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Immunoglobulin G1 Enzyme-Linked Immunosorbent Assay for Diagnosis of Johne's Disease in Red Deer (*Cervus elaphus*)

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This study was designed to develop a customized enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of Johne's disease (JD) in farmed deer. Two antigens were selected on the basis of their superior diagnostic readouts: denatured purified protein derivative (PPDj) and undenatured protoplasmic antigen (PpAg). ELISA development was based on the antigen reactivity of the immunoglobulin G1 (IgG1) isotype, which is a highly specific marker for mycobacterial disease seroreactivity in deer. Sensitivity estimates and test parameters were established using 102 *Mycobacterium paratuberculosis*-infected animals from more than 10 deer herds, and specificity estimates were determined using 508 uninfected animals from 5 known disease-free herds. A receiver-operated characteristic analysis determined that at a cut point of 50 ELISA units, there was a specificity of 99.5% and sensitivities of 84.0% with PPDj antigen, 88.0% with PpAg, and 91.0% when the antigens were used serially in a composite test. Estimated sensitivity was further improved using recombinant protein antigens unique for *M. paratuberculosis*, which identified infected animals that were unreactive to PPDj or PpAg. While 80% of animals that were seropositive in the IgG1 ELISA had detectable histopathology, the assay could also detect animals with subclinical disease. The test was significantly less sensitive (75%) for animals that were culture positive for *M. paratuberculosis* but with no detectable pathology than for those with pathological evidence of JD (>90%). When the IgG1 ELISA was used annually over a 4-year period in a deer herd with high levels of clinical JD, it eliminated clinical disease, increased production levels, and reduced JD-related mortality.

Johne's disease (JD) is a chronic enteritis found in ruminants, caused by infection with the bacterium *Mycobacterium paratuberculosis*, that produces major economic losses in livestock and dairy industries worldwide. Infection due to *M. paratuberculosis* manifests as a chronic inflammatory gastroenteritis, with epithelial thickening in the lower intestine causing malabsorption of nutrients and leading to wasting and eventual death in affected animals. In most ruminants, it can take several years for clinical symptoms of JD to present, highlighting the chronic nature of the disease. In deer, however, the process from infection to death can progress more rapidly, with animals dying from the disease as early as 8 months of age (21). This more acute presentation of pathology suggests that red deer (*Cervus elaphus*) may provide a more informative infection model to track immunological and etiological pathways of *M. paratuberculosis* infection. Outbreaks of JD have been reported in young deer (8 to 15 months), with death in >20% of animals; older animals can also sporadically present with clinical JD typical of that found in cattle and sheep, and this may be exacerbated by stress or aging (21). JD can be spread horizontally among adult animals and may also be spread pseudovertically during pregnancy. In some instances, viable *M. paratuberculosis* organisms have been isolated from the uterus and fetal tissues of cattle (15) and from fetal tissues of deer (29).

The control and eradication of JD in livestock remain worldwide problems, due to the long incubation time and the lack of sensitivity of diagnostic tests, especially for the diagnosis of subclinical *M. paratuberculosis* infection. Several antibody-based serodiagnostic tests that are effective to various degrees in farmed sheep, goats, and cattle have been developed (6, 23), but as yet, no definitive serodiagnostic test exists for the disease in farmed deer. Despite this, the potential utility of serodiagnosis for JD in cervids has been demonstrated in free-ranging animals (7, 28), although the specificity of such tests may be confounded by cross-reactivity due to immune sensitization of animals with environmental mycobacteria from the *Mycobacterium avium-M. intracellulare* complex (MAIC) (1–3).

Several immunodiagnostic tests have been described as tools for the control of mycobacterial diseases, such as bovine tuberculosis and JD, based on the broad range of the host's immune reactivity to the presence of virulent mycobacteria. Cell-mediated immunity is considered to be associated with protection against chronic intracellular infections, while humoral responses are generally considered to be more indicative of disease. Diagnostic testing for tuberculosis in cattle (19) and deer (14) shows that while enzyme-linked immunosorbent assays (ELISAs) that detect total immunoglobulin G (IgG) antibody responses are possible, those that target specific IgG

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antibody isotypes, IgG1 and IgG2, may have increased precision for the diagnosis of mycobacterial infections in ruminants. A study of immunodiagnostic tests for bovine paratuberculosis found that while IgG2 levels decreased as disease progressed, IgG1 levels did not increase significantly (18). Levels of IgG1 and IgG2 vary dramatically over the infection cycle in cattle, and the interpretation of diagnostic results that measure the reactivity of these antibodies may be influenced by the type of antigen used in the diagnostic assay (18). The antigens used by Koets et al. (18) were proteins and glycolipids (heat shock proteins and lipoarabinomannan) isolated from *M. paratuberculosis*. Other studies of seroresponsiveness in mycobacterial infection have shown that the antibody subclasses directed against protein antigens are predominantly IgG1 and IgG3 and that the response to carbohydrate antigens is predominated by antibodies of the IgG2 isotype (27), an observation confirmed in a study comparing cattle antibody responses to *M. paratuberculosis* lipoarabinomannan and heat shock proteins (18).

