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Sensitive and Specific Enzyme-Linked Immunosorbent Assay for Detecting Serum Antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in Fallow Deer

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The enzyme-linked immunosorbent assay (ELISA) is the diagnostic test most commonly used in efforts to control paratuberculosis in domestic ruminants. However, commercial ELISAs have not been validated for detecting antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in wild animals. In this study, we compared the sensitivities and specificities of five ELISAs using individual serum samples collected from 41 fallow deer with or without histopathological lesions consistent with paratuberculosis. Two target antigenic preparations were selected, an ethanol-treated protoplasmic preparation obtained from a fallow deer *M. avium* subsp. *paratuberculosis* isolate (ELISAs A and B) and a paratuberculosis protoplasmic antigen (PPA3) (ELISAs C and D). Fallow deer antibodies bound to the immobilized antigens were detected by using a horseradish peroxidase (HRP)-conjugated anti-fallow deer IgG antibody (ELISAs A and C) or HRP-conjugated protein G (ELISAs B and D). A commercially available assay, ELISA-E, which was designed to detect *M. avium* subsp. *paratuberculosis* antibodies in cattle, sheep, and goats, was also tested. Although ELISAs A, C, and E had the same sensitivity (72%), ELISAs A and C were more specific (100%) for detecting fallow deer with lesions consistent with paratuberculosis at necropsy than was the ELISA-E (87.5%). In addition, the ELISA-A was particularly sensitive for detecting fallow deer in the latent stages of infection (62.5%). The antibody responses detected with the ELISA-A correlated with both the severity of enteric lesions and the presence of acid-fast bacteria in gut tissue samples. In summary, our study shows that the ELISA-A can be a cost-effective diagnostic tool for preventing the spread of paratuberculosis among fallow deer populations.

Mycobacterium avium subsp. *paratuberculosis* is the causative agent of a slow and progressive granulomatous enteritis and lymphadenitis in ruminants, named Johne's disease (JD), or paratuberculosis. *M. avium* subsp. *paratuberculosis* has also been implicated as a causal or exacerbating agent of Crohn's disease, a chronic inflammatory bowel disease of humans characterized by transmural inflammation and granuloma formation (1–3). In addition, some recent reports suggested a link between *M. avium* subsp. *paratuberculosis* infection and type 1 diabetes (4, 5). *M. avium* subsp. *paratuberculosis* is responsible for considerable economic losses to the dairy and livestock industries worldwide because of a reduction in milk production, premature culling of infected animals, and increased replacement rates (6). Approximately 22% of all dairy herds and 8% of all beef herds in the United States have JD, causing an annual loss of >$200 million to the dairy industry alone (7). *M. avium* subsp. *paratuberculosis* has also been detected in a wide range of wild ruminants, including cervids, making JD eradication particularly difficult (8). The fallow deer (*Dama dama*) is a gregarious cervid and one of the world's most widely naturalized animals. Fallow deer are found not only in Eurasia but also in North and South America as farmed animals, in game parks, and often as feral populations (9). In Spain, *M. avium* subsp. *paratuberculosis* was first diagnosed in fallow deer in a free-ranging population in Asturias, northern Spain, where paratuberculosis appears to be endemic in fallow deer and in cattle (10–12). This fallow deer population shares pastures and waterholes with cattle, horse, sheep, and goats, and as a consequence, it is possible that domestic ruminants were the source of infection for this population (13).

The control of JD has proven to be difficult due to the nature of *M. avium* subsp. *paratuberculosis* infection. Animals become infected by ingesting *M. avium* subsp. *paratuberculosis*-contaminated milk, colostrum, feed materials, or water (14). Even when infected, several years are required before domestic ruminants show signs of disease and shed bacteria in their feces. Consequently, the majority of *M. avium* subsp. *paratuberculosis* infections are unnoticed and undiagnosed. However, severe progressive cases of paratuberculosis have been described in young (1- to 2-year-old) captive fallow deer, so it appears that this clinical disease is more common in young wild ruminants than in young cattle (8). Fast, accurate, easy-to-perform, and cost-effective JD screening tools in fallow deer are needed as practical means of controlling disease spread, especially if regulation conditions change and *M. avium* subsp. *paratuberculosis* is reclassified as a notifiable zoonotic agent. The fecal culture test is recognized as the gold standard antemortem test for diagnosing JD in domestic ru-

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minants, but it is costly, unable to detect subclinical infections, and requires up to 16 weeks to complete; also, like the PCR tests, it cannot distinguish between pass-through and colonizing bacteria (15, 16). For surveillance of cervids for JD, antibody-based diagnostic tests are preferred over fecal culture because deer are handled only once, assays can be performed for antibodies to many pathogens, and immediate processing of the sample is not required (17–19). However, current commercial serological tests for detecting *M. avium* subsp. *paratuberculosis* antibodies in domestic ruminant species are reported to be less sensitive than fecal culture and have not been validated for use with sera from all cervid species (20). Consequently, a specific ELISA for the rapid serodiagnosis of *M. avium* subsp. *paratuberculosis* infection in fallow deer is not available. Therefore, the aim of the current study was to compare the accuracy of a commercially available ELISA and four in-house ELISAs for diagnosing JD in fallow deer. The in-house assays used two different antigenic preparations and two different secondary antibodies. The specificity and sensitivity of each ELISA were estimated using serum samples from known disease-free fallow deer and further samples from fallow deer that had been confirmed to be infected with JD by histopathological examination of gastrointestinal tract tissues and associated lymph nodes at necropsy. The predictive ability of each ELISA to identify adult fallow deer in different stages of infection was investigated. Finally, the ELISA results were compared to the results from acid-fast staining, quantitative PCR (qPCR), and bacteriological culture.

MATERIALS AND METHODS

Ethics statement. All samples were taken from legally hunted fallow deer. The sampling procedures were approved by the Animal Ethics Committee of the Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA) (permit 082/2005) and by the Department of Environmental Affairs, Government of the Principality of Asturias, Spain.

