

Early Antibody Responses to Experimental *Mycobacterium bovis* Infection of Cattle

W. R. Waters,^{1*} M. V. Palmer,¹ T. C. Thacker,¹ J. P. Bannantine,¹ H. M. Vordermeier,²
R. G. Hewinson,² R. Greenwald,³ J. Esfandiari,³ J. McNair,⁴ J. M. Pollock,⁴
P. Andersen,⁵ and K. P. Lyashchenko³

United States Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Bacterial Diseases of Livestock Research Unit, Ames, Iowa¹; Veterinary Laboratories Agency, Weybridge, New Haw, Addlestone, Surrey, United Kingdom²; Chembio Diagnostic Systems, Inc., Medford, New York³; Bacteriology Department, Veterinary Sciences Division, Stormont, Belfast, United Kingdom⁴; and Statens Serum Institut, Copenhagen, Denmark⁵

Received 14 February 2006/Returned for modification 23 March 2006/Accepted 27 March 2006

Bovine tuberculosis persists as a costly zoonotic disease in numerous countries despite extensive eradication and control efforts. Sequential serum samples obtained from *Mycobacterium bovis*-infected cattle were evaluated for seroreactivity to mycobacterial antigens. Animals received *M. bovis* by aerosol, intratonsil, intranasal, or intratracheal inoculation. Assays included the multiantigen print immunoassay for determination of antigen recognition patterns, immunoblot analysis for sensitive kinetic studies, and the VetTB STAT-PAK test, a novel, rapid test based on lateral-flow technology. Responses to MPB83 were detected for all *M. bovis*-infected animals regardless of the route or strain of *M. bovis* used for inoculation. Other less commonly recognized antigens included ESAT-6, CFP-10, and MPB70. Responses to MPB83 were detectable as early as 4 weeks after inoculation, were boosted upon injection of purified protein derivatives for skin testing, and persisted throughout the course of each of the four challenge studies. MPB83-specific immunoglobulin M (IgM) was detected prior to MPB83-specific IgG detection; however, early IgM responses rapidly waned, suggesting a benefit of tests that detect both IgM- and IgG-specific antibodies. The VetTB STAT-PAK test detected responses in sera from 60% (15/25) of the animals by 7 weeks after challenge and detected responses in 96% (24/25) of the animals by 18 weeks. These findings demonstrate the potential for new-generation antibody-based tests for the early detection of *M. bovis* infection in cattle.

Tuberculosis (TB) in humans may result from exposure to any one of the tubercle bacilli included within the *Mycobacterium tuberculosis* complex (i.e., *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. pinnipedii*, and *M. microti*). *Mycobacterium bovis*, unlike *M. tuberculosis*, has a wide host range, is the species most often isolated from tuberculous cattle, and has several wildlife maintenance hosts, including the Eurasian badger (*Meles meles*), brush-tailed possum (*Trichosurus vulpecula*), and white-tailed deer (*Odocoileus virginianus*). Wildlife reservoirs have made *M. bovis* eradication from national herds in several developed countries, including the United Kingdom, New Zealand, and the United States, particularly difficult (3, 4, 16). Eradication campaigns in these countries have generally relied on test and removal, slaughterhouse surveillance, movement restriction, and/or wildlife reservoir control strategies. The tests most widely used for the detection of TB in humans and cattle include the measurement of delayed-type hypersensitivity (i.e., skin testing) to purified protein derivatives (PPDs) and/or in vitro assays for gamma interferon produced in response to mycobacterial antigen stimulation (i.e., Bovigam [Prionics AG, Schlieren, Switzerland] and Quantiferon Gold

[Cellestis Inc., Carnegie, Victoria, Australia]). These tests rely on early cell-mediated responses, a hallmark of TB immunopathogenesis. In contrast, the poor sensitivity of antibody-based tests has prevented the widespread use of these assays for the early detection of tuberculous cattle (14). Recent studies, however, have indicated that serum antibody to another mycobacterial infection of cattle (i.e., *M. avium* subsp. *paratuberculosis*) is detectable relatively early after experimental (18) and natural (6) infection, if sensitive immunoassays are applied.

The objectives of the present study were to evaluate antigen recognition patterns of the early antibody response using four different routes of administration to elicit respiratory *M. bovis* infection, to determine the contribution of immunoglobulin M (IgM) to the early response, and to evaluate the use of a novel and convenient test for the rapid detection of early-infected cattle. Routes, doses, and strains of inocula were chosen based on the predominant models used for evaluation of the immunopathogenesis of *M. bovis* infection of cattle.

MATERIALS AND METHODS

Calves, challenge inoculum, and necropsy. For aerosol challenge, nine female and castrated male Maine Anjou calves (~4 months of age) were obtained from a TB-free herd in Iowa, randomly assigned to two groups, and housed according to institutional guidelines of the National Animal Disease Center, Ames, Iowa (NADC), in a biosafety level 3 (BL-3) facility. One group ($n = 5$) received 10^5 CFU of *M. bovis* strain 95-1315. This strain was originally isolated from a

* Corresponding author. Mailing address: United States Department of Agriculture, Agricultural Research Service, National Animal Disease Center, P.O. Box 70, Ames, IA 50010-0070. Phone: (515) 663-7756. Fax: (515) 663-7458. E-mail: rwaters@nadc.ars.usda.gov.