The present study was undertaken with the aim of defining the serological reactivity of farmed red deer (*Cervus elaphus*) that had confirmed JD and/or were known to be infected with *M. paratuberculosis*. Initially, panels of sera from deer with clinical disease and/or confirmed *M. paratuberculosis* infection, as well as further samples from known disease-free animals, were used to estimate levels of specificity and sensitivity in an IgG1-based serodiagnostic test. The spectrum of IgG1 reactivities against a panel of unique recombinant *M. paratuberculosis* antigens was also described for these sera. Further, implementation of this assay as the basis for a test-and-cull management strategy was investigated in farmed adult and juvenile deer for its ability to reduce the proportion of seroreactive animals over time. Finally, the predictive ability of the test to identify low weight productivity in juvenile deer and to detect clinical and subclinical JD in adult deer was investigated.

MATERIALS AND METHODS

Animals. Blood samples were obtained from red deer (*Cervus elaphus*) from several farms throughout the South Island of New Zealand. Samples were submitted to the Disease Research Laboratory (University of Otago, Dunedin, New Zealand); within 24 h of bleeding, serum or plasma samples were separated and stored at −20°C until they were assayed. Farms with a history of JD were identified, via ongoing records of animals that continually exhibited poor condition and weight loss and that had been confirmed to be infected with JD by gross pathology and histopathology examination of gastrointestinal tract tissues and associated lymph nodes at necropsy, and *M. paratuberculosis* infection was confirmed by bacteriological culture. Samples of serum were sourced from at least 10 farms known to have a history of *M. paratuberculosis* infection and clinical JD based on these findings. The animals from which these samples were derived were subjected to gross pathological examination, bacteriological culture, and histological examination at necropsy to confirm JD status, as described in detail below. Among these animals, 102 deer with confirmed diagnoses of *M. paratuberculosis* infection were used as the basis for the foundation study to determine preliminary estimates of diagnostic assay sensitivity. While the majority of these animals had disease confirmed by histopathology, a number of culture-positive animals (30%) with no clinical signs of disease or histopathology were included in the data set as subclinically infected representatives. In addition, serum or plasma samples of a further 508 deer, from five different properties with no prior history or ongoing evidence of *M. paratuberculosis* infection, were submitted for use as controls in the determination of diagnostic assay estimates of specificity.

Necropsy. All animals were euthanized humanely using a captive bolt gun and processed in a registered deer slaughter plant under statutory regulations. Animals were subjected to gross pathological examination, and samples of the jejunal lymph nodes and the ileocecal lymph node, as well as tissue sections of jejunum, terminal ileum, and the ileocecal valve, were taken for bacteriological culture and histological examination to confirm JD status. Tissue samples removed from the animals were fixed in 10% buffered formalin and embedded in paraffin wax. Sections were cut at 4 to 5 μm using a rotary microtome. Duplicate slides were stained using an automatic stainer (Shandon Linisatin GLX) with hematoxylin and eosin and Ziehl-Neelsen stains. Histological lesions were graded on a numerical scale of 0 to 3 using criteria outlined previously (24):

(i) Grade 1. Focal granulomata were evident within the lamina propria or excised lymphoid tissue, in the presence or absence of acid-fast organisms (AFOs).

(ii) Grade 2. Focal granulomata were evident within lymphoid tissue, with granulomata extending into the lamina propria tissues of the gastrointestinal tract, in the presence or absence of AFOs.

(iii) Grade 3. Gross lesions were evident on macroscopic examination of lymph nodes and gut tissues, with widespread inflammatory infiltrates throughout the lamina propria and submucosae; AFOs were present at one or more tissue sites.

Protein antigens. A number of commercial source and recombinant complex mycobacterial antigens were used in the diagnostic study. Paratuberculosis protoplasmic antigen (PpAg), derived from the *M. avium* subsp. *avium-M. avium* subsp. *paratuberculosis* complex, was obtained from Allied Monitor, Inc. (Fayette, MO). Purified protein derivative (Johnin PPD [PPDj]) of *M. paratuberculosis* was prepared by CIDC Lelystad (The Netherlands) from *M. paratuberculosis* (strain 85B) culture supernatant as reported previously (18), according to Office International des Epizooties standard methodology.

Methods for the production of the recombinant proteins used in this study have been described earlier (3). Briefly, a bovine clinical isolate of *M. paratuberculosis* was used as the template source for PCR amplifications. *M. avium* subsp. *paratuberculosis*-specific sequences were cloned in *Escherichia coli* and produced as maltose binding protein (MBP) fusions and polyhistidine-tagged proteins. Fusion proteins were overexpressed and purified by passage on amylose resin columns (MBP fusions) or on TALON resin columns (His-tagged proteins). The recombinant antigenic targets used in ELISAs are outlined in Table 1; these proteins were used in ELISAs at a concentration of 2 μg/ml. MBP alone was included in assays which tested recombinant MBPs. The background ELISA reactivity to MBP for individual serum samples was subtracted from the values obtained with recombinant MBPs.

