THE ELISA TECHNIQUE FOR DIAGNOSIS OF SEVERE TUBERCULOSIS IN DEER AND EXOTIC RUMINANTS.

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ABSTRACT

An ELISA test for the detection of antibody to *M.bovis* has been developed recently in our laboratory. This uses a purified antigen (MB) extract from M.bovis and a new system for detection of deer immunoglobulin (antibody). It is very sensitive in identifying animals with serious Tb infection though less precise in diagnosing low grade lesions. Because it is relatively cheap it can be used to screen ST (-) animals within infected herds for more efficient identification of animals with skin test 'anergy'. It is also used routinely in our laboratory to further enhance the discrimination of BTB testing for disease diagnosis in herds with ST (+) animals and diagnosed Tb. In a few exceptional herds with spurious *M bovis* reactivity; (ST (+), CCT (+) & BTB (+), without confirmed evidence of Tb lesions at autopsy), the ELISA can be applied as an ancillary screen, following skin testing to increase the specificity of immunodiagnostic tests which used PPD antigens.

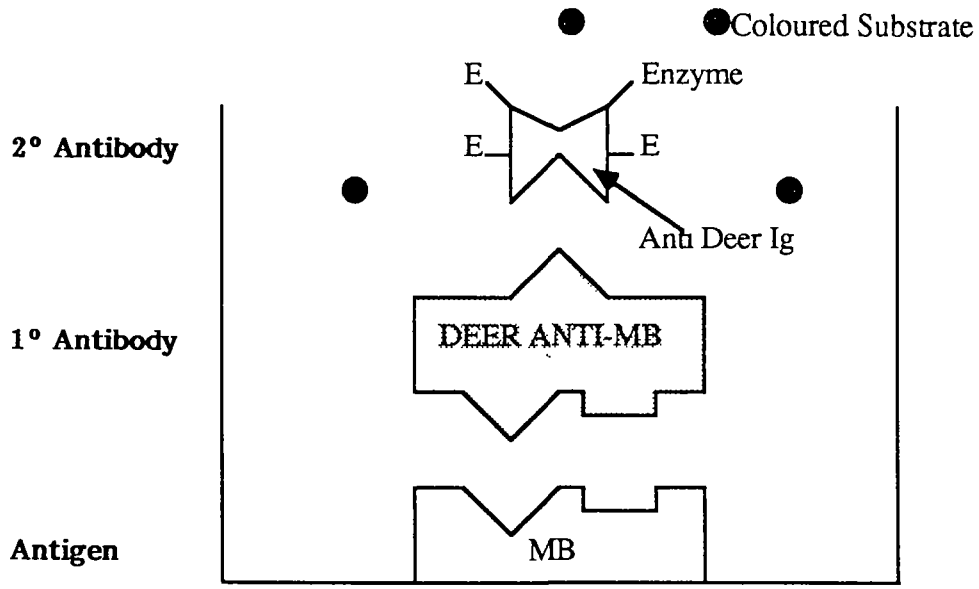
INTRODUCTION

The ELISA test (Engvall and Ruoslahti, 1979) has become a widely established immunoassay for the detection of antibody. It has the advantage that it can be automated and used to screen relatively large numbers of samples for antibody detection and it can be used quantitatively to estimate the amount of antibody contained in a given sample. The principle of the assay is outlined in Figure 1, and basically involves the use of horse radish peroxidase linked 2^{o} antibody specific for deer 1^{o} immunoglobulin to detect the presence of an antibody antigen complex with solid-phase system. Hence the name enzyme-linked immunosorbent assay (ELISA).

Whereas a critical standardization of all components of the system is required to establish optimal conditions for assaying a given antibody, the limiting factors which define the quality of the assay in any given application, is the purity of the available antigen used in the assay and the quality of the reagents which is used to detect the antibody under test. Critical attention to the above factors has allowed us to develop an ELISA assay appropriate for detecting antibodies to *M. bovis*

FIGURE 1.

