Evaluation of an Enzyme-Linked Immunosorbent Assay for Detection of Antibodies to Bovine Leukemia Virus in Serum and Milk

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The results of examination of 375 bovine serum and 150 bovine milk samples for detection of bovine leukemia virus infection by the immunodiffusion technique were compared with those of an enzyme-linked immunosorbent assay (ELISA). It was concluded that the ELISA is another useful method for the detection of antibodies to bovine leukemia virus in serum and milk. The ELISA provides a quantitative result and has the advantage of being more sensitive and less time-consuming than the conventional immunodiffusion technique.

Enzootic bovine leukosis is the most important neoplastic disease of cattle. Clinical signs of bovine leukemia virus (BLV) infection (lymphocytosis and lymphoid tumors) appear after a long incubation period during which the presence of antiviral antibodies in serum and milk of infected cattle is the only constant feature of BLV infection.

Viral antibodies produced by BLV-infected cattle include antibodies directed against the nonglycosylated viral components, mainly the major internal protein p24 (24,000 Da), and antibodies recognizing the viral envelope glycoproteins gp51 (51,000 Da) and gp30 (30,000 Da) (8).

Several immunological tests, such as the radioimmunoassay (3) and counterimmunoelectrophoresis (7), have been investigated for detection of antibodies against BLV. However, these methods require complex equipment, are expensive, and are not well suited for mass-screening purposes. Recently, the enzyme-linked immunosorbent assay (ELISA) has been applied for detection of antibodies against BLV by using the BLV particles as antigens adsorbed to the wells of microtiter plates (9). However, this technique usually yields high background values, mainly because of the adsorption of nonviral contaminants of the BLV preparation to the walls of the wells. To overcome this disadvantage, a purified preparation of the envelope glycoprotein gp51 of BLV (6) and the use of purified anti-gp51 monoclonal antibodies (8) were reported. However, the results obtained are not entirely satisfactory, and their routine use in clinical laboratories is therefore restricted. This article reports our evaluation of an ELISA with commercially available equipment and reagents for detection of bovine immunoglobulin G antibody specific for the envelope glycoprotein gp51 antigen of BLV in serum and milk. Results obtained by ELISA procedure were compared with those obtained using the immunodiffusion (ID) technique (1, 4, 10), which is currently used as a diagnostic standard for BLV infection.

Antigen. The gp51 antigen of BLV was prepared as described elsewhere (8) and was kindly supplied by the Institut Vaccinal du Docteur Pourquier, Montpellier, France. Protein content was measured by the method of Bradford (2) by reference to a calibration line obtained with bovine serum albumin (BSA) (IBF Biotechnics, Villeneuve-la-Garenne, France) as a standard.

Serum and milk specimens. Serum and milk specimens from cattle with established BLV infection were obtained from the Institut Vaccinal du Docteur Pourquier. The diagnosis was based on the appropriate clinical syndrome and laboratory evidence of infection. For the latter purpose, the ID test based on the detection of antibodies to BLV was performed as described elsewhere (5) by using the gp51 as an antigen. Bovine serum and milk samples previously tested by the ID technique and shown to be positive or negative were used for the ELISA experiment. The negative and positive control samples (giving a negative and positive reaction in the ID test, respectively) were from cattle without or with appropriate clinical signs from geographic regions in which BLV infection was nonendemic and endemic, respectively.

ELISA. Polystyrene microtitration plates (Maxisorp F 96 Immuno Plate; Nunc) were used as the solid phase for the assays. A total reaction volume of 0.1 ml was used in all microtitration wells, and each experiment was set up in duplicate. All washes were performed four times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T). The substrate solution for peroxidase, containing 10^{-2} M o-phenylenediamine (Sigma) and 10^{-3} M H_2O_2, was prepared in citrate phosphate buffer (0.1 M; pH 5.5). After incubation for 15 min at 37°C, the reaction was stopped by the addition of 100 µl of 4 N H_2SO_4. The optical density at 492 nm (OD_{492}) was measured in a microplate colorimeter (Titertek Multiskan MC; Flow Laboratories). Results are expressed as the difference in OD_{492} (ΔOD_{492}) between experimental and control (optical density obtained for the wells in which gp51 was not present) samples.

For the assay, wells of the microtitration plates were coated with gp51 (400 ng per well) in sodium carbonate buffer (0.05 M; pH 9.6), except for control wells, in which gp51 was not present. After incubation overnight at 4°C, the plates were washed. The coated plates were usually used immediately but could be stored at 4°C for up to 2 months without a decrease in reactivity.

