# BOVINE LEUKOSIS - RECENT DEVELOPMENTS WITH USE OF MONOCLONAL ANTIBODIES AND ELISA TESTS

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ABSTRACT

A variant of the enzyme-linked immunosorbent assay (ELISA) technique involving the use of a specially selected monoclonal antibody against the BLV envelope glycoprotein gp51 yields a highly sensitive, practical and cheap method for the detection of Bovine Leukemia virus (BLV) antibodies, gp51 antigen and for the measurement of antigenic variations in the gp51 molecule.

## INTRODUCTION

The enzootic form of bovine leukosis, a contagious, typical herd disease, is induced by bovine leukemia virus (BLV), a retrovirus which is exogenous to the bovine species.

Serological surveys of the cattle population seem to be the only approach for the early detection of BLV infection and the adequate basis for an efficient policy of eradication of the disease. Because the antibodies against the envelope glycoprotein BLV gp51 appear earlier after experimental infection and their titre is consistently higher than the antibodies against the internal protein p24, a test involving the gp51 antigen is the most sensitive method for the early detection of BLV infection (for review, see Burny et al., 1980).

In our laboratory, liquid phase radioimmunoassay (LPRIA) involving iodinated gp51 has repeatedly been shown to be the most sensitive test (Mammerickx et al., 1980; Portetelle et al., 1980). An optimised agar gel immunodiffusion test involving gp51 and other BLV proteins shows good agreement with LPRIA, detects anti-BLV antibodies soon after seroconversion and is moreover efficient for eradication of bovine leucosis (Mammerickx et al., 1978).

The recent development of enzyme immunoassays has elicited great interest among scientists concerned with the seroepidemiology of bovine

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leukosis, but so far, results have been disappointing. (Hoff-Jorgensen, 1980; Ressang et al., 1980; Manz et al.,1981; Altaner et al., 1982; Graves et al., 1982; Maris et al., 1983).

The basic principles of enzyme immunoassays are similar to those developed for quantitative radioimmunoassays but the measurement of the enzyme activity replaces the counting of radioactivity (De Savingny et al., 1980). The heterogeneous enzyme immunoassays, popularly named ELISA (enzyme-linked immunosorbent assay), take advantage of a solid phase to separate the free enzyme-labelled antigen or antibody from the specific antigen-antibody complexes containing the enzyme-labelled antigen or antibody. Enzyme immunoassays which do not imply the use of a solid phase are based on antibody-mediated changes in enzyme activity. This procedure is defined as a homogeneous enzyme immunoassay. At present, only ELISA appears to be applicable in viral diagnosis (Avrameas, 1983).

An efficient ELISA procedure implies a high degree of specific binding versus a low degree of non-specific binding to the solid phase used. These performance characteristics are dependent to a great extent on the binding characteristics and the purity of immunoreactants involved. The availability of immunoglobulin preparations with every molecule directed against an antigen of choice should improve the kinetics of the antigen-antibody reaction and thus the sensitivity and the specificity of immunoassays.

For this reason, investigators concerned with immunoassay systems are particularly interested in the evolving technology related to the production and development of monoclonal antibodies (Yolken, 1983). The potential advantages of monoclonal antibodies for immunoassay systems are numerous and are summarised elsewhere (Van Zaane, this book; Langone and Van Vunakis, 1983).

Although monoclonal antibodies can be utilised in a wide variety of immunoassays including radioimmunoassays and immunofluorescent assays, our work has focused on adapting monoclonal antibodies to the ELISA technology in BLV research.

The principle of the use of ELISA test is largely accepted in veterinary laboratories since these assays use stable and non-radioactive reagents. This stability can also take maximal advantage of the high consistency offered by the homogeneous and monospecific nature of monoclonal antibodies.

PRINCIPLE OF THE METHOD

For this study, a variant of the classical ELISA technique is described, which involves the use of a gp51-specific monoclonal antibody. From a panel of 15 monoclonal antibodies directed against gp51, the antibody providing maximal exposure of gp51 antigenic sites recognised by bovine antisera was selected. This specially selected purified anti-gp51 monoclonal antibody is adsorbed to the wells of a microtitre plate to which it specifically binds the antigen contained in a non-purified BLV preparation.

