Serial ELISA and Skin Testing in Deer for Diagnosis of *Mycobacterium bovis* Infection

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Introduction

Development of diagnostic tests for Tb in deer has posed a number of major technical challenges. Whereas the single intradermal skin test (ST) (Beatson, *et.al.* 1984) has been successful in identifying infected herds, it has limited sensitivity (80-85%) (Carter, *et.al.* 1984) and an inability to detect some seriously infected ST(-) '*anergic*' animals, so the ST may fail to identify every infected animal within a diseased herd. The need to develop second line screening tests which can diagnose ST(-) '*anergic*' animals in Tb infected herds has remained as a unique challenge for Tb diagnostics in deer. Not only must the second line test be capable of detecting seriously diseased animals which are ST(-) but it should also be sufficiently cheap to allow for its use as a whole herd screening test in large groups of animals.

Earlier studies (Griffin and Cross, 1986, 1989) in our laboratory have demonstrated that no single test has sufficient sensitivity and specificity to identify every infected animal and concurrently produce acceptable specificity, to ensure the safe salvage of animals showing non-specific ST(+) reactivity to saprophytic mycobacteria. Our solution to this problem has been the development of composite tests which measure complementary facets (cellular and antibody) of immunity, which focus on different aspects of the disease response. We have developed (Griffin and Cross, 1986, 1989, Griffin and Buchan, 1993) a blood test for Tb (BTB) which measures specific cellular reactivity using lymphocyte transformation (LT) and antibody production using ELISA. Technical parameters and specifications for application and interpretation of the LT, ELISA and BTB are as given by Griffin, *et.al.* (1993). The BTB test has both high levels of sensitivity (>95.0%) and specificity (98.6%) (Griffin and Buchan 1993) and can be used to accurately diagnose tuberculosis due to *M.bovis*, or salvage animals with non-specific reactivity due to *M.avium* or other saprophytic mycobacteria. While the BTB is a very accurate test, its high cost ($100.00) precludes its use as a whole herd test, with the result that it is used largely as an ancillary test in relatively small numbers of ST(+) animals. The cost of this test can be justified when it is used selectively in parallel with ST to look at ST(+) animals where False(+) ST reactivity due to non-specific sensitisaton is suspected. It can also be used on 'elite' stock, or imported animals, where risk from Tb infection must be excluded with a high degree of confidence. When used selectively even in infected herds, the precise information obtained from BTB can be used to identify risk from infection in residual ST(-) animals from the herd.

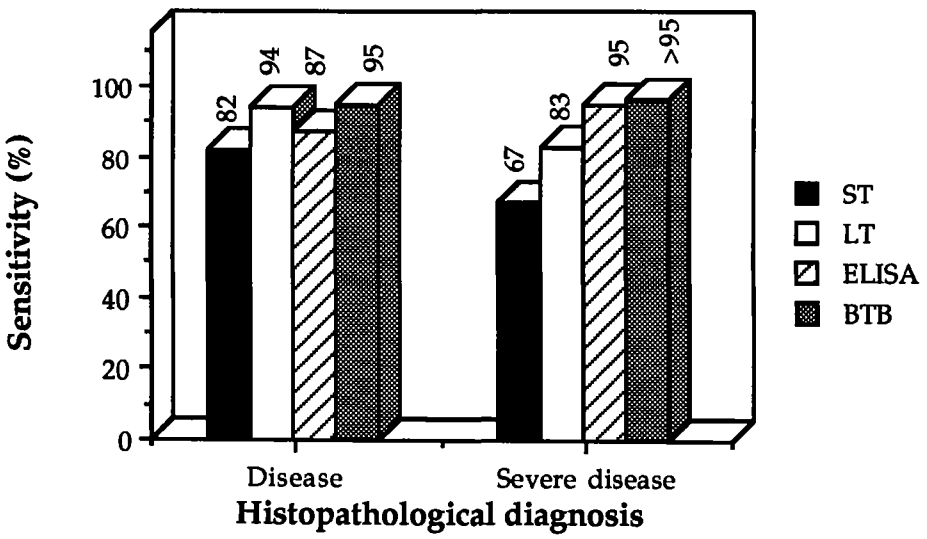
While this test can accurately discriminate between Tb due to *M.bovis* and non-specific reactivity and can be justified as an ancillary test in ST(+) animals, its cost precludes its use as a Tb screening test in conventional deer herds where its superior sensitivity could be exploited. This limits the value of BTB to target infected ST(-) '*anergic*' animals in Tb infected herds. During development of the BTB we noted (Griffin and Buchan, 1989, Griffin, Nagai and Buchan, 1991) that the ELISA component of the BTB when used alone gave a high predictive value for diagnosing Tb, with a direct correlation between the level of ELISA reactivity and the severity of disease.