THE ELISA



In our ELISA assay for Tb we use a unique protein isolated for *M. bovis*, unique to this species. We have also used monoclonal and conventional enzyme conjugated 2^{o} antibodies which specifically detect deer antibody (immunoglobulin) and allow us to assay specific antibodies in deer in a highly specific way. The system can be used to detect total antibody levels or IgG and IgM classes of antibody.

PPD consists of a mixture of antigens, some of which are shared between other species of Mycobacteria, so it is not appropriate to use such antigens alone in the study of specific mycobacterial disease. Crude antigen extracts from mycobacterial species or antigen complexes such as PPD has proved unsatisfactory for use in antibody detection systems for diagnosis of tuberculosis in farmed animals (Corner & Pearson, 1978; Auer, 1987). This is borne out by independent studies (Auer & Schleehauf, 1988; Ritacco *et al.* 1987) in cattle and deer (Sutton *et al.* 1985), where ELISA has been used for diagnosis of tuberculosis. The consistent findings by all of these workers are that the ELISA has value in detection of tuberculosis, but it has a restricted sensitivity (67%) There are also limitations in specificity, because a large number (18%) of uninfected animals appeared positive in this test (False +), limiting its value for use as a diagnostic test in the field.

Whereas antibody is not regarded as important in the protection of an individual against tuberculosis following infection, it may have value for identifying immunologic reactivity associated with proliferating Tb disease. Using an ELISA for detection of tuberculosis in deer (de Lisle *et al.* 1984), it was recognised that whereas specificity was a problem, the test appeared to have

potential in detecting serologically positive reactivity in seriously affected animals with clinical tuberculosis. Similar studies in humans have also confirmed that whereas antibody tests are not highly sensitive in the detection of all forms of tuberculosis, they may be valuable in the detection of serious proliferative tuberculosis, such as generalised Tb or miliary tuberculosis.

The ELISA technique for Tb diagnosis in deer as developed in our laboratory, has used a highly purified *M. bovis* antigen extract (MB), and a sensitive antiglobulin detection system which allows for the monitoring of relatively small concentrations of antibody to highly specific antigen. This has allowed us to improve the specificity of the assay very considerably and allowed us to use a significant dilution of animal serum under test, which obviates the problems caused by non-specific reactivity due to high levels of cross-reactive antibody which is present in concentrated serum. The earlier reported studies (de Lisle *et al*, 1984) identified this problem where high levels of cross-reactivity were found using PPD antigens, when serum above a concentration of 1 in 80 was used in the assay system. Because of this we have used a minimal dilution of 100 in the antibody detection system. We have standardized the ELISA assay to utilise very small quantities of highly purified antigen (MB), with high dilutions of antibody, for detection of specific antibody to *M. bovis*.

RESULTS

To date we have carried out ELISA tests on serum samples obtained from more than 2,000 animals. Summary findings from 300+ animals subjected to autopsy are given in Figure 2. The animals have been obtained from herds known to harbour *M. bovis* and infection has ranged within the cohorts studied from infection rates of greater than 40%, to infection rates of less than 1%. The data is presented as group-mean titres given in ELISA units for the individual animals. The animals included in this analysis were obtained from 6 geographically separate herds. This included herds with mixed *M.avium* and *M.bovis*, animals experimentally infected with *M.bovis* and herds harbouring significant levels of Tb due to *M. bovis*.

Following autopsy the animals were segregated into four discrete disease categories based on histologic and macroscopic findings.

0. **NVL** - animals which showed no macroscopic lesions at autopsy and no evidence of tuberculosis on culture of lymphatic tissue.
1. **Low grade Tb lesions** - animals in which histologic evidence of tuberculosis was found in a small lesion within a single lymph node.
2. **Moderate grade Tb lesions** - animals had lesions at one or more sites with caseous or calcified consistency, and histology compatible with Tb granuloma.
3. **Severe Tb lesions** - animals which showed multiple caseous or liquifactive lesions, or animals showing generalised pleural involvement.

