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Comparison of Agar Gel Immunodiffusion Test, Enzyme-linked Immunosorbent Assay and Western Blotting for the Detection of BLV Antibodies

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With 3 figures

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Summary

An indirect enzyme-linked immunosorbent assay (ELISA) for the diagnosis of bovine leukaemia virus (BLV) infection was developed and compared with the agar gel immunodiffusion test (AGIDT). Western blotting (WB) was used as confirmatory test. ELISA and AGIDT had specificities that were comparable with that of WB, however, ELISA showed a higher sensitivity than AGIDT. The ELISA was useful for screening a large number of samples, whereas WB was important for detecting the antibody response against the individual BLV-proteins. Different types of positive serological reactions were discerned in WB, that correlated with reactions of sera in AGIDT and ELISA. The most important antigen in WB and ELISA was the BLV protein p24, whereas the BLV glycoproteins gp51 and gp30 were of special importance in AGIDT. The relevance of repeatedly testing the antibody response in BLV-infected herds for control and eradication programmes using assays with higher sensitivity than AGIDT was demonstrated.

Introduction

Bovine leukaemia virus (BLV) is an economically important pathogen infecting a large number of bovines in the American continent. Control and eradication programmes are mainly based on serological diagnosis by agar gel immunodiffusion test (AGIDT), followed by separation or removal of infected animals (Johnson and Kaneene, 1992). However, the relatively low sensitivity of the AGIDT might favour the occurrence of low titre BLV-infections in clinically normal herds. This is of remarkable importance in herds which had been tested repeatedly for some time by AGIDT (Manz and Bauer, 1985), and might be relevant in countries, where the management of cattle and the presence of bloodsucking vectors promote the spreading and maintenance of blood-transmitted viral diseases (Ferrer, 1980).

In retroviral infections, the enzyme-linked immunosorbent assay (ELISA) has been used mainly as a screening test, whereas western blotting (WB) has been chosen as reference confirmatory assay (Poiesz et al., 1997). In ELISA the non-specific reactions are relatively common and difficult to distinguish from specific ones, whereas in WB the detection of antibodies against the individual viral proteins allows the resolution of specific from non-specific reactions (Sherman et al., 1995). However, ELISA is as sensitive as WB, relatively inexpensive, easy to standardize and especially useful to screen a large number of samples.

Even though there are several ELISA protocols described and compared with AGIDT in the literature, the demonstration of improved sensitivity and specificity of ELISA using WB as confirmatory test has not been reported to the authors' knowledge. The clearly distinguishable

positive reactions in WB supports the use of this assay as a reference test for the validation of ELISA for the diagnosis of BLV-infections. Similar conclusions with other retroviral systems have been reached (Poiesz et al., 1997). The intention of this work was to develop a simple, specific and sensitive ELISA for the detection of antibodies from naturally BLV-infected bovines, using WB as confirmatory test.

Materials and Methods

Cells and BLV-antigens

BLV was grown in either, chronically infected foetal lamb kidney (FLK) cells or in infected and cloned bat lung (BatCl2) fibroblasts as described by Van Der Maaten and Miller (1976) and Graves and Ferrer (1976), respectively. The BLV-antigen was concentrated from supernatant fluids by clarification at 15 500 ***g*** and 4°C for 20 min, followed by sedimentation at 106 000 ***g*** and 4°C for 120 min in a Hitachi centrifuge (rotor RP 50T-198; Hitachi Scientific Instruments, Mountain View, CA). Pellet antigens (BLV-FLK, BLV grown in FLK cells and BLV-BatCl2, BLV grown in BatCl2-cells) were resuspended in 0.01 M phosphate buffer, 0.15 M NaCl (PBS), pH 7,2 and kept at –20°C until used. Protein content was determined as described by Lowry et al. (1951).

Bovine sera

Control sera: Bovine sera from 54 Holstein dairy cows were collected from BLV-free herds from the state of Hessen in Germany and kindly donated by Dr Manz. Two BLV-negative sera (one bovine viral diarrhoea virus (BVDV) positive and one BVDV-negative), and sera from three experimentally BLV-infected bovines (one BVDV positive and two BVDV negative) from Pennsylvania were kindly donated by Dr Ferrer. Seven sera from Holstein cows with lymphosarcoma were obtained from the Pathology Unit of the Veterinary School, Universidad Nacional, Costa Rica.

