Assessment of the Sensitivity and Specificity of Enzyme-Linked Immunosorbent Assay (ELISA) for the Detection of Mycobacterial Antibodies in Bovine Tuberculosis

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With 2 figures

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Summary

An ELISA which detects bovine circulating IgG mycobacterial antibodies using M. bovis PPD as antigen was assessed. PPDs prepared from unheated cultures of two M. bovis strains were compared with autoclaved bovine PPD; the latter was found to be a more reliable antigen.

Ninety per cent of bacteriologically confirmed tuberculous cattle were detected by this method (18/20), whereas 89.8% of negative reactions (44/49) were observed in healthy cattle from a tuberculosis-free area. Antibody levels of tuberculin negative cattle from an endemic area did not differ significantly from antibody levels of cattle from a tuberculosis-free area.

An almost complete qualitative correlation was observed between instrumental and visual readings.

In the present trial, the sensitivity and specificity of the ELISA were similar to those of the tuberculin skin test at its best. In addition, operative advantages and low cost of the ELISA make of it a valuable tool for the diagnosis of bovine tuberculosis.

Key words: Bovine tuberculosis, mycobacterial antibodies, ELISA

Introduction

Bovine tuberculosis is still a problem of both public health and economic importance in large areas of the world. The detection of tuberculosis infection in live cattle is based exclusively on the tuberculin skin test. This is a time-consuming and costly procedure because of the movement of animals and the veterinary services involved. In addition, different interpretation criteria and subjective factors may lead to discrepancies in the results.

The potential use of antibodies against mycobacterial antigens as a diagnostic tool in human and animal disease has been explored with variable success (1, 4, 7, 14). A number
of serological methods for detecting tuberculous cattle have been described, such as complement fixation, bentonite flocculation, kaolin agglutination, indirect immunofluorescence and gel precipitation (2, 13, 16, 19, 21). None of them achieved the desired specificity and sensitivity or possessed the operative advantages required for large scale use.

Recently, THOEN et al. (17, 18) applied a modified enzyme-linked immunosorbent assay (ELISA) to the detection of mycobacterial antibodies in the sera of cattle naturally and experimentally exposed to *M. bovis*. To our knowledge, however, the specificity and sensitivity of the method remain unexplored.

An ELISA that detects bovine circulating IgG mycobacterial antibodies using PPD as antigen has been developed in our laboratory. The aim of the present study was to assess its sensitivity and specificity in view of its possible application to the diagnosis of bovine tuberculosis in the field.

**Material and Methods**

**Antigens:** The following antigens were evaluated:

i) *M. bovis* tuberculin PPD, batch 1—85, 5.5 mg/ml in pH 7.0 phosphate buffered saline (PBS), 0.5% phenol solution, prepared at CEPANZO from autoclaved strain AN5 culture filtrates on Dorset Henley medium, by the trichloroacetic acid precipitation method according to GREEN and modified by H. Huitema (8, 11). This PPD was standardized against the European Economic Community Standard for bovine PPD in guinea pigs (20).

ii) A PPD prepared from strain AN 5 in the above-mentioned medium, without autoclaving the cultures. Mycobacteria were killed by treatment with 1% phenol at 37°C for 3 days and filtered (3). The protein content of this PPD solution was 0.80 mg/ml.

iii) A PPD prepared from BCG strain, following the same method as ii) and with a final protein concentration of 0.80 mg/ml.

The biological potency of unheated PPDs was tested by a six-point assay in previously sensitized guinea pigs using *M. bovis* PPD batch 1—85 as reference. Results were statistically evaluated by Finney’s standard method for parallel-line assays (5). Relative potencies of PPD strain AN 5 and BCG were 30 % and 102 %, respectively.

Antigen concentrations ranging from 0.80 to 0.02 mg/ml in carbonate buffer pH 9.6 were assayed during the optimization of the method.

**Sera:** Serum samples were collected from 120 cattle grouped as follows:

a) Forty-nine healthy animals from a tuberculosis-free area (Campo DILFA, Uruguay).

b) Fifty-one tuberculin negative cattle from a tuberculosis endemic area (Province of Buenos Aires, Argentina) under a voluntary programme of bovine tuberculosis control and eradication.

c) Twenty cattle with macroscopic tuberculous lesions at necropsy selected at a slaughterhouse. Samples of lesions were collected for bacteriological studies. Serum samples were obtained from the intracardiac clot.

Sera were kept at −20°C until used. The optimal serum dilution was found to be 1:200 and was used throughout the optimization of the ELISA and the analysis of the above-mentioned samples. Positive and negative control sera, kindly provided by Dr. C. THOEN, were included in triplicate in each test.