The above classification was establised to determine if the quality of antibody response could be related to severity of disease as well as detection of disease in individual animals. The analysis as given in Figure 2 shows a high degree of correlation between the severity of disease and level of antibody to MB found in the serum of the infected animal.

Figure 2

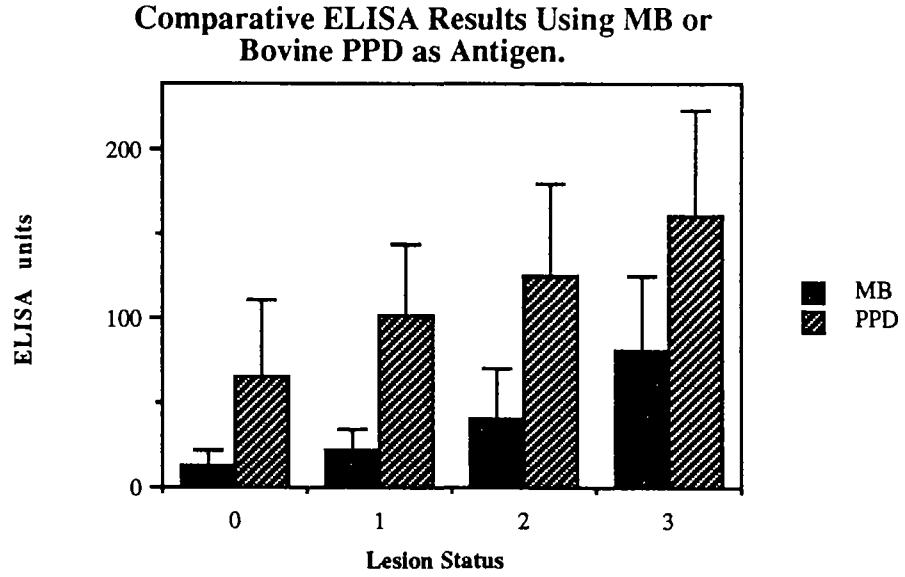


Table 1 ELISA Reactivity in Animals Tested Against MB and PPD Antigens

AUTOPSY STATUS

	0	1	2	3	1+2+3
Number Tested	68 (34)	22 (16)	28 (24)	45 (36)	95 (76)
Number ELISA +ve	13 (14)	17 (13)	27 (19)	45 (33)	89 (65)
% ELISA Positive	19% (41)	77% (81)	96% (79)	100% (92)	94% (86)
Range of Titres	2.3- 62 (17-191)	10-56 (52-198)	10-132 (47-232)	16-213 (36-298)	10 -213 (36-298)

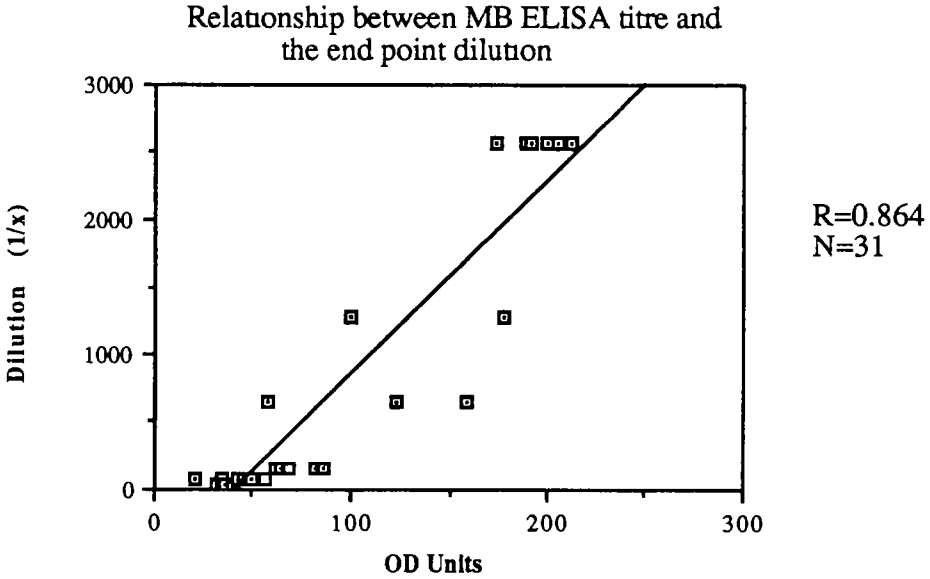
* Figures in brackets represent the number of animals tested using Bovine-PPD.