Animals. The target population was a free-ranging fallow deer population in the unfenced public-owned hunting preserve of El Sueve, Asturias, Spain. Fallow deer were introduced into this region in 1960, and in 2006, the population was estimated to be approximately 500. The estimated prevalence of fallow deer with paratuberculosis-associated histopathological lesions in this population was estimated to be 29% (11). Our study group included 25 adult fallow deer legally hunted from 2004 through 2007 and with *M. avium* subsp. *paratuberculosis* infection confirmed by histopathology and immunohistochemistry. In addition, a further 16 adult fallow deer without histopathological lesions were included in the data set. Blood and gut tissue sampling was carried out after hunting by trained veterinarians. Blood samples were collected from the chest cavity immediately after the fallow deer were shot. The samples were allowed to coagulate and were centrifuged and stored at −20°C until they were assayed.

Histopathological analysis. Tissue sections of proximal and distal jejunum, proximal and distal ileum, and samples of the ileocecal valve and the jejunal and ileocecal lymph nodes were collected from the 41 hunted fallow deer at necropsy and processed for bacteriological culture and histopathological examination. The tissue samples removed from the animals were fixed in 10% neutral buffered formalin and dehydrated through graded alcohols and xilol before being embedded in paraffin wax. Several sections were cut from each tissue sample at 4 μm using a microtome and subsequently stained with hematoxylin and eosin (HE) and Ziehl-Neelsen (ZN) stains. The stained sections were examined by light microscopy for pathological lesions and for the presence of acid-fast bacteria (AFB). According to their location and extension, the histopathological lesions were classified into focal, multifocal, and diffuse categories (11). The focal lesions consisted of small granulomas, mainly located in the jejunal and ileal lymph nodes. The multifocal lesions consisted of well-demarcated granulomas in the intestinal lymphoid tissue and also in the intestinal lamina propria. The diffuse lesions were characterized by severe granulomatous enteritis and lymphadenitis. According to the cells present in the infiltrate and the amount of AFB, diffuse lesions were subdivided into diffuse intermediate and diffuse multibacillary lesions. Diffuse intermediate lesions consisted of large numbers of lymphocytes and macrophages with small numbers of AFB. Langhans giant cells were present but always in lower numbers than the diffuse multibacillary lesions. Large numbers of AFB were detected in the diffuse multibacillary lesions, and a granulomatous infiltrate consisting of epithelioid cells, lymphocytes, macrophages, and numerous Langhans giant cells was observed. The four types of histopathological lesions were further grouped into one of two categories using epidemiological criteria outlined previously, latent forms (focal lesions) and patent forms (multifocal and diffuse lesions) (21).

***M. avium* subsp. *paratuberculosis* detection from paraffin-embedded tissues by real-time quantitative PCR.** The isolation of genomic DNA from fixed paraffin-embedded samples was performed using the SpeedTools tissue DNA extraction kit, according to the manufacturer's instructions (Biotools, Madrid, Spain). Real-time qPCR to detect the single-copy F57 insertion sequence of *M. avium* subsp. *paratuberculosis* was performed in duplicate from 150 ng of purified DNA using the ParaTB Kuanti-VK kit, according to the manufacturer's instructions (Vacunek, Bizkaia, Spain). According to the manufacturer, this test has 100% specificity and sensitivity for detecting *M. avium* subsp. *paratuberculosis* and did not yield any false-positive signals on several non-*M. avium* subsp. *paratuberculosis* bacterial species tested. The kit uses a F57 TaqMan probe labeled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5′ end and primers that specifically amplify the F57 *M. avium* subsp. *paratuberculosis*-specific insertion sequence. Inhibition of the amplification reaction is ruled out by including an internal hybridization probe labeled with 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxy fluorescein succinimidyl ester (JOE) at the 5′ end and specific primers. This internal amplification control molecule is coamplified alongside the F57 diagnostic target in a duplex format. Quantification of the *M. avium* subsp. *paratuberculosis* titer was accomplished by preparing a standard curve using serial dilutions of bacteria. Real-time qPCR amplifications were performed using the ABI Prism 7500 detection system (Applied Biosystems, Carlsbad, CA), with the following conditions: 1 cycle of denaturation at 95°C for 10 min, 45 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 60 s. The results were analyzed with the ABI Prism software version 1.4. Only the samples with a typical amplification curve, a curve with a linear growing part, followed by plateau, and with threshold cycle (C_T) values of <38 were considered positive.

Bacteriological culture. A pool of gut samples (2 g) was decontaminated with 38 ml of hexadecylpyridinium chloride at a final concentration of 0.75% (Sigma, St. Louis, MO) and homogenized in a stomacher blender. After 30 min of incubation at room temperature, 15 ml of the suspension was transferred to a new tube and incubated overnight for decontamination and sedimentation. Approximately 200 μl of the suspension was taken from the layer of the suspension near the sediment and inoculated in duplicate into two slants of Herrold's egg yolk medium (HEYM) (Becton, Dickinson, and Company, Sparks, MD) and into two slants of Lowenstein-Jensen (LJ) medium (Difco, Detroit, MI), both supplemented with 2 mg/liter of Mycobactin J (Allied Monitor, Fayette, MO), as previously described (22). The tubes were incubated at 37°C in a slanted position with loose caps to allow the surface of the medium to dry. After 1 week, the caps on the slants were tightened and the cultures were observed every 4 weeks and considered negative if no bacterial growth was observed after 20 weeks. The samples were considered positive if one or more colonies with a morphology typical of *M. avium* subsp. *paratuberculosis* was observed in one or more culture tubes. Positive colonies were confirmed by PCR amplification of the IS*900* *M. avium* subsp. *paratuberculosis* insertion sequence, as described previously (23).