**Bacteriological studies:** Specimens were minced in a tissue grinder, decontaminated by adding 4% sodium hydroxide, and cultured on Löwenstein-Jensen and Stonebrink media. *M. bovis* was isolated from all of the 20 specimens collected at necropsy. Species identification was based on previously described tests (12).

**Conjugate:** Affinity purified goat anti-bovine IgG (H + L chain) coupled to horseradish peroxidase was obtained commercially (KIRKEGAARD-PERRY, Gaithersburg, MD. U. S. A.) and titrated every time a new lot was needed. Aliquots were kept at −20°C and diluted when required. Optimal dilutions ranged between 1:200 and 1:400 in PBS pH 7.3 with 1% bovine serum albumin (BSA) (Fraction V, Pentex, Miles Ltd., Elkhart, Indiana 46515, U. S. A.).

**Substrate:** The working substrate solution was prepared immediately prior to use by combining 50 μl of 2,2-azinobis (3-ethyl benzthiazoline sulphonic acid) (Sigma Chemical Co) stock solution (21.6 mg/ml), 50 μl of H₂O₃, 8 M and 12 ml of citrate buffer pH 4.

**ELISA procedure:** 50 μl of antigen were dispensed into each of the 96 wells of polystyrene microtitre plates (Immulon, II, Dynatech Laboratories, Alexandria, Va) and incubated at 4°C overnight. After discarding the contents, the plates were washed three times with 0.1% BSA, 0.05 %
Tween 20 PBS. Test sera diluted 1:200 in 1% BSA, 0.05% Tween 20 PBS, were added in triplicate to the antigen-coated wells and the plates were incubated at 37°C for one hour in a moist chamber. Triplicates of positive and negative control sera were also included in each plate. After repeating the washing procedure, 50μl of conjugate were added to each well and the plates were incubated and washed as described previously.

One hundred μl of the substrate solution was added to each well and incubated for 15 minutes at room temperature in the dark. The enzymatic reaction was stopped by adding 100μl of hydrofluoric acid 0.1 M, pH 3.3.

Optical densities (OD) were assessed photometrically at 405 nm on a micro ELISA reader calibrated against a blank in which the serum sample was replaced by 1% BSA, 0.05% Tween 20 PBS.

Statistical analysis: The "t" test was used to compare mean values of antibody levels of the three group of cattle.

The optical density cut-off value was determined by logistic regression analysis.

Results

Antigen evaluation: Autoclaved M. bovis PPD produced more consistent and discriminative ELISA reactions than the phenol treated and unheated M. bovis PPDs. The former was therefore adopted for the rest of the studies at an optimal concentration of 0.55 mg/ml in carbonate buffer pH 9.6.

Antibody determination in cattle sera

O.D. values of sera from cattle of the tuberculosis-free area ranged from 0.030 to 0.340 (mean ± SD: 0.115 ± 0.060); values for the tuberculin-negative herd from the tuberculosis endemic area did not differ significantly (mean ± SD: 0.135 ± 0.070; range 0.050 to 0.350, p > 0.1) (Fig. 1).

On the other hand, the OD of sera from cattle confirmed as tuberculous ranged from 0.100 to 1.030 (mean ± SD: 0.620 ± 0.280, p < 0.001).

![Fig. 1. Seric IgG antimycobacterial antibody levels in three groups of cattle as determined by enzyme linked immunosorbent assay (ELISA). Each point represents one animal. Horizontal bars indicate mean optical density values with two standard deviations](image-url)
A cut-off value of 0.200 was calculated on the basis of the correct classification of infected and tuberculosis-free cattle for any given O. D. value (Fig. 2). This value was very close to that obtained by the addition of two standard deviations of the mean of the non-infected group (0.115 ± 0.120 = 0.235).

Ninety percent (18 out of 20) of the sera from infected cattle had antibody levels above the cut-off point, whereas values for 89.8% (44 out of 49) of the sera from the tuberculosis-free herd were below. Values below the cut-off point were observed in 43 out of 51 (84.3 %) sera from the tuberculin negative cattle from the endemic area. Sera from the three groups of cattle with an OD of 0.200 were considered neither positive nor negative, and were therefore excluded in the calculation of the above mentioned percentages.

In addition to negative and positive control sera, to facilitate the qualitative evaluation of results in visual readings a serum dilution with an OD of 0.200 was selected as “borderline” value and included in each plate. An almost complete qualitative correlation was observed between instrumental and visual readings.

Discussion

Since the development of heterologous immunoenzymatic techniques, the advantages of their application to the serodiagnosis of infectious diseases have been emphasized, particularly their high sensitivity, the considerable savings arising from their use as a microtechnique, and the possibility of their partial or total automation.

