Development of a Recombinant Protein-Based Enzyme-Linked Immunosorbent Assay for Diagnosis of *Mycoplasma bovis* Infection in Cattle

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*Mycoplasma bovis* causes a range of diseases in cattle, including mastitis, arthritis, and pneumonia. However, accurate serological diagnosis of infection remains problematic. The studies described here aimed to identify an antigen that might be used to develop a more specific and sensitive diagnostic assay. A 226-kDa immunogenic protein was consistently detected in Western blots by antibodies in sera from calves experimentally infected with *M. bovis*. This protein was shown to be a membrane protein with lipase activity and was named mycoplasma immunogenic lipase A (Mila). Different regions of MilA were expressed in *Escherichia coli* as glutathione S-transferase (GST) fusion proteins and recombinant products from the amino-terminal end shown to have strong immunoreactivity with *M. bovis*-specific bovine sera. The most immunoreactive fusion protein, GST-MilA-ab, was used to develop indirect IgM and IgG enzyme-linked immunosorbent assays (ELISAs). The IgM ELISA detected *M. bovis*-specific IgM antibody 2 weeks after infection with 97.1% sensitivity and had a specificity of 63.3%, while the IgG ELISA detected *M. bovis*-specific IgG 3 weeks after infection with 92.86% sensitivity and had a specificity of 98.7%, demonstrating that the IgG ELISA has potential for use as a sensitive and specific assay for detecting infection in cattle.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** *Mycoplasma bovis* strain 3683, originally isolated from a joint lesion in a calf in Queensland, Australia, was grown at 37°C for 17 h in mycoplasma culture medium. The pGEX-4T-1 plasmid was used for cloning different regions of milA, and *Escherichia coli* JM109 cells were used to express the recombinant glutathione S-transferase (GST)–mycoplasma immunogenic lipase A (Mila) proteins.

**Serum samples from experimentally infected calves.** (i) **Experiment 1.** Sixteen 1-month-old Friesian-cross calves were weighed and allocated into one of two groups. Group 1 consisted of 4 animals exposed only to *mycoplasma* culture medium, while group 2 consisted of 12 calves exposed to an overnight culture of *M. bovis* strain 3683 twice on days 1 and 3, with each calf receiving an estimated dose of 10^5.2 color-changing units (CCU), using an aerosol exposure method described previously (17). The calves in groups 1 and 2 were held in separate facilities. Blood samples were taken from all the calves on days 0, 10, 17, and 24 after infection. All calves were euthanized and necropsied at day 24 and examined for lesions as described previously (17).

(ii) **Experiment 2.** Thirty-five 1-month-old Friesian-cross calves were stratified according to their weight and then randomly allocated into 1 of 2 groups. Group 1, the uninfected group, consisted of 5 calves exposed to an aerosol of mycoplasma culture medium, and group 2 consisted of 30...
calves exposed to an aerosol of M. bovis strain 3683. Infection and sampling were performed as described for experiment 1.

All calves in the two experiments were tested for bovine viral diarrhea virus and M. bovis before the commencement of the experiments and found negative, as described previously (17). The calves were monitored for clinical signs throughout the experimental period. In both experiments the calves in group 2 were subclinically infected but had lobular pulmonary consolidation detectable at necropsy. Calves in group 1 had no detectable lung lesions. Cultures from swabs from the trachea, bronchi, and lung lesions isolated no specific pathogens other than M. bovis from the calves in group 2 and no specific pathogen was isolated from the calves in group 1.

The two experiments were conducted with the approval of the University of Melbourne Animal Ethics Committee, application numbers 0911327 and 1111970.

**SDS-PAGE and Western blot analysis.** The whole-cell proteins of M. bovis strain 3683 and of M. agalactiae strain PG2 were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels, and Western blotting was performed as described previously (18). The blots were probed with a pool of M. bovis-specific calf sera collected from group 2 (experiment 1) calves on day 24, then with horseradish peroxidase (HRP)-conjugated sheep anti-bovine specific calf sera collected from group 2 (experiment 1) calves on day 24, diluted 1:500 in 5% sheep serum in phosphate buffered saline (PBS). Dilutions of positive-control, negative-control, and test sera were made in 2.5% sheep serum in 5% sheep serum in phosphate buffered saline (PBS). Dilutions of positive-control, negative-control, and test sera were made in 2.5% sheep serum in PBS. Dilutions of positive-control, negative-control, and test sera were made in 2.5% sheep serum in PBS.

