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Research Article

Overt *Mycobacterium avium* subsp. *paratuberculosis* Infection: An Infrequent Occurrence in Archived Tissue from False TB Reactor Cattle in Michigan, USA

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The objective of this study was to retrospectively determine whether or not cattle from the state of Michigan which were classified as bovine tuberculosis reactors, based on currently approved field and laboratory testing methods, were overtly infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Included in this study were 384 adult cattle submitted to the Diagnostic Center for Population and Animal Health over a seven-year period. Cattle were tested utilizing standard methods to confirm that all cattle were lesion and culture negative for infection with *Mycobacterium bovis* at postmortem examination. Retrospective analysis of formalin-fixed, paraffin-embedded sections of ileum and ileocecal lymph node were evaluated by histopathology, acid-fast staining, and PCR assays to detect MAP. Overall, only 1.04 percent of cattle showed overt infection with MAP on visual examination of sections of ileum and/or ileo-cecal lymph node. This increased slightly to 2.1 percent of cattle likely infected with MAP after additional testing using a PCR assay. Based on these results, we found no evidence that overt infection with MAP plays a major role in the false tuberculosis reactor test results for cattle examined in this study.

1. Introduction

The identification of bovine tuberculosis (TB) in white-tailed deer in Michigan in 1994, and the subsequent identification of TB in cattle, has resulted in a long-term surveillance program for TB in cattle [1]. To date, 50 cattle herds have been found in Michigan that contained one or more *M. bovis* infected animals [2]. Thousands of cattle have been tested as suspect reactors on the caudal fold test (CFT) and comparative cervical test (CCT) or gamma interferon assay (γ-IFN), but only 138 cattle have been found infected with *M. bovis*. The large number of cattle found as false-positive reactors on field and laboratory tests, compared with the relatively small number of cattle eventually diagnosed as TB positive, is a reflection of the specificity of the currently approved antemortem diagnostic procedures when disease prevalence is low. Development of improved antemortem screening tests for detection of cattle and other species infected with *Mycobacterium bovis (M. bovis)*, the causal agent of bovine tuberculosis, has been the subject of recent research [3–10]. This activity is driven by the less than optimal sensitivity and specificity of currently approved diagnostic tests for antemortem detection of TB.

Diagnostic tests currently approved to screen for bovine tuberculosis in the USA include the CFT, the CCT, and the whole blood γ-INF assay [11]. The skin tests measure a cell-mediated immune response (delayed type hypersensitivity response) stimulated by an injection of purified protein derivative (PPD) obtained from cultured *M. bovis*. The whole blood γ-INF assay also measures a cell mediated response

(production of γ-INF by lymphocytes) after stimulation with PPD. The content of PPD is a nonstandardized and variable complex mixture of various antigenic components prepared from cultures of mycobacteria [6, 12, 13]. Many of the antigens in PPD are shared among the various species of pathogenic and nonpathogenic mycobacteria. Thus, there is concern that previous exposure of cattle, or concurrent infection of cattle, with mycobacteria other than *M. bovis* will affect the sensitivity and/or specificity of current diagnostic assays [6, 12, 14–20]. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is widespread in Michigan, and infection of cattle with that organism may affect the outcome of currently approved tests for TB [19, 21, 22].

The relatively high prevalence of cattle herds infected with MAP and cattle infected with MAP in Michigan, and the low prevalence of TB in cattle examined postmortem, prompted us to conduct a retrospective study to determine whether overt infection with MAP was an important cause of false-positive reactors in currently approved tests for TB. Because TB in cattle is a regulatory disease that has zoonotic potential, postmortem examination of cattle that are suspect for TB is focused on collection of tissues known to be targeted by *M. bovis,* and collection of additional tissues for use other than diagnosis of TB is not standard practice.To determine infection of cattle with MAP, we were limited to formalin-fixed, paraffin-embedded distal ileum and ileo-cecal lymph node. Our purpose was to examine those available tissues for microscopic lesions consistent with infection with MAP, to identify acid-fast stained organisms within the tissues, and to substantiate infection with MAP using polymerase chain reaction (PCR) assays.

2. Materials and Methods

2.1. Cattle Selection Criteria. Cattle included in this retrospective study were from 13 contiguous counties within the north-east portion of the lower peninsula of Michigan. This region of the state is under an active surveillance program for bovine tuberculosis, as small numbers of infected cattle are detected periodically in that area [23]. Cattle designated for postmortem examination were removed from the herd the day before postmortem examination and transported to an isolated and secured holding facility. All cattle in the study were examined postmortem for bovine tuberculosis at the Diagnostic Center for Population and Animal Health (DCPAH), Michigan State University, between June 7, 2001 and May 1, 2008. The cattle were adult animals (greater than two years old) and mostly female (96%). There was a distinct predominance of dairy cattle compared to beef cattle (2 : 1). Finally, only cattle purchased by the State of Michigan for purposes of tuberculosis surveillance were included. All cattle in the study were found negative for overt infection with *M. bovis* using standardized postmortem diagnostic methods [24, 25]. Lymphoid tissues from all cattle were submitted to the National Veterinary Services Laboratory (NVSL) in Ames, Iowa, for confirmation of the histopathologic lesions and for culture for *M. bovis*. The cattle were grouped according to outcome of the primary caudal fold skin test (CFT) and the secondary comparative cervical skin test or whole blood gamma interferon assay for bovine tuberculosis. Group 1 consisted of 189 cattle that were false-positive reactors on both primary and secondary tests. Group 2 consisted of 122 cattle that were false-positive reactors on the CFT only. Group 3 consisted of 73 cattle which were negative on the CFT and were examined postmortem without a secondary test being performed; this group was considered the negative control group. The cattle included in groups 2 and 3 were from tuberculosis positive herds that were being depopulated or were cattle that had been exposed to animals that had bovine tuberculosis and were being examined postmortem for bovine tuberculosis.

2.2. Necropsy and M. bovis Diagnostics. Cattle were transported alive to the DCPAH, where they were euthanized by overdosage of intravenous barbiturates. The same diagnostic protocols were followed for all animals. Gross postmortem examinations were conducted with attention directed to examination of the animals' lungs, lymph nodes, and ileal-cecal-colic junction. Lymph nodes were harvested by anatomic region (cranial, thoracic, and abdominal), along with a section of terminal ileum. Lymph nodes were serially sectioned for gross examination. Portions of each lymph node and ileum were fixed in 10% neutral-buffered formalin for histopathology, while other portions of the same lymph nodes were shipped fresh on ice packs to the Tuberculosis Laboratory, National Veterinary Service Laboratories (NVSL), Ames, Iowa, for mycobacterial isolation and identification using previously described techniques [24, 25]. Formalin-fixed samples of lymph nodes and distal ileum were embedded in paraffin, sectioned at 5 um, and routinely processed for both hematoxylin and eosin (H&E) and Ziehl-Neelsen acid fast staining. Sections of stained tissue were examined microscopically for granulomatous inflammation and for presence of acid-fast bacilli.

2.3. Tissue Processing and DNA Extraction. Three serial sections, 20 μm thick, were cut from each block of paraffin-embedded distal ileum. The tissue sections were placed into a sterile 1.5 mL microcentrifuge tube and stored at room temperature until processed for extraction of DNA. Between blocks of paraffin-embedded tissue, the knife blade of the microtome was wiped clean with an absorbent tissue impregnated with a 10% solution of household bleach in 0.01 M phosphate buffered saline solution (pH 7.2). For DNA extraction, 1 section of paraffin-embedded ileum and ileo-cecal lymph node from each animal was placed in a microcentrifuge tube, using a sterile toothpick. The remaining sections of paraffin-embedded tissue were stored at room temperature for use as needed. Extraction of DNA and PCR were performed using previously described methods and PCR primers with slight modification [26, 27]. Briefly, a 1.5 mL microcentrifuge tube containing one section of paraffin embedded ileum was centrifuged at 16,000 $\times$ g for 1 minute at 24°C to collapse the tissue section. Approximately 200 μL of a 0.5% solution of polyoxyethylene-sorbitan monolaurate (Tween 20) in DNase and RNase free molecular

biology grade water was added to each microcentrifuge tube. The tubes were then subjected to 2 cycles of boil and snap freeze using first a 10-minute incubation at 100°C followed immediately by a 3-minute immersion into a dry ice-ethanol bath. Finally, the tubes were incubated an additional 10 minutes at 100°C and centrifuged at 3,000 × g at 4°C to pellet tissue debris and float the melted paraffin to the surface. The paraffin layer was removed with a sterile toothpick, and 5 μL of the liquid phase was aspirated and inoculated into a 200 μL PCR tube containing PCR primers and 20 μL PCR reaction mixture. The DNA extraction method was tested on archived formalin-fixed, paraffin-embedded sections of ileum and ileo-cecal lymph node from 25 cattle not submitted to DCPAH for TB postmortem examination and confirmed infected with MAP by bacterial culture. The tissue blocks had been archived from 5 to 8 years at the time of DNA extraction, and the number of acid-fast organisms observed in these sections varied from numerous to none. Only sections of tissues from one culture positive animal produced negative results on PCR assay. Multiple paraffin-embedded blocks of tissues from that animal were examined microscopically, and the block that tested negative on PCR assay lacked visible acid-fast organisms.

2.4. Polymerase Chain Reaction (PCR) Assays. The PCR for MAP used primers from the IS900 sequence, 5′-CCGCTA-ATTGAGAGATGCGATTGG and 5′-AATCAACTCCAG-CAGCGCGGCCTCG, and yielded a product of 229 base pairs. This PCR was done on all 384 samples of ileum and ileo-cecal lymph node. Subsequently, 271 representative samples of ileum from all 3 groups of cattle (including all tissues that tested positive for MAP) were subjected to a PCR assay designed to detect the *M. avium* group of organisms. The PCR primers used for that assay were from the gene for 16s ribosomal RNA, 5′-AGAGTTTGATCCTGGCTCAG and 5′-ACCAGAAGACATGCGTCTTG, and yielded a product of 193 base pairs. The PCR reaction mixture included a PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dATP, dGTP, dCTP, and dTTP, 1.25 units HotStar Taq polymerase, 0.4 pmol/μL of each primer for IS900 or 1.0 pmol/μL of each primer for the *M. avium* group, and 5 μL of sample DNA. The PCR reaction conditions were 1 cycle of 95°C for 15 min, 50 cycles of 94°C for 1 minute, 65°C (MAP) or 61°C (*M. avium*) for 15 sec, 72°C for 2 min, followed by 1 cycle of 72°C for 10 min. PCR amplification products were analyzed by gel electrophoresis using a 1.5% agarose gel in sodium borate buffer with 0.5 mg/mL of ethidium bromide mixed into the molten gel [28].

2.5. Statistical Analysis. Associations between PCR-confirmed MAP status and *M. bovis* test status (primary and secondary test reactors, primary (CFT) reactors only, and primary test negative cattle) were measured with odds ratios for *M. bovis* test reactors in comparison with *M. bovis* test negative cattle, and Fisher's Exact Test was used to determine whether these associations were statistically significant ($P < .05$).

3. Results

3.1. Group 1 Cattle. This group of cattle were found suspect for TB on sequential primary and secondary screening tests for bovine tuberculosis. The second of the sequential screening tests, either the CCT or gamma interferon assay, is designed to reduce the number of false-positive reactors that may be attributed to previous infection of the animal with the *M. avium* group of mycobacteria. Of 189 cattle that were positive reactors on both primary and secondary tests for bovine TB, only 1 cow was positive by PCR for MAP (Tables 1 and 2). Gross or microscopic lesions consistent with Johne's disease were not observed in tissues from that cow or in tissues from any other animal in Group 1 (Table 2). Also, acid fast organisms were not found in sections of the ileum or in ileo-cecal lymph nodes from any animal in Group 1. The PCR assay designed for detection of the *M. avium* group of organisms was negative for all cattle tested in this group (Tables 1 and 2).

3.2. Group 2 Cattle. Cattle in this group were false-positive reactors on the CFT and were negative on a secondary test. Cattle that are reactors on the CFT and are negative on a second screening test for infection with *M. bovis* may have been infected with mycobacteria in the *M. avium* group or to environmental mycobacteria and not *M. bovis*. Thus, cattle in Group 2 should have been at higher risk for infection with MAP or with other members of the *M. avium* group of mycobacteria than cattle in Group 1. Three of 122 cattle in Group 2 were positive for infection with MAP by PCR (Tables 1 and 2). Two of those 3 cattle had gross lesions of Johne's disease, consisting of mild to moderate thickening of the terminal ileum wall (Table 2). These two animals also had microscopic granulomatous lesions consistent with Johne's disease including visible acid fast organisms in the ileum and granulomatous lymphadenitis in the ileo-cecal lymph nodes. Additionally, tissue sections from these animals were positive by PCR for the *M. avium* group of bacterium (Tables 1 and 2). The third animal that tested positive for MAP using PCR assay lacked gross or microscopic lesions consistent with Johne's disease and lacked visible acid fast organisms in sections of ileum or ileo-cecal lymph node. This cow was negative by PCR for the *M. avium* group of mycobacteria. None of the remaining 122 cattle in Group 2 had lesions consistent with Johne's disease, had visible acid fast organisms in sections of tissue, or were positive by PCR assay for MAP or the *M. avium* group of mycobacteria.

3.3. Group 3 Cattle. This group consisted mostly of cattle culled from TB-infected herds, but also included some cattle that moved from TB-infected herds to other herds prior to detection of TB in the herd of origin. Reasons for the animals being culled included ill thrift, lameness, mastitis, or other chronic disease conditions. The cattle in this group had been administered the CFT but were not positive reactors on the CFT. Four of the 73 cattle were found positive for MAP by PCR assay, and two of those cattle also were positive on PCR assay for the *M. avium* group of mycobacteria

Table 1: Summary of results of various screening tests for bovine tuberculosis and for results of PCR assays.

M. bovis screening test result	Number of cattle tested	MAP PCR positive	Percent MAP positive	*M. avium* PCR positive
(1) CFT and CCT positive	189	1	0.5%	0
(2) CFT positive	122	3	2.5%	2
(3) CFT negative (Control group)	73	4	5.5%	1
Totals	384	8	2.1%	3

(Tables 1 and 2). None of those 4 cows had gross lesions consistent with Johne's disease, but acid fast organisms were found in sections of ileum from one cow positive by PCR assay for both MAP and the *M. avium* group of mycobacteria (Tables 1 and 2). None of the remaining 69 cattle in this group had lesions consistent with Johne's disease, had visible acid-fast organisms in sections of tissue, or were positive by PCR assay for the *M. avium* group of mycobacteria.

3.4. Statistical Analysis. There was not a statistically significant difference between the CFT false-positive cattle in Group 2 and CFT negative cattle in Group 3. In addition, the differences between the primary and secondary test reactor group (Group 1) and the CFT negative cattle (Group 3) was close to statistically significant ($P = .0511$) (Table 3).

4. Discussion

In the current study, we attempted to determine if overt infection of cattle with MAP was a common cause of false-positive reactions in currently approved field and laboratory tests for bovine tuberculosis. This was a retrospective study that made use of samples from 384 cattle examined postmortem and diagnosed free of bovine TB. We used a series of diagnostic assays that included gross examination of the ileum for thickening, microscopic examination of hematoxylin and eosin stained sections of distal ileum and ileo-cecal lymph node for histiocytic or granulomatous infiltrates, microscopic examination of acid-fast stained sections of ileum and ileo-cecal lymph node for detection of stained organisms, and finally PCR. This diagnostic approach is similar to current recommendations for detection of Johne's disease postmortem [29].

Our results were similar with recent studies that assessed formalin-fixed, paraffin-embedded tissues for surveillance for overt Johne's disease in randomly selected cattle at slaughter [30, 31]. Very few cattle ($n = 4$, 1%) in the current study had lesions and/or presence of acid fast stained organism that would be considered consistent with overt infection with MAP. Because bovine TB is a regulatory disease and samples of fresh tissues are shipped to the NVSL for final diagnosis, bacterial culture from feces or tissue was not attempted at the DCPAH in the current study. It is likely that use of culture techniques or PCR assays on fresh tissue would have resulted in identification of additional cattle infected with MAP in the absence of overt lesions [30, 31].

Further testing of tissue samples, using a PCR assay for the IS900, yielded positive results from an additional 4 cattle that did not show lesions or acid-fast organisms in tissues. This finding was not surprising because PCR likely is a more sensitive indicator of earlier infection with MAP than either gross or histologic lesions [32]. The PCR assay was based on IS900, a multiple copy transposable element commonly used as a target for detection of MAP [33]. This insertion sequence has been identified in mycobacteria other than MAP [34, 35]. Hence, it is possible that some of the 8 cattle that were positive on PCR may have been simultaneously infected with mycobacterium other than MAP. An attempt was made in the current study to identify cattle infected with mycobacteria other than MAP, using a group-specific PCR assay that can detect most members of the *M. avium* group, including MAP. However, that PCR assay only yielded positive results when acid-fast stained organisms were detected in tissue and when the PCR assay for MAP yielded positive results. Thus we failed to conclusively identify an animal currently infected with a mycobacterium other than MAP. A previous study also found that the PCR assay for the *M. avium* group of organisms yielded fewer positive results using formalin fixed-tissue than the PCR assay for MAP [26].

Based on antemortem laboratory tests, the prevalence of MAP in Michigan's dairy herds has been estimated recently to be about 50% with rates of infection of individual cattle estimated between 5 and 15% for most herds and infection rates higher than 15% in some herds [21, 22, 36]. The prevalence of MAP in Michigan's beef cattle herds is thought to be substantially lower than the prevalence in dairy herds. Approximately one third of the cattle in the current study were beef cattle, and none of the cattle suspected as being infected with MAP in the current study were beef cattle. The inclusion of a substantial number of beef cattle likely lowered rates of infection with MAP detected in the current study.

The cattle in Groups 1 and 2 were reactors in 1 or more field and/or laboratory tests for detection of TB in cattle. If infection with MAP influenced the results of field or laboratory tests for TB by increasing the number of false-positive reactors, cattle in Groups 1 and/or 2 likely would have higher rates of infection with MAP than cattle in Group 3, which consisted of cattle that were negative for TB on the CFT test. That outcome was not observed. Instead, infection with MAP, or other organisms possessing IS900, was identified in 5.5% cattle in Group 3, compared with 2.5% of cattle in Group 2 and 0.5% of cattle in Group 1 (Table 1). Recent reports indicate that previous or current infection with mycobacteria other than the tuberculosis group of mycobacteria adversely affects currently approved field and laboratory tests for detection of bovine TB, resulting in higher rates of false-negative tests [14, 15, 34]. Due to bovine TB being a regulatory disease, we did not attempt to detect *M. bovis* in the group of cattle that were in contact with TB-infected cattle, but had tested negative for TB on the CFT test.

Formalin fixation is known to cause DNA degradation which can compromise use of PCR assays. It is recommended to use PCR primer sets that amplify short sequences

Table 2: Summary of pathology and PCR results in MAP infected animals.

Study group	*M. bovis* test result	H & E histo. result ileum	Acid -fast result ileum	H & E histo. result lymph node[a]	Acid-fast result lymph node[a]	MAP PCR	*M. avium* PCR	Breed	TB status of herd
Group 1	Primary and secondary test positive.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Jersey	Neg.
Group 2	CFT Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Jersey	Pos.
Group 2	CFT Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Holstein	Pos.
Group 2	CFT Pos.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Holstein	Pos.
Group 3	CFT Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Holstein	Neg.
Group 3	CFT Neg.	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.	Holstein	Pos.
Group 3	CFT Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Holstein	Pos.
Group 3	CFT Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Holstein	Pos.

[a] Lymph node was ileo-cecal lymph node.

Table 3: Odds ratios for *M. bovis* test reactors compared to *M. bovis* test negative cattle, by PCR-confirmed MAP status.

Study group	MAP PCR positive			Fisher's exact test *P* value*	Odds ratio	
	Total number	No. of Positive	Percent		Estimate	95% C I
(1) Primary and secondry test reactors	189	1	0.5	.0511	0.11	0.01–1.06
(2) CFT reactor	122	3	2.5	.4281	0.43	0.09–2.00
Groups 1 and 2 combined	311	4	1.3	.0458	0.22	0.05–0.92

* Fisher's exact test compares the difference in the odds ratios between the bovine TB test group in that row verses the bovine TB test negative group.

(less than 200 bp) when formalin-fixed tissues are assayed [37]. The PCR primer sets used in the current study amplified targets of about 200 bp in length, so our assays were at the recommended upper limit for target detection in formalin-fixed tissue. This might have affected our results and reduced the number of cattle that were found infected with MAP. However, the effect of formalin fixation of tissue would occur across all groups of cattle. The relatively low number of primary test negative cattle in this study reduced the statistical power to the point where we could not confirm statistical significance between groups of cattle. The annual number of cattle designated for TB postmortem examination that are test negative of bovine TB is small in Michigan; hence, expanding that group of cattle was not possible in the time frame of the current study.

What other factors might be contributing to the high numbers of false-positive skin test cattle? This retrospective study only evaluated one possible *Mycobacterium* sp. and its presence at a specific regional site (the terminal ileum and ileo-cecal lymph node); exposure of the animal to other environmental *Mycobacteria* spp. located in other anatomic sites is one possible factor [18]. Another cause may be nonmycobacterial infections in tested cattle, such as *Nocardia* spp. [18]. Immunization of cattle for Johne's disease or with experimental *M. bovis* BCG vaccines—neither of these vaccinations are allowed in Michigan—may also cause false skin test results [18]. Finally, this false reactor rate may simply be intrinsic to the skin tests used. When the cattle population is this far advanced along the road to disease eradication such as is the current situation in the United States in general and Michigan in particular, using a screening test with very high sensitivity may necessitate living with a lower specificity as we attempt to detect the last few remaining infected individuals [18]. While there is no argument that it would be desirable to increase the specificity of current skin testing methods to detect *M. bovis* in cattle, cross-reaction with MAP does not appear to be a major limiting factor in the tests' utility based on this study.

In conclusion, the methods used in this study found few cattle infected with MAP and failed to find a positive association between an infection with MAP and false positive reactions in field and/or laboratory tests for bovine TB.

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