Bovine antibodies reacting then with gp51 can be detected by an enzyme-coupled antibovine immunoglobulin immunoreactant. If we use monoclonal antibodies directed against the different independent epitopes on the gp51 molecule as anti-gp51-peroxidase conjugate, we can search for BLV gp51 or BLV particles in complex media. If we first adsorb each of the monoclonal antibodies on the solid-phase, select for the gp51 in a non-purified preparation and then reveal the presence of gp51 with a mixture of the different monoclonal antibodies conjugated to the peroxidase, we can define antigenic variations in different BLV isolates (Fig. 1).

IMPORTANT TECHNICAL CONSIDERATIONS

Our ELISA tests use an antibody immobilised on a solid-phase. Finally, the enzyme-labelled antibodies react with the specific antibody-antigen complex but also in a non-specific way with eventual antigenic structures adsorbed to the solid phase.

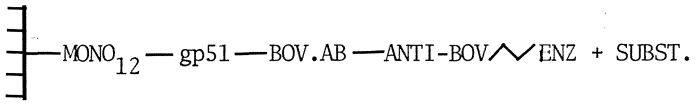
The specificity, the sensitivity and the reproducibility of the ELISA tests depend on several factors: the solid phase, the monoclonal antibody, the purity of this preparation, the antigen preparation, the saturation protein, dilutions of serum used, incubation time and temperature, reaction volumes, non-ionic detergent, properties of the enzyme-antibody conjugate, enzyme substrate.

1. Choice of the monoclonal antibody:

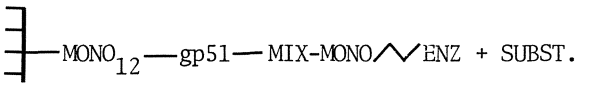
The production of monoclonal anti-gp51 antibodies has been described previously (Bruck et al., 1982 a, b). Monoclonal reagents used in this study were purified from mouse ascitic fluids by DEAE-Affigel blue (BIORAD) chromatography (Bruck et al., 1982c) and stored in aliquots at -80°C.

The monoclonal reagent used to bind gp51 was chosen from a panel of 15 monoclonal antibodies directed against 8 independent epitopes on the gp51 molecule.

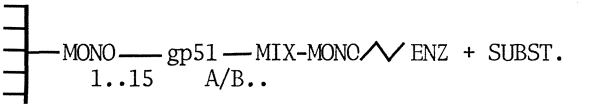
1. DETECTION OF BOVINE ANTIBODIES




2. DETECTION OF gp51 ANTIGEN



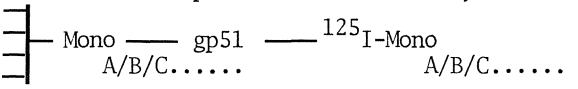

3. DETECTION OF gp51 ANTIGENIC VARIATIONS




MONO : Monoclonal antibody.
1...12 : Number.
BOV.AB : Bovine antibodies.
MIX : Mixture of monoclonal antibodies.
A/B : Different BLV isolates.
ANTI-BOV: Antibovine immunoglobulins.
ENZ : Enzyme (peroxidase or β-galactosidase).
SUBST : Enzyme substrate.

Fig. 1 Schematic representation of the ELISA tests.

Since the anti-gp51 antibodies produced by infected cattle were shown to be directed mainly against a limited region on gp51 (Portetelle et al., 1980) and more precisely sites, F,G,H defined with monoclonal antibodies (Bruck et al., 1983), the choice of the monoclonal antibody to bind gp51 to the solid phase was based upon obtaining optimal exposure of the relevant antigenic sites F,G,H to the bovine antibodies. This was determined in a sandwich solid phase radioimmunoassay as below:




With the monoclonal antibody GA12 directed against site E, the optimal exposure of the bound antigen was obtained for the binding of monoclonal antibodies against sites F,G,H and thus for the binding of bovine natural antibodies. Minimal non-specific binding and minimal background were also observed with this monoclonal antibody (Portetelle et al., 1983b).

