

Longitudinal study of ELISA seroreactivity to *Mycobacterium avium* subsp. *paratuberculosis* in infected cattle and culture-negative herd mates

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Abstract. Two thousand nine hundred fifty-two serum samples, collected once or twice annually from 545 cows of known fecal culture status were tested for antibodies to *Mycobacterium avium* subsp. *paratuberculosis* using a commercially available enzyme-linked immunosorbent assay (ELISA) test. Overall, 13.5% of the samples from 282 infected cows had positive ELISA results, but when tested multiple times, 38.3% of the cows had at least 1 serum sample with positive results. Among 263 fecal culture–negative cows, 98.1% of the serum samples had negative ELISA results, but when tested multiple times, 7.8% of the cows had at least 1 positive ELISA sample. Fecal culture was positive on a test before the first positive ELISA in 50 cows, ELISA was positive before fecal culture in 12 cows, and in 38 cows, both tests became positive at the same testing time. An additional 174 cows were positive on fecal culture and always negative on ELISA until culled. For cows that had ELISA sample:positive (S/P) ratios below the cutoff point, the change in S/P between sequential tests was evaluated to determine whether a rise in S/P could predict infection status. In this study, change in S/P was not a useful predictor of infection status in seronegative cows.

Key words: Cattle; ELISA; Johne’s disease; *Mycobacterium*; paratuberculosis.

Introduction

Paratuberculosis (Johne’s disease) is a chronic enteric infection of cattle and other ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Clinical signs of the disease in cattle include diarrhea, weight loss, and edema, but most infected animals show no clinical signs. Identification and removal of infected animals, along with management efforts to reduce transmission, are important facets of many control programs. In the National Johne’s Disease Voluntary Control Program, testing of sera for antibodies to MAP using enzyme-linked immunosorbent assay (ELISA) is recommended for identifying infected and uninfected herds, and as a screening test to identify possibly infected individual animals for additional testing.¹⁰

The sensitivity and specificity of ELISA tests have been studied extensively.^{1,3,4,9,12} The sensitivity of the test depends on the population of cattle tested. In one study, the sensitivity of a commercially available ELISA was 87% when applied to cows with advanced infection and clinical signs, but only 15% when applied to subclinical animals shedding low numbers of MAP in feces.⁹ These results suggested that seropositivity developed later in infection, compared with fecal

shedding, but sequential testing of animals was not performed to confirm this. Also, the age of onset of fecal shedding may be related to dose and age of exposure to MAP. This in turn could influence the timing of the onset of antibody production. One objective of the study reported here was to compare onset of fecal shedding and onset of antibody production in cows with multiple samples over time.

The repeatability of ELISA results on individual samples has been evaluated.^{2,9} Laboratory to laboratory, plate to plate, and well to well variability have been calculated. Although overall repeatability for MAP ELISAs has been found to be acceptable (coefficient of variation 10% or less between plates or different runs of the assay for the same serum sample), samples just above or just below the cutoff point, when retested, may yield the opposite dichotomous results.^{2,9} An additional source of variability in ELISA results is the actual concentration of antibodies in the animal’s serum, which may fluctuate over time. In one study, when seropositive cattle were retested at a later date, 39.5% tested negative.⁶ Cows with initial ELISA results just above the cutoff were more likely to switch classifications than those with results further above the cutoff.⁶ The infection status of the cattle in that study was not confirmed by organism detection methods such as fecal culture. Another objective of the present study was to determine the frequency with which infected cows and fecal culture-negative cows “switch” ELISA results when tested on multiple, sequentially obtained samples.

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The result of an ELISA is a continuous variable (optical density [OD], or a ratio of test sample OD to a control sample OD), which is dichotomized as positive or negative by comparison to a cutoff point. In one report, ELISA OD results that were just below the recommended cutoff point, although classified as negative, were 15 times more likely to come from infected cattle than uninfected cattle, based on likelihood ratios.² Previous assessments of ELISA sensitivity were based on analysis of 1 sample from each animal tested. We chose to investigate the possibility that multiple test results from individual animals might provide additional information. For example, if a subsequent ELISA result shows a rise in OD, but not yet above the cutoff, does that rise in OD suggest a greater likelihood of infection? The third objective of this study was to compare change in OD values over time for infected and uninfected cattle to determine whether a change in OD would provide additional diagnostic information.

Materials and methods

Samples. Samples from our laboratory's serum collection were used for this study. Fecal and serum samples had been collected from 12 dairy herds known to be infected with MAP during the period 1984–2003. Herds were participating in a voluntary Johne's disease control program that involved annual (3 herds) or twice-annual (9 herds) collection of samples. Herds ranged in size from 50 to 160 milking head (median = 60), and all animals ≥ 24 months of age were sampled at each visit. Feces were cultured for MAP at the time of collection, and serum was frozen at -20°C until ELISA analysis was performed in 2004. Feces (2 g/sample) were cultured on 4 slants each of Herrolds Egg Yolk medium using a centrifugation–double incubation method to enhance sensitivity and reduce contamination.¹¹ However, in the herd from which samples were collected before 1987, 30 infected cows were identified using a sedimentation method. Farmers were provided with fecal culture results, but were not required to cull infected cows.

Cows with ≥ 2 serum and fecal samples, collected at least 6 months apart, were chosen for the study. Laboratory records were reviewed and cows were classified as infected if any of that animal's fecal culture results yielded a positive result, and animals were classified as fecal culture–negative if all fecal culture results were negative. Samples ($n = 2,952$) were analyzed from 545 cows. Of these cows, 295 had 5 or more samples available, 90 had 4 samples, 97 had 3 samples, and 63 had 2 samples. There were 282 infected cows, with 1,461 samples, and 263 fecal culture–negative cows with 1,491 samples.

ELISA testing. A commercially available ELISA test for MAP antibodies^a was used according to the manufacturer's recommendations, except that samples were run in single, rather than duplicate, wells to mimic common practice in diagnostic laboratories and reduce study costs. All samples from an individual cow were tested on the same 96-well plate. Samples were thawed immediately before analysis.

The 2,952 serum samples were tested during a 4-week period by the same operator. For each sample, the ratio of the patient sample OD to the positive control sample OD (S/P ratio) was determined. Samples with $S/P \geq 0.25$ were classified as positive according to the manufacturer's recommendation.

Data analysis. The percentage of infected cows with at least 1 positive ELISA result (relative sensitivity), and the percentage of fecal culture–negative cows with all negative ELISA results (relative specificity) were determined, and the 95% confidence interval (CI) was calculated. Because the fecal culture–negative cows were not from test-negative herds, the possibility exists that some of those animals were actually infected but not shedding MAP in feces, or shedding MAP at a level below the limit of detection. For that reason, the term *relative specificity* was used, because true specificity can only be determined using uninfected herds. The relationship between time of first fecal-positive result and time of first seropositive result was determined. The age at onset of fecal MAP shedding (which might relate to dose and age of exposure to MAP) was compared for those infected cows that were always ELISA-negative, versus those that had an ELISA-positive result, using the Student's *t*-test. The null hypothesis was rejected when $P < 0.05$.

For each individual cow's sequential serum samples, the change in S/P ratio ($\Delta S/P$) was calculated for each pair of consecutive tests, by subtracting the earlier S/P from the later S/P. Also, for each cow, a maximum $\Delta S/P$ was calculated by choosing the 2 samples (not necessarily sequential) among all those recorded that gave the maximum difference in S/P for that cow. An analysis was performed to determine the utility of $\Delta S/P$ to predict fecal culture status. For this analysis, only serum samples with an ELISA S/P ratio below the manufacturer's cutoff, 0.25, were used, because the objective was to determine whether seronegative cows could be identified as infected based on a rise in S/P ratio; once the S/P ratio is above the cutoff, there is no need to further enhance sensitivity by using $\Delta S/P$. By varying the $\Delta S/P$ cutoff incrementally, the percentage of infected cows above the cutoff (relative sensitivity compared to fecal culture), and percentage of fecal culture–negative cows above the cutoff (false positive rate, relative to fecal culture) were determined for various $\Delta S/P$ cutoff values, and receiver operator characteristic (ROC) curves were constructed. The area under the ROC curve was determined using the trapezoidal method.⁵

Results

Of the 1,461 serum samples from infected cows, 197 (13.5%) gave positive ELISA results. Of the 282 infected cows, 108 (relative sensitivity 38.3%, CI = 32.6%–44.2%) had at least 1 serum sample with positive ELISA results; 174 infected cows (61.7%) were negative on all ELISA tests. Fifty-nine of the infected cows were classified as "heavy" shedders on the basis of a fecal culture result of >75 colonies per culture tube. Of those 59, 45 (76.2%, CI = 63.4%–86.3%) were ELISA-positive on or before the time that heavy shedding was detected, an additional 5 were ELISA-

negative but became ELISA-positive on a subsequent test, and 9 remained ELISA-negative at all bleed dates. By contrast, of the remaining 223 infected cows that were “light” to “moderate” shedders (<70 colonies/tube), only 58 (26.0%, CI = 20.4%–32.2%) had 1 ELISA-positive sample. The highest ELISA S/P values came from the cows that exhibited heavy fecal shedding. Ninety-three percent of the serum samples with $S/P > 2.0$ were from heavy-shedding cows, contrasted with 41% of the samples with $1.0 < S/P < 2.0$ and 20% of the samples with $0.25 < S/P < 1.0$.

Of the 108 seropositive infected cows, 8 were detected by ELISA before the first positive fecal culture, but those cows were from the group whose corresponding fecal sample were cultured using only the less-sensitive sedimentation method. For the remaining 100 cows, 50 were fecal culture-positive at least 1 test before becoming ELISA-positive (median, 1.0 year earlier; range, 0.5 to 3 years), 38 were positive for the first time on fecal culture and serum ELISA concurrently, and 12 were ELISA-positive at least 1 test before being detected by fecal culture (median, 0.5 year earlier; range, 0.5–2 years). There were 174 cows that were detected positive on fecal culture but were negative on every ELISA until culling. There was no difference between age at onset of first detectable fecal shedding between the ELISA-positive cows and those that were always ELISA-negative.

Of the 1,491 samples from fecal culture-negative cows, 29 (1.9%) gave positive ELISA results. Of 263 uninfected cows, 243 (92.3%) were negative on all ELISA tests. The ELISA results of the 20 seropositive fecal culture-negative cows were studied in more depth. One of the 20 cows was ELISA-positive on all subsequent tests, and 2 cows had no subsequent tests after the positive result. The remaining 17 cows were positive on only that 1 serum sample and were ELISA-negative on subsequent tests. For 11 of those 17 cows, the single positive ELISA result was close (within 0.1 unit) to the manufacturer’s S/P cutoff point of 0.25. The 29 serum samples were retested using a different ELISA system.^b Results were positive for 13 samples (9 cows) and negative for the remaining 16 samples (11 cows).

The sequential ELISA results of infected cows were examined to determine the frequency of reverting to seronegative after a positive result. For 50 of the 108 infected cows with a positive ELISA, the cow was culled after the positive ELISA, and there were no subsequent tests. Of the 58 remaining infected cows that had testing repeated after the first positive ELISA, 44 (75.8%) remained positive on all subsequent tests. For 4 of the 14 cows that reverted to ELISA-negative, the S/P value on the test before reverting to seronegative was close (within 0.1 unit) to the cutoff S/P of

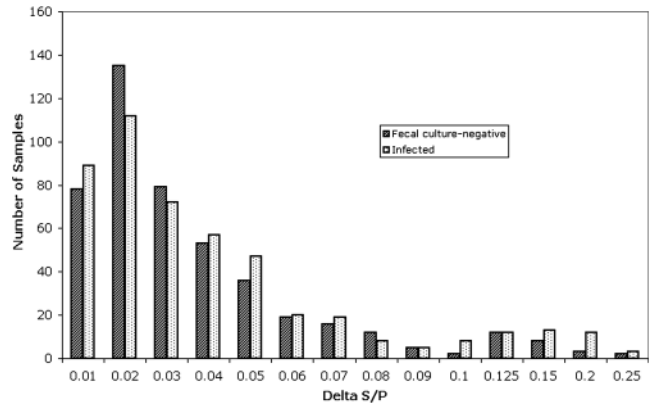


Figure 1. Distribution of $\Delta S/P$ ratio (change in ELISA S/P ratio) for sequential serum samples for cows infected with *M. avium* subsp. *paratuberculosis*, and fecal culture-negative cows. Infection status was determined by fecal culture on Herolds Egg Yolk Medium. Chart was truncated to show only samples for which the $\Delta S/P > 0$.

0.25; in 2 cases the S/P was > 1.0 . All 6 of the cows that had additional samples after the seronegative result were positive again on later tests. When the 14 ELISA-negative samples were retested with a different ELISA system,^b 13 had negative results.

The frequency distribution of sequential $\Delta S/P$ values for samples below the S/P cutoff revealed poor discrimination between infected and uninfected cows (Fig. 1). The area under the ROC curve for serial $\Delta S/P$ and maximum $\Delta S/P$ were 0.54 and 0.52, respectively (data not shown).

Discussion

Most previous studies of ELISA performance relative to fecal culture have involved single, concurrently collected samples as opposed to multiple, longitudinal samples as in the present study. Despite the differences in these studies, the results show similar performance. The overall sensitivity of the ELISA, relative to fecal culture, is reported at 27% to 45%.^{1,3,4,9,12} However, the sensitivity is highly dependent on the population sampled. The ELISA detected 75% of heavy shedders in one study, compared with 15% of low shedders.^{4,9} Similarly, in the current study, 76.2% of heavy shedders and 26.0% of moderate and light shedders had positive ELISA results.

In this study, 1.9% of serum samples from fecal culture-negative cows had positive ELISA results, similar to the specificity reported in previous studies.^{1,3,4,7,9,12} However, 7.7% of fecal culture-negative cows had at least 1 positive ELISA result, which is higher than might be expected based on previously reported specificity range of 98% to 100%. The slightly higher sensitivity for low shedders and lower relative specificity in this study is probably due to a combination of factors. First, in the current study, each

animal was tested multiple times, and the animal was classified as ELISA-negative only if all tests were negative, whereas previous studies involved a single sample per animal. Although multiple tests over time increase the chance of detection of an infected animal, this would also increase the chances of a false-positive result. Another potential cause for lower relative specificity is that fecal culture-negative cows in this study were not from uninfected herds. It is possible that some of these 20 cows were misclassified as uninfected because they were not shedding MAP, or were shedding below the limit of detection. Alternatively, some cows may have been exposed to MAP and developed low levels of antibody without developing infection and fecal shedding. Finally, some of the false-positive ELISA results may have been due to analytic error, because samples were not tested in duplicate wells as per the manufacturer's recommendations, and repeat analysis of the samples gave negative results in some cases. Although these samples represent a small percentage of the overall test panel, these results emphasize the importance for diagnostic laboratories to consider retesting positive samples, especially if the samples are initially run in single rather than duplicate wells. Furthermore, it is important for veterinarians to consider the potential for false-positive results, particularly when tests are applied to low prevalence herds, in which the positive predictive value will be lower.

Because most studies have shown that many light-shedding infected cattle are seronegative, it has been presumed that as MAP infection progresses, fecal shedding precedes development of antibodies in most cases. The results of this study would support that conclusion. In this study, of 282 cows that were shedding MAP in feces, 174 were negative on every ELISA until culling, and fecal shedding preceded detection of antibody in an additional 50 (total of 224 of 282 cows, 79.4%, CI = 74.2%–83.9%). Initial detection was concurrent in 38 of 282 cows (13.4%, CI = 9.7%–18.0%) and was first on ELISA in 20 of 282 (7.1%, CI = 4.4%–10.7%). But of the 20 cows that were positive on ELISA before fecal culture, 8 were fecal-tested using only the less-sensitive sedimentation method.

Serum ELISA results that fluctuate from positive to negative are a potential source of frustration for veterinarians and producers. In a previous study, when 157 seropositive cattle were resampled 77 to 600 days after the first test, 62 (39.5%) had negative ELISA results on the second sample.⁶ The study reported here, although employing more tests per cow, showed similar results. In all, 31 of 76 (42.1%, CI = 29.6%–52.6%) cows that were tested again after the first ELISA-positive had a subsequent negative ELISA result. In the previous study, fecal culture status of the cows was not determined. In the present study, the rate

of reversion in culture-negative cattle 17/18 (94.9%, CI = 72.7%–99.8%) was much higher than that for culture-positive cattle 14 of 58 (24.1%, CI = 13.9%–37.2%).

There are several possible reasons for fluctuation in ELISA status. These could include false-positive results on the first test or false-negative results on the second test, or actual fluctuation in antibody production by the cow; a combination of these factors probably contributed to the results of this study. The 2 infected cows that had S/P ratios >1.0 and then became ELISA-negative were heavy fecal shedders, and it is possible that in some cows in later stages of the infection, antibody production may decline. Concentration of serum immunoglobulin G into the colostrum during the periparturient period could also result in a decline in serum antibody concentration,⁸ but calving dates were not available to test this hypothesis. In many of the uninfected cows, as noted above, it appears that the positive results were close to the cutoff, and inherent variation in the test procedure or in the cow's antibody concentration more readily resulted in the change in ELISA status. In the previous study, reversion to seronegative status on the second sample was more likely with lower S/P values on the first sample.⁶

One objective of the study was to determine whether knowledge of previous ELISA results could improve detection of infected cattle with S/P results below the cutoff. A previous study demonstrated that cattle with OD values just below the cutoff point still had a likelihood ratio of 15× for infection.¹ Our hypothesis was that as infected cattle begin to produce antibodies, the rise in antibody levels could be detected before the level of antibodies exceeded the test cutoff. To that end, the change in S/P for each sequential test, as well as the maximum change in S/P over all tests was compared for culture-positive and culture-negative cows. Unfortunately, neither sequential Δ S/P nor maximum Δ S/P was able to discriminate well between the 2 groups of cattle, as evidenced by the low area under the ROC curves. An area under the curve of 1.0 represents a test with perfect discrimination, and an area of 0.50 represents a test that discriminates no better than chance alone.⁵ Thus, the Δ S/P method, with an area under the ROC curve of 0.54, discriminates poorly between culture-negative and culture-positive cows. For example, if animals with a rise in S/P > 0.1 were considered positive, 51 seronegative culture-positive cows would have been identified. However, 44 culture-negative cows would have also been identified as positive. With a more stringent cutoff, using a rise in S/P > 0.20, only 12 additional culture-positive cows are identified as positive, and 7 culture-negative cows would also be identified. From these results, it appears

that comparing current ELISA results with previous results provides minimal advantage to simply comparing the current result to the S/P ratio cutoff point of 0.25 to determine serologic status of individual animals.

The results obtained for this study, which employed a single, commercially available ELISA test, may not be applicable for other assays that employ different antigens or differ otherwise in performance. However, agreement between the ELISA kit used in this study and 4 other commercially available ELISA assays was 80.4% to 94.0%.³ Clearly, regardless of which ELISA is employed, sensitivity is best for detection of heavy fecal shedders. Fecal culture detects infected animals earlier in the course of disease, but ELISA does identify those animals that are shedding the large numbers of MAP into the environment. The choice of whether to use ELISA, fecal culture, or a combination of detection methods (or no testing) in a Johne's disease control program depends on herd management, objectives, and economic considerations.

In summary, serial serum sampling, while increasing the sensitivity to detect infected cattle by ELISA, also increases the chance of a false-positive result. The change in S/P ELISA results over time was not a useful predictor of infection status in seronegative cows. When diagnostic laboratories apply the commercially available ELISA using a single sample well per serum sample, positive results should be verified by repeat testing to minimize the likelihood of a false-positive result.

Acknowledgements

The authors wish to thank Elizabeth Aksim, Anne Monson, and Alison Wolfram for technical assistance with this project. This study was supported by a grant from Veterinary Services, USDA-APHIS (Grant 03-9100-0796-GR) and the Pennsylvania Department of Agriculture.

Sources and manufacturers

- a. HerdChek® *Mycobacterium paratuberculosis* antibody ELISA Kit, IDEXX Laboratories, Westbrook ME.
- b. Parachek®, Biocor Animal Health, Omaha, NE.

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