Test sera. Forty sera were collected from beef herds (Zebu crossbreeds) in the low BLV-prevalence regions of Costa Rica. One hundred and forty sera were collected from dairy herds (mainly Holstein and Jersey breeds) in the high BLV-prevalence regions of Costa Rica (Jiménez et al., 1995). These 140 sera could be further divided into two groups: group I, composed of 97 sera from animals tested by AGIDT for exportation purposes; and group II, composed of 43 sera from a herd subjected to a voluntary BLV-control programme based on the removal of AGIDT-positive animals during 32 months.

Conjugate

Affinity-purified rabbit anti-bovine IgG(H + L) (Sigma, St. Louis, MO, USA) was conjugated with peroxidase as described by Nakane and Kawaoi (1974). The determination of the optimal dilution of the conjugate was shown previously (Dolz, 1990). The conjugate dilution used in BLV-ELISA was 1 : 4000, in WB 1 : 8000.

Serological tests

Agar gel immunodiffusion test. AGIDT was performed in bacteriological polystyrene plates poured with a layer, 3.5 mm thick, of 0.8 % type IV agarose, dissolved in 0.05 M Tris-HCl, 8.5 % NaCl, pH 7.2. The pattern of six peripheral and one central well including the control sera was that recommended in the 'Leukassay B' (Pittman Moore, Washington Crossing, NJ, USA). The antigen wells were filled with 25 μl of BLV-FLK antigen, containing 13.5 μg protein, 0.1 % Triton X-100.

Enzyme linked immunosorbent assay. The ELISA protocol described by Dolz (1990) was used throughout all experiments. Microtitre plates were coated with 200 μl BLV-BatCl2 antigen (0.5 μg protein/ml) dissolved in 0.05 M carbonate buffer, pH 9,6, containing 0.025 % Triton X-100 and incubated for 16 h at 37°C. The plates were washed three times with washing buffer (PBS, 0.2 % casein hydrolysate, 0.1 % Tween-20), and bovine control and test sera diluted 1 : 50 in PBS (containing 1 M NaCl, 0.1 % Tween-20, 2 % Foetal Bovine Serum) were added to the wells and incubated 1 h at 37°C. After washing the plates, the conjugate diluted in PBS, 0.1 % Tween-20 was added to each well and incubated for 2 h at 37°C. After washing, peroxidase substrate (10 mg of *o*-phenyl-endiammine dissolved in 1 ml of ethanol, diluted to 100 ml of bidistilled water, containing 200 μl of 3 % H_2O_2) was added, and the optical density (OD) recorded at 405 nm in a Microplate Reader, model MR-700 (Dynatech, Chantilly, VA).

Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and WB for BLV was performed as previously described (Dolz, 1990). For continuous blots (8 cm long × 1.5 mm wide), 0.5 ml of BLV-FLK antigen containing 750 μg of protein were used. Post-coating was performed for 6 h with PBS, 4 % casein hydrolysate and the blot rinsed with PBS, 0.2 % casein hydrolysate, 0.1 % Tween-20. Control and test sera diluted 1 : 100 in PBS, 1 M NaCl, 0.1 % Tween-20 were added to the strips (2 mm wide) and incubated overnight at room temperature. The nitrocellulose strips were washed three times as described above and conjugate diluted in PBS, 0.1 % Tween-20 was added. After 4 h incubation, the strips were washed three times. Peroxidase substrate 3,3′-diamino-benzidine was added until coloured bands developed.

Results

Both BLV-antigens propagated in either FLK or BatCl2 cells were suitable for serological tests. Since some lots of BLV-FLK antigens gave non-specific reactions in ELISA, BLV-FLK was used in AGIDT and WB, while BLV-BatCl2 antigen was used preferentially in ELISA.

