

Single-Antigen Serological Testing for Bovine Tuberculosis[∇]

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Antibody responses are useful indicators of *Mycobacterium bovis* infection of cattle. Tests for such responses often use multiple *M. bovis* antigens as detection probes. This is recommended because responses to single antigens may be too variable for consistent diagnosis. However, the use of multiple antigens increases costs and the risk of false-positive results. As an alternative, the SeraLyte-*Mbv* system detects responses to a single *M. bovis* antigen, MPB83, by using a chemiluminescent testing platform with a high degree of analytical sensitivity. Testing with the SeraLyte-*Mbv* system was conducted in a blinded fashion with sera from experimentally infected and control cattle. To assess the species specificity of the single-antigen test, the sample included sera from animals infected with *M. bovis* ($n = 27$), *M. kansasii* ($n = 4$), *M. avium* subsp. *paratuberculosis* ($n = 11$), *M. avium* subsp. *avium* ($n = 12$), and uninfected animals ($n = 15$). Upon unblinding of the results, the sensitivity of the SeraLyte-*Mbv* system relative to the results for animals with known *M. bovis* infection was 89%. Consistent with the conservation of MPB83 sequences within the genus *Mycobacterium*, all 4 *M. kansasii*-infected animals tested positive with the SeraLyte-*Mbv* system and all 23 *M. avium*-infected animals tested negative. Blinded analysis of 30 serum samples collected from nine animals at various time points postinfection indicated 100% sensitivity after ≥ 3 months postinfection. All 15 uninfected samples in the blinded sample set tested negative with the SeraLyte-*Mbv* system. Unblinded analysis of sera from an additional 895 animals in 10 accredited bovine tuberculosis-free states revealed 98% specificity overall. The results support the feasibility of single-antigen testing for bovine tuberculosis with the SeraLyte-*Mbv* system.

Cellular immune responses, such as gamma interferon release upon T-cell stimulation, are commonly used as indicators of *Mycobacterium tuberculosis* complex infection of cattle, humans, and other mammals. As an alternative indicator, serological responses offer several potential advantages. These include the rapidity with which such responses can be detected and the stability of antibodies during sample transport, storage, and handling.

Diagnostic tests for serological responses often use panels of two or more *M. tuberculosis* complex antigens as detection probes (multiantigen testing). This is recommended because the responses to single antigens are thought to be too variable for consistent diagnosis (1–3, 6–8, 13, 16). In studies conducted with infected cattle, serum recognition of individual antigens has been reported to vary from animal to animal (1, 4, 5). MPB83 is among the most consistently recognized antigens in animals infected with *M. bovis*. Despite this, serological responses to MPB83 alone have rarely exhibited good sensitivity for use for the diagnosis of *M. bovis* infection (2–4, 11, 14, 16). This problem is mitigated by multiantigen testing with additional well-characterized antigens, such as ESAT6, CFP10, and Acr1, among others. In multiantigen strategies, positive responses to subsets of *M. tuberculosis* complex antigens are considered diagnostic of infection. However, reliance on multiple antigens increases assay costs and increases the risk of cross-reactivity

with immunoglobulins directed against orthologous antigens expressed by other bacteria.

An antigen recognition pattern is influenced by the analytical sensitivity of the detection systems used to look for it. When an infected animal's serological response to an antigen falls below a system's detection threshold, then the response is scored as absent. Analysis of the same animal by a more sensitive assay could yield a positive result for that antigen. Thus, an attractive alternative to multiantigen testing is to use a single-antigen probe in a testing system with a high degree of analytical sensitivity. In order to test this hypothesis, we used the PriTest SeraLyte-*Mbv* system to test for antibodies to a single antigen, MBP83, in sera from experimentally infected and control cattle. The SeraLyte-*Mbv* system uses advanced chemiluminescence-based chemistry and optics for the highly sensitive detection of antibody binding to antigens.

MATERIALS AND METHODS

Samples from experimentally infected cattle. Analysis with the SeraLyte-*Mbv* system was conducted in a blinded fashion with 90 frozen serum samples originating from 69 different animals. The samples were derived from three previous studies. The blinded analysis did not distinguish between the samples from the three studies.

Thirty-two of the 90 samples came from a previous study of immune responses to *M. bovis* antigens in cattle experimentally infected with *M. bovis* or *M. kansasii* (15). Sera included samples from *M. bovis*-infected calves ($n = 18$), *M. kansasii*-infected calves ($n = 4$), and noninfected calves ($n = 10$). Each sample in this group came from a distinct animal. The challenge dosages were 4×10^4 CFU for *M. bovis* strain 95-1315 and 4×10^8 CFU for *M. kansasii* strain 03-6931. The inocula were instilled directly into both tonsillar crypts of sedated calves, as previously described for the inoculation of white-

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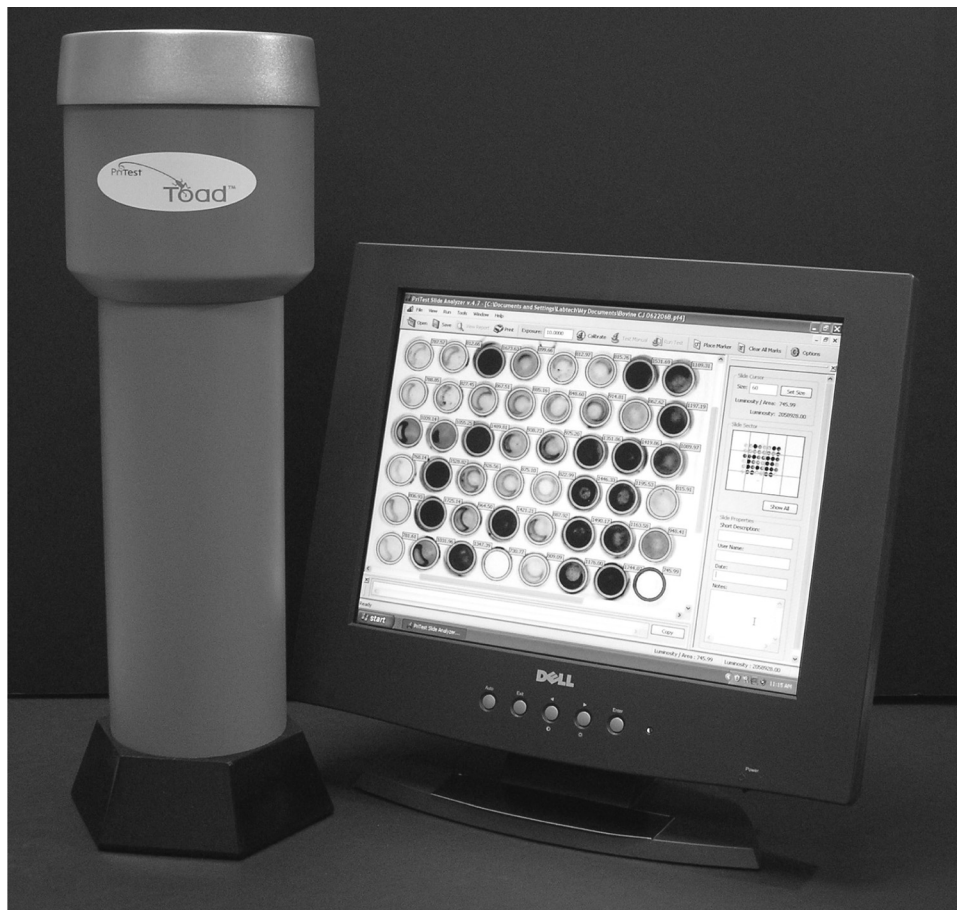


FIG. 1. Total optical assay device (TOAD) with a typical digital image of a 48-well readout.

tailed deer (10). Approximately 4.5 months after inoculation, all cattle were euthanized and examined as described previously (15). All *M. bovis*-infected animals were positive by the caudal fold test (CFT), histology, and culture at the time of euthanasia. The *M. kansasii*-infected animals were positive by CFT but not by the other methods (15).

Twenty-eight of the 90 samples came from a previous study of infections caused by *M. bovis*, *M. avium* subsp. *avium*, and *M. avium* subsp. *paratuberculosis* (12). Sera in this group came from nonchallenged calves ($n = 5$), *M. avium* subsp. *avium*-challenged calves ($n = 12$), and *M. avium* subsp. *paratuberculosis*-challenged calves ($n = 11$). Each sample came from a distinct animal.

The *M. bovis*-infected animals were positive by CFT, histology, and culture at the time of euthanasia. The *M. avium*-infected animals were positive by a modified comparative cervical tuberculin test and a modified gamma interferon test, and the *M. avium* subsp. *paratuberculosis*-infected animals were differentiated by the use of culture of fecal samples.

The remaining 30 samples came from a previous aerosol infection study of bovine tuberculosis (TB) (9). Healthy Maine Anjou calves (age, 4 months) were experimentally infected with *M. bovis* by an aerosol method. The sera from this group included preinfection (negative) samples ($n = 2$) and samples collected monthly for up to 4 months postinfection ($n = 28$). The 30 samples in this group came from nine different animals, all of which were positive by the tuberculin skin test at 3 months postinfection (9). Each sample was collected at a distinct monthly time point pre- or postinfection, and the blinded analysis did not provide information on the collection time point or the source animal. The results obtained for samples collected 3 months postinfection were used to calculate the overall sensitivity and specificity of the SeraLyte-*Mbv* system for the blinded sample.

Samples from uninfected animals in TB-free U.S. states. State laboratories and private donors kindly provided sera from a total of 895 uninfected cattle. The animals lived in accredited TB-free U.S. states (South Dakota, Kansas, Nebraska, Iowa, Montana, Florida, Mississippi, Maine, Georgia, and Oregon), and therefore, natural *M. bovis* infection was likely to be very rare

within this group. Analysis with the SeraLyte-*Mbv* system was conducted by use of a protocol similar to that applied to the samples from the experimentally infected animals. Because the sample sources were known, this analysis was not blinded.

Analysis with SeraLyte-*Mbv* system. The SeraLyte-*Mbv* technology (PriTest Inc., Redmond, WA) uses ferrite (magnetic iron) beads covalently attached to the MPB83 antigen. The ferrite-bound antigen binds to antibody formed against MPB83 in infected animals. To run the 2-h test, the ferrite conjugates are first incubated for 30 min with diluted sera and are then collected at a magnetic port. The ferrite conjugates are washed to remove residual serum, again collected, and then suspended in a solution of chicken anti-bovine primary antibody. The anti-bovine primary antibody binds to the bovine serum antibody associated with the antigen-coated beads. Biotinylated rabbit anti-chicken secondary antibody is immediately added and binds to chicken anti-bovine antibody. After a 20-min incubation, the ferrite conjugates are collected, washed, and then suspended in a streptavidin-horseradish peroxidase solution for 10 min. The ferrites are again collected, washed three times, and suspended in a Luminol peroxide chemiluminescent reagent and then transferred to a cassette for imaging of the emitted photons. The intensity of the emitted signal is proportional to the concentration of MPB83-specific serum antibody bound to the ferrite conjugate.

The SeraLyte-*Mbv* system attains its unique sensitivity in part by combining a very responsive charge-coupled-device camera with novel imaging software (PriTest Inc.) to differentiate MPB83 antibody-positive sera from sera devoid of MPB83 antibody. The camera is incorporated into a total optical assay device (PriTest Inc.), shown in Fig. 1 with a typical digital image of a 48-well readout. Imaging analysis is optimized by the use of proprietary algorithms for calibration and leveling that enhance the signal and discriminate it from the background noise.

Study design. Ninety samples stored frozen at the Agricultural Research Service (ARS) in Ames, IA, for up to 3 years were assigned code numbers that

TABLE 1. Conservation of *M. bovis* antigens in *Mycobacterium* species

Organism	% Amino acid identity by BLAST analysis (expected value)					
	MPB83 (Mb2898)	ESAT6 (Mb3905)	CFP10 (Mb3904)	Acr1 (Mb3423)	MPB64 (Mb2002c)	PPE68 (Mb3903)
<i>M. tuberculosis</i>	100 (4e-121)	100 (2e-46)	100 (3e-49)	100 (0.0)	100 (5e-131)	100 (0.0)
<i>M. kansasii</i>	76 (2e-83)	97 (2e-45)	95 (2e-40)	ND ^a	ND	ND
<i>M. avium</i>	ND	ND	ND	80 (0.0)	54 (4e-63)	50 (8e-27)
<i>M. smegmatis</i>	40 (1e-22)	71 (7e-30)	61 (5e-19)	72 (0.0)	43 (2e-47)	62 (8e-60)

^a ND, no homolog with the expected value of <1e-10 was detected.

provided no information on infection status. Although samples were derived from three earlier studies, this information was not contained in the coded sample identifiers. Samples were delivered to PriTest Inc. for blinded analysis with the SeraLyte-Mbv system, which yield a relative luminosity unit (RLU) value for each sample. After all measurements were completed, the sample identifications were unblinded. The percent sensitivity and specificity relative to the results for animals with unblinded infection status were calculated at signal thresholds ranging from 10 to 100 RLUs.

RESULTS

The SeraLyte-Mbv system was developed in-house by PriTest Inc. Early versions utilized a CFP10-ESAT6 fusion conjugate to detect serological responses to these two *M. tuberculosis* complex antigens. Second-generation tests substituted MPB83 for CFP10-ESAT6 because of the higher reported incidence of MPB83 seroreactivity in cattle and other mammals infected with *M. tuberculosis* complex species, including *M. bovis* (2-4, 11, 14, 16). Preliminary analyses with nonblinded sample sets from cattle confirmed that reactivity to MPB83 is a more consistent indicator of infection than reactivity to the CFP10-ESAT6 fusion when the SeraLyte-Mbv system is used (data not shown).

Analysis with the single-antigen SeraLyte-Mbv system was conducted with sera from cattle that were experimentally infected with *M. bovis* (n = 27), *M. kansasii* (n = 4), *M. avium* subsp. *paratuberculosis* (n = 11), or *M. avium* subsp. *avium* (n = 12), as well as uninfected animals (n = 15). Bioinformatic analysis with the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>) predicted that the *M. avium* subspecies do not produce antigens with strong homology (≥50% amino acid identity) to MPB83, whereas *M. kansasii* does (Table 1). Therefore, *M. bovis*- and *M. kansasii*-infected animals were expected to be positive for MPB83 antibody,

while *M. avium* (*M. avium* subsp. *avium* or *M. avium* subsp. *paratuberculosis*)-infected animals and uninfected animals were expected to be negative. The same expectation applies to any serological test that includes MPB83 as a probe (2-4, 11, 14, 16).

For the purpose of comparison, Table 1 also shows the homology among five other antigens commonly used in multiantigen testing for serological reactivity to *M. bovis* antigens (13, 16). Significant sequence conservation is common among the >100 species of *Mycobacterium*, even when the sequences of *M. bovis* antigens are compared to the sequences of antigens of relatively distantly related species, such as *M. smegmatis*. This phenomenon is one rationale for single-antigen testing.

Upon unblinding of sample identifications, sensitivity and specificity values relative to infection status were calculated for sensitivity thresholds ranging from 10 to 100 RLUs. Good test performance was evident at most threshold values, with the highest sensitivity being seen at lower threshold values (Table 2). Optimal performance was observed when values ranging from 50 to 80 RLUs were applied. At a threshold value of 50 RLUs, the SeraLyte-Mbv system correctly detected 24 of 27 samples from *M. bovis*-infected animals, which corresponds to 89% sensitivity (Table 3).

Four of four samples from *M. kansasii*-infected animals were also found to be positive, as expected, given the conservation of the *M. kansasii* ortholog to MPB83. We are not aware of other studies that have examined the cross-reactivity of *M. bovis* antigens with *M. kansasii*-infected animals. However, the tuberculin skin test as well as most reported serological tests include MPB83 as an antigen, and therefore, similar cross-reactivities should be expected.

The SeraLyte-Mbv system correctly excluded all 15 samples from uninfected animals, which corresponds to 100% specificity. It also correctly excluded all 23 samples from

TABLE 2. Sensitivity and specificity as functions of detection threshold value

Threshold value (RLUs)	% Sensitivity	% Specificity
10	100	53
20	93	82
30	93	88
40	90	89
50	89	100
60	89	100
70	85	100
80	85	100
90	81	100
100	81	100

TABLE 3. Sensitivity and specificity of SeraLyte-Mbv analysis of sera collected from 69 animals^a

Organism in serum sample	No. of isolates	No. of samples with the following result with the SeraLyte-Mbv system:	
		Positive	Negative
<i>M. bovis</i>	27	24	3
<i>M. kansasii</i>	4	4	0
<i>M. avium</i> subsp. <i>paratuberculosis</i>	11	0	11
<i>M. avium</i> subsp. <i>avium</i>	12	0	12
Uninfected	15	0	15

^a A threshold value of 50 RLUs was used.

TABLE 4. Detection of MPB83 antibody in samples taken over time postinfection with *M. bovis*

Animal no.	SeraLyte- <i>Mbv</i> result at the following time points postinfection (mo)				
	0 ^a	1	2	3	4
1	Negative	NT	Negative	Positive	NT ^b
2	Negative	NT	Positive	Positive	Positive
3	NT	NT	Positive	Positive	Positive
4	NT	NT	Positive	Positive	Positive
5	NT	Positive	Positive	Positive	Positive
6	NT	Positive	Positive	Positive	Positive
7	NT	NT	Positive	Positive	Positive
8	NT	NT	Positive	Positive	Positive
9	NT	NT	Positive	Positive	Positive

^a Preinfection samples.

^b NT, not tested.

M. avium subsp. *avium*- and *M. avium* subsp. *paratuberculosis*-infected animals, as expected, given the absence of MPB83-like antigens in *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium*. The absence of cross-reactivity with these ubiquitous pathogens illustrates the specificity of single-antigen testing.

Prior to unblinding, all samples that were positive at a threshold value of 50 RLUs were retested twice at the same threshold value. All were positive in both repetitions, indicative of good reproducibility.

In order to assess the infection time course over which the MPB83 antigen was detectable with the SeraLyte-*Mbv* system, an analysis was conducted with serum samples ($n = 30$) collected at multiple time points from the nine animals in the aerosol infection study (9). As shown in Table 4, most samples collected 1, 2, 3, or 4 months after infection with *M. bovis* were positive by analysis with the SeraLyte-*Mbv* system. The sole false-negative sample was collected from animal 1 at 2 months postinfection. In total, 27 of the 28 infected samples in this analysis tested positive by use of the SeraLyte-*Mbv* system (sensitivity, 96%). The two preinfection samples analyzed were negative.

In order to test the specificity of the system with a larger sample set, analysis with the SeraLyte-*Mbv* system was conducted with an unblinded sample of sera from 895 uninfected cattle in 10 accredited TB-free U.S. states. The protocol used

for this analysis was similar to that applied to the blinded sample, except that earlier-generation reagent cassettes and antigen conjugation chemistries were used. Positive control samples consisting of sera from infected cattle were uniformly positive at the applied RLU threshold value. The results were negative for 875 of the 895 samples (Table 5), which corresponds to a specificity of 98%.

DISCUSSION

Humoral immune responses offer several theoretical advantages over cellular responses when one is screening for specific microbial infections. These include the rapidity with which antibodies can be detected in serum and the stability of the antibodies during sample transport, storage, and handling. For the detection of *M. tuberculosis* complex infection, the standard approach of tuberculin skin testing has the disadvantage of requiring a follow-up reading of the delayed-type hypersensitivity response 2 to 3 days after antigen application. In contrast, antibodies can be detected in a single analysis, provided that a test has a sensitivity sufficient for the detection of a wide range of antibody titers.

Diagnostic tests for serological responses most often use panels of multiple *M. tuberculosis* complex antigens as detection probes (multiantigen testing). This is recommended because the responses to single antigens are thought to be too variable for consistent diagnosis (1–3, 6–8, 13). However, the present study indicates that an assay platform with a high degree of analytical sensitivity can detect responses to a single antigen, MPB83, in 89% of cattle experimentally infected with *M. bovis* or *M. kansasii*, a related pathogen that expresses a homologous antigen.

MPB83 is among the most consistently recognized antigens in cattle and other mammals infected with *M. bovis* and is a component of most or all serological tests for cattle that have been described (2–4, 11, 14, 16). Therefore, all such tests would be expected to cross-react with *M. kansasii*, as seen in the present study. The false-positive detection of *M. kansasii* infection can be problematic; however, the problem appears to be rare, as indicated by the high degree of specificity (98%) observed for the sera from 895 cattle from 10 accredited TB-free U.S. states that were tested. Nonetheless, secondary tests that exclude *M. kansasii* infection may become useful adjuncts to the tuberculin skin test as

TABLE 5. Specificity of unblinded SeraLyte-*Mbv* testing of sera from 895 TB-free animals

Sample source	State(s) of origin	No. of samples:	
		Tested with SeraLyte- <i>Mbv</i> system	With positive results
Cooperative Brucellosis Laboratory, Pierre, SD	South Dakota, Kansas, Nebraska, Iowa, Montana	300	6
The Live Oaks Diagnostic Laboratory, FL	Florida	99	2
Veterinary Diagnostic Laboratory, KS	Kansas	100	1
Private donor	Mississippi	99	0
Private donor	Maine	98	5
University of Georgia Veterinary Diagnostic and Investigational Laboratory, Tifton, GA	Georgia	99	1
Oregon Department of Agriculture, Salem, OR	Oregon	100	5
Total	10 states	895	20

well as serological testing. The 20 false-positive serum samples in our sample might reflect *M. kansasii* infection, or alternatively, they may reflect a rare exposure to *M. bovis* from unknown sources. Overall, the method exhibited a high degree of specificity, as expected of a single-antigen test.

With the exception of a study conducted with a small number of experimentally infected animals (14), we are not aware of previous studies (blinded or otherwise) in which serological responses to MPB83 alone were seen in $\geq 89\%$ of samples from infected animals. It is possible that the detection methods used in earlier studies (2–4, 11, 14, 16) did not have sufficient analytical sensitivity. The SeraLyte-*Mbv* system detected responses to MPB83 as early as 1 month postinfection in two animals; however, the limited analysis whose results are shown in Table 4 suggests that testing at 3 months after aerosol infection may be needed for consistent detection.

Multiantigen testing has the potential to increase diagnostic sensitivity, but it can also create new challenges. Test costs increase proportionately with increasing numbers of antigen reagents, as does the risk of cross-reactivity with immunoglobulins directed against homologous antigens expressed by nontuberculous mycobacteria. As seen in Table 1, this problem can extend beyond *M. kansasii* for some antigens. Moreover, the requirement for a precise signal threshold for each antigen may adversely affect the robustness of the assay. For example, a recent study conducted with a large number of nonblinded bovine serum samples and 13 *M. bovis* antigens generated excellent results (16). However, careful calibration was needed to identify signal threshold values for each of the 13 antigens. It is not yet known how well such algorithms will function when the test is applied in a blinded fashion to diverse samples by diverse users.

Like multiantigen tests, single-antigen tests require optimization of the signal threshold value. However, optimization should be considerably simpler for single-antigen tests. This is illustrated by the robust range of detection threshold values (30 to 80 RLUs) that delivered sensitivities and specificities equaling or exceeding 85%. Values outside of that range delivered an elevated sensitivity or specificity, but not both. Such values may nonetheless be useful for specific applications, such as preliminary screening.

The strengths of the present study include its blinded structure and the use of cattle known to be infected with nontuberculous *Mycobacterium* species. The limitations include the sample size and the use of experimentally infected animals, which may have higher antibody titers than naturally infected animals. Concerns about titer are somewhat offset by the fact that the samples had been stored frozen for up to 3 years prior to analysis, which may have diminished the antibody titers in some samples. Nonetheless, blinded analysis of a larger sample of naturally infected and uninfected cattle is needed to more fully evaluate the single-antigen approach.

In conclusion, the results of this blinded analysis support the feasibility of using a single *M. tuberculosis* complex antigen to detect the serological responses to bovine TB. If it is further supported by the findings of expanded studies conducted with naturally infected animals, then single-antigen testing may constitute a more specific and cost-effective alternative to multiantigen testing for bovine TB.

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