ELISAs and estimated sensitivity/specificity analysis. A standard ELISA protocol described previously (31) and then modified for deer (12) was used in this study, with variations in the antigens and antibodies used, as stated specifically. Ninety-six-well microtiter plates (MaxiSorp; Nunc, Denmark) were coated with 50-μl antigen preparations diluted in carbonate buffer (pH 9.6) at a final concentration of 5 μg/ml for complex mycobacterial antigens or 2 μg/ml for recombinant antigens. After incubation overnight at 4°C, unbound antigen was removed from the plates by washing them six times in phosphate-buffered saline

TABLE 1. Summary of the recombinant *M. paratuberculosis* proteins used in this study

Coded antigen no.	Recombinant *M. paratuberculosis* protein
1	MAP0105c
2	MAP1636c
3	MAP2756c
4	MAP3437c
5	MAP2963c
6	MAP0855
7	MAP2762c
8	MAP1345
9	MAP2753
10	MAP (gene 217)
11	MAP0860c
12	MAP (gene 217)
13	MAP3817c
14	MAP2751
15	MAP0338c
16	MAP0858
17	MAP3732c
18	MAP3736c
19	MAP2154c
20	MAP2416c
21	MAP2121c (major membrane protein)
22	MAP0261c
23	MAP0904 (Csp/1)

containing 0.05% Tween 20 (wash buffer). Nonpreabsorbed test serum samples were diluted in wash buffer and added to separate wells for each antigen, incubated for 1 h at 37°C, and then washed a further six times. Unconjugated mouse monoclonal antibody specific for cervine IgG1 (9-f-98) (14) was then added and incubated for 1 h at 37°C, and unbound antibody was removed by washing six times. Antibody binding was visualized using a polyclonal goat anti-mouse IgG horseradish peroxidase-conjugated tertiary antibody (Biosource International, Camarillo, CA) and an *o*-phenylenediamine dihydrochloride substrate system, as described previously (11, 14). The reaction was stopped by the addition of H_2SO_4, and the absorbance was read at 490 nm using an automated microplate reader (model 3550; Bio-Rad). Optical densities (OD) were converted to ELISA units (EU) by subtracting the OD of known negative serum samples from the OD of the test serum and multiplying the result by 100.

Data obtained from ELISA of 102 animals with culture-confirmed JD and 508 test-negative animals from herds with no reported JD cases were entered into an online receiver-operated characteristic (ROC) analysis program (9).

RESULTS

Initial estimates of assay sensitivity and specificity. ROC curves were initially prepared to estimate specificity values for two selected antigens (PPDj and PpAg). Having established the levels of test reactivity (cut points) that could produce high levels of estimated specificity for individual antigens, we determined estimated sensitivity values for a group of infected animals using the corresponding cut points that gave estimated 100% specificity values for the uninfected animals. Using PPDj as a target antigen, the ELISA showed an estimated specificity of 100% at a cut point of 60 EU for PPDj antigen tested against serum from the 508 uninfected animals (Fig. 1A). At the equivalent cut point, the test had an estimated sensitivity of 81% for 102 *M. paratuberculosis*-infected animals. With PpAg as the target antigen, an estimated specificity of 100% was obtained at a cut point of 40 EU for PpAg (Fig. 1B). At this cut point, the estimated sensitivity was 85%.

The ELISA results obtained with PpAg and PPDj as target antigens were combined into serial test analyses (Table 2), which indicated that estimates of specificity and sensitivity could be improved using a composite assay. Table 2 data show EU values for PPDj and PpAg scaled to give different test specificities (97.0 to 100%) with composite sensitivities of 87 to 97%. Thus, these antigens can be used in combination to improve overall estimates of sensitivity at any given level of estimated specificity above 98.0%. When cut points of 40 EU for PpAg and 60 EU for PPDj (the cut points for an estimated 100% specificity) were used in this serial test, overall estimated sensitivity values of 87% were established for this set of animals. An estimated specificity of 99.5% was obtained with cut points of 24 EU for PpAg and 43 EU for PPDj. Using cut points of 50 EU for the two antigens tested serially, the estimated sensitivity was 85.3%, and the estimated specificity was 99.8%.

Recombinant protein antigens. Having determined the initial parameters of estimated sensitivity and specificity of the ELISA using complex protein antigen sources, we next wanted to investigate the responses of deer sera using recombinant proteins as antigenic targets. The series of recombinant *M. paratuberculosis* proteins outlined in Table 1 were screened against sera from 10 noninfected animals from herds with no prior history of JD and 10 *M. paratuberculosis*-infected animals from farms with a history of JD. The subset of 10 animals with confirmed *M. paratuberculosis* infection was chosen precisely because a proportion (2/10) of the animals were unreactive to both of the conventional test antigens (PPDj and PpAg) in the IgG1 ELISA. All antigens were used at a concentration of 2 μg/ml, and the results of the IgG1 ELISA are given in Fig. 2. With negative values subtracted, the results in Fig. 2 show that proteins 1, 2, and 11 were reactive in noninfected control animals and that other proteins (6, 7, 13–22) were seroreactive in *M. paratuberculosis*-infected animals and low in noninfected animals. Thus, although reactive, some recombinant proteins from *M. paratuberculosis* do not appear to be serologically specific. EU values for recombinant proteins were invariably lower than those found for complex *M. paratuberculosis* proteins (PPDj and PpAg), and most of the recombinant antigens produced low reactivity (<40 EU) in uninfected animals. In contrast, PPDj and PpAg antigens generated EU values that were >50 in 80% of *M. paratuberculosis*-infected animals.

In a diagnostic context, IgG1 ELISA sensitivity was investigated in more detail in the same subgroup of 10 known *M. paratuberculosis*-infected deer as described above (Table 3). Although the individual recombinant protein antigens provided a lower sensitivity than that of the complex proteins overall (Table 3), those culture-positive animals that were not detected using PPDj or PpAg were detected by screening their sera for IgG1 reactivity against recombinant antigen 7 (MAP2762c). This preliminary finding suggests that it may be possible to increase the diagnostic potential of an IgG1 ELISA by employing composite complex- and recombinant-antigen screening.