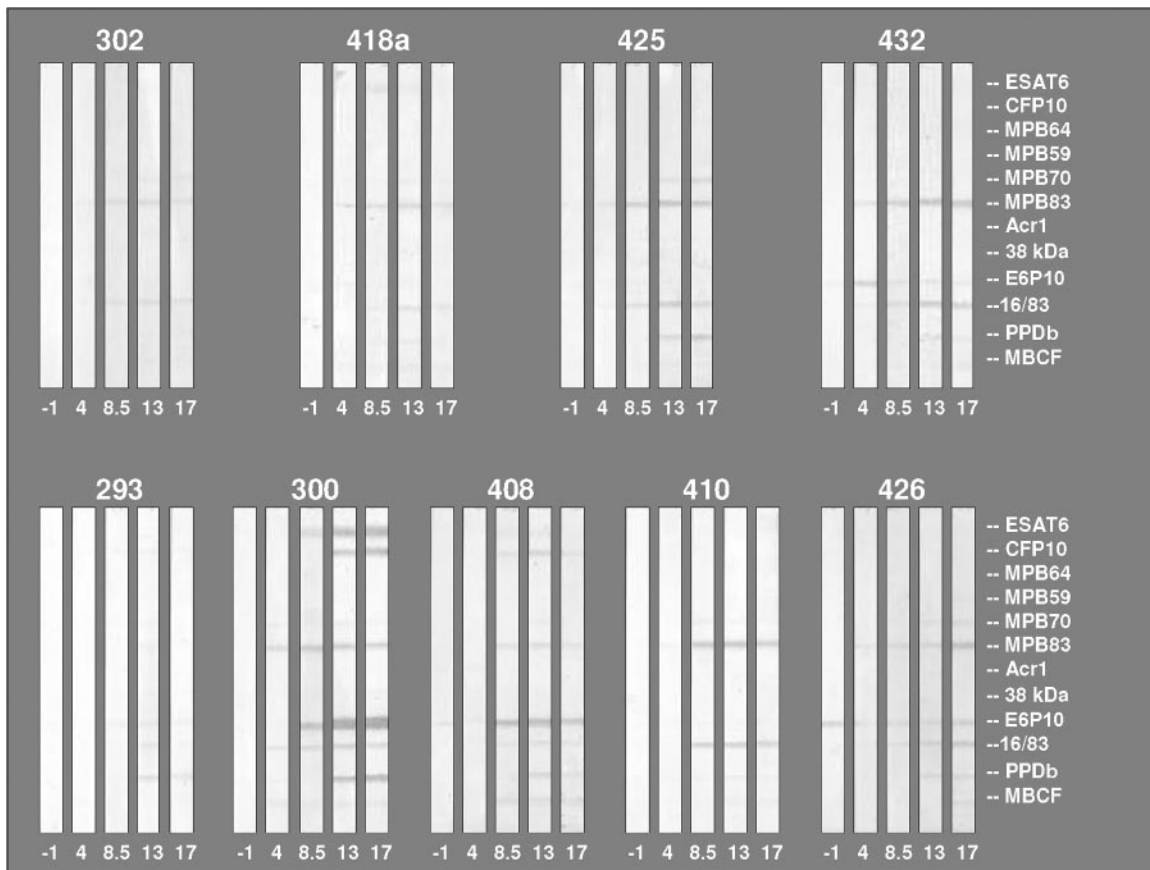


FIG. 1. Antigen recognition patterns by antibody from cattle inoculated with *Mycobacterium bovis* via the aerosol route. Cattle received either 10^5 CFU of *M. bovis* strain 95-1315 (animals 302, 418a, 425, and 432) or 10^5 CFU of *M. bovis* strain HC2005T (animals 293, 300, 408, 410, and 426), and serological reactivity to *M. tuberculosis* complex antigens was determined by multiantigen immunoprint assay of sequential serum samples. Antigens are indicated in the far-right margin. For each panel of five nitrocellulose strips, animals are indicated in the upper margins, and weeks after challenge are indicated in the lower margins. E6P10 refers to the ESAT-6:CFP10 fusion protein. 16/83 refers to 16-kDa alpha-crystallin/MPB83 fusion protein. PPDb refers to *M. bovis* PPD. MBCF refers to *M. bovis* culture filtrate.

white-tailed deer in Michigan (15). The other group ($n = 4$) received 10^5 CFU of *M. bovis* strain HC2005T. This strain was originally isolated from a dairy cow in Texas (19). The challenge inoculum consisted of mid-log-phase *M. bovis* isolates grown in Middlebrook 7H9 medium supplemented with 10% oleic acid-albumin-dextrose complex (Becton Dickinson Microbiology Systems, Franklin Lakes, NJ) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO). To harvest tubercle bacilli from the culture medium, bacteria were pelleted by centrifugation at $750 \times g$, washed twice with phosphate-buffered saline solution (PBS) (0.01 M, pH 7.2), and diluted to the appropriate density for use as an inoculum in 2 ml of PBS. The inoculum was sonicated for 5 s prior to nebulization to disperse clumps of bacteria. Enumeration of bacilli was done by serial dilution plate counting on Middlebrook 7H11 selective medium (Becton Dickinson). Aerosol inoculation procedures were performed as described previously (11). Briefly, cattle were restrained, and the challenge inoculum was delivered by nebulization into a mask covering the nostrils and mouth. The nebulization apparatus consisted of a compressed air tank, a jet nebulizer, a holding reservoir, and a mask (Trudell Medical International, London, Ontario, Canada). Compressed air at 25 lb/in² was used to jet nebulize the challenge inoculum (2 ml *M. bovis* in PBS) directly into the holding reservoir. Upon inspiration, the nebulized inoculum was inhaled through a one-way valve into the mask and directly into the nostrils. A rubber gasket sealed the mask securely to the muzzle, preventing the leakage of inoculum around the mask. Expired air exited through one-way valves on the sides of the mask. The nebulization process continued until all of the inoculum, a 1-ml PBS wash of the inoculum tube, and an additional 2 ml of PBS were delivered (~12 min). Strict BL-3 safety protocols were followed to protect personnel from exposure to *M. bovis*.

For intratonsillar challenge, eight male ~3-month-old Holstein calves were obtained from a TB-free herd in Wisconsin and housed at the NADC according to institutional guidelines and approved animal care and use protocols in a BL-3 facility. The challenge inoculum (4×10^4 CFU of *M. bovis* strain 95-1315 diluted in 0.2 ml of phosphate-buffered saline [0.15 M, pH 7.2]) was instilled directly into both tonsillar crypts of sedated calves as described previously for inoculation of white-tailed deer (10).

For intratracheal challenge, 6-month-old Holstein/Holstein-cross calves were obtained from TB-free herds and housed at the Animal Services Unit, Veterinary Laboratory Agencies, Weybridge, United Kingdom, in a BL-3 facility. Calves received 4×10^4 CFU of *M. bovis* strain AF 2122/97 (a field isolate from Great Britain) by intratracheal instillation as previously described (17).

