Association between caudal fold tuberculin test responses and results of an ELISA for *Mycobacterium avium* subsp *paratuberculosis* and mycobacterial culture of feces in tuberculosis-free dairy herds

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**Objective**—To evaluate associations between *Mycobacterium avium* subsp *paratuberculosis* (MAP) and caudal fold tuberculin (CFT) test results in cattle.

**Design**—Longitudinal and cross-sectional evaluations.

**Animals**—1 California (approx 3,600 cows) and 3 Colorado (approx 640, 1,190, and 1,480 cows) dairy herds considered free of *Mycobacterium bovis* infection.

**Procedures**—In the California herd, the association between CFT response and MAP status was determined with ELISA and mycobacterial culture of feces within 1 year before and after CFT testing. The association between CFT and MAP status in all herds was modeled with mixed-effects logistic regression.

**Results**—In the California herd, significantly higher odds of being classified as suspect by CFT were found for cows with results of MAP ELISA negative before and positive after CFT testing (OR, 5.6) and cows positive before and after CFT testing (OR, 8.1). Higher odds were found for cows positive for mycobacterial culture of feces before and negative for culture after CFT testing (OR, 4.6) and cows negative for mycobacterial culture of feces before and positive for culture after CFT testing (OR, 13.2). All herds had higher odds of being classified as suspect by CFT testing for cows with positive results for ELISA (OR, 2.9) or mycobacterial culture of feces (OR, 5.0), compared with cows with negative results of the same tests.

**Conclusions and Clinical Relevance**—A strong association was found between positive MAP test results and being classified as a suspect by CFT testing. Within-herd MAP prevalence may affect specificity of CFT testing for tuberculosis in cattle. (*J Am Vet Med Assoc* 2014;244:582–587)
Mycobacteria from the Mycobacterium avium complex, including MAP, have been implicated as a cause of cross-reactivity for the CFT test. Mycobacterium avium subsp. paratuberculosis is the etiologic agent of paratuberculosis (Johne's disease), a chronic gastrointestinal disease in ruminants. Prior observational and experimental studies have suggested that exposure to MAP vaccines and other organisms of the Mycobacterium complex may cause results for the CFT test that would result in classification of the animal as a suspect. Findings from studies on the ability of M. bovis infection to interfere with the ability to diagnose MAP infection (and with MAP infection to interfere with the ability to diagnose M. bovis infection) indicate that cross-reactions affect test results in both directions. Although there was no evidence of an association between CFT test results and MAP status in another study, it is possible that exposure to other environmental mycobacteria can sensitize cattle to bovine tuberculin and induce a nonspecific response to the CFT test. Furthermore, serologic diagnosis of MAP in some herds may also be complicated by false-positive ELISA results because of exposure of cattle to high numbers of environmental mycobacteria. The objective of the study reported here was to evaluate the association between results of an MAP ELISA and mycobacterial culture of feces with CFT test responses in 4 tuberculosis-free dairy herds.

**Materials and Methods**

**Animals**—Herd differences were selected on the basis of participation in the National Johne's Disease Demonstration Herd Project, test results of routine slaughter surveillance for tuberculosis, intensive housing systems, and availability of CFT and MAP test results for individual cows. Of 58 dairy herds that participated in the National Johne's Disease Demonstration Herd Project, 4 (1 in California and 3 in Colorado) met the inclusion criteria for the present study. The California dairy herd (herd 1) consisted of Jersey cattle (approx 2,900 cows in 2004 and 3,600 cows in 2008) and was located in the central valley of the state. Cows were housed in freestalls bedded with composted manure. Cows were fed a total mixed ration and had no access to pasture. All replacement females were born on the premises, and approximately 50% were reared off-site at calf ranches during the study period. Two of the California dairy herds consisted of Holstein cattle (1,188 cows in herd 2 and 1,480 cows in herd 3). The remaining California herd (herd 4) consisted of 637 cows, of which approximately 75% were Holsteins and 25% were Jerseys. The Colorado herds housed cows in freestalls with access to drylots but no access to pasture. Herds 2 and 3 had home-raised replacements and raised heifers on-site. Herd 4 obtained replacements from various sources, and female cattle born at the dairy were reared off-site at a heifer ranch.

**CFT testing**—The CFT test was performed on all cows at the California dairy herd in April 2004 and March 2008 as part of epidemiological investigations following the detection of tuberculosis in other California dairies. In Colorado, dairy herds were tested for tuberculosis every 3 years (a state requirement that is more stringent than federal regulatory requirements). The CFT test was performed on cattle of herd 2 in April and May 2005 and July 2008, cattle of herd 3 in October 2005 and November 2008, and cattle of herd 4 in November 2005 and April 2009. Briefly, 0.1 mL of bovine PPD tuberculin was injected intradermally into the caudal fold at the base of the tail. The injection site was visually inspected and digitally palpated 66 to 78 hours after PPD injection. A cow with swelling at the site of injection was classified as a suspect. As reported by USDA APHIS, the CFT test was performed on cows classified as suspects by CFT testing; the CCT test was performed within 10 days after the CFT injection. All cows from the 4 herds had negative results for any CCT tests conducted following CFT tests.

**Serum ELISA for MAP**—A blood sample was collected from the coccygeal vein of each cow of the 4 herds into 10-mL tubes that did not contain anticoagulant. Samples were allowed to clot, and serum was tested with a commercial ELISA kit. Optical densities were measured and reported as sample-to-positive ratios, which were determined as follows: sample-to-positive ratio = (optical density of blood sample – optical density of negative control sample)/(optical density of positive control sample – optical density of negative control sample). A sample-to-positive ratio ≥ 0.25 was considered as a positive result, as recommended by the manufacturer of the test kit.

Since 2003, cows from the California dairy herd were routinely tested for MAP with the ELISA (serum samples) and mycobacterial culture of feces at the onset of their nonlactating (dry) period. In addition, whole-herd ELISA for MAP were conducted on serum samples obtained in March 2003, October 2007, and October 2008 and included all dry and lactating cows in the herd on the test date.

Colorado herds were tested yearly for MAP with the ELISA (serum samples) and mycobacterial culture of feces. Testing was initially conducted in cows that had calved at least once; subsequently, samples were collected from cows at various times throughout the production cycle, depending on the owner's preference for a testing strategy. In herd 2, all cows that were at 90 days of gestation were tested with mycobacterial culture of feces followed by an ELISA at the end of lactation. In herd 3, all cows were tested with mycobacterial culture of feces and an ELISA at the end of lactation. In herd 4, mycobacterial culture of feces and an ELISA were performed quarterly; cows between 90 and 180 days of lactation on test day were included.

**Mycobacterial culture of feces for MAP**—At the time of blood collection, 25 g of feces was obtained from the rectum of each cow. A new obstetric sleeve was used for each cow. Mycobacterial culture of feces was performed as a whole-herd test or on a subset of the herd at various production stages.

Samples from the California dairy herd were submitted to the California Animal Health and Food Safety Laboratory, Tulare, Calif, where an automated liquid culture system was used. Briefly, samples were mixed with a brain-heart infusion broth with antimicrobials followed by incubation in egg yolk at 37°C for 6 weeks.
At the end of incubation, all samples were screened for acid-fast bacilli by Ziehl-Neelsen staining and a PCR assay that amplified a segment of the IS900 gene.\(^{12}\) Results were reported as the number of days until a positive result and were further categorized as positive (positive result at \(\leq 42\) days after initiation of culture) or negative (no positive result for \(> 42\) days after initiation of culture).

Samples from the 3 Colorado herds were submitted to the Rocky Mountain Regional Animal Health Laboratory, Denver, where another automated liquid culture system\(^*\) was used. Fecal samples were mixed with a brain-heart infusion broth with antimicrobials followed by incubation in egg yolk at 37°C for 7 weeks. When the system signaled that a sample was positive twice during incubation, the samples were tested for the presence of acid-fast bacilli by Ziehl-Neelsen staining and a PCR assay that amplified a segment of the heat-shock protein gene. Samples were considered MAP positive if the system signaled twice before the end of the 49-day incubation period and there were positive results for both acid-fast staining and the PCR assay. If the system signaled twice before the end of the 49-day incubation period and there were acid-fast bacilli detected with acid-fast staining, but there were negative results for the PCR assay, the organisms were considered to be acid-fast organisms other than MAP.

**Statistical analysis**—The association between sequential MAP test results and a cow being classified as a suspect on CFT testing was investigated for MAP testing conducted within 12 months before and after CFT testing. The OR and 95% CI were computed separately for the serum ELISA and mycobacterial culture of feces before and after CFT testing. The OR was computed as the ratio of the odds of a cow being classified as a suspect by CFT testing were higher in a cow with negative results for the ELISA or mycobacterial culture of feces before and after CFT testing (reference category). On the other hand, an OR \(< 1\) indicated the odds of a cow being classified as a suspect by CFT testing were higher in a cow with negative results for the ELISA or mycobacterial culture of feces before and after CFT testing than the odds of a cow in a specific MAP sequential testing category.

To assess agreement between results of the MAP ELISA and mycobacterial culture of feces, Cohen \(\kappa\) coefficients,\(^{13}\) which measure the agreement beyond chance, were calculated. Cows that had a record of mycobacterial culture of feces and the ELISA during the 12-month period before and after CFT testing were used for this calculation.

Mixed-effects logistic regression was used to model CFT test results and was based on binary test results (positive or negative) of both the MAP ELISA and mycobacterial culture of feces. Lactation number and the most recent results for the MAP ELISA and mycobacterial culture of feces obtained during the 12-month period before and after CFT testing, regardless of whether the most recent result was obtained before or after CFT testing, were evaluated as fixed effects. Herd was modeled as a random effect to account for lack of independence of observations within each herd. All fixed effects and 2-way interactions were tested for significance (\(P < 0.05\)), and only significant terms were retained in the model. The OR and 95% CI were computed for parameter estimates in the final model. Significance of a random effect was evaluated with a likelihood ratio test that compared the model with and without inclusion of the random effect. Model goodness-of-fit was assessed by evaluation of extreme values in the Pearson residual histogram. Statistical analyses were conducted with statistical software.\(^4\) To evaluate whether the estimated OR was dependent on the time window for MAP tests results, the analysis was repeated with those records obtained during a 6-month period that encompassed the CFT testing.

**Results**

**Animals**—The number of cows that had records with complete information for lactation number and MAP ELISA results during the 12-month period before and after CFT testing was 4,924, 2,054, 1,869, and 777 for herds 1, 2, 3, and 4, respectively. The percentage of cows with positive results for the MAP ELISA included in the analysis was 4.0%, 3.8%, 7.8%, and 7.9% for herds 1, 2, 3, and 4, respectively. Results of mycobacterial culture of feces obtained during the 12-month period before and after CFT testing were available for 4,093, 1,565, 363, and 681 cows for herds 1, 2, 3, and 4, respectively. Results of mycobacterial culture of feces were positive for 6.7%, 2.4%, 2.0%, and 5.1% cows in herds 1, 2, 3, and 4, respectively.

A total of 6,258 cows (4,082, 1,474, 196, and 506 for herds 1, 2, 3, and 4, respectively) had both an MAP ELISA and mycobacterial culture of feces during the 12-month period before and after CFT testing as well as complete information on lactation number and were included in the logistic regression analysis. Cohen \(\kappa\) coefficient between results of the MAP ELISA and
mycobacterial culture of feces was 0.32 (95% CI, 0.25 to 0.39), which indicated a low level of agreement beyond chance. A total of 1.7%, 0.4%, 3.4%, and 2.0% of cows were classified as suspects during CFT testing for herds 1, 2, 3, and 4, respectively. None of the cows classified as suspects during CFT testing had positive results for the CCT test, which provided additional evidence that the 4 herds were free of M bovis infection.

Association between results of sequential MAP ELISA and mycobacterial culture of feces before and after CFT testing—In the California dairy herd, 1,585 (32.2%) cows had results of 2 consecutive ELISAs available during the 12-month period before and after CFT testing. The median interval between an MAP ELISA and CFT testing was 56 and 198 days before and after CFT testing, respectively. For cows that had positive ELISA results before and after CFT testing, the odds of being classified as suspects by CFT testing were 8.1 (95% CI, 2.9 to 22.7) times as high as those for cows with negative ELISA results before and after CFT testing. Cows with negative ELISA results before CFT testing that had positive ELISA results after CFT testing had higher odds (OR, 5.6; 95% CI, 2.3 to 13.6) of being classified as a suspect by CFT testing than cows with negative ELISA results before and after CFT testing (Table 1).

A separate analysis was performed to evaluate whether the OR differed for the MAP ELISA status before or after CFT testing. Cows with positive ELISA results before CFT testing had higher odds (OR, 3.9; 95% CI, 1.4 to 10.3) of being classified as a suspect by CFT testing, compared with the odds for cows with negative ELISA results before and after CFT testing. Cows with negative ELISA results before CFT testing had higher odds (OR, 6.2; 95% CI, 2.7 to 13.2) of being classified as a suspect by CFT testing, compared with the odds for cows with negative ELISA results after CFT testing.

In the California dairy herd, results of mycobacterial culture of feces during the 12-month period before and after CFT were available for 572 (11.6%) cows. The median interval between mycobacterial culture of feces for MAP and CFT testing was 235 and 153 days before and after CFT testing, respectively. Cows that had positive results for mycobacterial culture of feces before and after CFT testing had higher odds (OR, 6.2; 95% CI, 0.7 to 54.0) of being classified as a suspect by CFT testing, compared with the odds for cows that had negative results for mycobacterial culture of feces before and after CFT testing; however, these odds did not differ significantly. The strongest association (OR, 13.2; 95% CI, 3.2 to 55.1) was for cows that had negative results for mycobacterial culture of feces before CFT testing and positive results for mycobacterial culture of feces after CFT testing (Table 1).

### Discussion

In the present study, there was a significant association between CFT test responses and results of an MAP ELISA and mycobacterial culture of feces in 4 dairy herds considered free of tuberculosis (on the basis of epidemiological evidence, including slaughter surveillance of culled cows and negative CCT test results).

<table>
<thead>
<tr>
<th>MAP test status</th>
<th>ELISA</th>
<th>Mycobacterial culture of feces</th>
</tr>
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<tbody>
<tr>
<td>Before CFT testing</td>
<td>After CFT testing</td>
<td>No. of cows classified as suspect by CFT testing</td>
</tr>
<tr>
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<td>Positive</td>
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</tr>
<tr>
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<td>19</td>
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<tr>
<td>Total</td>
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<td>31</td>
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A sample-to-positive ratio > 0.25 was considered a positive result, as recommended by the manufacturer of the ELISA. Results of liquid mycobacterial culture of feces were categorized as positive (positive result at ≤ 42 days after initiation of culture) or negative (no positive result for > 42 days after initiation of culture).

Reference = Reference category for comparison of ORs. — = Not applicable.
The association between being classified as a suspect by CFT testing and sequential MAP results, as assessed by use of an ELISA and mycobacterial culture of feces, was evaluated to determine whether temporal changes in MAP test status would affect CFT test results. In addition, the association of an MAP test result (mycobacterial culture of feces and ELISA) and the odds of being classified as a suspect by CFT testing was evaluated.

In several studies, investigators have found that *M. avium* and *M. bovis* share proteins that induce cross-reactions in diagnostic tests. This effect could result in biased imprinting memory of the T-lymphocyte response toward *M. avium* antigens, compared with the response to *M. bovis* antigens.15–17

The ELISAs for MAP have low sensitivity, which may be attributed in part to the facultative intracellular nature of the organism and the host immune response to MAP.10,18 Although specificity of the ELISA used in the present study has been reported as > 97%,19 the ELISA may yield false-positive results for cows exposed to mycobacteria other than MAP. For example, in another study,20% of cattle with natural *M. bovis* infection had false-positive results for the MAP ELISA. In the present study, some of the positive results for the MAP ELISA after CFT testing may have been induced by PPD inoculation.8

When mycobacterial culture of feces was used to evaluate MAP status of cows, there was a significant association for those cows that had positive results for mycobacterial culture of feces before CFT testing and negative results after CFT testing (OR, 4.6) and cows that had negative results for mycobacterial culture of feces before CFT testing and positive results after CFT testing (OR, 13.2). Although the odds for cows that had positive results for mycobacterial culture of feces before and after CFT testing (OR, 6.2) were not significant, there was only a single cow with positive CFT test results in this category, so the estimate for this particular category was imprecise. It may have been more difficult to interpret the outcome for longitudinal analysis of results of mycobacterial culture of feces than ELISA results because intermittent shedding of MAP is a well-recognized phenomenon, especially in cattle that shed low numbers of organisms.16

In the present study, 2 liquid culture systems were used for mycobacterial culture of feces for the Colorado and California herds. Both methods provide comparable recovery of mycobacteria in general, although MAP has not been specifically evaluated. Therefore, we assumed that these 2 methods had comparable sensitivity and specificity.

To assess the use of a 12-month period in our analysis, which can arguably be excessively long for the evaluation of potential interference or cross-reaction with antibody response and fecal shedding of MAP in a cow, we repeated the analysis with records obtained during the 6-month period before and after CFT testing. The OR for the association of CFT and MAP results was similar for both periods, but the CIs were wider for the 6-month analysis, likely because of the smaller number of records available. Results for culture of feces for MAP are poorly associated with MAP ELISA results,21–23 which is expected given that an ELISA detects antibodies and culture of feces detects the presence of the mycobacteria in feces. In the present study, a low level of agreement (Cohen κ, 0.32) between the 2 tests was found, which is consistent with results of other studies. The temporal patterns of antibody production and shedding of organisms differ depending on disease stage and individual animal responses. Therefore, it was not surprising that results of the ELISA and mycobacterial culture of feces were independent significant predictors of a suspect CFT result in the logistic regression model.

For the California dairy herd, records available for the longitudinal analysis represented only 32% (ELISA) and 11.6% (mycobacterial culture of feces) of the entire herd because of the analysis requirement for sequential results of the ELISA or mycobacterial culture of feces during the 12-month period before and after CFT testing. This loss of records was attributable to the fact that whole-herd CFT testing was not always performed within the 12-month period in which an MAP test was performed. Inclusion of a subsample of the herd could potentially have resulted in biased OR estimates if the sample differed from the overall herd with regard to the distribution of the MAP or CFT test results. An ELISA was performed on serum samples obtained from all cows at the end of lactation and also for 3 whole-herd tests independent of prior MAP test results. Similarly, a CFT test was conducted on all cows in the herd. The owner did not cull cows on the basis of MAP test results. Therefore, it is reasonable to assume that there was no substantial selection bias in the sample of cows included in the longitudinal analysis.

When interpreting specificity estimates of the CFT test for the detection of tuberculosis in cattle, other herd characteristics that affect the accuracy of CFT testing should be considered. In another study, tuberculosis testing in paratuberculosis-free herds resulted more frequently in false-positive CFT test responses for breeding farms with dairy cattle than for beef cattle farms, for production systems with free or mixed housing than for those with tie stalls, and for cattle 2 to 3 years old versus cattle > 3 years old. On the other hand, among factors affecting MAP ELISA performance is the stage of lactation (ie, number of days in lactation), although results of studies24–27 are contradictory. Additionally, sensitivity of the MAP ELISA increases with age of cattle24 and is greater for Jerseys versus larger breeds and for high-parity (> 4) cows,26 whereas mycobacterial culture of feces has no age-related effects on sensitivity.24 However, the specificity of the CFT test in the present study was consistent with estimates reported elsewhere.28 Testing for *M. bovis* infection with the IFN-γ assay, which is approved only for use as a secondary test in cows classified as suspects by CFT testing, was not conducted in the 4 herds during the study period; thus, the association between results of the IFN-γ assay, the MAP ELISA, and mycobacterial culture of feces could not be evaluated.

The CFT test detects whether an animal has been previously sensitized by exposure to mycobacteria, and secondary tests are then used to differentiate responses attributable to *M. bovis* exposure from responses attributable to *M. avium* exposure. The secondary tests add to
the expense of tuberculosis testing in cattle. In a previous study, there were equivocal findings related to the association between results for MAP and CFT testing, but we found a strong association between these two test results in the present study. More than 90% of US dairy herds are expected to have positive results when tested for MAP, and herds with a high prevalence of MAP infection could have a substantial effect on testing costs because of the need for more secondary testing.

Development of more specific alternative screening tests or investigation of alternative testing protocols that involve the use of existing tests may offer options for reducing costs associated with increased secondary testing. However, evaluation of testing costs and the data on comparative specificity of IFN-γ and CFT testing also warrant consideration when making testing decisions.

Use of multiple diagnostic tests for the detection of tuberculosis in cattle has been proposed for herds with dual infections. In the present study, a significant association was detected between CFT test responses and MAP status (as assessed on the basis of results of serum ELISA and results of mycobacterial culture of feces). This association was likely attributable to cross-reaction between antigens shared by these mycobacteria and used in the respective tests. The potential decrease in diagnostic specificity of the CFT test should be considered when testing strategies are selected for use in control programs, especially in herds with a high prevalence of MAP infection.

References
13. Gilot P, Cocito C. Comparative analysis of three sensitins used in cutaneous testing for tuberculous and paratuberculosis in cattle. JAVMA, Vol 244, No. 5, March 1, 2014 Scientific Reports 587

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