Sequential case studies of the potential for control of JD in a heavily infected herd via serological screening. (i) Study 1. The performance of the IgG1 ELISA was first evaluated in one deer herd, comprising 434 adult female deer (hinds) and 268 fawns (juvenile deer), from a farm with a history of significant clinical losses due to *M. paratuberculosis* infection and clinical JD in all groups of animals. Breeding hinds were sampled longitudinally over a 4-year period (2002 to 2005), and the data are shown in Fig. 3. Following each testing episode, subgroups of animals were selected randomly for confirmation of JD (data not shown) by histopathology and tissue culture. Each animal was classified as diseased, infected, or uninfected based on histopathology and microbial culture. In the initial phase of the study, a high cutoff point (>100 EU) was set for the IgG1 ELISA, and animals with higher reactivities were culled. Six months after commencement, all animals with reactivities between 50 and 100 EU (or above) were culled. The proportion of ELISA-positive reactors in the mixed-age hinds decreased from 40% in 2002 to <3% in 2005. However, as retrospective ROC analysis showed that values of 50 EU could more precisely define a positive diagnostic reaction (as described above), the initial clearance of infection from the adult stock was compromised, as some infected but nondetected animals had remained in the herd for up to 6 months after the initial testing. Reactivity in the mixed-age stags from this farm was similar to that found in the hinds, and similar clearance rates of infection were seen over the sequential sampling period (data not shown). Among the subsample of culled hinds in this study that gave IgG1 values of >50 EU, 29% showed no histopathology, although *M. paratuberculosis* was recovered from tissue samples by microbial culture (i.e., subclinical infection).

The ELISA was applied to determine if diagnostic intervention and elective culling of adult and juvenile animals would

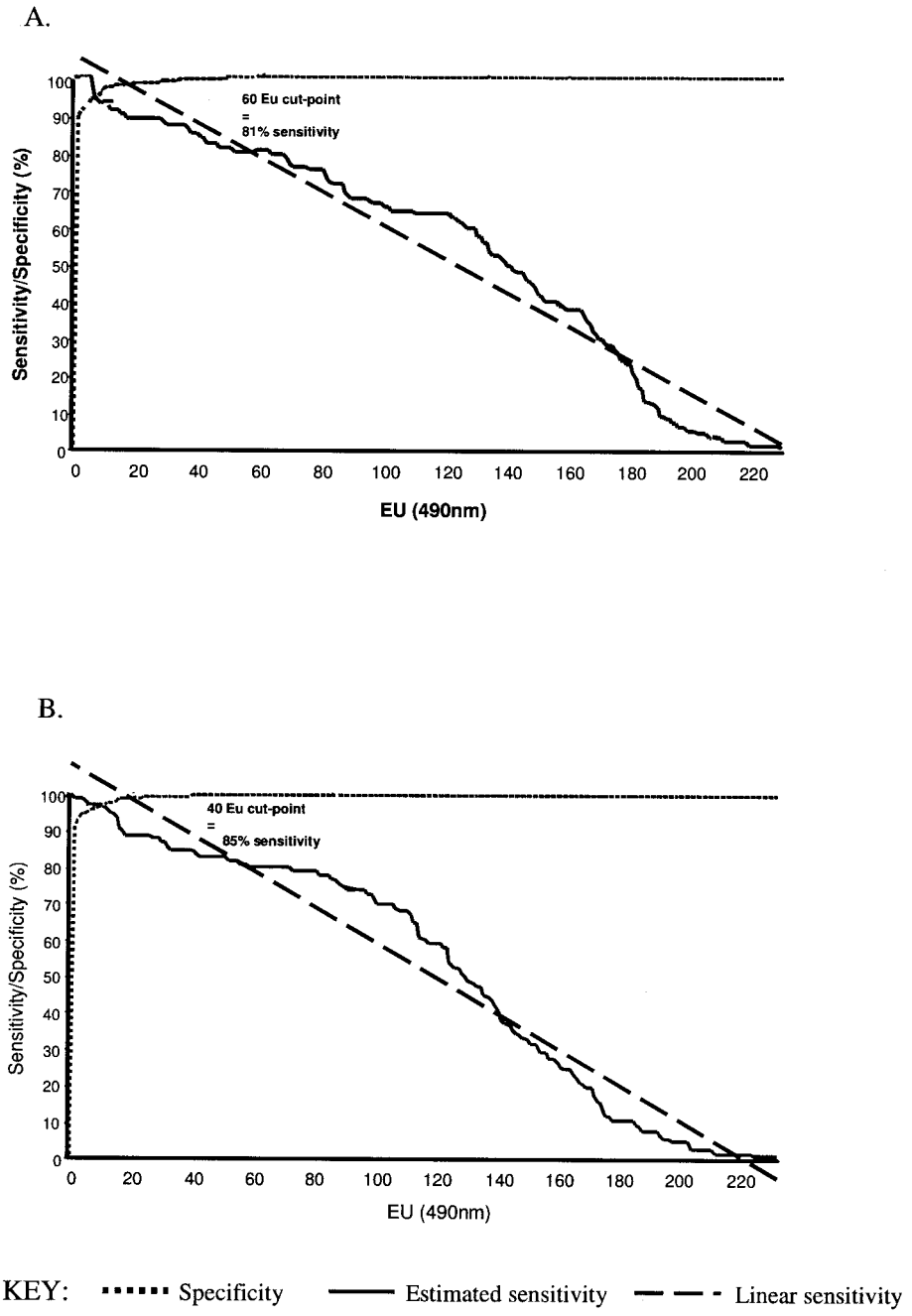

FIG. 1. ROC curve analysis of IgG1 ELISA. Estimated sensitivity and specificity values using complex *M. paratuberculosis* protein antigens. Serum samples from 102 deer with confirmed *M. paratuberculosis* infection, as well as 508 samples obtained from deer herds with no prior history of JD, were assayed by IgG1 ELISA using PPDj (A) or PpAg (B) as the target antigen. Data were entered into a ROC analysis program; numerals refer to the calculated ELISA cut points and estimated test sensitivity values corresponding to 100% estimated test specificity for each antigen.

