

Association of fecal shedding of mycobacteria with high ELISA-determined seroprevalence for paratuberculosis in beef herds

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Objective—To evaluate the seroprevalence of paratuberculosis by use of 2 commercial ELISAs in association with prevalence of fecal shedding of mycobacteria within beef cattle herds.

Design—Cross-sectional field study.

Animals—Six beef herds (affected herds; 522 cattle) with and 3 geographically matched herds (181 cattle) without high seroprevalence of paratuberculosis.

Procedures—Blood and fecal samples were collected from adult cattle and assessed for serum anti-*Mycobacterium avium* subsp *paratuberculosis* (MAP) antibodies with 2 commercial ELISA kits and submitted for bacterial culture for MAP and environmental bacteria (termed environmental mycobacteria) via a radiometric method, respectively. Species of mycobacterial isolates were identified, and sensitivities and specificities of the 2 ELISAs were compared.

Results—Compared with comparison cattle, cattle from affected herds were 9.4 times as likely to have environmental mycobacteria isolated from feces. Among the 6 affected and 3 comparison herds, the proportions of cattle shedding environmental mycobacteria were 0.225 (range, 0.1 to 0.72) and 0.04 (range, 0 to 0.06), respectively. Although relative MAP-detection specificities (compared with bacterial culture of feces) were different between the 2 ELISAs, sensitivities were not. Nine environmental mycobacterial species were identified from participating herds. All affected herds apparently had ≥ 1 bovid infected with MAP, although MAP was not isolated from any cattle in comparison herds.

Conclusions and Clinical Relevance—In beef herds with persistently high rates of false-positive ELISA results, which may be associated with recovery of environmental mycobacteria from feces, organism detection via bacterial culture of feces or PCR assay should direct paratuberculosis control measures. (*J Am Vet Med Assoc* 2007;230:890–895)

Enzyme-linked immunosorbent assays for serum antibodies against MAP have been used extensively for purposes of monitoring and control of paratuberculosis in cattle because they are inexpensive and widely available, provide results rapidly, and have sufficient sensitivity and specificity for use as a screening tool. Early-generation ELISAs for paratuberculosis had low specificity, but the development of the absorbed ELISA that incorporates *Mycobacterium phlei* antigens to remove cross-reactive antibodies has improved ELISA specificity substantially.¹

The 2 ELISAs for paratuberculosis that are most widely used in the United States have reported specificities of 95.3% and 99.7% when used to evaluate a large

ABBREVIATIONS

MAP	<i>Mycobacterium avium</i> subsp <i>paratuberculosis</i>
CI	Confidence interval

number of well-characterized samples.² However, within some herds, the apparent false-positive result rate can be substantially higher than that expected.^{3,4} Exposure to certain mycobacteria has been shown to cause false-positive reactions in intradermal tuberculin tests in cattle.⁵⁻⁷ In a long-term study⁸ of US Navy recruits performed during the middle of the past century, it was shown that people from certain geographic regions had a greater probability of yielding positive results in intradermal tuberculin tests. By use of PPD-S (the standard tuberculin purified-protein derivative) as well as PPD-B (derived from a *Mycobacterium intracellulare* isolate), extensive mapping of the results indicated a high rate of positive intradermal tuberculin test results among people from the southern part of the United States. Reaction to PPD-B was particularly prevalent in residents of South Texas and the Coastal Bend region.⁸ Several environmental mycobacteria have been isolated from coastal and inland waterways on the Atlantic and Gulf Coast of the United States.⁹ The regional distribution of these organisms corresponds with the regional distribu-

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tion of false-positive tuberculin test results identified in the US Navy recruit study.

On the basis of our clinical and laboratory experiences, we have formed a conclusion that there can be an apparently high rate of MAP infection, as determined serologically, among cattle in certain herds despite the facts that those animals do not develop clinical disease and MAP organisms are not subsequently isolated from them. Non-MAP mycobacteria were isolated from the feces of cattle in some of these herds. We hypothesized that environmental mycobacteria (ie, non-MAP organisms shed in feces) were able to induce an immune response that cross-reacted with antigens in the ELISAs for paratuberculosis. The purpose of the study reported here was to evaluate the seroprevalence of paratuberculosis by use of 2 commercial ELISAs in association with prevalence of fecal shedding of mycobacteria within herds of beef cattle.

Materials and Methods

Herd selection—Beef cattle herds in which the seroprevalence of paratuberculosis was $\geq 15\%$ and comparison herds without high seroprevalence of paratuberculosis (determined on the basis of results of previous testing by use of an appropriate serum ELISA) were chosen for study. The study was approved by the College of Veterinary Medicine Clinical Research Review Committee at Texas A&M University, and informed consent was obtained from the owners prior to the participation of each herd. Samples were collected from all herds except 1 (herd 1-A) by the investigators as part of a prevalence survey⁴ performed 3 years prior to the present study. Herds were defined as affected if the ELISA-determined seroprevalence of paratuberculosis was $\geq 15\%$ without isolation of MAP or if ELISA results were considered inconsistent with incidence of clinical cases of paratuberculosis identified by the herd veterinarian. Comparison herds were selected on the basis of geographic proximity to an affected herd and prior ELISA results indicative of low seroprevalence of paratuberculosis. Selection of the herds was performed without knowledge of the breed. All of the affected herds were *Bos indicus* (5) or *B indicus* composite (1) breeds, whereas all of the control herds were *Bos taurus* breeds.

Sample collection and testing—Collection of samples of blood via venipuncture of the coccygeal vein and feces from the rectum was performed by private practice veterinarians or by one of the investigators (AJR). In 5 herds, all females that had previously given birth to a calf were evaluated. In 1 herd, the bred heifers were also tested. All bulls that were ≥ 2 years old in all herds were evaluated. Both the blood and fecal specimens were packed in ice and shipped by overnight express courier to the John's Testing Center in Madison, Wis, within 24 hours of collection. Serum was

separated and analyzed by use of 2 commercial ELISA kits (ELISA-A^a and ELISA-B^b). Microbial culture of feces was performed via radiometric culture methods in a modified commercial liquid medium^c as previously described.¹⁰ Briefly, the medium was supplemented with mycobactin J, egg yolk suspension, and antimicrobials. Fecal samples were decontaminated with 1.0% hexadecylpyridinium chloride and concentrated by filtration. The resulting filter membrane was placed into radiometric culture medium and evaluated weekly for growth by use of an ionization detector.⁴ For acid-fast organisms, a PCR assay for the *IS900* gene insertion element was used to identify MAP. Mycobacterial isolates that were negative for *IS900* were termed environmental mycobacteria and identified via high-performance liquid chromatography of extracted mycolic acids¹¹; the maximum number of *IS900*-negative mycobacterial isolates submitted for identification was 10 isolates/herd.

Statistical analysis—By use of available software,^e prevalence ratios with exact 95% CIs were calculated to quantify the association between herd type (affected or comparison) and the isolation of environmental mycobacteria from feces of cattle. A prevalence ratio was calculated as the proportion of cattle in affected herds yielding an environmental mycobacterial isolate in their feces divided by the corresponding proportion of cattle from the 3 comparison herds. The proportions of bovids from which environmental mycobacteria were isolated were also compared between affected and comparison herds by use of a Mann-Whitney test in commercially available software.^f The nonparametric Mann-Whitney test was necessary because the simple prevalence ratio

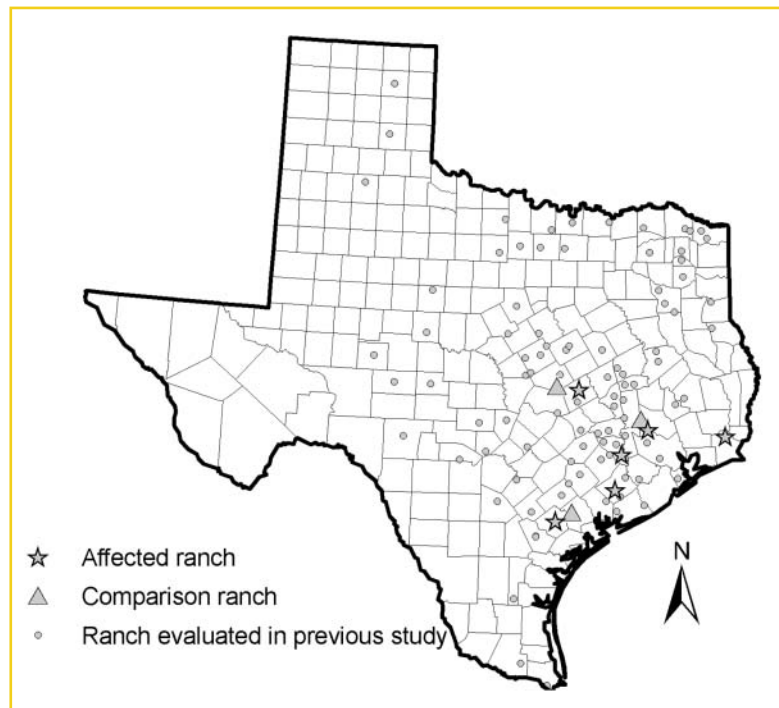


Figure 1—Distribution of ranches in Texas that were studied previously,⁴ and ranches that had unusually high seroprevalence for paratuberculosis (affected herds) and geographically matched ranches that had low seroprevalence for paratuberculosis (comparison herds) in a study of fecal shedding of mycobacteria.

does not account for the clustering of animals within herds. Thus, a statistically conservative approach was taken to compare median prevalences at the herd level by use of a nonparametric test. Sensitivities and specificities of the 2 ELISAs were estimated relative to results of bacterial culture of feces and compared by use of a McNemar χ^2 test.¹² A value of $P < 0.05$ was considered significant.

Results

Six affected herds (522 cattle) met the criteria for enrollment, and the owners agreed to participate in the study. Three geographically matched comparison herds (181 cattle) were chosen that were 24, 35, and 42 km (approx 15, 22, and 26 miles) from an affected herd (Figure 1). Cattle from affected herds

Table 1—Within-herd prevalence estimates for paratuberculosis determined on the basis of results of 2 ELISAs (A and B) and isolation of MAP and environmental (non-MAP) mycobacteria on 9 Texas beef cattle ranches during 2003.

Herd	No. of cattle in herd	No. of cattle with positive ELISA-A results (%)	No. of cattle with positive ELISA-B results (%)	No. of cattle yielding MAP from feces (%)	No. of cattle yielding environmental mycobacteria from feces (%)
Affected*					
1-A	132	30 (23)	5 (4)	2 (2)	16 (12)
2-A	89	41 (46)	8 (9)	6 (7)	9 (10)
3-A	65	22 (34)	7 (11)	1 (2)	10 (15)
4-A	60	45 (75)	24 (40)	4 (7)	43 (72)
5-A	126	39 (31)	14 (11)	4 (3)	38 (30)
6-A	50	5 (10)	1 (2)	1 (2)	18 (36)
Comparison†					
1-C	45	2 (4)	1 (2)	0	2 (4)
2-C	52	2 (4)	0	0	3 (6)
3-C	84	5 (6)	0	0	0

*A herd was classified as affected when $\geq 15\%$ of the cattle yielded positive results via ELISA-A on previous evaluation and either MAP was not isolated from microbial cultures of feces or there was no paratuberculosis problem in the herd as determined by the herd veterinarian (herd 1-A). †A herd from a similar geographic location as an affected herd was classified as a comparison herd when no cattle yielded positive results via ELISA-A on previous evaluation.

Table 2—Results of microbial culture of feces with regard to environmental (non-MAP) mycobacteria from beef cattle in 6 affected* and 3 comparison† herds.

Herd group	No. of samples with environmental mycobacterial isolates	No. of samples with no environmental mycobacterial isolates‡	Total No. of samples
Affected (522 cattle)	134	380	514
Comparison (181 cattle)	5	175	180
Total (703 cattle)	139	555	694

Culture results were not available for 4 bovids in herd 1-A, 4 bovids in herd 4-A, and 1 bovid in herd 3-C. ‡Includes bacterial cultures of feces from which no mycobacteria (MAP or environmental organisms) were isolated and those in which bacterial and fungal contamination precluded further characterization. See Table 1 for remainder of key.

Table 3—Relative sensitivity (%) and specificity (%) of 2 serum ELISAs for paratuberculosis in a cross-sectional study of beef cattle in 6 affected* and 3 comparison† herds.

ELISA	Herd group					
	All		Affected		Comparison	
	Sensitivity (95% CI)	Specificity (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
A	71.4 (44.5–90.2)	71.1§ (66.8–75.2)	71.4 (44.5–90.2)	71.1§ (66.8–75.2)	NA	95.0§ (91.0–97.5)
B	57.1 (31.1–80.4)	95.2 (93.3–96.7)	57.1 (31.1–80.4)	93.4 (90.8–95.5)	NA	99.4 (97.3–100.0)

Sensitivity was calculated relative to isolation of MAP from a single fecal sample collected on the same day. Herd 4-A was not included in these calculations because of the inability to match fecal and blood samples because of sample identification errors. §Estimate for ELISA-A was significantly ($P < 0.05$) different from the estimate for ELISA-B within a herd group. NA = Not applicable. See Table 1 for remainder of key.

were more likely to be seropositive for paratuberculosis and have environmental mycobacteria isolated from their feces (Table 1). For ELISA-A, the median proportion of cattle with positive ELISA results in the affected herds was 0.325 (range, 0.10 to 0.75) and in the comparison herds was 0.04 (range, 0.04 to 0.06). For ELISA-B, the median proportion of cattle with positive ELISA results in the affected herds was 0.1 (range, 0.02 to 0.4) and in the comparison herds was 0 (range, 0 to 0.02). The proportion of cattle for which environmental mycobacteria were isolated from feces among the affected herds was 0.225 (range, 0.1 to 0.72) and among the comparison herds was 0.04 (range, 0 to 0.06). This difference was significant ($P = 0.02$; Mann-Whitney test). The proportion of cattle for which MAP was isolated from the feces among the affected herds was 0.025 (range, 0.02 to 0.07); no MAP was isolated from the comparison herds. Data were cross-classified by herd type, and the prevalence ratio was 9.4 (95% CI, 3.9 to 22.6; Table 2). Compared with results of microbial culture of feces, the relative sensitivities of the 2 ELISAs were not different, but specificities were significantly different (Table 3). Twenty-one percent (19/91) of cattle that were shedding environmental mycobacteria in feces yielded positive results via ELISA-A only at the time of sample collection; the analogous proportion for ELISA-B was 8% (7/91 bovinds). On 1 ranch, 39 cattle that were shedding non-MAP organisms and 2 bovinds that were shedding both MAP and environmental mycobacteria in their feces could not be linked to their individual serologic results. Nine environmental mycobacterial species were identified (Table 4). Sixteen non-MAP isolates were classified as *Mycobacterium avium* complex, and 1 was classified as *Mycobacterium terrae* complex. The *M avium* complex includes *Mycobacterium scrofulaceum*, *M avium* subsp *avium*, and *M intracellulare*.

Discussion

Results of the present study have indicated an association between high seroprevalence for paratuberculosis (determined via ELISA) in beef herds and the frequency of isolation of non-MAP mycobacteria from the feces of those cattle. Therefore, exposure to mycobacteria in the environment may explain the occurrence of the unusually high rate of MAP-positive ELISA results among cattle in some herds from which the rate of MAP isolation is not correspondingly high. The isolation of environmental mycobacteria was 9.4 times as likely from cattle in affected herds, compared with the comparison herds. The CI for this ratio is not valid (too narrow) because it does not account for the clustering inherent in these data. However, the point estimate is the same, and the more conservative nonparametric test determined that seroprevalence of paratuberculosis (determined via ELISA) was not the same in affected and comparison herds in our study.

All affected herds had at least 1 bovid that was shedding MAP in feces. Therefore, not all of the positive ELISA results were false-positive results. The reported sensitivity of bacterial culture of feces for detection of MAP infection among mature cattle is approximately 40%.¹³ In the present study, 18 cattle yielded positive results for MAP via bacterial culture of feces. This implies a true infection rate of approximately 45 cattle. The sensitivity of either ELISA-A or ELISA-B for detection of cattle with MAP (determined via bacterial culture of feces) was estimated to be approximately 30%; therefore, one would have expected 30% of 45 or approximately 14 true-positive ELISA results from cattle in this population. In fact, 182 and 59 cattle were identified as MAP positive by use of ELISA-A and ELISA-B, respectively. Therefore, even though the affected herds were infected with MAP at low levels, it is not reasonable to suspect that the positive ELISA results in these herds

Table 4—Herd-level prevalence of environmental mycobacteria in feces obtained from beef cattle in 6 affected* and 3 comparison† herds.

Variable	Herd group									Total
	Affected						Comparison			
	1-A	2-A	3-A	4-A	5-A	6-A	1-C	2-C	3-C	
No. of cattle from which feces were collected	128	89	65	56	126	50	45	52	83	694
No. of environmental isolates	16	9	10	43	38	18	2	3	0	139
No. of environmental isolates for which identity was not determined	7	0	0	28	21	8	0	0	0	64
No. of environmental isolates for which identity was determined	9	9	10	15	17	10	2	3	0	75
No. of isolates by species										
<i>Mycobacterium asiaticum</i>	2	0	0	0	5	0	0	0	0	7
<i>Mycobacterium avium</i>	0	4	0	2	0	0	0	0	0	6
<i>Mycobacterium avium</i> complex	0	1	2	4	5	2	2	1	0	17
<i>Mycobacterium celatum</i>	0	1	0	0	0	4	0	1	0	6
<i>Mycobacterium gordonae</i>	0	0	0	0	0	0	0	1	0	1
<i>Mycobacterium intracellulare</i>	0	1	0	1	1	0	0	0	0	3
<i>Mycobacterium kansasii</i>	0	1	0	0	0	2	0	0	0	3
<i>Mycobacterium mucogenicum</i>	2	0	0	0	0	0	0	0	0	2
<i>Mycobacterium nonchromogenicum</i>	1	0	0	0	0	0	0	0	0	1
<i>Mycobacterium scrofulaceum</i>	4	0	8	8	6	2	0	0	0	28
<i>Mycobacterium terrae</i> complex	0	1	0	0	0	0	0	0	0	1

||Includes all non-MAP mycobacteria.
See Table 1 for remainder of key.

were attributable solely to paratuberculosis. False-positive results were clustered in affected herds. Assuming a specificity of 95% and 99%, respectively, for ELISA-A and ELISA-B, one would expect to obtain approximately 26 false-positive test results with ELISA-A and 5 false-positive test results with ELISA-B among the 522 cattle in the affected herds. Clearly, in the affected herds, these 2 ELISAs did not perform as expected. However, if we assume that the unaffected herds were free of MAP infection, the specificities of the ELISAs were similar to those reported previously.² The false-positive result rates of the 2 ELISAs in the present study were not similar. This difference may be explained by differences in the antigens and reagents between the 2 assays; however, these technical details are proprietary. Also, the manufacturers of these tests make periodic changes in the antigens and reagents. Therefore, the results obtained in years prior to and following the study may be different.

In 4 of the 5 herds from which samples had also been analyzed 3 years earlier,⁴ the seroprevalences of paratuberculosis (determined via the same serum ELISA) at this time were higher. The differences with time may reflect a more intensive sampling strategy used in the present study (all mature cattle in study herds were evaluated, compared with 50 cattle/herd in the previous study⁴). Isolation of MAP from cattle in the affected herds was not unexpected in the present study. Clinical paratuberculosis had been reported in the past for some of the affected ranches in the present study, but no cases were ever confirmed via bacterial culture of feces. In the previous study,⁴ only fecal samples from cattle with positive results via ELISA were submitted for bacterial culture, and these samples were tested only after the feces had been frozen. Also, the laboratory and culture methods were not the same in the previous and present studies. Other factors that influence exposure of cattle to environmental mycobacteria may include weather or other environmental conditions. Furthermore, the ELISAs were performed in different laboratories for the previous and present studies.

Interference with results of intradermal tuberculin tests in humans by atypical (environmental) mycobacteria and isolation of those same types of mycobacteria from the environment have suggested a regional distribution of these organisms.^{8,9} The affected herds in the present study were located in the same regions where interference (ie, abnormally high rates of false-positive tests) with results of intradermal tuberculin testing in humans has been identified. However, the comparison herds were also located within this same region. There appears to be a clustering effect within herds even within the affected region; this could be attributable to topographic features, dietary factors, or unidentified management factors among ranches. However, such differences were not apparent among the herds and ranches enrolled in our study. Interestingly, the breed distribution in the affected and comparison herds of our study was not the same. No consideration was given to breed when the comparison herds were selected; in fact, the investigator who derived the list of potential comparison herds was not aware of the breed of cattle in the selected herds. Nevertheless, 5 of the 6 affected herds were comprised of Brahman (*B indicus*) cattle,

and 1 affected herd was comprised of Beefmaster cattle, whereas all 3 comparison herds were comprised of *B taurus* breeds. The difference in the breed distribution between affected and comparison herds might be attributable to chance, or there may be a breed predisposition associated with *B indicus* for harboring or shedding non-MAP mycobacteria or for developing serum antibodies against environmental mycobacteria. A possible relationship between the exposure to and shedding of environmental mycobacteria and resistance or susceptibility to MAP infection has not been studied to the authors' knowledge.

The percentage of cattle that were shedding environmental mycobacteria and yielded positive results via ELISA-A (21%) or via ELISA-B (8%) at the time of sample collection was relatively small. This finding is consistent with our clinical experiences. The association between seroprevalence of paratuberculosis and prevalence of environmental mycobacteria in feces on a herd basis is high, whereas the association between seroprevalence of paratuberculosis and shedding of those organisms in feces on an individual cow basis is not. This suggests that the presence of environmental mycobacteria in feces of cattle is a pass-through phenomenon and not indicative of infection. When non-MAP mycobacteria are in the environment, the organisms may be just as likely to be found in the feces of a bovid with a positive result to an ELISA for paratuberculosis as they are in the feces of a bovid with a negative test result. The reason for the response of some cattle to environmental mycobacteria serologically, the duration of that serologic response, and the reason for the presence of environmental mycobacteria in the feces of certain cattle are unknown.

Mycobacterium scrofulaceum was the most prevalent of the non-MAP organisms isolated in our study. Falkingham et al⁹ reported a high rate of isolation of *M scrofulaceum* from aquatic environments along the southern coastal area including Texas, and it is possible that surface water is a source of exposure for resident cattle. In the present study, the second most prevalent isolate was *M avium* complex. Some isolates were reported simply as *M avium* complex because of the limitations of high-performance liquid chromatography to clearly differentiate among members of the *M avium* complex. Included in this category are some isolates of *M avium*, *M scrofulaceum*, and *M intracellulare*, which could not be differentiated from one another. Therefore, the prevalence of some or all of these 3 species was underestimated. Experimental inoculation of cattle with the environmental mycobacteria *M avium*, *M scrofulaceum*, and *M intracellulare* is capable of eliciting serum antibody immune responses.^{5,14}

Overall, our data have indicated that some herds of beef cattle have a high rate of false-positive ELISA results for paratuberculosis. Furthermore, those false-positive results are associated with recovery of environmental mycobacteria from bovine feces. Therefore, when high rates of positive ELISA results for paratuberculosis are identified in herds with little or no clinical evidence of paratuberculosis or microbiological evidence of MAP infection, false-positive results attributable to exposure to environmental mycobacteria should be suspected.

Verification of the MAP infection rate within a herd should be attempted by use of a different ELISA or via microbial culture of feces. In herds with persistently high rates of false-positive ELISA results, organism-detection-based tests should be used to direct measures for paratuberculosis control.

- a. HerdChek, IDEXX Laboratories Inc, Westbrook, Me.
- b. ParaCheck, CSL/Biocr, Omaha, Neb.
- c. BACTEC-12B BD Diagnostic Systems, Franklin Lakes, NJ.
- d. BACTEC 460, Johnson Laboratories, Towson, Md.
- e. Epi Info, version 6.04, CDC, Atlanta, Ga.
- f. SPSS, version 11.5 for Windows, SPSS Inc, Chicago, Ill.

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