2. Choice of the purification method for monoclonal antibodies:

The use of DEAE-Affigel Blue chromatography for the purification of monoclonal antibodies from ascitic fluids (Bruck et al., 1982a) leads to minimal non-specific binding in our ELISA tests (Portetelle et al., 1983b) probably because proteins involved in non-specific binding are retained on the column. It is thus important to use the purest antibody preparations available to coat the plastic surface.

3. Choice of the microtitre plate:

Microtitre plates specially treated to increase their fixation capacity (for use in enzyme immunoassay) are not suitable in our ELISA system for the detection of bovine antibodies to BLV with an excess of enzyme-labelled anti-immunoglobulin reagent. High background values and high non-specific binding were observed with these plates: we use normal microtitre plates to ensure optimal specificity (Portetelle et al., 1983b).

However, the treated plates are suitable for the detection of gp51 antigen or gp51 antigenic variations because maximal absorption of the monoclonal antibody to the well provides maximal sensitivity of these tests.

4. Choice of an inert protein:

To minimise non-specific binding and background, sites on the solid-phase are saturated with an inert protein. We have tested several sources of inert protein: bovine serum albumin (BSA), gelatin and serum from different animal species. In our system, only BSA (Sigma A9647 or other commercial BSA with equivalent quality) seems to give satisfactory results. Fraction V of BSA from certain preparations may contain contaminants which cross-react with bovine immunoglobulins and the conjugate. With other proteins, high cross-reactivity with the anti-bovine immunoglobulin conjugate is observed (gelatin contains fragments of bovine immunoglobulins).

5. Properties of the enzyme-protein conjugate:

Conjugate effectiveness depends on both the enzyme and the antibody used, and also on the conjugation procedure chosen to link the two, and plays a very important role in the development of ELISA tests. This is especially true for the detection of bovine antibodies to BLV. However, in the case of gp51 detection with the monoclonal antibodies mixture labelled

with peroxidase, no special problem exists, probably because of the purity and the homogeneity of the monoclonal antibody preparations.

5.1 Enzymes:

We use only two enzymes in our system: horseradish peroxidase and E. coli β-galactosidase. These enzymes possess a high substrate turnover number, do not lose much of their activity after linkage to the antibody and remain stable at room temperature throughout the periods usually required for ELISA assays. In commercial preparations, the highest specific activity (and thus purity) is generally observed with peroxidase. However, β-galactosidase is known to give the lowest non-specific binding (Avrameas, 1983).

5.2 Coupling procedures:

Antibodies (Ab) are coupled by the periodate-oxidation method to the peroxidase (POD) preparation in a molar ratio POD: Ab = 2.5:1 (Portetelle et al., 1983a). The SH groups of β-galactosidase (GAL) can be linked to Fab fragments with a thiol-reacting agent (molar ratio 1:1) (O'Sullivan and Marks, 1981).

These coupling procedures produce a homogeneous population of conjugates which are able to detect the presence of bovine antibodies with the maximum of sensitivity. It has been reported that a higher molecular ratio in the conjugates increases the sensitivity but also increases non-specific reactions (O'Sullivan et al., 1981).

5.3 Choice of the anti-bovine immunoglobulins antibodies:

So far, we have tested three different anti-bovine antibodies conjugates for their capacity to yield the minimal non-specific binding in the detection of specific bovine antibodies.

1. Fab fragments from goat anti-bovine immunoglobulins - POD.
2. Fab fragments from goat anti-bovine immunoglobulins - GAL.
3. Fab fragments from rabbit anti-goat immunoglobulins - POD.

All of these Fab preparations are purified by affinity chromatography on bovine immunoglobulins immobilised on AH-Sepharose (Portetelle et al., 1983a).

We have tested conjugates (1) and (2) on a large scale (Mammerickx et al., 1983), the best results were observed with the β-galactosidase

conjugate. Conjugate (3) seems as promising as the β-galactosidase conjugate (unpublished observations).

Protein A from Staphylococcus aureus appears to be a poor reagent for detecting specific bovine antibodies, as it possesses a very low affinity for bovine immunoglobulins.

Conjugates based on the biotin-streptavidin or avidin interaction yielded a higher amplification of the specific signal but a simultaneous increase of the non-specific signal.