have an impact on the incidence of clinical disease or production parameters in the young animals within the affected herd. Fawns were first tested at 6 to 8 months of age, and those that tested negative were tested again at 12 to 15 months. The outcome of this test is given in Fig. 4 and Table 4. A number of ELISA-positive animals were selected at random to confirm JD by histopathology and tissue culture (data not shown). As pathology was considered to be the definitive end point for the diagnosis of JD, routine fecal culture to establish shedding was not carried out in this study. Serological screening indicated a progressive decline in the incidence of antibody-positive fawns coincident with the implementation of the test-and-cull management regimen. Among the fawns, there was a group (>10%) that became seropositive at 12 to 15 months during the 2002 sampling period, indicating active spread of infection throughout the juvenile group. By contrast, only one seropos-

TABLE 2. Estimated specificity and sensitivity values for *M. paratuberculosis* IgG1 ELISA[a]

Stated specificity (%)	Estimated-sensitivity (%)		
	Single-antigen tests		Composite test
	PpAg	PPDj	PpAg + PPDj
100.0	85	81	87
99.5	88	84	91
99.0	90	89	94
98.5	94	89	96
98.0	96	90	97
97.5	96	92	97
97.0	96	92	97

[a] Data represent estimated assay sensitivities across a range of defined specificities for ELISA using single complex-antigen sources (PPDj or PpAg) and a composite test employing both antigens in series (data derived from ROC curves).

itive 6-month-old animal was seen in 2004, although a small proportion (4%) of the fawns converted to seropositivity at 12 to 15 months (Fig. 4). No fawns presented with clinical disease in 2004. An attempt was made to correlate test status with growth production figures and mortality. Table 4 indicates that those animals testing negative in the ELISA on two consecutive occasions (at age 6 to 8 months and again at 12 to 15 months) had the lowest death rates and the highest production figures among the juvenile group.

(ii) Study 2. The IgG1 ELISA was applied with two different deer herds (A and B) that had large cohorts (>150) of stags (adult male deer) used for velvet harvesting and that had reported significant losses due to JD. The test was used to identify animals that were seroreactive; these animals were electively slaughtered and subjected to a full histopathological evaluation postmortem. Data are presented in Tables 5 and 6.

Results in Table 5 show data for groups of adult stags from the test herds A and B. The majority (80 to 87%) of ELISA-positive animals from both herds had detectable histopathology at necropsy follow-up. Traditional use of immunodiagnostic ELISAs for *M. paratuberculosis* shows that while antibody-based tests are relatively effective (>90%) in diagnosing clinically diseased animals, they are limited in diagnosing subclinical disease or infection. In an attempt to determine how seroreactivity in deer relates to infection or disease severity, test performance was further evaluated in a series of 250 deer (mixed age/sex) obtained from more than 10 infected herds. Cohorts of animals, whether ELISA positive or negative, were necropsied, and their tissues were examined by microbiological culture and histopathology. In this data set, 100 animals were lesion negative (but culture positive), and 150 animals were lesion positive; 75 of these lesion-positive animals (histopathologically scores, 1, 2, or 3) were confirmed by tissue culture as positive for *M. paratuberculosis*. The performance values for the ELISA are given in Table 6. The results indicate that while animals with detectable pathology were diagnosed with a high degree of estimated sensitivity (>90%), animals with no detectable pathology but which were culture positive for *M. paratuberculosis* were diagnosed with a lesser (but still relatively high) degree of estimated sensitivity (>75%).

DISCUSSION

Whereas JD in domestic cattle and sheep has been well documented for more than a century, it has been recognized only more recently as an emerging economic problem in farmed deer (10, 20, 21). In New Zealand, there has been an increase in the incidence of JD in farmed deer in recent years (21), and it is estimated that the prevalence of the disease in New Zealand farmed deer herds is around 5% (8). Current methods of JD diagnosis are inadequate. Cultivation of *M. paratuberculosis* from fecal samples is presently the most precise test; however, this is costly and can take several weeks, limiting its use as a practical diagnostic tool. Further, a recent report has indicated that while individual fecal culture can provide a diagnostic sensitivity of 67.5% in farmed deer in toto

