p24 were detected, however, anti-gp51 antibodies were detected weakly or not at all (Fig. 2, lanes 1–4). The lack of reactivity with gp51 correlated with AGID-negative and ELISA- doubtful reactions.

DISCUSSION

The use of conventional serological assays in BLV diagnosis still give inconsistent results, predominantly false-positive or negative reactions. Western blot immunoassay has been suggested as a suitable confirmatory test [6, 13]. However, the use of an appropriate BLV antigen seems to be crucial for the usefulness of this method [14]. The discrepancy between the results obtained in the conventional serological and in the Western blot assays regarding the gp51-binding antibodies has been reported previously [15, 16]. Although in sera from the infected animals the reaction with *gag*-related protein p24 is very clear, the reaction against gp51 may be weaker or absent. The lack of

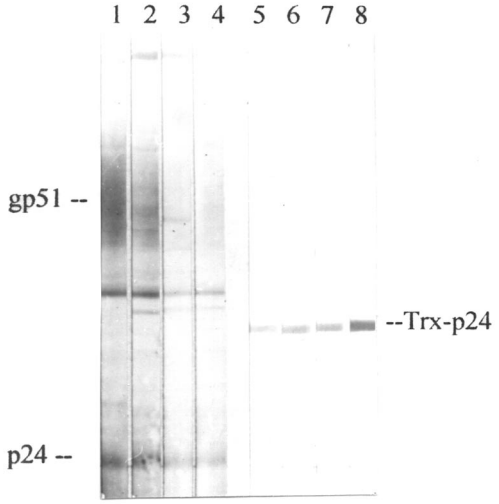

Figure 2. Western blot analysis of sera from BLV-infected cattle using different antigen preparations.

A sucrose gradient-purified BLV virus from FLK-BLV cell culture supernatant (lanes 1–4) and the purified fusion protein Trx-6His-p24 (lanes 5–8) separated by 10% SDS/PAGE were transferred to a Hybond C filter. The filters were incubated with the four sera which had given inconsistent results by the ELISA and AGID, and were subjected to Western blot analysis as described previously [12].

this reactivity in Western blot analyses, noted in our study, could be explained by the loss of conformational epitopes (F, G, H) of gp51 following disruption by the denaturing conditions of SDS/PAGE analysis [17]. The presence of bovine serum albumin and the insignificant amount of glycoproteins in the antigen preparation were

also considered as the factors contributing to the lack of immunostaining of gp51 in Western blots [14]. While in naturally infected animals anti-p24 antibodies were found less frequently and at lower titres than anti-gp51, their detection by Western blot analysis was consistent, despite the use of crude BLV antigen containing both p24 and gp51 [6, 13]. This was in accordance with our Western blot analysis using both viral and recombinant fusion antigens, although the immunostaining with the latter was more clear.

In conclusion, the immunobloting assay with recombinant p24 fusion protein as an antigen, is an efficient confirmatory test for the presence of specific antibodies in sera samples from BLV infected animals. Particularly in these cases when the animals may be considered negative because lack of the reaction with gp51. The fusion protein Trx-6xHis-p24 is expressed in *E. coli* as a soluble form at high concentration, circumventing the inefficient production of the retroviral antigens from the cell cultures. Furthermore, the detection of anti-p24 antibodies could potentially distingish the naturally infected from vaccinated animals, if BLV envelope glycoproteins were used as an immunogen.

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