ANCILLARY TESTS IN EPIDEMIOLOGICAL INVESTIGATIONS OF TUBERCULOSIS IN DEER.

J Frank T Griffin, Glenn S Buchan, John P Cross,
Christie Rodgers.

Deer Research Laboratory, Department of Microbiology, University of Otago, P O Box 56, Dunedin.

INTRODUCTION

Skin Test Herd Screening

Throughout the past ten years there has been considerable debate as to the efficacy of different testing systems for diagnosis and control of Tb within farmed deer herds in New Zealand and more latterly in Europe. The single cervical skin test (ST), which uses 0.1mg of bovine PPD injected intradermally at a single site, has been used as the primary screening test for Tb diagnosis in cattle and deer. The test is read 72 hours post inoculation, and any visible, measurable or palpable reaction found at the skin site is taken as positive evidence of reactivity. It has been estimated that the ST, has under field conditions in deer herds a sensitivity of 85% in diagnosing Tb and specificity of 98.5% in excluding Tb (Carter et al 1986). It is accepted that considerable variation may be found in the test performance under different farming conditions.

In experimental studies carried out by de Lisle et al (1984) on a group of fifty stags experimentally infected with *M.bovis*, it was shown that 36% of uninoculated animals had reactivity to bovine tuberculin when any type of test reaction was taken as positive. Using an increase in skin thickness at the site of inoculation of greater than 2.5mm it was found that 0/50 animals reacted to bovine PPD prior to experimental inoculation. These experimental studies have highlighted the prospect of significant wastage of non tuberculous animals in herds due to false (+) skin test reactivity.

On New Zealand deer farms where no evidence of *M.bovis* infection has been found, but consistently high levels of reactivity to the ST are reported, these are frequently false (+) reactions. Whereas non-specific reactivity can be excluded by an increase in skin thickness of greater than 2.5mm it seriously impairs the ability of skin test to diagnose Tb, or its sensitivity. de Lisle et al (1984) data shows that the test has a sensitivity of 82% when all skin test reactions are taken as positive. However, the sensitivity drops to 45% when a baseline reading of 2.5mm for skin test thickness increase is taken. Experience using the ST in Britain has shown that this test identifies significant numbers of reactor animals where no subsequent evidence for *M.bovis* can be found at necropsy. Such experiences have led to UK deer veterinarians to look for alternative tests which yield higher levels of specificity and avoid undue wastage of non diseased animals (Griffith 1989). The primary objective of any immunodiagnostic screening test for disease must be to identify reactive animals with the highest level of precision to ensure that infected stock are identified. The high degree of sensitisation of deer with mycobacteriae presents a challenge in properly categorising disease risk in reactor animals (Griffin & Cross, 1989).

The ST (+) Animal: An Asset !

The dilemma is that skin test readings which give appropriate sensitivity, may produce large numbers of non specific reactions in herds not harbouring disease. This

has led many farmers and veterinarians to regard reactors to ST as a liability which must be tolerated in the course of a Tb management programme. We suggest that the ST reactor deer should be regarded in a totally different light if effective Tb diagnosis is to be carried out. The ST reactor animal should be considered to be the key asset in the implementation of a Tb diagnostic
programme to identify or exclude *M.bovis*. Appropriate and detailed study of the ST reactor animals within a herd should provide additional insights for disease diagnosis. Comprehensive examination of a group of reactors will allow the veterinarian to accurately diagnose Tb if present or to exclude Tb if reactions are due to non specific mycobacterial sensitisation. Complete data from a reactor group will also provide background information which acts as a mirror to reflect the risk of disease in the residual ST negative stock within a herd.

Test and Slaughter : Cost - Effectiveness.

Traditionally the approach to Tb diagnosis has been to apply the ST on a whole herd basis and then slaughter all ST positive reactors. The likely outcome from slaughter of ST reactors is that lesions may be identified in only a proportion of the ST(+) reactors. Should such lesions be found it is then mandatory to carry out comprehensive histological and bacteriological examinations of the lesions to accurately confirm the presence of *M.bovis*. Lesions similar to those found in Tb may also be produced by agents such as Actinobacillus, so an accurate and complete laboratory diagnosis is necessary to confirm the presence of *M.bovis* in ST positive reactors.

