BLOOD TESTING FOR DIAGNOSIS OF TB WITHIN A NATIONAL CONTROL PROGRAMME

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Intradermal skin testing in the diagnosis of cervine tuberculosis

The pioneering studies (Beatson, Hutton and de Lisle, 1984) on the use of tuberculin skin testing for the control of tuberculosis in an infected deer herd in New Zealand were carried out between 1978 and 1983. During this period 3620 individual animal tests were carried out during 29 different testing episodes, at which 107 reactors were identified and slaughtered. Gross lesions, consistent with Tb infection, were found in 82 (77%) of the skin test reactors. Of 68 animals which died unexpectedly throughout the study, 25 (37%) had Tb lesions, and of these 15 (60%) had generalised Tb (GTB). Up to the time that the herd was depopulated in 1983, 43/326 (13%) of the animals which were negative to skin testing had tuberculosis diagnosed at autopsy. In the early test series; 0.1ml of 1mg/ml bovine PPD was used for cervical testing, but in the later part of the programme, 2mg/ml bovine PPD was used for the intradermal cervical skin test (ST). Application of the comparative cervical test (CCT) gave consistently poor results, with only 26% of ST reactors giving a positive CCT. It is recognised that this level of CCT reactivity may have been compromised somewhat due to suppression caused by the application of the prior ST.

A subsequent study which involved active experimental infection of deer with *M. bovis*, de Lisle et al. (1984) demonstrated that the single intradermal skin test had a sensitivity of 86% (36/44), in detecting *M. bovis* infected deer, when positive skin test reactivity was taken as the presence of a visible or palpable skin test reaction. When an increase of ≥2.5mm in skin thickness was taken as evidence of reactor activity, the test showed only a 45% (20/44) sensitivity in detecting tuberculous animals. This data highlights the necessity of assessing reactors as all animals showing any evidence of skin test reactivity, if acceptable levels of sensitivity are to be achieved. A parallel study carried out on experimentally infected animals (Carter et al., 1984) indicated that repeated short interval (3 weeks) testing, significantly reduced the sensitivity of the ST. They also showed that effective skin testing was influenced markedly by the quality of animal restraint, lighting and the preparation of the skin site prior to testing. An added complication is that deer have thin skin (1-3mm), so extra care must be taken to ensure that the antigen used for skin testing is applied

intradermally rather than subcutaneously. Unless adequate shaving of the hair is carried out prior to testing, an intradermal inoculation may not be achieved, or even following appropriate inoculation diffuse or oedematous positive reactions may easily be overlooked. This has meant that application of skin testing in deer is more technically demanding than similar tests in cattle.

Since the onset of herd testing for tuberculosis and the slaughter of ST reactors in New Zealand deer herds, it became obvious that the use of the single intradermal skin test (ST) produced an unacceptably large percentage (60-70%) of False (+) reactors, due to high levels of sensitisation of deer with atypical *Mycobacteria*. Because of the large number of small reactions also found in non-infected deer, Carter et al. (1984), stressed that care should also be exercised if False (+) reactions are to be eliminated. Widespread exposure of deer to *M. avium* and *M. paratuberculosis* (de Lisle and Havill, 1985; Griffin, 1988a) would infer that sensitisation of farmed deer to cross reactive shared mycobacterial antigens demands caution in the development of techniques to ensure that acceptable levels of specificity can be achieved using tuberculin skin testing in deer.

Official statistics on New Zealand deer Tb testing in 1985 (Carter et al., 1986) identified 2594 (1.45%) reactors among 178,788 animals tested. Based on a validity of 30% it was suggested that the likely prevalence of tuberculosis in farmed deer would be 0.5%. It was also felt (Carter et al., 1986) that the occurrence of False (+) reactions in herds with no history of tuberculosis, had caused many farmers to lose confidence in the standard intradermal skin test (ST). This problem was further exaggerated by the prevailing test conditions which have applied in New Zealand since 1985, in which no compensation was paid for slaughter of reactor animals, and because involvement of farmers in the scheme was voluntary.

With this in view a CCT was introduced (Carter et al.,1985; 1986) to improve the specificity of tuberculin skin testing. Using animals infected experimentally with *M. bovis*; 1mg/ml bovine PPD for testing, and 0.5mg/ml avian PPD, they demonstrated that the CCT had a sensitivity of 92%; using a 2mm increase in skin thickness and a bovine $\geq$ avian reaction, as indicative of a positive reaction. They noted significant post test suppression (40%) of skin test reactivity, if CCT was applied within 28 days of ST and some (11%) suppression at a 60 day test interval. Mean skin thickness for bovine PPD, was reduced from 7.49mm at Day 0 to 2.44mm at Day 60. Whereas a 120 day interval showed no post-test suppression, it was suggested that a 90 day test interval could provide a reasonable compromise.