Skin Test and ELISA for Tb diagnosis in deer

Recognising that complementary laboratory assays of cellular and humoral immunity in the BTB can act as additive tests to give high levels of sensitivity and specificity it was decided that the skin test, which measures cellular immune reactivity *in vivo* could be used in series with the ST with the potential to improve Tb diagnosis in deer.

Evidence for the potential of ELISA given in Figure 1, shows the data obtained from *M.bovis* Tb infected deer herds, in which disease was first diagnosed by culture of *M.bovis* and confirmed in other animals by histological diagnosis only. Three hundred and forty five animals from 54 tuberculous herds are included in this database. All these animals had tuberculosis diagnosed by their lesions following slaughter which were typical of Tb. Diseased animals were segregated into two groups; firstly animals with one or two small lesions (*Disease*) or those with three or more lesions, including animals with generalised tuberculosis (GTB) (*Severe disease*).

Figure 1. Sensitivity of ST, LT, ELISA and BTB to diagnose Tb in farmed deer with histolopathology compatible with tuberculosis.

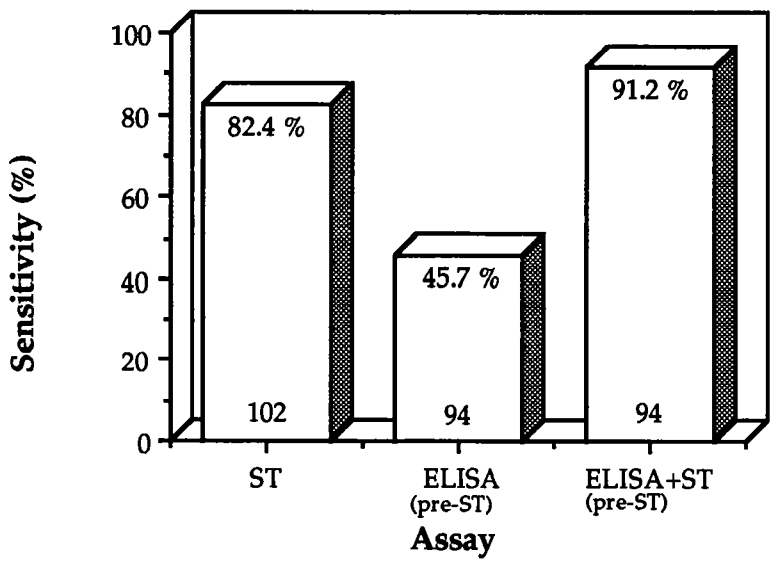


These results show that there was a trend for cellular assays (ST or LT) to be less sensitive in animals with increasing disease severity; ST: 82% → 67%; LT: 94 → 83%. In contrast, the ELISA showed increased sensitivity which appeared to relate to disease severity (87% → 95%). The data also shows that when ELISA was combined with LT to produce the BTB there were similarly high levels of sensitivity (95%) in animals with either moderate or severe disease. In the BTB test, animals with either LT(+) or ELISA(+) reactions are classified as BTB(+). ELISA and LT results were additive as diseased animals missed by one test were usually positive to the second test.

The data given in Figure 1 was derived from animals which had disease diagnosed only by histological assessment. It was considered necessary to repeat these studies using animals where Tb had been diagnosed using *M.bovis*(+) cultures as the '*gold standard*' for diagnosis. Results given in Figures 2 and 3 are from a group of 102 animals from a Tb infected herd where Tb was diagnosed in each animal using *M.bovis* culture following the slaughter of all the animals in a whole herd depopulation programme.