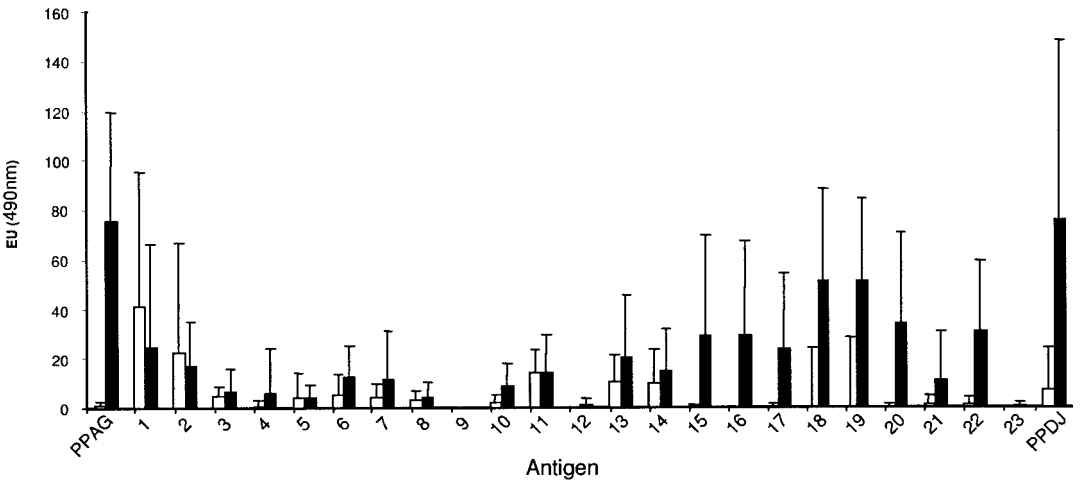

FIG. 2. IgG1 antibody responses in *M. paratuberculosis*-infected and noninfected deer against a panel of recombinant *M. paratuberculosis* antigens. Sera from 10 *M. paratuberculosis*-infected deer (black bars) and 10 noninfected deer (white bars) were screened by IgG1 ELISA against a range of recombinant *M. paratuberculosis* antigens. Responses against complex protein antigens (PpAg and PPDj) are shown for comparison. Data represent mean EU ± standard errors of the mean.

TABLE 3. Effect of using single or combined complex and recombinant antigens on the diagnostic sensitivity of IgG1 ELISAs in *M. paratuberculosis*-infected deer[a]

Infected animal	Mean EU value using:										
	Complex antigens		Recombinant antigens								
	PPDj	PpAg	3	4	7	10	15	16	17	20	21
1	**210**	**145**	0	0	0	**23**	**67**	**76**	**71**	**65**	**63**
2	**86**	**102**	0	0	0	0	**75**	**84**	**81**	**64**	0
3	**54**	43	4	0	0	0	**27**	**26**	**39**	**37**	0
4	**70**	**79**	4	6	17	6	0	0	0	0	11
5	**120**	**135**	14	**57**	0	11	0	0	0	0	0
6	**173**	**56**	2	0	2	**24**	0	0	0	0	14
7	7	20	**30**	0	**46**	17	0	0	2	**59**	0
8	32	**64**	0	0	0	4	3	0	12	0	0
9	0	**94**	0	0	0	0	**112**	**90**	10	**103**	0
10	8	15	11	0	**49**	2	5	13	**24**	13	19

[a] IgG1 ELISAs were undertaken using sera from 10 known *M. paratuberculosis*-infected deer, with screening of reactivity against complex (PPDj, PpAg) or recombinant *M. paratuberculosis* antigens. Data represent mean EU values for each assay. Data in bold indicate cases predicted to be positive by the diagnostic assay (using cut points of 50 EU for complex antigen sources and 20 EU for recombinant antigens). Estimated sensitivity values were generally low for the recombinant antigens, except for antigen 7 (MAP2762c), which detected *M. paratuberculosis*-infected animals 7 and 10 that had been missed using the complex-antigen sources.

(25), the concurrent sensitivity for individual fecal culture is only 47% among deer that have been diagnosed as culture positive for *M. paratuberculosis* from homogenized gut tissue specimens at necropsy. This indicates that animals may harbor *M. paratuberculosis* infection in enteric tissues without detectable fecal shedding, limiting the diagnostic potential of fecal culture as an indicator of subclinical infection. Serological screening offers the potential for rapid and effective individual-level screening of *M. paratuberculosis* status, with a view to implementing a test-and-cull management strategy similar to that used to control bovine tuberculosis in domestic livestock. Estimated sensitivity values of 80% have been reported for an absorbed ELISA in a small sample of free-ranging Tule elk in California (22), with most animals showing no evidence of clinical JD.

Considering the close phylogenetic relatedness between mycobacteria of the MAIC and *M. paratuberculosis*, one particular aim of the current project was to determine if test parameters of antigen-specific tests that had acceptable levels of specificity for routine *M. paratuberculosis* diagnosis in deer could be developed. Preliminary results indicated a high degree of serological reactivity against complex *M. paratuberculosis*, PPDj, and PpAg. The present study focused on the seroresponsiveness of the IgG1 isotype antibody to these antigens in deer, based on our previous experience with serodiagnostics for *Mycobacterium bovis* tuberculosis in deer, which has identified IgG1 as a sensitive indicator of *M. bovis* infection and tuberculosis disease status (5, 14). Subsequent implementation of this test with large groups of confirmed *M. paratuberculosis*-infected and noninfected control deer in the present study provided evidence that the most effective cut point would be 50 EU, delivering an estimated specificity of 99.5% and estimated sensitivities of 88.0% with PPDj antigen and 84.0% with PpAg. Currently, the only ELISA-based methodologies for JD diagnosis in livestock involve preabsorbing test serum with a sap-

