Early Detection of Circulating Antigen and IgM-Associated Immune Complexes during Experimental *Mycobacterium bovis* Infection in Cattle

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ABSTRACT The presence of circulating antigen in cattle experimentally infected with *Mycobacterium bovis* was demonstrated using dual-path platform (DPP) technology. The antigen capture immunoassays employed rabbit polyclonal antibody recognizing predominantly *M. tuberculosis* complex-specific epitopes and were able to detect soluble substances and whole cells of mycobacteria. The antigen found in serum appeared to be mostly bound to IgM, but not to IgG, within the immune complexes formed at early stages of *M. bovis* infection. The antigen was also detected in bile and urine, indicating possible clearance pathways. The data correlation analyses supported the idea of the role of IgM responses in antigen persistence during *M. bovis* infection. The antigen was detectable in serum months prior to detectable antibody seroconversion. This proof-of-concept study suggested the potential for improved immunodiagnostics for bovine tuberculosis.

KEYWORDS *Mycobacterium bovis*, tuberculosis, antigen, antibody, immune complexes

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Antigen detection in blood and alternative biological fluids has long been explored as an attractive diagnostic approach (1–3). The presence of pathogen-derived substances and/or circulating immune complexes (CIC) has been demonstrated in viral, bacterial, fungal, and parasitic infections (4–8). Commercial products have been developed for direct antigen detection in a variety of human infectious diseases, such as malaria, influenza, tuberculosis (TB), human immunodeficiency virus (HIV) infection, hepatitis B virus infection, and dengue fever (9–14), as well as in many animal infections, including equine influenza, porcine epidemic diarrhea, canine or feline heartworm, feline leukemia, and feline immunodeficiency virus infection (15–19). However, no blood-based antigen detection test is available for *Mycobacterium bovis* infection.

Bovine TB control is largely dependent on the quality and reliability of antemortem tests. The diagnostic methods currently approved for use in cattle have serious limitations. The intradermal tuberculin test has suboptimal sensitivity and inconsistent performance (20, 21), while the available blood assays lack the required accuracy and often show significant variability in different geographic areas (22–24). Serologic methods may be additionally useful to identify *M. bovis*-infected cattle (21, 22), but the existing antibody assays lack diagnostic sensitivity, especially in early infection, and thus require improvement.

The aim of this proof-of-principle study was to determine if circulating antigen can be detected in bovine TB using Chembio dual-path platform (DPP) technology. We designed several DPP assay configurations to measure levels of free and antibody-
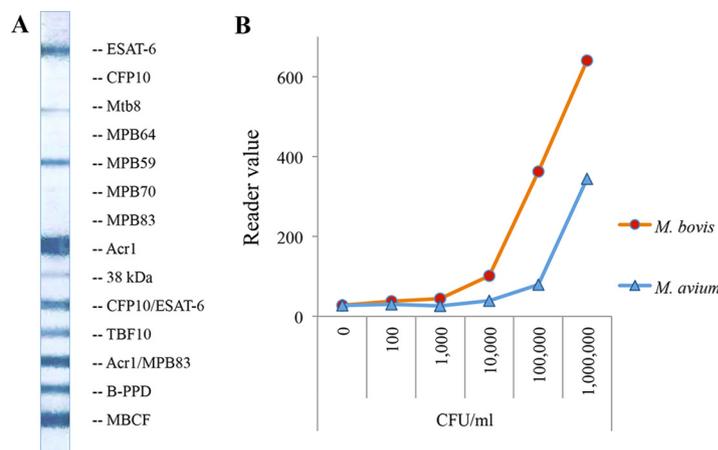
FIG 1 Immunoreactivity profile of the antigen detection reagent used in the DPP Ag assay. (A) Recognition of *M. tuberculosis* complex antigens in MAPIA by the rabbit antibody against *M. tuberculosis* PPD. MAPIA was performed as described in Materials and Methods. Immobilized antigens are shown on the right margin. Visible bands on the strips indicate the presence of IgG antibody to the corresponding antigen. (B) Detection of *M. bovis* (circles) or *M. avium* (triangles) by DPP Ag assay.

bound mycobacterial antigen as well as of IgM and IgG antibodies in serum samples serially collected from *M. bovis*-inoculated cattle. Results demonstrated the feasibility of rapid antigen detection during *M. bovis* infection, the association between antigen circulation and IgM responses, and the potential for development of improved sero-diagnostics capable of earlier identification of infected animals.

