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Comparison of two enzyme-linked immunosorbent assays for diagnosis of *Mycobacterium avium* subsp. *paratuberculosis*

S. L. B. McKenna¹, D. C. Sockett, G. P. Keefe, J. McClure, J. A. VanLeeuwen, H. W. Barkema

Abstract. Enzyme-linked immunosorbent assays (ELISAs) are used in Johne's disease (JD) control programs as a first screening for presence of the disease in a herd. A high sensitivity of the ELISA is therefore important, yet the commonly used ELISAs have relatively low sensitivity. The inclusion of an absorption phase, although improving specificity, potentially decreases sensitivity. Sera and feces of 383 adult dairy cows in 8 herds were used to compare the test characteristics of an absorbed and a nonabsorbed indirect ELISA for the detection of JD. The absorbed ELISA is based on a protoplasmic antigen, whereas the nonabsorbed uses a lipoarabinomannan-based antigen. The potential advantage of the nonabsorbed ELISA is that it may be less specific and more sensitive. Two herds certified free of JD were used to compare the specificity of the ELISAs. The other herds used to compare sensitivity were either infected with *Mycobacterium avium* subsp. *paratuberculosis* or had unknown status. Using fecal culture as a gold standard, the diagnostic specificity for the absorbed and nonabsorbed ELISAs were 98.4% and 87.9%, respectively. The diagnostic sensitivity was 72.4% and 65.5% for the absorbed and the nonabsorbed ELISA, respectively. Furthermore, a comparison using a fecal DNA probe as the comparison standard resulted in both ELISAs having a sensitivity of 61.9%. Agreement between the 2 ELISAs was moderate, with a kappa statistic of 0.58. The nonabsorbed ELISA did not have a higher sensitivity and had a lower specificity than the absorbed ELISA. Therefore, in this population, there was no advantage gained with using the nonabsorbed ELISA.

Key words: Culture; ELISA; Johne's disease; kappa; paratuberculosis; specificity.

Johne's disease (JD) is a disease of ruminants that causes chronic granulomatous enteritis. The causative organism is *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*). Ef-

forts are being made to manage JD through herd-level and national control programs; however, there are difficulties in accurately diagnosing animals infected with *Mptb*. Fecal culture for *Mptb* yields results with 100% specificity (and subsequently 100% positive predictive value [PPV]), but traditional culture methods can take up to 3 months for results.⁹ Therefore, more rapid tests such as an enzyme-linked immunosorbent assay (ELISA), which are subsequently cheaper, are more desirable. However, the sensitivity of ELISAs for *Mptb* ranges from 15% to 88%,^{2–4} depending on level of fecal shedding, with a specificity as high as 97.9%.⁷

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Table 1. Distribution of animals for each herd along with *Mycobacterium avium* subsp. *paratuberculosis* status and ELISA test results ($n = 383$).

Farm	No. of test cows	Herd size	No. <i>Mptb</i> -positive cows (%) ^a	No. <i>Mptb</i> PCR-positive cows (%) ^a	Absorbed ELISA-positive (%) ^b	Nonabsorbed ELISA-positive (%) ^b
A	67	67	0 (0)	—	1 (1.5)	6 (9.0)
B	49	49	0 (0)	—	5 (10.2)	8 (16.3)
C	73	73	46 (63.0)	38 (52.0)	23 (31.5)	27 (37.0)
D	28	111	12 (42.8)	8 (28.6)	17 (15.3)	16 (14.4)
E	20	218	1 (5)	—	13 (6.0)	11 (5.0)
F	78	78	18 (23.1)	10 (12.8)	13 (16.7)	24 (30.1)
G	51	282	17 (33.0)	—	42 (14.9)	26 (9.2)
H	17	66	10 (58.8)	7 (41.1)	13 (19.6)	15 (22.7)
Total	383	944	104 (27.1)	63 (16.4)	127 (12.8)	133 (13.7)

* Fecal culture and PCR-positive percentages based on number of cows tested.

† ELISA-positive percentages based on whole herd.

Initially, ELISAs for JD had a low diagnostic specificity, which resulted in a high proportion of test-positive animals being false positives.¹ Inclusion of an absorption step, where serum was preabsorbed with *Mycobacterium phlei* to remove potential cross-reacting antibodies, improved specificity.¹² Current commercial ELISAs for *Mptb* include an absorption phase; however, there is a potential decrease in sensitivity because of absorption of antibodies present for *Mptb*. As part of JD control programs, using an ELISA with a higher sensitivity would be beneficial. In a study performed in Germany with a nonabsorbed ELISA^a modified for milk, the sensitivity was reported to be 60.9% with a specificity of 94.6%.¹¹ However, there were no comparisons with absorbed ELISAs in the same population of animals. The objective of this study was to compare the sensitivity and specificity of the ELISA developed in Europe without an absorption phase with the absorbed ELISA commonly used in North America, using fecal culture and polymerase chain reaction (PCR) test results on Wisconsin dairy cattle as gold standard tests.

During 1999, serum and fecal samples from 383 adult dairy cows from 8 Wisconsin dairy herds (Table 1) were used to compare the 2 ELISAs. Two of the herds (herds A and B, $n = 116$) were certified free of JD as part of the State of Wisconsin Voluntary Johne's Disease Control Program and had no history of clinical JD or of any *Mptb* fecal culture-positive cows. The other 6 herds either had unknown *Mptb* status or infected herd status. Four of the herds (herds D, E, H, and G) had submitted serum samples of the whole herd previously for absorbed ELISA evaluation. These herds then resubmitted serum samples from test-positive cows along with a fecal sample for retesting and confirmation, and the retest data were used for this study.

Serum samples were split and *Mptb* serostatus was determined using the absorbed ELISA^b at the Wisconsin Veterinary Diagnostic Laboratory in Madison, Wisconsin, and the nonabsorbed ELISA^a at the Svanova research facility in the Uppsala Science Park, Sweden. Both are indirect ELISAs that use different antigens; the absorbed ELISA uses a protoplasmic antigen, and the nonabsorbed ELISA uses a purified extract of lipoarabinomannan (LAM). Fecal cultures were performed on Herrold egg yolk media at the Wisconsin Veterinary Diagnostic Laboratory using the method de-

scribed previously.⁸ For 196 samples in which there still were fecal samples available (herds C, D, F, and H), a PCR assay was performed using a DNA Probe as described by the manufacturers instructions.^c

Analysis of data was done using Stata 7.0®.^d Specificity and sensitivity calculations along with exact confidence intervals (CIs) were generated using the "diagt" command. Agreement of the 2 ELISAs was tested using a kappa statistic using the "kagof" command. Estimations of PPV based on various levels of apparent prevalence were calculated using Excel.^e

Fecal cultures for *Mptb* on the 2 JD-free herds (herds A and B) were all negative (Table 1). In these herds, the absorbed ELISA identified 110 of the 116 cows tested as negative, resulting in a specificity of 94.8% (95% CI 89.1, 98.1), whereas the nonabsorbed ELISA identified 102 cows as negative for a specificity of 87.9% (95% CI 80.6, 93.2). Of the 267 cattle tested in the other 6 herds, 87 (32.6%) yielded a positive culture for *Mptb*, and 63 of 196 (32.1%) fecal samples tested by PCR were also positive for *Mptb*. Combining results in a parallel manner, 104 cows (39.8%) were positive on either culture or PCR (Table 1).

When calculating the sensitivity of the ELISAs using the 104 cows that were positive on either PCR or culture as true disease positive, the absorbed ELISA identified 65 cows as positive for a sensitivity of 62.5% (95% CI 52.5, 71.8), and the nonabsorbed ELISA identified 59 cows for a sensitivity of 56.7% (95% CI 46.7, 66.4). The sensitivity results for the ELISAs compared with the 87 fecal culture-positive cows were 72.4% (95% CI 63.0, 81.8) for the absorbed ELISA and 65.5% (95% CI 55.5, 75.5) for the nonabsorbed ELISA. The absorbed and nonabsorbed ELISA had the same sensitivity when compared with PCR, 60.3% (95% CI 47.2, 72.4) by identifying 38 of 63 positive cows (Table 1).

In Table 2, a summary is given for agreement of diagnostic tests using kappa statistics. The kappa statistic comparing the absorbed ELISA and the nonabsorbed ELISA was 0.58 (95% CI 0.09, 0.66), showing that the 2 tests had a 58% agreement on the status of the samples beyond agreement by chance alone. The agreement determined between the ELISAs and fecal culture was lower, with the kappa value for the nonabsorbed ELISA being 0.34 (95% CI -0.03,

Table 2. Comparison of kappa values and agreement for various combinations of diagnostic tests for *Mycobacterium avium* subsp. *paratuberculosis*.

	Observed agreement (%)	95% CI for agreement	kappa	95% CI for kappa
Nonabsorbed versus absorbed ELISA	81.2	76.9, 85.0	0.58	0.09, 0.66
Nonabsorbed ELISA versus PCR	64.8	57.7, 71.5	0.25	0.06, 0.38
Nonabsorbed ELISA versus culture	72.3	67.6, 76.8	0.34	0.00, 0.42
Absorbed ELISA versus PCR	73.0	66.2, 79.0	0.39	0.03, 0.52
Absorbed ELISA versus culture	77.0	72.5, 81.1	0.44	0.00, 0.52
PCR versus culture	79.6	73.3, 85.0	0.54	0.03, 0.66
Nonabsorbed ELISA versus PCR + culture	68.9	64.0, 73.5	0.28	0.04, 0.37
Absorbed ELISA versus PCR + culture	73.6	68.9, 78.0	0.38	0.00, 0.47

0.42) and the absorbed ELISA of 0.44 (95% CI -0.01 , 0.52). The agreement was lower still when cattle were classified using results from culture or PCR (testing parallel) with a kappa for the nonabsorbed ELISA of 0.28 (95% CI -0.04 , 0.37) and the absorbed ELISA of 0.38 (95% CI -0.02 , 0.47). Fecal culture and PCR had moderate agreement with a kappa of 0.54 (95% CI 0.03, 0.66).

Using the specificity and sensitivity estimates derived from using parallel testing (fecal or PCR positive), values were calculated for PPV of the 2 ELISAs at various levels of apparent prevalence. The PPV was then plotted against apparent prevalence to demonstrate the effect that specificity would have on PPV. At low apparent prevalence, the nonabsorbed ELISA had a lower PPV and did not meet the PPV of the absorbed ELISA until an apparent prevalence of 63% (Fig. 1).

When evaluating diagnostic accuracy on any test, it is important to use samples that accurately represent disease status. The use of animals from farms that have had a series of negative test results over a period of time is the usual source of negative animals to assess specificity for JD. There is the potential for inclusion of animals infected with *Mptb* in this test population; however, the use of animals with repeated negative culture results¹⁰ or animals that have had a long-standing history in a negative herd certification program are commonly used as negative gold standards.⁵

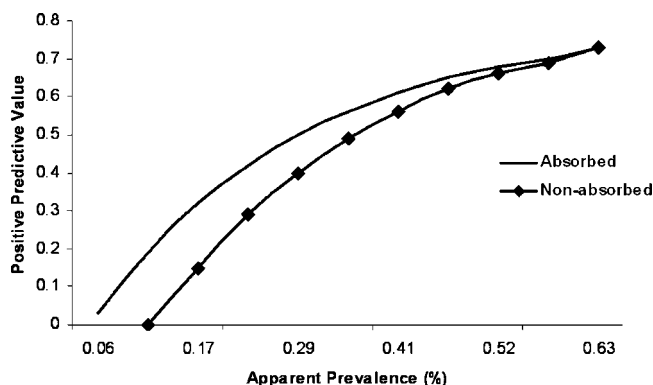
To analyze specificity in the case of this dataset, 116 animals from 2 JD-free herds were used. The absorbed ELISA

identified more animals as negative in a negative herd with a specificity of 94.8%, whereas the nonabsorbed ELISA had a specificity of 87.9%. The exclusion of an absorptive stage in the nonabsorbed ELISA is a component of this decreased specificity. The nonabsorbed ELISA does not use *M. phlei* antigens to absorb nonspecific antibodies; therefore it is expected to have a lower diagnostic specificity. However, it would also be expected that with a decrease in specificity, there would be a gain in sensitivity or at least a significant increase in the number of positive results, which was not demonstrated with this data.

The 2 ELISAs evaluated had similar sensitivities ($P > 0.05$, overlapping 95% CI) when based on fecal culture and PCR alone or on parallel testing of both fecal culture and fecal PCR. Although there was no statistically significant difference in sensitivity of the 2 tests, the nonabsorbed ELISA did identify more cattle as positive and some of these positives may be nonshedding infected animals. When PCR was the sole comparison standard, the 2 ELISAs had the exact same sensitivity, which was likely a chance occurrence because they did not identify the same animals as positive and because the kappa value was quite low. The estimates of sensitivity were slightly higher than those in previous studies,^{8,10} but would be similar to estimates of sensitivity found in more recent studies for animals that were more likely to be fecal shedders.⁴

Overall, there was poor agreement beyond that because of chance between all diagnostic methods used in this study. The only combination that derived moderate agreement beyond that because of chance was the 2 ELISAs compared with each other (kappa value of 0.58) and when the fecal culture and PCR were compared with each other (kappa value of 0.54). The absorbed ELISA identified 127 samples as positive and the nonabsorbed ELISA identified 133. They agreed on 94 samples as being positive and disagreed on 72 samples. Neither ELISA performed well in comparison with fecal culture or PCR, either interpreted in series or parallel, with kappa values that were not significantly greater than zero. This signifies that the amount of agreement between the ELISAs and PCR was very close to what one would predict based on just chance alone.

To implement a JD control program that has a testing component, the test used should be performed quickly and inexpensively and have high sensitivity to ensure identification of infected animals at all stages of disease. In the

**Figure 1.** Estimated PPVs of 2 ELISAs with increasing apparent herd *Mycobacterium avium* subsp. *paratuberculosis* prevalence, on the basis of calculated sensitivity and specificity.

absence of a confirmatory test, a high specificity is also needed to achieve a high PPV at low prevalence to ensure that cattle identified are truly positive. Therefore, it is imperative to determine whether there is an advantage to using a nonabsorbed ELISA instead of an absorbed ELISA.

One difference between the 2 ELISAs tested was the absorption step, but another difference was the antigens used. A recent study using an ELISA based on a polysaccharide antigen in the 32- to 42-kD range consistent with LAM, provided enhanced sensitivity in comparison with an ELISA based on protein antigens.⁷ In that study, both ELISAs contained an absorption phase. In general, ELISAs without an absorption phase have been associated with decreased specificity; therefore, absorbed ELISAs have become more commonly used.¹ Although the study presented here cannot determine which component affected the performance, it is still important to identify whether one ELISA may have a diagnostic advantage over the other.

The nonabsorbed ELISA did identify more animals as positive. Some of the animals detected by the nonabsorbed ELISA may in fact be in the earlier stages of disease, yet not fecal shedding. The sensitivities reported in this study for the ELISAs would more accurately be described as estimates for identifying *Mptb* fecal shedders and not necessarily *Mptb* infected cattle. It could be advantageous to identify animals that may have *Mptb* antibodies but are not fecal shedding. As established in the authors' slaughterhouse study,⁶ animals can have lymph nodes that are organism positive on culture, yet are negative on ileum culture, and have no evidence of infection on the basis of histology, indicating these animals are in the early stages of disease. If the organisms were in the lymph system at a level detectable by culture methods, antigens may be present for the immune system to develop antibodies. Therefore, these animals may be serologically positive but fecal culture negative, a state that would be advantageous to identify before fecal shedding commences.

The nonabsorbed ELISA had a lower specificity than the absorbed ELISA. This has implications for the accurate use of the test and its PPV. The value of a positive nonabsorbed ELISA result on an individual animal is diminished, especially in lower prevalence herds. In Fig. 1, this point is illustrated. At low to moderate apparent prevalence, the nonabsorbed ELISA has a lower PPV than the absorbed ELISA, and in fact it is not until extremely high prevalence that the PPV becomes equal. Although the nonabsorbed ELISA identified more animals as test-positive animals, it had a lower sensitivity than the absorbed ELISA, and higher numbers of positive animals identified by the nonabsorbed ELISA would appear to be animals that are erroneously called positive. It can be concluded from this study that the nonabsorbed ELISA had a lower specificity compared with the absorbed ELISA without a gain in sensitivity. Testing a low preva-

lence herd with the nonabsorbed ELISA would result in more false-positive results, thereby limiting the usefulness of the nonabsorbed ELISA in control programs.

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Sources and manufacturers

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- b. Herdchek[®] ELISA, IDEXX Laboratories, Westbrook, ME.
- c. HerdChek[®] DNA Probe, IDEXX Laboratories, Westbrook, ME.
- d. Stata Corporation, College Station, Texas, TX.
- e. Excel, Microsoft Corporation, Bellevue, WA.

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