For the ELISA of sera, the samples were tested at a 20-fold dilution in PBS-T containing skim milk powder (0.2%; Gloria S.A., Paris, France) (PBS-T-SMP) and incubated for 1 h at 37°C. The plates were then washed, and a 1-in-1,000 dilution of horseradish peroxidase-labelled rabbit anti-bovine immunoglobulin G (Dakopatts) in PBS-T-SMP
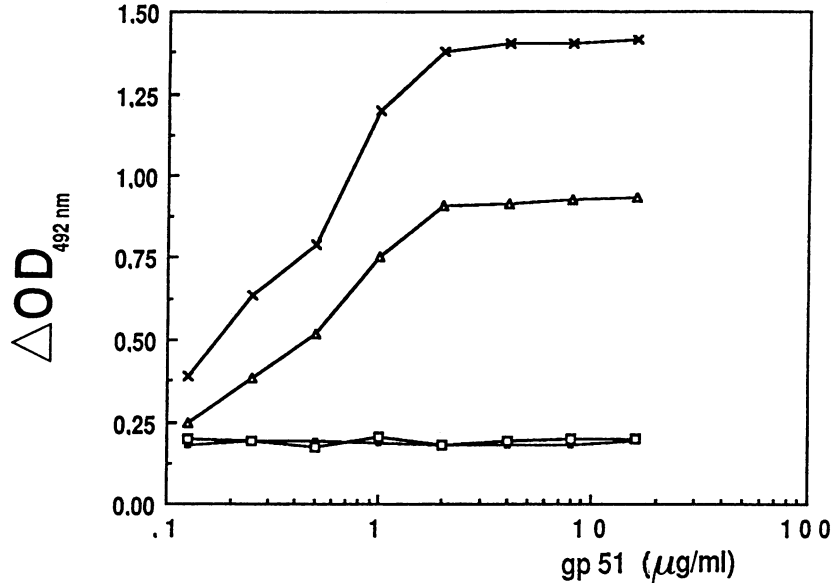
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FIG. 1. Effect of concentration of gp51 coated on wells on immune responses. Symbols: ■, negative control serum, diluted 1:20; ×, positive control serum, diluted 1:20; □, negative control milk, undiluted; △, positive control milk, undiluted.

was added. After being incubated again for 1 h at 37°C, the plates were washed, and substrate solution was added.

For the ELISA of milk samples, the samples were left undiluted and incubated for 1 h at 37°C. The plates were then washed, and a 1-in-1,000 dilution of horseradish peroxidase-labelled rabbit anti-bovine immunoglobulin G in PBS-T containing 0.05% BSA (PBS-T-BSA) was added. After incubation for 1 h at 37°C, the plates were washed, and substrate solution was added.

The differences in OD for duplicate samples were averaged. Serum or milk specimens yielding a ΔOD_{492} greater than 0.26 were considered positive, whereas specimens with a ΔOD_{492} lower than this value were scored as negative. This cutoff provided maximum sensitivity and specificity.

Determination of the optimal reaction conditions for the ELISA. (i) **Amount of gp51.** The choice of the optimal concentration of gp51 used for the coating onto the well was based on obtaining maximal absorbance readings for BLV-positive control samples and minimal background values for BLV-negative control samples. As shown in Fig. 1, for all concentrations of gp51 tested, there was no increase in the background values from BLV-negative control samples. An optimal level of discrimination between positive and negative control samples was obtained with gp51 in the range of 2 to 16 μg/ml. We found that in this range, there was no increase in immune responses to BLV-positive samples. The binding sites were saturated on the solid phase. A gp51 working concentration of 4 μg/ml (400 ng per well) was
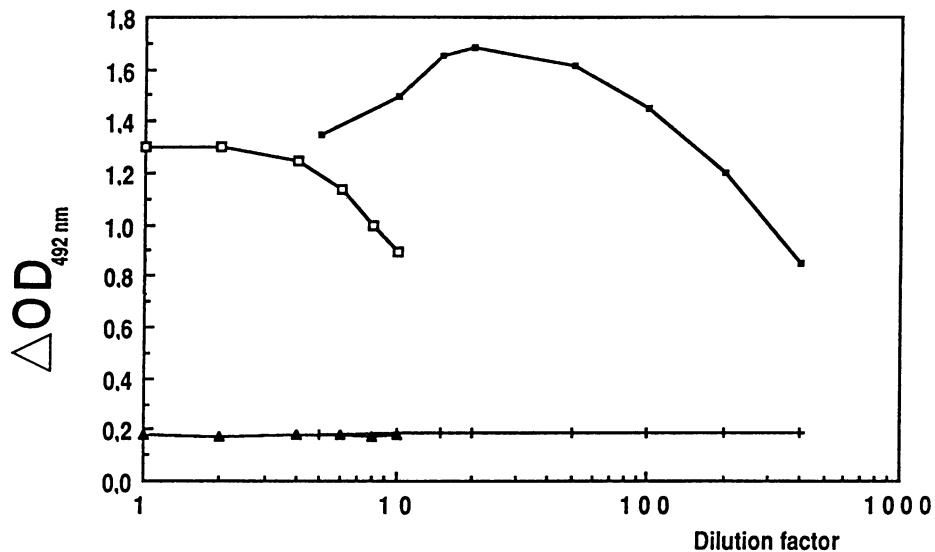