***M. avium* subsp. *paratuberculosis* antigenic preparations.** Two antigenic preparations were tested in the ELISA screening: an ethanol-

treated protoplasmic preparation obtained from a fallow deer *M. avium* subsp. *paratuberculosis* isolate (A7) or a commercially available paratuberculosis protoplasmic antigen (PPA3) (Allied Monitor). For the production of A7, the SU-89 isolate of *M. avium* subsp. *paratuberculosis* was cultured in Middlebrook 7H9 medium (Becton, Dickinson and Company) supplemented with 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC) (Becton, Dickinson and Company) and 2 mg/liter of Mycobactin J (Allied Monitor). The culture was maintained at 37°C without shaking until it reached an optical density (OD) at 600 nm of 0.7. The bacterial cells were then centrifuged at 3,000 × *g* for 15 min. The pellet was then washed twice with phosphate-buffered saline (PBS), resuspended in 2 ml of PBS, and subjected to 6 cycles of freeze-thawing, followed by sonication in a Branson Digital Sonifier cell disruptor. The bacterial lysates were then centrifuged at 35,000 × *g* for 20 min at 4°C in a 3K30 centrifuge to remove whole bacterial cells and cell walls (Sigma-Aldrich, St. Louis, MO). The supernatant protoplasm was collected and incubated overnight at 4°C with two volumes of 80% ethanol. After centrifugation at 12,740 × *g* for 30 min at 4°C, the resultant protoplasmic extract was resuspended in 5 ml of phosphate-buffered saline (PBS) (pH, 7.2) and used to coat the corresponding ELISA plates.

Conjugates. Fallow deer IgG antibodies bound to the immobilized antigens were detected by using a horseradish peroxidase (HRP)-conjugated protein G that binds IgGs from a wide range of mammalian species (Thermo Scientific, Pierce Biotechnology, Rockford, IL) or an HRP-conjugated anti-fallow deer IgG polyclonal antibody. For preparation of the anti-fallow deer IgG antibody, fallow deer IgG was purified from a pool of serum samples collected from four animals using the Melon gel IgG spin purification kit, according to the manufacturer's instructions (Thermo Scientific). This kit uses a spin column procedure for IgG purification to remove nonrelevant proteins, such as albumin and transferrin, which are often present in serum samples in high abundance. The purified IgG was quantified using the Bradford Reagent (Sigma-Aldrich), adjusted to a concentration of 1 mg/ml by adding PBS, and then used to immunize two New Zealand White rabbits. The immunization protocol consisted of 4 intramuscular injections at days 7, 14, 28, and 35 using 0.5 mg of purified fallow deer IgG emulsified in Freund's incomplete adjuvant (Sigma-Aldrich). The last boost on day 42 was intravenous and without Freund's adjuvant. Each immunized rabbit was bled on day 50, and its blood was used for the purification of the anti-fallow deer IgG antibody by protein A-Sepharose chromatography using HiTrap affinity columns (GE Healthcare, Uppsala, Sweden). Purified anti-IgG antibody (5 mg) was conjugated with 5 mg of HRP, in accordance with the method of Tijssen and Kurstak (24).

ELISAs. ELISAs A and B both employed as a coating antigen an ethanol-treated protoplasmic preparation from a fallow deer *M. avium* subsp. *paratuberculosis* isolate (A7) and the anti-fallow deer IgG antibody or protein G as a conjugate, respectively. ELISAs C and D used commercial PPA3 and the anti-fallow deer IgG antibody or protein G, respectively. Fallow deer serum samples were also tested by a commercially indirect ELISA (ELISA-E), according to the manufacturer's instructions (ID.vet Innovative Diagnostic, Montpelier, France). The commercially available ELISA-E uses a purified *M. avium* subsp. *paratuberculosis* extract as a coating antigen and anti-ruminant IgG conjugate validated for detecting antibodies against *M. avium* subsp. *paratuberculosis* in cattle, sheep, and goats. In order to reduce cross-reactivity with environmental mycobacteria, cross-reactive antibodies were absorbed by mixing 100 μl of each individual fallow deer serum sample with 100 μl of a *Mycobacterium phlei* saline suspension (5 g/liter) (Allied Monitor) for 18 h at 4°C. The optimal concentrations of serum samples and HRP-conjugated anti-fallow deer IgG polyclonal antibody were determined by performing a preliminary checkerboard ELISA using serum samples from three animals with a positive or negative result by histopathology. Once the optimum concentrations of target serum and conjugate were determined, serum samples from the 41 fallow deer included in the study were analyzed with ELISAs A, B, C, D, and E.

ELISAs A, B, C, and D were performed in duplicate in 96-well microtiter plates (Corning, NY) coated with 100 μl of A7 or PPA3 diluted in 0.1 M sodium carbonate-bicarbonate coating buffer (pH, 9.6) (Sigma-Aldrich) at a final concentration of 5 μg/ml. After incubation for 18 h at 4°C, unbound antigen was removed by washing each well five times with 200 μl of PBS with 0.05% Tween 20 (PBS-T). The plates were then blocked for 1 h at room temperature with blocking solution consisting of 1% yeast extract in PBS-T. The plates were then washed three times with PBS-T, and 100 μl of each of the 41 preabsorbed test serum samples diluted 20 times in blocking solution (vol/vol) was added into separate wells for each antigenic preparation. After 1 h of incubation at 37°C, the plates were washed five times with 200 μl of PBS-T. Each well was then inoculated with 100 μl of HRP-conjugated protein G or HRP-conjugated rabbit anti-fallow deer IgG polyclonal antibody, both diluted 1:1,500 in blocking solution and incubated at 37°C for 1 h. Unbound conjugate was removed after five washes with 200 μl of PBS-T, and then antibody binding was visualized by adding 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate to each well. The plates were incubated for 10 min at room temperature (RT) in the dark, and the reactions were stopped by adding 50 μl of 3 N H_2SO_4 per well. The optical density (OD) in each well was measured at 450 nm by an ELISA plate reader (model 680; Bio-Rad, Hercules, CA).