5.4 Choice of the enzyme substrate:

Several different chromogenic and fluorogenic substrates are available (Avrameas, 1983). β-galactosidase activity can be easily determined with O-nitrophenyl-B-D-galactose, using a spectrophotometer at 410 nm.

In the case of peroxidase, several chromogenic donors have been used for their spectrophotometric measurement. The first results were obtained with O-phenylenediamine, a substance that gives highly sensitive and reliable measurements at 492 nm (Portetelle et al., 1983a; Mammerickx et al., 1983). Other highly sensitive marker substances, like ABTS, O-dianisidine and tetramethylbenzidine are also available. Tetramethylbenzidine appears to give a high specific signal versus a low non-specific signal and is a non-hazardous substance (Table 1). Chloro-1-naphthol,

TABLE 1 Effect of the peroxidase substrate on the specificity of the BLV ELISA test for the detection of specific bovine antibodies.

| Substrate | Index value |
|---|---|
| O-Phenylenediamine | 100 |
| O-Dianisidine | 118 |
| Tetramethylbenzidine | 158 |

Conjugate : Fab fragments from rabbit antigoat Ig-POD.
Serum dilution tested : 1/200.
Index value : Specific OD for reference positive serum divided by specific OD of reference negative serum.
Data are expressed as percentage of the minimal index value observed.

diaminobenzidine, diethylcarbazole yield poor sensitivity in the detection of POD activity. Chromogenic substrates with spontaneous oxidation should

be avoided (brown colour of O-phenylenedianine).

## 6. Determination of the optimal reaction conditions

### 6.1 Binding of monoclonal antibody:

The quantity of antibody fixed to the solid-phase is of critical importance in the establishment of an efficient ELISA test. Coating at high antibody concentration (300 ng per well) enhances the specific binding of gp51 antigen in the case of gp51 detection (law of mass action) and thus the sensitivity. A dose of 100 ng monoclonal antibody per well is optimal for the detection of bovine antibodies to BLV (Portetelle et al., 1983a); if 300 ng are added, more gp51 is involved in the titration test and the sensitivity is reduced. The use of less than 100 ng of monoclonal antibody increases the sensitivity of the test but requires longer incubation of the antigen - bovine antibody reaction and also increases the risk of non-specific binding with some bovine sera.

### 6.2 Binding of gp51 antigen:

Precoated plates (monoclonal antibody - gp51) for the detection of bovine antibodies to BLV can be prepared in advance and stored (at 4°C for at least one month). Four day culture supernatant of a BLV-infected fetal lamb kidney cell line (FLK) (Van Der Maaten et al., 1976) is used as a source of antigen. The culture medium is MEM supplied with non-essential amino-acids, kanamycin and 10% heat inactivated fetal calf serum. Minimal non-specific binding and background are observed with fetal calf serum versus newborn calf serum.

Cell debris are eliminated by centrifugation and filtration (0.45 µ); 50 µl of supernatant (more than 10 ng gp51) is mixed with 50 µl saturation buffer (2% bovine serum albumin in a high phosphate - 0.2M - concentration buffer to minimise pH variation) and incubated for a minimum time of 24 h. This relatively long incubation time necessary for maximal yield of the system is required because of the low antigen concentration used and the low avidity of monoclonal antibody GA12 for gp51 (Bruck et al., 1982a). For the detection of gp51, a maximum volume of 200 µl containing the saturation buffer and the non-ionic detergent Tween 80 (maximum 2%) is generally used and incubated 16 h at 4°C.

### 6.3 Influence of reaction volume, detergent, time and temperature:

We have repeatedly shown that incubation at room temperature or 37°C

appears to be deleterious to the gp51 antibody reaction (Portetelle et al., 1980; 1983a). Reaction of antibody with gp51 is more complete when the incubation temperature is 4°C. A standard incubation time of 4 h is generally used for the conjugates. Monoclonal antibody is generally coated in a 50 µl volume. Incubation of the bovine serum dilution in 200 µl slightly decreases the sensitivity of the detection test (16 h as standard incubation time), but most interestingly also the non-specific adsorption of negative BLV serum: non-specific immunoglobulin bound to the upper part of the well is not revealed by addition of 50 µl of conjugate.

