AMERICAN SOCIETY FOR MICROBIOLOGY | Clinical and Vaccine Immunology®

DIAGNOSTIC LABORATORY IMMUNOLOGY

Check for updates

Identification of Novel Antigens Recognized by Serum Antibodies in Bovine Tuberculosis

Konstantin P. Lyashchenko,[a] Adrian Grandison,[a] Karen Keskinen,[a] Alina Sikar-Gang,[a] Paul Lambotte,[a] Javan Esfandiari,[a] Gregory C. Ireton,[b] Aarthy Vallur,[b] Steven G. Reed,[b] Gareth Jones,[c] H. Martin Vordermeier,[c] Judy R. Stabel,[d] Tyler C. Thacker,[d] Mitchell V. Palmer,[d] W. Ray Waters[d]

Chembio Diagnostic Systems, Inc., Medford, New York, USA[a]; Infectious Disease Research Institute, Seattle, Washington, USA[b]; Tuberculosis Research Group, Animal and Plant Health Agency, Addlestone, United Kingdom[c]; National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Ames, Iowa, USA[d]

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

Received 29 August 2017 **Returned for modification** 19 September 2017 **Accepted** 30 September 2017

Accepted manuscript posted online 4 October 2017

Citation Lyashchenko KP, Grandison A, Keskinen K, Sikar-Gang A, Lambotte P, Esfandiari J, Ireton GC, Vallur A, Reed SG, Jones G, Vordermeier HM, Stabel JR, Thacker TC, Palmer MV, Waters WR. 2017. Identification of novel antigens recognized by serum antibodies in bovine tuberculosis. Clin Vaccine Immunol 24:e00259-17. https://doi.org/10.1128/CVI.00259-17.

Editor Patricia P. Wilkins, Parasitology Services

Address correspondence to Konstantin P. Lyashchenko, klyashchenko@chembio.com.

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

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,[c] Rv1463,[b] Rv1592c,[b] Rv1980c,[c] Rv2386c,[b] Rv2650c,[b] Rv2873,[c] Rv2875,[c] Rv3704c,[b] Rv3834c,[b] Rv3874[c]

[a]High 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).
[b]Protein produced at APHA as described previously (30).
[c]Protein 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: Protein	Name: Gene	Amino acid sequence identity (%): *M. bovis*	Amino acid sequence identity (%): *M. avium* subsp. *paratuberculosis*	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	*mpt83*	100	<30	28	9	37/42 (88.1)	NA
2	Rv2875	*mpt70*	100	<30	28	9	37/42 (88.1)	NA
3	Rv2650c	*rv2650c*	89	41	9	9	18/42 (42.9)	Yes
4	Rv1463	*rv1463*	100	90	9	9	18/42 (42.9)	Yes
5	Rv3834c	*serS*	100	88	7	9	16/42 (38.1)	Yes
6	Rv0798c	*cfp-29*	100	87	9	7	16/42 (38.1)	Yes
7	Rv3704	*gshA*	100	74	9	6	15/42 (35.7)	Yes
8	Rv1592c	*rv1592c*	99	80	6	8	14/42 (33.3)	Yes
9	Rv3874	*cfp-10*	100	<30	10	3	13/42 (31.0)	No
10	Rv1196	*PPE18*	98	41	11	2	13/42 (31.0)	No
11	Rv1980c	*mpt64*	99	55	11	2	13/42 (31.0)	No
12	Rv2386c	*mbtI*	100	74	7	6	13/42 (31.0)	Yes

[a]The overall rate is presented as the number of cattle whose serum was reactive/total number of cattle tested (percent).
[b]Antibody reactivity detected prior to or in the absence of seroconversion with the DID38 fusion protein (Rv2875-Rv2873). NA, not applicable.
[c]Antibody reactivity detected at any time point during experimental infection ranging from 6 to 36 weeks after inoculation of *M. bovis*.
[d]Samples from *M. bovis*-infected CFT nonreactors (15).