When carrying out diagnostic tests which are used in series it is important to establish if there is interference or enhancement between tests. The data in Figure 2 showed the sensitivity of the ELISA and ST using blood taken just before application of the skin test.

Figure 2: Sensitivity of ST, pre-ST ELISA and the combined tests to diagnose *M.bovis*(+) deer

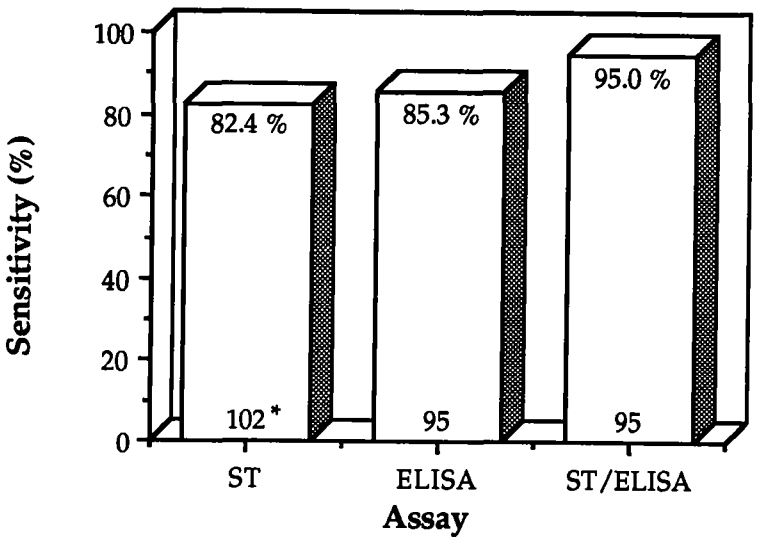


Results given in Figure 2 show the performance of ELISA when blood was taken immediately before the ST. In this case the sensitivity of the pre-ST ELISA was 45.7%. However, when pre-ST ELISA was used serially with ST the sensitivity of the ST alone (82.4%) was increased to 91.2% when ST/(+) or

ELISA(+) results were taken as positive for any given animals. Even though the pre-ST ELISA had a low sensitivity as a stand alone test, it still was positive for nine ST(-) animals, seven of which had either multiple lesions or GTB. On the other hand results from the *post mortem* examination also identified nine *M.bovis*(+) animals, which were negative for both tests [ST(-)/ELISA(-)]. One of these was NVL while eight had single lesions, of which five had extensive calcification.

The results given in Figure 3 show how the ST and ELISA tests performed using blood samples obtained ten days after skin test reading; the time recommended for sampling ST(+) animals for BTB.

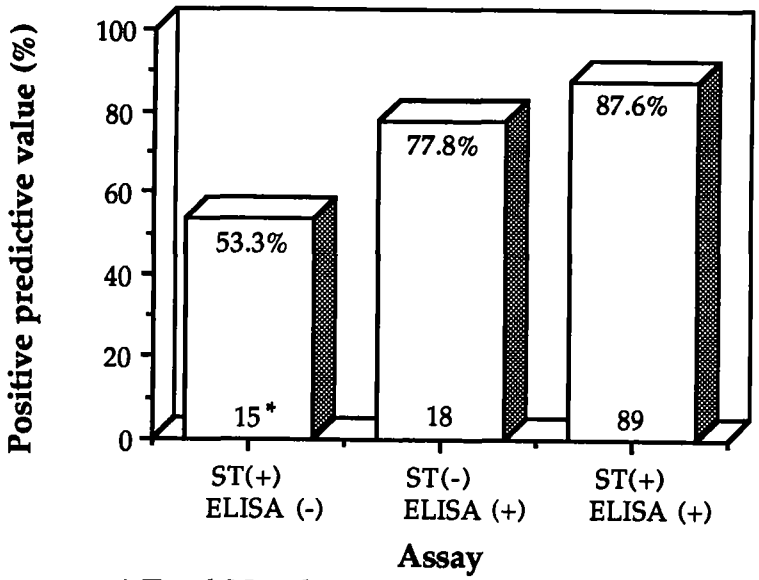
Figure 3: Sensitivity of ST, ELISA and a combination of both tests in a group of *M.bovis*(+) tuberculous deer



* Total number of animals analysed.