Using the minimal colourometric reading of 15 ELISA units to distinguish between positive and negative antibody titres, it can be seen that there is a significant change in the level of antibody detection across the four categories of animals. Following screening for positive antibody reactivity of a single dilution of serum, can be on individual samples to define the antibody titre (Figure 3) The ELISA test can be used quantitatively, using a single serum dilution providing attention is paid to reference values for the assay using positive and negative control serum from deer

Whereas a number of animals which did not show any lesions at autopsy (19%) were identified as antibody positive to MB using these criteria, this was not unexpected because many of the animals in question were from herds with a high incidence (>20%) of Tb and in direct contact with spreading infection so they may have been sensitised through exposure to *M. bovis*.

Animals from herds with a low incidence of Tb (>1%) showed a low incidence of false positives in the ELISA using MB. Among animals with low grade (1) lesions, 23% had antibody titres less than 20. The percentage of animals showing negative titres was 4% in animals with moderate grade disease (2), and all seriously infected (3) animals were ELISA (+).

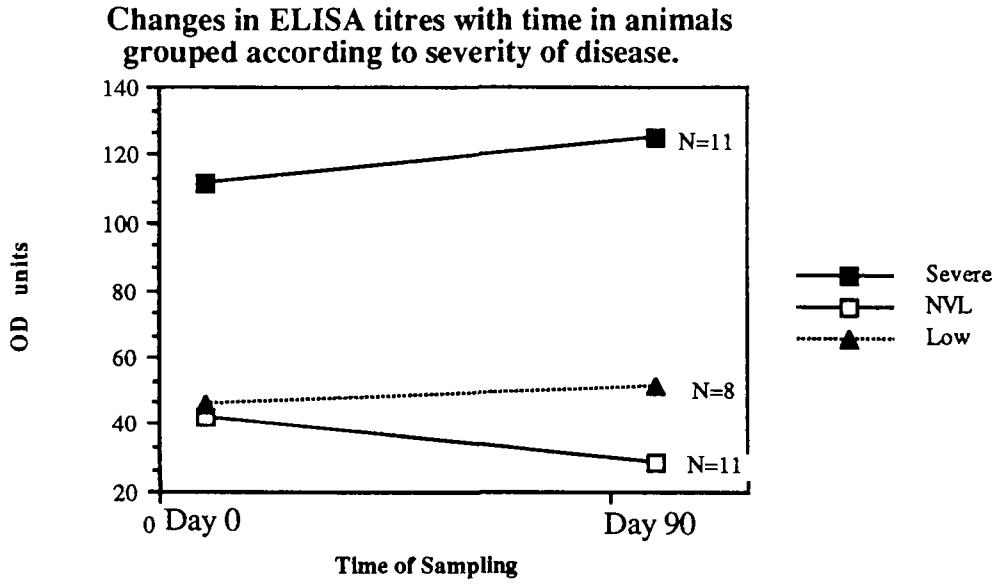
Figure 3.



The data presented in Figure 3 shows that a single antibody dilution (100) provides an ELISA reading which bears good correlation (R=0.864) to values obtained when conventional serological titres are carried out to obtain an end point (OD ≥ 20 - Figure 3).