An additional complication inherent in the CCT is that measured increases in skin test thickness are necessary to produce a specific test, whereas it has been demonstrated (de Lisle et al., 1984) that high levels of sensitivity with ST are found only when all visible or palpable skin test tuberculin reactions, are deemed positive for deer. They state that based on a sensitivity of 85% for ST

under field conditions, the likely sensitivity of composite ST and CCT should be in the region of 68%. The specificity of CCT under experimental conditions was found to be 99%. Of 1,157 deer tested on properties considered to be free of tuberculosis, 15 (1.3%) showed positive CCT bovine reactions.

Some caution must be exercised in extrapolating from the findings obtained from skin test parameters in deer, which have assessed sensitivity levels only using small numbers of animals infected experimentally with *M. bovis* . A more valid approach would be to carry out extensive autopsies on animals in herds known to be infected naturally with M. bovis, and to restrospectively assess sensitivity following examination of ST(+) and ST(-) animals. Similarly specificity data obtained from herds known to be free of Tb may not accurately reflect the complexity of reactivity which may prevail in herds with mixed infection. It is however likely that, at best, the performance of tuberculin skin testing in deer will not exceed the best levels found currently in cattle. Francis et al. (1978), who reviewed a number of skin test trials in cattle, quote the summary findings from a number of different studies, which give an average sensitivity of 81.8%, and a specificity of 96.3% for ST. They suggest that available evidence on CCT infers more limited sensitivity for this assay and the contraindication for its use as a primary diagnostic assay. Similarly, Wilson (1986) has recommended that the CCT should not be used in herds of deer known to be infected with *M. bovis*. Local studies (de Jong and Ekdahl, 1969), using CCT in cattle herds, have yielded a disappointingly low sensitivity (68.6%) for this assay under New Zealand conditions. This figure is significantly lower than the sensitivity figures (88.8%) for CCT in the UK, where it is used as the main diagnostic test (Francis et al., 1978).

Laboratory Tests for Tb diagnosis in deer

Since 1963 (Schreck, 1963; Permain et al., 1963), various research workers have evaluated the prospect of using lymphocyte stimulation assays to monitor tuberculosis in humans (Verma et al., 1974), primates (Charapas et al., 1970) and ruminants (Muscoplat et al., 1974). Whereas these workers have consistently found that lymphocyte stimulation *in vitro* using tuberculin provides superior discrimination for disease diagnosis, no diagnostic laboratory system has yet been produced which has been tested in a naturally infected host under field conditions. The most likely explanations for this are that laboratory tests, using sterile culture systems, are difficult to automate for widespread application, and longitudinal studies on disease diagnosis under field conditions are very expensive.

Our laboratory has developed the blood test for Tb (BTB) for use in deer (Griffin and Cross, 1986). The criteria used to develop this test considered that the development of a new diagnostic system, should result in improved levels

of sensitivity, specificity and validity, and yet be sufficiently robust so that it could have value within the known constraints which apply in the control of cervine tuberculosis. Rather than develop an assay using animals which were experimentally infected with *M. bovis* or *M. avium*, it was decided to focus the studies on farmed deer herds which had known levels of naturally occurring Tb infection due to *M. bovis*, mixed infection with *M. bovis* and *M. avium* or atypical reactions due to *M. avium*. Rather than use small groups of experimental animals, we have established a complete reference database of laboratory parameters using uninfected animals, against which the performance of diseased animals could be evaluated. To date our laboratory has examined more than 15,000 blood samples from more than 6,000 deer, and the findings have been included in the database, using grouping which include sex and age differences. Following testing, large numbers of animals have been autopsied to establish their disease status. Although this approach is very expensive it is the only valid means to develop a diagnostic system for natural infection, whereby parameters can be established which relate in a meaningful way to relevant field conditions.