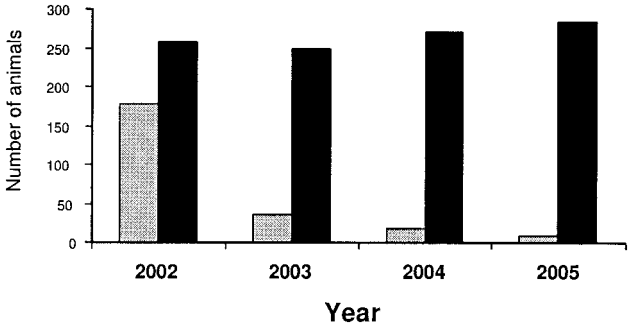

FIG. 3. Longitudinal changes in the proportion of test-positive and test-negative adult deer over a 4-year period following implementation of an IgG1-based ELISA test-and-cull management strategy. Regular serological screening for *M. paratuberculosis* infection was undertaken on a deer herd with a known history of JD. Screening commenced in 2002 (with an initial cut point of 100 EU) and proceeded over a 4-year period (with the cut point dropping to 50 EU after 6 months); test-positive animals were culled from the herd upon testing positive. Data represent longitudinal changes in the number of test-positive animals (gray bars) against test-negative animals (black bars) (data are shown for adult hinds only; data for stags not shown).

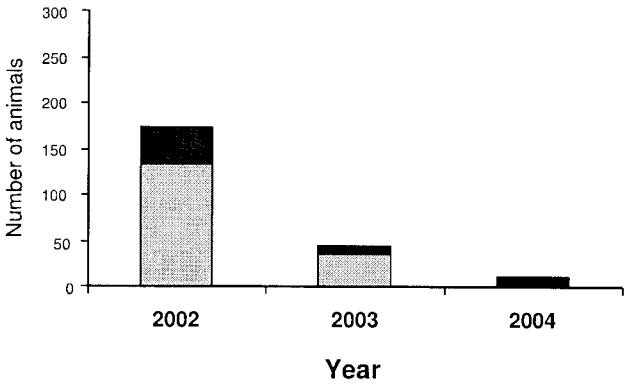

FIG. 4. Longitudinal changes in the number of IgG1 ELISA-positive young deer following implementation of an ELISA-based test-and-cull management strategy. Young deer (fawns) were screened by IgG1 ELISA to detect *M. paratuberculosis* infection at two ages, 6 to 8 months (gray bars) and 12 to 15 months (black bars). Data represent the number of fawns testing positive at each time point, following the implementation of a test-and-cull management strategy in 2002.

TABLE 4. Production values and death rates in young deer associated with IgG1 ELISA status[a]

Total no. of deer	IgG1 test result (age)	Death rate (%)	Mean body wt at 15 mo (kg)	% of Animals with wt of >95 kg at 15 mo
201	Neg (6–8 mo); neg (12–15 mo)	3	104	93
39	Neg (6–8 mo); pos (12–15 mo)	8	100	77
76	Pos (all)	43	90.3	30

[a] Young deer (fawns) were screened by IgG1 ELISA to detect *M. paratuberculosis* infection at two ages, 6 to 8 months and 12 to 15 months. Subsequent production characteristics and death rates in these animals were recorded and matched against the diagnostic status of animals at the two time points. Pos, ELISA positive; neg, ELISA negative.

rophytic mycobacterium (*Mycobacterium phlei*) prior to testing against mixed *M. paratuberculosis* antigens. Evaluation of the effectiveness of an absorbed ELISA for detection of *M. paratuberculosis* in sheep has been reported to provide an estimated specificity between 99% and 95%, with corresponding sensitivity values between 21.9% and 41.5% (26). Sensitivity was shown to vary between flocks with histological lesion type and condition score. An absorbed ELISA adapted for use with cattle serum showed a sensitivity of 64% in infected cattle over 3 years of age and only 38% for infected animals less than 2 years of age (17). The manifestation of JD in cattle and sheep typically takes several years of subclinical infection before clinical signs present. The faster and more florid onset of disease in deer could mean that antibody-based assays may prove more effective and be more sensitive for disease detection in younger animals.

In the present study, when the two antigens (PPDj and PpAg) were used in series in the IgG1 ELISA at cut points that gave an estimated 99.5% specificity, an estimated composite sensitivity (91%) was achieved that improved the diagnostic performance of the IgG1 ELISA more than that for either antigen alone. Because the two antigens used in combination provided results that gave superior estimated sensitivity, there is the question of whether a third antigen added to the composite test could further improve sensitivity. Studies were carried out to determine if recombinant proteins—specific to a gene region unique in the *M. paratuberculosis* genome (3)—could be used to increase the estimated test sensitivity without compromising specificity. A similar genome-based antigen identification approach has been tested previously (30) and used to develop a recombinant *M. paratuberculosis*-specific antigen-based ELISA for cattle, utilizing the recombinant polypeptide a362 from the immunodominant 34-kDa protein of the A36 complex as a target. The 34-kDa protein contains B-cell epitopes to all tested mycobacteria, but the a362 polypeptide is specific to *M. paratuberculosis*. The ELISA developed by Vannuffel et al. (30) in the mid-1990s had a reported estimated sensitivity of 70% and a specificity of 95% for *M. paratuberculosis* diagnosis in cattle, considerably more precise than the contemporary commercial ELISAs of that time. In the present study, only a relatively small number of recombinant proteins were shown to be seroreactive with deer IgG1. However, when used in combination with PpAg and PPDj in IgG1 screening, antigen 7 (MAP2762c) appeared to be complementary and capable of detecting *M. paratuberculosis*-positive animals that had been missed using PPDj or PpAg. MAP2762c represents a protein in the *M. paratuberculosis* genome for which there are no matches to other proteins. The adjacent gene to MAP2762c codes for a protein (MAP2761c) with strong homology to proteins found in both *M. bovis* and *Mycobacterium tuberculosis* that are in the PPE gene family of lipid-rich proteins found in mycobacteria. Evidence suggests that these proteins are associated with the bacterial cell's surface and provide diverse antigenic profiles that affect immunity (32). Whether the proximity of MAP2762c to this PPE protein homolog is significant is unknown, but with this data set of animals, MAP2762c appeared to offer promise for serial diagnostic tests, run in conjunction with the complex antigens PPDj and PpAg, to improve the estimated sensitivity of an IgG1-based ELISA for JD in deer. While the performance characteristics of the assay look promising, it is important to recognize that the values obtained are estimates of sensitivity and specificity. No study was carried out in which all animals, irrespective of their test status, were necropsied with microbiological and histological follow-up; therefore, the figures given here are likely to be higher than those expected when the test is used under field conditions.