The 40 sera collected from Zebu crossbreeds from the low BLV-prevalence regions of Costa Rica reacted negatively in AGIDT, ELISA and WB. From the 140 sera obtained from highland dairy cattle (*Bos taurus*) 47.1 % gave positive reactions and 14.3 % gave weak-positive reactions in AGIDT. However, the proportion of positive animals increased in ELISA to 82.1 % and in WB to 92.1 % (Fig. 1c). In the first group of 97 sera collected from animals tested for exportation purposes, the AGIDT yielded 60.8 % positive and 16.5 % weak-positive reactions, whereas the ELISA detected 88.7 % and the WB 94.8 % positive reactions (Fig. 1a). In the second group of 43 sera obtained from a herd which was subjected to a BLV-control programme based on AGIDT during 32 months, the AGIDT yielded 16.3 % positive and 9.3 % weak-positive reactions, whereas ELISA detected 67.4 % and WB 86 % positive reactions (Fig. 1b).

The WB was shown to be the most sensitive test. The sensitivity and specificity of the AGIDT with respect to the WB for the 140 sera were 51.2 % and 90.9 %, respectively, whereas the sensitivity and specificity of the ELISA with respect to the WB for the 140 sera were 89.1 % and 100 %, respectively. The specificity of ELISA and AGIDT was comparable with WB; however, the sensitivity of ELISA was closer to that of WB and superior to AGIDT.

Although BLV-negative and BLV-positive sera reacted in WB with non-specific bands of molecular weight ranging from 26 to 28 kDa and 60 to 80 kDa, they were easily resolved from BLV-specific bands (Fig. 2, lane a). The reactions presented by BLV-positive sera could be classified into three types: sera which recognized p24 in WB and reacted negatively or weakly positively in AGIDT (positive reaction type 1, Fig. 2, lane b); sera which recognized p24 and gp30 in WB and reacted negatively, weakly positively or positively in AGIDT (positive reaction type 2, Fig. 2, lane c); and sera which recognized p24, gp30 and gp51, some also detected lower molecular weight BLV-proteins (p15 and p12) in WB, and reacted weakly positively or positively in AGIDT (positive reactions type 3, Fig. 2, lane d).

All BLV-negative sera from Germany, BLV-negative sera from Pennsylvania and BLV-negative test sera from local cows only showed the non-specific bands ranging 26 to 28 kDa and 60 to 80 kDa described above, whereas the seven sera from Holstein cows with lymphosarcoma showed reactions against the major core protein p24, the glycoproteins gp51 and gp30 and some low molecular weight BLV-proteins (positive reactions type 3). In this respect, most of the BLV-positive sera of group I reacted in WB against p24 and gp30 (positive reactions type 2), whereas most of the sera in group II reacted only against p24 (positive reactions type 1). In the course of time, some sera in group II showed only a weak reaction against p24 in WB. It was possible to establish a relationship between weak reactions of sera against p24 in WB and the lack of serological detection in ELISA during this time. (Fig. 3).

In this group II it was also possible to demonstrate colostral antibodies with sera from five calves tested by AGIDT, ELISA and WB at various time. All animals (under 2 months of age) reacted positively in ELISA and WB on the first testing date, whereas four animals reacted positively and one calf weakly positively in AGIDT. Four calves that reacted positive in AGIDT showed reactions against p24, gp30, gp51 and some low molecular weight BLV-proteins,

