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STUDIES ON BOVINE LEUKOSIS

VI. ENZYME LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF ANTIBODIES TO BOVINE LEUKOSIS VIRUS

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Résumé

ÉTUDES SUR LA LEUCOSE BOVINE. VI. UTILISATION DU TEST ELISA POUR LA DÉTECTION DES ANTICORPS CONTRE LE VIRUS DE LA LEUCOSE BOVINE. — Les résultats de l'examen de 2784 échantillons de sérum par le test d'immunodiffusion en gel d'agarose (AGIDT-gp) ont été comparés à ceux du test ELISA. Une discordance a été enregistrée dans 0,5 % des cas. On peut en conclure que la technique ELISA est une autre méthode sérologique intéressante à ajouter à la liste des techniques séroplogiques actuellement disponibles pour la détection des anticorps contre le BLV. Les avantages de la technique sont discutés.

A diverse array of immunologic tests are known to-date for the serological diagnosis of enzootic bovine leukemia (EBL). Because of its simplicity, reproducibility and sensitivity the agar gel immunodiffusion test (AGIDT), using bovine leukemia virus glycoprotein (BLV gp) as antigen, has been selected as tool to be used for the serodiagnosis of EBL in the nine european community countries. The search for more sensitive techniques for large screening purposes is, however, continuing. A radioimmunoassay (RIA) is less suitable as it requires expensive equipment. Its application is therefore restricted to central fascilities. Immunofluorescence as practised in this Institute (Ressang et al., 1978) is helpful if the condition of the test sera is optimal. The evaluation of its end-results, especially with low-titer sera, is sometimes dependent on subjective assessment. The question arises if the enzyme linked immunosorbent assay as described by Engvall and Perlmann (1972) can serve as an alternative. The adaption of an indirect ELISA for the serology of EBL in this Institute was made possible through the work of one of us (Gielkens; manuscript in preparation).

This paper reports the results of a comparative study employing the ELISA and the AGIDT to examine the presence of antibodies to BLV in bovine sera. A small number of sera tested previously in the RIA (Dr. Levy and Dr. Devare) were also examined in both tests.
Materials and Methods

Antigen

Bat cells were obtained through courtesy of Prof. J.F. Ferrer, New Bolton Center, Univ. of Pennsylvania. The cells were kept in stationary cultures in 75 cm² disposable plastic flasks. The medium consisted of MEM Hanks’ solution to which was added 10 % inactivated foetal calf serum, 0.042 % NaHCO₃, 1 % glutamine, 1 % non-essential amino acids and 0.1 % lactalbumine. The cells were passaged every 4-5 days and medium was collected and stored at 4° C until used.

Two liter of medium was clarified by centrifugation at 2,000 rpm for 15 minutes and the virus was pelleted by a centrifugation at 9,000 rpm for 16 hours in a JA-14 rotor of a Beckman centrifuge. The pellet was resuspended in 10 mM Tris-HCl pH 7.4, 100 mM NaCl and 1 mM EDTA (TNE) and BLV was isolated after spinning through a cushion of 10 % sucrose (w/v) in TNE at 20,000 rpm (fixed rotor 40) for two hours. The isolated virus preparation was treated with an equal volume of ether for five minutes. Quick evaporation of the ether was accomplished by flushing with N₂. The antigen was divided in small aliquots (25-50µl) and stored at -20° C.

Sera

A total of 2,784 sera were examined. These could be sub-divided as follows:

a) 433 sera were obtained from the same number of cows known to be free from EBL as evidenced by clinical, hematological and AGIDT gp examinations. These sera were examined in the ELISA to determine the extinction values in normal sera;

b) 15 sera were courteously given by Dr. Daniel Levy (Lab. d’Immunologie et de Virologie des Tumeurs, Hopital Cochin, Paris) and 25 sera were a generous gift from Dr. S.G. Devare, N.I.H., Bethesda, Maryland, USA). Both groups of sera were examined in the RIA using labelled BLV p24 and BLV 24 plus gp 51 respectively;

c) 2,311 serum samples were derived from 17 farms that have been infected with EBL by import of cattle in the past. The sera came from the same number of animals.

AGIDT

The test was carried out as described (Res-sang et al., 1978). Antigen was obtained commercially (Pitman Moore, USA).