The value of IgG1-based serodiagnosis in the practical management of JD was investigated in two case studies involving

TABLE 5. Lesion rate in adult stags selected as seropositive by IgG1 ELISA in two deer herds[a]

Herd	No. of animals tested	No. of seroreactive IgG1 ELISA-positive animals	% of ELISA-positive animals with lesions at necropsy (%)
A	495	50	87
B	151	60	80

[a] Incidence of IgG1 test positivity was cross-tabulated with incidence of *M. paratuberculosis* lesions in stags from two separate *M. paratuberculosis*-infected deer herds (A and B).

TABLE 6. Relationship between the immunodiagnostic performance of the IgG1 ELISA and disease severity[a]

Test result	Histonegative, culture-positive animals	Result for lesion-positive animals of histopathology grade:		
		1	2	3
No. of seropositive animals/total no. of animals	77/100	39/43	37/40	67/67
Estimated sensitivity of ELISA (%)	77	91	93	100

[a] ELISA performance was evaluated in 250 mixed age/sex deer from 10 farms with prior and ongoing history of JD to determine how seroreactivity relates to infection or disease severity. Animals were subgrouped into histopathology-negative, culture-positive and histopathology positive animals (graded 1 to 3 in order of increasing severity).

on-farm observations of New Zealand deer herds with recurrent high incidences of JD among their stock. In study 1, a conservative cut point of 100 EU was initially employed to screen and identify seroreactivity among adult deer and to selectively remove those animals testing positive. Following ROC analysis of the ELISA database, the cut point designating a positive reaction was reduced to 50 EU. Culling all animals with reactivities of >50 EU for either PPDj or PpAg produced a progressive reduction in mortality, disease severity, and reactor rates among both adult and juvenile deer over an ensuing 4-year period. The level of seroreactivity was negatively correlated with performance indicators in juvenile animals, where deer that tested positive in the IgG1 ELISA showed a greatly increased death rate and decreased production, demonstrating that the screening had been effective in limiting the impact of JD in that herd. This result provides preliminary evidence that, if properly implemented and combined with appropriate farm management practices, an IgG1 ELISA could provide a useful ancillary tool to curb the spread of JD in deer and reduce the economic losses that accompany it. There is a justification for testing juvenile animals first at 6 to 8 months and again at 12 to 15 months of age to maximize disease control in seriously affected herds with clinical losses in young deer. The earlier observation that ELISA has the potential to diagnose infection in deer without any evidence of clinical JD (22) implies that *Cervidae* may provide more-readily detectable seroreactivity levels than the lower levels found in cattle (6). Thus, the practical on-farm implementation of an IgG1-based serodiagnostic test for JD in deer might include the scheduled screening of juvenile animals as they become susceptible to *M. paratuberculosis* infection.

The second case study aimed to identify serodiagnostic correlates of infection and disease in two *M. paratuberculosis*-infected deer herds. The majority of ELISA-positive deer proved to have JD lesions at necropsy, and it was noteworthy that the more advanced cases (histopathology score, 2 or 3) comprised animals for which the ELISA showed the highest estimated diagnostic sensitivity, suggesting that ELISA is extremely effective in detecting chronic disease. However, the IgG1-based ELISA also had an estimated sensitivity of 77% for those *M. paratuberculosis*-infected animals that were shown to be free of lesions. Early diagnosis and removal of subclinical cases are important components of successful test-and-slaughter management for chronic infections (4) and have formed the mainstay of tuberculosis control programs in North America, the British Isles, and New Zealand for several years. IgG1 antibodies generally represent a polarized T-helper-cell 2 (Th2)-biased humoral immune response, and we have previously reported that Th2-biased immune reactivity during mycobacterial infection in deer strongly denotes a disease-related immune response (13, 16); however, it is possible that further development and refinement of the IgG1-based ELISA might increase the sensitivity of the assay for the diagnosis of subclinical *M. paratuberculosis* infection. Collins et al. (6) have recently highlighted the need for rapid, more-precise, and cost-effective JD screening tools in livestock management as a practical means of controlling disease spread, especially if regulatory conditions change and *M. paratuberculosis* is reclassified as a notifiable zoonotic disease. Ongoing research in our laboratories is currently directed to fulfill that aim.

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