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

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

[a]The 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

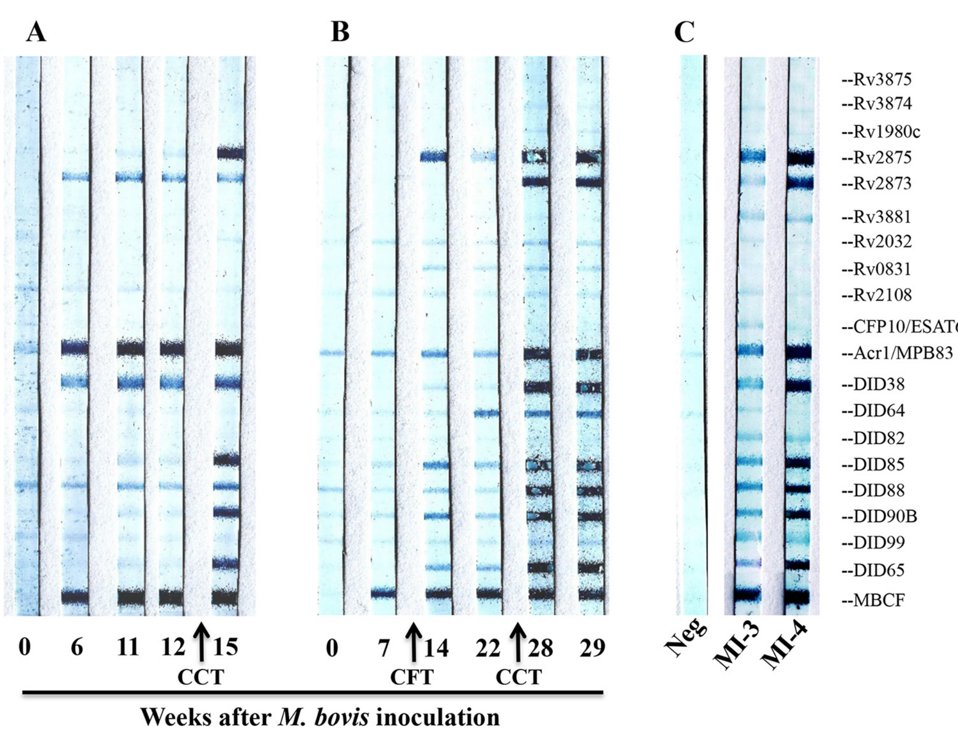

FIG 1 MAPIA reactivity of mycobacterial protein antigens produced at IDRI, USA, with serum samples from cattle experimentally or naturally infected with *M. bovis*. (A) Serial samples collected over time from calf 221 that had been aerosol inoculated with *M. bovis* strain 95-1315; (B) serial samples 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.