The BTB assay system

Because laboratory techniques allow for the more critical quantitation of immune reactivity in animals exposed to *Mycobacteria*, they offer the prospect of increased precision in the assessment of the individual animal's response to infection. Such assays allow for the more specific definition of the immunological reactions in the host, and can be used to distinguish reactions uniquely specific for *M. bovis*, *M. avium*, *M. paratuberculosis* or other saprophytic *Mycobacteria*. They also may allow for a distinction to be made between reactivity due to disease caused by *M. bovis*, and reactivity which results from infection without the establishment of Tb disease. Measurement of inflammatory reactions in the test animals adds further discrimination in identifying animals with lesions due to *M. bovis*, as opposed to immune clearance of bacteria from infected stock.

The basic assay used to define specific immunological function involves a modified lymphocyte transformation assay, in which mononuclear leukocytes are co-cultured with PPD in triplicate, for 5 days prior to pulsing with ^{3}H-thymidine. Cells are harvested 18 hours post labelling with thymidine using an automated cell harvester (PHD Cell Harvester), and the radiolabel uptake estimated as the counts per minutes (cpms) using a β-scintillation counter (LKB 1214 Rackbeta). Negative unstimulated controls, and mitogen (Concanavalin-A) stimulated positive controls are included in every assay. A range of PPD is used to identify specific reactions due to *M. bovis*, *M. paratuberculosis*, *M. avium* or saprophytic mycobacteria. Repeat triplicate controls using bovine

tuberculin are incorporated into separate microculture plates to validate the level of bovine reactivity in every animal under assay.

Differential white blood cell counts which calculate the number of lymphocytes (LAN), and the number of neutrophils (NAN), and haematological values (HB; haemoglobin) are estimated using a Technicon H6000/C, standardised for blood of *Cervus elaphus*, calibrated against standard techniques. Plasma viscosity (PV) is estimated using a modified Harkness viscometer, using a 3.60% NaCl standard, and distilled water as a two point calibration. Fibrinogen (Fib) is calculated by thrombin clotting using a semi-automated technique, calibrated against a heat precipitation method (Cross, 1987).

The pattern of usage of the BTB, since offical recognition of the test by MAF in September 1987 is as given in Table 1. A total of 2378 animals have been tested and more than 6000 blood samples have been processed in the laboratory during the intervening period. A large amount of research has also been carried out in conjunction with the routine diagnostic work to study other aspects of immune reactivity in naturally infected animals and in experimental animals vaccinated with *M. bovis*. The significant change in the economic climate and equity base of deer farming has meant that there has been an increasingly selective use of the BTB since January 88, with an increasing number of clients submitting small numbers (1-3) of samples for testing. This has placed considerable extra responsibility on the laboratory when reporting findings, and we continue to exercise the greatest caution to ensure that there is firm evidence to diagnose or exclude *M. bovis* infection. If there is any element of doubt we always request a confirmatory sample before completing a diagnosis.

Table 1
Application of the BTB test between Oct 87 and July 88.

Total Number of Herds Tested	Total Number of Animals Tested	Sample Size per Herd 1-5	6-20	>20
109	2378	72	21	16

The basis for using the BTB as indicated in the submission forms from veterinarians is given in Table 2. These findings are interesting in that they highlight that the paramount objective of the client is to salvage stock, even when *M. bovis* infection has already been confirmed within a herd. It appears likely therefore that test specificity is the most important consideration in current demand for the BTB. Of the 52 herds, with a previous history of *M. bovis* infection, from which samples were submitted, 35 (67%) had evidence of mixed infection with *M. bovis* and *M. avium*.

Table 2.
Reasons for Carrying out the BTB Test.

To Confirm Diagnosis of *M. bovis* following ST	To Confirm Non Specific Reactivity to ST	To Define the Status of CCT (+) Reactors
52 (47.7%)	40 (36.7%)	17 (15.6%)

A significant number of herds (16%) have submitted samples because of their concern for the specificity of reactivity in CCT(+) animals. The main body of submissions in this category come from herds with no history of *M. bovis* infection. The remaining submissions from CCT (+) reactors are from herds which have a recent history of *M. bovis* infection but because there has been low validity in detecting lesions at autopsy the veterinarian has come to doubt the specificity of the CCT(+) finding. Submissions from CCT(+) reactors which have been examined using the BTB demonstrate that of 120 CCT(+) reactor animals examined only 33 (27.5%) had reactivity which was specific for *M. bovis* PPD. It appears reasonable to conclude therefore that CCT(+) reactors in closed herds with a complete testing history which excludes *M. bovis,* may be due to False (+) CCT reactivity.