There was comparable sensitivity with the ST (82.4%) and ELISA (85.3%) in this sample. However, when the results of ST and ELISA were combined and all animals which were either ST(+) or ELISA(+) were classified as test positive then there was a marked increase in the overall sensitivity (95%) of the combined tests. The reason for this becomes obvious when the disease status of the animals are studied. The ELISA test alone identified 11 animals as Tb positive which were ST(-); six of these had GTB, three had suppurating lesions draining onto the skin, one had multiple lesions and one had a single lesion. This showed that the ELISA was capable of identifying seriously affected animals with skin test '*anergy*'. Five *M.bovis*(+) animals were ST(-) and ELISA(-) and all showed low levels of disease. Three of these five animals had single lesions and two were NVL.

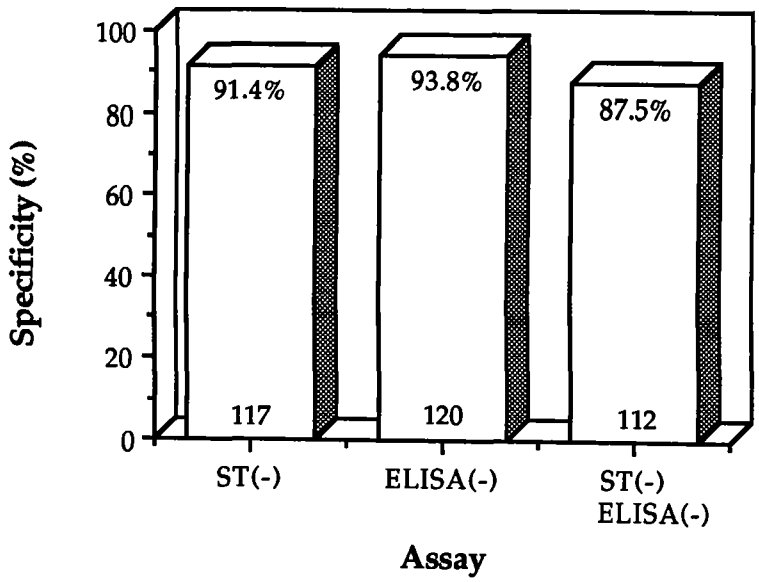
Figure 4: Positive Predictive Value for ELISA and ST in 106 *M.bovis*(+) animals and 125 *M.bovis*(-) animals



* Total Number test positive

Results given in Figure 4 show a high positive predictive value (PPV): (Number Test Positive/(Number Diseased) x 100 using the ELISA post-ST. The PPV for ELISA(+)/ST(-) animals (77.8%) was significantly higher ($p<0.01$) than that found in animals which were ELISA(-)/ST(+) [(53.3%)]. Even higher PPV's were seen in animals which were positive to both tests [(ELISA(+)/ST(+) - 87.6%)].

Figure 5: Specificity of ST and ELISA in a group of 128 *M.bovis*(-) animals from a heavily infected deer herd



The data given in Figure 5 shows that the ELISA had a specificity of 93.8% to identify *M.bovis*(-) NVL animals from a heavily infected deer herd. Data obtained using 218 animals from disease free herds gave a specificity of 100% for the ELISA (Griffin, *et.al.* 1993).

Discussion

The results from this study showed that the use of combined tests which measure cell mediated immune reactivity and antibody are additive and may be used as a composite to give new precision for Tb testing in deer. The ELISA can complement the LT when it is used in the BTB to study ST(+) animals, as can the ST, when used serially to examine ST(-) animals, in a whole herd test. The key advantage of the ELISA is that it can identify seriously diseased animals which are *anergic* to ST. The ELISA is also enhanced significantly by prior skin testing. The results given in Figure 2 and 3 show that the sensitivity of ELISA is increased (45.7 to 85.3%) significantly when blood is taken ten days post ST reading. It is obvious that exposure of infected deer to 0.1mg *M.bovis* PPD during ST acts as a booster antigen to induce antibody production in ELISA(-) animals or to increase the antibody levels in ELISA(+) deer. Similar enhancement of the ELISA has been found following ST in cattle (Lepper, *et.al.* 1973, Harboe, *et.al.* 1991). This is in marked contrast to the cellular assays (ST or LT), where skin testing causes a suppressed response for up to 60 days for repeat skin testing (Corrin, *et.al.* 1993) and for 10 days if LT (Griffin and Buchan, 1993) is used. The enhancement of antibody production post skin test can be exploited by carrying out the ELISA ten days or more post ST. Data from our laboratory shows that the elevated ELISA reactivity persists from 10-30 days post ST.