For intranasal challenge, two Friesian-cross calves of approximately 6 months of age were obtained from a Northern Irish herd with no history of tuberculosis infection for a minimum of the previous 5 years. The animals were housed in isolation at the Veterinary Sciences Division, Belfast, United Kingdom, under negative pressure and maintained according to local institutional and statutory requirements. The animals were challenged by direct instillation of approximately 10^7 CFU of a field strain of *M. bovis* (T/91/1378) into the nasal passages as previously described (9, 13).

At the conclusion of each of the four challenge studies, cattle were euthanized and examined for gross lesions. Various tissues were collected (e.g., tonsil; lung; liver; spleen; and lung-associated, head-associated, mesenteric, and prefemoral lymph nodes) for bacteriologic culture and microscopic examination, and detailed descriptions of these findings were presented elsewhere previously (9, 11-13, 17). All animals had gross and microscopic lesions compatible with *M.*

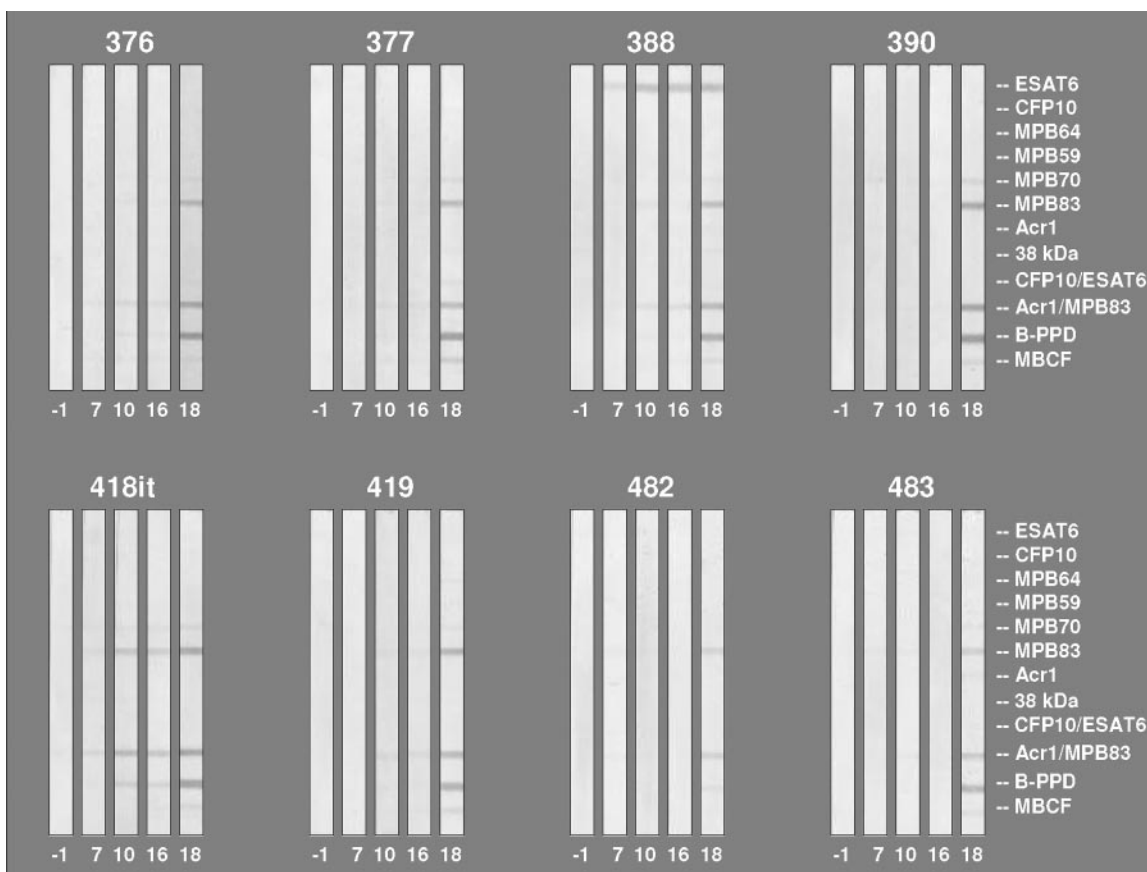


FIG. 2. Antigen recognition patterns by antibody from cattle inoculated with *Mycobacterium bovis* strain 95-1315 via intratonsillar instillation. Serological reactivity to *M. tuberculosis* complex antigens was determined by multiantigen immunoprint assay of sequential serum samples. Antigens are indicated in the far-right margin. For each panel of five nitrocellulose strips, animals are indicated in the upper margins, and weeks after challenge are indicated in the lower margins. E6P10 refers to the ESAT-6:CFP10 fusion protein. 16/83 refers to the 16-kDa alpha-crystallin/MPB83 fusion protein. PPDb refers to *M. bovis* PPD. MBCF refers to *M. bovis* culture filtrate.

bovis infection, and *M. bovis* was isolated upon culture of tissues from each animal.

Tuberculin skin test procedures. Cattle were tested for in vivo responsiveness to mycobacterial antigens by a tuberculin skin test. Calves receiving *M. bovis* via aerosol were injected with PPDs for the comparative cervical test (CCT) at ~8.5 weeks after inoculation. Calves receiving *M. bovis* via the intratonsillar route were injected with PPD(s) for the caudal fold test (CFT) at ~8 weeks after inoculation and for the CCT at ~16 weeks after inoculation. Calves receiving *M. bovis* via the intratracheal route were injected with PPDs for the CCT at 15 weeks after inoculation, with the test being performed as specified by EEC directive 80/219EEC, amending directive 64/422/EEC, annexe B (4a). Calves receiving *M. bovis* by the intranasal route were not skin tested. For the CFT, the thickness of the fold of skin on the right side of the tail head was measured and injected intradermally with 0.1 ml (100 μ g) of *M. bovis*-derived PPD. For the CCT, 100 μ g of *M. bovis* PPD and 40 to 50 μ g of *M. avium* PPD (amounts differ slightly between countries) were injected intradermally at separate clipped sites in the midcervical region according to guidelines described in the USDA APHIS circular 91-45-01 (16a). Tuberculin for skin test procedures were obtained from either the Brucella and Mycobacterial Reagents Section of the National Veterinary Services Laboratory, Ames, Iowa (aerosol and intratonsillar studies), or the Tuberculin Production Unit at the Veterinary Laboratory Agencies, Weybridge, United Kingdom (intratracheal studies). All animals in each of the challenge groups tested positive by skin test.