**Characterization of MilA.** The protein sequences of each of the recombinant proteins were aligned to predicted gene sequences of other mycoplasmas using Pfam (Sanger Institute) and BLASTP (NCBI [National Center for Biotechnology Information]).

For the specific detection of lipase activity, 2-fold dilutions (2 μg/mL, 1 μg/mL, 0.5 μg/mL, 0.25 μg/mL, and 0.125 μg/mL) of recombinant GST-Mila-ab were incubated at room temperature with 100 μg of 1,2-O-dilauryl-rac-glycerol-3-glutaric acid resorufin methyl ester (Sigma), as described previously (19). Similar concentrations of GST-P65, which has previously been shown to have lipase activity (19), and GST were used as positive and controls, respectively. The release of resorufin was detected using a Hybrid multimode microplate reader (BioTek) with absorbance measured at 572 nm each minute for 30 min.

**Optimized indirect IgG ELISA using GST-Mila-ab.** The ELISA method used was adapted from that described by Duffy et al. (20). Each well contained 1.2 μg of GST-Mila-ab and blocking was performed with 5% sheep serum in phosphate buffered saline (PBS). Dilutions of positive-control, negative-control, and test sera were made in 2.5% sheep serum in PBS. Dilutions of positive-control, negative-control, and test sera were made in 2.5% sheep serum in PBS.

**FIG 1** A 6-kbp region of the MBOVG45_710 (mila) gene was cloned as two 3-kbp fragments, AB and CD, and subcloned as four 1.5-kbp DNA fragments, A, B, C, and D, with the expressed gene sequences bounded by restriction endonuclease cleavage sites. In addition, two 2-kbp DNA fragments were amplified using primers containing appropriate restriction endonuclease cleavage sites: abF-BamHI (CACggatccATCAAAGACGTGA [binding site, 144 –166]) and abR-SalI (TTCgtcgacGG [binding site, 165 –188]). The modified DNA sequence to aid manipulation in codon usage. The synthetic gene sequence was designed to contain a TGA tryptophan codon was altered to TGG to conform with manufacturer’s instructions, and ligated into pGEX-4T-1 (GE Healthcare), which had been digested with BamHI and SalI. The milA-A, milA-B, milA-C, and milA-D DNA fragments were excised from pGEX-4T-1-mila-AB or pGEX-4T-1-mila-CD DNA as the template and 0.4 μM of the appropriate primers (Fig. 1) in a reaction volume of 50 μl containing 2 mM MgSO4, 100 μM each deoxynucleoside triphosphate, and 1.5 U of platinum Taq thermo-polymerase (Life Technologies). Cycling conditions included an initial denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 68°C for 2 min. After agarose gel electrophoresis the 1.8-kbp products were excised, purified, and ligated into pGEX-4T-1. The ligated plasmids were used to transform E. coli JM109 cells by electroporation. The transformants were selected on LB agar containing ampicillin (50 μg/mL), and selected clones were incubated for 3 h at 30°C in the presence of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Invitrogen). Expression of glutathione S-transferase (GST) fusion proteins from the recombinant plasmids was assessed by SDS-PAGE and Western blotting of whole-cell lysates. The fusion protein GST-Mila-ab was purified by affinity chromatography using glutathione-Sepharose 4B beads (GE Healthcare) and elution with free glutathione.

**Mass spectrometric analysis.** The proteins of M. bovis strain 3683 were separated by SDS-PAGE in a 7.5% polyacrylamide gel and stained with colloidal Coomassie blue (Life Technologies), and the major bands were separated by SDS-PAGE in a 7.5% polyacrylamide gel and stained with 10% polyacrylamide gels, and Western blotting was performed as described previously (18). The blots were probed with a pool of M. bovis-specific calf sera collected from group 2 (experiment 1) calves on day 24, then with horseradish peroxidase (HRP)-conjugated sheep anti-bovine IgG antibody (Bethyl Laboratories, Inc.), and binding was visualized using SIGMAFAST 3,3′-diaminobenzidine (DAB) according to the manufacturer’s instructions.

The protein sequences of each of the recombinant proteins were aligned to predicted gene sequences of other mycoplasmas using Pfam (Sanger Institute) and BLASTP (NCBI [National Center for Biotechnology Information]).
PBS-T (phosphate-buffered saline containing 0.05% [vol/vol] Tween 20). A 2-fold dilution series of the pool of *M. bovis*-specific calf sera collected from the group 2 calves (experiment 1) on day 24, from 1/75 to 1/9,600, as well as a single dilution of the pool of *M. bovis*-negative calf sera collected from the group 1 calves (experiment 1) on day 24 (1/300) and of test sera (1/300) were assayed on each plate in duplicates. HRP-conjugated sheep anti-bovine IgG heavy- and light-chain antibodies (Bethyl) were diluted at 1/2,000 in 2.5% sheep serum in PBS-T and used as the conjugate. After the final wash, 100 µL of 0.3 g 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (peroxidase substrate; KPL)/L was added to each well and the plate incubated for 7 min at room temperature. The reaction was stopped by the addition of 100 µL of 1% SDS to each well, and the absorbance of each well measured at 405 nm using a Hybrid multimode microplate reader (BioTek).

**FIG 2** Western blots of whole-cell proteins of *M. bovis* strain 3683 probed with sera from 6 calves (A through F) collected on day 0 (lanes 1) and day 24 (lanes 2). Uninfected, calves inoculated with mycoplasma culture medium; infected, calves inoculated with *M. bovis* strain 3683; arrow, protein of interest seen in strip 2 in infected calves.

Testing the sensitivity of the indirect IgG ELISA. Calf sera collected at different time points during experiments 1 and 2 were tested using the IgG ELISA protocol as described above. Using the program DeltaSoft 3 (Bio-metallic, Inc.), we calculated the antibody titers for each serum sample by plotting the optical density (OD) values on the standard curve generated using the dilutions of the positive-control serum pool on each plate. The cutoff value for the IgG ELISA was calculated using the test results of all the uninfected animals in both experiments, including all the samples from group 1 and the samples from day 0 from group 2 (a total of 78). The mean antibody titer plus 2 standard deviations (SD) of these uninfected animals was calculated and considered to be the cutoff value.

Test results from the two experiments were pooled to calculate the sensitivity and specificity of the IgG ELISA. For the calculations of sensitivity and specificity, all the calves that were exposed experimentally to *M. bovis* were considered to be positive and all the calves that had not been exposed to *M. bovis* strain 3683 probed with pooled *M. agalactiae* strain PG2 probed with pooled *M. bovis*-specific calf sera as the secondary antibody at a dilution of 1/2,000 and the enzymatic reaction incubated for 15 min. The calf sera from experiment 2 were tested in the IgM ELISA and the antibody titers were calculated as described for the IgG ELISA.

All statistical analyses were performed using Student’s *t* test in GraphPad Prism 5.

**RESULTS**

Identification of mycoplasma immunogenic lipase A. In the Western blot analysis, a single protein with a molecular weight greater than 170 kDa was recognized by serum antibodies from all the infected calves in experiment 1 at day 24 after infection. Sera from the uninfected calves and sera collected on day 0 from infected calves did not contain antibody recognizing this protein (Fig. 2). A similar-sized protein (226 kDa) was detected in whole-cell proteins of *M. bovis* strain 3683 and *M. agalactiae* strain PG2, but antibodies in infected calf sera bound only to the protein in *M. bovis* (Fig. 3). Mass spectrometric analysis identified the coding sequence as that of MBOVPG45_0710, which is predicted to be a 302.9-kDa membrane protein in *M. bovis* PG45 (NCBI accession number YP_004056499). Only the amino-terminal 60% of the remaining 40% not detected, resulting in the lower molecular weight estimated for the protein from SDS-PAGE.

Expression of recombinant MilA. The milA-AB, milA-CD, milA-A, milA-B, milA-C, milA-D, milA-ab, and milA-cd regions were successfully inserted into pGEX-4T-1 and introduced into *E. coli* JM109 cells. Recombinant GST-MilA-CD, GST-MilA-B, GST-MilA-C, GST-MilA-D, and GST-MilA-cd were highly expressed but did not react with *M. bovis*-specific calf sera. GST-MilA-AB and GST-MilA-A reacted weakly, but GST-MilA-ab (92.9 kDa) reacted strongly with *M. bovis*-specific calf sera (see Fig. S1 in the supplemental material). DNA sequencing established that all the cloned inserts had the expected sequences.

Characterization of MilA. The gene encoding MBOVPG45_
0710 (*milA*) is predicted to code for a 302 kDa-protein of 2,700 amino acids (aa) in the *M. bovis* reference strain PG45. Further analysis revealed that it contained a membrane-spanning region at its amino-terminal end, with the remainder of the protein predicted to be located extracellularly. Homologs were found in *M. bovis* strains Hubei-1 and HB0801, *M. agalactiae* strains 5632 and PG2, *Mycoplasma columbinum* strain SF7, and *Mycoplasma fermentans* strains JER and M64 (Table 1). Searches of the Pfam database identified an SGNH_hydrolase region at the amino terminal end (aa 1 to 375) that belonged to a family of GDSL-like lipases (Fig. 4). The SGNH_hydrolase region is characterized by the conserved catalytic site amino acids Ser, Gly, Asp, and His. The active site catalytic triad, Ser-His-Asp, is in block I (Ser 99) and block V (His 362 and Asp 360). Alignment of region 1 of MilA (aa 1 to 375) with other mycoplasma lipases demonstrated that in MilA, the Ser, Gly, Asp, and His residues in blocks I, II, III, and V were conserved (Fig. S2). In block II, Gly was conserved, while in block III, the sequence AXND was conserved in *M. bovis* PG45 and *M. bovis* Hubei-1, although in most of the hydrolase family this sequence is GXND, as it is in *M. fermentans*, *M. agalactiae*, and *M. columbinum*. In block V all mycoplasma MilA homologs had the DIHP sequence, instead of the more typical DXXHP sequence. Analysis of genome sequences adjacent to *milA* revealed the presence of rpoC, rpoB, a lipoprotein gene upstream and hypothetical protein, transposase, and aminopeptidase genes downstream (Fig. 4). The *milA* gene was located downstream of *rpoC* and *rpoB* in all genomes, the exception being the second copy of the gene in *M. bovis* strain Hubei-1. In most species, the genes downstream of the *milA* homolog differed from each other.

The recombinant GST-MilA-ab protein catalyzed release of resorufin from 1,2-O-dilauryl-rac-glycero-3-glutaric acid resorufin methyl ester over a 30-min period, as did the positive control GST-P65. The optical density at 572 nm (OD572) of reaction mixtures containing GST only did not change over the 30-min period, indicating that the negative control did not induce any release of resorufin (Fig. 5).

**Sensitivity of IgG ELISA in experimental studies.**

(i) **Experiment 1.** No calves in the uninfected group had detectable changes in antibody titer against GST-MilA-ab throughout the experiment. The calves in the infected group did not have a significant increase in antibody titer until 3 weeks after infection (Fig. 6 and Table 2). The mean IgG titer of uninfected calves was 23.4, so a cutoff of 47.4 (mean + 2×SD) was used to define positive test results. On day 10 after inoculation, none of the infected calves were positive, but by day 17, two animals were positive, and by day 24 eleven animals were positive.

(ii) **Experiment 2.** As in experiment 1, infected calves had high anti-GST-MilA-ab IgG titers by day 24 (Fig. 7 and Table 3). In the uninfected group, two calves had titers above the cutoff at day 10, but only one calf had titers that remained above the cutoff on days 21 and 22. The mean IgG titer of uninfected calves was 23.4, so a cutoff of 47.4 (mean + 2×SD) was used to define positive test results. On day 10 after inoculation, none of the infected calves were positive, but by day 17, two animals were positive, and by day 24 eleven animals were positive.
17 and 24. In the infected group, four calves were positive on day 17 and 28 were positive on day 24.

The specificity of the IgG ELISA was 98.7% and the sensitivities were 0%, 14.3%, and 92.8% on days 10, 17, and 24, respectively.

**Sensitivity of IgM ELISA in experimental studies.** Throughout experiment 2, calves in the uninfected group had no significant increases in IgM antibody titers against GST-MilA-ab, with the exception of a single calf on day 24. The mean IgM titer of the uninfected calves was 101.5 ± 88.16, so a cutoff of 277.8 was used to define positive test results. The infected group had a significant increase in IgM titers 2 weeks after infection (Table 4, Fig. 8). A single calf in the infected group was positive on day 0, 2 were positive on day 10, 13 were positive on day 17, and 19 were positive on day 24.

The specificity of the IgM ELISA was 97.1% and the sensitivities were 6%, 43.3%, and 63.3% on days 10, 17, and 24, respectively.

**DISCUSSION**

A novel 226-kDa protein, MilA, was identified in *M. bovis* strain 3683 by Western blotting and was shown to be immunoreactive using *M. bovis*-specific calf sera. Although several *M. bovis* proteins, including the Vsps and P48, have been described and characterized as highly immunogenic proteins (16), all of these are smaller than 100 kDa. This high-molecular-weight protein may have been overlooked previously due to its poor transfer onto Western blots and into two-dimensional gels. MilA was estimated to be 226 kDa, but the gene encoding it, MBOVPG45_0710 (*milA*), which was identified by mass spectrometric analysis of tryptic peptides derived from it, was predicted to encode a 302-kDa protein, suggesting that MilA may be cleaved into 2 or more smaller peptides, as is seen with the ciliary adhesin of *M. hyopneumoniae* (22).

A search of the Pfam database for MilA revealed that it belonged to the GDSL-like lipase family, but the presence of a number of differences in key motifs suggested that the mycoplasma lipases/hydrolases may comprise a distinct enzymatic family. Lipase activity has been detected in *M. gallisepticum, Acholeplasma laidlawii, M. hyopneumoniae*, and *M. mycoides* (19, 23, 24), but the lipase of *M. mycoides* (23) has no sequence similarity with MilA and its homologs. A number of putative lipase genes have been identified in the complete genome sequences of mycoplasmas (22, 25, 26), including *M. bovis* (13), but the activities of their products are yet to be confirmed, except for P65 of *M. hyopneumoniae*. This investigation is the first characterization of a lipase in *M. bovis*. Sequence analysis revealed that the homologs of MilA in other mycoplasmas also have the lipase domain in their amino terminal region and no identifiable conserved domains at their carboxyl ends.

It has been suggested that lipases play an essential role in the nutritional requirements of mycoplasmas (24) by hydrolyzing lipids in their environment and liberating the long-chain fatty acids for subsequent transport into the cell (23). However, no transport system genes could be identified close to *milA* or its homologs. The only conserved genes adjacent to these lipase genes were *rpoC* and *rpoB*. Therefore, if the fatty acids broken down by MilA are transported into the cell, it is not clear what transport system might be involved. Respiratory mycoplasmas with lipolytic function may reduce the function of surfactants that maintain normal lung function (19). Pulmonary surfactants are predominantly composed of lipids, fatty alcohols, and free fatty acids in the lung epithelium and can also act as antimicrobial agents (27). Therefore,
TABLE 3 Anti-\textit{M. bovis} IgG titers of calf sera from experiment 2

| Group   | Infection status | No. of calves | Mean ± SD of log_{10} anti-\textit{M. bovis} IgG titers at the indicated day after challenge^a |
|---------|------------------|--------------|------------------------------------------------------------------------------------------|
| 1       | Uninfected       | 5            | 1.4 ± 0.38 A                                                                            |
|         | Infected         | 30           | 1.15 ± 0.25 A, 1.3 ± 0.4 A, 2.3 ± 0.4 B                                                  |

^a Values marked with the same letter in the same column are not significantly different (\(P < 0.05\)).

esterase or lipase activity in a mycoplasmal respiratory pathogen may aid in their capacity to evade these antimicrobial effects and reduce the surfactant-mediated mycoplasmonic activity of alveolar macrophages (28, 29). Some bacterial lipases interfere with neutrophil function and have effects on immune function (30), while a lack of surfactant can induce pulmonary inflammation, increase neutrophil infiltration, and result in unreactive alveolar macrophages (29). Whether lipase activity plays a role in enhancing lung inflammation in \textit{M. bovis} infections is yet to be elucidated. The presence of a region similar to the carboxyl end of the protein in the homologs in other mycoplasmas suggests that this region may play a synergistic role related to the lipase function, but the function of this region in MilA is yet to be clarified.

This study has demonstrated that under experimental conditions an IgG ELISA based on a fragment of MilA is a fast, convenient, sensitive, and reproducible method to detect cattle infected with \textit{M. bovis}. The \textit{milA}-ab fragment was generated from the AB gene fragment and excluded the hydrophobic region at the start of the AB region (the first 100 bp), improving the expression and solubility of the recombinant protein, making it more suitable for larger-scale recombinant protein production. Compared to other published ELISAs for detection of antibodies against \textit{M. bovis}, the concentration of the recombinant GST-MilA-ab antigen needed (1.2 \(\mu\)g/well) was quite low, highlighting its sensitivity for use as an ELISA antigen. The dilution used to test sera (1/300) was higher, allowing more freedom to adjust the test sera dilutions and thus assess very low concentrations of antibody against \textit{M. bovis}. An increase in \textit{M. bovis}-specific IgG titers was detected 3 weeks after experimental infection, but increased \textit{M. bovis}-specific IgM antigens were detected as early as 2 weeks after infection. The IgG ELISA was found to have a higher sensitivity than the IgM ELISA, suggesting it would be more suitable as a diagnostic tool for field situations.

Most of the published descriptions of ELISAs to detect antibodies against \textit{M. bovis} were developed using \textit{M. bovis} whole-cell proteins as the coating antigen, resulting in an assay with greater sensitivity, but less specificity (31). Use of a single, highly immunoreactive protein is more likely to result in a specific ELISA. The most immunoreactive antigens in the \textit{M. bovis} cell membrane are the Vsps (variable surface proteins), but, while the Vsps may have diagnostic utility (32), they are subject to phase and antigenic variation, which may result in variations in the antibody response against them in individual infected cattle and compromise the sensitivity of the assay (33). Therefore, use of a protein such as MilA that appears not to be subject to phase or antigenic variation, but is still highly immunoreactive, is more likely to result in a useful diagnostic tool.

The cross-reactivity with other common pathogens in ruminants, especially \textit{M. agalactiae}, needs to be tested for any \textit{M. bovis} ELISA, as many ELISAs developed previously have shown cross-reactivity with antibodies induced by \textit{M. agalactiae} (10, 11, 34). This is probably attributable to the use of whole-cell proteins as antigens, as a mixture of many cellular proteins is more likely to give rise to false-positive reactions due to the presence of antibodies induced by commensal mycoplasmas (31). A similarly sized protein to MilA is found in \textit{M. agalactiae}, but it was not recognized in Western blots by \textit{M. bovis}-specific calf sera. Furthermore, sera collected before challenge did not bind to MilA in Western blots. Therefore, it appears likely that the recombinant protein will not be cross-reactive with antibodies induced by commensals in cattle. The presence of a similar protein to that of MilA-ab in \textit{M. agalactiae} PG2, with an amino acid identity of 454/584 (78%), suggests that the homolog in \textit{M. agalactiae} may be used to develop an IgG ELISA to screen for \textit{M. agalactiae} infection in sheep.

Earlier ELISAs developed to detect \textit{M. bovis} infection were more targeted toward testing for antibodies in milk (10, 34, 35), and most had limited sensitivity. In addition, there was a marked delay between the presence of detectable antibodies in serum and the detection of antibodies in milk (35). A few ELISAs have been developed to detect \textit{M. bovis} antigens in pneumonia and arthritic infections in cattle (36). However, the sensitivity of these antigen detection sandwich ELISAs could be affected by contamination (36, 37), recent antibiotic treatment, and the presence of clinically infected animals (38) or by inhibitory products in tissue samples or nasal swabs. The IgG ELISA developed here could be used to screen for \textit{M. bovis}-infected heifers before they join a clean milking herd or as a regular screening method to maintain \textit{M. bovis}-free herds for trade purposes (8). Furthermore, it could be used to test sera to screen for \textit{M. bovis} infection in dairy cattle. Further work examining the utility of this IgG ELISA in other \textit{M. bovis}-infected populations, such as dairy cows with subclinical intramammary infections and chronically infected animals, is needed. The IgG ELISA could also be used as a component in the assessment of animal trials of vaccines against \textit{M. bovis}, as it may assist in assessing the relative efficacy of different vaccine candidates in inducing an immune response to \textit{M. bovis}.

![Graph](http://cvi.asm.org/Downloaded from http://cvi.asm.org)
We thank C. Colson for care of the calves during the experiment. We declare that we have no conflicts of interest.

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