The facility to use an ELISA test after the ST, means that ST(+) animals can be retested by BTB or slaughtered following skin testing. BTB(+) results suggestive of serious disease, or levels of Tb lesions in animals *post mortem*, and the examination of the herd history allows the veterinarian and producer to decide if there is a risk from residual *anergic* ST(-) animals, before deciding to use an ELISA on the ST(-) animals. The 10-30 day time frame allows reasonable scope to accumulate appropriate information to include or exclude an ELISA as part of the management programme.

While ST has a significant enhancing effect on ELISA, the ELISA still identifies the majority of severely affected ST(-) animals when use concurrently with ST. ST enhancement of ELISA occurs more in animals with early infection or those with low or moderate levels of disease. The majority of such animals present as ST(+) during routine testing, so the inability of the pre-ST ELISA to identify them is not a major concern. Not alone is the ELISA capable of identifying seriously diseased animals but it also does not leave a significant reservoir of infected animals which are ST(-)/ELISA(-). This is vital if disease control is to occur in a timely fashion. The data given in Figure 2 shows that ST alone

would have left the producer with nine very seriously diseased ST(-) animals. In contrast, the combined ELISA/ST failed to identify only four single lesioned animals out of a total of 102 diseased animals (Figure 3). It is likely that these animals would not have acted as a reservoir of infectious spread in the immediate future and that they would have been identified in the next whole herd test, carried out 60-90 days later. ST alone would have left an infectious reservoir which may have infected many in contact animals prior to the next test. It is important to consider the nature of infection in test negative animals as well as the incidence of infected animals, when working through a disease eradication programme. The need to identify and slaughter seriously diseased animals at the earliest possible time is paramount in successful disease control. Use of ELISA ten days post ST offers such an opportunity, where its high positive predictive value allows it to diagnose seriously diseased deer.

Whereas the ST and ELISA provide a very sensitive whole herd test strategy for Tb eradication, the levels of specificity of ELISA (Figure 5) mean that the ELISA did not incriminate a significant number of non-diseased animals or cause undue wastage, even in a herd with a high incidence of Tb lesions (39%). While the ELISA had an 'estimated' specificity of 93.8% in *M.bovis*(-) animals from the diseased herd, it had a specificity of 100% in the 218 disease-free animals tested independently (Griffin, *et.al.* 1993). It is accepted that accurate specificity figures must always be derived from animals in disease-free herds as it can never be stated with certainty that an *M.bovis*(-) animal in an infected herd is truly disease-free.

The animals in this study had been skin tested a number of times prior to the ELISA test and there was no evidence that ST caused significant seroconversion to ELISA(+) when non-infected animals were repeatedly skin tested prior to ELISA testing. All 218 animals from disease free herds used in the other database had been tested repeatedly with ST and still showed 100% ELISA(-) responses (Griffin, *et.al.* 1993).

These results show that when ELISA is used in Tb infected herds it is a powerful tool to identify '*anergic*' ST(-) animals, which may harbour serious infection. The ELISA test is designed to identify infection in ST(-) animals and must not be used to salvage ST(+) animals. The BTB should be used to clarify the status of ST(+) animals where its high specificity and sensitivity allows it to be used to salvage a ST(+) animal as a non-reactor. With ST(+) animals, the challenge for the ancillary test (BTB/CCT) is to accurately identify why the animal produced the initial ST reaction. Our evidence suggests that BTB can identify reactions due to *M.bovis* with a high degree of sensitivity (>95%) while it can also salvage animals with non-specific mycobacterial reactivity with a high degree of specificity (98.6%) (Griffin and Buchan, 1993, Griffin, *et.al.* 1993). The ELISA offers an important new strategy to identify seriously infected animals which are ST(-) while the BTB can be used to clear animals which have false ST(+) reactions due to non-specific sensitisation.

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