Accepting the relatively low incidence of Tb within the national herd it is more likely that no lesions will be found by slaughtering ST positive because the initial skin test reactivity was caused by non specific mycobacterial sensitisation. Should a farmer slaughter a group of reactors and find no lesions, it is possible that a proportion of these animals may not be classic non specific reactors but in fact have reactivity due to *M.bovis* exposure in the absence of lesions. *M.bovis* infection may be present in animals not showing lesions, so the assumption that all NVL reactors may not have been exposed to *M.bovis* may lull the farmer and veterinarian into a false sense of security as to the true status of the herd. Apart from an inaccurate diagnostic outcome animals slaughtered as ST positive reactors are down-graded and a minimal carcass price ($2/kg) is paid for individual reactor animals.

Together these observations suggest that skin testing and slaughter of reactors as the main line for disease management may be doubly wasteful, through slaughter of non diseased animals with low cash returns, and the failure to obtain precise diagnostic information to allow the farmer to accurately evaluate the disease status of his herd. We consider it short sighted to apply a skin test and slaughter policy as the sole vehicle for Tb diagnosis in deer herds.

ANCILLARY TESTS FOR TB DIAGNOSIS IN DEER HERDS

Ancillary Immunodiagnostic tests may be carried out on ST positive deer to produce a more effective system for disease management. Tests used in conjunction with the ST should introduce a new element of specificity to accurately exclude non specific reactors but retain a level of sensitivity to diagnose *M.bovis* within a ST reactor group. If an appropriate ancillary test is applied to ST positive animals it should be possible to identify true *M.bovis* reactions and target animals for slaughter. All animals with specific reactivity to *M.bovis* should be subject to slaughter and if lesions are found on necropsy these should be examined histologically and bacteriologically to confirm *M.bovis*. Should no lesions be found at necropsy in animals considered to have reactivity specific for *M.bovis* pooled lymph nodes should be submitted for

bacteriological culture to identify the presence of *M.bovis*. If comprehensive laboratory testing fails to diagnose *M.bovis* in a group of animals considered to have evidence of specific immune reactivity to *M.bovis* then extra caution should be entertained in the application of subsequent tests to identify false negative animals not reactive to the earlier ST. Whereas False(-) animals may not test positive to repeat skin tests it is likely that they will act as a reservoir of infection and sensitise other animals which will still act as ST(+) sentinels in the skin test programme.

Commonly ancillary tests will identify non specific sensitisation to organisms other than *M.bovis* in ST(+) animals. When non-specific sensitisation is confirmed in each of a group of ST(+) animals then the farmer can retain these animals as non reactors or if they wish have them slaughtered as non reactors and recover standard carcass value ($6/kg). In such situations the use of ancillary tests provide extra information to define the specificity of reactivity which allows a farmer to effectively salvage stock or cull animals without any penalty at slaughter. This approach is both cost effective at a monetary level and provides key information which reassures the farmer that non specific sensitisation may be present on his property and allows him to make an informed evaluation of subsequent tests. Using this approach the farmer can pursue a disease management programme which maximises the chances of diagnosing true disease and minimises wastage due to non specific sensitisation.

CHOICE OF ANCILLARY TESTS FOR TB DIAGNOSIS IN DEER

Comparative Cervical Testing (CCT)

The CCT has been used widely in countries which have a low incidence of tuberculosis in farmed animals. This has become necessary because of the considerable wastage in skin testing programmes using ST when there is a very low level of *M.bovis* in farmed stock. While CCT provides increased specificity over the ST there is a contingent loss in sensitivity in accurately diagnosing tuberculosis. It is considered that this trade off is acceptable in farming environments where Tb is at low levels in cattle herds. Because of problems with the specificity of ST considerable emphasis has been placed on the use of CCT as an ancillary test for Tb diagnosis in farmed deer.

An evaluation of CCT has been carried out by Carter et al (1986) using a group of deer experimentally infected with *M.bovis*. In their studies they used 0.1ml of a 1.0mg/ml solution of Bovine PPD at one cervical site, and 0.1ml of a 0.5mg/ml solution of Avian PPD at a second site. A skin thickening of 2.0mm or greater was considered to be positive if the reaction of the bovine site was equal to or greater than the avian site. Using this technique they claim that CCT has a sensitivity of 92% for accurate diagnosis of Tb. They qualify these findings by stating that whereas the ST had a sensitivity of 95% under similar experimental conditions it had a sensitivity of 85% under field conditions. By extrapolation it was likely that the CCT sensitivity found under experimental conditions would likely not hold up under field conditions and they suggested that the CCT should have a sensitivity of 80% under field conditions. To date no accurate field data has been produced on the performance of CCT in naturally infected deer herds.