Preliminary reports by Thoen et al. (17, 18) on the ELISA detection of mycobacterial antibodies in cattle exposed to M. bovis encouraged us to explore further the applicability of this method to the diagnosis of bovine tuberculosis in the field. Results reported in the present study indicated that 90.0 % of the tuberculous and 89.8 % of the healthy cattle were correctly classified when their sera were tested with the ELISA. Similar sensitivity and specificity are achieved by the tuberculin skin test under optimal conditions (6). If the
ELISA results are confirmed in further trials, its operative advantages would make it an obvious choice for screening studies in areas where tuberculosis is endemic.

Immunodepression due to intercurrent infection, advanced disease, and old age may hamper antibody response in tuberculous cattle and give false negative results, as is the case with cutaneous hypersensitivity reactions. Non-detectable IgG antibody levels should also be expected to occur at very early stages of the disease.

Tuberculin skin tests performed in exposed and non-exposed cattle have been reported to elicit a humoral immune response measurable by kaolin agglutination, gel precipitation and ELISA (16, 18, 21). This has been confirmed at our laboratory, with the ELISA test here reported, in cattle experimentally infected with *M. bovis* (data not included) and would account for false positive results in areas where tuberculin skin testing is a common practice, such as those selected for our study. However, sensitization to tuberculoproteins, if it did occur, did not affect the present ELISA results, since a well-defined cut-off point was observed between antibody levels of infected and non-infected cattle. On the other hand, since the humoral response to tuberculin stimulus occurs earlier and is more dramatic in infected animals, ELISA testing of sera after skin testing may provide further diagnostic evidence when undefined results are obtained.

Antibody levels of a carefully controlled herd from an endemic area did not differ significantly from those from a tuberculosis-free area. These findings give further reliability to the test since its cut-off value seems not to fluctuate according to the epidemiological status of the area under study. In fact, since no comparative study of the ELISA and skin tests had been performed, the ELISA positive results observed in the tuberculin negative cattle from an endemic area might be ascribed either to a lower specificity of the ELISA or to a lower sensitivity of the skin test.

Antibody levels above the cut-off point in the tuberculosis-free herd should be considered instead truly false positive results. Mycobacterial antibodies are expected to cross-react broadly with antigens from the same genus and with a few other microorganisms (22), and so, sensitization to other microorganisms than *M. bovis* may account for false positive results in ELISA. Lastly, the spontaneous occurrence of bovine autoantibodies which cross-react with *M. bovis* antigens has been reported, although their prevalence and their presumptive interference with the serological detection of tuberculous infection in cattle have not been explored (15).

Significantly, none of the false positive results greatly exceeded the cut-off point, which suggests that the antigens responsible for unspecific stimulation, whichever they may be, can elicit only a weak antibody response to *M. bovis* in non-tuberculous cattle. Simultaneous ELISA testing of weak positive sera with antigenic extracts from various mycobacteria might reveal a specific anti *M. bovis* response, provided that differences in antibody titres were substantial.

Maximal specificity is required if the method is to be used in the last stages of an eradication programme, when the number of false positive cattle may largely surpass that of truly infected cattle. The specificity of tests as accurate as ELISA relies almost entirely on the antigen employed. Heat treatment inherent to conventional PPD preparations has been reported to impair antigenicity of tuberculoproteins (9, 10). Extracts of unheated mycobacteria killed with phenol have been proposed as more specific antigens for cattle tuberculin skin testing (3). In our experience, however, when PPDs prepared from unheated cultures of two *M. bovis* strains were compared with autoclaved PPD, the latter proved to be a more reliable ELISA antigen, with the additional advantage that it is standardized and readily available.

To our knowledge, this is the first report that defines the sensitivity and specificity of ELISA for the detection of bovine tuberculosis by testing it in non-tuberculous and bacteriologically confirmed tuberculous cattle. A broader project is now under way at our laboratory with cattle chosen at random at the slaughterhouse 72 hours prior to slaughter in which the results of bacteriological diagnosis are being compared with those of tuberculin skin tests and serum antibody levels obtained with the ELISA.
Zusammenfassung

Bestimmung der Sensitivität und Spezifität eines enzymgebundenen Immunadsorptionstests (ELISA) zum Nachweis von mykobakteriellen Antikörpern bei boviner Tuberkulose

Ein ELISA zum Nachweis von zirkulierenden, bovinen IgG-Antikörpern mit *M. bovis* als Antigen wurde überprüft. Autoklaviertes bovines PPD erwies sich, verglichen mit PPDs aus nicht erhitzten Kulturen zweier *M. bovis*-Stämme, als geeignetes Antigen.


In der vorliegenden Untersuchung waren die Ergebnisse des ELISA bezüglich Sensitivität und Spezifität ähnlich denen des Tuberkulin-Hauttestes. Darüber hinaus machen die technischen Vorteile und geringen Kosten den ELISA zu einem nützlichen Hilfsmittel für die Diagnostik der bovinen Tuberkulose.

References