Considerable extra research has been carried out to determine whether the BTB can be used for the early detection of *M. bovis* infection in herds where *M. bovis* is known to be present at significant levels. The results given in Table 3 show the findings obtained from four infected herds where the BTB was used, after clearance of ST reactors which had shown a significant incidence of disease. The results show that if the BTB is used it will detect a significant number (10%) of animals showing specific reactivity to Bovine-PPD which do not react to ST. Repeat testing of these animals 90 days later showed that whereas the incidence of ST reactors had increased with time (3.1% to 10.4%), there was a greater increase in the numbers of BTB positive animals (14.7 to 21.0%). There is little doubt therefore that the BTB can detect reactivity to *M. bovis* much earlier than the ST, and in a significantly larger number of animals. This information is of considerable value in segregating 'at-risk' BTB (+) animals, early in a management programme, and in identifying False(-) ST reactors, which would pose a hazard by spreading infection among non-reactor animals.

Table 3.

BTB and early diagnosis of *M. bovis* infection.

Day 0		Day 90		
BTB(+)	ST(+)	BTB(+)	ST(+)	*M. bovis* confirmed at autopsy *
106/720 (14.7%)	22/720 (3.1%)	148/708 (21.0%)	73/702 (10.4%)	99/148 (67.0%)

*Animals were selected for autopsy on the basis of a confirmed positive BTB to *M. bovis*.

Because the BTB can detect exposure to *M. bovis* much earlier than the ST it is necessary to determine if animals showing early BTB(+) reactivity will subsequently develop *M. bovis* disease. Such information would allow for the early culling of 'high-risk' animals to facilitate early management of *M. bovis* infection. The autopsy findings from a series of 153 animals which came from four separate farms with known *M. bovis* infection are given in Table 4. Sequential blood samples were taken and the results obtained from the initial blood sample and the pre-autopsy sample taken a minimum of 90 days later were compared. Animals which sustained a high level (B4>10,000cpms) of BTB reactivity throughout the sampling period had an extremely poor prognosis and 80% of the diseased animals were in this category, while only 27% of the uninfected animals had this pattern of reactivity. Of the animals with consistent low grade BTB reactivity only 5% had lesions at autopsy whereas 67% of the disease free BTB reactive animals were in this category. The main observation from this study is that if animals show an early and persistently high grade BTB response there is the strong link between BTB reactivity and active Tb disease, and a poor prospect of salvaging uninfected animals.

Table 4.
Use of Sequential Samples for BTB, in the Management of M. bovis infection.

BTB Status			Disease Status at Autopsy *	
Day 0		≥Day 90	Lesions or *M. bovis* Isolated	NVL and no *M. bovis* Isolated
High	-	High	77(79.4%)	15(16.1%)
High	-	Low	4(4.1%)	4(4.3%)
Low	-	High	11(11.3%)	12(13.0%)
Low	-	Low	5(5.2%)	62(66.7%)

* Animals were grouped as diseased or Tb free after autopsy and laboratory examination post mortem.

Another interesting observation has emerged from our longitudinal sampling of animals repeatedly over long periods (18 months). This study has showed that South Island animals sampled during July and August tend to have reduced reactivity in the BTB, suggesting an association between reduced immunological reactivity and climatic or nutritional stress during winter (Griffin, 1988b). Further experimental studies are looking critically at changes in reactivity throughout the year. ST reactivity would also indicate that a higher incidence of False (-) ST animals are found at the same time. Whereas the BTB results can be used quantitatively to predict the emergence of tuberculosis in adult deer there appear to be some exceptions to this rule. We find that weaner animals show a lower grade of reactivity to Tb, for the first 6 months, compared with mature stock. We regard any specific BTB reaction to *M. bovis* in young animals with suspicion and would recommend that all such animals should be culled or segregated. This low response to BTB in weaners is also mirrored by their poor granulomatous reactivity and potentially high levels of AFB at autopsy. Because there is no data available on the efficacy of the ST in fawns it is not possible to comment objectively. It is vital therefore that some caution is necessary when applying the ST to fawns from reactor hinds or in herds with known *M. bovis* disease. A detailed research study is being carried out to look critically at the immunological reactivity in young deer following weaning, using the ST and BTB.

The current programme of routine diagnosis is being enhanced by the associated research programme which is looking at many facets of immune reactivity. It is hoped that we can report new research findings in the coming year which will further extend the scope of our laboratory systems for the management of tuberculosis and other diseases in farmed deer.

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† The BTB is registered under NZ Patent No. 214400, with Patent applications pending in Europe, EEC, US, Canada and Australia.

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Figure 3:
The abilty of a single blood test (BTB) to predict lesion status at autopsy.