In other studies carried out by these authors using herds considered free of *M.bovis* they produced specificity values of 98.5% for the CCT. Subsequently it has been suggested that the CCT should be approved for field use in New Zealand because of its sensitivity of 89.7% and its specificity of 98.7% (Corrin et al, 1990).

Data from other studies using CCT under field conditions have produced highly varied findings. Field studies carried out in Britain on fifty deer tested under farm conditions (Stuart et al, 1988) found the CCT to have a sensitivity of 80% and a specificity of 61%. A comprehensive evaluation of the use of CCT for diagnosis of Tb

in UK farm herds by Phillips (1990) gave sensitivity values of 70% for Tb diagnosis in farmed deer. A recent field study carried out by Griffith (1989) who tested 34 hinds in a herd heavily infected with *M.bovis* showed the CCT to have a sensitivity of 31% and a specificity of 88%. Studies from our laboratory (Griffin et al, 1990) examining a population of 90 deer from a herd which was depopulated showed a sensitivity of 65% and a specificity of 64% for the CCT. Together these results show the high levels of variation in both the sensitivity and specificity of CCT under field conditions. It is considered that the CCT does not give appropriate levels of sensitivity for effective Tb diagnosis so it has been recommended that CCT should not be used in herds known to be considered at risk from Tb or having had a recent diagnosis of tuberculosis. An official publication from New Zealand Ministry of Agriculture states that "deer showing reactivity to bovine tuberculin should be considered as infected with *M.bovis* unless there is good evidence to the contrary, i.e the principle of guilt until proven innocent should be applied.". It further states "if the epidemiologic assessment is not thorough the results of any CCT applied will be more difficult to interpret and the accuracy of diagnosis reduced.", and that "the CCT should be used to assist in determination of non specificity not *M.bovis* infections.". The composite recommendations from New Zealand Veterinary Association Deer Branch Officers and New Zealand Ministry of Agriculture and Fisheries Officers state that the CCT should not be used in herds known to be infected with *M.bovis* or considered likely to harbour *M.bovis*.

Other considerations which influence the value of the CCT as an ancillary test, is that a minimum period of 90 days must elapse between the application of ST and a subsequent CCT. Data from Carter et al (1986) shows that the level of reactivity found to CCT is significantly reduced if animals are tested at 28 days or 60 days following the application of the ST. While there was no increase in reactivity between day 28 and day 60 post ST, no evidence of impairment of skin test reactivity was found at 120 days following application of ST. It has been recommended that, as a compromise to limit management constraints, a 90 day interval following skin testing is appropriate for the application of the CCT.

The CCT requires accurate measurement of skin thickening and vernier calipers should be used to produce accurate measurements. Whereas it is possible to produce reproducible measurements under experimental conditions this is harder to achieve under field conditions where restraint, lighting and handling facilities are less appropriate. This places considerable responsibility on the testing veterinarian to assure that all animals with an increase of 2mm in skin thickness are deemed to be positive CCT reactions.

In the experience of our laboratory, animals with questionable CCT reactions may have high levels of specific immunologic reactivity to *M.bovis* using other laboratory tests. Seriously diseased animals may produce negative or marginal CCT reactions. The ability of the CCT to identify an infected herd, which has a high incidence of tuberculosis is without question. However, the ability of the CCT to diagnose each diseased animal in a herd with a low incidence of disease is much more open to debate. A recent example has shown that a group of 27 skin test reactors at an accreditation ST, were re-tested clear with CCT. Within one week of the CCT negative findings in the ST(+) group, 2 animals died from clinical tuberculosis. Further laboratory tests carried out on the ST(-) CCT(-) animals showed a number with reactivity to *M.bovis* and Tb lesions. This finding highlights the danger that *M.bovis* infected animals can easily be missed by using CCT to clear ST positive animals when there is the false assumption that disease is absent from a herd.

Blood Test for Tuberculosis (BTB)

Work began in our laboratory in 1985 to look at laboratory techniques for diagnosis of tuberculosis in farmed deer. Results from the initial pilot studies evaluated

lymphocyte transformation of deer blood mononuclear cells with bovine and avian PPD and a measurement of inflammatory cells and proteins (Griffin and Cross,1986). Subsequent work (Griffin and Cross, 1987) carried out on an infected herd which was depopulated, and a number of farmed deer herds known to harbour *M.bovis* disease showed that the lymphocyte transformation test alone had a sensitivity of 95% and specificity of 92% for Tb diagnosis of farmed deer. In recent years over 30,000 deer blood samples have been analysed by the laboratory and necropsy findings evaluated for more than 3000 deer slaughtered throughout this programme (Griffin and Cross, 1989).

Refinement of the original lymphocyte transformation technique and measurement of inflammatory parameters and the more recent introduction of an ELISA antibody assay (Griffin and Buchan, 1989) has produced a composite blood test for Tb (BTB) which has demonstrably superior sensitivity and specificity for diagnoses of Tb in farmed deer. A comprehensive audit of our data has shown that the lymphocyte transformation assay alone has a sensitivity of 93% and a specificity of greater than 95% for diagnosis of Tb in naturally infected farmed deer under a diverse range of farming conditions. When ELISA and inflammatory parameters are included with lymphocyte transformation data, superior sensitivity and specificity can be obtained for the BTB assay.

Whereas the principles of the BTB assay evaluate cellular and humoral immune reactivity to avian and bovine PPD and are conceptually similar to the CCT a number of major differences are evident between the BTB and the CCT. As the BTB involves the examination of immunologic and inflammatory markers in peripheral blood it offers a number of significant advantages over the CCT. The major advantages are that the BTB has superior sensitivity in diagnosing Tb and a high degree of specificity to identify non infected animals. The BTB involves laboratory manipulation of blood rather than intradermal inoculation of antigens so an array of assays can be carried out on a single blood sample to produce a quantitative laboratory assay which can measure relative reactivity to bovine or avian tuberculin. Repeat blood tests can be carried out at will without any interference caused by earlier tests.

Major advantage of the BTB is that it can detect disease earlier and identify seriously diseased animals more effectively than conventional skin testing. We have been surprised to find that the heavily diseased animals have extremely high levels of immunologic and inflammatory reactivity when tested in the laboratory even though they may be ST or CCT negative. As this is the group of animals which are classically False (-) and 'anergic' to skin testing it was both surprising and rewarding to find that they were identifiable by the BTB. Whereas a minimal period of 90-100 days is required between repeated skin testing to avoid suppression of in vivo reactivity a similar lag period is not necessary prior to carrying out BTB assays. Our laboratory (Griffin and Cross, 1987) has shown that the BTB assay can be carried out effectively at an interval of 14 days following the application of a skin test.

Ancillary tests such as the BTB which has high levels of sensitivity, while retaining a high level of specificity, offers unique advantages for deer Tb testing. When the BTB is used in conjunction with the standard skin test (ST) the results give a high degree of confidence that where true *M.bovis* activity is present among ST reactors this can be identified specifically by the BTB. Where ST reactivity is shown to be due to non specific sensitisation this also becomes clearly evident from use of the BTB on ST positive reactors. The high degree of confidence obtained using a combination of ST and BTB allows for an accurate evaluation to be made on the status of ST positive reactors to effectively diagnose Tb where present or actively exclude it when absent. This technique has been used in many infected deer herds throughout New Zealand to advance a selective culling programme or salvage non reactor animals. The short time required between skin testing and blood testing allows for the early application of this ancillary test where there is an unknown history or reason to suspect *M.bovis* infection

within a herd. This offers a further advantage in disease monitoring by allowing the early interception of disease and the implementation of appropriate management systems to control or exclude Tb from the herd. The extremely rapid spread of Tb within some deer herds makes it mandatory that comprehensive Tb testing be employed at the greatest possible frequency to ensure that Tb can be identified and brought under control.

The foundation studies on the application of cervical skin testing (ST) to control Tb within infected deer herds (Beatson et al,1984) have shown that it may be impossible to eradicate Tb from a deer herd using skin testing alone. It has been recognised within New Zealand and more recently in the UK (Griffith, 1989) that whereas the skin test will identify a Tb infected herd of deer it may not be possible to eradicate Tb from some deer herds using skin testing alone. Use of laboratory tests such as BTB provide an important alternative strategy for diagnosis and management of Tb within infected herds and salvage of non specific reactor stock in herds free of *M.bovis*. The skin test and the BTB have been used to eradicate Tb from a number of Tb infected, NZ deer herds.

FACTORS WHICH INFLUENCE DIAGNOSTIC PRECISION OF TB TESTS.

From the outset it has been recognised that the application of immunodiagnostic tests for Tb diagnosis in farmed deer is uniquely challenging. Extreme care must be exercised in the preparation of skin test sites and caution exercised in the application of intradermal inoculations of tuberculin into deer skin. The apparently increased susceptibility of farmed deer to tuberculosis and the fulminant and active form of infection found in some animals infers that the immunological responsiveness of farmed deer to Tb may be different from more resistant species such as cattle. Such differences have made it impossible to extrapolate from the large body of literature obtained from skin testing of cattle for control of tuberculosis.

The observations made from the earlier studies on deer skin testing (ST) suggest that any skin test reactivity at the site of tuberculin inoculation must be taken as a positive reaction so that acceptable levels of sensitivity can be obtained using the ST as a screen test for tuberculosis in farmed deer. Field use of CCT suggests it should not be applied as a test in herds known to harbour Tb infection or deer herds with an unknown history. The large database obtained using the BTB in naturally infected deer herds studied using the BTB suggests that this assay is an appropriate ancillary test for diagnosis or exclusion of Tb from farmed deer herds.

The central parameters which contribute to the value of any immunodiagnostic test system for disease, are its sensitivity to accurately diagnose disease and its specificity to accurately exclude disease. These parameters are measurably affected by the incidence of disease within a herd, the numbers of animals tested, and the presence or absence of mixed infection with *M.bovis* and other species of mycobacteria. Effective disease diagnosis can only be carried out if all animals within the herd are tested and standardisation is used in test execution, interpretation and application.

Repeat Tests

It is widely accepted that the predictive value of diagnostic tests for disease can be measurably improved by carrying out repeat tests. Combining a primary screening skin test with an ancillary test which has a high degree of sensitivity and specificity would offer an acceptable repeat test protocol. The BTB fulfills these criteria and is an ideal ancillary test for use with ST in deer herds. By contrast the questionable sensitivity of the CCT makes it a less than ideal ancillary Tb test for deer. The appropriate use of paired tests will accurately validate or exclude disease with maximal efficiency. For effective disease management it is mandatory that repeat tests be carried out on all skin

test reactor animals to ensure that appropriate information is available before submitting any reactor animals for slaughter.

An example of the predictive value of a single test such as the skin test or a combination of tests involving skin tests and BTB is given in Table 1. This data shows that assuming the sensitivity of 85% and a specificity of 98.5% for the skin test significant wastage of animals will occur when a large number of animals are screened for Tb. Assuming an incidence of one diseased animal per thousand, 1585 animals will appear as skin test positive if 100,000 animals are screened using the ST. At such a disease incidence the true number of infected animals will be 100 whereas 1585 animals are implicated as diseased animals using the ST alone. This represents a predictive value of 7.8% in diagnosing tuberculosis with a wastage of 1500 animals in the single test schedule. The application of an ancillary test such as the BTB to the 1585 ST(+) animals significantly improves the precision of testing, where 79 animals are detected as diseased while only 32 are slaughtered as false positives. Together these tests improve the precision of diagnosing disease up to 78% and allow for effective salvage of 1468 animals in the implementation of the test schedule. At the same time the high sensitivity of BTB does not introduce unnecessary levels of false negative reactivity as 79 versus 85 diseased animals are identified by the combined tests. Additional BTB data on the specific patterns of reactivity and level of *M.bovis* reactivity in the true ST(+) reactors provides information which also allows for a reflection on disease prospects in residual ST negative stock within the respective herds. This data shows that use of a single test and slaughter policy is extremely wasteful because of its low predictive value in identifying true disease animals within groups of reactors from a single test.

Table 1

Predictive Value of Tests.

	TRUE (+)	TRUE (-)	TOTAL	PREDICTIVE VALUE
1º SKIN TEST (+)	85	999	1084	7.8%
2º BTB TEST (+)	80	22	102	78%

A similarly compelling case may be made for the use of re-testing of animals which yield equivocal or imprecise reactions to quantitative tests such as CCT or BTB. Because of the error inherent in measuring skin test reactivity or immunologic reactivity in the laboratory it is necessary that where equivalent reactions are found between bovine and avian tests such animals should be deemed as equivocal and re-tested to define their true reactivity. Whereas post skin test suppression makes it inappropriate to carry out repeated CCTs it is feasible to carry out a BTB test on animals which produce equivocal reactions to CCT or BTB. Such problems do not arise with the ST where an unequivocal positive result is given to any visible, measurable or palpable reaction at the site of bovine PPD inoculation. Nonetheless repeat ancillary tests on ST positive

animals will maximise the efficiency of disease detection and minimise wastage of non disease animals with non specific sensitisation.

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