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Mycobacterium avium subsp. *paratuberculosis* Antibody Response, Fecal Shedding, and Antibody Cross-Reactivity to *Mycobacterium bovis* in *M. avium* subsp. *paratuberculosis*-Infected Cattle Herds Vaccinated against Johne's Disease

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Vaccination for Johne's disease with killed inactivated vaccine in cattle herds has shown variable success. The vaccine delays the onset of disease but does not afford complete protection. Johne's disease vaccination has also been reported to interfere with measurements of cell-mediated immune responses for the detection of bovine tuberculosis. Temporal antibody responses and fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis*, the causative agent of Johne's disease, were measured in 2 dairy cattle herds using Johne's disease vaccine (Mycopar) over a period of 7 years. Vaccination against Johne's disease resulted in positive serum *M. avium* subsp. *paratuberculosis* antibody responses in both herds, and the responses persisted in vaccinated cattle up to 7 years of age. Some vaccinated animals (29.4% in herd A and 36.2% in herd B) showed no serological reactivity to *M. avium* subsp. *paratuberculosis*. *M. avium* subsp. *paratuberculosis*-specific antibody responses were also detected in milk from Johne's disease-vaccinated animals, but fewer animals (39.3% in herd A and 49.4% in herd B) had positive results with milk than with serum samples. With vaccination against *M. avium* subsp. *paratuberculosis*, fecal shedding in both dairy herds was reduced significantly ($P < 0.001$). In addition, when selected Johne's disease-vaccinated and -infected animals were investigated for serological cross-reactivity to *Mycobacterium bovis*, no cross-reactivity was observed.

Johne's disease in cattle is a chronic disease caused by *Mycobacterium avium* subsp. *paratuberculosis*. In the United States, the disease causes estimated losses of $200 million every year (1). Control of Johne's disease is achieved by testing, culling, and improving biosecurity and herd management (2). Vaccination, using killed inactivated vaccines, has also been attempted for disease control. The vaccines are said to afford protection by delaying the onset of clinical disease, but the protection against infection in cattle is not complete (3, 4). Vaccination with a calfhood vaccine is prescribed for replacement heifers and male calves. Currently available vaccines have the major disadvantages that they cause granulomas, can result in accidental vaccination of humans, and are said to interfere with the bovine tuberculosis (TB) skin test (4). Furthermore, the true cost benefits of vaccinations are unknown, although vaccinations historically have been shown to have economic value (1, 5).

Information regarding the efficiency of Johne's disease vaccination in cattle herds is scarce, and cross-reactivity in bovine TB tests has been shown to be a problem in vaccinated cattle, small ruminant, and cervid herds (6–12). Additional testing using the comparative cervical test (CCT) or gamma interferon (IFN-γ) measurement helps to determine whether reactivity seen with skin-based screening is specific, but the follow-up testing is often laborious and time-consuming (4, 6).

To study the effects of vaccination in cattle, we selected two dairy herds receiving Johne's disease vaccination in the wake of natural disease, and we studied *M. avium* subsp. *paratuberculosis*-specific antibody responses and fecal shedding of *M. avium* subsp. *paratuberculosis* in these two herds. Vaccination or infection with *M. avium* subsp. *paratuberculosis* has been shown to result in interference in cell-based bovine TB assays (6–12). With the recent availability of new serological assays to detect bovine TB, the cross-reactivity of *M. avium* subsp. *paratuberculosis*-specific antibodies in response to Johne's disease infection or vaccination was investigated using the IDEXX *Mycobacterium bovis* antibody enzyme-linked immunosorbent assay (ELISA), which is based on two antigens (MPB83 and MPB70) (13).

MATERIALS AND METHODS

Sample populations. Two herds (designated A and B) that were receiving vaccinations and were part of the Pennsylvania Johne's Disease Demonstration Herd Project were selected and were studied for 7 years (2004 to 2010).

Sampling plan. Paired blood and fecal samples were collected from all ≥24-month-old animals, both lactating and nonlactating, in both herds, in order to evaluate the *M. avium* subsp. *paratuberculosis* antibody responses and shedding status of the individual animals. The two types of samples were collected from each animal in the herd on the same day, identified using unique nontransferable identification numbers, and sent on ice to the laboratory in Harrisburg, Pennsylvania, for processing. Sampling was carried out annually for 7 years, 11 to 13 months after the

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previous sampling. A total of 952 animal samplings were carried out, 594 from vaccinated animals and 358 from unvaccinated animals.

In select years, Dairy Herd Improvement Association (DHIA)-collected milk samples were also obtained from all lactating cows. These samples were obtained from the DHIA test date closest to the time of the fecal and blood sampling in selected years. Since both herds were on a monthly sampling schedule, the milk samples were always obtained within 2 weeks of the blood and fecal samples but not on the same day. The milk samples were tested to evaluate the levels of *M. avium* subsp. *paratuberculosis* antibody responses.

Initial and yearly assessments were conducted for both herds, and management changes were recorded. Both farms were tested annually beginning in 2004 (year 1) and continuing through 2010 (year 7). Vaccination was included as part of disease control measures on both farms.

Vaccination and management changes. According to state regulations, caudal fold tuberculin testing was performed on all calves prior to the initiation of vaccination. All test results were negative. Calves were vaccinated by the herd veterinarians with 0.5 ml of killed whole-cell vaccine (Mycopar), administered subcutaneously in the brisket area, before 35 days of age. Herd A began receiving vaccination against Johne's disease prior to the initiation of this study; therefore, by year 1 of the study, there were 116 vaccinated animals >24 months of age in the herd. The proportions of vaccinated animals (versus unvaccinated animals) sampled in this herd ranged from 63.7% (74/116 animals) in 2004 to 100% (122/122 animals) in 2007. Herd B initiated *M. avium* subsp. *paratuberculosis* vaccination late in 2002 and did not have any vaccinated animals >24 months of age by the time of the first sampling in 2004. In year 7, 98% of the animals (49/50 animals) that underwent sampling had received the calfhood vaccination.

In addition to vaccination, herd A implemented numerous changes designed to minimize the probability of new infections, based on a Johne's disease risk assessment (http://www.johnesdisease.org/Risk%20Assessment%20&%20Management%20Plans%20for%20Johne%27s.pdf). The most significant changes included improved hygiene of the maternity area, prompt removal of the calves from the maternity area, and development of a protocol to calve any test-positive cows in a separate location. In addition, sick animals were not housed in the maternity area, and colostrum from any test-positive or untested animals was not used for heifer calves. Almost all fecal culture-positive animals were culled from the herd shortly after diagnosis, although animals with very low levels of shedding occasionally were retained for longer periods. Special attention was paid to these animals, to monitor them for clinical signs, and precautions (see above) were taken to minimize the risk of new infections resulting from these animals.

Numerous risk areas were identified in herd B by means of the risk assessment. However, this farm elected to make very few substantive changes in their management practices, although positive animals were identified. Some attempts were made to not have heavy shedders or clinically ill animals calve in the maternity area. Sick animals were generally not housed in the maternity area, although this did occur. For financial reasons, herd B retained many of its serum- and fecal-culture-positive animals, unless there were concurrent reasons to remove the animals (e.g., high somatic cell counts or other disease concerns) or they began showing clinical signs. Most but not all replacements for herds A and B were home-raised.

ELISAs. The IDEXX *Mycobacterium paratuberculosis* ELISA kit (HerdChek *M. pt.* kit) for detection of *M. avium* subsp. *paratuberculosis*-specific antibodies in serum and the IDEXX *Mycobacterium bovis* ELISA kit for *M. bovis* (IDEXX, Westbrook, ME) were used in this study. ELISAs were conducted following the manufacturer's directions, with a 1:20 dilution of 100 μl of serum for the IDEXX HerdChek *M. pt.* kit and a 1:50 dilution of 100 μl of serum for the *M. bovis* ELISA kits. Antibody responses against *M. avium* subsp. *paratuberculosis* in milk and serum samples from vaccinated cattle were compared. Milk samples (100 μl at a 1:20 dilution) from selected animals from both herds (440 samples from herd A and 190 samples from herd B) were tested using the Prionics Paracheck ELISA (Prionics, Switzerland). This kit is approved for testing both serum and milk samples. Positive and negative results were determined according to the kit instructions. Quantitative data were expressed as sample/positive (S/P) ratios for the IDEXX assays or sample/negative values for the Prionics assay (Prionics, Switzerland).

Agar slant cultures. Two grams of feces was decontaminated by the double incubation-centrifugation method, as described previously (14). This material was used to inoculate 4 slants of Herrold's egg yolk medium containing 2 mg/liter mycobactin J (Becton, Dickinson & Co., MD). *M. avium* subsp. *paratuberculosis* colonies were confirmed by using acid-fast staining and an IS*900* PCR assay.

Statistical analyses. Proportions of vaccinated and unvaccinated animals were calculated for each category in different study years. The effects of vaccination on serological responses and *M. avium* subsp. *paratuberculosis* shedding were analyzed with chi-square analysis. Relative risks from *M. avium* subsp. *paratuberculosis* exposure of vaccinated and unvaccinated groups were calculated. Correlation coefficients (r) were calculated for correlations between the serum and milk ELISA results, to understand the relationship. Serum S/P ratios for selected animals ($n = 14$) were plotted as scatter or line plots, to show antibody responses following vaccination.

RESULTS

Antibody responses and fecal shedding. Chi-square statistical analysis showed that vaccination was strongly associated with positive serum responses and reduction of fecal shedding of *M. avium* subsp. *paratuberculosis* in both herds ($P < 0.001$). Unvaccinated cattle in herd A showing no evidence of infection were predominately seronegative for *M. avium* subsp. *paratuberculosis* antibodies, as indicated by comparing positive ELISA results for the total and vaccinated animal categories (Table 1). Significant proportions of vaccinated animals showed antibody responses (ranging from 32.7% to 70.9% in different years) and were found to be fecal culture negative for *M. avium* subsp. *paratuberculosis* ($P < 0.001$). The rate of seropositivity was highest in 2006, when >70.4% of animals tested positive (Table 1). A substantial number of vaccinated animals remained serologically negative (29.4% in herd A). At the start of the study, the within-herd prevalence rate was 6%. In the vaccinated group, fewer animals shed *M. avium* subsp. *paratuberculosis* in feces, and those that were ELISA positive shed fewer organisms (maximum, 3.2%). The whole-herd fecal culture-positive rate was also maintained at a low level. Some animals were fecal culture positive but remained seronegative in response to both *M. avium* subsp. *paratuberculosis* infection and vaccination. Table 1 shows the total fecal culture-positive and ELISA-positive results in response to either vaccination or infection. For some animals that showed seropositive responses, antibody levels did drop below the cutoff value of 0.25 or to the negative range, as determined by S/P ratios (Fig. 1A). However, for many other animals that could be traced for several years in both herds A and B, animals remained positive for serum *M. avium* subsp. *paratuberculosis* antibodies in the fifth year of the study or 7 years following vaccination (Fig. 1B).

The second herd, herd B, initially showed considerable rates of infection (>20% within-herd prevalence) in both the vaccinated and unvaccinated groups. The fecal shedding rate was 23.2% in 2004, at the start of the study, and remained above 20% until 2006, with several high-level shedders being identified within the herd (Table 1). ELISA positivity rates ranged between 51 and 100% among vaccinated animals in any given study year. Overall, 36.2% of vaccinated animals showed no ELISA reactivity. Fecal shedding dropped from an initial rate of greater than 20% to less than 10%

TABLE 1 *Mycobacterium avium* subsp. *paratuberculosis* annual antibody responses and fecal shedding in *M. avium* subsp. *paratuberculosis*-infected cattle herds receiving *M. avium* subsp. *paratuberculosis* vaccination with management changes (herd A) or without management changes (herd B)

Herd and yr	No. in herd[a]	No. (%)[b]						
			Total		Vaccinated		ELISA positive	
		Vaccinated	ELISA positive	Fecal culture positive	ELISA positive	Fecal culture positive	Fecal culture positive	Vaccinated fecal culture positive
A								
2004	116	74 (63.7)	49 (42.2)	7 (6)	47 (63.7)	0	2	0
2005	116	105 (90.5)	74 (63.7)	1 (0.8)	73 (69.5)	0	0	0
2006	115	110 (95.6)	78 (67.8)	3 (2.4)	78 (70.9)	1 (0.9)	0	0
2007	122	122 (100)	40 (32.7)	2[c]/3 (2.4)	40 (32.7)	2[c]/3 (2.4)	2	2
2008	125	125 (100)	65 (52.0)	3[c]/4 (3.2)	65 (52)	3[c]/4 (3.2)	2	2
2009	128	94 (73.4)	49 (38.2)	2[c]/3 (2.3)	49 (52.2)	1 (1)	0	0
2010	128	66 (51.5)	35 (27.3)	1 (0.7)	34 (51.5)	0	0	0
B								
2004	43	0	3 (6.6)	10 (23.2)	0	0	3	0
2005	45	2 (4.4)	7 (15.5)	8[c]/11 (24.4)	2 (100)	1 (50)	3	1
2006	52	30 (57.6)	30 (57.6)	8[c]/11 (21.1)	25 (83.3)	4[c]/5 (16.6)	9	3
2007	61	49 (80.3)	27 (44.2)	3[c]/4 (7.2)	26 (53.0)	2[c]/3 (6.1)	3	3
2008	55	52 (94.5)	33 (60)	3[c]/5 (9.0)	32 (61.5)	3[c]/4 (7.6)	4	2
2009	52	51 (98)	32 (61.5)	5[c]/6 (11.5)	31 (60.7)	5[c]/6 (11.7)	6	4
2010	50	49 (98)	25 (50)	2 (4.0)	25 (51.0)	2 (4.0)	2	1

[a] The numbers of animals >24 months of age are indicated for each study year.

[b] Total and vaccinated ELISA- and *M. avium* subsp. *paratuberculosis* fecal culture-positive animals are indicated as ELISA positive and fecal culture positive, respectively. Animals that were ELISA positive and were *M. avium* subsp. *paratuberculosis* fecal culture positive in the total and vaccinated groups are indicated as fecal culture positive and vaccinated fecal culture positive, respectively, under ELISA positive.

[c] New infections for each year are indicated, showing totals for new infections and total positive results.

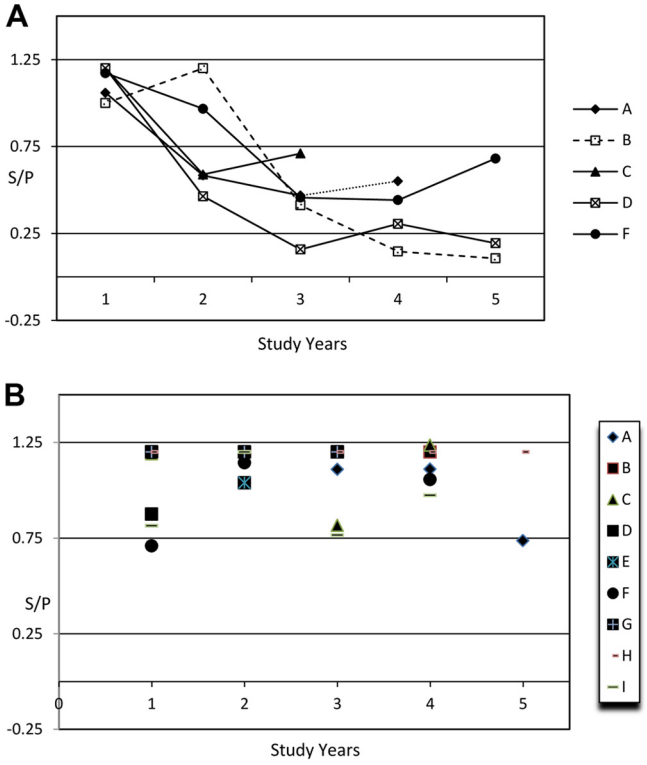

FIG 1 Serological responses to Johne's disease vaccination. (A) Line plot showing the trend of serum S/P ratios (*y* axis) tested annually during the 5-year study period (years 1 to 5), from >2 years to 7 years of age (*x* axis), for selected animals ($n = 5$; animals A, B, C, D, and F). (B) Scatter plot showing the trend of serum S/P ratios (*y* axis, with maximum capped values of 1.2) tested during the 5-year study period (years 1 to 5), from >2 years to 7 years of age (*x* axis), for selected animals ($n = 9$; animals A to I).

in subsequent years. Vaccinated animals were found not to be fully protected in herd B, as was the case in herd A. Several animals (range, 4.0 to 50%) were found to be shedding *M. avium* subsp. *paratuberculosis* and were identified as high shedders even after being vaccinated. Overall, the numbers of *M. avium* subsp. *Paratuberculosis*-shedding animals decreased with time. In herd B, similar to herd A, some animals were fecal culture positive for *M. avium* subsp. *paratuberculosis* but were serologically negative in both the vaccinated and infected groups.

Serum and milk antibody responses. Positive correlations between the serum and milk antibody responses were noticed for both vaccinated herds (herd A, $r = 0.69$; herd B, $r = 0.63$). Many vaccinated animals showed measurable *M. avium* subsp. *Paratuberculosis*-specific antibody responses in serum but showed no specific antibodies for *M. avium* subsp. *paratuberculosis* in milk. In herd A, 440 sample pairs collected from lactating vaccinated animals were tested with milk and serum ELISAs; 173 (39.3%) were found to be positive for *M. avium* subsp. *paratuberculosis*-specific antibody responses by serum testing but were negative by milk testing. In herd B, among the 141 vaccinated animals sampled, 51 (49.0%) tested positive in serum testing but negative when milk was analyzed for *M. avium* subsp. *paratuberculosis*-specific responses. There was no major difference in serum and milk antibody responses among the unvaccinated animals ($n = 104$), including the animals that were shedding *M. avium* subsp. *paratuberculosis*. For 5/104 animals (4.8%) in herd A and 7/125 animals (5.6%) in herd B, although the animals were shedding *M. avium* subsp. *paratuberculosis*, both milk and serum ELISAs failed to detect infected animals among the unvaccinated animals. In the same unvaccinated category, 93% of animals sampled for all 3 tests (fecal, serum, and milk testing) were negative.

Serological cross-reactivity to *Mycobacterium bovis*. Sera that were strongly positive for *M. avium* subsp. *paratuberculosis* antibody responses (S/P ratios) due to infection or vaccination were tested with a *M. bovis* ELISA. Our results showed no reactivity with the *M. bovis* ELISA, although the animal sera were positive for *M. avium* subsp. *paratuberculosis* antibodies. Twenty serum samples with S/P ratios of >0.25, 10 serum samples with S/P ratios of <0.25 from animals vaccinated against *M. avium* subsp. *paratuberculosis*, and 16 serum samples with S/P ratios of >0.25 from *M. avium* subsp. *paratuberculosis*-infected animals that were serologically positive were included in this comparison. None of the sera showed any reactivity to *M. bovis*.

DISCUSSION

Vaccination of calves with a killed vaccine has not been shown to completely prevent *Mycobacterium avium* subsp. *paratuberculosis* infection but is considered to be an effective tool for controlling the spread of disease (8). Another study showed reductions in herd infection rates but claimed that similar reductions were possible without vaccination with changes in management practices (3). Our study describes in detail observational findings for the two *M. avium* subsp. *paratuberculosis*-vaccinated herds. The infection levels in both of these study herds dropped, albeit slowly, when extensive management changes were not incorporated along with the vaccination strategy. However, due to multiple confounding factors during the study period, including management changes on the two farms, culling, herd replacements, and the inability of currently available immunological tests to distinguish vaccinated animals from infected animals, the observed protection could not be attributed only to vaccination, although it appears that vaccination has some protective role. The role of vaccination in protection against Johne's disease would be strengthened with data comparisons between vaccinated and unvaccinated herds. A control herd was not included in the study. However, a Johne's disease-infected herd (543 animals) that was participating in a demonstration herd project but did not receive vaccination that was followed during the same study period did not show any drop in infection levels during the study period (2006 to 2011). This herd maintained within-herd infection rates of approximately 10% for the entire study period (E. Hovingh, unpublished data). Another important observation for both study herds was the overall reduction in herd infection levels with the passage of time, not just in the vaccinated group. Among other factors, this was probably due to the risk of organism introduction being reduced in the unvaccinated group because of decreased infection rates in the vaccinated group (15). At least within the time frame of the current study, it was evident that Johne's disease infection could not be completely eliminated by vaccination alone, with or without management changes in the study herds.

Immunologically, *M. avium* subsp. *paratuberculosis* vaccination of cattle has been shown to result in *M. avium* subsp. *paratuberculosis*-specific gamma interferon (IFN-γ) responses in vaccinated calves by 7 days and specific antibody responses in 80% of calves by 3 to 6 months (16, 17). The strong antibody levels reported by those authors were sustained throughout the 12-month study period (16, 17). Because the infection is mainly intracellular, antibodies may not be able to confer direct protection, but the antibodies have been shown to confound detection of bovine TB (6, 7, 11). In the study groups, the antibody responses against *M. avium* subsp. *paratuberculosis* among vaccinated animals were maintained for several years, particularly if the study subjects started with stronger antibody responses. The antibody responses did not always correlate with protection. One of the issues in fully assessing the protective effects of antibodies is the current inability to distinguish vaccine immunity from immunity resulting from infection. Using the S/P ratio as an indicator of antibody immunity, at least 70% of all vaccinated cattle, if not clearly positive (above the 0.25 cutoff value), showed some level of measurable antibody response. The remainder of the vaccinated animals failed to show any measurable antibody response. Some of the ELISA-negative sera were also found to be nonreactive when tested with *M. avium* subsp. *paratuberculosis* agar gel immunodiffusion (AGID) testing. In fact, the AGID test results were negative even when *M. avium* subsp. *paratuberculosis* IDEXX ELISA results were positive, indicating either that the AGID test may be detecting a different class of *M. avium* subsp. *paratuberculosis* antibodies or that the two tests use different antigens. It is possible that some vaccinated animals in our study became seronegative by the time they were first tested (>2 years) but this appears to be less likely, as other authors have also reported failed antibody responses or antibody anergy in calves, measured even soon after vaccination (16, 18). Antibody reactivity in vaccinated animals did decline over the study period, but failed antibody responses to vaccination point to the involvement of some other immune mechanism. The reason why some animals show anergic antibody responses, which has also been reported earlier, is not fully understood and has not received much attention (16, 18). The anergic responses could result either from fetal exposure to *M. avium* subsp. *paratuberculosis* resulting in immunotolerance to the *M. avium* subsp. *paratuberculosis* antigens or the maternal antibody responses in calves inhibiting antibody responses in the vaccinated animals. The development of fetal regulatory T cells responsible for immunotolerance resulting from exposure to maternal alloantigens has been demonstrated earlier (19). Similar findings have also been reported for other infections upon fetal exposure (20), with T-cell responses reported to be normal but B-cell or antibody responses being affected.

In the vaccinated animals, milk and serum antibody responses were found to be positively correlated but milk frequently tested negative even when serum results were positive. The difference between milk and serum reactivity does not appear to be because of the two different ELISA kits used to test the two different specimen types. The two kits have been reported to have comparable sensitivities and only a slight difference in specificities (21). Differences in serum and milk *M. avium* subsp. *paratuberculosis* antibody reactivities have also been reported for infected cattle, and differences in infected cattle have been explained based on parity, stage of lactation, and dilution effects, with a likelihood of cattle testing *M. avium* subsp. *paratuberculosis* antibody positive when tested between 4 and 12 weeks of parturition (22). In infected animals, antibody production from local lymphoid systems adds to the total *M. avium* subsp. *paratuberculosis* antibody response, as these tissues have been shown to be infected (23); in Johne's disease-vaccinated cattle, the contribution of local immunity to milk antibodies against *M. avium* subsp. *paratuberculosis* using inactivated vaccine is likely negligible.

Antibody responses resulting from Johne's disease vaccination or infection are said to increase the risk of nonspecificity for TB testing in cattle, sheep, and deer herds (6–8, 10, 11, 24). Widespread use of Johne's disease vaccination can pose a problem for

TB surveillance. Cattle and deer vaccinated with killed *M. avium* subsp. *paratuberculosis* vaccine have been reported to demonstrate antibody responses to *M. avium* subsp. *paratuberculosis* and also to *M bovis* (6, 12). Using the new IDEXX *M. bovis* assay (using MPB70 and 83 antigens), we did not see any cross-reactive antibody responses against bovine TB in vaccinated cattle in *M. avium* subsp. *paratuberculosis*-vaccinated or -infected animals. Our findings are supported by another study, in which testing of deer herds with modified ELISA tools did not show any cross-reactions in chromatographically based enzyme immunoassays (6). The loss of cross-reactivity appears to be tied to use of the antigens MPB70 and MPB83 in the new ELISAs. These antigens have shown promise for improved diagnosis of bovine TB (13). MPB70 antigen, in particular, can identify TB-infected cattle late in the infection cycle, and MPB83 detects early antibody responses. Detection of antibodies to these antigens is boosted by skin tuberculin testing when cattle are infected with bovine TB (25). Although animals were not tested in our study soon after tuberculin skin testing, animals that received both tuberculin testing and vaccination against Johne's disease did not show any seroreactivity to *Mycobacterium tuberculosis* despite showing positive responses to *M. avium* subsp. *paratuberculosis*. In a previous study in which vaccination with *M. avium* subsp. *paratuberculosis* was followed by tuberculin skin testing, the specificity of *M. tuberculosis* antigens in serological assays was not compromised by *M. avium* subsp. *paratuberculosis* vaccination (7).

Vaccination can be a useful strategy for the management of Johne's disease but is not used frequently due to the associated risks (4). Concerns about *M. avium* subsp. *paratuberculosis* vaccination interfering with the interpretation of diagnostic tests for *M. avium* subsp. *paratuberculosis* or bovine TB are valid. Current *M. avium* subsp. *paratuberculosis* vaccines do not afford full protection but do offer economic promise while better vaccines are being researched (26, 27). The newer serological bovine TB ELISAs may help address some of the concerns about *M. avium* subsp. *paratuberculosis* vaccines interfering with TB testing, but this needs to be further evaluated in geographically diverse settings. Until vaccines that afford better protection are developed, using good management practices on farms and reducing the numbers of high-level shedders, controlling vertical transmission, and making sure that only testing-negative animals enter the herd are some approaches to decrease infection levels on farms.

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