FIG. 2. Dilution curves of samples at various dilutions in PBS-T-SMP (for serum) or PBS-T-BSA (for milk). Symbols: ■, positive control serum; +, negative control serum; □, positive control milk; △, negative control milk.

TABLE 1. Comparison of ID and ELISA results in
detection of antibodies against BLV

Group[a]	No. of serum samples (n = 375)	No. (%) of serum samples positive by:		No. of milk samples (n = 150)	No. (%) of milk samples positive by:	
		ID	ELISA		ID	ELISA
1	180	0 (0)	0 (0)	70	0 (0)	0 (0)
2	45	0 (0)	25 (56)	30	0 (0)	15 (50)
3	150	150 (100)	150 (100)	50	50 (100)	50 (100)

[a] Group 1, samples from cattle without appropriate clinical signs of BLV infection; groups 2 and 3, samples from cattle with appropriate clinical signs of BLV infection.

selected and used for coating onto the wells throughout this study.

(ii) **Establishment of the working dilution of samples.** The ideal ELISA should provide a quantitative answer derived from a single reading at a determined dilution of the samples rather than from a titration curve. We chose a working dilution of samples at which both the positive control samples and the negative control samples (which influence the sensitivity and discriminating potency of the ELISA) gave reliable results. As shown in Fig. 2, a 1:20 dilution of serum and undiluted milk samples seemed suitable for the ELISA.

Evaluation of the ELISA procedure. In the ELISA system, the mean background value (ΔOD_{492}) of the dilution system (when no serum or milk was added) was 0.13. The highest ΔOD_{492} obtained when a series of 20 negative control serum samples and 10 negative control milk samples was tested was less than twice the background value (i.e., <0.26). Therefore, we considered this value a suitable cutoff; serum or milk samples yielding a ΔOD_{492} greater than 0.26 were considered positive, whereas those with a ΔOD_{492} less than this value were scored as negative. A comparison of the results obtained by the ID test and ELISA is shown in Table 1. A total of 375 serum and 150 milk samples previously tested by the ID technique were subjected to the ELISA. The 180 serum and 70 milk specimens that were from cattle without clinical signs and that were negative by the ID method were also negative by ELISA. Of 45 serum and 30 milk samples from animals judged to be infected (on the basis of appropriate clinical signs) but which yielded negative results in the ID test, 25 serum and 15 milk samples were found to be positive by the ELISA (56 and 50%, respectively). All of the 150 serum and 50 milk samples from cattle with appropriate clinical signs that were positive by ID (100%) also were positive by ELISA.

Discussion. Although the same antigen, gp51, was used for both the ID test and ELISA, the ELISA may have detected antibodies different from those measured by ID. ELISA may have detected subclasses of immunoglobulin G which were not reactive in the ID test. The ELISA was more sensitive than the ID assay. The ELISA detected antibodies against BLV in 25 of 45 serum samples and in 15 of 30 milk samples that were from cattle with appropriate clinical signs of BLV infection and that were negative by ID.

Because anti-gp51 antibodies appear earlier than other antibodies after experimental infection (8), most laboratories routinely use the gp51 ID assay for early detection of BLV infection. However, the ID assay suffers from a lack of sensitivity and nonquantitative results. Interpretation of ID results is sometimes subjective, and its reliability depends to a great extent on the training and experience of the laboratory personnel. Despite the small number of samples examined, the results of this study demonstrated that the ELISA is a sensitive and specific test for the diagnosis of BLV infection. The ELISA requires a minimal time for setup (only 2 h to complete the test), and it is easy to interpret the quantitative results obtained. In addition, the precoated plates can be prepared in advance and stored without a decrease in reactivity.

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