RESULTS

Design and characterization of antigen capture DPP assays. In three antigen-detecting test configurations, undefined mycobacterial antigen is captured by the rabbit anti-*M. tuberculosis* antibody, followed by signal visualization with colloidal gold nanoparticles coupled with the same rabbit antibody, making an immune sandwich in the DPP Ag assay, or with goat anti-bovine IgM antibody in the DPP IgM-CIC assay or with goat anti-bovine IgG antibody in the DPP IgG-CIC assay. As demonstrated by multiantigen print immunoassay (MAPIA), the rabbit anti-*M. tuberculosis* antibody had a broad spectrum of IgG reactivity to various mycobacterial protein antigens (Fig. 1A). The DPP Ag assay displayed dose-dependent detection of *M. tuberculosis* lipoarabino-mannan (LAM) with a wide dynamic range of 10 to 1,000 ng/ml (data not shown) or of *M. bovis* culture at 10^4 to 10^6 CFU/ml (Fig. 1B). The test was able to detect *M. avium* subsp. *avium* cells but only at a >10-fold-higher density than *M. bovis* cells. Thus, the reactivity profile of the antibody selected for the antigen capture DPP tests included both protein and nonprotein antigens, with predominant recognition of *M. tuberculosis* complex-specific epitopes.

Antibody responses in *M. bovis*-infected cattle. Serum samples serially collected from 7 *M. bovis*-inoculated cattle were used to analyze antibody responses and circulating antigen. All samples from infected calves produced positive results in single cervical tests (SCT) and comparative cervical tests (CCT), developed gross and micro-scopic lesions (necrotic and mineralized granulomas) in multiple organs, and had *M. bovis* isolated from the tissues (Table 1), thus demonstrating advanced disease. All of these animals showed IgM and IgG responses to MPB70/MPB83 by week 14 postinoc-ulation (Table 2), but only 2 of them produced IgG antibody to CFP10/ESAT-6 and none had IgM antibody to this antigen (data not shown). IgM and IgG responses to MPB70/MPB83 appeared elevated following SCT and CCT administered at weeks 13 and 30 postinoculation, respectively. The IgM levels peaked 1 week after bovine protein purified derivative (PPD) injection, while the IgG antibody boost required 2 weeks to occur (Table 2 and Fig. 2).

TABLE 1 Tuberculin skin test and pathology results obtained from *M. bovis*-inoculated cattle[a]

Animal ID[b]	SCT result[c] (mm)	CCT result[d] (mm)	Gross lesion score[e]	
			Lymph nodes[f]	Lungs[g]
8	13.5	8.0	2	0
51	43.2	29.5	3	5
53	29.1	28.0	3	5
54	24	51.5	3	3
55	23	19.5	3	4
56	36.7	38.0	3	5
57	11.9	7.0	3	4

[a]*Mycobacterium bovis* was isolated from a pool of thoracic lymph nodes following procedures used for clinical samples at the National Veterinary Services Laboratory, Ames, IA. *M. bovis* was identified in all cultures.

[b]ID, identification number.

[c]SCT was administered at week 13 postinoculation; results are shown as the change in skin thickness 72 h after bovine PPD injection.

[d]CCT was administered at week 30 postinoculation; results are shown as the difference between reactions to bovine PPD and to avian PPD measured 72 h after bovine PPD injection.

[e]Gross lesion scoring system was used as described by Vordermeier et al. (25).

[f]Tracheobronchial and mediastinal lymph nodes were examined for gross pathology using the following scoring scale: 0, no necrosis or visible lesions; 1, one small focus (1 to 2 mm in diameter); 2, several small foci; 3, extensive necrosis.

[g]Lungs were examined for gross pathology using the following scoring scale: 0, no visible lesions; 1, no external gross pathology but lesions seen upon slicing; 2, <5 visible lesions of <10 mm in diameter; 3, ≥5 visible lesions of <10 mm in diameter; 4, >1 distinct visible lesion of >10 mm in diameter; 5, gross coalescing lesions. All animals except animal 8 had visible lesions in all lobes.

Antigen and CIC detection in *M. bovis*-infected cattle. The ability of DPP IgM-CIC and DPP IgG-CIC assays to detect the respective analytes was demonstrated in a pilot experiment with IgG-CIC and IgM-CIC preformed by spiking antibody-containing specimens with *M. bovis* culture filtrate (MBCF). As shown in Fig. 3, with accumulation of specific IgM and IgG antibodies over the course of *M. bovis* infection, the detectability of free antigen added to the respective serum samples was gradually reduced, presumably due to antibody-mediated competitive inhibition, while signals obtained in DPP IgM-CIC and DPP IgG-CIC assays reciprocally increased.

Antigen and IgM-CIC were found in sera from 4/7 *M. bovis*-inoculated animals, but, surprisingly, none of them produced detectable IgG-CIC at any time point of infection (Table 2). Figure 2 shows results obtained from two calves, animal 51 and animal 54, displaying distinct patterns of the antigen and antibody kinetics. In the other two antigenemic animals, animals 56 and 57, the antigen levels were not significantly affected by the SCT administered at week 13 postinoculation, but they were notably increased after the CCT administered at week 30 postinoculation, similarly to that found in calf 51 (Fig. 2A). In contrast, animal 54 showed a sharp spike of the antigen and IgM-CIC levels at weeks 14 to 17 postinoculation concurrently with the SCT-boosted IgM response, followed by rapid decline and less evident boost of the antigen signal after the CCT administration at week 30 postinoculation (Fig. 2B).

A strong ($r = 0.929$) correlation was found between DPP Ag and DPP IgM-CIC readouts obtained with sera serially collected from the antigenemic cattle, suggesting that the DPP Ag assay can detect IgM-bound antigen as well. Indeed, upon pretreatment of selected antigen-positive samples with 50 mM 2-mercaptoethanol (2-ME) or heating at 90°C to dissociate IgM-CIC, the DPP Ag signal was abolished (data not shown).

We also noticed that the SCT-boosted IgM responses at week 14 postinoculation of *M. bovis* were inversely correlated with antigen levels measured at week 45 ($r = -0.787$). The top three early IgM producers in the group showed no antigenemia in advanced disease, whereas the lowest IgM response found in animal 56 was associated with the highest antigen level (data not shown). This finding suggests that IgM can serve as a negative correlate to predict antigen persistence at later stages of *M. bovis* infection. No correlation between antigen circulation and IgG antibody responses was

TABLE 2 Reader data obtained for various DPP assays with serum samples collected from *M. bovis*-inoculated cattle at selected time points

Animal ID	Wk postinfection	Antibody to MPB70/MPB83		*M. bovis* antigen		
		IgM	IgG	Free	IgM-CIC	IgG-CIC
8	0	10	0	0	0	0
	14	**517**	**292**	0	0	0
	15	**408**	**813**	0	0	0
	31	**341**	**782**	0	36	0
	36	**115**	**753**	0	0	0
51	0	0	0	0	31	0
	14	**254**	**79**	**73**	**77**	0
	15	**190**	**305**	20	**69**	0
	31	**319**	**716**	**98**	**109**	0
	36	**162**	**742**	**459**	**229**	0
53	0	0	0	0	0	0
	14	**793**	**271**	0	0	0
	15	**487**	**792**	0	0	0
	31	**731**	**812**	0	0	0
	36	**187**	**861**	0	0	0
54	0	0	0	0	0	0
	14	**494**	0	**788**	**712**	0
	15	**314**	**123**	**772**	**629**	0
	31	**292**	**448**	**191**	**64**	0
	36	**226**	**423**	**63**	36	0
55	0	5	0	0	26	0
	14	**622**	**394**	27	0	0
	15	**319**	**314**	0	16	0
	31	**417**	**689**	0	0	0
	36	**94**	**538**	0	0	0
56	0	0	0	0	0	0
	14	**99**	**91**	**63**	**137**	0
	15	32	**118**	37	**109**	0
	31	**299**	**750**	**368**	**361**	0
	36	**72**	**712**	**485**	**387**	0
57	0	0	0	0	0	0
	14	**208**	**75**	**56**	0	0
	15	**249**	**42**	**77**	0	0
	31	**184**	**578**	**447**	**111**	0
	36	**53**	**174**	**99**	12	0

[a]Positive results (corresponding to a cutoff value of 40 RLU) are shown in bold.

observed. Collectively, the results indicate a close inverse association between early IgM antibody responses and the presence of *M. bovis* antigen later in the course of disease.

Antigen and antibody detection in alternate specimens. Bile and urine samples collected from the *M. bovis*-infected cattle at necropsy (45 weeks postinoculation) were also tested by DPP IgM, DPP IgG, and DPP Ag assays in comparison with the matching sera. As shown in Table 3, IgM antibody to MPB70/MPB83 was found in serum from 6/7 animals and in bile from 1 of them but not in urine. All calves remained DPP IgG seropositive at necropsy, while only 2/7 animals had measurable levels of IgG antibody in bile and none had it in urine. In contrast, *M. bovis* antigen was found in all 7 infected cattle, whether in serum (4/7), bile (3/7), or urine (5/7). The antigen distribution patterns revealed animal-to-animal variation, with only one calf (animal 54) showing DPP Ag-positive results for all three specimen types (Table 3). Incidentally, that calf developed the strongest post-SCT IgM serum antibody response in the study, and the response was associated with the highest levels of antigen and IgM-CIC detected at
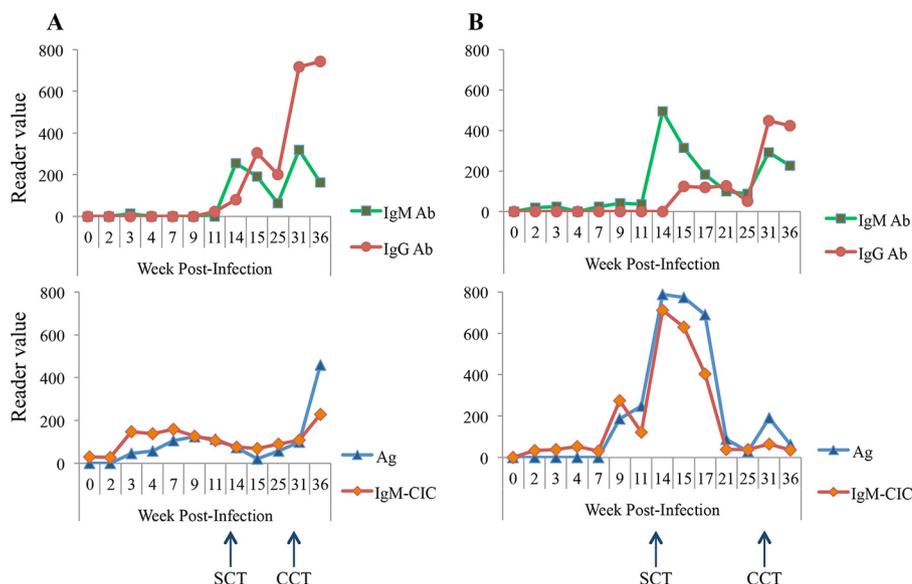
FIG 2 Kinetics of antibody responses, circulating antigen, and IgM-CIC in *M. bovis* infection. (A) Animal 51. (B) Animal 54. Assays were performed as described in Materials and Methods. (Top panels) Serum levels of IgM (squares) and IgG (circles) antibodies were measured by DPP IgM and DPP IgG assays, respectively. (Bottom panels) Serum levels of *M. bovis* antigen (triangles) and IgM-CIC (diamonds) were measured by DPP Ag and DPP IgM-CIC assays, respectively. Arrows indicate the time points for administration of SCT and CCT at weeks 13 and 30 postinfection, respectively.

week 14 postinoculation (Fig. 2B). Moreover, while serum antigen levels in that animal significantly declined by the end of experiment, the urinary antigen level was still the highest in the group (Table 3).

Animal 51 was the only infected calf that showed the presence of IgM and IgG antibody in both serum and bile. Therefore, we used the opportunity to compare the antibody reactivity profiles by MAPIA. Results demonstrated qualitative similarities for the IgM and IgG binding patterns obtained with serum and bile from calf 51 (Fig. 4). Notably, that animal showed the highest level of *M. bovis* antigen in bile in the group.

Diagnostic potential of antigen detection. Antibody seroconversion detected in all 7 *M. bovis*-infected cattle by week 14 postinoculation was characterized by rising levels of IgM and IgG isotypes seen typically 1 to 2 weeks after SCT administration (Fig. 2). In comparison, serum antigen and IgM-CIC were first detected in 4/7 cattle at weeks 2 to 9 postinoculation or 6 to 12 weeks prior to the onset of antibody responses (Table 4). The results suggest that combining antigen and antibody detection in one assay may significantly improve diagnostic sensitivity at early stages of *M. bovis* infection.

DISCUSSION

In the present study, we evaluated a large collection of serum samples serially obtained from 7 cattle after *M. bovis* infection to demonstrate the feasibility of rapid detection of antigens, free and/or within CIC, in comparison with the antibody responses. The antigen and IgM-CIC, but not IgG-CIC, appeared in serum early during *M. bovis* infection and prior to detectable antibody seroconversion. Accumulation of circulating antigen over time mirrored the evolution of IgM responses, with most illustrative examples observed shortly after the skin tests when the levels of antigen, free IgM antibody, and IgM-CIC were concurrently boosted. Intriguingly, the antigenemia measured at advanced disease was inversely correlated with IgM responses produced in early infection.

Considering the close relationship between IgM and *M. bovis* antigen detection in conjunction with the fact that DPP Ag and DPP IgM-CIC utilized the same immuno-capture reagent, we hypothesized that the DPP Ag assay can detect not only free but also IgM-bound antigen via unengaged epitopes that may be recognized by the rabbit
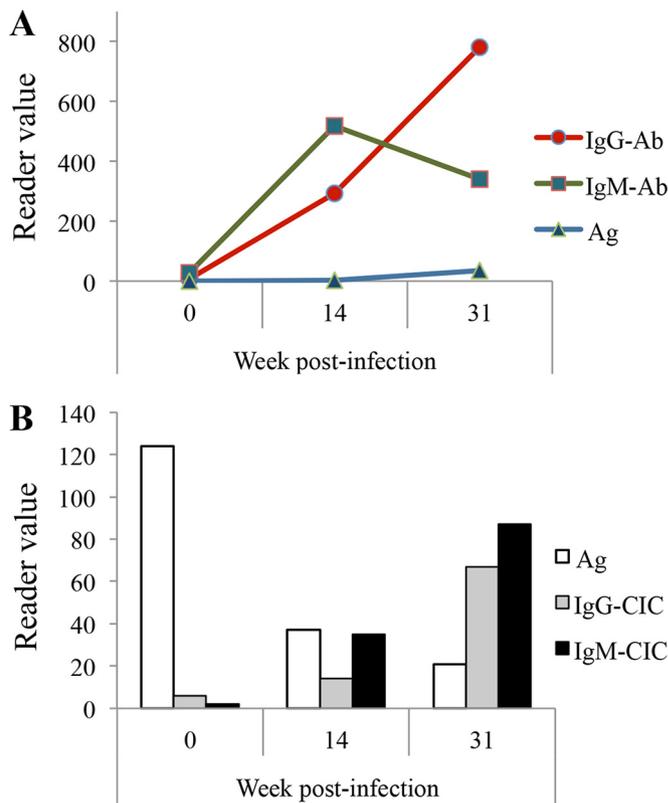
FIG 3 Detection of IgM-CIC and IgG-CIC preformed *in vitro* by mixing MBCF with bovine serum samples collected during experimental *M. bovis* infection. (A) Antigen (triangles) and free IgM (squares) and IgG (circles) antibodies to MPB70/MPB83 in untreated sera from antigen-negative animal 8. (B) Antigen (open bars), IgG-CIC (gray bars), and IgM-CIC (solid bars) in sera from animal 8 spiked with 10 μg/ml MBCF.

antibody. Further, it is conceivable that in the latter scenario, upon achieving the optimal antigen-antibody ratio and in the absence of competing inhibition by immune IgG, the analytical sensitivity of antigen detection can be significantly enhanced, as the epitope density on the surface of lattice-like IgM-CIC is amplified (3), due to the large size and pentameric structure of multivalent IgM antibody, as depicted in Fig. 5. In support of this assumption, we found that serum pretreatment for IgM fragmentation or CIC dissociation diminished the antigen detection signal. This observation was in contrast to the well-established enhancing effect of CIC-dissociating procedures on the detection of antibody-bound antigens in human diseases (7, 12).

Presumably, at early stages of *M. bovis* infection the replicating mycobacteria can release soluble products into circulation at levels initially below the test threshold for

TABLE 3 Semiquantitative DPP detection of *M. bovis* antigen and free antibodies to MPB70/MPB83 in different types of biological specimens collected from cattle at necropsy (45 weeks postinfection)

	No. of specimens[a]								
	DPP IgM			DPP IgG			DPP Ag		
Animal ID	Serum	Bile	Urine	Serum	Bile	Urine	Serum	Bile	Urine
8	**45**	0	0	**515**	7	8	32	12	**179**
51	**505**	**243**	0	**738**	**509**	2	**125**	**133**	36
53	**86**	0	0	**621**	**247**	4	26	15	**117**
54	**66**	0	0	**744**	3	4	**42**	**66**	**493**
55	31	0	0	**600**	6	9	33	**103**	3
56	**81**	0	0	**718**	4	5	**114**	34	**394**
57	**96**	0	0	**181**	3	6	**52**	29	**103**

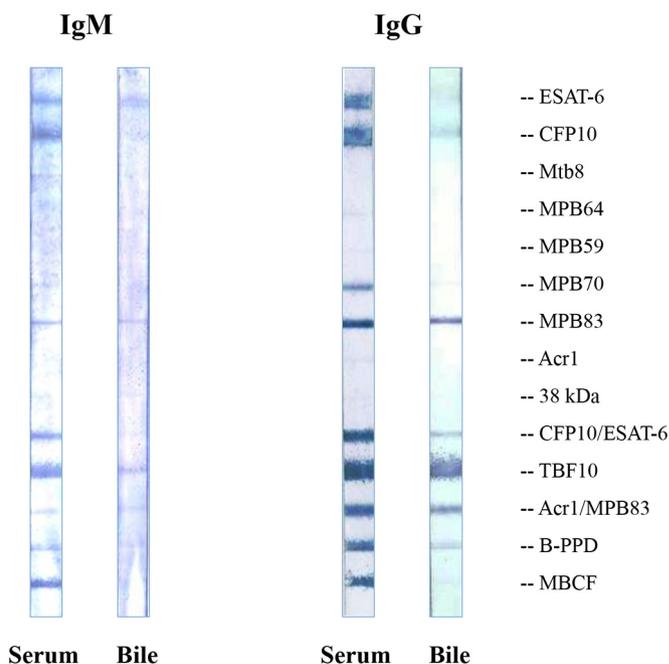[a]Positive results (corresponding to a cutoff value of 40 RLU) are shown in bold.

FIG 4 Comparative characterization of reactivities of IgM (left panels) and IgG (right panels) by MAPIA in serum and bile collected from calf 51 at necropsy (45 weeks postinfection). MAPIA was performed as described in Materials and Methods. Immobilized antigens are shown on the right margin. Visible bands on the strips indicate the presence of IgM or IgG antibodies to the corresponding antigens.

free-antigen detection, which hence remains elusive until an adaptive IgM response develops. Recent studies have demonstrated circulating LAM in tuberculous humans at previously undetectable levels via use of high-density lipoprotein (HDL) pulldown discs, thereby demonstrating association of *M. tuberculosis* antigens with host carrier molecules (e.g., HDL, CIC, etc.), which may be used to augment immunodetection (26). The presence of mannosylated LAM and *M. bovis* peptides in blood of tuberculous cattle has also been recently reported (27, 28), although the studies did not analyze relationships between the circulating antigens and antibody responses. Further, in addition to specific antibodies to *M. bovis*, natural (preexisting) IgM or polyreactive IgM induced by the pathogen (29, 30) may be involved in IgM-CIC formation, thus contributing to the suggested mechanism of naturally amplified antigen detection.

Finding no IgG-CIC despite the presence of circulating components, antigen, and IgG antibodies does not preclude the possibility of their real-time formation in *M. bovis* infection. The technical functionality of the DPP IgG-CIC assay was proven by demonstrating its ability to identify bovine IgG-CIC preformed *in vitro*. Factors that probably contributed to the negative results obtained for IgG-CIC detection in serum in the

TABLE 4 Initial detection of circulating *M. bovis* antigen relative to IgG seroconversion in experimentally infected cattle

| Animal ID | Wk postinoculation of detection of: | | Time difference (wks) |
	Antibody	Antigen	
8	14	ND[a]	NA[b]
51	14	3	11
53	14	ND	NA
54	15	9	6
55	14	ND	NA
56	14	7	7
57	14	2	12

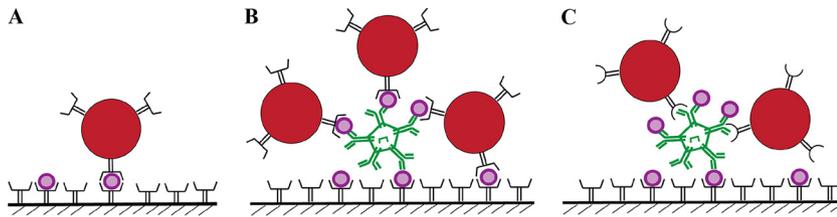[a]ND, not detected.
[b]NA, not applicable.

FIG 5 Schematic of IgM-amplified detection of circulating *M. bovis* antigen. (A) Free-antigen capture by DPP Ag assay. (B) IgM-bound antigen capture by DPP Ag assay. (C) IgM-bound antigen capture by DPP IgM-CIC assay. In each DPP version, *M. bovis* antigen (small circles) was captured by the use of purified rabbit antibody to *M. tuberculosis* immobilized on nitrocellulose strip (test line). Signal is visualized by colloidal gold nanoparticles (large circles) coupled with the same rabbit antibody in the DPP Ag assay (A and B) or with goat antibody to bovine IgM in the DPP IgM-CIC assay (C).

present study may include the following: (i) competitive inhibition due to similar epitope recognition repertoires of the rabbit antibody used in the assay and the bovine IgG antibodies produced during infection; (ii) inability of bivalent IgG to act like multivalent IgM antibody capable of amplifying antigen signal, as suggested above; and (iii) faster clearance from circulation through the powerful FcR-mediated pathway (2, 3).

Indeed, it has been reported that IgG-CIC can be efficiently absorbed by high-affinity receptors for the IgG constant domain (FcγR) expressed on various types of innate immune cells (31), thus accelerating transportation of IgG-CIC to the tissues involved in the recycling metabolism (3). In contrast, success in identifying a specific receptor for IgM (FcμR) has long been elusive (32). After numerous attempts failed to demonstrate it on blood cells, FcμR was finally identified in mice and humans but only on lymphocytes, thus making it unique among all known FcRs (33, 34). There is no published evidence for the existence of FcμR in cattle. Considering the striking disparity between FcγR and FcμR expression levels on blood cells in other species and the difficulty of entry of large-size IgM into tissue fluids (30), we speculated that bovine IgM-CIC might escape clearance and circulate longer than IgG-CIC. Accumulation of CIC containing predominantly IgM has been documented in human TB and Lyme disease (1, 4, 6, 35).

Since *M. bovis* antigen was also found in bile and/or urine of the infected cattle, we concluded that the clearance of *M. bovis* products was mediated by the antibody-dependent mechanisms involving hepatobiliary and urinary systems. The organs participating in the CIC uptake include the lungs, spleen, kidneys, and liver, with the liver known to be a key player in the elimination of microbial substances, particularly in the presence of specific antibodies (2). Elevated concentrations of IgM- and IgA-containing CIC in bile were reported for human patients diagnosed with primary sclerosing cholangitis (36). Mouse experiments showed that LAM injected intravenously disappeared from circulation much faster if monoclonal IgM antibody to LAM was first administered to the animals (37). Consequently, increased levels of LAM were found in the liver and bile of those mice, similarly to our results reported here. Since LAM is known to be a potent immunomodulatory factor affecting CD1-restricted T cells and contributing to immune evasion (38), specific IgM antibody responses leading to timely immune clearance of LAM and other antigens released during *M. bovis* infection may play a protective role, thus supporting the growing body of evidence for the antibody-mediated immunity against TB (39).

As with other infectious diseases, one of the shortcomings of the serodiagnostic approach in bovine TB is the absence of specific antibody at early stages of infection (40, 41). The detection of *M. bovis* antigen and IgM-CIC prior to the manifestation of measurable IgM and IgG responses may help overcome this limitation. Early IgM-CIC were found in 3/4 antigenemic animals without detectable free IgM antibody, suggesting masking of paratopes on IgM molecules bound to circulating antigen. Importantly, unlike the antibody response, the onset of antigenemia (including IgM-CIC) was not dependent on the bovine PPD injection. Therefore, integration of the antigen detection

component in the serologic assays is expected to significantly reduce the diagnostic window of false-negative results prior to antibody seroconversion during *M. bovis* infection. This approach has been previously proposed for diagnosis of acute-phase infections in humans, such as HIV infection, hepatitis B, or pulmonary histoplasmosis (5, 9, 42). Simultaneous detection of p24 antigen and HIV antibody has been successfully implemented in development of the fourth-generation screening immunoassays with superior sensitivity, particularly in identifying recent infections (14).

Given the multiplexing capability of the DPP technology, this platform is well suited to designing a rapid animal-side TB test to combine detection of circulating antigen and detection of free antibody. The diagnostic synergy suggested for these biomarkers in the present proof-of-concept study will have to be validated on large numbers of well-characterized specimens from cattle naturally infected with *M. bovis* and other mycobacteria and from TB-free populations. Further research will also determine whether the presence and levels of circulating antigen are associated with disease progression. Future analytical studies on the CIC composition will identify novel TB biomarkers that may lead to development of more-accurate diagnostic tools for earlier detection of *M. bovis* infection in cattle and other host species.

MATERIALS AND METHODS

Animals and *M. bovis* challenge. Holstein steers ($n = 7$) were obtained from a TB-free herd in IA and housed in a biosafety level 3 (BSL-3) facility at the National Animal Disease Center, Ames, IA, according to Institutional Biosafety and Animal Care and Use Committee guidelines. All calves received 8×10^3 CFU of *M. bovis* by aerosol at ~11 months of age. Virulent *M. bovis* (95-1315; USDA Animal Plant and Health Inspection Service [APHIS] designation) was originally isolated from a white-tailed deer in Michigan, USA (43). A low (≤3)-passage-number culture was prepared in Middlebrooks 7H9 liquid media (Becton Dickinson, Franklin Lakes, NJ) supplemented with 10% oleic acid-albumin-dextrose complex (OADC) plus 0.05% Tween 80 using standard techniques (44). For aerosol infection, *M. bovis* was delivered to restrained calves by nebulization of the inoculum into a mask (Equine AeroMask; Trudell Medical International, London, Ontario, Canada) as described in detail previously (45).

Tuberculin skin test procedures and assessment of lesions. A single cervical test (SCT) and comparative cervical test (CCT) were administered to each steer at 13 and 30 weeks after *M. bovis* challenge, respectively, as described previously (41). All calves were euthanized ~11.5 months after challenge by intravenous administration of sodium pentobarbital. Tissues collected from the lungs, liver, and lymph nodes (mandibular, parotid, medial retropharyngeal, mediastinal, tracheobronchial, hepatic, and mesenteric) were examined for gross lesions and processed for microscopic analysis and isolation of *M. bovis* as described previously (25, 46). Bile and urine specimens were also collected at necropsy. Qualitative assessment of mycobacterial colonization was performed via standard mycobacterial culture techniques (47) using Middlebrook 7H11 selective agar plates (Becton Dickinson) incubated for 8 weeks at 37°C as well as IS*6110* real-time PCR for confirmation of colonies as previously described (48). Strict biosafety protocols, including BSL-3 containment upon initiation of *M. bovis* challenge in animal rooms and standard laboratory practices for handling *M. bovis* cultures and samples from *M. bovis*-infected animals, were followed to protect personnel from exposure to *M. bovis* throughout the study.

Multiantigen print immunoassay (MAPIA). The assay was performed as previously described (49). The antigen panel consisted of 12 recombinant proteins of *M. tuberculosis* and 2 native antigen preparations of *M. bovis*, including the following: ESAT-6 and CFP10 proteins as well as hybrids CFP10/ ESAT-6 and alpha-crystallin/MPB83 (Acr1/MPB83) from Statens Serum Institut (Copenhagen, Denmark); MPB59, MPB64, MPB70, and MPB83 proteins as well as bovine protein purified derivative (B-PPD) tuberculin and *M. bovis* culture filtrate (MBCF) from the Veterinary Sciences Division of the Agri-Food & Biosciences Institute (AFBI) (Stormont, United Kingdom); Mtb8 and polyepitope fusion TBF10 (developed by Corixa Corp., Seattle, WA, USA); and the 38-kDa protein and Acr1 (from Standard Diagnostics, Seoul, South Korea). Rabbit IgG antibody bound to the immobilized antigens was detected by peroxidase-conjugated protein G (Sigma, St. Louis, MO, USA), whereas cattle IgM and IgG antibodies were detected by incubation of strips with peroxidase-labeled antibody to bovine IgM (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and with peroxidase-conjugated protein G (Sigma, St. Louis, MO, USA), respectively. MAPIA bands were developed with 3,3′,5,5′-tetramethyl benzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and evaluated visually, with a band of any intensity being read as an antibody-positive reaction.

Dual-path platform (DPP) assays for antibody and antigen detection. Bovine IgM and IgG antibodies were measured by DPP IgM and DPP IgG assays, respectively, using fusion proteins MPB83/ MPB70 and CFP10/ESAT-6, as described previously (41, 46). For antigen detection, we designed DPP Ag assay employing rabbit polyclonal antibody generated against *M. tuberculosis* PPD (ViroStat, Westbrook, ME, USA) both as a capture reagent immobilized on a test line and as a signal detector coupled with colloidal gold nanoparticles by the use of the Chembio standard conjugation procedure. Key parameters, including the immunocapture line, signal detection system, and serum dilution, were optimized in pilot experiments using MBCF at various concentrations. For functional evaluation of DPP Ag, we used lipoarabinomannan (LAM) purified from *M. tuberculosis* CDC1551 (Mossman Associates, Blackstone, MA,

USA) and cultures of *M. bovis* 95-1315 and *M. avium* subsp. *avium* 3988 (bovine isolate) grown in Middlebrook's 7H9 media (NADC, Ames, IA, USA) supplemented with 10% OADC plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO, USA). For detection of circulating immune complexes (CIC) containing *M. bovis* antigen and IgM or IgG antibodies, we designed DPP IgM-CIC and DPP IgG-CIC assays. The immunocapture test lines in these two assays were identical to that employed in the DPP Ag assay, whereas the colloidal gold nanoparticles were coated with goat anti-bovine IgM or goat anti-bovine IgG antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) in the DPP IgM-CIC and DPP IgG-CIC assays, respectively. Serum samples were tested at a 1:4 dilution in assay running buffer. Results were recorded 20 min after adding diluted serum sample. Using an optical reader, reflectance of the test line was measured in relative light units (RLU), as previously described (41, 46).

Treatment of specimens. The procedure to denature serum IgM with 2-mercaptoethanol (2-ME) described for use in humans (50) was modified for use with cattle sera. Briefly, 90 μl of bovine serum was mixed with 10 μl of 0.5 M 2-ME, subjected to vortex mixing, and incubated for 1 h at 37°C before testing. For CIC dissociation, serum was heated at 90°C for 6 min (7), followed by centrifugation to collect supernatant for testing.

Data analysis. For test interpretation, a cutoff value was established as the mean plus 3 standard deviations in a pilot study using a DPP reader with 7 preinfection and 20 additional negative-control samples from noninfected calves of the same breed, gender, and age. For all the DPP assays used in the present study, reactivity above 40 RLU was considered a positive result. Correlation coefficients were calculated using VassarStats software (51).

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