Data analysis. Five antibody detection-based ELISAs were compared for their ability to detect *M. avium* subsp. *paratuberculosis*-specific antibodies in fallow deer with histopathological lesions compatible with JD and with a positive immunostaining result. Although the sensitivity estimates reported in the present study are relative sensitivity estimates, i.e., are relative to the histopathology results, we use the term sensitivity throughout the study. The OD values obtained with 25 *M. avium* subsp. *paratuberculosis*-positive and 16 negative serum samples were subjected to four-graph receiver operating characteristic (FG-ROC) analysis, which is a plot of the test sensitivity (Se) and specificity (Sp) for each threshold (cutoff) value (25). The plot also calculates the semisum of sensitivity and specificity ([Se + Sp]/200), or diagnostic value, the ratio of specificity to sensitivity (specificity discrimination index [SpDI]), and the ratio of sensitivity to specificity (sensitivity discrimination index [SeDI]), along with the 95% confidence intervals. The cutoff values that yielded the highest diagnostic value were selected as the optimal cutoff values for each ELISA. The selected cutoff values were located in a region in which small changes in its numerical value did not substantially change the semisum of sensitivity and specificity.

Differences in the mean ODs of the serum samples collected from the infected and noninfected fallow deer were statistically analyzed with the least square of means (LSMEANS) statement using Student's *t* test with the Tukey-Kramer adjustment for multiple comparisons (SAS Proc General linear model [GLM] SAS procedure; SAS Institute, Inc., Cary, NC).

The overall agreement for categorical assay interpretation (positive or negative) between pairs of diagnostic assays was evaluated with the agree option of the TABLES statement in the SAS Proc FREQ (SAS Institute, Inc.). The coefficient of agreement (kappa) was interpreted as follows: κ of 0.00 to 0.20, poor; κ of 0.21 to 0.40, fair; κ of 0.41 to 0.60, moderate; κ of 0.61 to 0.80, good; and κ of 0.81 to 1.00, excellent. The Fisher option of the TABLES statement in SAS Proc FREQ was used to compare the frequencies of categorical variables, such as the presence or absence of JD-associated lesions, the presence or absence of latent and patent lesions, ZN-positive versus ZN-negative staining results, qPCR-positive versus qPCR-negative results, and culture-positive versus culture-negative results. For all analyses, differences were considered significant at a P value of <0.05.

The current study is in accordance with the Standards for Reporting of Animal Diagnostic Accuracy Studies for paratuberculosis (STRADAS-paraTB) guidelines for improving the quality of reporting test accuracy studies for paratuberculosis (26).

RESULTS

Histopathological findings and *M. paratuberculosis* detection in gut tissues by bacteriological culture and qPCR. Histopatho-

TABLE 1 Histopathological findings, bacteriological culture, Ziehl-Neelsen (ZN) staining, and qPCR of gut tissues collected from 41 hunted fallow deer

Animal	Lesion type	ZN stain result	qPCR[a]	Culture result	ELISA OD results[b]				
					A	B	C	D	E
89	Diffuse multibacillary	Positive	9.41	Positive	**2.777**	**2.152**	**1.353**	**0.389**	**0.509**
108	Diffuse multibacillary	Positive	0	Negative	**1.987**	**0.520**	**0.572**	0.094	0.209
138	Diffuse multibacillary	Positive	17,753.2	Negative	**2.395**	**0.640**	**1.659**	**0.402**	**2.316**
87	Diffuse multibacillary	Positive	3.64	Negative	**2.160**	**1.189**	**0.783**	**0.319**	0.231
92	Diffuse intermediate	Positive	2.91	Negative	**2.807**	**1.766**	**2.552**	**1.237**	**0.428**
67	Diffuse intermediate	Positive	0	Negative	1.167	**0.836**	**0.801**	**0.285**	**0.346**
74	Diffuse intermediate	Positive	0	Negative	**2.672**	**1.094**	**2.013**	**0.642**	**0.659**
90	Multifocal	Positive	0	Negative	**2.488**	**1.152**	**1.072**	**0.200**	**0.419**
105	Multifocal	Negative	0	Negative	**2.231**	**1.031**	**0.903**	0.149	**1.601**
99	Focal	Negative	0	Negative	**1.744**	0.203	0.446	0.052	**0.403**
118	Focal	Negative	0	Negative	**1.722**	0.253	**1.053**	0.067	**0.347**
119	Focal	Negative	0	Negative	**1.813**	0.122	**0.734**	0.082	0.150
123	Focal	Negative	0	Negative	0.397	0.084	0.221	0.048	0.130
63	Focal	Negative	13.29	Negative	0.603	0.250	0.247	0.069	**0.250**
134	Focal	Negative	0	Negative	0.649	0.117	0.244	0.061	0.140
70	Focal	Negative	0	Negative	**2.777**	**1.758**	**1.155**	**0.574**	**0.518**
77	Focal	Negative	0	Negative	**2.114**	**0.527**	**0.849**	**0.150**	0.214
86	Focal	Negative	0	Negative	**2.333**	**0.550**	**1.022**	**0.155**	**0.333**
88	Focal	Negative	0	Negative	0.599	0.113	0.327	0.054	0.142
91	Focal	Negative	5.48	Negative	**2.193**	**0.533**	**0.736**	0.118	**0.291**
48	Focal	Negative	0	Negative	1.169	0.401	0.261	0.063	**0.258**
49	Focal	Negative	0	Negative	**2.173**	**1.223**	**0.751**	**0.150**	**0.425**
51	Focal	Negative	0	Negative	**1.698**	0.385	**0.589**	0.075	**0.262**
52	Focal	Negative	0	Negative	0.990	**0.933**	0.484	**0.203**	**0.361**
58	Focal	Negative	0	Negative	**2.934**	**2.121**	**1.086**	**0.348**	**0.334**
117	Negative	Negative	0	Negative	1.452	0.106	0.537	0.051	**0.835**
120	Negative	Negative	0	Negative	0.191	0.083	0.093	0.037	**0.270**
124	Negative	Negative	ND	Negative	0.317	0.064	0.154	0.039	0.107
125	Negative	Negative	ND	Negative	0.281	0.092	0.165	0.064	ND
126	Negative	Negative	ND	Negative	0.438	0.082	0.236	0.047	0.239
128	Negative	Negative	ND	Negative	0.562	0.100	0.240	0.047	0.178
129	Negative	Negative	ND	Negative	0.727	0.104	0.210	0.052	0.196
130	Negative	Negative	ND	Negative	0.939	0.205	0.260	0.055	0.196
131	Negative	Negative	ND	Negative	0.912	0.093	0.281	0.050	0.147
137	Negative	Negative	ND	Negative	0.207	0.115	0.163	0.124	0.132
140	Negative	Negative	ND	Negative	0.465	0.091	0.220	0.039	0.116
135	Negative	Negative	ND	Negative	0.824	0.263	0.441	0.121	0.097
136	Negative	Negative	ND	Negative	0.670	0.287	0.292	0.111	0.227
113	Negative	Negative	ND	Negative	1.306	0.419	0.520	0.063	0.177
107	Negative	Negative	ND	Negative	0.237	0.105	0.132	0.044	0.156
127	Negative	Negative	ND	Negative	0.221	0.093	0.161	0.076	0.131

[a] qPCR results expressed as DNA copy numbers. ND, not determined.
[b] *M. avium* subsp. *paratuberculosis*-specific antibodies detected by each ELISA are shown as mean OD values. Serum samples with OD readings shown in bold type were considered positive.

logical examination, ZN staining, bacteriological culture, and qPCR of gut tissue samples from the 41 fallow deer included in the study are summarized in Table 1. Sixty-one percent of the fallow deer had histopathological lesions in their gut tissues consistent with JD (25/41). The distribution of the lesions showed a preponderance of fallow deer with focal lesions (64% [16/25]). In order to confirm that the histopathological lesions were *M. avium* subsp. *paratuberculosis* specific, intestinal and lymph node sections from all fallow deer with a positive histopathological result were subjected to immunohistochemical staining, as previously described (11). Using immunohistochemistry, we were able to specifically detect *M. avium* subsp. *paratuberculosis* in intestinal and/or lymph node sections of all the fallow deer with a positive histopathological result (data not shown). AFB were not detected by ZN staining of the gut tissue samples from animals with focal lesions. In contrast, AFB were detected by ZN staining in 88% (8/9) of the animals with multifocal and diffuse lesions.

Although AFB were detected in 32% of the animals with histopathological lesions consistent with paratuberculosis (8/25), only one animal with lesions had an *M. avium* subsp. *paratuberculosis* load in gut tissues in sufficient quantity to be detectable by bacteriological culture (4% [1/25]). qPCR was positive in the gut tissue samples of six animals with lesions (24% [6/25]). All the animals without histopathological lesions (39% [16/41]) showed negative bacteriological culture and ZN staining results. Similarly to the distribution of AFB, *M. avium* subsp. *paratuberculosis* detection

TABLE 2 Sensitivities, specificities, diagnostic values, and cutoffs of ELISAs A, B, C, D, and E

ELISA	Antigen	Conjugate	Sensitivity (%)	Specificity (%)	Diagnostic value[a]	Cutoff
A	Antigen A7	α-fallow deer IgG	72	100	0.8600	1.530
B	Antigen A7	Protein G	64	100	0.8200	0.445
C	PPA-3	α-fallow deer IgG	72	100	0.8600	0.545
D	PPA-3	Protein G	52	100	0.7600	0.150
E	PPD	α-bovine IgG	72	87.50	0.7975	0.240

[a] Diagnostic values are the semisums of sensitivity and specificity.

rates by bacterial culture and qPCR greatly increased with the severity of the lesions.

ELISA diagnostic performance. Four in-house ELISAs (A, B, C, and D) and a commercially available ELISA kit, named ELISA-E, were evaluated for *M. avium* subsp. *paratuberculosis* serum antibody detection with respect to the absence or presence of detectable histopathological lesions consistent with JD at postmortem examination. The optimal concentrations of serum samples and anti-fallow deer IgG conjugate that provided the best signal-to-noise ratio were determined previously (see Table S1 in the supplemental material). The signal-to-noise ratio is the ratio of the ODs of a positive control and a negative control at specific serum and conjugate concentrations (27). Serum samples from three animals with and without histopathological lesions consistent with JD were selected as positive and negative controls and tested at 1:20 and 1:40 dilutions in combination with four dilutions of the anti-fallow deer IgG antibody (1:250, 1.500, 1:1,000, and 1:1,500). The best signal-to-noise ratio between the mean OD readings of the serum samples collected from the three animals with and without histopathological lesions was obtained when a 1:20 dilution of serum and a 1:1,500 dilution of the anti-fallow deer IgG conjugate were used. With these serum and conjugate dilutions, the mean OD values obtained with positive serum samples were approximately 7 times greater than that of negative serum samples when the antigenic preparations A7 (ratio, 7.066) or PPA-3 (ratio, 7.785) were used (see Fig. S1 in the supplemental material). Based on these results, a 1:20 dilution of serum samples and 1:1,500 of conjugate were used on subsequent experiments.

In order to select the optimal cutoff values that differentiate uninfected and infected animals, a panel of 41 serum samples from fallow deer with and without histopathological lesions consistent with JD were tested with ELISAs A, B, C, D, and E. The mean ODs measured with each ELISA were subjected to four-graph receiver operating characteristic (FG-ROC) analysis, which is a plot of the test sensitivity and specificity for each cutoff value (see Fig. S2 in the supplemental material). The plot also calculates the semisum of the sensitivity and specificity, or diagnostic value. A cutoff value expressed in the OD_{450} that yielded the highest semisum was selected as the optimal cutoff for ELISAs C (0.545), D (0.150), and E (0.240). In the ELISA-A plot, three cutoff values between 1.520 and 1.660 had the highest semisum (0.8600) and, therefore, the intermediate value was selected as the optimal cutoff for this ELISA (1.530). The ELISA-B plot showed two peaks with the highest semisum (0.8200). Because optimal cutoff values must be located in a region in which small changes in their numerical value do not substantially change the semisum, 0.445 was finally selected as the optimal ELISA-B cutoff. Once the cutoff for each ELISA was determined, the serum samples from the 41 fallow deer included in the study were classified as ELISA positive or negative according to their corresponding ODs (Table 1).

Sensitivity and specificity estimates for each the five ELISAs at their selected cutoff values are presented in Table 2. The specificities of ELISAs A, B, C, and D for detecting fallow deer with lesions consistent with JD did not differ (100%; Fisher's test, $P > 0.05$), and the four assays had higher specificity than ELISA-E (87.5%; Fisher's test, $P = 0.00002$). Overall, the sensitivities among the five ELISAs ranged from 52% for ELISA-D to 72% for ELISAs A, C, and E. The ELISA-D had significantly lower sensitivity than all other assays (Fisher's test, $P < 0.05$). Using the antigenic preparation A7 as a coating antigen and HRP-protein G as a conjugate, the ELISA-B showed an estimated specificity of 100% and a sensitivity of 64% for the infected animals. The estimated sensitivity was further improved in the ELISA-A (72%) by using a specific anti-fallow deer IgG antibody, which was able to identify infected fallow deer that were undetected when using protein G as a conjugate. Although ELISAs A, C, and E had the highest sensitivity (72%), ELISAs A and C using the anti-fallow deer IgG conjugate were more specific (100%) for detecting fallow deer with JD lesions at necropsy than the commercially available ELISA-E (87.5%). In addition, ELISAs A and C had the highest diagnostic value (0.8600) compared to those of the other ELISAs.

Associations between antibody detection and presence of histopathological lesions. The mean OD values measured with ELISAs A, B, C, and D showed higher readings among animals with lesions than among those without lesions (*t* test, $P < 0.05$). In contrast, the ELISA-E failed to show significant differences in the mean OD readings between the serum samples from animals with and without lesions. All the ELISAs, including the ELISA E, showed statistically significant differences in OD readings between the animals without lesions and those with diffuse multibacillary lesions, which suggested that as tissue damage increased, antibody production significantly increased as well. Only when the ELISA-A was used, significant differences were observed between the mean OD readings of the serum samples from animals without lesions and with all types of lesions, including focal, multifocal, diffuse intermediate, and diffuse multibacillary (*t* test, $P = 0.0005$, 0.0054, 0.0020, and 0.0002, respectively). Significant differences between the antibody levels of the serum samples collected from animals without lesions and from animals in the latent stages of infection (with focal lesions) were only statistically significant when ELISAs A and C, both using the anti-fallow deer IgG antibody, were used (*t* test, $P = 0.0005$ and 0.0273, respectively).

The number of serum samples detected with each ELISA according to the presence or absence of histopathological lesions is presented in Table 3. Antibody production was detected with ELISAs A and C in 72% of the animals with JD-associated lesions of any type (18/25). Conversely, antibody responses were not detected in 28% of the animals with JD-associated lesions (7/25). Our results also indicated that the seropositive rate increased significantly with the degree of tissue damage. For instance, serum

TABLE 3 Fallow deer that tested positive by ELISAs A, B, C, and D and by the commercial bovine paratuberculosis ELISA-E according to the presence of focal, multifocal, or diffuse lesions, or according to the presence of latent or patent forms

Histopathology and lesion type	No. of deer tested positive/no. total deer by ELISA:				
	A	B	C	D	E
Fallow deer without lesions	0/16	0/16	0/16	0/16	2/16
Fallow deer with histopathological lesions					
Focal	10/16	7/16	9/16	6/16	11/16
Multifocal	2/2	2/2	2/2	1/2	2/2
Diffuse intermediate	2/3	3/3	3/3	3/3	3/3
Diffuse multibacillary	4/4	4/4	4/4	3/4	2/4
Total	18/25	16/25	18/25	13/25	18/25
Kappa[a]	0.6674	0.5812	0.6674	0.4581	0.5633
P value	<0.0001[b]	<0.0001[b]	<0.0001[b]	0.0003[b]	0.0003[b]
Fallow deer with histopathological forms[c]					
Latent	10/16	7/16	9/16	6/16	11/16
Patent	8/9	9/9	9/9	7/9	7/9
Total	18/25	16/25	18/25	13/25	18/25
Kappa[d]	0.9110	1.000	1.000	0.8175	0.6528
P value	<0.0001[b]	<0.0001[b]	<0.0001[b]	<0.0001[b]	0.0022[b]

[a] Coefficient of agreement (kappa) between the ELISA results and the presence of focal, multifocal, or diffuse lesions.
[b] Statistically significant association between the ELISA results and the presence/absence of lesions or the presence/absence of latent or patent forms (Fisher's test).
[c] Histopathological lesions were grouped into two categories, latent (focal lesions) and patent forms (multifocal and diffuse lesions).
[d] Coefficient of agreement (kappa) between the ELISA results and the presence of latent or patent forms.

samples from all the animals with multifocal and diffuse lesions were positive when tested with ELISAs B and C. In contrast, all the ELISAs had lower sensitivities for detecting animals with focal lesions. More precisely, ELISAs A, B, C, D, and E detected anti-*M. avium* subsp. *paratuberculosis* antibodies in the serum samples of 10, 7, 9, 6, and 11 animals, respectively, from a total of 16 animals with focal lesions. The ELISA-E detected antibodies in two animals without histopathological lesions; therefore, it was less specific than the other four tested ELISAs. Taken together, our results indicate that the ELISA-A was more specific and sensitive than the other four tests for detecting fallow deer with diffuse and multifocal lesions (specificity, 100%; sensitivity, 88%) and moderately sensitive for detecting animals with focal lesions (specificity, 100%; sensitivity, 62.5%).

The coefficients of agreement (kappa) between the results of the ELISAs and the presence or absence of lesions consistent with JD are also included in Table 3. While ELISAs A and C showed good agreement ($\kappa = 0.6674$), ELISAs B, D, and E had moderate agreement ($\kappa = 0.5812$, 0.4581, and 0.5633, respectively). Better agreement between the ELISA results and the histopathological findings was obtained when the lesions were grouped into latent (focal lesions) or patent forms (multifocal and diffuse lesions), with ELISAs A, B, C, and D showing excellent agreement ($\kappa = 0.9110$, 1.000, 1.000, and 0.6528, respectively) and the ELISA E showing good agreement ($\kappa = 0.6528$). The frequencies of animals with/without lesions and with/without latent or patent forms were statistically analyzed, and the corresponding *P* values are also presented in Table 3. The associations between the positive/negative ELISA results and the presence/absence of JD-associated lesions or presence/absence of latent or patent forms were all statistically significant (Fisher's test, $P \leq 0.0022$).

Associations between antibody detection and positive ZN staining, qPCR, and bacteriological culture of gut tissues. ZN staining, qPCR, and bacteriological culture of the gut tissue samples collected from the 41 fallow deer included in our study are summarized in Table 4 according to the serological status of the animals estimated with each of the five ELISAs. The assay agreements (kappa) between the ELISAs and each of the other three diagnostic tests are also presented in Table 4. While ELISAs B, C, and D showed moderate agreement with ZN staining ($\kappa = 0.5495$, 0.4730, and 0.5605, respectively), ELISAs A and E showed fair and poor agreement, respectively. The associations between the results obtained with ELISAs A, B, C, and D and the ZN staining results

TABLE 4 Pairwise comparison of antibody detection-based tests for JD and Ziehl-Neelsen staining, qPCR, and bacteriological culture of gut tissues collected from 41 fallow deer included in the study

Test combination	No. of deer tested positive/no. total deer by ELISA:				
	A	B	C	D	E
ELISA$^+$/ZN$^+$	7/8	8/8	8/8	7/8	6/8
ELISA$^+$/ZN$^-$	11/33	8/33	10/33	6/33	14/33
Total	18/41	16/41	18/41	13/41	20/41
Kappa[a]	0.3676	0.5495	0.4730	0.5605	0.2077
P value	0.0133[b]	0.0001[b]	0.0004[b]	0.0005[b]	0.1300
ELISA$^+$/qPCR$^+$	5/6	5/6	5/6	4/6	5/6
ELISA$^+$/qPCR$^-$	13/21	11/21	13/21	9/21	15/21
Total	18/27	16/27	18/27	13/27	20/27
Kappa	0.1250	0.1940	0.1250	0.1681	0.0649
P value	0.6279	0.3497	0.6279	0.3845	1.0000
ELISA$^+$/culture$^+$	1/1	1/1	1/1	1/1	1/1
ELISA$^+$/culture$^-$	17/40	15/40	17/40	12/40	19/40
Total	18/41	16/41	18/41	13/41	20/41
Kappa	0.0619	0.0752	0.0619	0.1022	0.0512
P value	0.4390	0.3902	0.4390	0.3171	0.4978

[a] Kappa is the coefficient of agreement between each pair of diagnostic test results.
[b] Statistically significant association between tests (Fisher's test).

were all statistically significant (Fisher's test, $P \leq 0.0133$). The agreement between the results obtained with each of the five ELISAs and the qPCR or culture results was poor (κ, <0.20), and their associations were not statistically significant (Fisher's test, $P > 0.05$).

DISCUSSION

In the present study, we compared the sensitivities and specificities of a commercially available ELISA (ELISA-E) and four in-house ELISAs for detecting anti-*M. avium* subsp. *paratuberculosis* antibodies in samples from free-ranging fallow deer. The in-house assays used two different antigenic preparations and different secondary antibodies. ELISAs A and B both employed an ethanol-treated protoplasmic preparation from a fallow deer *M. avium* subsp. *paratuberculosis* isolate (A7) as a coating antigen and anti-fallow deer IgG antibody or protein G as a conjugate, respectively. ELISAs C and D used commercial PPA3 as a coating antigen and anti-fallow deer IgG antibody or protein G as an HRP conjugate, respectively. As pathology is considered to be the definitive endpoint for diagnosing JD, the sensitivity, specificity, and diagnostic value of each ELISA were estimated using serum samples collected from 41 fallow deer with and without histopathological lesions consistent with JD. This allowed us to test serum samples from animals without histopathological lesions consistent with JD and from animals in different stages of the disease for determining the sensitivity and sensibility parameters of each ELISA. However, we have to recognize that histopathological examination fails to detect early *M. avium* subsp. *paratuberculosis* infections. Animals recently infected with *M. avium* subsp. *paratuberculosis* lack detectable histopathological changes resulting from infection. Detectable lesions and significantly high levels of specific antibodies usually appear in the more advanced stages of infection. On the other hand, due to the reduced number and small size of the focal lesions, this type of lesion can sometimes be missed by histopathological analysis in truly infected animals. We categorized the infected fallow deer into four groups based on histopathological observation. It should be pointed out that the proportion of animals in each histopathological category might not reflect what is seen in a whole free-ranging population.

Our results clearly demonstrate that the ELISA-E, a commercial ELISA designed to detect antibodies against *M. avium* subsp. *paratuberculosis* in domestic ruminants, had lower specificity for detecting antibodies in fallow deer than that of the four in-house ELISAs, which had zero false-positive results. The lower specificity of the ELISA-E (87.5%) than that of the other four tests (100%) suggests that the anti-bovine IgG secondary antibody included in the ELISA-E had limited specificity for fallow deer IgGs. Similarly, the low affinity of the Idexx ELISA kit anti-bovine conjugate for elk, bison, and caribou IgGs makes it inappropriate to test samples from these animal species (18). The diagnostic sensitivity (72%) and diagnostic value (0.8600) of ELISAs A and C, both which used the anti-fallow deer IgG antibody as a conjugate, were much greater than those of ELISAs B and D, which used protein G. These findings agree with those reported by Pruvot et al. (18), who found that the protein G affinity for elk and caribou IgGs was lower than it was for cattle. The same trend was previously shown for red deer and reindeer in comparison to cattle (28). In our study, the ELISA-A sensitivity for fallow deer with naturally acquired *M. avium* subsp. *paratuberculosis* infection diagnosed by histopathology (72%) appears within the range of the sensitivity estimates reported for other cervid species, such as red deer (77%) and elk (68% to 73%) (29).

The ODs measured with ELISAs A, B, C, and D showed significantly higher readings among animals with lesions than among those without lesions (*t* test, $P < 0.05$). In contrast, the ELISA-E failed to show significant differences in the OD readings between serum samples from animals with and without lesions. Significant differences were observed between the mean OD readings of the serum samples from animals without lesions and from animals with focal, multifocal, diffuse intermediate, and diffuse multibacillary lesions only when the ELISA-A was used (*t* test, $P = 0.0005$, 0.0054, 0.0020, and 0.0002, respectively). It was significant that the most advanced cases, animals with diffuse multibacillary lesions, comprised animals for which all the ELISAs showed higher antibody levels than the antibody levels in animals without lesions. Although the likelihood of having anti-*M. avium* subsp. *paratuberculosis* antibodies is greater with more advanced disease, our results also demonstrate that the presence of antibodies may also occur at the latent stages of infection. In fact, differences between the antibody levels of serum samples collected from animals without lesions and from animals in the latent stages of infection (animals with focal lesions) were statistically significant when ELISAs A and C were used (*t* test, $P = 0.0005$ and 0.0273, respectively). In agreement with our data, previous studies in deer suggested that subclinically affected deer produced higher levels of seroreactivity (IgG1) than was previously shown in cattle or sheep (29–31). Overall, our results are in agreement with those of other studies on the detection of mycobacterial infections in domestic and wild ruminants, where ELISA detected the most severe infections but showed lower sensitivity for detecting animals in the latent stages of infection (20, 25). The ELISA-A that employs as a coating antigen an ethanol-treated protoplasmic preparation from a fallow deer *M. avium* subsp. *paratuberculosis* isolate (A7) had the greatest estimated sensitivity for *M. avium* subsp. *paratuberculosis*-infected animals with latent forms (62.5% [10/16]). This result might be due to the fact that ethanol-extracted *M. avium* subsp. *paratuberculosis* antigens can be used to diagnose JD with high sensitivity (15, 32). Biochemical characterization of the *M. avium* subsp. *paratuberculosis* extracted antigens present in the antigenic preparation A7 was not performed in the current study. Associations between the antibody responses detected with each of the five ELISAs (positive or negative) and histopathological findings (presence or absence of lesions) were estimated. ELISAs A, B, C, D, and E did not significantly differ in their classification of lesion-positive animals as ELISA positive (Fisher's test, $P > 0.05$). Kappa statistics indicated a good level of agreement for ELISAs A and C and moderate agreement between the positive results from ELISAs B, D, and E and the presence of lesions. A better level of agreement between the ELISA results and histopathological findings was obtained when the lesions were grouped into latent (focal) or patent forms (multifocal and diffuse), with ELISAs A, B, C, and D showing an excellent level of agreement ($\kappa = 0.9110$, 1.000, 1.000, and 0.6528, respectively), and the ELISA E showed good agreement ($\kappa = 0.6528$). These results suggested that the classification of the lesions into latent and patent forms reduces the misclassification of lesions and allows for a better agreement between the ELISA and histopathological results.

Moderate concordances were found between the results of the ZN staining and ELISAs B, C, and D ($\kappa = 0.5495$, 0.4730, and 0.5605, respectively). In contrast, ELISAs A and E had fair and

poor agreement ($\kappa = 0.3673$ and 0.2077, respectively). The agreement between the results obtained with each of the five ELISAs and the qPCR or culture results were poor (κ, <0.20), and their associations were not statistically significant (Fisher's test, $P \geq 0.05$). Therefore, a positive result with ELISA could not be associated with a positive bacteriological culture and/or qPCR result. We can hypothesize that animals with apparent false-positive results by ELISA were truly infected with *M. avium* subsp. *paratuberculosis*, but bacteriological culture and qPCR failed to detect the organism. A second plausible explanation for the ELISA-positive findings in culture-negative and PCR-negative animals is that *M. avium* subsp. *paratuberculosis* exposure triggers antibody production without progressive infection (20). The inherent difficulty of culturing some *M. avium* subsp. *paratuberculosis* strains might be responsible for more animals being detected by qPCR than by culture. This hypothesis is supported by the fact that the only *M. avium* subsp. *paratuberculosis* isolate recovered from the tissues of the infected fallow deer, the SU-89 isolate, took longer to be detected in the Bactec MGIT 960 liquid culture system at a given inoculum size than did other C-type *M. avium* subsp. *paratuberculosis* strains isolated from cattle or wild animals (33).

In conclusion, our study demonstrates that the ELISA-A, which used an ethanol-treated protoplasmic preparation from a fallow deer *M. avium* subsp. *paratuberculosis* isolate as a coating antigen and a polyclonal anti-fallow deer IgG antibody outperformed the other four ELISAs. The ELISA-A was very sensitive for detecting fallow deer with patent pathology (88%) and moderately sensitive for detecting animals in the latent stages of infection (62.5%). Based on diagnostic sensitivity and specificity, the ELISA-A was superior for detecting anti-*M. avium* subsp. *paratuberculosis* antibodies in serum samples from fallow deer compared to a commercial ELISA routinely used for the diagnosis of bovine paratuberculosis. Our results also demonstrate a strong association between ELISA reactivity in fallow deer and the presence of both histopathological lesions and acid-fast bacteria in gut tissues. This coupled with the low cost inherent in ELISA technology offers a first screening tool in paratuberculosis surveillance that is able to segregate fallow deer populations into infected and not infected animals. Since herd-to-herd assay variation is an important consideration when judging assay performance, further validation of the ELISA-A using a larger number of fallow deer serum samples from different geographic areas is needed. Although the number of tested samples was limited, our study showed that the ELISA-A can be a cost-effective tool for preventing the spread of paratuberculosis among fallow deer population and for reducing the potential transmission of *M. avium* subsp. *paratuberculosis* between fallow deer and livestock in extensive grazing systems.

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