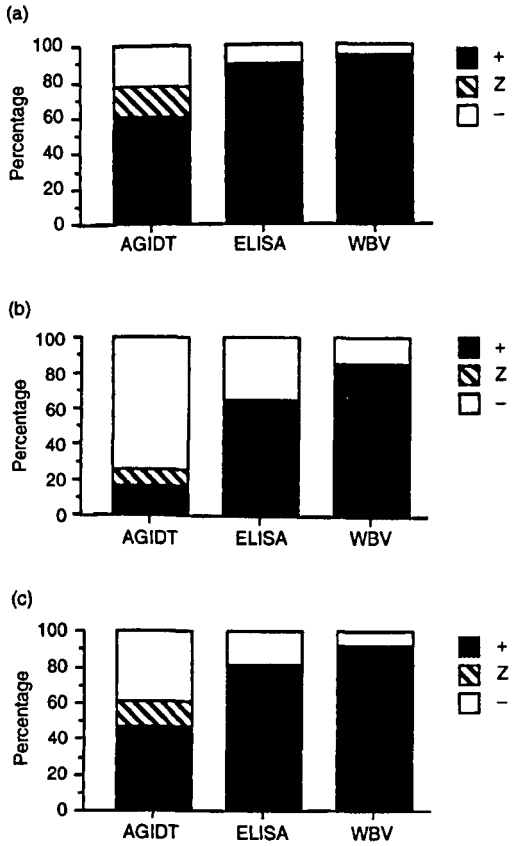

Fig. 1. Comparison of reactions of bovine sera from the highlands from Costa Rica in AGIDT, ELISA and WB. Ninety-seven sera were tested for exportation purposes for the first time for BLV antibodies (a); 43 sera were obtained from a herd subjected to a BLV-control programme based on AGIDT (b). The reactions of the 140 sera were shown in (c). (+, positive reactions; Z, weak-positive reactions; –, negative reactions).

whereas the weakly positively reacting serum in AGIDT showed only reaction against p24 in WB. Six months later all animals presented BLV-negative reactions in WB, ELISA and AGIDT.

Discussion

The results with AGIDT with 140 sera of dairy cattle collected in the highlands of Costa Rica (47.1 % positivity) were in accordance with those from other authors (Rodriguez et al., 1980; Jiménez et al., 1995). However, the proportion of positive animals increased to 82.1 % and 92.1 % when ELISA and WB were used, respectively. Even though, there are several reports which suggest that AGIDT has a lower sensitivity compared with ELISA, which is in accordance to our results, there are, to the authors' knowledge, no data about the sensitivity and specificity of ELISA for the diagnosis of BLV-infection compared with WB. The ELISA described in this work showed high specificity (100 %) compared with WB, since no false positive reactions were found among the group of negative sera obtained from Germany, USA and Costa Rica.

The use of the AGIDT failed in the herd which was subjected to a BLV-control programme based on AGIDT during 32 months. A possible explanation could be that the antigen used in

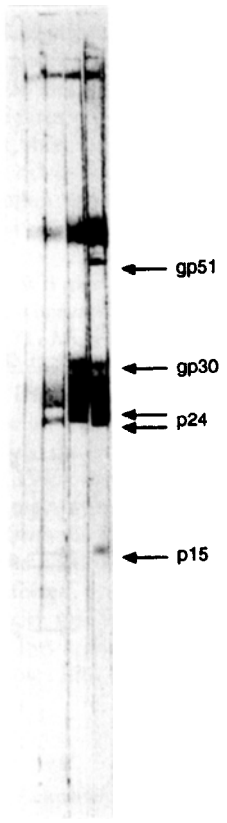

Fig. 2. Description of the different reactions of BLV-negative and BLV-positive sera in WB (lane a = negative reaction, lane b = positive reaction type 1, lane c = positive reaction type 2 and lane d = positive reaction type 3).

this assay consisted mainly of p24 antigen. According to Behrens et al. (1982) the gp51-antigen is of essential importance in AGIDT. In contrast with this are the investigations of Grundboek et al. (1986), Ristau et al. (1988) and Molloy et al. (1990) about a sensitive of p24-AGIDT, p24-RIA and p24-ELISA for the diagnosis of BLV-infections. The results of Bonilla (1985), who compared the AGIDT used in this work with the 'Leukassay B', determined a lower sensitivity for the latter. The author explained these results, indicating that the commercial ID-test could not detect antibodies others than anti-gp51. The present authors' observations using WB, were that most of the sera of the herd which was subjected to a BLV-control programme based on AGIDT showed mainly antibodies against p24.

The results obtained with the AGIDT in this herd that was tested at various times suggest that the antibody content in the sera was under the detection limit of the assay. The same behaviour was described by Manz and Bauer (1985). They showed a failure of the AGIDT compared to ELISA, in herds controlled over a long time with AGIDT. Most of the negative sera by AGIDT were low titre reactors in ELISA. The fluctuation in serological detection of positive sera by ELISA in the herd that was tested at various times (where animals became BLV-ELISA negative, although they were positive in previous and subsequent bleedings) could be due to temporary titre falls, for example in relation with birth, or otherwise find no explanation in the literature (Forschner and Keyserlingk-Eberius, 1980). In WB, these sera showed a weak but still detectable reaction against p24. This observation is relevant, stressing