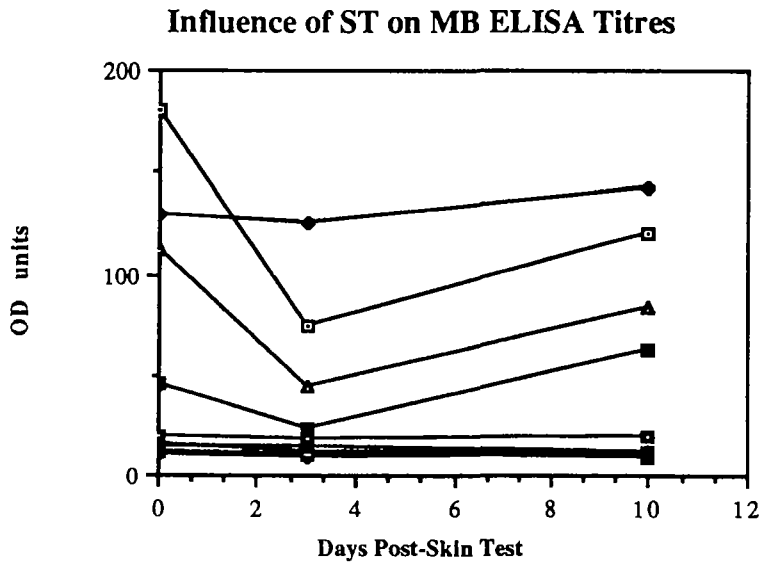
In this way the ELISA can be considered as a quantitative assay. This is borne out by developmental ELISA titration curves obtained using representative blood samples containing low, moderate or high levels of antibody. In each case there was a linear change in OD in the dilution range $^1/_{50}$ to $^1/_{200}$. A median figure of $^1/_{100}$ was chosen to provide a screening dilution which would accomodate a wide range of antibody levels.

Figure 4.



Changes in ELISA titres obtained from lesion positive animals suffering from different degrees of Tb is presented in Figure 4. Samples were obtained over a 3 month interval and the summary findings suggest that animals with severe levels of Tb have consistently high titres or increasing titres in paired samples. The titres obtained from animals with low grade disease were also sustained over the three month period though the levels were generally much lower than those found in the more seriously diseased animals. The titre found in ELISA (+) animals with no visible lesions tended to be lower than the diseased groups, and a number of NVL animals showed a drop in titres over the sampling period.

Figure 5.



In an attempt to define what influence skin testing might have on antibody, ELISA titres were also measured at the time of applying a skin test (ST), on reading the test (D+3) and ten days after testing (D+10). The findings in Figure 5 show that animals with high ELISA titres generally showed a significant drop in titre at the time for reading the ST. Whereas this drop did not cause a reversion from + to - status, the level of interference could complicate ELISA measurements on blood samples obtained on reading the skin test. The ELISA titres had generally recovered towards pre-ST levels by D+10. These findings infer that an interval of ten days should elapse between skin testing with bovine PPD and blood sampling when MB is used in ELISA tests.

ROLE FOR ELISA TEST FOR Tb DIAGNOSIS IN DEER

Whereas we have not carried out sufficient assays to identify the true sensitivity of the test in detecting tuberculous animals it appears from the above findings that the assay may be of real value in screening skin test negative animals relatively inexpensively to detect seriously affected animals where disease is considered to be present within a herd. We do not consider that the ELISA can be used as a first line screen because of its questionable sensitivity in detecting low grade disease. It would appear more relevant that this test be applied in conjunction with other tests to identify the overall disease prevalence within of a herd. At present we routinely carry out ELISA assays on all blood samples submitted for the BTB test and find the ELISA is particularly valuable in identifying animals with relatively low grade BTB reactivity specific for *M. bovis*. Terminally ill animals have impairment of reactivity in the non-specific (ConA) positive control, which is used to assess the overall quality of each animal's lymphocytic response in the BTB. Whereas the overall lymphocytic impairment can be due to unrelated disease, parallel use of the BTB and an ELISA, which yields a positive titre with MB, alerts the laboratory to the likelihood of serious disease due to *M. bovis*.

