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Diagnostic Accuracy of a *Mycobacterium phlei*-Absorbed Serum Enzyme-Linked Immunosorbent Assay for Diagnosis of Bovine Paratuberculosis in Dairy Cows

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The purpose of this study was to describe the responses of sera from five groups of cattle to an enzyme-linked immunosorbent assay (ELISA) for paratuberculosis by using serum absorbed with *Mycobacterium phlei* at a single working dilution. The infection status of the cattle was determined by fecal culture. Cattle with different levels of exposure (high versus low prevalence and test negative) and disease manifestation (clinically suspect infection versus subclinical infection) were examined, as follows: (i) two paratuberculosis-negative herds; (ii) a fecal culture-confirmed, clinically suspect cases of paratuberculosis; (iii) cows from a paratuberculosis-infected herd with a high infection rate, as determined by fecal culture, but with no clinical cases at the time of sampling; (iv) cows from three paratuberculosis-infected herds known to have paratuberculosis diagnosed on the farm (low infection rate determined by fecal culture); and (v) one fecal culture-negative herd with known serologically positive cattle. Results generally showed a decreased ELISA response when absorbed rather than nonabsorbed serum from each animal was used. The results of the fecal culture confirmed clinically suspect cases, which were analyzed in relation to the amount of colonies isolated from the animals on fecal culture (0, +, ++, +++, ++++, and above). There was a significant increase in the ELISA response for animals with heavy *Mycobacterium paratuberculosis* shedding (++++ or above), when both unabsorbed and absorbed sera were used, compared with the response in animals that were fecal culture negative or that shed *M. paratuberculosis* at lower levels (less than ++++) ($P < 0.05$). The effects on sensitivity and specificity by using different cutoff points for the five groups of cattle with different levels of exposure is described, since sera were not discretely segregated into distinct groups of positive and negative samples. The specificity of the ELISA in the two fecal culture-negative herds was 100% at an ELISA cutoff of an optical density (OD) of 0.1 and above for absorbed serum. For unabsorbed serum the specificity was 62.9% at a similar cutoff value. Similarly, the specificity of the fecal culture-negative, serologically positive herd increased from 37.5 to 72.2 at an ELISA cutoff value of 0.1 to 0.2 (OD) by using absorbed versus unabsorbed serum and from 75.0 to 94.4 at an ELISA cutoff value of 0.2 to 0.3 (OD).

Serological testing for paratuberculosis has, for the most part, suffered from a lack of diagnostic accuracy, as recently reviewed by Chiodini et al. (3). One way of attempting to increase diagnostic specificity is to use test serum absorbed with *Mycobacterium phlei* in an enzyme-linked immunosorbent assay (ELISA) as described by Yokomizo et al. (12). Briefly, by this the methodology 50 μl of test serum mixed with 950 μl of a heat-killed salt suspension (5 mg [dry weight]/ml) of *M. phlei* was used. The mixture was allowed to react at room temperature for 1 h and was then centrifuged at 1,200 × *g* for 30 min at 4°C.

Milner et al. (7) described a modification of the serum absorption technique described above by adding 40 mg of *M. phlei* to 1 ml of a 1/100 dilution of serum in ELISA buffer and left at 4°C overnight. Immediately before use, *M. phlei* was removed by centrifugation at 12,000 × *g* for 5 min. Both techniques were investigated in our laboratory in pilot experiments, and results were found to be comparable by either method. The method of Milner et al. (7) was chosen, since the overnight processing made it easier to plan the laboratory work load from day to day. The only modification made to the method of Milner et al. (7) was that instead of using live *M. phlei* for absorption, organisms that were heat killed by autoclaving at 121°C and a pressure of 15 lb/in^2 for 15 min were used. In other pilot studies, no difference with the results of the method of Milner et al. (7) was found when killed *M. phlei* organisms were used.

The purpose of this study was to describe the responses of five different groups of cattle to an ELISA for paratuberculosis by using serum absorbed with *M. phlei* at a single working dilution.

MATERIALS AND METHODS

Study herds. Sera from five groups of cattle were tested. All cattle were tested by fecal culture for the presence of *M. paratuberculosis*. The groups of cattle were as follows: group 1, two herds determined to be paratuberculosis negative by fecal culture (n = 62); group 2, clinically suspect cases of paratuberculosis confirmed by fecal culture (n = 40); group 3, cows from a herd with a high paratuberculosis infection rate determined by fecal culture but with no clinical cases at the time of the testing (n = 105); group 4, cows from three herds subclinically infected with herds paratuberculosis confirmed by fecal culture (n = 6) and diagnosed on the farm at the time of the testing (n = 120); and group 5, one fecal culture-negative herd with no previous diagnosis of

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paratuberculosis by fecal culture but with known serologically positive cattle determined by the complement fixation test (n = 72).

All cows in the study belonged to one of the following three breeds: SDM (Danish Holstein), RDM (Red Danish Milkrace), or Jersey. All animals older than 12 months were tested three times at 6-month intervals. The two paratuberculosis-3 negative herds (group 1) had no history of paratuberculosis. Group 2, fecal culture-confirmed suspect cases, was composed of bovine fecal samples submitted to the National Veterinary Diagnostic Laboratory, Copenhagen, Denmark, by different cattle owners. There were no clinical cases of paratuberculosis during the 18-month study period in the three herds infected subclinically with paratuberculosis (group 4). This was also true for the fecal culture-negative, serologically positive herd (group 5).

Fecal and serum samples. Samples were collected from the herds described above during a previous study (2), with the exception of the 40 samples from clinically suspect cases, which were obtained just prior to this study. Fecal samples were taken from the rectum by using disposable gloves, and blood was drawn from the jugular vein. Processing of the material in the laboratory was started 18 to 24 h after sampling, and fecal cultures were performed at 24 to 48 h after sampling.

Serum was separated by centrifugation and aspiration and was stored at −70°C for later testing.

Absorption treatment. For absorption of test sera, the method described by Milner et al. (7) was used. The only modification of the procedure was the use of heat-killed *M. phlei* organisms (which were autoclaved for 15 min at 121°C and 15 lb of pressure per in^2), which were found to be as effective as live organisms in pilot experiments. Forty-milligrams (wet weight) of *M. phlei* was added to 1 ml of a 1/100 dilution of serum in ELISA buffer (see below), and the solution was stored at 4°C overnight. Immediately before use, *M. phlei* was removed by centrifugation at 12,000 × g for 5 min.

Fecal culture. Fecal samples were cultured by the protocol used by the National Veterinary Diagnostic Laboratory (1). Three grams of feces was suspended in a 4% solution of sodium hydroxide and a 5% solution of oxalic acid containing 0.1% malachite green. After centrifugation, the sediment was suspended in 4 ml of 0.15 M NaCl, and 0.1 ml of the suspension was inoculated into each of four tubes with Lowenstein-Jensen medium containing 0.16 g of mycobactin P (National Veterinary Diagnostic Laboratory), 4 g of sodium pyruvate (Merck, Darmstadt, Germany), 0.5 g of cycloheximide (Sigma Chemical Co., St. Louis, Mo.), 0.2 g of chloramphenicol, and 200,000 U of benzylpenicillin-sodium (Leo Pharmaceutical Products, Copenhagen, Denmark) (1).

Counting of colonies was done after incubation for 16 weeks at 37°C. Nonchromogenic, slowly growing colonies were classified as *Mycobacterium paratuberculosis*, and subcultures were made on Lowenstein-Jensen media with and without mycobactin P. Mycobactin P dependence and colony morphology were the diagnostic criteria for *M. paratuberculosis*. Results of fecal culture were expressed as the number of viable *M. paratuberculosis* colonies per gram of feces processed in the four culture tubes in the following categories: +, <150 colonies per g of feces; ++, 151 to 700 colonies per g of feces; +++, 701 to 1,500 colonies per g of feces; and ++++, 1,500 colonies per g of feces to too many colonies to count.

Antigens used in ELISA. Sonic extracts of *M. paratuberculosis* ATCC 19698 were used as antigens. Two hundred milligrams (wet weight) of the organism was suspended in 20 ml of phosphate-buffered saline (0.5 M NaCl, 5.8 mM $Na_2HPO_4 \cdot 2H_2O$, 3.1 mM K_2HPO_4), sonicated at 4°C during two periods of 15 min each, and centrifuged at 10,000 × g for 30 min at 4°C. The supernatant was harvested (4 mg/ml of protein), divided into aliquots, and stored at −70°C until use. The *M. phlei* strains (strains 1 to 4) that are routinely used for mycobactin P production at the National Veterinary Serum Laboratory were used to absorb serum (unpublished data).

ELISA serology. An indirect ELISA was used to detect antibodies to *M. paratuberculosis* by using the antigen in a dilution of 1:400 (0.1 μg of protein) in 100 μl of a carbonate buffer (0.1 M Na_2Co_3 [pH 9.6]) in polystyrene ELISA plates (Maxisorp immunoplates; Nunc, Roskilde, Denmark) and incubating overnight at 4°C. Plates were rinsed by flooding the plates three times (2 min each time) with a wash solution containing phosphate-buffered saline and 0.1% Tween-20 (pH 7.5) at room temperature. Test sera were diluted 1:400 in ELISA buffer (wash solution and 0.5% gelatin [Difco Laboratories, Detroit, Mich.]), and 100 μl was applied to each well. Plates were incubated for 60 min at 37°C and rinsed three times as described above, and 100 μl of a 1:400 dilution of rabbit anti-bovine immunoglobulin G (heavy and light chains) conjugated to horseradish peroxidase (Dakopatts, Copenhagen, Denmark) in ELISA buffer was added to each well. The plates were incubated for 1 h and rinsed as described above. One hundred microliters of substrate solution (2 mg of 1,2-phenylenediamine dihydrochloride) was added to 12 ml of 0.1 M citric acid–$Na_2HPO_4 \cdot 12H_2O$, 5 μl of 30% H_2O_2 (pH 5.0; Difco), and the plates were read in a spectrophotometer (Microplate Kinetic reader; Molecular Devices Corp.) at a wavelength of 495 nm 10 min after adding the substrate, after the reaction was stopped with a 0.5 M solution of H_2SO_4. Positive and negative reference sera (National Serum Veterinary Laboratory, Copenhagen, Denmark) were run on each plate. Both sera were used routinely as positive and negative controls in the serology diagnostic laboratory for complement fixation testing for paratuberculosis (serum from a fecal culture-positive cow with naturally occurring Johne's disease and serum from a cow without Johne's disease). Each sample was run in duplicate on each plate, and each serum sample from the same animal was used unabsorbed as well as absorbed with *M. phlei*. Results were expressed as a proportion of the positive reference serum (5). In this report, ELISA response refers to the average optical density (OD) of a sample expressed as a percentage of the OD of the positive reference serum sample.

Data analysis. The end result of an ELISA run at a single serum dilution is in quantitative (OD) units and is not a qualitative (positive or negative) result. Sensitivity was defined as the rate of positive ELISA results for fecal culture-positive cows, and specificity was defined as the rate of negative ELISA results for of fecal culture-negative cows. Sensitivities and specificities were calculated at different cutoff levels for the ELISA response for paratuberculosis in the herds studied. The positive predictive value was defined as the rate of true-positive ELISA (fecal culture positives) among all ELISA-positive results, and the negative predictive value was defined as the rate of true-negative ELISAs (fecal culture negatives) among all ELISA-negative results. The Student's t test was used for comparison of means (Epistat Services, Richardson, Tex.). A probability value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Mean ELISA values for the five study herds. The mean ELISA values for the two paratuberculosis-negative herds (n = 62) were calculated to be 19.9 by using unabsorbed serum and 12.1 by using absorbed serum (Table 1).

The results for the fecal culture-confirmed, clinically suspect cases (n = 40) were analyzed in relation to the number of colonies isolated from the animals, determined by + through ++++, and, thereby, the amount of shedding in that animal at the time that the fecal sample was taken for culture (Table 1).

The herd with the fecal culture-positive infection rate of 88 of 105 (83.8%) was likewise analyzed in relation to the number of colonies isolated from the animals (Table 1).

Results for the three subclinically infected herds that had two fecal culture-positive cows each (for one herd, a + shedder and a ++ shedder; for two herds, two + shedders each) and the fecal culture-negative, serologically positive herd (n = 72) are also given in Table 1.

Sensitivity and specificity at different cutoff levels for the positive ELISA value for paratuberculosis. Since the frequency distribution of fecal culture-negative and -positive cattle demonstrates a considerable overlap in the distribution of the ELISA response between positive and negative cattle, selection of the optimum cutoff point for positive and negative results was not intuitively obvious, since the sera did not always segregate into two groups. The ELISA cutoff valve that provides the point where the sum of diagnostic sensitivity and specificity is maximized can be calculated by adding the sensitivity and specificity for each chosen ELISA cutoff value. This allows more than one cutoff value between positive and negative samples, interpreting calculated sensitivities and specificities over a wide range of ELISA cutoff values.

Results for the calculated sensitivities and specificities at different cutoff levels for the positive ELISA value by using absorbed serum for the herds studied are given in Table 2. The sensitivity of the ELISA in the fecal culture-confirmed, clinically suspect cases (group 2) was 82.5 at an ELISA cutoff of <0.1 (OD), as seen in Table 2. The corresponding positive predictive values were 100%, because every animal in group 2 was fecal culture positive.

The specificity of the ELISA in the two fecal culture-negative herds (group 1) was 16.1 at an ELISA cutoff of <0.1 (OD) and then rose to 100%, starting at a cutoff of 0.1 (OD) and above with a corresponding negative predictive value of 100%, because every animal in group 1 was fecal culture negative. The specificity of the fecal culture-negative, serologically positive herd (group 5) was 22.2 at <0.1 (OD) (Table 2).

The sensitivity of the ELISA in the herd with a fecal culture-positive infection rate of 83.8% (group 3) was 98.9 at a cutoff of <0.1 (OD), and the positive predictive value was 83.6%. The specificity was 47.1 at 0.1 to 0.2 (OD), 94.1 at 0.2 to 0.3, and 100% at 0.3 to 0.4 and above (Table 2).

Similarly, the diagnostic sensitivity in the three subclinically infected herds (group 4) were 66.7 at a cutoff of <0.1 (OD) (positive predictive value, 3.5%); on the basis of only six cases of + and ++ shedders; Table 2). The specificity of the ELISA with absorbed sera is also shown in Table 2.

Results with sera from the same herds but with unabsorbed sera are presented in Table 3. Generally, the specificity was higher for the different groups of cattle serum tested when *M. phlei*-absorbed serum was used compared with that when unabsorbed serum was used, as can be seen

TABLE 1. Mean ELISA values for five study herds

Group	Mean ELISA values for the following colonies[a]:									
	0		+		++		+++		++++	
	Absorbed	Unabsorbed	Absorbed	Unabsorbed	Absorbed	Unabsorbed	Absorbed	Unabsorbed	Absorbed	Unabsorbed
Two paratuberculosis-negative herds (n = 62; group 1)	12.1 ± 2.3[b] (62)	19.9 ± 4.8[c] (62)								
Clinically suspect cases (n = 40; group 2)			7.5 ± 2.8[d] (4)	11.0 ± 3.2[d] (4)	12.5 ± 2.5[d] (2)	17.0 ± 1.6[b] (2)	17.2 ± 1.1[b] (6)	19.8 ± 2.8[b] (6)	25.0 ± 2.3[c] (28)	28.8 ± 3.1[c] (28)
High-prevalence herd (n = 105; group 3)	19.9 ± 3.1[d] (17)	24.6 ± 1.5[b] (17)	17.6 ± 7.0[b] (38)	21.6 ± 5.4[b] (38)	19.6 ± 6.6[b] (8)	23.0 ± 6.8[b] (8)	23.5 ± 5.8[b] (14)	27.1 ± 4.3[b] (14)	33.9 ± 2.2[c] (28)	38.6 ± 2.1[c] (28)
Three subclinically infected herds (n = 120; group 4)	18.1 ± 11.8[b] (116)	21.1 ± 8.4[b] (116)	17.4 ± 8.9[b] (3)	20.4 ± 9.1[b] (3)	20.6 (1)	25.3 (1)				
Fecal culture-negative, serologically positive herd (n = 72; group 5)	16.9 ± 4.1[b] (72)	24.7 ± 4.5[c] (72)								

[a] Mean ELISA values are expressed as a percentage of the OD of a positive reference serum sample. Absorbed, absorbed (*M. phlei*) serum; unabsorbed, unabsorbed (*M. phlei*) serum. Values in parentheses are number of cattle. Means in the same row with different superscripts (*b* to *d*) are significantly different ($P < 0.05$).

TABLE 2. Sensitivity and specificity at different cutoff levels for the positive ELISA value for paratuberculosis for sera absorbed with *M. phlei*

ELISA value[a]	No. of cattle in the indicated groups that were fecal culture:							Sensitivity (%) in group[b]:			Specificity (%) in group[c]:			
	Positive			Negative										
	3	4	2	3	4	5	1	3	4	2	3	4	5	1
<0.1	1	2	7	0	5	16	10	87/88 (98.9)	4/6 (66.7)	33/40 (82.5)	0/17 (0)	5/114 (4.4)	16/72 (22.2)	10/62 (16.1)
0.1–0.2	45	2	13	8	79	36	52	42/88 (47.7)	2/6 (33.3)	20/40 (50.0)	8/17 (47.1)	84/114 (73.7)	52/72 (72.2)	62/62 (100)
0.2–0.3	23	2	14	8	23	16	0	18/88 (20.5)	(0)	6/41 (15.0)	16/17 (94.1)	107/114 (93.9)	68/72 (94.4)	62/62 (100)
0.3–0.4	8	0	4	1	3	2	0	10/88 (11.4)	(0)	2/40 (5.0)	17/17 (100)	110/114 (96.5)	70/72 (97.2)	62/62 (100)
0.4–0.5	6	0	1	0	2	1	0	4/88 (4.5)	(0)	1/40 (2.5)	(100)	112/114 (98.2)	71/72 (99.1)	62/62 (100)
0.5–0.6	1	0	1	0	1	0	0	3/88 (3.4)	(0)	(0)	(100)	113/114 (99.1)	71/71 (99.1)	62/62 (100)
0.6–0.7	1	0	0	0	0	1	0	2/88 (2.3)	(0)	(0)	(100)	113/114 (99.1)	72/72 (100)	62/62 (100)
0.7–0.8	2	0	0	0	1	0	0	(0)	(0)	(0)	(100)	114/114 (100)	72/72 (100)	62/62 (100)
0.8–0.9	0	0	0	0	0	0	0	(0)	(0)	(0)	(100)	114/114 (100)	72/72 (100)	62/62 (100)
>0.9	0	0	0	0	0	0	0	(0)	(0)	(0)	(100)	114/114 (100)	72/72 (100)	62/62 (100)
All sera	88	6	40	17	114	72	62							

[a] Mean ELISA value expressed as a percentage of the OD of a positive reference serum sample.
[b] Number of positive ELISA results at each cutoff level/number of positive fecal cultures × 100.
[c] Number of negative ELISA results of each cutoff level/number of negative fecal cultures × 100.

by comparing the data in Tables 2 and 3. However, test sensitivity was not similarly affected by the absorption, as seen in Tables 2 and 3.

Because the mean ELISA values were higher in the ++++ shedders than they were in +, ++, and +++, shedders, as shown in Table 1, indicating that an animals had to shed ++++ for ELISA values to correlate with fecal culture test results, the ELISA sensitivity was calculated for heavy shedders (++++) by using absorbed (Table 4) and unabsorbed (Table 5) serum. For the high-prevalence herd, the diagnostic sensitivity increased from 47.7 at 0.1 to 0.2 (OD) to 82.1 when only animals which were ++++ shedders were used (compare the data in Tables 2 and 4). This same trend was seen for clinically suspect cases that shed ++++ and above, suggesting that the increase in diagnostic sensitivity of the ELISA is due to the higher antibody levels detectable by the ELISA in heavily shedding animals (>1,500 colonies per g of feces). The correlation between the increased sensitivity of the ELISA with heavy shedding of organisms, as detected by fecal culture, was not remarkably affected by using absorbed (Table 4) and unabsorbed (Table 5) sera.

DISCUSSION

The problem with immunoassays in general is the overlap in the signal obtained with specimens from diseased and nondiseased populations. If there is some overlap between the ELISA results for positive and negative cattle, as there is in many ELISAs for paratuberculosis, then information is lost when the results are expressed in a dichotomous, yes or no manner. A method of reporting results that uses the information available from a quantitative ELISA response was used in this study.

The diagnostic potential of an ELISA with serum absorbed with *M. phlei* versus that of an ELISA with nonabsorbed serum was investigated in herds with known fecal culture status. We confirmed the results of Milner et al. (7, 8, 10), Yokomizo and colleagues (11, 12), and Lopez (6) that

TABLE 3. Sensitivity and specificity at different cutoff levels for the positive ELISA value for paratuberculosis for unabsorbed sera

ELISA value[a]	No. of cattle in the indicated groups that were:							Sensitivity (%) in group[b]:			Specificity (%) in group[c]:			
	Positive			Negative										
	3	4	2	3	4	5	1	3	4	2	3	4	5	1
<0.1	0	0	5	0	2	2	0	(0)	6/6 (100)	35/40 (87.5)	(0)	2/114 (1.8)	2/72 (2.8)	(0)
0.1–0.2	23	3	13	6	55	25	39	65/88 (73.9)	3/6 (50.0)	22/40 (55.0)	6/17 (35.3)	57/114 (50.0)	27/72 (37.5)	39/62 (62.9)
0.2–0.3	36	3	10	7	42	27	20	29/88 (33.0)	(0)	12/40 (30.0)	13/17 (76.5)	99/114 (86.9)	54/72 (75.0)	59/62 (95.2)
0.3–0.4	15	0	8	3	7	14	3	14/88 (15.9)	(0)	4/40 (10.0)	16/17 (94.1)	106/114 (93.0)	68/72 (94.4)	62/62 (100)
0.4–0.5	6	0	3	1	2	1	0	8/88 (9.1)	(0)	1/40 (2.5)	17/17 (100)	108/114 (94.3)	69/72 (95.8)	62/62 (100)
0.5–0.6	1	0	0	0	0	1	0	2/88 (2.3)	(0)	1/40 (2.5)	(100)	108/114 (94.3)	70/72 (97.2)	62/62 (100)
0.6–0.7	0	0	1	0	0	1	0	2/88 (2.3)	(0)	(0)	(100)	108/114 (94.3)	71/72 (98.6)	62/62 (100)
0.7–0.8	2	0	0	0	4	0	0	(0)	(0)	(0)	(100)	112/114 (98.2)	72/72 (100)	62/62 (100)
0.8–0.9	0	0	0	0	1	0	0	(0)	(0)	(0)	(100)	113/114 (99.1)	72/72 (100)	62/62 (100)
>0.9	0	0	0	0	1	0	0	(0)	(0)	(0)	(100)	114/114 (100)	72/72 (100)	62/62 (100)
All sera	88	6	40	17	114	72	62							

[a] Mean ELISA value expressed as a percentage of the OD of a positive reference serum sample.
[b] Number of positive ELISA results at each cutoff level/number of positive fecal cultures × 100.
[c] Number of negative ELISA results of each cutoff level/number of negative fecal cultures × 100.

TABLE 4. Sensitivity at different cutoff levels for the positive ELISA value for paratuberculosis for sera absorbed with *M. phlei* from ++++ fecal shedders

ELISA value[a]	No. of cattle in the following groups that were fecal culture positive:		Sensitivity (%) in group[b]:	
	3	2	3	2
<0.1	0	3	(0)	25/28 (89.3)
0.1–0.2	5	9	23/28 (82.1)	16/28 (57.1)
0.2–0.3	9	10	14/28 (50.0)	6/28 (21.4)
0.3–0.4	6	4	8/28 (28.6)	2/28 (7.1)
0.4–0.5	4	1	4/28 (14.3)	1/28 (3.6)
0.5–0.6	1		3/28 (10.7)	(0)
0.6–0.7	1		2/28 (7.1)	(0)
0.7–0.8	2		(0)	(0)
0.8–0.9	0		(0)	(0)
All sera	28	28		

[a] Mean ELISA value expressed as a percentage of the OD of a positive reference serum sample.
[b] Number of positive ELISA results at each cutoff level/number of positive fecal cultures × 100.

the ELISA with absorbed serum is as sensitive as the ELISA with unabsorbed serum but that the number of false-positive reactions is significantly decreased, as shown by the gains in specificity at lower ELISA cutoff values (Tables 2 and 3). The ELISA was able to diagnose paratuberculosis infections in animals that were heavy shedders but not in animals that were light shedders (Table 1). This is also documented in Tables 2 and 4. Test sensitivities were about the same in all fecal culture-positive cattle examined, without regard to the degree of fecal shedding (Table 2), and in heavy shedders only (Table 4). This indicated and confirms the findings of others that the ELISA serologic results mainly detect cattle with advanced infections (9).

Recently, Milner et al. (9) reported a sensitivity of 57% and a specificity of 98.9% using their *M. phlei*-absorbed ELISA when it was evaluated with samples from three herds known to have Johne's disease (9). The discrimination values between positive and negative ELISA responses were defined as 12 antibody units, as described previously (7). In this study, we calculated the sensitivities and specificities of the ELISA at different cutoff levels. The advantage of using this method is avoidance of the loss of information because of some overlap between the ELISA results for positive and negative cattle compared with the results expressed in a dichotomous, yes or no manner, as in the study of Milner et al. (9). We obtained a sensitivity of 82.5% in the fecal culture-confirmed, clinically suspect cases (group 2), 98.9% in the herd with a fecal culture-positive infection rate of 83.8% (group 3), and 66.7% in the three subclinically infected herds (ELISA cutoff, <0.1 [OD]).

Similarly, the specificity of the ELISA for the two fecal culture-negative herds (group 1) was 100% starting at a cutoff of 0.1 (OD) and above. For the herd with a fecal culture-positive infection rate of 83.8%, it was 100% starting at a cutoff of 0.3 to 0.4 (OD) and above.

The significantly higher mean ELISA titer values in heavily shedding animals (greater than ++++) determined in this study suggests that the ELISA primarily detects heavy shedders of the organism. This is supported by a recent study by Milner et al. (9), which indicated that infected cattle seroconverted at the same time as the onset of shedding of *M. paratuberculosis* in feces, as detected by fecal culture.

The importance of using different groups of cattle with different clinical stages of Johne's disease is supported by the results of present study as well as those of others (9). Our results support those of Milner et al. (9), that the ELISA with absorbed serum could be of value if it is used to detect heavy shedders of the organism so that those animals can be removed from an infected herd. This is important since Milner et al. (9) used a different strain of *M. paratuberculosis* (strain VRI 316) for antigen preparation than was used in this study and the studies tested cattle herds in Australia and Denmark, respectively. The high specificity of the ELISA with absorbed serum suggests that it may have a place in a Johne's disease control program. It identifies heavily shedding animals that should be removed at regular testing intervals from heavily infected cattle herds.

Recently, a diagnostic reagent kit, Johne's Absorbed Enzyme Immunoassay (Johne's kit; Diagnostic Division, Commonwealth Serum Laboratories, Parkville, Victoria, Australia), for the detection of paratuberculosis in cattle has become available. The Johne's absorbed enzyme immunoassay kit has a reported specificity of 99.8%, on the basis of results obtained by testing 997 serum samples from cattle from a geographical area known to be free of paratuberculosis. This kit was also recently evaluated by an independent laboratory in the United States (4). A test specificity of 99% was reported on the basis of testing 196 serum specimens from cattle without paratuberculosis. Results of our efforts to develop a serological diagnostic system for Johne's disease by using absorbed serum support the results of those studies.

TABLE 5. Sensitivity at different cutoff levels for the positive ELISA value for paratuberculosis for unabsorbed sera from ++++ fecal shedders

ELISA value[a]	Positive fecal culture		Sensitivity (%) in group[b]:	
	3**	2+	3	2
<0.1	0	2	(0)	26/28 (92.9)
0.1–0.2	3	10	25/28 (89.3)	16/28 (57.1)
0.2–0.3	4	6	21/28 (75.0)	10/28 (35.7)
0.3–0.4	10	7	11/28 (39.3)	3/28 (10.7)
0.4–0.5	5	3	6/28 (21.4)	(0)
0.5–0.6	4		2/28 (7.1)	(0)
0.6–0.7	0		2/28 (7.1)	(0)
0.7–0.8	1		1/28 (3.6)	(0)
0.8–0.9	1		(0)	(0)
All sera	28	28		

[a] Mean ELISA value expressed as a percentage of the OD of a positive reference serum sample.
[b] Number positive ELISA at each cutoff level/number of positive fecal cultures × 100.

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