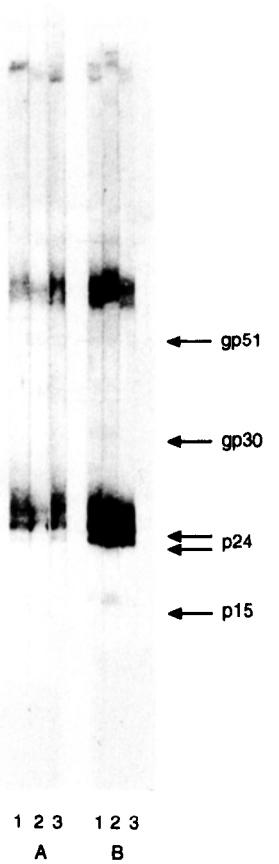

Fig. 3. Reactions of two BLV-positive sera in WB from the herd subjected to a BLV-control programme based on AGIDT. 1, blood sample from day 0; 2, blood sample 6 months later; 3, blood sample 18 months later. (Serum A reacted negatively in AGIDT over time, whereas in ELISA it reacted positively (1), negatively (2) and positively (3). Serum B reacted positively in AGIDT, ELISA and WB over time.

the importance of repeatedly testing and studying the dynamics of the humoral response in BLV-infected herds.

The WB described in this work showed a high sensitivity. In accordance with Bünger et al. (1994), Heeney et al. (1988), Ristau et al. (1988) and Walker et al. (1987) the p24 protein was the most important BLV-antigen concerning sensitivity and specificity of the assay. The WB has the advantages of easy proof of specificity (appearance of the p24-band), and that weak-positive reactions were still distinguishable, even when non-specific reactions appeared (Walker et al., 1987). The non-specific reactions that showed BLV-positive and -negative sera were reported by Walker et al. (1987) as non-specific reactions between the conjugate and the bovine immunoglobulin components of the cell culture medium, where BLV was grown.

Our observations are also in agreement with the WB results obtained by Heeney et al. (1988), who found that sera from BLV-infected cattle always reacted against p24, although sera from animals with lymphosarcoma detected a greater number of BLV-proteins, and with the results from Ristau et al. (1988), who demonstrated that BLV-experimentally infected calves preferentially developed antibodies against p24. In this respect the results of the present study do not agree with Walker et al. (1987) and Kittelberger et al. (1996), who claimed that the

absence of reactivity of sera from BLV-infected bovines against the major BLV-glycoprotein was due to the loss of the reactive epitopes in WB. In the experience of the present authors, those sera which recognized p24, gp30 and gp51 in WB were also precipitated in AGIDT. In these cases, it might be that reduced titres or lower affinity of antibodies against the different viral determinants are more relevant, than the loss of antigenic domains. In this respect, the finding of colostral antibodies in five calves in the herd under control is particularly important. Sera from four calves showed antibodies against different BLV-proteins, and reacted positive in AGIDT, whereas one sera reacted only against p24 in WB, and showed a weakly positive reaction in AGIDT.

The present experiments with WB demonstrated that the p24 antigen was the most important antigen in WB and ELISA, whereas the glycoprotein antigens gp51 and gp 30 seemed to be of special importance in AGIDT. Most of the sera reacted positively in AGIDT, when they showed antibodies against different BLV proteins, especially against the glycoproteins. Weak reactions of sera with p24 in WB led to fluctuation in the serological detection by ELISA.

In countries of high prevalence of BLV-infection such as Costa Rica, where the management of dairy herds and probably the presence of bloodsucking vectors promote the spreading of the virus (Jiménez et al., 1995), tests with relatively low sensitivity are not adequate for control programmes, since they may favour the maintenance of BLV in clinically normal cattle. The present results with sera from the herd under control strongly suggest, that control based solely on a low-sensitivity precipitation assay, might complicate the epidemiology by selecting animals which, although infected, do not produce detectable antibodies in AGIDT. For its high sensitivity, relative simplicity, rapidity, the ease of testing a large number of samples, objective reading and automation ELISA is considered to be the most adequate test for future survey and control programmes in Costa Rica.

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