MAPIA. The following recombinant antigens of *M. bovis* were purified to near homogeneity as the following polyhistidine-tagged proteins (Rv numbers according to the classification of Cole et al. [2] are in parentheses): ESAT-6 (Rv3875) and CFP-10 (Rv3874), produced at the Statens Serum Institut, Copenhagen, Denmark, and MPB59 (Rv1886c), MPB64 (Rv1980c), MPB70 (Rv2875), and

MPB83 (Rv2873), produced at the Veterinary Sciences Division, Stormont, Belfast, United Kingdom (7). Alpha-crystallin (Acr1 [Rv3391]) and the 38-kDa protein (PstS1 [Rv0934]) were purchased from Standard Diagnostics, Seoul, South Korea. Polyprotein fusions CFP-10/ESAT-6 and Acr1/MPB83 were constructed by overlapping PCR using gene-specific oligonucleotides to amplify the genes from *M. tuberculosis* H37Rv chromosomal DNA. The fused polygene PCR products were cloned into the pMCT6 *Escherichia coli* expression vector using SmaI/BamHI restriction enzymes. The polyproteins were purified to near homogeneity by exploiting the polyhistidine tag encoded by the vector. *M. bovis* culture filtrate (MBCF) was obtained from a field strain of *M. bovis* (T/91/1378; Veterinary Sciences Division, Belfast, United Kingdom) cultured in synthetic Sauton's medium. Multiantigen print immunoassay (MAPIA) was performed as described previously (8). Briefly, antigens were immobilized on nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) at a protein concentration of 0.05 mg/ml using a semiautomated airbrush printing device (Linomat IV; Camag Scientific Inc., Wilmington, Del.). The membrane was cut perpendicular to the antigen bands into 3-mm-wide strips. Strips were blocked for 1 h with 1% nonfat skim milk in PBS with 0.05% Tween 20 (PBST) and were then incubated for 1 h with serum samples diluted 1:40 in blocking solution. After being washed, strips were incubated for 1 h with peroxidase-conjugated protein G (Sigma) diluted 1:1,000 for IgG detection or with peroxidase-labeled antibody to bovine IgM (Kirkegaard & Perry Laboratories) diluted 1:500, followed by another wash step. Cattle antibodies bound to printed antigens were visualized with 3,3',5,5'-tetramethyl benzidine (Kirkegaard & Perry Laboratories). Results were evaluated by semiquantitative densitometry using Scion Image (version beta 4.0.2).

Immunoblot assay. Electrophoresis and immunoblot assays were performed using previously described procedures (1), with the following modifications.

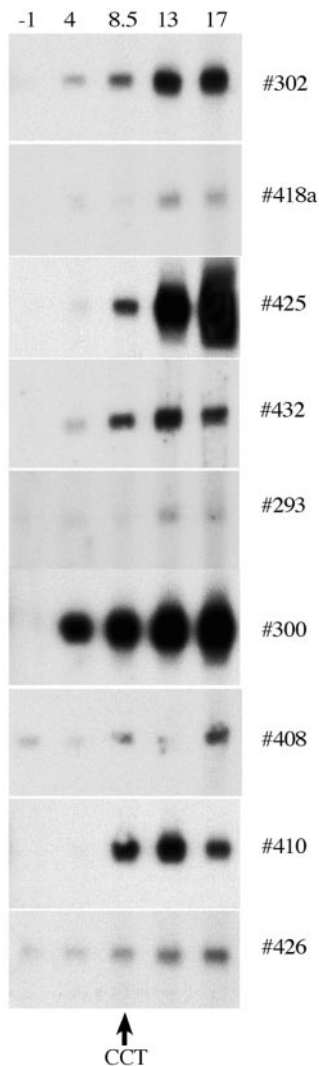


FIG. 3. Kinetics of the response to MPB83 by antibodies from cattle inoculated with *Mycobacterium bovis* via the aerosol route. Cattle received either 10^5 CFU of *M. bovis* strain 95-1315 (animals 302, 418a, 425, and 432) or 10^5 CFU of *M. bovis* strain HC2005T (animals 293, 300, 408, 410, and 426), and serological reactivity to MPB83 was determined by immunoblot analysis of sequential serum samples. Weeks after challenge are indicated in the upper margin, and animals are indicated in the right margin. In the lower margin, CCT indicates the time point for in vivo administration of purified protein derivatives (i.e., *M. avium* and *M. bovis* PPD) for the comparative cervical test (CCT).