Non-specific adsorption can be reduced but not excluded by including a non-ionic detergent in the medium during the incubation (2% for bovine serum) and washing steps (0.2%). We have used Tween 80, either alone (0.2% in conjugate dilution) or supplemented with BSA (2%) in other cases (see Appendix).

6.4 Influence of serum dilution:

The quantity of bovine antibodies fixed to the solid phase is of critical importance in the establishment of an efficient ELISA test. High concentrations of bovine antibody reduce specific binding and enhance non-specific binding (steric hindrance). When the dilution of bovine serum used is too low, the specific binding on the solid phase will be small and consequently the sensitivity will be reduced. It is important to find the optimal dilution for the ELISA system under investigation.

Sera from a known infected herd and from nineteen BLV-free herds were tested at four dilutions: 1/20, 1/60, 1/180, 1/540 at the same time with the β-galactosidase conjugate (2) (Mammerickx et al., 1983). The optical density was read and the animals are classified according to the optical density scored. The best results were observed with the dilution 1/60. At this dilution the two cattle populations were the most distinctly separated. The mean value of optical density (OD) observed with positive animals was in the range of 0.500-0.750 OD; the mean OD value for negative animals was in the range o.001-0.025 OD. A suitable cut-off value between the two cattle populations appeared to be situated at the optical density 0.150 for the serum dilution 1/60.

ADVANTAGES

We have found that specially selected monoclonal antibodies can be

used to obtain sensitive immunoassay systems. The main limitation of monoclonal antibody technology in immunoassays continues to be the tediousness of the procedures necessary for generation and selection of the hybridoma clones (Langone and Van Vunakis, 1983).

The system so designed for BLV research was shown to be as sensitive as gp51 LPRIA for the detection of bovine antibodies (Portetelle et al., 1983a); 10 pg gp51 per well can also be detected with our ELISA system although our classical radioimmunoassay can detect 50 pg. This difference is due to the stability and the homogeneity of the immunoreactants in the ELISA test and the accelerated radiolysis observed with gp51 if specific activity is higher than $2X10^6$ dpm/µg. The sensitivity of this system takes advantage of the increased concentration of specific antibody on the solid-phase offered by monoclonal antibodies (Table 2), and the increased range of antigen-binding sites offered by the polyclonal antibodies or the mixture of monoclonal antibodies as conjugates.

TABLE 2 Concentration of specific antibody in different preparations.

| Antibody preparation | Concentration of specific antibody (%) |
|---|---|
| Monoclonal | 100 |
| Affinity purified | 30(*) |
| Hyperimmune | 10(*) |

(*) Relative concentration, in comparison with the monoclonal antibody preparation.

Sensitivity and specificity are assured by the fact that the use of a monoclonal antibody rather than a polyclonal antibody for gp51 provides minimal interference with the glycoprotein antigen sites recognised by the bovine antibodies, ensures optimal presentation of antigen and reduces the non-specific binding observed with serum from immunised animals, which often contains antibodies to non-viral antigens. High background values are observed when BLV antigen is adsorbed directly onto the wells of microtitre plates probably because of the contamination of the BLV preparation by cellular antigens. Index values (mean value of OD for positive sera divided by mean value of OD for negative sera at the same dilution)

generally cited in the literature in this case were less than 2.0 (Graves et al., 1982). The use of purified gp51 as antigen in ELISA test would most probably overcome this disadvantage of high non-specific binding but would also significantly increase the cost of the test. Monoclonal antibodies adsorbed to the well specifically select for the antigen contained in a non-purified preparation. Since monoclonal antibody can be produced in unlimited amounts from ascitic fluids, our test thus also provides a cheap method for the detection of anti-BLV antibodies.

Standardisation and reproducibility of ELISA involving monoclonal antibody is very easy since monoclonal antibodies are homogeneous and monospecific reagents.

For the large scale detection of BLV infection with an ELISA system, it is necessary to automate the ELISA procedure (multiple washings, precision of the dilution, multiple incubation with different reagents) in order to obtain a diagnostic test which would be as practical as the immunodiffusion test for the detection of BLV specific antibodies in sera.