If there is to be real progress in the containment of tuberculosis as a disease within the national deer herd, it is imperative that the most sensitive techniques are used to screen herds for the presence of Tuberculosis. Because of this it is obvious that the single cervical skin test (ST) must continue to be used as the primary screening test for Tb diagnosis. Subsequent to identifying skin test positive reactors the specificty of their reactivity can then be checked with CCT or BTB. The logical second line test at present remains the BTB which has a very high degree of sensitivity (> 95%) in detecting tuberculosis. However, should it be established using ST and BTB that Tb is present at significant levels in a herd, and this is confirmed on autopsy, then False (-) ST reactors from the initial screen must always be a consideration. If a residual disease is suspected, then it is reasonable that a second inexpensive screen test be used to examine animals which have passed the initial skin test (ST). The ELISA, which is a relatively cheap test appears to offer the real prospect of a second line inexpensive screen test to look at large numbers of non-reactor animals to

diagnose tuberculosis. The data provided above shows that the ELISA screen can be particularly effective in identifying skin test negative animals which harbour serious proliferating tuberculosis. Whereas we are not advancing an overall statement on the sensitivity of the test for detecting all lesion-bearing animals, it can be stated with a high level of confidence that our ELISA assay is extremely sensitive in detecting animals with wide-spread tuberculosis. These are the category of animals which pose the greatest risk to failure of a test programme using conventional skin testing. Because of this when disease is considered an issue within a herd the ELISA would appear to have real merit as a second line screening test.

It should be noted that skin testing appears to influence ELISA titres (Figure 5) so it would be best if this test is not used until two weeks after skin testing. Further work is required to establish the true relevance of skin test interference and this is being done at present. There is no evidence that repeated skin testing will sensitise a negative animal to produce ELISA (+) titres although critical studies are required to validate this for deer.

The data used in Figure 2, has been obtained from herds with *M. bovis* infection alone, or mixed infection due to *M. avium* and *M. bovis*, and animals experimentally infected with *M. bovis*. We are confident that the findings presented will stand the test of application for the identification of seriously diseased tuberculous animals, over the national herd in general. Using the highly specific antigen (MB) it is likely that true *M. bovis* infection will be identifed consistently. By contrast, when PPD is used for ELISA a lower level of sensitivity (80%) is evident for animals with low or moderate grade lesions.

The test may also have a role in resolving problems where CCT or BTB reactivity to *M. bovis* PPD-B is found in herds where no evidence of *M. bovis* is diagnosed at autopsy. Because the ELISA uses a highly specific, though relatively expensive, *M. bovis* antigen extract, these animals can be identified as ELISA negative with MB antigen and the issue of cross reactive spurious *M. bovis* reactivity can be confirmed. In recent times one region of New Zealand has thrown up a whole series of ST(+) animals from different herds, which give positive reactions to PPD-B using the BTB or CCT as ancillary tests. Autopsy of reactor animals consistently fails to diagnose *M. bovis* lesions. Use of MB antigen in the ELISA allows us to identify such animals and limit the possible application of DCP status to such herds. Unfortunately the cost of producing MB antigen precludes its use in the BTB test, where large quantities (10ug) are required for a single animal's test. By contrast 0.1ug of antigen is sufficient for an ELISA test on an individual animal. Preliminary studies using MB antigen in the BTB have shown that whereas MB has an apparently higher specificity than PPD, it has consistently lower sensitivity (<80%) in detecting *M.bovis* lesions in deer. Further research is being carried out currently to evaluate the feasibility of more widespread use of MB antigen within our laboratory assays in general.

The ELISA assay has also been evaluated for Tb diagnosis in exotic ruminants. It has been shown to be capable of detecting antibody to *M.bovis*

compatible with Tuberculosis. High levels of MB specific ELISA antibody and BTB reactivity have been used to diagnose Tb in Austrian Oryx and Gazelle. The ELISA response has also been shown to specifically exclude non-specific skin test reactors with sensitisation due to *M.avium*. Earlier work has also identified that the ELISA and BTB can also be used to diagnose Tb in cattle.

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