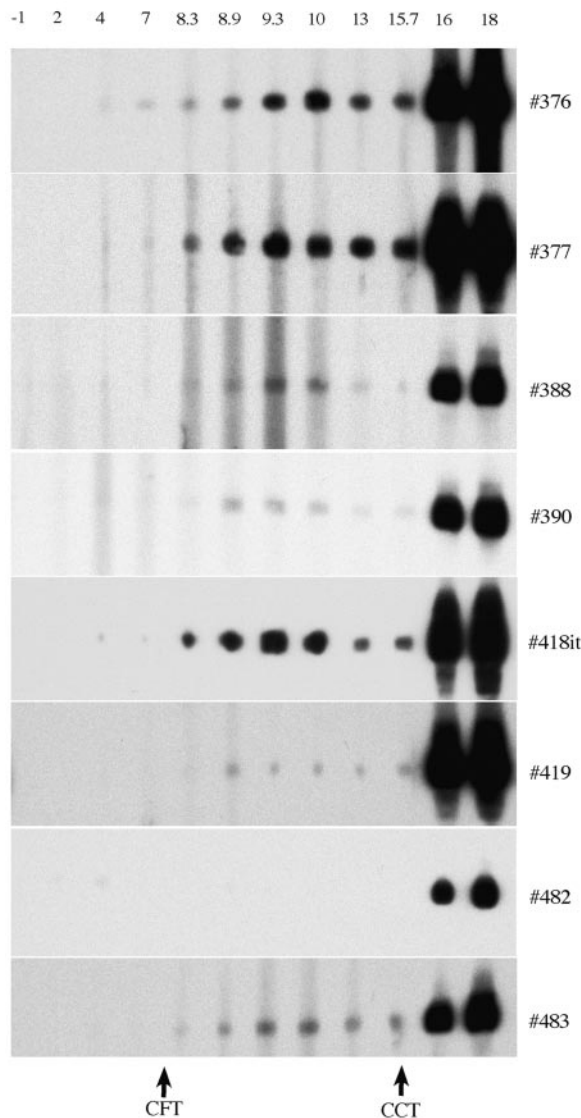


FIG. 4. Kinetics of the response to MPB83 by antibodies from cattle inoculated with *Mycobacterium bovis* strain 95-1315 via intranasal instillation. Serological reactivity to MPB83 was determined by immunoblot analysis of sequential serum samples. Weeks after challenge are indicated in the upper margin, and animals are indicated in the right margin. In the lower margin, CFT indicates the time point for the in vivo administration of *M. bovis* PPD for the caudal fold test (CFT), and CCT indicates the time point for the in vivo administration of purified protein derivatives (i.e., *M. avium* and *M. bovis* PPD) for the comparative cervical test (CCT).

Comparisons of the reactivities of serial serum samples against recombinant MPB83 antigen were conducted using a slot-blotting device (Bio-Rad, Richmond, Calif.). Antigen was electrophoresed through preparative 12% (wt/vol) polyacrylamide gels and transferred to nitrocellulose filters. These filters were placed in a blocking solution consisting of PBST and 2% (wt/vol) bovine serum albumin (PBST-BSA). After blocking, the filters were placed into a slot blot device, and individual serum samples, diluted 1:200 in PBST-BSA, were added to independent slots. After a 2-h incubation with gentle rocking, blots were washed three times with PBST and incubated with peroxidase-conjugated anti-bovine IgG heavy and light chains (Kirkegaard Perry Laboratories) diluted 1:80,000 in PBST-BSA for 1.5 h. Blots were again washed three times with PBST and developed for chemiluminescence in SuperSignal detection reagent (Pierce Chemical Co., Rockford, Ill.).

Lateral-flow assay. A rapid immunochromatographic assay, the VetTB STAT-PAK test, was recently developed by Chembio Diagnostic Systems, Inc., Medford, N.Y., to detect antibodies of three isotypes (IgM, IgG, and IgA) against mycobacterial antigens in various host species. The test employs a unique cocktail of *M. tuberculosis* or *M. bovis* antigens and a blue latex bead-based signal detection system. The ready-to-use disposable device consists of a plastic cassette containing a strip of nitrocellulose membrane impregnated with test antigen and laminated with several pads made of glass fiber and cellulose. Thirty microliters of sera and 3 drops of sample diluent are added sequentially to the sample pad. As the diluted test sample migrates to the conjugate pad, the latex particles conjugated to antigen bind antibody, if present, thus creating a colored immune complex. Driven by capillary forces, this complex flows laterally across the nitrocellulose membrane impregnated with specific antigen and binds to the immo-

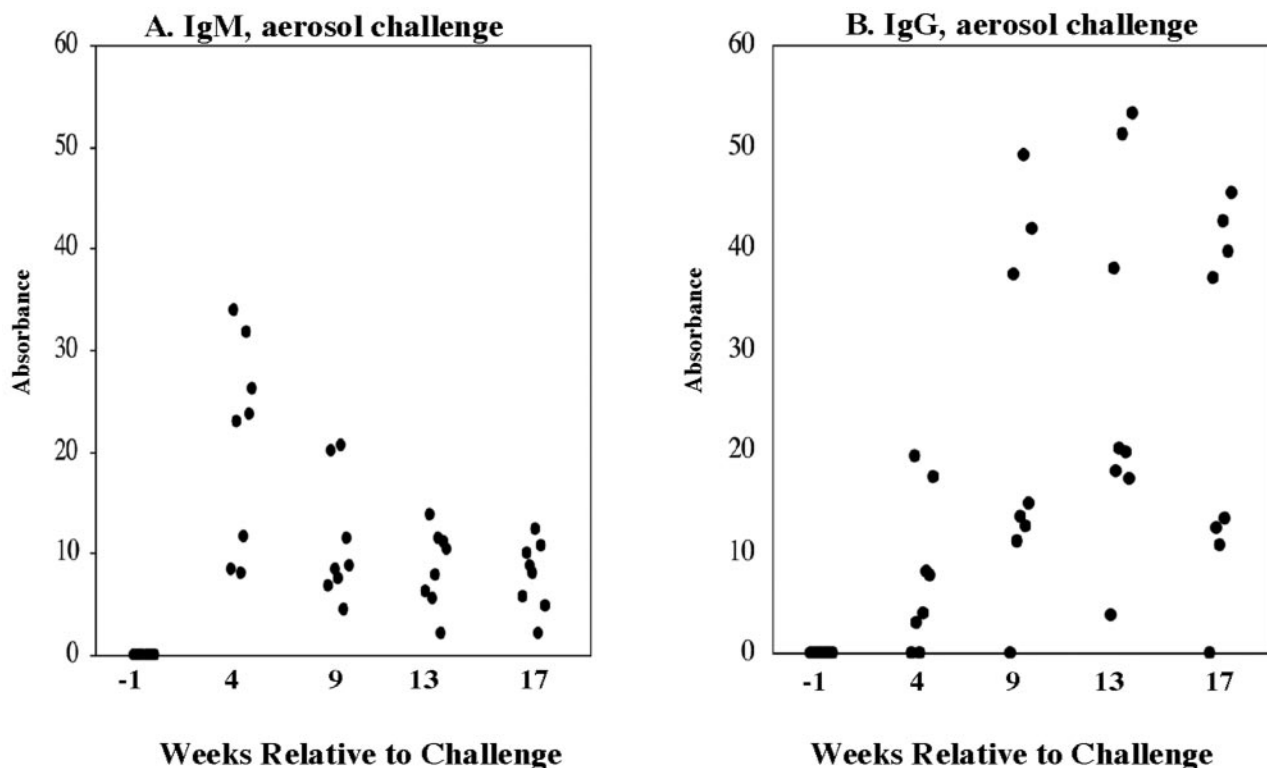


FIG. 5. Response kinetics and intensity of reactivity to MPB83 by IgM (A) and IgG (B) in sequential serum samples obtained from cattle inoculated with *Mycobacterium bovis* via the aerosol route. Semiquantitative densitometry using Scion Image (version beta 4.0.2) was used to determine the intensity of IgM (A) or IgG (B) reactivity to MPB83 printed onto nitrocellulose.

bilized antigen, producing a visible blue band in the test area of the device. In the absence of specific antibody, no band is visible in the test window. The liquid continues to migrate along the membrane, producing a similar blue band in the control area of the device, irrespective of the presence of specific antibody in the test sample, demonstrating that the test immunoreagents are functioning properly. Results are read at 20 min. Any visible band in the test area is considered positive.

RESULTS AND DISCUSSION

Antigen recognition patterns. A comparison of antibody responses from short-duration challenge studies using two different inoculation routes, aerosol and intratonsillar, revealed variable individual animal antigen recognition patterns, even within challenge treatment groups (Fig. 1 and 2). Similar variability was detected with sera from cattle receiving *M. bovis* by either intranasal or intratracheal instillation (data not shown). Despite the variability, MPB83, either alone or as a fusion partner with the 16-kDa alpha-crystallin protein (16/83), was clearly the serodominant target for each route of exposure and challenge strain. Other less commonly recognized antigens included ESAT-6, CFP-10, MPB70, *M. bovis* PPD, and MBCF.

Kinetics of response to MPB83. With the aerosol route of inoculation (Fig. 3), antibody to MPB83 was detected by immunoblot analysis as early as 4 weeks after inoculation in 7/9 animals (i.e., animals 300, 302, 408, 418a [very faint band], 425, 426, and 432). Faint bands, however, were detected with prechallenge sera from two animals (i.e., animals 408 and 426). Three days after injection of PPDs for the comparative cervical

skin test (i.e., CCT) at ~8.5 weeks after challenge, 8/9 aerosol-inoculated animals reacted to MPB83, although the response by animal 418a was very weak. With the intratonsillar route of inoculation (Fig. 4), antibody to MPB83 was detected at 4 and 7 weeks after inoculation in sera from 1/8 animals (i.e., animal 376) and 2/8 animals (i.e., animals 376 and 377), respectively. Antibody to MPB83 was not detectable in prechallenge sera in the intratonsillar group. Three days after injection of PPD for the caudal fold skin test (Fig. 4), responses to MPB83 were detected with sera from three additional animals (animals 418a, 483, and 388). By 7 days after PPD administration for the CFT (i.e., ~9 weeks), 7/8 animals had responses to MPB83. Injection of PPDs for the CCT greatly increased the level of antibody to MPB83 (Fig. 4).

With both aerosol and intratonsillar inoculation of *M. bovis*, the intensity of reactivity to MPB83 generally increased over time after inoculation, and responses for all animals were detected by 16 weeks after inoculation (Fig. 3 and 4). Without exception, antibody to MPB83 was elicited prior to that of either PPD or MBCF, both complex mycobacterial antigens (Fig. 1 and 2). Three animals developed responses to ESAT-6 and/or CFP-10 (Fig. 1 and 2), yet only animal 388 had antibody to ESAT-6 elicited prior to that of MPB83 (Fig. 2). Antibody to MPB70 was detectable only after injection of PPD for the skin test and not prior to that of MPB83 (Fig. 1 and 2).

To further characterize the response, antibody bound to MPB83 coated onto nitrocellulose was probed with anti-bovine

TABLE 1. Kinetics of the isotype response to MPB83 as detected by MAPIA and corresponding VetTB STAT-PAK test results

Animal	Wk after inoculation ^a	MAPIA result ^b		VetTB STAT-PAK test result ^{b,c}
		IgM	IgG	
193	0	-	-	-
	3	+	-	+
	6	+++	+++	+++
	12	+	+++	+++
	25	-	+++	+++
	48	-	++	++
	78	-	+++	+++
198	0	-	-	-
	3	+	-	+
	6	++	++	+++
	12	++	+++	+++
	25	-	+++	+++
	48	-	++	++
	78	-	+++	+++

^a Cattle received 10⁶ CFU of *M. bovis* strain AF2122/97 via intranasal administration.

^b Responses indicate the relative intensities of bands, with -, +, ++, and +++ indicating no, weak, moderate, and intense reactivities, respectively.

^c The test utilizes a unique cocktail of several recombinant antigens, including MPB83, ESAT-6, and CFP-10, optimized by evaluation of antigen recognition patterns using MAPIA.

IgM or protein G conjugate to distinguish between IgM and IgG responses (Fig. 5 and Table 1). Particularly with aerosol and intranasal inoculation, MPB83-specific IgM was detected early (Fig. 5 and Table 1). Notably, MPB83-specific IgM was detected in sera from all aerosol-inoculated animals by 4 weeks after challenge (Fig. 5) and in sera from intranasally inoculated animals by 3 weeks after challenge (Table 2). A rise to steady

TABLE 2. VetTB STAT-PAK test responses in cattle infected with *M. bovis* by aerosol or intratonsillar inoculation^a

Route	Wk after inoculation	Responder rate (no. of positive/total no. inoculated)
Aerosol ^b	-1	0/9
	4	8/9
	8.5	8/9
	17	8/9 ^f
Intratonsillar ^c	-1	0/8
	7	0/8
	10	3/8 ^f
	16	0/8 ^f
	18	8/8 ^f
Intratracheal ^d	0	0/6
	2	0/6
	5	5/6
	9	3/6
	13	4/6
	16	6/6 ^f
Intranasal ^e	0	0/2
	3	2/2
	6	2/2
	12	2/2

^a The VetTB STAT-PAK test utilizes a unique cocktail of several recombinant antigens, including MPB83, ESAT-6, and CFP-10, optimized by evaluation of antigen recognition patterns using MAPIA.

^b Cattle received 10⁵ CFU of *M. bovis* (either strain 95-1315 [*n* = 5] or HC2005T [*n* = 4]) by the aerosol route.

^c Cattle received 4 × 10⁴ CFU of *M. bovis* strain 95-1315 by instillation directly into the tonsillar crypts.

^d Cattle received 4 × 10⁴ CFU of *M. bovis* strain T/91/1378 via intratracheal administration.

^e Cattle received 10⁶ CFU of *M. bovis* strain AF2122/97 via intranasal administration.

^f After skin test.

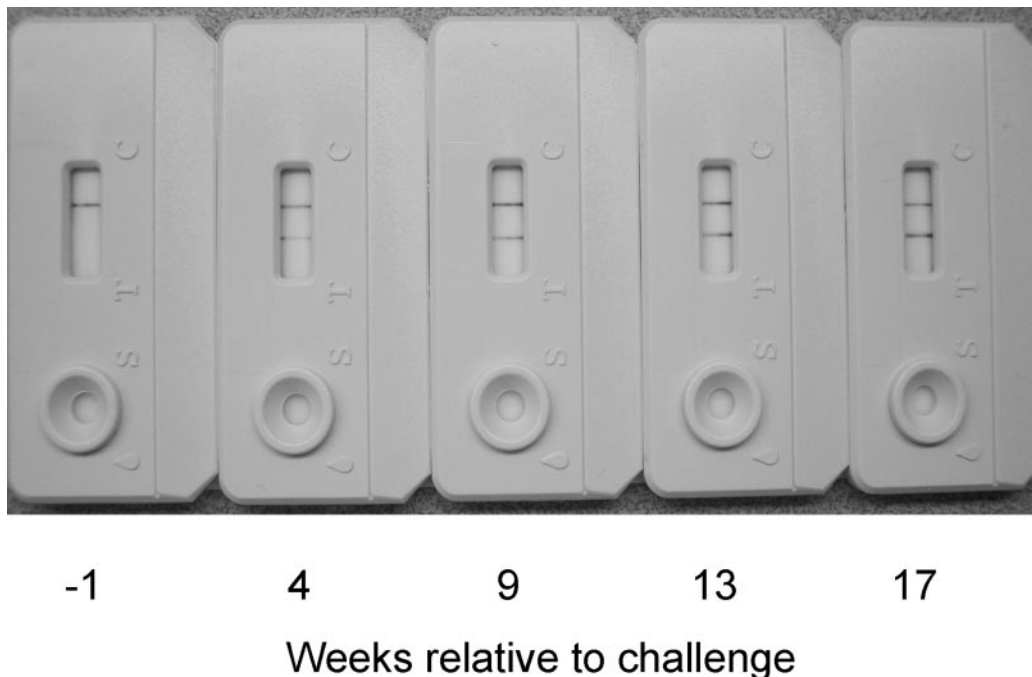


FIG. 6. Vet-TB STAT-PAK test results from sequential serum samples from a calf inoculated with *Mycobacterium bovis* via aerosol. The upper band of reactivity is an internal positive control indicating mobility of the serum sample and diluent across the membrane. The lower bands of reactivity visualized on samples obtained at 4, 8.5, 13, and 17 weeks after challenge indicate a positive reaction to the test antigens.

MPB83-specific IgG responses followed waning early IgM responses. Similar yet less-robust patterns of the kinetics of the isotype response to MPB83 were detected with sera from animals inoculated by the intratonsillar and intratracheal routes (data not shown).

Rapid test for early diagnosis. Results from MAPIA were used to select a cocktail of antigens for a novel lateral-flow immunoassay (i.e., VetTB STAT-PAK test) (Fig. 6) as described previously for use with samples from Eurasian badgers (5). This assay affords the detection of both IgM and IgG bound to specific antigen. Weak responses were detected as early as 3 weeks after intranasal challenge (i.e., corresponding to MPB83-specific IgM as detected by MAPIA), with increased intensity of the test band at 6 weeks after challenge and throughout the remainder of the study (Table 1). With aerosol challenge, responses were detected for 8/9 animals beginning at 4 weeks after challenge (Table 2). The one nonresponding animal (animal 293) had weak responses to MPB83 by immunoblot analysis and MAPIA. Similarly, the use of the VetTB STAT-PAK test detected responses with sera from 5/6 intratracheally inoculated animals at 5 weeks after challenge and from 6/6 animals at 16 weeks after challenge (Table 2). In contrast, only 3/8 and 0/8 animals inoculated by the intratonsillar route had detectable antibody responses with the VetTB STAT-PAK test at 10 and 16 weeks after challenge, respectively (Table 2). However, by 18 weeks after challenge (i.e., 2 weeks after injection of PPDs for the CCT), responses were detected with each of the eight animals.

In summary, antibody responses to MPB83 were detected by immunoblot analysis and/or MAPIA for all *M. bovis*-infected animals, regardless of the route or strain of *M. bovis* used for inoculation. Overall, 60% (15/25) of sera from experimentally infected cattle were reactive by the VetTB STAT-PAK test by 7 weeks after challenge, and 96% (24/25) of sera were reactive by 18 weeks. Responses to MPB83 were detectable as early as 4 weeks after inoculation, were boosted upon injection of PPDs for skin testing, and persisted throughout the course of each of the four challenge studies. MPB83-specific IgM was detected prior to MPB83-specific IgG detection; however, these early IgM responses rapidly waned, suggesting a benefit of tests that detect both IgM- and IgG-specific antibodies. Ongoing studies have demonstrated the potential for use of the VetTB STAT-PAK test as a "cow-side" test using whole-blood samples and for slaughter surveillance programs using diaphragm juice (i.e., extracellular muscle fluid). Despite the promising findings, caution should be used when extrapolating results from the present findings using moderate- to high-dose challenge studies to field situations. Additionally, animals in the present study were 6 months of age or younger at the time of challenge. It is unclear how age (e.g., neonate versus juvenile versus adult) would impact humoral responses, particularly in a field situation. Similar high sensitivities may not be detected under natural transmission conditions. Thus, additional studies are under way to evaluate these and other emerging antibody-based diagnostic platforms with samples from naturally infected cattle.

ACKNOWLEDGMENTS

We thank Jim Fosse, Peter Lasley, Jessica Pollock, and Shelly Zimmerman for excellent technical support.

REFERENCES

- Bannantine, J., and J. R. Stabel. 2000. HspX is present within *Mycobacterium paratuberculosis*-infected macrophages and is recognized by sera from some infected cattle. *Vet. Microbiol.* **76**:343–358.
- Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, et al. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**:537–544.
- Corner, L. A., M. A. Stevenson, D. M. Collins, and R. S. Morris. 2003. The re-emergence of *Mycobacterium bovis* infection in brushtail possums (*Trichosurus vulpecula*) after localised possum eradication. *N. Z. Vet. J.* **51**(2):73–80.
- Donnelly, C. A., R. Woodroffe, D. R. Cox, F. J. Bourne, C. L. Cheeseman, R. S. Clifton-Hadley, G. Wei, G. Gettinby, P. Gilks, H. Jenkins, W. T. Johnston, A. M. Le Fevre, J. P. McInerney, and W. I. Morrison. 2005. Positive and negative effects of widespread badger culling on tuberculosis in cattle. *Nature* **439**:843–846.
- European Economic Community. 1980. EEC directive 80/219, amending directive 64/432 annexe B. *Off. J. L047*:25–32.
- Greenwald, R., J. Esfandiari, S. Lesellier, R. Houghton, J. Pollock, C. Aagaard, P. Andersen, R. G. Hewinson, M. Chambers, and K. Lyashchenko. 2003. Improved serodetection of *Mycobacterium bovis* infection in badgers (*Meles meles*) using multiantigen test formats. *Diagn. Microbiol. Infect. Dis.* **46**:197–203.
- Koets, A. P., V. P. Rutten, M. de Boer, D. Bakker, P. Valentin-Weigand, and W. van Eden. 2001. Differential changes in heat shock protein-, lipaarabinomannan-, and purified protein derivative-specific immunoglobulin G1 and G2 isotype responses during bovine *Mycobacterium avium* subsp. *paratuberculosis* infection. *Infect. Immun.* **69**:1492–1498.
- Lightbody, K. A., R. A. Skuce, S. D. Neill, and J. M. Pollock. 1998. Mycobacterial antigen-specific antibody responses in bovine tuberculosis: an ELISA with potential to confirm disease status. *Vet. Rec.* **142**:295–300.
- Lyashchenko, K. P., M. Singh, R. Colangeli, and M. L. Gennaro. 2000. A multi-antigen print immunoassay for the development of serological diagnosis of infectious diseases. *J. Immunol. Methods* **242**:91–100.
- Neill S. D., J. Hanna, J. J. O'Brien, and R. M. McCracken. 1988. Excretion of *Mycobacterium bovis* by experimentally infected cattle. *Vet. Rec.* **123**:340–343.
- Palmer, M. V., D. L. Whipple, and S. C. Olsen. 1999. Development of a model of natural infection with *Mycobacterium bovis* in white-tailed deer. *J. Wildl. Dis.* **35**:450–457.
- Palmer, M. V., W. R. Waters, and D. L. Whipple. 2003. Aerosol delivery of virulent *Mycobacterium bovis* to cattle. *Tuberculosis (Edinburgh)* **82**:275–282.
- Palmer, M. V., W. R. Waters, T. C. Thacker, and K. P. Lyashchenko. 2006. Effects of different skin-testing regimens on gamma interferon and antibody responses in cattle experimentally infected with *Mycobacterium bovis*. *Clin. Vaccine Immunol.* **13**:387–394.
- Pollock, J. M., D. A. Pollock, D. G. Campbell, R. M. Girvin, A. D. Crookard, S. D. Neill, and D. P. Mackie. 1996. Dynamic changes in circulating and antigen-responsive T-cell subpopulations post-*Mycobacterium bovis* infection in cattle. *Immunology* **87**:236–241.
- Pollock, J. M., B. M. Buddle, and P. Andersen. 2001. Towards more accurate diagnosis of bovine tuberculosis using defined antigens. *Tuberculosis (Edinburgh)* **81**:65–69.
- Schmitt, S. M., S. D. Fitzgerald, T. M. Cooley, C. S. Bruning-Fann, L. Sullivan, D. Berry, T. Carlson, R. B. Minnis, J. B. Payeur, and J. Sikarskie. 1997. Bovine tuberculosis in free-ranging white-tailed deer from Michigan. *J. Wildl. Dis.* **33**:749–758.
- Schmitt, S. M., D. J. O'Brien, C. S. Bruning-Fann, and S. D. Fitzgerald. 2002. Bovine tuberculosis in Michigan wildlife and livestock. *Ann. N. Y. Acad. Sci.* **969**:262–268.
- U.S. Department of Agriculture. 1999. Bovine tuberculosis eradication uniform methods and rules, p. 1–34. U.S. Government Printing Office, Washington, D.C.
- Vordermeier, H. M., M. A. Chambers, P. J. Cogle, A. O. Whelan, J. Simmons, and R. G. Hewinson. 2002. Correlation of ESAT-6-specific gamma interferon production with pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine tuberculosis. *Infect. Immun.* **70**:3026–3032.
- Waters, W. R., J. M. Miller, M. V. Palmer, J. R. Stabel, D. E. Jones, K. A. Koistinen, E. M. Steadham, M. J. Hamilton, W. C. Davis, and J. P. Bannantine. 2003. Early induction of a humoral and cellular immune response during experimental *Mycobacterium avium* subsp. *paratuberculosis* infection of calves. *Infect. Immun.* **71**:5130–5138.
- Whipple, D. L., C. A. Bolin, and J. M. Miller. 1996. Distribution of lesions in cattle infected with *Mycobacterium bovis*. *J. Vet. Diagn. Investig.* **8**:351–354.