PRESENT AND FUTURE APPLICATIONS

Bovine material is known to be the most complicated material to be used in ELISA techniques because high non-specific binding is frequently observed.

A proper choice of the conditions used in our non-classical ELISA technique for BLV was necessary to render this test sensitive, specific and reproducible. The lack of need for antigen purification also makes this test a cheap test. These important technical considerations are probably also suitable for the large scale detection of other bovine viral antibodies or antigens eg rotaviruses and coronaviruses.

At the present time, the sensitivity of all ELISA tests greatly depends on the avidity of the antibodies used in the conjugate. To reduce the non-specific signal in the bovine system, it is absolutely necessary to use antibodies and more precisely Fab fragments isolated by immunoadsorption.

In the future, the use of conjugates with highly avid monoclonal antibodies directed against several epitopes on the bovine immunoglobulins might obviate the need for polyclonal antibodies isolated by immunoadsorption. Monoclonal antibodies can be utilised in a wide variety of immunoassays eg ELISA, immunofluorescence etc to overcome the disadvantages

inherent to the generation of antibodies in animals (presence of non-specific antibodies).

ELISA and monoclonal antibodies technology thus appear as two complementary new developments in veterinary research. This is especially true in BLV research, where they provide a basis for serological surveillance of the cattle population and also for the production of an experimental vaccine. Monoclonal antibodies allow an independent study of each epitope displayed by an antigenic molecule and thus facilitate the study of antigenic variation among different virus isolates as well as the study of an eventual gp51 subunit or a synthetic peptide to be used as a vaccine against BLV infection. BLV-neutralising monoclonal antibodies can be used as probes for the detection of these important epitopes in the development of a BLV vaccine.

## APPENDIX

## ELISA TEST FOR DETECTION OF BOVINE ANTIBODIES TO BLV

Some modifications were brought to the procedure previously described (Portetelle et al., 1983a).

Monoclonal antibody (100 ng per well) in 50 µl of PBS (pH 7.4; sodium phosphate 0.01 M, NaCl 0.15 M) was incubated for 16 h at 4°C in the wells of a 96-well microtitre plate (GIBCO 2-69620). Unbound antibody was discarded and the wells were washed twice with 200 µl of PBS containing 0.2% Tween 80 (washing buffer). Saturation buffer (50 µl of buffer pH 7.4, sodium phosphate 0.2 M, NaCl 0.15 M containing 2% bovine serum albumin and 0.06% azide) was added into each well; after 15 minutes, 50 µl of culture supernatant from BLV infected cell line was added and mixed. After at least 24 h incubation at 4°C, the antigen solution was discarded and the wells were washed twice with 200 µl of washing buffer. The sera to be tested were diluted (first dilution 1:20, dilution factor 1:3) in 200 µl of reaction buffer (PBS containing 2% bovine serum albumin, 2% Tween 80, 10% glycerol and 0.02% azide) and incubated overnight at 4°C.

The wells were then washed three times with 200 µl of washing buffer and 50 µl of the same buffer containing the POD-Fab or GAL-Fab anti-bovine immunoglobulin conjugate at a concentration of 35 ng Fab per well. The plate was incubated at room temperature for another 4 h and then washed four times with washing buffer. Bound peroxidase was revealed by adding 100 µl of freshly prepared substrate (0.04% chromogenic substrate and

0.02% peroxide) in 0.1 M Na $H_2$ $PO_4$. $H_2O$, pH5. Bound β-galactosidase was revealed by adding 100 µl of freshly prepared substrate (0.08% ONPG) in 0.1 M Na $H_2$ $PO_4$. $H_2O$, pH7, containing 1 mM $MgSO_4$ and 0.2 mM Mn $SO_4$. The mixture was allowed to react for 20 mins at room temperature in the dark (POD) or for 45 mins (GAL). The reaction was stopped by the addition of 100 µl 6N HCl (POD) or 100 µl 1M $Na_2$ $CO_3$ (GAL). The optical density was scored by a Microelisa Automatic Reader.

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