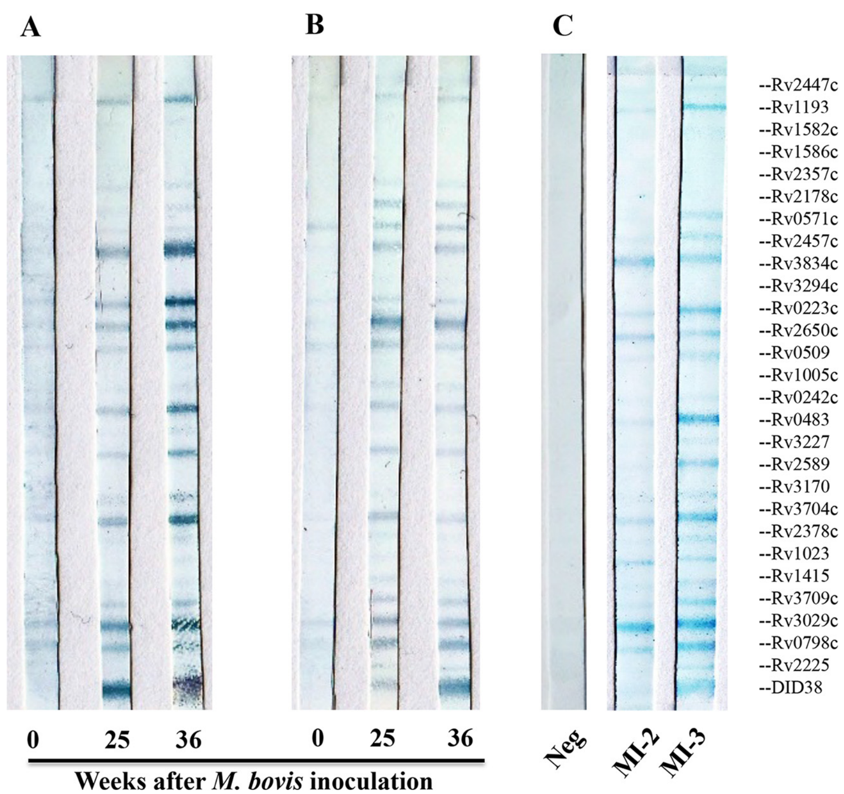

FIG 2 MAPIA reactivity of mycobacterial protein antigens produced at APHA in the United Kingdom with serum samples from cattle experimentally or naturally infected with *M. bovis*. (A and B) Serial samples collected from calves 51 and 57, respectively, prior to and after aerosol inoculation with *M. 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 polyepitope 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 polyepitope 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 polyepitope fusion proteins in MAPIA with serum samples from *M. bovis*-infected cattle[a]

Fusion protein name	Fusion protein composition[b]	Antibody reactivity: No. of cattle with the following whose sera were reactive: Experimental infection[d] (n = 31)	Naturally acquired disease[e] (n = 11)	Overall rate[c]
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)

[a]High 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).
[b]Known seroreactive proteins (included in Table 1) are shown in bold with underlining.
[c]The overall rate is presented as number of cattle whose serum was reactive/total number of cattle tested (percent).
[d]Antibody reactivity detected at any time point in the course of experimental infection ranging from 6 to 36 weeks after inoculation of *M. bovis*.
[e]Samples 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],

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

	No. of animals with antibody reactivity[b]								
Test antigen[a]	Animals with experimental *M. bovis* infection ($n = 36$)	Animals with natural *M. bovis* infection ($n = 38$)	Noninfected control animals ($n = 91$)	*M. avium* subsp. *paratuberculosis*-vaccinated animals ($n = 5$)	*M. avium* subsp. *paratuberculosis*-infected animals ($n = 8$)	*M. avium*-infected animals ($n = 8$)	% sensitivity[c]	% specificity[d]	% accuracy[e]
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)

[a]The 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.

[b]Antibody reactivity detected at any time point during experimental infection ranging from 6 to 36 weeks after inoculation of *M. bovis*.

[c]Data in parentheses represent the number of culture-confirmed animals that tested positive/total number of culture-confirmed animals tested.

[d]Data in parentheses represent the number of noninfected animals that tested negative/total number of noninfected animals tested.

[e]Data 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* (~10^4 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* (~10^9 CFU of strain TMC 702, a chicken isolate) or *M. avium* subsp. *paratuberculosis* (~10^8 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 IS*6110* 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.

ACKNOWLEDGMENTS

We thank Jeffrey Nelson for providing well-characterized cattle samples from the NVSL serum bank.

This work was partially supported by the Small Business Innovation Research Program of the USDA National Institute of Food and Agriculture (award no. 2016-33610-25688). H.M.V. is a Jenner Investigator. The work performed by use of the Gateway approach was funded by the Department for Environment, Food and Rural Affairs of the United Kingdom (grant SE3233).