ELISA

The indirect micro ELISA procedure was performed in polystyrene microtiter plates (Nutacon, Schiphol). The antigen was diluted in 0.05 M carbonate buffer, pH 9.6 and 100 µl was added to each well. The plate was covered with a plastic tape, kept at 37° C overnight and then stored at -20° C until required. Befor use the wells were washed with distilled water containing 0.05 % Tween 80. Washing was done with a home made device which, under 0.4 atm. pressure, flushed the wells with an excess of washing fluid. One hundred µl of sera at 1 : 10 dilutions were added and after the plate was covered with a plastic sheet it was kept overnight at 4° C. After washing 100 µl of the rabbit anti bovine IgG peroxidase conjugate (Nordic, Tilburg, The Netherlands) was pipetted and the plate kept for one hour at 37° C. The wells were washed again and 100 µl of the enzyme substrate was added. The latter consisted of purified 5 amino salicylic acid dissolved in a solution containing 10 mM phosphate (pH 6.9) and 0.1 M EDTA (pH 6.9) adjusted to pH 6.0. Incubation was at room temperature for 30 minutes. The reaction was stopped with 50 µl 0.2 % sodium azide. Absorbance of the reddish brown colour was read 60 minutes thereafter at 449 nm with a vitatron spectrophotometer. A total of 72 test sera and one serum known to be free from antibodies to BLV (negative control) were examined in each run. Each plate also include a test of a serial two-fold dilution (1 : 80 to 1 : 10240) of a positive control serum, a substrate control (antigen coated well containing only substrate), a conjugate control (antigen coated well to which conjugate and substrate were added) and system control (uncoated wells with positive or negative serum to which conjugate and substrate was given). Serum and conjugate dilutions were made in phosphate buffered saline (PBS, pH 7.1) which further contained 0.05 % Tween 80, 0.03 M EDTA (pH 7.0) and 1 % lamb serum.

ELISA competition test

For the determination of the specificity of the immunological reaction in the ELISA, a competition test was performed. For this purpose, a BLV gp antigen preparation (Pitman Moore, USA) previously dialyzed against
PBS, was diluted to give a final protein concentration of 2 mg x ml⁻¹. This antigen preparation was added to an equal volume of test serum, diluted 1:5 in PBS and the mixture was incubated for one hour at 37°.

After preincubation, blocking of virus-specific antibodies was measured by using the indirect ELISA.

As a control, positive and negative reference sera preincubated in the presence or absence of BLV gp antigen, was included in this assay.

**Results**

The distribution of the extinction values in the indirect ELISA of 433 sera from clinically normal, non-EBL affected cows are given in Table 1.

The majority (63.05%) of normal sera had absorbance values between 0.2 - 0.4 and only 3.23% were in the 0.6 - 0.8 range.

It was therefore decided to consider sera with an E₄₉₉ value higher than 0.8 to be positive.

The outcome of the ELISA and AGIDT gp with 2311 sera is presented in Table 2.

There was a good agreement between the results of both tests. A discrepancy was seen in twelve (0.5%) cases.

The forty sera which were tested in the RIA had results identical to those of the ELISA and the AGIDT employing gp. All the positive sera (26 samples) had E₄₉₉ values higher than 1.0. Negative samples scored below 0.5.

**Discussion**

Based on the results obtained with the ELISA it can be stated that this technique is another useful serological method that can be added to the list of established tests presently available for the demonstration of antibody to BLV. Its results compared very well with those of the AGIDT gp. A discrepancy in the outcome of both tests was found in only 0.5%.

The question whether the ELISA is an alternative to the RIA for the demonstration of antibodies to BLV can only be answered by a comparative test preferably with samples collected in an EBL infected farm (for sensitivity) and in an EBL free one (for specificity). The impression exists that for the detection of antibodies to BLV, the ELISA equals the indirect FAT as regards sensitivity and specificity. The results of the former can, however, be read more objectively by spectrophotometry. Besides the ELISA can be mechanized.

We are presently testing other methods of antigen preparation and it seems that simple, less laborious techniques such as precipitation with polyethylene glycol are equally suitable. A crucial part of the ELISA is the background staining in some negative sera. This can be greatly overcome by thorough washing, right choice of microtiter plates and an optimal working dilution of the conjugate. The latter can be found out by checkerboard titration. The addition of 1% lamb serum in the dilution fluid of the serum and the conjugate is also of much help. The overnight incubation at 4° C with serum can be reduced to 2-3 hours incubation at 37° C with equal results.

An explanation for the discrepancy in 12 samples (0.5%) is difficult to find. The sera from the six cows positive in the AGIDT but negative in the ELISA could not be retested as the animals were already slaughtered. From the six ELISA positive, AGIDT negative sam-
ples only three sequent sera could be reexamined. One of these became positive in the AGIDT. Of the remaining two sera the extinction values were reduced over 50 % as measured in the ELISA competition test, after preincubation with BLV gp antigen. This indicated that the ELISA reaction with the discrepant sera was specific.

It is quite possible that the discrepancy was due to the different class of antibody demonstrated in both tests.

Another possibility of the cause of the discrepancy was the difference in the representation of gp-51 and p24 in the BLV preparation used for the ELISA as compared with the Pit man Moore antigen.

Summary

The results of examination of 2 784 sera in the AGIDT-gp were compared with those of the ELISA. A discrepancy was seen in 0.5 % of the cases. It was concluded that the ELISA was another useful serological method added to the list of serological techniques presently available for the detection of antibodies to BLV. The advantages of the technique are discussed.

References