



Identification of Novel Antigens Recognized by Serum Antibodies in Bovine Tuberculosis

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ABSTRACT Bovine tuberculosis (TB), caused by *Mycobacterium bovis*, remains an important zoonotic disease posing a serious threat to livestock and wildlife. The current TB tests relying on cell-mediated and humoral immune responses in cattle have performance limitations. To identify new serodiagnostic markers of bovine TB, we screened a panel of 101 recombinant proteins, including 10 polyepitope fusions, by a multiantigen print immunoassay (MAPIA) with well-characterized serum samples serially collected from cattle with experimental or naturally acquired *M. bovis* infection. A novel set of 12 seroreactive antigens was established. Evaluation of selected proteins in the dual-path platform (DPP) assay showed that the highest diagnostic accuracy (~95%) was achieved with a cocktail of five best-performing antigens, thus demonstrating the potential for development of an improved and more practical serodiagnostic test for bovine TB.

KEYWORDS antigen, antibody, *Mycobacterium bovis*, serodiagnosis, tuberculosis

Bovine tuberculosis (TB) is an important zoonotic disease caused by *Mycobacterium bovis*, a member of the *M. tuberculosis* complex (1). Although *M. bovis* is most often isolated from tuberculous cattle, it has a broad range of susceptible host species, including humans (2–4). The control of TB in cattle is therefore difficult due to the existence of wildlife reservoirs of *M. bovis*, such as white-tailed deer in the United States, Eurasian badgers in Great Britain, wild boars in Spain, and brushtail possums in New Zealand (5–8).

The antemortem diagnostic methods currently approved for use in cattle have limitations. The intradermal tuberculin test has suboptimal sensitivity and inconsistent performance (1, 9, 10), while the available blood-based assays, such as the Thermo Fisher Scientific Bovigam TB kit or the Idexx *M. bovis* antibody (Ab) test, lack the required accuracy and show a significant variability when used in different geographic areas (11–13). Given the ease of sample collection and the test procedure, antibody detection assays may be useful to identify *M. bovis*-infected cattle (1, 14, 15), but the existing methods require improvement.

The primary goal of the present study was to identify novel seroreactive antigens of *M. bovis*. Using a multiantigen print immunoassay (MAPIA) and well-characterized serum samples serially collected from cattle with experimental or naturally acquired *M. bovis* infection, we screened a panel of 101 recombinant proteins of *M. tuberculosis*, including 10 polyepitope fusions. The performance of MAPIA-selected candidates was also evaluated in pilot experiments using the dual-path platform (DPP) technology to

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TABLE 1 Distribution of *M. tuberculosis* proteins, ranked by antibody reactivity rates, obtained in MAPIA with serum samples from 42 *M. bovis*-infected cattle^a

Reactivity rate (%)	No. of proteins	Protein names
0	32	Rv0016c, ^b Rv0070c, ^b Rv0242c, ^b Rv0486, ^b Rv0792c, ^b Rv0800, ^b Rv1005c, ^b Rv1127c, ^b Rv1128c, ^b Rv1246, ^c Rv1582c, ^b Rv1586c, ^b Rv1789, ^c Rv1813c, ^c Rv1985, ^c Rv2075, ^c Rv2178c, ^b Rv2357c, ^b Rv2378c, ^b Rv2382c, ^b Rv2389, ^c Rv2447c, ^b Rv2450, ^c Rv2885c, ^b Rv2992c, ^b Rv3088, ^b Rv3227, ^b Rv3294c, ^b Rv3478, ^c Rv3734c, ^b Rv3871, ^c Rv3876 ^c
1–10	17	Rv0363, ^c Rv0571c, ^b Rv0577, ^c Rv0888, ^b Rv1023, ^b Rv1270c, ^c Rv1415, ^b Rv1559, ^b Rv1702c, ^b Rv1926, ^c Rv2457c, ^b Rv2608, ^c Rv2801, ^c Rv2858c, ^b Rv2866, ^c Rv3127, ^c Rv3865 ^c
11–20	9	Rv0379, ^c Rv1180, ^b Rv1371, ^b Rv1945, ^b Rv2032, ^c Rv2623, ^c Rv3121, ^c Rv3615, ^c Rv3875 ^c
21–30	13	Rv0223c, ^b Rv0831c, ^c Rv1288, ^c Rv1738, ^c Rv1818, ^c Rv2108, ^c Rv2141c, ^b Rv2225, ^b Rv2589, ^b Rv2945, ^c Rv3170, ^b Rv3709c, ^b Rv3873 ^c
>30	12	Rv0798c, ^b Rv1196, ^b Rv1463, ^b Rv1592c, ^b Rv1980c, ^c Rv2386c, ^b Rv2650c, ^b Rv2873, ^c Rv2875, ^c Rv3704c, ^b Rv3834c, ^b Rv3874 ^c

^aHigh nonspecific binding was found with the following proteins (which were excluded from the data analyses): Rv0483, Rv0509, Rv1193, Rv2280, Rv3029c, and Rv3340, which were produced at APHA as described previously (30), and Rv3614 and Rv3881, which were produced at IDRI as described previously (21).

^bProtein produced at APHA as described previously (30).

^cProtein produced at IDRI as described previously (21).

demonstrate the feasibility of developing an improved and more practical blood test for bovine TB.

RESULTS

Antigen characterization. Using MAPIA, we screened a panel of 101 recombinant proteins of *M. tuberculosis/M. bovis*, including 10 polyepitope fusions developed in our previous studies on human TB serology. Serum samples were obtained from 31 cattle that had been aerosol inoculated with *M. bovis* strain 95-1315 or 10-7428 in four independent experiments (serially collected during infection) and from 11 tuberculous cattle with naturally acquired disease that were tuberculin skin test nonreactors (15). An emphasis was placed on antigen candidates capable of eliciting relatively early antibody responses and/or showing complementary reactivity to the Rv2875 (MPT70) and Rv2873 (MPT83) proteins, known to be predominantly recognized by antibodies in *M. bovis* infection (16, 17).

Out of 91 single proteins, 51 (~56%) displayed specific IgG reactivity in one or more *M. bovis*-infected animals and 32 were nonreactive, whereas 8 proteins produced high levels of nonspecific IgG binding and therefore were excluded from the data analysis (Table 1). Only 12 proteins were recognized with a frequency of >30%. Tables 2 and 3 show the performance characteristics of these 12 antigens. Figures 1 and 2 provide examples of the MAPIA results obtained in the antigen-screening experiments with

TABLE 2 Characterization of top 12 MAPIA-reactive antigens recognized by serum antibodies from *M. bovis*-infected cattle

Animal no.	Name		Amino acid sequence identity (%)		Antibody reactivity in MAPIA			
					No. of cattle with the following whose sera were reactive:			
					Experimental infection ^c (n = 31)	Naturally acquired disease ^d (n = 11)	Overall rate ^a	Complementary to DID38 ^b
1	Rv2873	<i>mpt83</i>	<i>M. bovis</i>	<i>M. avium</i> subsp. <i>paratuberculosis</i>	28	9	37/42 (88.1)	NA
2	Rv2875	<i>mpt70</i>	100	<30	28	9	37/42 (88.1)	NA
3	Rv2650c	<i>rv2650c</i>	89	41	9	9	18/42 (42.9)	Yes
4	Rv1463	<i>rv1463</i>	100	90	9	9	18/42 (42.9)	Yes
5	Rv3834c	<i>serS</i>	100	88	7	9	16/42 (38.1)	Yes
6	Rv0798c	<i>cfp-29</i>	100	87	9	7	16/42 (38.1)	Yes
7	Rv3704	<i>gshA</i>	100	74	9	6	15/42 (35.7)	Yes
8	Rv1592c	<i>rv1592c</i>	99	80	6	8	14/42 (33.3)	Yes
9	Rv3874	<i>cfp-10</i>	100	<30	10	3	13/42 (31.0)	No
10	Rv1196	<i>PPE18</i>	98	41	11	2	13/42 (31.0)	No
11	Rv1980c	<i>mpt64</i>	99	55	11	2	13/42 (31.0)	No
12	Rv2386c	<i>mbtI</i>	100	74	7	6	13/42 (31.0)	Yes

^aThe overall rate is presented as the number of cattle whose serum was reactive/total number of cattle tested (percent).

^bAntibody reactivity detected prior to or in the absence of seroconversion with the DID38 fusion protein (Rv2875-Rv2873). NA, not applicable.

^cAntibody reactivity detected at any time point during experimental infection ranging from 6 to 36 weeks after inoculation of *M. bovis*.

^dSamples from *M. bovis*-infected CFT nonreactors (15).

TABLE 3 Antibody profiles obtained with top 12 seroreactive antigens in cattle naturally infected with *M. bovis*

Animal no.	Seroreactivity to the following protein ^a :											
	Rv2873	Rv2875	Rv2650c	Rv1463	Rv3834c	Rv0798c	Rv3704	Rv1592c	Rv3874	Rv1196	Rv1980c	Rv2386c
755	+	+	+	+	+	+	+	+	+	-	-	+
272	+	+	+	-	+	+	+	+	+	-	+	+
976	+	+	+	+	+	+	+	+	-	-	-	+
857	-	-	+	+	+	-	-	+	-	-	-	+
676	+	+	+	+	+	-	+	+	-	-	-	+
889	+	+	+	+	+	+	+	+	-	+	-	-
352	+	+	-	-	+	+	-	-	-	-	-	-
855	-	-	+	+	+	+	-	+	-	-	-	-
370	+	+	-	+	-	-	-	-	-	-	-	-
364	+	+	+	+	+	+	+	+	-	-	-	+
809	+	+	+	+	-	-	+	-	+	+	+	-

^aThe results obtained for each antigen by MAPIA with serum from *M. bovis*-infected CFT nonreactors are shown as antibody positive (+ with gray shading) or negative (- with no shading).

Infectious Disease Research Institute (IDRI) and Animal and Plant Health Agency (APHA) proteins, respectively.

The Rv2875 and Rv2873 proteins were confirmed to be the major serological targets in *M. bovis* infection. While both antigens elicited IgG antibodies in ~88% of tuberculous cattle, the seroconversion times obtained for the two proteins varied from animal to animal and were more or less affected by the tuberculin test-induced antibody boost, depending on the experimental design (Fig. 1A and B). The next 10 best-performing antigens recognized in animals with experimental and naturally acquired *M. bovis* infections showed the potential for added serodiagnostic value, with individual

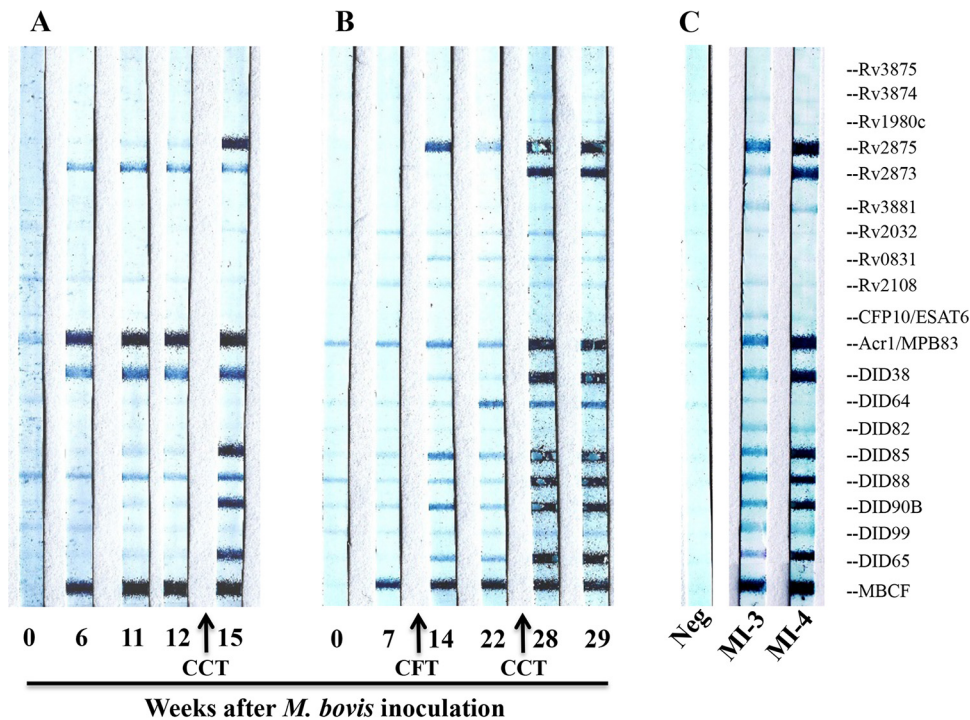


FIG 1 MAPIA reactivity of mycobacterial protein antigens produced at IDRI, USA, with serum samples from cattle experimentally or naturally infected with *M. bovis*. (A) Serial samples collected over time from calf 221 that had been aerosol inoculated with *M. bovis* strain 95-1315; (B) serial samples collected over time from calf 1210 that had been aerosol inoculated with *M. bovis* strain 10-7428; (C) serum samples from one negative control (Neg) and two tuberculous CFT nonreactors (MI-3 and MI-4) identified in a Michigan *M. bovis*-affected herd (15). MAPIA was performed as described in Materials and Methods, and the positions of the immobilized proteins are shown on the right margin. Visible bands on the strips indicate the presence of an IgG antibody to the corresponding antigens. Arrows, the relative time points of administration of PPD for CFT and CCT.

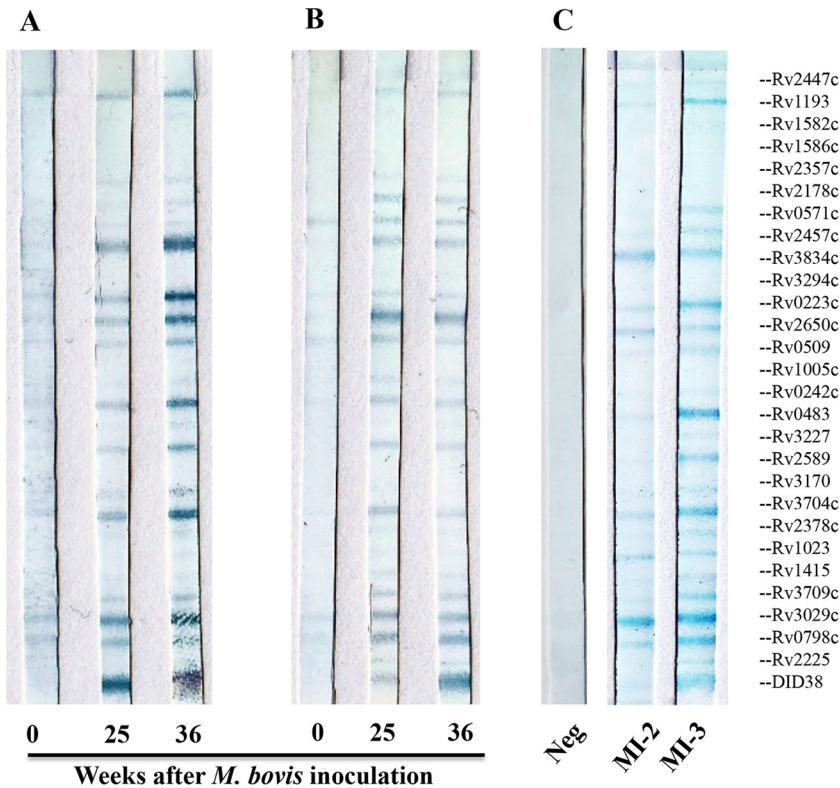


FIG 2 MAPIA reactivity of mycobacterial protein antigens produced at APHA in the United Kingdom with serum samples from cattle experimentally or naturally infected with *M. bovis*. (A and B) Serial samples collected from calves 51 and 57, respectively, prior to and after aerosol inoculation with *M. bovis* strain 95-1315 and injection of PPDs for CCT at 13 and 30 weeks after *M. bovis* inoculation; (C) serum samples from one negative control (Neg) and two tuberculous CFT nonreactors (MI-2 and MI-3) identified in a Michigan *M. bovis*-affected herd (15). MAPIA was performed as described in Materials and Methods, and the positions of the immobilized proteins are shown on the right margin. Visible bands on the strips indicate the presence of an IgG antibody to corresponding antigens.

reactivity rates ranging from 31 to 43% (Tables 2 and 3). When combined, the top 12 antigens provided a cumulative sensitivity of 95.2%. Antibody kinetics, the response magnitude, and antigen recognition patterns varied among the infected animals (Fig. 1 and 2; Table 3), suggesting that integration of new candidates identified in the present study may reduce the diagnostic window, known to be a limitation of the serological detection of bovine TB (1, 10).

Some of the single proteins selected in the present study were also components of the polypeptide fusions available for serological evaluation (Table 4). As expected, DID38, DID65, and DID85, each of which included Rv2873 and/or Rv2875, showed superior seroreactivity over the other fusions in MAPIA studies (Fig. 1). Table 4 shows the results obtained for the four best-performing fusions (i.e., DID38, DID65, DID85, and CFP10–ESAT-6), with antibody recognition rates ranging from 33 to 88%. Six fusion proteins produced high levels of nonspecific IgG binding and were excluded from the data analysis. Serum from four calves in the experimental infection group ($n = 31$) and from one animal in the naturally acquired infection group ($n = 11$) did not react with the four best-performing fusions. Combinatorial analysis of the antibody reactivity profiles shown by the newly identified proteins of serodiagnostic value may lead to the design of highly reactive polypeptide fusions.

Pilot evaluation of selected antigens in DPP assay. Based on the MAPIA results, we selected the five best-performing single proteins (Table 2) and the top two fusions (Table 4) to demonstrate a proof of principle of their diagnostic performance in an independent antibody assay, such as an assay in the DPP format. The single proteins were printed as a cocktail onto DPP strip membranes, whereas the fusions were

TABLE 4 Performance of polypeptide fusion proteins in MAPIA with serum samples from *M. bovis*-infected cattle^a

Fusion protein name	Fusion protein composition ^b	Antibody reactivity		Overall rate ^c
		No. of cattle with the following whose sera were reactive:		
		Experimental infection ^d (n = 31)	Naturally acquired disease ^e (n = 11)	
DID38	Rv2875-Rv2873	27	10	37/42 (88.1)
DID65	Rv2875 -Rv0934- Rv3874	26	10	36/42 (85.7)
DID85	Rv2875 -Rv0831-Rv2032	27	9	36/42 (85.7)
CFP10-ESAT-6	Rv3874 -Rv3875	8	6	14/42 (33.3)

^aHigh nonspecific binding was found with the following fusion proteins (which were excluded from the data analyses): Acr1-MPT83 (Rv2031-Rv2873), DID64 (Rv2031-Rv0934-Rv387), DID82 (Rv1980c-Rv3619-Rv38814), DID88 (Rv2873-Rv1980c-Rv3881), DID90 (Rv2873-Rv0934-Rv2032), and DID99 (Rv1980c-Rv2108-Rv3881).

^bKnown seroreactive proteins (included in Table 1) are shown in bold with underlining.

^cThe overall rate is presented as number of cattle whose serum was reactive/total number of cattle tested (percent).

^dAntibody reactivity detected at any time point in the course of experimental infection ranging from 6 to 36 weeks after inoculation of *M. bovis*.

^eSamples from *M. bovis*-infected CFT nonreactors (15).

immobilized as separate test lines. To determine seroreactivity rates, we used an extended collection of bovine serum samples derived from *M. bovis* challenge experiments (36 animals) and from naturally infected cattle (38 animals) diagnosed with TB in three states within the United States (Michigan, Texas, and New Mexico). For assessment of specificity and potential cross-reactivity, we used serum from cattle experimentally inoculated with *M. avium*, cattle vaccinated against or infected with *M. avium* subsp. *paratuberculosis*, and noninfected control animals from TB-free herds. Among the three DPP assay versions evaluated (Table 5), the five-protein cocktail showed the highest estimate of diagnostic accuracy (~95%), with no cross-reactivity being detected in samples from cattle groups vaccinated against paratuberculosis or infected with non-TB mycobacteria. DPP reader values obtained for the five-protein cocktail with 67 seropositive samples from *M. bovis*-infected cattle (Table 5) ranged from 53 to 1,026 relative light units (RLU), whereas the 2 false-positive serum samples showed DPP readings of 48 and 87 RLU.

DISCUSSION

Bovine TB remains a serious threat to livestock in the United States and worldwide. The existing tests for cattle have operational, logistic, and diagnostic accuracy limitations. Current antemortem tests for bovine TB include measurement of delayed-type hypersensitivity (i.e., skin testing) to purified protein derivative (PPD) and/or an *in vitro* assay for gamma interferon produced in response to mycobacterial antigen stimulation (i.e., the Bovigam assay [Thermo Fisher Scientific, NY]). These tests rely on early cell-mediated immune (CMI) responses, a hallmark of immunopathogenesis in bovine TB (1, 9, 11).

A serodiagnostic approach constitutes an attractive alternative to the current antemortem tests for bovine TB, because antibody detection assays are generally simple, rapid, reproducible, inexpensive, and relatively noninvasive. In contrast to the intradermal tuberculin test, serology does not require multiple interventions, is much less subjective for interpretation, and does not interfere with the immune status of the tested animals. Importantly, serological assays can detect cattle with *M. bovis* infection that are anergic to CMI-based tests (15, 18, 19). Despite the attractive operational advantages of serology, numerous attempts to develop a reliable antibody test for *M. bovis* infection in cattle have been disappointing (9, 11).

In the present study, we screened a large number of potentially useful serological candidates by MAPIA, identified a new set of antibody-reactive antigens, and demonstrated the feasibility of developing a more sensitive serological assay for bovine TB. The vast majority of proteins in this collection have never been evaluated in *M. bovis*-infected cattle. The panel of 12 best-performing proteins included 4 antigens known to be recognized by serum antibodies in bovine TB (i.e., Rv1980c [MPB64],

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TABLE 5 Diagnostic performance of three DPP prototype versions using selected antigens

Test antigen ^a	No. of animals with antibody reactivity ^b							% sensitivity ^c	% specificity ^d	% accuracy ^e
	Animals with experimental <i>M. bovis</i> infection (n = 36)	Animals with natural <i>M. bovis</i> infection (n = 38)	Noninfected control animals (n = 91)	<i>M. avium</i> subsp. <i>paratuberculosis</i> -vaccinated animals (n = 5)	<i>M. avium</i> subsp. <i>paratuberculosis</i> -infected animals (n = 8)	<i>M. avium</i> -infected animals (n = 8)				
Protein cocktail	35	32	2	0	0	0	90.50 (67/74)	98.20 (110/112)	95.20 (177/186)	
DID38	33	30	1	0	0	0	85.10 (63/74)	99.10 (111/112)	93.50 (174/186)	
DID65	30	30	2	0	0	0	81.10 (60/74)	98.20 (110/112)	91.40 (170/186)	

^aThe protein cocktail includes the top five proteins, Rv2873, Rv2875, Rv2650c, Rv1463, and Rv3834c (Table 1); DID38 includes the Rv2873 and Rv2875 sequences; and DID65 includes the Rv2873, Rv0934, and Rv3874 sequences.

^bAntibody reactivity detected at any time point during experimental infection ranging from 6 to 36 weeks after inoculation of *M. bovis*.

^cData in parentheses represent the number of culture-confirmed animals that tested positive/total number of culture-confirmed animals tested.

^dData in parentheses represent the number of noninfected animals that tested negative/total number of noninfected animals tested.

^eData in parentheses represent the number of true-positive and true-negative results for all tested animals/total number of animals tested.

Rv2873 [MPB83], Rv2875 [MPB70], and Rv3874 [CFP10]) (17, 20) and 8 previously unknown antigens.

Advances in genomic and proteomic research have made it possible to provide a large-scale characterization of TB biomarkers eliciting antibody responses in humans and nonhuman primates (21–23). The studies defined the *M. tuberculosis* immunoproteome to be approximately 10% of the bacterial proteome enriched for membrane-associated and extracellular proteins, which can lead to the development of more accurate serodiagnostic tools (24). Interestingly, the set of 13 serological targets found to be predominantly associated with active TB in humans (22) included only 2 proteins, Rv1980c and Rv2873, identified in the present study among the top 12 antigens recognized by antibodies in *M. bovis*-infected cattle.

Our previous research on bovine TB serology using enzyme-linked immunosorbent assay (ELISA), Western blotting, and MAPIA has demonstrated that (i) antibody responses to *M. bovis* involve multiple antigens, including MPB64, MPB70, MPB83, CFP10, and ESAT-6, with remarkable animal-to-animal variations in reactivity patterns (20); (ii) IgM and IgG antibodies can be detected in blood 3 to 4 and 4 to 6 weeks, respectively, after *M. bovis* inoculation (2, 17); (iii) individual seroreactivity profiles may evolve with disease progression (20); (iv) the variability and evolution of the humoral immune response may be addressed by use of multiantigen cocktails to maximize the diagnostic sensitivity of serological assays (17, 20, 25); (v) antibody responses in *M. bovis*-infected cattle are boosted following PPD injection for skin testing (3, 25); and (vi) at advanced disease stages, antibody levels positively correlate with lesion severity (25, 26).

In recent years, several groups have reported on antibody tests for bovine TB developed in six different immunoassay formats employing recombinant antigens (12, 14, 17, 19, 27, 28). One product, the Idexx *M. bovis* Ab test, has received USDA licensure and OIE approval for use for bovine TB diagnosis. This is an ELISA detecting serum antibody to the MPB70 and MPB83 antigens with a sensitivity of 63% and a specificity of 98% (12). The test specificity is compromised in cattle vaccinated for Johne's disease (29) or infected with *M. kansasii* (12).

To develop a more accurate blood test for bovine TB, we propose to combine the rational antigen selection strategy presented here with the innovative DPP technology. This report describes the identification of novel mycobacterial proteins of immunodiagnostic importance. Using selected antigens, we demonstrated the feasibility of improved serodiagnosis of bovine TB. The results provide further evidence that combinations of carefully selected specific antigens are required to develop a highly sensitive blood test for TB in cattle. This concept will be validated in our ongoing work on the development of new polyepitope fusion antigens incorporating newly discovered seroreactive proteins of diagnostic value.

MATERIALS AND METHODS

Antigens. We used 91 recombinant proteins of *M. tuberculosis* expressed and purified at APHA and IDRI using two different strategies as previously described (21, 30). The APHA proteins were produced via the Gateway approach, whereas the IDRI antigens were preselected on the basis of a high amino acid sequence identity with *M. bovis* protein sequences and a relatively low identity with *M. avium* subsp. *paratuberculosis* protein sequences. In addition, we tested 10 polyepitope fusions constructed in our previous studies on human TB serology, as described previously (21), to maximize protein expression and epitope accessibility. Each fusion included at least one TB-specific protein, such as MPT64 (Rv1980c), MPT70 (Rv2875), MPT83 (Rv2873), or CFP10 (Rv3874), known to be recognized by antibodies of *M. bovis*-infected cattle (17, 20).

Animals and specimens. Serum samples were obtained from Holstein steers that had been aerosol infected with *M. bovis* (~10⁴ CFU of strain 95-1315 or 10-7428) in four experiments performed as described previously (2, 3, 31) in a biosafety level 3 (BSL-3) facility at the National Animal Disease Center, Ames, IA, in accordance with Institutional Biosafety and Animal Care and Use Committee guidelines. Serial samples were collected at multiple time points postinoculation. For the caudal fold test (CFT), all calves received 0.1 ml (100 µg) of *M. bovis* purified protein derivative (PPD) intradermally in the right caudal skin fold adjacent to the tail head (applied 89 days after challenge) according to guidelines described in USDA APHIS circular 91-45-011 (32). For the comparative cervical test (CCT), calves received 0.1 ml (100 µg) of *M. bovis* PPD and 0.1 ml (40 µg) of *M. avium* PPD intradermally at separate clipped sites in the midcervical region according to guidelines described in USDA APHIS circular 91-45-011 (32). Balanced PPDs for CCT were obtained from the Brucella and Mycobacterial Reagents section of the

National Center for Animal Health, Ames, IA. Serum samples were also obtained from 16 cattle at ~22 weeks after inoculation with *M. avium* subsp. *avium* (~10⁹ CFU of strain TMC 702, a chicken isolate) or *M. avium* subsp. *paratuberculosis* (~10⁸ CFU of strain 167, a bovine isolate) and from 5 calves at ~52 weeks after vaccination with the Mycopar vaccine for Johne's disease (0.5 ml of the heat-killed whole-cell suspension of *M. avium* subsp. *paratuberculosis* in oil subcutaneously), as described previously (33, 34).

Serum specimens were obtained from 38 cattle with TB naturally acquired in three *M. bovis* outbreaks within the United States. In New Mexico, 27 animals in a dairy herd were diagnosed with *M. bovis* infection by detection of tuberculous lesions upon postmortem examination, followed by isolation of *M. bovis* via mycobacterial culture at the National Veterinary Services Laboratories (NVSL), Ames, IA (35). In Michigan and Texas, 11 tuberculous cattle were identified to be CFT nonreactors. Disease in these animals was confirmed by the presence of gross lesions and positive results by histopathology, mycobacterial culture, and/or IS6110 PCR, as previously described (15). The negative-control group included 51 samples obtained prior to experimental infection from cattle in the NADC studies and 40 serum samples collected from tuberculin skin test nonreactors in two TB-free herds. Serum samples were stored frozen at -70°C until needed for serological assays.

MAPIA. The antigens were printed onto a nitrocellulose membrane, and the multiantigen print immunoassay (MAPIA) was performed as previously described (25). Bovine IgG antibodies were detected by incubation of strips with peroxidase-conjugated protein G (Sigma, St. Louis, MO, USA). MAPIA bands were developed with 3,3',5,5'-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and evaluated visually, with a band of any intensity being read as an antibody-positive reaction.

DPP assay. Bovine IgG antibodies against selected antigens were detected using protein A/G (Thermo Fisher Scientific) as described previously (15). Serum samples were tested at a dilution of 1:10 in assay running buffer. Results were recorded 20 min after addition of a diluted serum sample. Using an optical reader, we measured the reflectance of the dual-path platform (DPP) test lines, with values above 40 RLU being considered a positive result, as described previously (36).

Data analysis. Antigen reactivity rates and serology data were stratified according to the results available from the postmortem diagnostic tests. Sensitivity was defined as the proportion of culture-confirmed animals that tested positive. Specificity was calculated as the proportion of noninfected animals that tested negative. Accuracy was defined as the proportion of all true-positive and true-negative results for all tested animals.

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