CLINICAL AND VACCINE IMMUNOLOGY, May 2009, p. 613–620 Vol. 16, No. 5
1556-6811/09/$08.00+0 doi:10.1128/CVI.00461-08

Rapid Mycobacterial Liquid Culture-Screening Method for *Mycobacterium avium* Complex Based on Secreted Antigen-Capture Enzyme-Linked Immunosorbent Assay▽

Sung Jae Shin,[1] Kelly Anklam,[2] Elizabeth J. B. Manning,[2] and Michael T. Collins[2]*

Department of Microbiology and Infection Signaling Network Research Center, College of Medicine, Chungnam National University, Daejeon 301-747, South Korea,[1] and Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, 2015 Linden Drive, Madison, Wisconsin 53706-1102[2]

Received 3 December 2008/Returned for modification 27 December 2008/Accepted 19 February 2009

Sensors in automated liquid culture systems for mycobacteria, such as MGIT, BacT/Alert 3D, and Trek ESP II, flag growth of any type of bacteria; a positive signal does not mean that the target mycobacteria are present. All signal-positive cultures thus require additional and often laborious testing. An immunoassay was developed to screen liquid mycobacterial cultures for evidence of *Mycobacterium avium* complex (MAC). The method, called the MAC–enzyme-linked immunosorbent assay (ELISA), relies on detection of MAC-specific secreted antigens in liquid culture. Secreted MAC antigens were captured by the MAC-ELISA with polyclonal anti-*Mycobacterium avium* subsp. *paratuberculosis* chicken immunoglobulin Y (IgY), detected using rabbit anti-MAC IgG, and then revealed using horseradish peroxidase-conjugated goat anti-rabbit IgG. When the MAC-ELISA was evaluated using pure cultures of known mycobacterial ($n = 75$) and nonmycobacterial ($n = 17$) organisms, no false-positive or false-negative MAC-ELISA results were found. By receiver operator characteristic (ROC) analysis of 1,275 previously identified clinical isolates, at the assay optimal cutoff the diagnostic sensitivity and specificity of the MAC-ELISA were 92.6% (95% confidence interval [95% CI], 90.3 to 94.5) and 99.9% (95% CI, 99.2 to 100), respectively, with an area under the ROC curve of 0.992. Prospective evaluation of the MAC-ELISA with an additional 652 clinical samples inoculated into MGIT ParaTB medium and signaling positive per the manufacturer's instructions found that the MAC-ELISA was effective in determining those cultures that actually contained MAC species and warranting the resources required to identify the organism by PCR. Of these 652 MGIT-positive cultures, the MAC-ELISA correctly identified 96.8% (of 219 MAC-ELISA-positive cultures) as truly containing MAC mycobacteria, based on PCR or high-performance liquid chromatography (HPLC) as reference tests. Only 6 of 433 MGIT signal-positive cultures (1.4%) were MAC-ELISA false negative, and only 7 of 219 MGIT signal-negative cultures (3.2%) were false positive. The MAC-ELISA is a low-cost, rapid, sensitive, and specific test for MAC in liquid cultures. It could be used in conjunction with or independent of automated culture reading instrumentation. For maximal accuracy and subspecies-specific identification, use of a confirmatory multiplex MAC PCR is recommended.

Members of the *Mycobacterium avium* complex (MAC) are a family of intracellular bacterial pathogens causing significant disease in both animals and humans. The complex contains four subspecies of *M. avium*: *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *hominissuis*, and *M. avium* subsp. *silvaticum* (24, 35). *Mycobacterium intracellulare* is also a member of the complex (20, 35).

The clinical importance of MAC infection has increased in recent decades because of the greater population of immunocompromised individuals with longer life expectancies, immunosuppressive chemotherapy, and the spread of human immunodeficiency virus infection (8, 20, 25, 27). With AIDS patients, the incidence of disseminated mycobacterial infection caused by MAC strains can reach up to 50% (19). Although these mycobacterial infections are not often characterized to subspecies, it appears that *M. avium* subsp. *hominissuis* is most often involved with AIDS patients (3, 4, 18, 24, 35). In addition, *M. avium* subsp. *hominissuis* causes infection in a subset of patients without an obvious immune defect (13) or underlying pulmonary disease and in children with lymphadenitis or cystic fibrosis (31). In virtually all cases, these organisms are believed to be of environmental origin: surface water, tap water, soil, dust, or food (22, 24, 29, 38). *M. avium* subsp. *avium*, ubiquitous in the environment and more virulent than *M. avium* subsp. *hominissuis*, is distinguished by the insertion element IS*901* (24). While capable of infecting multiple animal species, *M. avium* subsp. *avium* is commonly isolated from birds as one of the causes of avian tuberculosis (26, 32). *M. avium* subsp. *silvaticum*, also called the "wood pigeon bacillus," is uncommonly isolated but reported to cause enteritis in ruminants as well as disseminated infection in other hosts (33).

M. avium subsp. *paratuberculosis* infection causes paratuberculosis (Johne's disease) characterized by chronic granulomatous enteritis in animals, most often ruminants (9, 21). This organism grows very slowly in vitro (slower than most "slow-growing" mycobacteria), is dependent on mycobactin for growth in vitro, and is alone in containing IS*900* in its genome

* Corresponding author. Mailing address: 2015 Linden Drive, Madison, WI 53706-1102. Phone: (608) 262-8457. Fax: (608) 265-6463. E-mail: mcollin5@wisc.edu.

▽ Published ahead of print on 4 March 2009.

(15, 16, 23). *M. avium* subsp. *paratuberculosis* has a broad host range and is implicated by some in the pathogenesis of Crohn's disease in humans (1, 12). The inability of *M. avium* subsp. *paratuberculosis* to produce the siderophore mycobactin renders it incapable of replication in the environment, with the possible exception of inside free-living amoeba, and so it is considered an obligate parasite of animals and possibly humans (6). Paratuberculosis has emerged as a common and costly disease for the dairy industry (16). Surveys indicate that at least 68% of U.S. dairy herds are *M. avium* subsp. *paratuberculosis* infected (36).

Microbiological culture remains a mainstay for diagnosis of mycobacterial infections, since it has greater sensitivity than PCR-based methods and yields the living isolates necessary for antibiotic susceptibility testing and molecular epidemiology. Because culture on conventional solid bacteriological media is laborious and slow, liquid culture-based mycobacterial detection systems, such the Bactec, MGIT, Trek ESP, and BacT/Alert 3D systems, have become commonplace in clinical laboratories, offering the advantages of automation and shorter detection times from clinical samples (5, 7, 17, 37). However, a positive signal during culture with any of these systems is simply a nonspecific indication of any sort of microbial growth (37). Thus, specimen processing and decontamination protocols to selectively kill nonmycobacterial microflora in the clinical or environmental samples are key components for an effective assay (7, 34). Although a number of different protocols have been described (7, 11, 28, 34), a standard protocol specifically designed for optimal recovery of MAC has not yet been established.

Numerous PCRs are performed in our laboratory in response to these signal-positive cultures; in the last year, approximately 45% did not contain the pathogen of interest, MAC (unpublished data). This sample management approach is inefficient and labor-intensive.

To better focus PCR resources on those cultures most likely to contain MAC, a novel enzyme-linked immunosorbent assay (ELISA) was designed to detect secreted MAC antigens in culture medium fluid. This assay, called the MAC-ELISA, was then evaluated for analytical and diagnostic specificity and sensitivity, first using pure cultures and then cultures derived from clinical samples.

MATERIALS AND METHODS

Bacterial strains, cultures, and preparations of antigens. To develop a MAC antigen-capture ELISA with anti-MAC antibody as the solid phase, a number of organism cultures were prepared. MAC strains were selected to encompass the most clinically important *M. avium* subspecies, using both type strains and clinical strains. Antibodies were produced by immunization of rabbits (immunoglobulin G [IgG]) and chickens (IgY) with *M. avium* subsp. *paratuberculosis* and MAC culture filtrate antigens (CFA). All bacterial strains used for antibody production and tested in this study are listed in Table 1. Briefly, *M. avium* subsp. *paratuberculosis* ATCC 19698, *M. avium* subsp. *paratuberculosis* JTC114, and *M. avium* subsp. *paratuberculosis* JTC303 were cultivated in modified Watson-Reid (mWR) (pH 6.0) broth media supplemented with 2 μg/ml of mycobactin J (Allied Monitor, Fayette, MO). The static cultivation was performed by inoculating 100 μl of 10^9 CFU/ml seedlot culture into the cell culture flask (75 cm^2; canned neck; Corning Inc., NY) containing 50 ml of mWR broth medium for 10 weeks at 37°C in 5% CO_2 humidified conditions.

M. avium subsp. *paratuberculosis* CFA were harvested and pooled as previously described (30). *M. avium* subsp. *avium* strain ATCC 35712 and *M. avium* subsp. *hominissuis* strain 104 were cultured in mWR for 6 weeks at 37°C to obtain and pool MAC antigens.

Cellular extracts (CE) were used to remove by absorption cross-reactive antibodies from the rabbit anti-MAC IgG and chicken anti-MAC IgY as previously described (30). To prepare CE antigens (CEA), *Mycobacterium intracellulare* ATCC 13950, *M. intracellulare* ATCC 25122, and *Mycobacterium scrofulaceum* ATCC 19981 were cultivated in mWR broth for 4 weeks at 37°C. *Mycobacterium phlei* ATCC 11758 and *Mycobacterium terrae* ATCC 15755 strains were cultivated in mWR for 2 weeks at 37°C.

To evaluate antibody specificity, other non-MAC mycobacterial strains were cultured in 7H9 broth supplemented with 10% OADC (oleic acid-albumin-dextrose-catalase; Becton Dickinson, Sparks, MD) for 2 to 4 weeks at 37°C (Table 1). Nonmycobacterial strains were grown in Luria-Bertani (LB) broth.

For preparation of CEA from each bacterium grown in mWR, 7H9 or LB broth was prepared as previously described (30). The concentration of proteins in each CFA and CEA preparation was determined by a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

Antibody production. MAC (two strains) and *M. avium* subsp. *paratuberculosis* (three strains) antigen pools were made to immunize rabbits and chickens. Briefly, 250 μl of each antigen was pooled, adjusted to a final concentration of 1,000 μg/ml, and stored as 1-ml aliquots at −20° until use. After mixing 250 μl of each filtrate, each pool was adjusted to a final concentration of 1,000 μg/ml and stored as 1-ml aliquots at −20° until use. A total of four chickens and four rabbits were used for production of antibody, two each for anti-*M. avium* subsp. *paratuberculosis* and anti-MAC.

At each immunization, laying chickens were inoculated with 500 μl of CFA mixed with an equal volume of Freund's incomplete adjuvant (FIA). The first immunization was given subcutaneously. Subsequent immunizations were given intramuscularly, the first 2 weeks later and the remaining four at 1-week intervals. Eggs from each hen were collected daily after the second immunization, labeled, and stored at 4°C until use.

The IgY was precipitated from egg yolk by adding 1 volume of 40% polyethylene glycol 8000 (Sigma) in phosphate-buffered saline (PBS) to 3 volumes of egg yolk and then centrifuged at 13,000 × *g* for 20 min (2). The purified IgY was then dialyzed four times with 1 liter 10 mM PBS.

Immunization of rabbits for production of rabbit anti-*M. avium* subsp. *paratuberculosis* and anti-MAC antibody followed essentially the same protocol as that used for chickens, with slight modification. Briefly, each rabbit was intradermally inoculated with 500 μg/ml CFA pool in an equal volume of FIA. The subsequent three immunizations were done by subcutaneous inoculation of 250 μg/ml of the CFA pool in an equal volume of FIA at 2-week intervals. After the first and third immunizations, the serum antibody levels for each antigen were measured by an ELISA. After the fourth immunization, serum was harvested from each rabbit. Rabbit IgG purification was then performed using an ImmunoPure (G) IgG purification kit (Pierce), according to the manufacturer's instructions.

Both chicken IgY and rabbit IgG were pure, as evidenced by a single band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis comparable to those of the commercial antibody controls (data not shown). The yield was 4 to 5 mg/ml of IgY from a single egg and 10 ml of 2 to 3 mg/ml rabbit IgG by BCA assay.

Enhancement of antibody specificity. The specificity of rabbit anti-*M. avium* subsp. *paratuberculosis* IgG and anti-MAC IgG were enhanced by absorption by both *M. phlei* and *Escherichia coli* antigens; chicken anti-*M. avium* subsp. *paratuberculosis* IgY and anti-MAC IgY were enhanced by absorption with *M. phlei* antigens. Briefly, 100 μg of purified IgY was mixed with 10^7 CFU/ml of *M. phlei* ATCC 11758 and incubated at 4°C overnight. The mixture was then filtered using a 0.2-μm syringe filter (Nalgen). The filtered antibody was dialyzed in 10 mM PBS three times, and the final concentration of absorbed anti-*M. avium* subsp. *paratuberculosis* and anti-MAC IgY was determined using the BCA protein assay. As intact mycobacterial cells alone were not sufficient for removal of the cross-reactivity of rabbit anti-*M. avium* subsp. *paratuberculosis* and anti-MAC IgG with other bacteria, CEA of both *M. phlei* ATCC 11758 (500 μg/ml) and *E. coli* DH5α (200 μg/ml) were used to absorb cross-reactive rabbit antibodies. Only absorbed chicken IgY and rabbit IgG were employed in the final assay (referred to as "chicken anti-*M. avium* subsp. *paratuberculosis* IgY" and "chicken anti-MAC IgY" and "rabbit anti-*M. avium* subsp. *paratuberculosis* IgG" and "rabbit anti-MAC IgG").

Specificity of anti-MAC antibody preparations before and after absorption. Reactivity of chicken anti-MAC IgY and rabbit anti-MAC IgG were tested by an ELISA both before and after absorption, using multiple mycobacterial CFA and CEA. Briefly, 2 μg/ml of test antigen was coated on the wells of a 96-well plate (Maxisorp; Nalge Nunc International, Rochester, NY) by overnight incubation at 4°C. After the wells were washed three times with wash buffer (KPL, Gaithersburg, MD), they were blocked with 10% normal goat serum (Sigma, St. Louis, MO) at room temperature (RT) for 2 h. Either (i) 100 μl of 2 μg/ml absorbed

TABLE 1. Bacterial strains used to assess specificity of MAC-ELISA[c]

Species or organism	Total no. of strains	Reference strains included in total no. tested	Sources other than ATCC[a]	Detection time with MAC-ELISA (wk)[b]
Mycobacterium spp.				
M. avium subsp. *paratuberculosis*	13	ATCC 19698, K-10	JTC	3
M. avium subsp. *avium*	4	ATCC 35712, ATCC 25291	JTC	1 or 2
M. avium subsp. *hominissuis*	6	104	JTC, EPA, WSLH	1 or 2
M. intracellulare	9	ATCC 13950, ATCC 25122	JTC, EPA, WSLH	1
M. silvaticum	1	ATCC 49884		3
M. abscessus	1	ATCC 19977		N
M. asiaticum	4	ATCC 25276	JTC	N
M. bovis	3	ATCC 19210	JTC	N
M. celatum	4	ATCC 51130	JTC	N
M. flavescens	2	ATCC 14474	JTC	N
M. fortuitum	2	ATCC 49404	WSLH	N
M. gordonae	2	ATCC 14470	JTC	N
M. kansasii	3	ATCC 12478	JTC	N
M. lentiflavum	2	ATCC 51985	WSLH	N
M. malmoense	1	ATCC 29571		N
M. marinum	2	ATCC 927	WSLH	N
M. nonchromogenicum	1	ATCC 19530		
M. phlei	1	ATCC 11758		N
M. scrofulaceum	7	ATCC 19981	JTC	N
M. simiae	2	ATCC 25275	WSLH	N
M. smegmatis	2	ATCC 14468, mc^2155		N
M. terrae	3	ATCC 15755	JTC	N
Nonmycobacterial species				
Aeromonas hydrophila	1		WSLH	N
Corynebacterium pseudotuberculosis	1		JTC	N
Enterococcus faecalis	1	ATCC 29212	WSLH	N
Enterobacter aerogenes	1		WSLH	N
Escherichia coli	4	ATCC 25922	WSLH	N
Klebsiella pneumoniae	1		WSLH	N
Proteus vulgaris	1		WSLH	N
Pseudomonas aeruginosa	1		WSLH	N
Unidentified fungi	6		JTC	N
Total	92			

[a] Isolates were identified using mutiplex PCR and HPLC. JTC, Johne's Testing Center, Madison, WI; EPA, Environmental Protection Agency, Cincinnati, OH; WSLH, Wisconsin State Laboratory of Hygiene, Madison, WI.

[b] Detection time indicates the amount of time for CFA to be completely detected in the assay, when a starting inoculum of 10^2 CFU/ml of all strains was cultivated in 7H9 broth, up to 8 weeks. N, not detected.

[c] ATCC, American Type Culture Collection, Manassas, VA.

or nonabsorbed anti-MAC IgY or (ii) 100 μl of 1:4,000 diluted absorbed or nonabsorbed rabbit anti-MAC IgG was added to each well and then incubated at RT for 30 min while being shaken at 60 rpm. After the wells were washed five times with wash buffer, horseradish peroxidase (HRP)-conjugated rabbit anti-IgY (GenTel) at a dilution of 1:4,000 or HRP-conjugated sheep anti-rabbit IgG (Vector) at a dilution of 1:5,000 was added to each well and incubated for 30 min at RT. Plates were washed five times with wash buffer (KPL), after which 100 μl of TMB substrate (TMBE-500; Moss Inc., Pasadena, MD) was added to each well, and then they were incubated for 1 min at RT. The reaction was then stopped by addition of 100 μl stop solution (KPL). The optical density (OD) of the final reaction in each well was measured at 450 nm using an ELISA reader (μQuant; Bio-Tek Instruments Inc., Winooski, VT).

Development of optimal MAC-ELISA protocol. Critical reagents in the MAC-ELISA are (i) the solid phase capture antibody, chicken anti-*M. avium* subsp. *paratuberculosis* IgY; (ii) the test substance, mycobacterial broth culture fluid potentially containing secreted MAC antigens; (iii) the detector antibody, rabbit anti-MAC IgG; and (iv) the conjugate, HRP-conjugated goat anti-rabbit IgG (Vector) (see Fig. 2). The concentrations and volumes of all critical antibody components were optimized for analytical sensitivity and specificity by reagent titration individually and in various combinations with culture fluid from pure cultures of *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. intracellulare*, *M. scrofulaceum*, *M. phlei*, *M. terrae*, and *Corynebacterium pseudotuberculosis* (data not shown). The final MAC-ELISA protocol was as follows. Plates (96 well, Maxisorp; Nalge Nunc International) were first coated with 10 μg of capture antibody, chicken anti-MAC IgY, and diluted in coating buffer (KPL) by overnight incubation at 4°C. After the wells were washed three times with wash buffer (KPL), all wells were blocked with 10% normal goat serum (Sigma) at RT for 2 h. Medium (100 μl) from the liquid cultures to be tested was next added to each well. After 1 h at RT with shaking (60 rpm), the plate was again washed three times with wash buffer. The detector antibody, rabbit anti-MAC IgG (100 μl of 0.5 μg/ml) was added to each well and incubated 30 min at RT. Wells were again washed three times with washing buffer (KPL). Then, 100 μl of HRP-conjugated goat anti-rabbit IgG (Vector) at a dilution of 1:5,000 was added to all wells and incubated for 30 min at RT. Plates then were washed five times with wash buffer (KPL). Last, 100 μl TMB substrate (TMBE-500; Moss Inc.) was added to each well and was followed by a 1-min RT incubation, after which the reaction was stopped by adding 100 μl of stop solution (KPL) to each well. The OD of the final reaction in each well was measured at 450 nm using an ELISA reader (μQuant; Bio-Tek Instruments, Inc.). On each plate there were two positive controls in duplicate (*M. avium* subsp. *paratuberculosis* and MAC culture fluid) and three negative controls (PBS, MGIT medium, and *M. phlei* culture fluid). The cutoff value for a positive assay is determined as two times the mean OD of the three negative-control wells.

Analytical sensitivity and specificity of the MAC-ELISA. CF from pure liquid cultures of both mycobacterial and nonmycobacterial strains were tested. To estimate MAC-ELISA analytical sensitivity, twofold serial dilutions of CF (16.0 to 0.0078 μg/ml) from each type strain were tested. Assay results were compared with positive controls (culture fluid from *M. avium* subsp. *paratuberculosis* ATCC

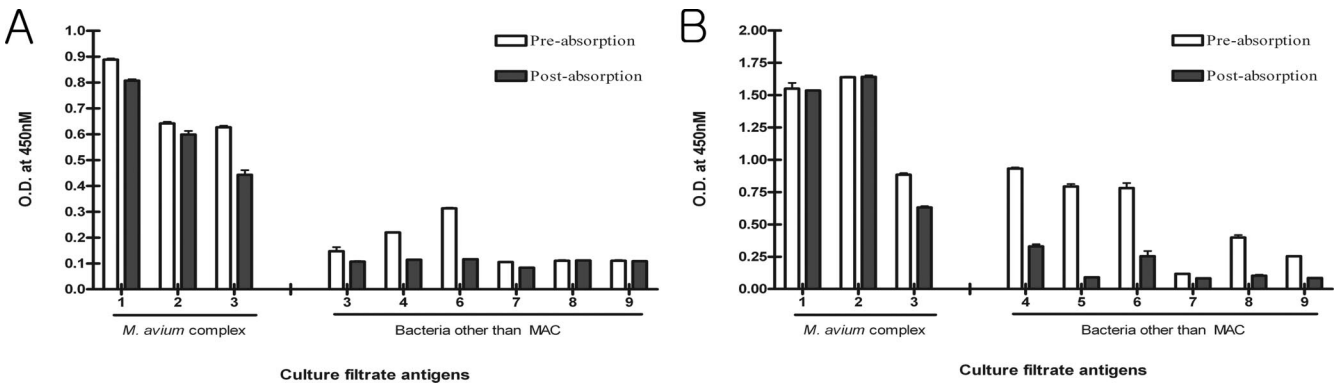

FIG. 1. Comparison of single antibody cross-reactivity pre- and postabsorption. Chicken anti-MAC IgY (A) and rabbit anti-MAC IgG (B). Lanes for both panels: 1, *Mycobacterium avium* subsp. *paratuberculosis* ATCC 19968; 2, *Mycobacterium avium* subsp. *avium* ATCC 35712; 3, *Mycobacterium intracellulare* ATCC 25122; 4, *Mycobacterium phlei* ATCC 11758; 5, *Mycobacterium terrae* ATCC 15755; 6, *Mycobacterium scrofulaceum* ATCC 19981; 7, *Corynebacterium pseudotuberculosis* clinical isolate; 8, *Escherichia coli* ATCC 25922; 9, a mixture of environmental bacteria, including *Aeromonas hydrophila*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Proteus vulgaris*.

19698 and *M. avium* subsp. *avium* ATCC 35712) and negative controls (culture fluid from *M. phlei* and *M. terrae*). To determine MAC-ELISA analytical specificity and optimal timing for testing, culture fluid from 7H9 broth cultures of mycobacteria was collected weekly, up to 8 weeks (Table 1). Briefly, 10^2 CFU of each mycobacterial strain was inoculated into 10 ml of Middlebrook 7H9 broth (Difco, Sparks, MD) supplemented with 0.5% glycerol and 10% OADC (Middlebrook), and incubated at 37°C for 8 weeks. For *M. avium* subsp. *paratuberculosis* strains, 2 μg/ml of mycobactin J (Allied Monitor, Fayette, MO) was also added to the culture medium for optimal growth. Nonmycobacterial strains were grown in LB medium (Table 1). *Corynebacterium pseudotuberculosis* was grown in brain heart infusion broth. Culture fluid from all strains, along with positive and negative controls, was tested weekly by MAC-ELISA, as described above.

Assessment of MAC-ELISA versus MGIT960 ParaTB culture system for pure cultures. Duplicate tubes of MGIT ParaTB medium (Becton Dickinson) were inoculated with serial dilutions of *M. avium* subsp. *paratuberculosis* ATCC 19698, *M. avium* subsp. *avium* ATCC 35712, *M. phlei* ATCC 11758, or *M. terrae* ATCC 15755. Briefly, undiluted stock cell suspension (1.0 ml) was added to 9.0 ml of 10 mM PBS (pH 7.2), and 10-fold serial dilutions were made in 10 mM PBS (pH 7.2), with vortexing between each dilution step resulting in 10^0 to 10^7 CFU/ml of each of the four mycobacterial strains. From each dilution, 100 μl was inoculated into MGIT ParaTB medium (Becton Dickinson, Sparks, MD). Each MGIT tube contained 7 ml of modified Middlebrook 7H9 broth base, with mycobactin J and fluorescent indicator measuring changes in oxygen concentration embedded in silicone on the bottom of the tube. Per the manufacturer's instructions, each tube was supplemented with 800 μl MGIT ParaTB supplement (BD), 500 μl egg yolk (BD), and 100 μl VAN antibiotic cocktail, resulting in final concentrations of 10 μg/ml vancomycin, 40 μg/ml amphotericin B, and 60 μg/ml nalidixic acid. All MGIT ParaTB medium tubes were incubated at 37°C in a MGIT 960 instrument. Tubes were removed when the machine signaled them positive, based on changes in the indicator. For each MGIT-positive tube, culture fluid (100 μl) was then tested by a MAC-ELISA, with results analyzed in relationship to the time to detection (incubation time in days until signal positive) for each culture.

Preliminary assessment of the MAC-ELISA using well-defined clinical cultures. A total of 1,275 animal fecal, tissue, water, and soil samples yielding acid-fast stain-positive organisms were tested using the MAC-ELISA. This set was obtained from 684 clinical cultures in modified Bactec 12B medium and 591 clinical cultures in MGIT ParaTB medium. Once a liquid culture instrument signaled positive, acid-fast staining was done on the cultures and contamination was checked by inoculation into 5% sheep blood agar plates. Final identification of mycobacterial isolates was done using as a reference method a multiplex PCR simultaneously targeting mycobacterial 16S DNA and four insertion elements, IS*900*, IS*901*, IS*1311*, and IS*1245* (Johne's Testing Center, Madison, WI), with reference strains as controls. Ultimately these 1,275 clinical samples yielded 340 MAC and 344 non-MAC mycobacteria from modified Bactec 12B medium and 305 MAC and 286 mycobacteria other than MAC from MGIT ParaTB medium. The optimal cutoff, sensitivity, and specificity of the MAC-ELISA were determined by receiver operator characteristic (ROC) curve analysis.

Validation of the MAC-ELISA to triage MGIT signal-positive cultures. Prospectively, 652 consecutive clinical samples (animal feces, tissues, water, or soil) were processed for *M. avium* subsp. *paratuberculosis* isolation according to the manufacturer's recommendations using the MGIT ParaTB medium. The first time the MGIT 960 instrument signaled a tube "positive," it was removed from the instrument, vortexed, and reinserted in the machine. After the tube signaled positive a second time (or if it signaled positive within 1 week of the 49-day incubation protocol), the MAC-ELISA was performed. For MAC-ELISA-negative cultures, acid-fast staining (Ziehl-Neelsen) on culture fluid smears independently assessed the presence of mycobacteria. The multiplex PCR was used to verify the identity of mycobacteria in all acid-fast stain-positive and MAC-ELISA-positive MGIT cultures. In any cases of discrepancy between MAC-ELISA and multiplex PCR results, two assays were used to clarify the true identity of mycobacterial isolates: IS*900* nested PCR for *M. avium* subsp. *paratuberculosis* (greater analytical sensitivity than the multiplex) and HPLC of cell wall mycolic acids for all other mycobacteria (Wisconsin State Laboratory of Hygiene, Madison, WI) (14).

Statistical analysis. Specificity and sensitivity were evaluated by ROC curves. MAC-ELISA OD values before and after antibody absorption were compared by the *t* test. Differences in OD values between MAC cultures and cultures with mycobacteria other than MAC were compared by the Mann-Whitney test. Statistical analyses were done using statistical software (GraphPad Prism version 4.03 for Windows; GraphPad Software, San Diego CA).

RESULTS

Anti-MAC antibody specificity. Prior to absorption with heterologous antigens, both chicken anti-*M. avium* subsp. *paratuberculosis* IgY and anti-MAC IgY showed cross-reactivity to other mycobacteria, such as *M. scrofulaceum*, *M. phlei*, and *M. terrae*. After absorption with *M. phlei* cells, the cross-reactivity to those mycobacteria disappeared without significant decrease in reactivity (ELISA OD) to target *M. avium* subsp. *paratuberculosis* and MAC mycobacteria (Fig. 1A). Rabbit anti-MAC IgG and anti-*M. avium* subsp. *paratuberculosis* IgG both cross-reacted with nonmycobacteria as well as all mycobacteria tested. After absorption with the CEA from *M. phlei* and *E. coli*, however, this cross-reactivity decreased significantly without appreciable change in reactivity to secreted antigens of *M. avium* subsp. *paratuberculosis* or MAC (Fig. 1B). The absorbed chicken and rabbit anti-MAC and anti-*M. avium* subsp. *paratuberculosis* retained strong reactivity to both MAC and *M.*

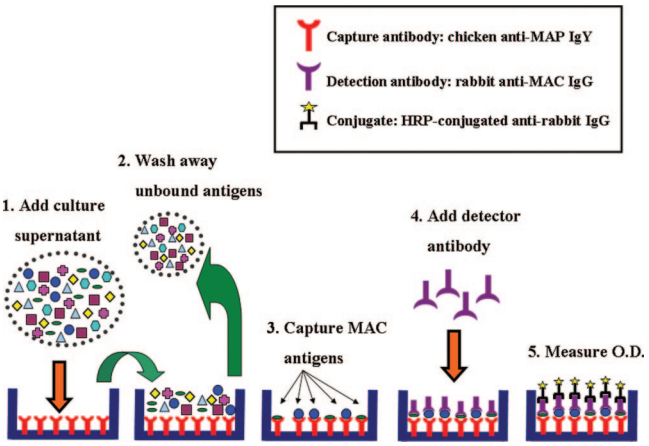

FIG. 2. Schematic diagram of the MAC-ELISA procedure. Key reagents for each step of the MAC-ELISA are indicated in the figure key. MAP, *Mycobacterium avium* subsp. *paratuberculosis*; O.D., optical density.

avium subsp. *paratuberculosis* and moderate reactivity to *M. intracellulare* and could not distinguish among these members of the MAC.

Development of MAC-ELISA. Numerous combinations and concentrations of chicken and rabbit anti-*M. avium* subsp. *paratuberculosis* and anti-MAC were tested during development of the MAC-ELISA. The combination providing optimal sensitivity and specificity for detection of secreted MAC antigens in liquid cultures required use of chicken anti-*M. avium* subsp. *paratuberculosis* IgY for antigen capture and rabbit anti-MAC IgG for captured-antigen detection, together with a suitable commercial conjugate to detect rabbit antibody binding (data not shown) (Fig. 2). Although the antibodies were produced using selected subspecies of the MAC, they did not discriminate among MAC subspecies nor between *M. avium* and *M. intracellularae*. The final assay is thus complex specific, but not species or subspecies specific, and therefore is referred to as the MAC-ELISA.

MAC-ELISA specificity and sensitivity for pure cultures. Culture fluid obtained weekly from 92 mycobacterial and nonmycobacterial strains were tested. After 8 weeks of incubation, no mycobacteria outside the MAC triggered a positive MAC-ELISA (Table 1). All MAC members (13 *M. avium* subsp. *paratuberculosis*, 4 *M. avium* subsp. *avium*, 6 *M. avium* subsp. *hominissuis*, 1 *M. avium* subsp. *silvaticum*, and 9 *M. intracellularae* strains) became MAC-ELISA positive between 1 and 4 weeks of incubation in Middlebrook 7H9, when the starting inoculum was 10^2 CFU. The specificity and sensitivity of the MAC-ELISA were enhanced by use of absorbed antibodies (Fig. 3). Assay accuracy using anti-*M. avium* subsp. *paratuberculosis* IgY for antigen capture and anti-MAC IgG for antigen detection was superior to all other antibody combinations (data not shown). The MAC-ELISA analytical sensitivity was 0.03125 μg/ml *M. avium* subsp. *paratuberculosis* CFA (Fig. 3B) and 0.0625 μg/ml *M. avium* subsp. *avium* CFA (Fig. 3C) when two times the negative-control OD (*M. phlei* culture fluid) was used as the cutoff for a positive test.

Optimal incubation time for detection and detection limit. Time to detection as reported by the MGIT 960 instrument

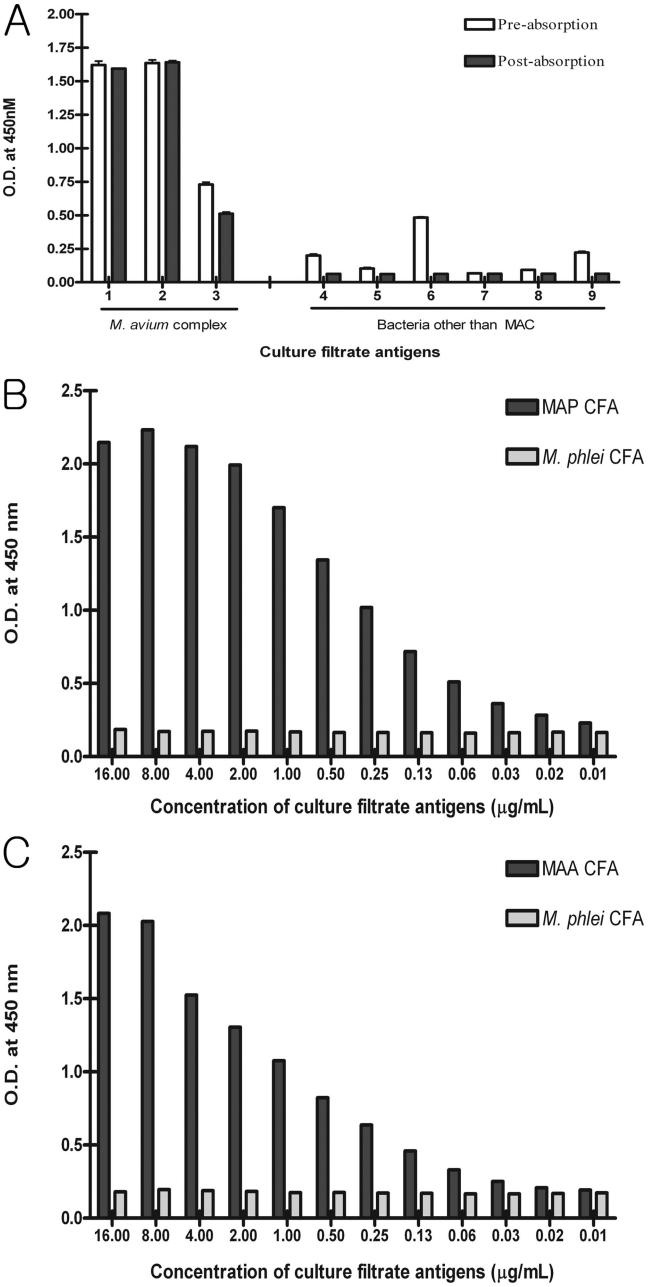

FIG. 3. Analytical sensitivity of the MAC-ELISA. (A) Enhanced specificity and sensitivity of the MAC-ELISA by absorption of chicken anti-*M. avium* subsp. *paratuberculosis* IgY capture antibody and rabbit anti-MAC IgG detector antibody. Lanes: 1, *Mycobacterium avium* subsp. *paratuberculosis* ATCC 19968; 2, *Mycobacterium avium* subsp. *avium* ATCC 35712; 3, *Mycobacterium intracellulare* ATCC 25122; 4, *Mycobacterium phlei* ATCC 11758; 5, *Mycobacterium terrae* ATCC 15755; 6, *Mycobacterium scrofulaceum* ATCC 19981; 7, *Corynebacterium pseudotuberculosis* clinical isolate; 8, *Escherichia coli* ATCC 25922; 9, a mixture of environmental bacteria, including *Aeromonas hydrophila*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Proteus vulgaris*. (B) Analytical detection limit of the MAC-ELISA using purified *M. avium* subsp. *paratuberculosis* (MAP) CFA. (C) Analytical detection limit of the MAC-ELISA using purified *M. avium* subsp. *avium* (MAA) CFA.

TABLE 2. Comparison of time to positive culture between MAC-ELISA and MGIT cultures

Inoculum CFU/ml	Time to positive culture (days)[c]					
	M. avium subsp. *paratuberculosis* JTC303		*M. avium* subsp. *avium* ATCC 35712		*M. phlei* ATCC 11758	
	MGIT[a]	MAC-ELISA[b]	MGIT	MAC-ELISA	MGIT	MAC-ELISA
10^6–10^7	4.8	7	3.5	7	0.7	ND
10^5–10^6	7.2	7	5.3	7	2.6	ND
10^4–10^5	10.1	14	6.9	7	4.3	ND
10^3–10^4	12.8	21	8.7	14	6.0	ND
10^2–10^3	17.1	21	10.6	14	10.4	ND
10^1–10^2	21.5	28	12.7	21	14.8	ND
10^0–10^1	39.1	35	15.8	21	ND	ND
10^{-1}–10^0	ND	ND	ND	ND	ND	ND

[a] The MGIT 960 instrument measures fluorescence as an indication of microbial growth hourly.
[b] Culture fluid was tested by the MAC-ELISA weekly.
[c] ND, not detectable by the MAC-ELISA up to 56 days of incubation.

and incubation time to positive MAC-ELISA were similar, given that the MGIT instrument read cultures hourly and culture fluid was tested by the MAC-ELISA only weekly. The MAC-ELISA detection limit for *M. avium* subsp. *paratuberculosis* and MAC was 10^1 CFU/ml. Culture fluid from *M. phlei* never triggered a positive MAC-ELISA (Table 2).

ROC analysis of the MAC-ELISA using well-defined clinical cultures. A significant difference in MAC-ELISA OD values was observed between clinical cultures containing MAC and non-MAC mycobacteria ($P < 0.0001$) (Fig. 4A). The cutoff value for maximum assay accuracy was determined by ROC curve analysis. The assay sensitivity and specificity were 92.6% (95% confidence interval [95% CI], 90.3 to 94.5) and 99.9% (95% CI, 99.2 to 100), respectively, with an area under the ROC curve (AUC) of 0.992 (Fig. 4B).

Clinical application of MAC-ELISA. The MGIT 960 instrument signaled growth in 652 clinical cultures; MAC-ELISA indicated that MAC species were present in 219 (33.6%) of them. Among these 219 cultures, 212 were confirmed as containing MAC organisms (96.8% [210 *M. avium* subsp. *paratuberculosis* and 2 MAC]). The other seven were found to contain mycobacteria other than MAC, for a false-positive MAC-ELISA rate of 3.2% (7/219) (Fig. 5).

The remaining 433 MGIT-positive cultures were MAC-ELISA negative (66.4%). Of these, 426 (98.4%) did not contain acid-fast bacteria, suggesting a high rate of false-positive signals by the MGIT system. Seven of the 433 MGIT-positive but MAC-ELISA-negative cultures (1.6%) had acid-fast bacteria identified as *M. avium* subsp. *paratuberculosis* ($n = 6$) or non-MAC mycobacteria ($n = 1$), resulting in a false-negative rate of 6/433 (1.4%) (Fig. 5). More than 500 MGIT signal-negative cultures as well as uninoculated culture medium were also MAC-ELISA negative (data not shown).

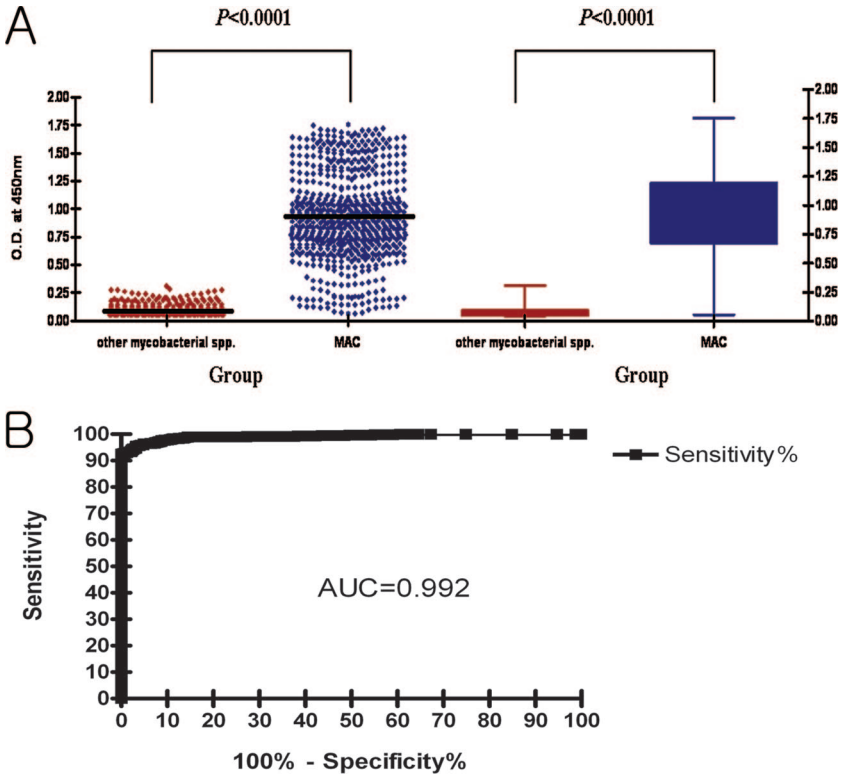

FIG. 4. Preliminary assessment of the MAC-ELISA using 1,275 well-defined clinical cultures. (A) Scatter plot (left) of MAC-ELISA OD values for MAC and mycobacteria other than MAC. Each spot represents the MAC-ELISA OD value for a single culture, and the horizontal bar represents the mean OD of the group. Bar and whisker plot (right) of MAC-ELISA OD values. The boxes represent ±standard errors of the means, and the error bars represent 95% CIs. (B) ROC analysis of the scatter plot data. AUC, area under the curve.

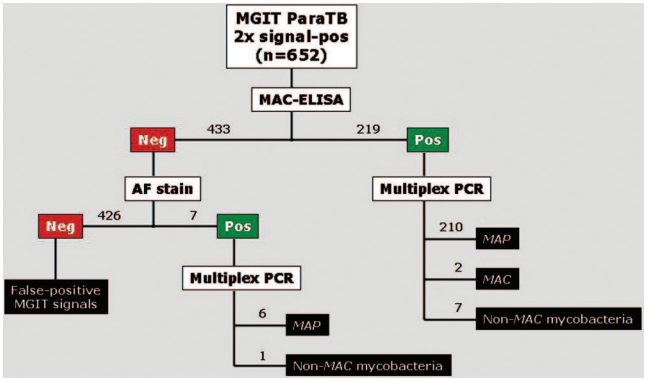

FIG. 5. Clinical application of the MAC-ELISA in conjunction with the MGIT ParaTB medium and MGIT 960 instrument. Neg, negative; Pos, positive; AF, acid-fast; *MAP*, *Mycobacterium avium* subsp. *paratuberculosis*; 2x signal-pos, second time tube signaled positive (see Materials and Methods).

DISCUSSION

Antibodies produced by immunization of chickens or rabbits with MAC or *M. avium* subsp. *paratuberculosis* CFs yielded high titers of purified anti-mycobacterial egg yolk IgY or serum IgG, respectively. The specificity of these antibody preparations for MAC mycobacteria was significantly enhanced by absorption with antigens from heterologous organisms: *M. phlei* for the chicken IgY and both *M. phlei* and *E. coli* for rabbit IgG. These antibodies proved useful reagents in the design of a highly specific assay for MAC antigens secreted during culture in liquid media: Middlebrook 7H9, WR, modified Bactec 12B, or MGIT ParaTB medium.

Identification of MAC at the subspecies level would be clinically relevant because of differences in epidemiology and pathogenicity (6, 35). However, members of the MAC have few reliable phenotypic characteristics that allow identification by conventional methods. Even the most common reference method for mycobacterial identification, HPLC chromatograms of mycolic acid cell wall extracts, fails to reliably distinguish among MAC subspecies (10, 35).

Automated liquid culture systems, such as Bactec 460, MGIT 960, Trek ESP II, and BacT/ALERT, in combination with specific specimen-processing protocols, are designed to triage clinical cultures, that is, distinguish cultures that contain microorganisms of interest from those that do not. The goal of such systems is to help clinical laboratories focus organism identification resources on those cultures with the highest probability of containing clinically relevant mycobacterial pathogens.

The MAC-ELISA is an effective tool for selecting MGIT ParaTB medium cultures for additional testing by PCR; 96.8% of 219 cultures so selected contained MAC mycobacteria, while only 6 of 652 MGIT-positive cultures (0.9%) were MAC-ELISA false negative (Fig. 5). With the panel of clinical samples evaluated, use of the MAC-ELISA to screen MGIT-positive cultures before application of PCR correctly avoided unnecessary PCR testing of 64.3% (419) of MGIT signal-positive cultures. Acid-fast staining of cultures is also effective at culture screening but is more labor-intensive, requires technical skill, and is not objective. A limitation of the study was that clinical samples were from animals being assessed for Johne's disease and thus strongly biased toward recovery of *M. avium* subsp. *paratuberculosis*. Further evaluation in a human clinical mycobacteriology laboratory setting is necessary. The MAC-ELISA may provide a low-cost, rapid, objective, sensitive, and specific test for MAC in signal-positive cultures in automated mycobacterial detection systems.

ACKNOWLEDGMENTS

This work was funded by the Johne's Testing Center, School of Veterinary Medicine, University of Wisconsin—Madison, by a grant of the Korea Science and Engineering Foundation (KOSEF) through the Infection Signaling Network Research Center (R13-2007-020-01000-0) at Chungnam National University and by the Korea Science and Engineering Foundation (KOSEF) through MOST grant R01-2007-000-10702-0.

REFERENCES

1. **Abubakar, I., D. Myhill, S. H. Aliyu, and P. R. Hunter.** 2008. Detection of *Mycobacterium avium* subspecies *paratuberculosis* from patients with Crohn's disease using nucleic acid-based techniques: a systematic review and meta-analysis. Inflamm. Bowel Dis. **14:**401–410.
2. **Akita, E. M., and S. Nakai.** 1993. Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic *E. coli* strain. J. Immunol. Methods **160:**207–214.
3. **Aronson, T., A. Holtzman, N. Glover, M. Boian, S. Froman, O. G. Berlin, H. Hill, and G. Stelma, Jr.** 1999. Comparison of large restriction fragments of *Mycobacterium avium* isolates recovered from AIDS and non-AIDS patients with those of isolates from potable water. J. Clin. Microbiol. **37:**1008–1012.
4. **Ashford, D. A., E. Whitney, P. Raghunathan, and O. Cosivi.** 2001. Epidemiology of selected mycobacteria that infect humans and other animals. Rev. Sci. Tech. **20:**325–337.
5. **Badak, F. Z., D. L. Kiska, M. O'Connell, C. M. Nycz, C. Hartley, S. Setterquist, and R. L. Hopfer.** 1997. Confirmation of the presence of *Mycobacterium tuberculosis* and other mycobacteria in mycobacterial growth indicator tubes (MGIT) by multiplex strand displacement amplification. J. Clin. Microbiol. **35:**1239–1243.
6. **Biet, F., M. L. Boschiroli, M. F. Thorel, and L. A. Guilloteau.** 2005. Zoonotic aspects of *Mycobacterium bovis* and *Mycobacterium avium-intracellulare* complex (MAC). Vet. Res. **36:**411–436.
7. **Carricajo, A., N. Fonsale, A. C. Vautrin, and G. Aubert.** 2001. Evaluation of BacT/Alert 3D liquid culture system for recovery of mycobacteria from clinical specimens using sodium dodecyl (lauryl) sulfate-NaOH decontamination. J. Clin. Microbiol. **39:**3799–3800.
8. **Chaisson, R. E., R. D. Moore, D. D. Richman, J. Keruly, T. Creagh, et al.** 1992. Incidence and natural history of *Mycobacterium avium*-complex infections in patients with advanced human immunodeficiency virus disease treated with zidovudine. Am. Rev. Respir. Dis. **146:**285–289.
9. **Cocito, C., P. Gilot, M. Coene, M. de Kesel, P. Poupart, and P. Vannuffel.** 1994. Paratuberculosis. Clin. Microbiol. Rev. **7:**328–345.
10. **Dei, R., E. Tortoli, A. Bartoloni, M. T. Simonetti, and E. Lillini.** 1999. HPLC does not differentiate *Mycobacterium paratuberculosis* from *Mycobacterium avium*. Vet. Microbiol. **65:**209–213.
11. **Dundee, L., I. R. Grant, H. J. Ball, and M. T. Rowe.** 2001. Comparative evaluation of four decontamination protocols for the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from milk. Lett. Appl. Microbiol. **33:** 173–177.
12. **Feller, M., K. Huwiler, R. Stephan, E. Altpeter, A. Shang, H. Furrer, G. E. Pfyffer, T. Jemmi, A. Baumgartner, and M. Egger.** 2007. *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's disease: a systematic review and meta-analysis. Lancet Infect. Dis. **7:**607–613.
13. **Field, S. K., D. Fisher, and R. L. Cowie.** 2004. *Mycobacterium avium* complex pulmonary disease in patients without HIV infection. Chest **126:**566–581.
14. **Glickman, S. E., J. O. Kilburn, W. R. Butler, and L. S. Ramos.** 1994. Rapid identification of mycolic acid patterns of mycobacteria by high-performance liquid chromatography using pattern recognition software and a *Mycobacterium* library. J. Clin. Microbiol. **32:**740–745.
15. **Green, E. P., M. L. Tizard, M. T. Moss, J. Thompson, D. J. Winterbourne, J. J. McFadden, and J. Hermon-Taylor.** 1989. Sequence and characteristics of IS*900*, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucleic Acids Res. **17:**9063–9073.
16. **Harris, N. B., and R. G. Barletta.** 2001. *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. Clin. Microbiol. Rev. **14:**489–512.
17. **Hillemann, D., E. Richter, and S. Rusch-Gerdes.** 2006. Use of the BACTEC Mycobacteria Growth Indicator Tube 960 automated system for recovery of mycobacteria from 9,558 extrapulmonary specimens, including urine samples. J. Clin. Microbiol. **44:**4014–4017.

18. **Hoffner, S. E., G. Kallenius, B. Petrini, P. J. Brennan, and A. Y. Tsang.** 1990. Serovars of *Mycobacterium avium* complex isolated from patients in Sweden. J. Clin. Microbiol. **28:**1105–1107.
19. **Horsburgh, C. R., J. Gettings, Jr., L. N. Alexander, and J. L. Lennox.** 2001. Disseminated *Mycobacterium avium* complex disease among patients infected with human immunodeficiency virus, 1985–2000. Clin. Infect. Dis. **33:**1938–1943.
20. **Inderlied, C. B., C. A. Kemper, and L. E. Bermudez.** 1993. The *Mycobacterium avium* complex. Clin. Microbiol. Rev. **6:**266–310.
21. **Manning, E. J., and M. T. Collins.** 2001. *Mycobacterium avium* subsp. *paratuberculosis*: pathogen, pathogenesis and diagnosis. Rev. Sci. Tech. **20:**133–150.
22. **Matlova, L., L. Dvorska, W. Y. Ayele, M. Bartos, T. Amemori, and I. Pavlik.** 2005. Distribution of *Mycobacterium avium* complex isolates in tissue samples of pigs fed peat naturally contaminated with mycobacteria as a supplement. J. Clin. Microbiol. **43:**1261–1268.
23. **Merkal, R. S., and B. J. Curran.** 1974. Growth and metabolic characteristics of *Mycobacterium paratuberculosis*. Appl. Microbiol. **28:**276–279.
24. **Mijs, W., P. de Haas, R. Rossau, T. Van der Laan, L. Rigouts, F. Portaels, and D. van Soolingen.** 2002. Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. avium for bird-type isolates and '*M. avium subsp. hominissuis*' for the human/porcine type of *M. avium*. Int. J. Syst. