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**Rural Industries Research and
Development Corporation**

*Diagnostic Tests
for Johne's Disease
in Deer*

A report for the Rural Industries
Research and Development
Corporation

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Foreword

Johne's disease was initially detected in a Victorian dairy herd in 1925. Since then it has infected an estimated 20% of Victorian dairy herds and during the 1980's and 90's has become established in sheep flocks in NSW and Victoria. Johne's disease is caused by an infection of the gut by *Mycobacterium avium* subsp *paratuberculosis*, all domestic and many wild ruminant species are known to be susceptible. In 2000, deer with subclinical and clinical forms of Johne's disease were detected for the first time in Australia.

National programs for the control of bovine and ovine Johne's disease have been established by the livestock industries and animal health authorities. These programs established Standard Definitions and Rules in an attempt to reduce the spread of the disease by zoning, providing market assurance programs and reducing the impact on farms by reducing the prevalence of infection. These control strategies are dependent on effective diagnostic technology. Currently the approved diagnostic test for Johne's disease in live deer is individual faecal culture. In contrast, strategies for pooled faecal culture have been developed and approved for flock diagnosis in sheep and the ELISA is used extensively in cattle.

This research project developed and evaluated a modified commercial ELISA and pooled faecal culture for the detection of *Mycobacterium avium* subsp *paratuberculosis* infection in deer. The report details the experimental development of the tests, the performance of the tests in infected and non-infected deer herds and discusses the application of the tests for detection of disease in individual animals, detection of infected herds and for market assurance program applications.

Benefits from the project are directed towards increased productivity and Johne's disease control strategies concurrent with other industries.

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This report, adding to RIRDC's diverse range of over 900 research publications, forms part of our Deer R&D program, which aims to promote an Australian deer industry which is profitable and efficient mainstream agricultural enterprise, based on the growth and development of participants, producing internationally competitive premium products.

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Abbreviations

AGID	Agar gel immuno diffusion test
ANOVA	Analysis of variance
ANZSDP	Australia and New Zealand Standard Diagnostic Procedures
CFU/g	Colony forming units per gram
CI	Confidence interval
DMAP	Deer market assurance program
ELISA	Enzyme linked immuno-sorbent assay
FC	Faecal culture
HEYM	Herrolds egg yolk media
HP	Histopathology
IFC	Individual faecal culture
IgG	Immuno globulin G
JD	Johne's disease
MAP	Market assurance program
MPN	Most probable number
OD	Optical density
OIE	Office of International des Epizooties
PCR	Polymerase chain reaction
PFC	Pooled faecal culture
SCAHLs	sub Committee of Animal Health Laboratories
TC	Tissue culture
TGROC	Two graph receiver operator curve

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Executive Summary

Johne's disease (JD) was originally described in 1894 in Germany when the cause of a chronic wasting disease afflicting a cow was identified as an infection by a tuberculosis like bacteria. In 1925 Johne's disease was detected in a Victorian dairy herd. Since then an estimated 20% of Victorian dairy herds have been infected and during the 1980's and 90's infection has become established in sheep flocks in NSW and Victoria. In 2000, deer with subclinical and clinical forms of JD were detected for the first time in Australia.

JD is caused by an infection in regions of the gut by *Mycobacterium avium* subsp *paratuberculosis* (*M. paratuberculosis*), all domestic and many wild ruminant species are known to be susceptible. Infection is usually established in young animals that will harbour the organism for many years. The infection spreads within specific regions of the intestine and is eventually excreted in increasing numbers in faeces before the animal finally succumbs to clinical disease. Experience in New Zealand indicates that JD in deer herds may be seen as 2 possible syndromes, (a) sporadic cases in mixed age groups, and (b) outbreaks affecting up to 15% of young animals (Macintosh, 2002).

To allow livestock industries and animal health authorities to establish national programs for control of JD, effective diagnostic technology is required. Currently the only approved diagnostic test for JD in live deer is individual faecal culture (IFC). By contrast, strategies for pooled faecal culture (PFC) have been developed and approved for flock diagnosis in sheep and the ELISA is used extensively for cattle.

This project evaluated two tests for the diagnosis of JD in live deer. PFC and a modified ELISA were evaluated by comparison with IFC, agar gel immunodiffusion (AGID), culture of tissues (TC) and histopathology (HP). Sera for ELISA and AGID testing and faeces for culture were collected from 1222 deer from 7 properties on which JD had been diagnosed and 474 deer from 2 properties with no evidence of the disease. Of the 1705 animals sampled, 390 animals were sampled at slaughter where faeces and tissues were collected for culture and fixed tissues for HP. HP examination was performed on selected samples based on TC or IFC results

The most sensitive method for confirming JD in deer was TC collected after slaughter. The HP examination was slightly less sensitive as it detected only 71% deer tested positive by TC. Among tests suitable for the diagnosis of JD in live animals, the IFC and ELISA detected 47% and 38-40% respectively of deer identified as infected by TC. These findings are consistent with previous reports of IFC and ELISA for the diagnosis of JD in cattle (Hope *et. al.*, 2000).

The concentration of *M. paratuberculosis* excreted in faeces of infected deer demonstrated considerable variation. The numbers of the organism ranged from 10^1 to 10^6 colony forming units per gram of faeces (CFU/g) in faeces from deer with subclinical infection. Animals with clinical JD excreted on average 5×10^6 *M. paratuberculosis* in a gram of faeces. The detection limit (analytical sensitivity) of culture for IFC was 10-50 (CI 4-324) CFU/g. By comparison, culture of faecal samples for PFC 5 (one infected and four non-infected deer) was able to detect 200 (95%CI 30-621) CFU/g and the detection limit for PFC 10 or 20 was approximately 10^4 CFU/gm of faeces.

The sensitivity of detecting an infected animal by PFC was reduced compared to IFC. The estimated sensitivity for PFC 5 compared to TC (estimated from IFC of 47% cf TC) was 32% for PFC 5 and 27% for PFC 10 and 20. Infected animals that were difficult to detect by PFC were animals with subclinical infection and less than 200 CFU/g of faeces. In a pooled faecal trial of a JD positive property where the prevalence of infection was 25%, PFC was positive for *M. paratuberculosis* at PFC 5 and 20 (PFC 10 not performed). However, it is unlikely that PFC of more than 5 deer would be effective in detecting infection as a whole herd test where herd

prevalence was less than 4% in a herd of 200 animals unless one or more animals were shedding above the detection limit.

An initial evaluation of the commercially available bovine ELISA kit (Parachek) demonstrated that the concentration of a conjugate used in this kit was found to be unsuitable for the diagnosis of JD in deer. The usefulness of more concentrated bovine conjugate, as well as Protein G and deer conjugates was investigated. Following optimisation experiments, each conjugate was further evaluated using sera from 172 deer with bacteriological and/or histopathological evidence of infection and in 210 IFC negative deer from two herds with no history of JD. The results were interpreted using three cut points: 0.1, 0.2 and 0.3 based on optical density (OD) readings at 450 nm. The use of the 0.2 cut point appeared to provide the optimal balance between sensitivity and specificity. The specificities and sensitivities obtained with the three different conjugates were similar. The sensitivities of the ELISA at 0.2 cut point were 38.1, 38.6 and 40.3% for the concentrated bovine conjugate, Protein G conjugate and deer conjugate, respectively. The specificities at this cut point were 99% for the concentrated bovine conjugate, 97.6% for the Protein G conjugate and 96.7% for the deer conjugate. The sensitivity of the AGID estimated against the results of TC was 20.5%. The specificity of the AGID using 72 sera from IFC negative deer was 100%.

For herd testing to provide market assurance for low risk of JD, diagnostic testing strategies are selected to detect infection in herds with 95% confidence of a prevalence of at least 2%. However the ability to detect disease at such a level of confidence and prevalence is dependent on the size of the herd and sensitivity of the test. Based on epidemiological statistics, results from this trial indicate that the IFC, ELISA, PFC of 5 and PFC 10-20 would identify JD at the 95% confidence in a herd of 200 deer with minimal prevalence of infection of 2.4%, 3%, 3.9% and 5%, respectively. It is unlikely that the AGID would be suitable for deer JD Market Assurance Program (MAP) as it is unlikely to identify animals not also identified by ELISA or faecal culture.

The findings of this study indicate that serological and bacteriological tests for JD in deer have similar performance to the tests used in cattle and sheep. Consequently it is recommended that these tests as modified for deer are appropriate to be adopted as standard diagnostic techniques and with consideration of the epidemiological limits with respect to the sensitivity of each test, are eligible for use in a deer industry MAP.

1. Introduction

Project Synopsis

Serological and PFC tests have been conducted across a large number of positive and negative animals and properties for the purpose of evaluating tests for the detection of *M. paratuberculosis* infection in deer. The performance of each test is described together with recommendations for their appropriate use and limitations when used as individual animal and herd tests specifically for use in Market Assurance Testing. A report detailing the test performance and methodology will be provided for consideration and endorsement by SCAHLS and Veterinary Committee for the test to be included in the Australia and New Zealand Standard Diagnostic Procedures (ANZSDP). Characterised specimens will be included in the National Serum Reference Panel maintained by the Office International des Epizooties (OIE) reference laboratory for JD for use in test quality assurance and test validation.

Background, relevance and potential benefits

JD is a chronic infection of ruminants by *M. paratuberculosis*. Infection results in thickening of the epithelium in the lower part of the small intestine leading to malabsorption of nutrients. This eventually leads to starvation for the animal and ultimately death. There is no therapeutic recourse, once the infection is established the disease will be fatal. JD in Australia has been present in dairy herds and sheep flocks for several decades, has been found in Alpaca over the last ten years and has recently (2000) been diagnosed in Victorian deer herds. Experience overseas indicates that deer are fully susceptible to both sheep and cattle strains of *M. paratuberculosis* (De Lisle G.W. & Collins D.M., 1993). Deer infected with *M. paratuberculosis* may have different expressions of disease ranging from acute severe symptoms in young animals to mild localised granulomatous reactions in animals with sub clinical infection (Mackintosh et al, 1999).

JD is a difficult disease to detect in the early stages of infection, it is more likely to be detected in older animals by ELISA or faecal culture. Over several years the infection spreads within the gut, the organism begins to be shed in the faeces and the humoral immune system is stimulated. Experience with cattle indicates faecal shedding can often precede an ELISA positive test result. Thus, more contamination of the environment may occur when relying on culling by ELISA results alone.

Serological tests for JD have been developed and evaluated for detecting infection in cattle and sheep, but species differences mean that bovine/ovine JD antibody tests are not directly suitable for deer specimens and there is little information on serological responses in infected deer. Deer are a susceptible species to both ovine and bovine strains of *M. paratuberculosis* and while infection in deer in Australia has been of the bovine strain, no antigenic differences have been demonstrated between ovine and bovine strains. When strain typing is required it is done at the molecular level on organisms from culture. Development of an ELISA test with a sensitivity and specificity similar to the bovine test would provide an economical option for identifying infectious animals for JD control in deer farms with a greater than 2% prevalence (Brett, 1998). The existing CSL bovine ELISA technology was the basis on which the JD antibody detection in deer serum was developed.

The currently approved diagnostic tests available for the diagnosis of JD in deer are IFC and necropsy with HP examination and TC. In addition to the requirement for suitable tests to assist the control of JD on infected farms there is also a need for tests that have the capacity to ensure freedom from infection. This is required to manage the risk of spread of infection and minimise impairments to movement and trade. Recently PFC has been accepted as an alternative to serological testing in the sheep MAP.

A MAP for the deer industry based on herd screening provided by serology or PFC testing will provide for a higher level of confidence in live deer trade by establishing negative disease status for herds with consecutive negative herd tests for JD and for the detection of infected animals from affected herds.

2. Objectives

To produce improved diagnostic capability for detection of JD in deer and provide reliable information on performance of tests. Review test performance for suitability in JD control strategies and for testing in a Deer Market Assurance Program (DMAP).

Stated Objectives and Achievements;

Develop a modified absorbed ELISA test for the detection of *M. paratuberculosis* infection in deer. Evaluate the sensitivity and specificity of the modified ELISA against IFC and/or TC as the gold standard.

To assess the AGID against the modified ELISA as a potential MAP test against 300 deer sera.

Estimate the numbers of organisms excreted by sub clinical and clinically infected animals.

To assess PFC at 3 different pooling rates in comparison with IFC and to estimate the sensitivity of PFC.

Conduct field testing of PFC on positive and negative properties.

All objectives were met. In addition the assessment of the AGID test included a review of historical testing from 839 deer sera tested by AGID and Protein G ELISA.

Recommendations of *M. paratuberculosis* diagnostic testing in deer have been prepared for acceptance by SCAHLS, Veterinary Committee and RIRDC for potential use in a DMAP.

3. Methodology

Standards and Definitions

The infection status of each deer was established using one or more of three standard tests

- i. Culture of faeces, and/or
- ii. Culture of tissues, and/or
- iii. Histopathological examination.

Faeces and sera from deer with an established infection were used in subsequent experiments for development and evaluation of the ELISA and PFC. Serum samples were only selected for use in the sensitivity panel for evaluation of ELISA sensitivity if they were positive by any of the 3 standard tests. Serum samples were only selected for use in the specificity panel for evaluation of ELISA specificity if they and all other samples from that same property were negative by one or more of the 3 standard tests, with no test positive.

A property (herd) was deemed infected if one or more animals tested from the property were positive for *M. paratuberculosis* by any of the 3 standard tests. A property was deemed uninfected for this study if there had been no history or evidence of JD on the property and all animals were negative by IFC in a whole herd test.

A test may be applied to an individual animal or an entire herd. As such test performance in this report is discussed in terms of the ability of a test to detect an individual animal or as a herd test, ie. the ability of a test such as PFC to detect an infected herd.

Specimen Collection

Blood, faeces and tissues from *M. paratuberculosis* infected and non-infected deer were obtained from previous diagnostic submissions, through collection of specimens at abattoirs and from whole herd on-farm testing. Blood and faecal specimens were obtained on-farm from five herds and at slaughter in abattoirs on four occasions.

During abattoir sampling the entire alimentary tract was removed at slaughter and mesenteric lymph nodes, ileum, ileo-caecal valve and colon samples were collected immediately. Tissues for culture were collected into separate containers for lymph nodes and gut, tissues for HP were collected then fixed directly into 10% formalin. Collection of tissue specimens was performed on stainless steel tables and risk of cross contamination was minimised by washing all surfaces and instruments with hot water and disinfectant between animals.

At the abattoir blood was collected directly from the jugular vein into 200 mL containers immediately after slaughter. Blood was collected from live animals on-farm by jugular venepuncture using 10 mL vacuum blood tubes. Faecal samples were collected by manual extraction from the rectum using a new glove for each animal.

Culture of Tissues and Faeces

Faeces and tissues (lymph nodes, ileum, ileo-caecal valve, colon) were cooled immediately after collection and stored at -80°C prior to culture. Processing of samples for culture was performed by double incubation and radiometric BACTEC culture for *M. paratuberculosis* in accordance with the Australian and New Zealand Standard Diagnostic Procedures (ANZSDP). Positive BACTEC cultures were subcultured onto 2 Herrolds Egg Yolk Media slopes (HEYM), with and without mycobactin as well as into a new BACTEC culture without egg yolk. Positive subcultures were confirmed by mycobactin dependence on the HEYM slopes and IS900 PCR. Following culture, the remainder of the samples were stored at

-20°C. Many faecal samples that demonstrated *M. paratuberculosis* by culture were subsequently retrieved from storage for PFC and quantification trials.

Determination of the Concentration of M. paratuberculosis in Individual Faeces

Estimation of the number of *M. paratuberculosis* organisms excreted in the faeces of infected animals was undertaken in 5 faecal culture experiments representing 1000 BACTEC cultures. Faeces from 39 infected deer were retrieved from -20°C storage and processed in triplicate. Ten-fold dilutions to 10⁻⁴ or 10⁻⁶ were prepared from each replicate and each dilution inoculated into BACTEC culture. The concentration of organisms was estimated using the Most Probable Number (MPN) technique (Cochran, 1950) and using the estimate of analytical sensitivity of culture as 10 to 50 CFU/g (Whitlock and Rosenberger, 1990). Faeces that cultured positive in their initial individual culture but were negative in culture attempts following -20°C storage were deemed to have few *M. paratuberculosis* organisms and CFU/g estimation was conservatively based on the original isolation assuming a single positive at neat concentration.

Pooled Faecal Culture

Pooling rate trials were performed in 4 experiments totalling 208 individual cultures from 52 deer that had previously tested positive by IFC. Thirty-two of the 52 faeces were successfully cultured in pooling trials following storage at -20°C, hence pooling trial results are based on these 32 samples. PFC was investigated by serial dilution of known infected faeces with known negative faeces. One gram of faeces from an infected deer was pooled with 4, 9 or 19 grams of faeces from negative deer in PFC 5, 10 and 20 and cultured. Pools were processed for faecal culture by the PFC method as used for pooled ovine faecal culture (Whittington *et al.*, 2000). The positive faeces used in each pool were also individually cultured in parallel with the pools by radiometric culture as previously described.

Pooled Faecal Culture Field trial of a Positive Property

Faecal samples were obtained from 239 deer on a known JD positive deer property that had a herd prevalence of 25.5% by IFC. Samples were processed as IFC and combined as PFC 5 and 20 in the order in which they had been sampled. In total 76 PFC 5 and 19 PFC 20 were prepared.

Pooled Faecal Culture Field trial of a Negative Property

The specificity field trial used 370 deer faeces from 3 known JD negative deer properties and processed as PFC 5, 10 and 20. From Property A, 140 faecal samples were processed as 28 PFC 5, and 14 PFC 10. One hundred faecal samples were processed from Property B and 130 from Property C. Two PFC 20 were prepared from each property. In total 74 PFC 5, 37 PFC 10 and 6 PFC 20 were cultured.

Serology

ELISA Development

Modification of the commercial bovine ELISA (PARACHEK, CSL) for JD was undertaken using a range of deer sera from known positive and negative animals as determined by HP, IFC or TC using three different conjugates; concentrated bovine conjugate, Protein G conjugate and rabbit anti-deer IgG conjugate. Initially, the 3 conjugates were tested at 5 dilutions using 12 sera. Following selection of appropriate conjugate dilutions, seven subsequent trials utilised samples of serum from a further 288 deer of positive and negative infection status. The number of dilutions was reduced by selection of the optimal dilution range from the previous trial. From the seven ELISA trials, sera suitable for use as positive and negative controls were selected based on the discrimination of known positive sera from known negative sera given an appropriate incubation time. Final dilutions used for each conjugate were; Protein G 1:16000, deer conjugate 1:1000, bovine conjugate 1:500. An approximate total of 70 ELISA plates were used, representing 6500

individual serological tests. The relationship between optical density values and the disease status of the animals was evaluated in the tests for sensitivity and specificity.

ELISA Sensitivity and Specificity

The sensitivity and specificity of the ELISA was determined for each conjugate at the final dilutions determined in the ELISA development. Positive and negative control sera used were selected from the sera used in the ELISA development procedure and the sensitivities and specificities determined at three optical density cut points, ie. OD of negative control + 0.1 or +0.2 or +0.3. The cut points chosen were based on the existing established cut points currently in use for the cattle and sheep ELISA.

The 172 sera used in the sensitivity trial were selected for use on the basis of a positive test result by either IFC, TC or HP. Sensitivity for each conjugate was calculated as the percentage of animals interpreted to be positive for JD by the modified ELISA from these 172 samples.

The specificity trial was initially performed on samples of serum from 526 deer collected from three properties considered to be free of infection. However, during the whole herd testing, four animals from one of these herds were detected as infected with *M. paratuberculosis* by IFC. Subsequently ELISA results from all animals in this herd and any deer where IFC provided no result due to overgrowth were excluded from the analysis of specificities. The final specificity of the ELISA was calculated on the basis of results obtained in 210 IFC-negative deer from two herds with no history of JD. These had no bacteriological and/or histological evidence of *M. paratuberculosis* infection by either TC, FC or HP.

Agar Gel Immuno Diffusion test (AGID)

AGID sensitivity and agreement estimates were performed on the 172 sera used previously for ELISA sensitivity testing plus 4 new sera from animals with histological and/or bacteriological evidence of *M. paratuberculosis* infection. Levels of agreement were calculated between AGID and Protein G, deer and bovine ELISA. AGID specificity was estimated using 72 of the sera used for the ELISA specificity evaluation.

Estimates of agreement between AGID and Protein G ELISA using historical data were performed using 839 sera from infected properties received as diagnostic accessions. The Protein G ELISA used for testing these sera was a preliminary version of the final Protein G ELISA protocol. The Protein G conjugate concentration for the preliminary protocol was used at 1:12,000, slightly higher concentration than the final dilution of 1:16,000.

Analysis of Results

Measurement of Agreement - Kappa Analysis

The measurement of agreement between results of the ELISA and AGID, TC, IFC and HP was determined using Kappa statistics (Martin et al., 1987). Kappa analysis makes a comparison of the results for one test with the results of the second and calculates the degree of concordance between the tests. Data for the 2 tests are displayed in a 2x2 table and results of comparisons are presented on a scale between -1 to 1, 1 being perfect agreement, 0 equals no agreement other than that what can be attributed by chance. The Fisher exact test was performed to indicate if the agreement is significant based on the sample size at the 95% significance level.

ELISA and AGID

The sensitivity and specificity of the ELISA and AGID were determined using the method described by Sackett et al. (1985) and the Two Graph Receiver Operator Curve (TGROC) method (Greiner, 1995, Greiner *et. al.*, 1995).

For comparative analysis of the OD values of the ELISA and IFC, TC and HP, ELISA OD's were adjusted by dividing them by the mean OD of the plate's positive controls. The correlations between the adjusted OD values (concentration of antibodies) measured by each conjugate was determined using the Pearson method. The comparisons of concentrations of antibodies in deer with typical JD lesions, animals with equivocal lesions and infected animals with no detectable lesions, as well as those measured in FC positive/TC positive and FC negative/TC positive animals were performed using the t test or ANOVA and Tukey's multiple comparison test.

Pooled Faecal Culture

Relative sensitivity of PFC was determined against matching IFC (Equation 1). Estimated sensitivity of PFC relative to TC was determined by calculating the proportion of animals detected by PFC compared to IFC and multiplying this proportion by the IFC/TC of 0.47 (Equation 2). Analytical sensitivity of PFC was estimated from the results of pools where the concentration of the organisms was estimated by MPN in parallel with the PFC.

$$1) \quad \frac{\text{No. of PFC +ve}}{\text{No. of IFC +ve}} \times 100 = \%$$

$$2) \quad \frac{\text{No. of PFC +ve}}{\text{No. of IFC +ve}} \times 0.47 \times 100 = \%$$

4. Results

Tissue culture, individual faecal culture and histopathology

Of the 172 deer with confirmed JD, 166 were examined by IFC, 110 were tested by TC and 68 were submitted for histopathological examination. *M. paratuberculosis* was detected in faeces in 112 (67.5%) of the 166 animals tested by IFC. In comparison, among the 110 infected deer examined by TC, 102 (92.7%) tested positive and of 68 examined for HP 34 (50%) were positive. Of the 101 deer examined by both TC and IFC, 93 tested positive by TC and only 50 were detected by IFC ($P < 0.0001$). This resulted in a poor agreement between FC and TC results as determined by Kappa statistics (Table 1). Relative to TC, IFC had a sensitivity of 47%.

Table 1. Results of individual faecal culture and tissue culture in 101 infected deer that were examined by both tests.

		Tissue culture		
		+ve	-ve	Total
Individual faecal culture	+ve	44	6	50
	-ve	49	2	51
Total		93	8	101
Kappa level of agreement = -0.08				
Fisher exact test $P < 0.0001$				

Granulomatous lesions with acid fast organisms typical of JD were detected in 36 (53%) of the 68 deer submitted for histopathological examination. Sixteen (24.2%) deer had equivocal granulomatous lesions without acid-fast organisms. No lesions were detected in the remaining 16 animals (23.5%). TC was significantly ($P = 0.0029$) more sensitive than HP at 70.2% (Table 2). In comparison, HP was slightly more sensitive than IFC (Table 3).

Table 2. Results of histopathology and tissue culture in 50 infected deer that were examined by both tests.

		Tissue culture		
		+ve	-ve	Total
Histopathology	+ve	33	2	35
	-ve	14	1	15
Total		47	3	50
Kappa level of agreement = 0.012				
Fisher exact test $P = 0.0029$				

Table 3. Results of histopathology and individual faecal culture in 50 infected deer that were examined by both tests.

		Histopathology		
		+ve	-ve	Total
Individual faecal culture	+ve	24	6	30
	-ve	11	9	20
	Total	35	15	50

Kappa level of agreement = 0.261
Fisher exact test P = 0.3873

ELISA

The sensitivities for the ELISA at 0.1 cut-point with three different conjugates was similar and ranged from 44.9% (bovine conjugate), 46.0% (protein G conjugate) and 51.1% (deer conjugate) (Table 4). The specificity of the ELISA at 0.1 cut-point were also similar; 97.6% for the bovine conjugate, 92.9% for the protein G conjugate and 89% for the deer conjugate (Table 5). Changing the cut-point from 0.1 to 0.2 and 0.3 to discriminate between positive reactions resulted in an improved specificity and decreased sensitivity. For all three conjugates, the sensitivities at the 0.3 cut-point were almost identical; 34.1% for the bovine conjugate, 35.2% for the protein G conjugate and 36.4% for the deer conjugate. Similarly, the specificities at this cut-point were also almost the same; 99.5% for the bovine conjugate, 98.6% for the protein G conjugate and 99.5% for the deer conjugate (Figure 1).

Table 4. Sensitivity of the ELISA.

Calculated sensitivities (%) of the ELISA with various conjugates* and end points for a positive interpretation.

Cut-point	Protein G conjugate	Deer conjugate	Bovine conjugate
0.1	46.0 (36.0 – 56.3)	51.1 (40.8 – 61.0)	44.9 (35.0 – 55.3)
0.2	38.6 (29.4 – 49.2)	40.3 (30.3 – 50.3)	38.1 (28.5 – 48.3)
0.3	35.2 (25.7 – 45.1)	36.4 (26.6 – 49.3)	34.1 (24.8 – 44.2)

*The sensitivities were calculated on the basis of results obtained by the ELISA in 172 deer with bacteriological and/or histopathological evidence of JD. 95% confidence intervals indicated in parentheses.

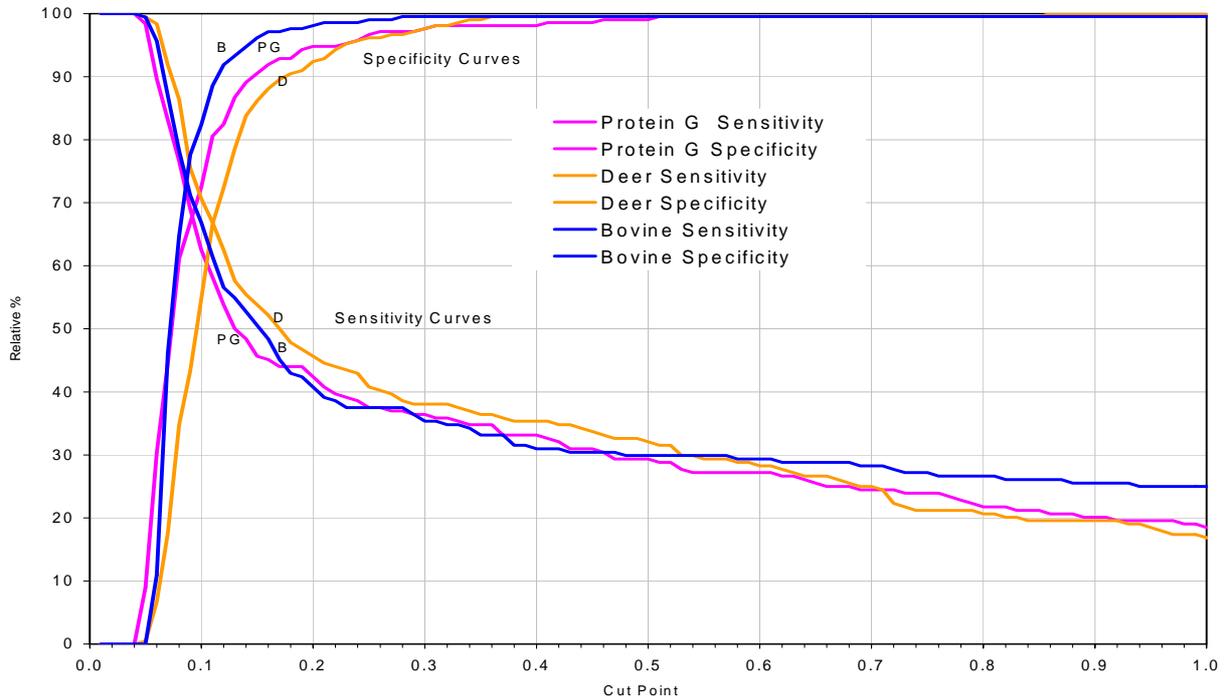
Table 5. Specificity of the ELISA.

Calculated specificities (%) of the ELISA with various conjugates* and end points for a positive interpretation.

Cut-point	Protein G conjugate	Deer conjugate	Bovine conjugate
0.1	92.9 (86.0 – 97.0)	89.0 (81.0 – 94.0)	97.6 (92.5 – 99.4)
0.2	97.6 (92.5 – 99.4)	96.7 (91.5 – 99.4)	99.0 (94.6 – 100)
0.3	98.6 (94.0 – 99.5)	99.5 (96.0 – 100)	99.5 (96.0 – 100)

*The specificities were calculated on the basis of results obtained by the ELISA in 210 IFC-negative deer from two herds with no history of JD. 95% confidence intervals indicated in parentheses.

Figure 1. Two graph receiver operator curves illustrating comparative sensitivity and specificity for the Protein G (PG), Bovine (B) and Deer (D) conjugates. The sensitivity curve begins at 100% and the specificity curve begins at 0%. Average negative control (0.07) is added to a desired cut point to give a cut point (eg. 0.2+0.07=0.27) from which sensitivity and specificity can be read from the graph.



Removing the optical densities for the 11 animals with clinical signs reduced the sensitivity of each of the conjugates by approximately 2-5%. The sensitivity for detection of subclinical animals was approximately; 42.4% and 30.3% for the bovine conjugate, 41.8% and 29.7% for the protein G conjugate and 48.5% and 32.7% for the deer conjugate at cut points 0.1 and 0.3 respectively. The ELISA at 0.1 cut-point failed to detect at least 50% of the 112 deer tested positive by FC, regardless of the conjugate (Table 6). The ELISA detected fewer faecal shedders when the cut-point was raised.

Table 6. The percentage of 112 IFC-positive deer detected by the ELISA

Cut-point	Protein G conjugate	Deer conjugate	Bovine conjugate
0.1	47.3	52.7	44.6
0.2	37.5	40.2	37.5
0.3	34.8	35.7	34.8

There was a strong agreement between the ELISA results obtained with protein G conjugate, bovine conjugate and deer conjugate in 172 deer with bacteriological and/or histopathological evidence of JD (Table 7). In addition, the correlation between the concentrations of antibodies measured by each conjugate was also very strong (protein G conjugate vs bovine conjugate, $r=0.9386$, $P<0.0001$; deer conjugate vs bovine conjugate, $r=0.9423$, $P<0.0001$; deer conjugate vs protein G conjugate, $r=0.9699$, $P<0.0001$).

Table 7. The level of agreement between the ELISA's results obtained with protein G conjugate, bovine conjugate and deer conjugate in 172 deer with bacteriological and/or histopathological evidence of JD.

Cut-point	No. animals tested positive and negative		No. concordant results	
	Protein G conjugate		Deer conjugate	
0.1	+ve	n= 80	78	(0.88)
	-ve	n= 92	84	
0.2	+ve	n= 66	65	(0.93)
	-ve	n= 106	101	
0.3	+ve	n= 61	60	(0.95)
	-ve	n= 111	108	
	Bovine conjugate		Protein G conjugate	
0.1	+ve	n= 77	72	(0.85)
	-ve	n= 95	87	
0.2	+ve	n= 66	62	(0.90)
	-ve	n= 106	102	
0.3	+ve	n= 60	58	(0.94)
	-ve	n= 112	109	
	Deer conjugate		Bovine conjugate	
0.1	+ve	n= 86	75	(0.85)
	-ve	n= 86	84	
0.2	+ve	n= 70	65	(0.93)
	-ve	n= 102	101	
0.3	+ve	n= 63	59	(0.94)
	-ve	n= 109	108	

Figures in brackets indicate the level of Kappa agreement.

By comparison, in the group of 210 deer considered to be non-infected by whole herd IFC, a good agreement was only observed between the ELISA's results obtained with protein G conjugate and deer conjugate (Table 8). The level of agreement between the protein G conjugate and bovine conjugate and between the deer conjugate and bovine conjugate was low to moderate. In addition, the correlation between concentrations of antibodies was stronger between the protein G conjugate and deer conjugate ($r= 0.911$, $P<0.0001$) compared to that measured between the protein G conjugate and bovine conjugate ($r= 0.7851$, $P<0.0001$) and the deer conjugate and bovine conjugate ($r= 0.7849$, $P<0.0001$).

Table 8. The level of agreement between the results obtained by the ELISA with protein G conjugate, ELISA with bovine conjugate and ELISA with deer conjugate in 210 deer without evidence of JD.

Cut-point	No. animals tested positive and negative		No. concordant results	
	Protein G conjugate		Deer conjugate	
0.1	+ve	n=15	13	(0.65)
	-ve	n=195	185	
0.2	+ve	n=5	4	(0.66)
	-ve	n=205	202	
0.3	+ve	n=3	1	(0.5)
	-ve	n=207	207	
	Bovine conjugate		Protein G conjugate	
0.1	+ve	n=5	4	(0.38)
	-ve	n=205	194	
0.2	+ve	n=2	1	(0.28)
	-ve	n=208	204	
0.3	+ve	n=1	1	(0.5)
	-ve	n=209	207	
	Deer conjugate		Bovine conjugate	
0.1	+ve	n= 23	5	(0.332)
	-ve	n= 187	187	
0.2	+ve	n= 7	2	(0.448)
	-ve	n= 203	203	
0.3	+ve	n= 1	1	(1)
	-ve	n= 209	209	

Figures in brackets indicate the level of Kappa agreement.

According to the results of confirmatory bacteriological and/or histological examination, the infected animals could be allocated to the following categories: (i) IFC and TC positive, (ii) IFC positive but TC negative, (iii) TC positive but IFC negative, (iv) TC and IFC negative (HP positive). A higher percentage of FC-positive animals gave positive reactions in the ELISA compared to FC-negative deer (Table 9).

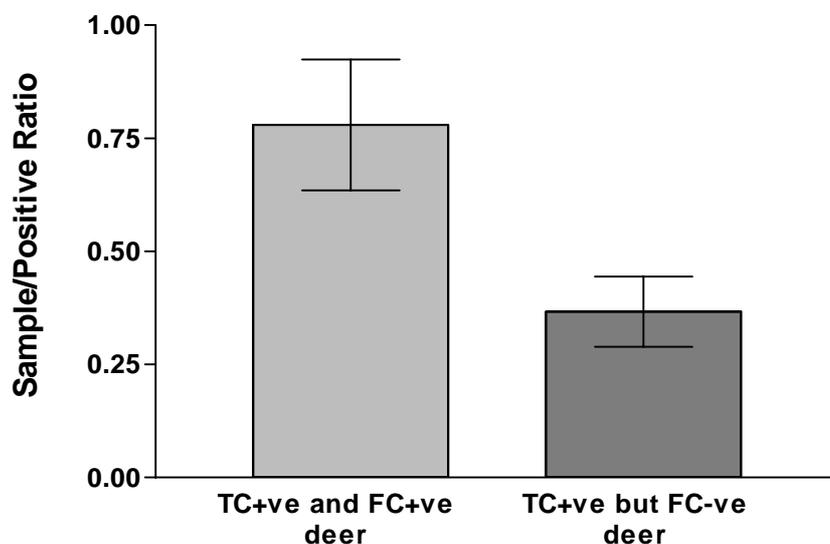
Table 9. Distribution of ELISA reactors in 101 infected deer that were examined by both individual faecal culture and tissue culture.

		Percentage of deer tested positive by ELISA								
		Protein G conjugate			Deer conjugate			Bovine conjugate		
		Cut-point			Cut-point			Cut-point		
		0.1	0.2	0.3	0.1	0.2	0.3	0.1	0.2	0.3
TC&FC+ve	n=44	52%	46%	39%	55%	46%	43%	52%	41%	39%
TC+ve/FC-ve	n=49	41%	35%	31%	41%	37%	33%	39%	35%	29%
FC+ve/TC-ve	n=6	67%	50%	50%	83%	33%	16%	67%	50%	50%
TC&FC-ve*	n=2	0	0	0	0	0	0	50%	0	0
TOTAL	n=101									

*both animals had histopathological evidence of JD

In addition, the optical densities (concentrations of antibodies) were significantly higher ($P=0.0112$) in FC and TC-positive deer compared to those measured in TC-positive but FC-negative animals (Figure 2).

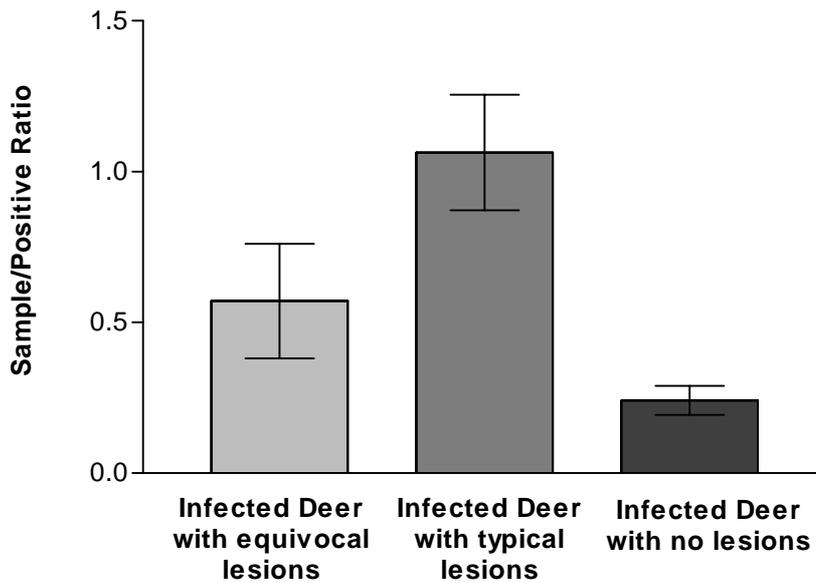
Figure 2. Mean ELISA sample/positive OD ratio assayed with protein G conjugate* in 44 IFC and TC-positive deer, 49 tissue culture-positive but IFC-negative animals.



*The results of the analysis of the ELISA's with the deer and bovine conjugates were concordant to those obtained with the ELISA with protein G conjugate.

The concentrations of antibodies were also significantly higher ($P < 0.01$) in deer with typical lesions with acid-fast organisms compared to those measured in infected animals without lesions (Figure 3). In addition, the concentrations of antibodies were also considerably higher in deer with typical lesions than in animals with equivocal lesions without acid fast organisms.

Figure 3. Mean ELISA sample/positive OD ratio assayed with protein G conjugate* in 30 deer with lesions typical of JD, 16 deer with equivocal lesions and 15 infected animals with no lesions.



*The results of the analysis of the ELISA's with the deer and bovine conjugates were concordant to those obtained with the ELISA with protein G conjugate.

AGID

The sensitivity of the AGID when tested on 176 sera (172 sera used for ELISA sensitivity testing plus 4 extra sera) from deer with evidence of JD was determined to be 20.5% (12.7–29.2%), slightly better than half that of the ELISA by any of the conjugates at cut point 0.2. The AGID only detected 32 of the 176 sera from deer with evidence of JD where the ELISA detected between 67–71 at cut point 0.2 (Table 10). Furthermore, results of initial serological testing using 839 diagnostic samples showed a much lower number of positive interpretations than compared to Protein G ELISA (preliminary protocol) (Table 11). AGID indicated 28 of 839 diagnostic sera to have a positive reaction compared to the ELISA with 132 at cut point 0.1 or 88 positive interpretations at cut point 0.2.

The level of agreement between the ELISA and the AGID on the sensitivity sera was moderate ranging from 0.479 for the deer conjugate to 0.537 for the Bovine conjugate (Table 10). Of the 32 positive animals detected by AGID, 31 were also detected by the ELISA, only 1 serum was detected by AGID that was not detected by the ELISA. Agreement between tests on the diagnostic sera was lower at 0.394 at cut point 0.2 (Table 12).

Table 10. The level of agreement between results obtained by the AGID test with results obtained from the ELISA with protein G conjugate, ELISA with deer conjugate and ELISA with bovine conjugate in sera from 176 deer with evidence of JD.

ELISA Cut point	Protein G n=176			Deer n=176			Bovine n=176		
	0.1	0.2	0.3	0.1	0.2	0.3	0.1	0.2	0.3
Both Tests Positive	35	34	33	36	34	34	35	34	32
ELISA Pos/AGID Neg	46	34	29	54	37	30	44	33	28
ELISA Neg/AGID Pos	1	2	3	0	2	2	1	2	4
Both Negative	94	106	111	86	103	110	96	107	112
Kappa	0.44	0.527	0.559	0.394	0.479	0.567	0.456	0.537	0.552
Fisher exact test P	= <0.0001 for all conjugates and dilutions								

The number of concordant positive results between the AGID and ELISA was only reduced by up to 3 when the cut point is raised from 0.1 to 0.3 for both the sensitivity sera and the diagnostic sera. This indicates that the AGID is reacting mostly to the sera that have a high optical density and therefore a high concentration of serum antibodies to *M. paratuberculosis*. Of the 176 sensitivity sera, 11 sera were from animals with clinical signs of which 9 were detected by AGID. ELISA detected all 11 animals with clinical signs.

The AGID gave no positive reactions to any of the 72 sera from deer that had tested negative by IFC and TC, indicating 100% specificity. In comparison, the ELISA gave positive reactions in 7 sera from the same batch at cut point 0.1, and 2 sera were ELISA positive at cut points 0.2 and 0.3 to one or more of the conjugates. The level of agreement between the 2 tests on the diagnostic samples was calculated to be 0.394 at the ELISA cut point of 0.2 (Table 12).

Table 11: Results from AGID and the preliminary Protein G ELISA for 839 deer sera.

	Protein G ELISA			AGID
	0.1	0.2	0.3	
Total Pos	132	88	77	28
Total Negs	707	751	762	805
Total Inconclusive	n/a	n/a	n/a	6
Total Number Tested	839	839	839	839

Table 12. Level of agreement between the AGID and the preliminary Protein G ELISA for 839 diagnostic submissions.

ELISA cut point	Protein G ELISA		
	0.1	0.2	0.3
Both Positive	26	24	23
ELISA Pos/AGID Neg	103	61	51
ELISA Neg/AGID Pos	2	4	5
Both Negative	702	744	754
ELISA P/inconc AGID's	3	3	3
ELISA N/inconc AGID's	3	3	3
Kappa	0.292	0.394	0.423
Fisher exact P =	<0.0001 for all dilutions		

Faecal Culture

Quantification of *M. paratuberculosis* Organisms Excreted in Faeces

The range of estimated number of organisms in faeces from infected deer varied from less than 20 to over 10^6 CFU/g (Table 13). Twenty-seven of the 39 animals tested (69%) were shedding approximately 2000 CFU/g or less (Figure 4). Eight deer (20%) were shedding 10^6 or greater CFU/g, the 4 remaining deer were shedding between approximately 10^4 to 5×10^5 CFU/g.

Thirty two of the 39 deer tested had subclinical infection. Of which twenty-seven had less than 2×10^3 CFU/g faeces (Figure 4). Within the group of deer with subclinical infection, 25 were shedding less than 200 CFU/g. One deer had 10^4 CFU/g and the four remaining deer with subclinical infection were shedding greater than 10^6 CFU/g, possibly indicating that these four animals had more advanced disease and extensive lesions. The range of excretion of *M. paratuberculosis* in the deer with subclinical infection could be roughly assigned into two groups, low and high shedders. The 27 low shedders (70%) were shedding between 10^1 and 2×10^3 CFU/g with 1 animal shedding 10^4 and 4 high shedders were shedding greater than 10^6 CFU/g with no medium subclinical shedders in the range of 10^4 - 10^6 CFU/g detected.

Seven deer with clinical signs were shedding a range of concentrations from 10^3 to 10^7 at an average of 5.3×10^6 . Three deer were shedding 10^3 , 10^4 and 10^5 CFU/g, the remaining four deer were shedding 10^6 and 10^7 CFU/g.

Figure 4. Distribution of the concentration of *M. paratuberculosis* detected in the faeces of deer with sub clinical (n=32) and clinical infections (n=7).

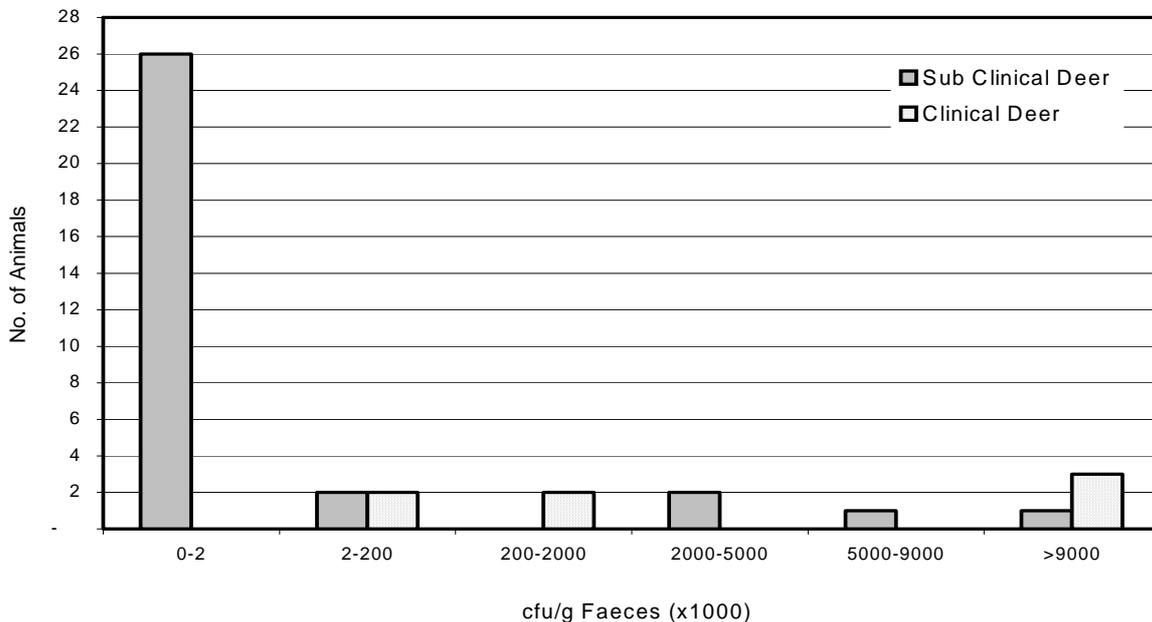


Table 13. The number of CFU/g determined in 39 faeces from deer with sub clinical and clinical *M. paratuberculosis* infection. The “Estimated Concentration CFU/g” allows for an analytical sensitivity of 10-50 CFU/g.

Animal Id	Test Status	Histopathology	Estimated Concentration CFU/g faeces	95 % Confidence Limits
921-30	SC, FP, TP, HP, EN, AN	Typical	20	3 – 60
921-39	SC, FP, TP, HP, EN, AN	Typical	20	3 – 60
921-42	SC, FP, TN, HP	Typical	20	3 – 60
921-44	SC, FP, TP, HP	Typical	20	3 – 60
921-50	SC, FP, TN, HP	Typical	20	3 – 60
921-73	SC, FP, TP, HP	Typical	20	3 – 60
920-11	SC, FP, TP, HP	Typical	20	3 – 60
920-14	SC, FP, TP, HP, EN	Typical	20	3 – 60
4341-26	SC, FP, TP, EN	Negative	20	3 – 60
4341-14	SC, FP, TP, EN, AN	Typical	20	3 – 60
4341-12	SC, FP, TP, EN, AN	Typical	20	3 – 60
4660-9	SC, FP, EN, AN		20	3 – 60
4660-19	SC, FP, EN, AN		20	3 – 60
4660-20	SC, FP, EN, AN		20	3 – 60
4660-33	SC, FP, EN, AN		20	3 – 60
4660-101	SC, FP, EN, AN		20	3 – 60
4660-122	SC, FP		20	3 – 60
4660-133	SC, FP, EP, AN		20	3 – 60
4660-23	SC, FP, EN, AN		72	4 – 324
4660-32	SC, FP, EN, AN		72	4 – 324
4660-144	SC, FP, EP, AN		72	4 – 324
4660-26	SC, FP		148	24 – 470
4660-14	SC, FP		186	30 – 621
4660-15	SC, FP		186	30 – 621
4660-24	SC, FP, EN, AN		186	30 – 621
920-1	SC, FP, TP, HP, EN, AN	Typical	10 ³	204 – 3648
4660-91	SC, FP, EP, AN		2x10 ³	432 – 7388
4660-103	SC, FP, EP, AN		10 ⁴	10 ⁵ – 1.3 x10 ⁵
4660-89	SC, FP, EP, AN		4.3 x10 ⁶	10 ⁶ – 1.1x10 ⁷
4341-36	SC, FP, TP, HP	Typical	4.6 x10 ⁶	2 x10 ⁶ – 18 x10 ⁶
4341-11	SC, FP, TP, HP, EP, AP	Typical	8.4 x10 ⁶	2 x10 ⁶ – 32x10 ⁶
920-9	SC, FP, TP, HP, EP, AP	Typical	1.48 x10 ⁷	3.5 x10 ⁶ – 46 x10 ⁶
4483	CL, FP, TP, HS	Equivocal	2x10 ³	700 – 10 ⁴
4330	CL, FP		4.6 x10 ⁵	2 x10 ⁴ – 4.1 x10 ⁶
344	CL, FP, TP, HP, EP, AP	Typical	7.1 x10 ⁴	5 x10 ³ – 3.2 x10 ⁵
5785-0030	CL, FP, TP, HP, EP, AP	Typical	1.8 x10 ⁶	4x10 ⁵ – 6.1 x10 ⁶
5785-0010	CL, FP, TP, HP, EP, AP	Typical	1.0 x10 ⁷	4 x10 ⁷
5785-0085	CL, FP, TP, HP, EP, AP	Typical	1.0 x10 ⁷	4 x10 ⁷
5785-0060	CL, FP, TP, HP, EP, AP	Typical	1.48 x10 ⁷	3.5 x10 ⁶ – 4.6 x10 ⁷

SC = Sub clinical FP = Faecal culture positive HP = Histopathology positive HS = Histopathology suspicious
CL = Clinical EP = ELISA positive Typical = Granulomatous reactions and acid fast organisms
EN = ELISA negative Equivocal = Pathological changes suggestive of *M. paratuberculosis* infection
TP = Tissue culture positive TN = Tissue culture negative AN = AGID Negative AP = AGID Positive
Empty cells = not performed

Pooled Faecal Culture – Pooling Rate Trials

Pooled Faecal Culture Sensitivity

Faeces from 32 deer that were previously demonstrated by IFC to contain *M. paratuberculosis* were used in PFC trials. Of the 32 deer faeces, 24 (75%) were re-isolated by individual culture, 16 (50%) in PFC 5, 14 (44%) in PFC 10 and 20 (Table 14). While PFC 10 and 20 detected the same number of animals, 10 animals were detected in common and 4 from each pool size were independent. Some variation in isolation of *M. paratuberculosis* occurred for six faecal specimens where IFC was negative but *M. paratuberculosis* was cultured in either PFC 5, 10 or 20. The variation is possibly due to low bacterial numbers, uneven distribution in the sample and/or the variable effect of chemical decontamination on altering the viability of *M. paratuberculosis*. Bacterial numbers close to the detection limit will also give variable results.

The ability of PFC to detect a single infected deer in a deer herd was reduced compared to IFC. Given the sensitivity of IFC was 47% compared to TC and PFC 5 detected 67% of the animals detected by IFC, then the estimated sensitivity for PFC 5 is 32%. PFC 10 and 20 detected 58% of the animals detected by IFC, the estimated sensitivity for PFC 10 and 20 is 27% (Table 15).

Infected animals that were difficult to detect by PFC were animals with subclinical infection and less than 200 CFU/g of faeces (Table 16). Out of 18 faecal samples from deer with subclinical infection detected by IFC, 9 (50%) were detected in PFC 5, 6 (33%) and 7 (39%) were detected in PFC 10 and 20 respectively. The estimated sensitivity for PFC of 5 and 10-20 animals for subclinical deer would be approximately 23.5% and 16.5%. TC and HP was not conducted on all animals from which faeces were used in PFC so it is difficult to make meaningful comparisons of pooled faecal results and the severity of lesions.

Table 14. The number of faecal culture positive animals detected by individual faecal culture, PFC 5 deer, PFC 10 and PFC 20 deer.

	No. of Animals	Individual Positive FC	Pool 5 FC +ve	Pool 10 FC +ve	Pool 20 FC +ve
Subclinical	24	18 (75%)	9 (38%)	6 (25%)	7 (29%)
Clinical	8	6 (75%)	7 (88%)	8 (100%)	7 (88%)
Total	32	24 (75%)	16 (50%)	14 (44%)	14 (44%)

Table 15. Detection of single positive deer faeces by PFC 5 deer, PFC 10 and PFC 20 deer as a % of individual faecal culture.

	Individual Positive FC	Pool 5 FC +ve	Pool 10 FC +ve	Pool 20 FC +ve
Subclinical	18	50%	33%	39%
Clinical	6	116%	133%	116%
Total	24	67%	58%	58%

The results of the MPN calculation on faeces used for pooling indicate that deer with subclinical infection were shedding much fewer numbers of organisms than deer with clinical infection (Table 16). Where *M. paratuberculosis* was not detected in PFC, the number of was less than 100 CFU/g of faeces. One deer with subclinical infection was shedding large numbers of organisms and was subsequently detected in all faecal pools. One deer with clinical signs was shedding low numbers of organisms and was detected in only one pool (Pool 10). This deer was also only equivocal by histopathological examination. The estimated analytical sensitivity for detecting a single infected animal by PFC 5 was at least 200 CFU/g in faeces from an infected animal. For PFC 10 and 20 animals, at least one animal per pool with 10⁴ CFU/g of faeces was required, however there was a paucity of data for animals that were used for PFC and MPN between 10³ and 10⁴ CFU/g and the detection limit maybe slightly less.

Pooled Faecal Culture Field Trial - JD Positive Property

IFC was performed on 239 deer from a known infected property of which 38 animals were IFC positive, a herd prevalence of 25.5%. Faeces were retrieved from storage and processed as PFC 5 and 20 in the order in which they had been sampled. Of the 76 PFC 5 prepared, 38 encompassed at least 1 sample positive by IFC, of these 12 of the 38 pools were culture positive for *M. paratuberculosis*. Of the nineteen PFC 20 prepared, 16 pools encompassed 42 samples detected as positive by IFC. Eleven of the 16 pools were culture positive for *M. paratuberculosis*.

Of the five infected properties from which faecal samples were drawn for PFC evaluations, all five properties were detected by at least one culture of pooled faeces. Data to evaluate PFC as a herd test was not available however given the analytical detection limit from 10² to 10⁴ CFU/g, PFC could be expected to have acceptable sensitivity for detecting infected herds.

Pooled Faecal Culture - Specificity and Overgrowth Rate

Of the IFC negative samples pooled into 74 PFC 5, 37 PFC 10 and 6 PFC 20 and processed for radiometric culture, all pools were culture negative for *M. paratuberculosis* at the end of the incubation period.

On average the overgrowth rate of faecal and TC by other organisms was equivalent to the overgrowth rate of culture for bovine sample at 5%. The pattern of overgrowth in IFC culture appeared to correlate with batches of faeces submitted to the laboratory. Accessions of faecal samples that had high overgrowth rates on the initial culture also had high overgrowth rates when re-cultured following storage. It appears to be an animal related factor, possibly diet that encourages higher than average overgrowth rates.

Table 16. Results of pooling faeces from 32 infected deer shedding *M. paratuberculosis* organisms with faeces from faecal culture negative deer.

Animal Id	Test Status	Histopathology	Estimated No. of cfu/g faeces	95 % Confidence Limits	Pooled Faecal Culture Rate
2271-19	SC, FP				N, 5, 20
4660-114	SC, FP, EN, AN				N
4660-118	SC, FP, EN, AN				20
4660-122	SC, FP		20	3 – 60	10
4660-127	SC, FP, EN, AN				N, 5, 10, 20
4660-113	SC, FP, EP, AP				N
4660-142	SC, FP, EP, AP				N, 5, 10, 20
4660-125	SC, FP, EP, AN				5
921-59	SC, FP, TN, HP	Typical			N
4341-43	SC, FP, TP, HP	Typical			20
4341-38	SC, FP, TP,HP	Typical			N
4341-39	SC, FP, TP,HP	Typical			N
921-64	SC, FP, TP, HS, EP, AP	Equivocal			N, 5
920-12	SC, FP,TP, HN, EN, AN	Negative			20
921-69	SC, FP,TP, HP, EP, AP	Typical			10
4660-23	SC, FP, EN, AN		72	4 – 324	N
4660-144	SC, FP, EP, AN		72	4 – 324	N
4660-26	SC, FP		148	24 – 468	N
4660-14	SC, FP		186	30 – 621	N
4660-15	SC, FP		186	30 – 621	N
4660-24	SC, FP, EN, AN		186	30 – 621	N, 5
4660-91	SC, FP, EP, AN		2140	432 – 10 ⁴	N, 5, 10
4660-103	SC, FP, EP, AN		10 ⁴	10 ³ – 1.3x10 ⁵	N, 5
4660-89	SC, FP, EP, AN		4.3 x10 ⁶	10 ⁶ – 1.1x10 ⁷	N, 5, 10, 20
5428	CL, FP, TP				N, 5, 10, 20
4483	CL, FP, TP, HS	Equivocal	3x10 ³	700 – 10 ⁴	10
4343-2	CL, FP, TP, HP, EP, AN	Typical			5, 10, 20
02/129	CL, FP, TP, HP, EP	Typical			N, 5, 10, 20
5785-0030	CL, FP, TP, HP, EP, AP	Typical	1.8 x10 ⁶	4x10 ⁵ – 6.1 x10 ⁶	N, 5, 10, 20
5785-0010	CL, FP, TP, HP, EP, AP	Typical	1.0 x10 ⁷	4 x10 ⁷	N, 5, 10, 20
5785-0085	CL, FP, TP, HP, EP, AP	Typical	1.0 x10 ⁷	4 x10 ⁷	N, 5, 10, 20
5785-0060	CL, FP, TP, HP, EP, AP	Typical	1.48 x10 ⁷	3.5 x10 ⁶ – 4.6 x10 ⁷	N, 5, 10, 20

SC = Sub clinical FP = Faecal culture positive HP = Histopathology positive HS = Histopathology suggestive
 CL = Clinical FN = Faecal culture negative HN = Histopathology negative Blank cells = no data
 EP = ELISA positive Typical = Granulomatous reactions and acid fast organisms
 EN = ELISA negative Equivocal = Pathological changes suspect of *M. paratuberculosis* infection
 TP = Tissue culture positive TN = Tissue culture negative AN = AGID Negative AP = AGID Positive
 N = Individual culture (Neat) 5 = PFC 5 10 = PFC 10 20 = PFC 20

Issues Relating to Cold Storage

Loss of viable organisms during frozen storage is well known and documented for all types of micro-organisms. Numbers of viable *M. paratuberculosis* have been shown to reduce by 54% to 62% in faeces during the first 3 weeks of frozen storage at -70°C (Richards, 1977) and viable numbers will continue to decrease over one to two years storage (Kim and Kubica, 1973). As storage of *M. paratuberculosis* at -70°C is more effective at maintaining viability than -20°C (Kim and Kubica, 1973, Cousins D., 2003, pers comm.), it is standard laboratory procedure to store all samples prior to testing at -70°C if testing cannot proceed within 24 hrs. If culture is to occur within 24 hours samples are stored at 4°C to avoid freeze-thawing effects. Following culture, samples are routinely stored at -20°C . Although loss of viability is known to occur, total loss is restricted only to samples that contain an initial low concentration of organisms.

In this trial the MPN and PFC experiments used faeces from a total of 91 animals that were positive for *M. paratuberculosis* by IFC. The samples were originally stored at -70°C prior to processing for IFC and stored at -20°C following. After retrieval from -20°C storage, *M. paratuberculosis* was re-cultured from 54 (60%) faeces and was not recovered from 37 (40%) faeces.

The reduced recovery following storage was confined to faeces of deer with subclinical infection. Of the 7 samples from deer with clinical signs in the MPN trial, all faeces were re-cultured by IFC. Failure to reisolate *M. paratuberculosis* from faecal samples was attributed to low initial concentrations and losses that are known to occur during storage and the decontamination procedure that reduced the concentration below the detection limit. For the purpose of MPN calculations these samples were estimated to contain less than 60 CFU/g.

The number of organisms estimated by MPN may be an under-estimate by an order of magnitude. The figures reported in tables 13 and 16 account for this potential loss (see methods).

5. Discussion

TC demonstrated the highest test sensitivity with approximately 92% of the infected animals positive, compared to 66% by HP and 49% by IFC. Agreement between TC with HP and IFC was low, the results indicated that TC detected 93% and 88% of IFC and HP positive animals respectively. However, TC detected an additional 53% and 33% positive animals. HP and IFC detected a similar population of infected animals, of which the majority was a subset of the TC positive population. If TC was considered to be the gold standard for these experiments, IFC detected less than half the infected animals, although TC was not performed on all the animals tested by IFC.

The commercial ELISA kit “PARACHEK” was suitable for testing deer specimens after modifying the conjugate. The sensitivity of the modified ELISA was determined to be between 38.1% to 40.3% using an optical density cut-point 0.2 greater than the negative control, or 34.1% to 36.4% using cut-point of 0.3. The sensitivity of the 3 conjugates was similar but the deer conjugate was consistently most sensitive, by 6.2% at cut-point 0.1 and by 2.3% at cut-point 0.3, with protein G only slightly more sensitive than the concentrated bovine conjugate at 0.3. The sensitivity of the modified ELISA with optimal conjugate concentration is similar to sensitivity for the ELISA for testing cattle and sheep (Hope *et al.*, 2000).

The concentrated bovine conjugate consistently had the highest specificity, with Protein G and deer conjugates demonstrating similar specificities. Protein G was slightly higher at cut-point 0.2 and the deer slightly higher at cut-point 0.3. To achieve 99% specificity or greater using the ELISA, a cut-point of 0.2 for bovine conjugate or 0.3 with the deer conjugate was required but the sensitivity reduced to 34–36%. Protein G specificity achieved only 98.6% at cut-point 0.3. By comparison, the recommended end points for the CSL PARACHEK ELISA are for bovine sera 0.1, specificity 99.1% – 99.9% and for ovine sera 0.2, specificity 98.5 – 99.7% (Hope *et al.* 2000).

Deer appear to be more susceptible to infection by other mycobacterial organisms, for example *M. avium* and *M. bovis*, and it may be expected that deer would produce a larger range of IgG antibodies that are not removed by absorption with the *M. phlei* antigens. These antibodies will be present and cross-react with the *M. paratuberculosis* antigen in the ELISA. This may be a contributing factor to the lower specificity and higher sensitivity of the deer conjugate (rabbit α deer IgG) than for the other conjugates. To achieve optimal reactions the bovine conjugate used in this trial was a more concentrated form than that supplied in the commercial kit, thus the bovine conjugate from the commercial kit as currently supplied is not suitable for performing a deer ELISA.

Correlation of optical density values from non-infected deer was moderate between protein G and deer (91%), but Protein G and deer conjugates were both low when compared with the concentrated bovine conjugate (78%). There was less agreement between results from the 3 conjugates for non-specific reactions than for true positive reactions. These differences are observed in the level of agreement measured by Kappa analysis where agreement between deer and protein G conjugates was high for true negatives and false positives. Both conjugates detected more false positives than the bovine conjugate at cut-points 0.1 and 0.2, the deer conjugate detected the most. Optical density values for serum from positive animals was more strongly correlated across all 3 conjugates (93.8%-96.9%) than with the sera from non-infected deer (78.4%-91.1%), protein G and deer conjugates the most closely concordant. The deer conjugate detected a higher proportion of true positives that were not detected by the other conjugates in a similar percentage to the extra false positives detected in the specificity test.

AGID was shown to have the lowest sensitivity at 20.5%. The AGID appeared to detect only animals with high concentrations of serum IgG antibody *ie.* high ELISA optical densities indicating that these animals were those with more advanced infection status. Studies in cattle have demonstrated similar estimates of sensitivity at 18.9%, 20%, 26.6% by JD AGID (Sherman *et al.*,

1990, Spangler *et al.*, 1988, Sockett *et al.*, 1992). An increase in AGID test sensitivity and duration of the disease has been demonstrated in cattle by Sherman (1989) who found the AGID test sensitivity increased from 28.8% during herd testing to 47% at time of slaughter. AGID is generally regarded as having high specificity which was indicated in this trial were the seven sera samples identified as false positive by the ELISA conjugates were not identified by the AGID. High specificity of AGID JD tests has been demonstrated in other species such as testing of ovine sera for JD (Hope *et al.*, 2000). However in regions where *M. avium* or *M. bovis* are prevalent such as New Zealand, specificity for testing with deer sera will be reduced due to the test's inability to discriminate between infection from three organisms (Macintosh, 1998).

Results from ELISA testing were more closely correlated with IFC than TC by a factor of 2. The optical densities of the ELISA were higher for deer that were excreting more organisms in faeces and for deer exhibiting clinical signs of JD. This suggests the ELISA is more likely to detect the more infectious deer. Increases in optical density of sera have been positively correlated with excretion of the organism in faeces of cattle (Reichel *et al.* 1999). The probability of a high optical density or positive IFC is most likely dependent on the length of time and severity of infection. Our experience with JD in cattle, however, suggests that it is unpredictable whether faecal culture or ELISA will be first to demonstrate positive results as infection progresses.

IFC had a relative sensitivity of 47% compared to TC, PFC 5 detected 67% of the IFC positive deer, an overall sensitivity of 32% for animals of all infection status. PFC 10 and 20 detected similar numbers of IFC (54-58%) thus PFC 10 and 20 had an overall sensitivity of 26%. The animals that were not detected in PFC were the subclinical animals shedding low numbers of organisms. This is indicated by a 50% reduction in detection of subclinical deer in PFC 5 while there was no reduction in detection of clinical animals. Given that MAP testing is intended to find subclinical infection in herds, the estimated sensitivity of detecting subclinical deer by faecal culture with PFC 5 and 10-20 deer would be approximately 23.5% and 16.5% respectively. It is possible that increased sensitivity of PFC may have been achieved if faeces had been cultured without storage. Experiments with cattle faeces indicate the minimal number of *M. paratuberculosis* organisms required for detection is in the order of 10 to 100 (Whipple *et al.*, 1992, Whitlock & Rosenberger 1990) given losses during the decontamination process on top of losses from frozen storage.

The advantage of faecal culture is that it detects animals shedding organisms. The disadvantages of faecal culture for the diagnosis of JD or certification of freedom are the length of time for results (8-10 weeks for negative findings) and cost of IFC. Faecal culture will only detect infected animals shedding bacteria and not those with early stages of infection prior to excretion of organisms. When animals with early subclinical infection commence shedding of *M. paratuberculosis* in faeces, detection by faecal culture can be variable as the concentration of organisms is low and intermittent. These animals have a greater chance of remaining undetected by PFC due to its reduced sensitivity compared to IFC. To identify an individual infected deer from a positive PFC, all individuals in the positive pool need to be retested, subclinical animals with low numbers of organism in their faeces are most at risk of remaining undetected. Although PFC as a herd test was not able to be evaluated in this project, each of the 5 properties from which positive faeces were used for PFC experiments produced at least one positive PFC. Given the analytical sensitivity of PFC, detection of an infected herd requires only one subclinical animal shedding at least 10^2 CFU/g for PFC 5 or approximately 10^4 CFU/g for PFC 10 and 20. In the case of overgrowth by other organisms, the culture is tested by PCR for the presence of *M. paratuberculosis* DNA albeit with a reduced sensitivity.

The cost advantage of using PFC compared to IFC would be approximately up to 50% depending on a laboratory's pricing structure. The disadvantage of PFC is the delay of up to 20 weeks for a result, and when a *M. paratuberculosis* positive culture is detected, a new round of testing is required to resolve which animal in the pool is infected. While IFC would provide for the highest sensitivity of the live animal tests, the cost of testing large numbers of animals at \$55 per animal may be prohibitive. The cost of ELISA testing at \$9.90 per animal would provide the best cost and sensitivity combination. ELISA testing would be approximately 20% the cost of IFC, 50% of the

cost of PFC 5 and about equivalent cost to PFC10. PFC 20 cultures would provide the cheapest option at approximately 50% the cost of the ELISA but with just over half the sensitivity for detecting an individual animal. If PFC were applied biannually it will provide an higher level of confidence in negative results but take away the cost advantage.

For market assurance programs to be effective they require a high degree of confidence in negative results. Most JD MAP's stipulate 95% confidence of detecting 1 infected animal in a herd with a prevalence of 2%. To achieve a 95% confidence limit by PFC 5, 550 animals are required (sensitivity 23.5%). Testing herds of less than these numbers will mean less certainty in a negative herd test result (Appendix 1). For ELISA testing (sensitivity 38%), a herd size of 300 animals or greater is required for testing. However, as the majority of animals will be subclinical, the effective sensitivity of the ELISA will also be reduced. After several years of testing, the Victorian Test and Cull program for dairy farms estimates the effective ELISA sensitivity to be 10-20% when applied as a whole herd test. Although test and cull together with improved management practices can be effective in reducing JD in medium to high prevalence herds, it is generally accepted that due mostly to the relative insensitivity of JD diagnostic tests, new strategies are needed for the long term plan to eradicate the disease.

6. Implications and Recommendations

The performances of IFC and ELISA for JD testing in deer are equivalent to JD testing for cattle and sheep. PFC has lower sensitivity for identifying individual animals but is expected to be useful as a herd test given the statistical limits outlined previously. AGID is considered unsuitable as a MAP test as faecal culture or the modified ELISA will also identify infected animals identified by AGID.

Given that the performance of both the absorbed ELISA, IFC and PFC of 5, 10 or 20 animals are equivalent to comparable tests used for other species and MAP's, the tests were found to be suitable for the diagnosis of JD in deer. Consequently absorbed ELISA, IFC and PFC are recommended tests for JD market assurance testing in deer herds.

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Appendix 1

Statistical limits of detection of disease in an infected population based on test sensitivity assuming 100% specificity (MacDiarmid, 1988).

Table A1: Minimum herd size required in a whole herd test to achieve a 95% confidence of detecting 1 infected animal at the sensitivity of each test, assuming 100% specificity.

Test	Test Sensitivity	Minimum Herd Size
Individual Faecal Culture	47%	250
Pool 5 Faecal Culture	32%	390
Pool 10, 20 Faecal Culture	26%	500
ELISA	38%	310
AGID	20%	675
Pool 5 for sub clinical animals	23.5%	550
Pool 10-20 for sub clinical animals	16%	850

Table A2: Minimum prevalence of disease required to be present in an infected herd of 200 animals to achieve a 95% confidence of detecting 1 infected animal at the sensitivity of each test, assuming 100% specificity.

Test	Test Sensitivity	Minimum Disease Prevalence Detectable in a 200 animal herd
Individual Faecal Culture	47%	2.4%
Pool 5 Faecal Culture	32%	3.9%
Pool 10, 20 Faecal Culture	26%	5.0%
ELISA	38%	3.1%
AGID	20%	6.7%
Pool 5 for sub clinical animals	24%	5.6%
Pool 10-20 for sub clinical animals	16%	8.6%