REFERENCES

1. Schiller I, Oesch B, Vordermeier HM, Palmer MV, Harris BN, Orloski KA, Buddle BM, Thacker TC, Lyashchenko KP, Waters WR. 2010. Bovine tuberculosis: a review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. Transbound Emerg Dis 57:205–220. https://doi.org/10.1111/j.1865-1682.2010.01148.x.
2. Waters WR, Thacker TC, Nelson JT, DiCarlo DM, Maggioli MF, Greenwald R, Esfandiari J, Lyashchenko KP, Palmer MV. 2014. Virulence of two strains of *Mycobacterium bovis* in cattle following aerosol infection. J Comp Pathol 151:410–419. https://doi.org/10.1016/j.jcpa.2014.08.007.
3. Waters WR, Palmer MV, Stafne MR, Bass KE, Maggioli MF, Thacker TC, Linscott R, Lawrence JC, Nelson JT, Esfandiari J, Greenwald R, Lyashchenko KP. 2015. Effects of serial skin testing with purified protein derivative on the level and quality of antibodies to complex and defined antigens in *Mycobacterium bovis*-infected cattle. Clin Vaccine Immunol 22:641–649. https://doi.org/10.1128/CVI.00119-15.
4. Ramdas KE, Lyashchenko KP, Greenwald R, Robbe-Austerman S, McManis C, Waters WR. 2015. *Mycobacterium bovis* infection in humans and cats in same household, Texas, USA, 2012. Emerg Infect Dis 21:480–483. https://doi.org/10.3201/eid2103.140715.
5. McNair J, Welsh MD, Pollock JM. 2007. The immunology of bovine tuberculosis and progression toward improved disease control strategies. Vaccine 25:5504–5511. https://doi.org/10.1016/j.vaccine.2007.02.037.
6. Palmer MV. 2007. Tuberculosis: a reemerging disease at the interface of domestic animals and wildlife. Curr Top Microbiol Immunol 315:195–215.
7. Naranjo V, Gortazar C, Vicente J, de la Fuente J. 2008. Evidence of the role of European wild boar as a reservoir of *Mycobacterium tuberculosis* complex. Vet Microbiol 127:1–9. https://doi.org/10.1016/j.vetmic.2007.10.002.
8. Musoke J, Hlokwe T, Marcotty T, du Plessis BJ, Michel AL. 2015. Spillover of *Mycobacterium bovis* from wildlife to livestock, South Africa. Emerg Infect Dis 21:448–451. https://doi.org/10.3201/eid2103.131690.
9. Buddle BM, Livingstone PG, de Lisle GW. 2009. Advances in ante-mortem diagnosis of tuberculosis in cattle. N Z Vet J 57:173–180. https://doi.org/10.1080/00480169.2009.36899.
10. Bezos J, Casal C, Romero B, Schroeder B, Hardegger R, Raeber AJ, López L, Rueda P, Domínguez L. 2014. Current ante-mortem techniques for diagnosis of bovine tuberculosis. Res Vet Sci 97:S44–S52. https://doi.org/10.1016/j.rvsc.2014.04.002.
11. Pollock JM, Buddle BM, Andersen P. 2001. Towards more accurate diagnosis of bovine tuberculosis using defined antigens. Tuberculosis 81:65–69. https://doi.org/10.1054/tube.2000.0273.
12. Waters WR, Buddle BM, Vordermeier HM, Gormley E, Palmer MV, Thacker TC, Bannantine JP, Stabel JR, Linscott R, Martel E, Milian F, Foshaug W, Lawrence JC. 2011. Development and evaluation of an enzyme-linked immunosorbent assay for use in the detection of bovine tuberculosis in cattle. Clin Vaccine Immunol 18:1882–1888. https://doi.org/10.1128/CVI.05343-11.
13. Trost B, Stuber T, Surujballi O, Nelson J, Robbe-Austerman S, Smith NH, Desautels L, Tikoo SK, Griebel P. 2016. Investigation of the cause of geographic disparities in IDEXX ELISA sensitivity in serum samples from *Mycobacterium bovis*-infected cattle. Sci Rep 6:22763. https://doi.org/10.1038/srep22763.
14. Green LR, Jones CC, Sherwood AL, Garkavi IV, Cangelosi GA, Thacker TC,

Palmer MV, Waters WR, Rathe CV. 2009. Single-antigen serological testing for bovine tuberculosis. Clin Vaccine Immunol 16:1309–1313. https://doi.org/10.1128/CVI.00028-09.
15. Waters WR, Vordermeier HM, Rhodes S, Khatri B, Palmer MV, Maggioli MF, Thacker TC, Nelson JT, Thomsen BV, Robbe-Austerman S, Bravo Garcia DM, Schoenbaum MA, Camacho MS, Ray JS, Esfandiari J, Lambotte P, Greenwald R, Grandison A, Sikar-Gang A, Lyashchenko KP. 2017. Potential for rapid antibody detection to identify tuberculous cattle with non-reactive tuberculin skin test results. BMC Vet Res 13:164. https://doi.org/10.1186/s12917-017-1085-5.
16. Lightbody KA, McNair J, Neill SD, Pollock JM. 2000. IgG isotype antibody responses to epitopes of the *Mycobacterium bovis* protein MPB70 in immunised and in tuberculin skin test-reactor cattle. Vet Microbiol 75:177–188. https://doi.org/10.1016/S0378-1135(00)00215-7.
17. Waters WR, Palmer MV, Thacker TC, Bannantine JP, Vordermeier HM, Hewinson RG, Greenwald R, Esfandiari J, McNair J, Pollock JM, Andersen P, Lyashchenko KP. 2006. Early antibody responses to experimental *Mycobacterium bovis* infection of cattle. Clin Vaccine Immunol 13:648–654. https://doi.org/10.1128/CVI.00061-06.
18. Coad M, Downs SH, Durr PA, Clifton-Hadley RS, Hewinson RG, Vordermeier HM, Whelan AO. 2008. Blood-based assays to detect *Mycobacterium bovis*-infected cattle missed by tuberculin skin testing. Vet Rec 162:382–384. https://doi.org/10.1136/vr.162.12.382.
19. Casal C, Infantes JA, Risalde MA, Díez-Guerrier A, Domínguez M, Moreno I, Romero B, de Juan L, Sáez JL, Juste R, Gortázar C, Domínguez L, Bezos J. 2017. Antibody detection tests improve the sensitivity of tuberculosis diagnosis in cattle. Res Vet Sci 112:214–221. https://doi.org/10.1016/j.rvsc.2017.05.012.
20. Lyashchenko KP, Pollock JM, Colangeli R, Gennaro ML. 1998. Diversity of antigen recognition by serum antibodies in experimental bovine tuberculosis. Infect Immun 66:5344–5349.
21. Ireton GC, Greenwald R, Liang H, Esfandiari J, Lyashchenko KP, Reed SG. 2010. Identification of *Mycobacterium tuberculosis* antigens of high serodiagnostic value. Clin Vaccine Immunol 17:1539–1547. https://doi.org/10.1128/CVI.00198-10.
22. Kunnath-Velayudhan S, Salamon H, Wang HY, Davidow AL, Molina DM, Huynh VT, Cirillo DM, Michel G, Talbot EA, Perkins MD, Felgner PL, Liang X, Gennaro ML. 2010. Dynamic antibody responses to the *Mycobacterium tuberculosis* proteome. Proc Natl Acad Sci U S A 107:14703–14708. https://doi.org/10.1073/pnas.1009080107.
23. Kunnath-Velayudhan S, Davidow AL, Wang HY, Molina DM, Huynh VT, Salamon H, Pine R, Michel G, Perkins MD, Xiaowu L, Felgner PL, Flynn JL, Catanzaro A, Gennaro ML. 2012. Proteome-scale antibody responses and outcome of *Mycobacterium tuberculosis* infection in nonhuman primates and in tuberculosis patients. J Infect Dis 206:697–705. https://doi.org/10.1093/infdis/jis421.
24. Burbelo PD, Keller J, Wagner J, Klimavicz JS, Bayat A, Rhodes CS, Diarra B, Chetchotisakd P, Suputtamongkol Y, Kiertiburanakul S, Holland SM, Browne SK, Siddiqui S, Kovacs JA. 2015. Serological diagnosis of pulmonary *Mycobacterium tuberculosis* infection by LIPS using a multiple antigen mixture. BMC Microbiol 15:205. https://doi.org/10.1186/s12866-015-0545-y.
25. Lyashchenko K, Whelan AO, Greenwald R, Pollock JM, Andersen P, Hewinson RG, Vordermeier HM. 2004. Association of tuberculin-boosted antibody responses with pathology and cell-mediated immunity in cattle vaccinated with *Mycobacterium bovis* BCG and infected with *M. bovis*. Infect Immun 72:2462–2467. https://doi.org/10.1128/IAI.72.5.2462-2467.2004.
26. Waters WR, Whelan AO, Lyashchenko KP, Greenwald R, Palmer MV, Harris BN, Hewinson RG, Vordermeier HM. 2010. Immune responses in cattle inoculated with *Mycobacterium bovis*, *Mycobacterium tuberculosis*, or *Mycobacterium kansasii*. Clin Vaccine Immunol 17:247–252. https://doi.org/10.1128/CVI.00442-09.
27. Jolley ME, Nasir MS, Surujballi OP, Romanowska A, Renteria TB, De la Mora A, Lim A, Bolin SR, Michel AL, Kostovic M, Corrigan EC. 2007. Fluorescence polarization assay for the detection of antibodies to *Mycobacterium bovis* in bovine sera. Vet Microbiol 120:113–121. https://doi.org/10.1016/j.vetmic.2006.10.018.
28. Whelan C, Whelan AO, Shuralev E, Kwok HF, Hewinson G, Clarke J, Vordermeier HM. 2010. Performance of the Enferplex TB assay with cattle in Great Britain and assessment of its suitability as a test to distinguish infected and vaccinated animals. Clin Vaccine Immunol 17:813–817. https://doi.org/10.1128/CVI.00489-09.
29. Coad M, Clifford DJ, Vordermeier HM, Whelan AO. 2013. The consequences of vaccination with the Johne's disease vaccine, Gudair, on diagnosis of bovine tuberculosis. Vet Rec 172:266. https://doi.org/10.1136/vr.101201.
30. Jones GJ, Khatri BL, Garcia-Pelayo MC, Kaveh DA, Bachy VS, Hogarth PJ, Wooff E, Golby P, Vordermeier HM. 2013. Development of an unbiased antigen-mining approach to identify novel vaccine antigens and diagnostic reagents for bovine tuberculosis. Clin Vaccine Immunol 20:1675–1682. https://doi.org/10.1128/CVI.00416-13.
31. Palmer MV, Waters WR, Whipple DL. 2002. Aerosol delivery of virulent *Mycobacterium bovis* to cattle. Tuberculosis (Edinb) 82:275–282. https://doi.org/10.1054/tube.2002.0341.
32. USDA, APHIS. 2007. Bovine tuberculosis eradication: uniform methods and rules (circular APHIS 91-45-011), p 1–29. U.S. Government Printing Office, Washington, DC.
33. Stabel JR, Waters WR, Bannantine JP, Lyashchenko K. 2011. Mediation of host immune responses after immunization of neonatal calves with a heat-killed *Mycobacterium avium* subsp. *paratuberculosis* vaccine. Clin Vaccine Immunol 18:2079–2089. https://doi.org/10.1128/CVI.05421-11.
34. Stabel JR, Waters WR, Bannantine JP, Palmer MV. 2013. Disparate host immunity to *Mycobacterium avium* subsp. *paratuberculosis* antigens in calves inoculated with *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium*, *M. kansasii*, and *M. bovis*. Clin Vaccine Immunol 20:848–857. https://doi.org/10.1128/CVI.00051-13.
35. Connell KM. 2008. Report of the Committee on Tuberculosis, p 580–581. Abstr 112th Annu Meet United States Anim Health Assoc, Greensboro, NC. http://www.usaha.org/upload/Proceedings/2008_USAHA_Proceedings.pdf.
36. Lyashchenko KP, Greenwald R, Sikar-Gang A, Sridhara AA, Johnathan A, Lambotte P, Esfandiari J, Maggioli MF, Thacker TC, Palmer MV, Waters WR. 2017. Early detection of circulating antigen and IgM-associated immune complexes during experimental *Mycobacterium bovis* infection in cattle. Clin Vaccine Immunol 24:e00069-17. https://doi.org/10.1128/CVI.00069-17.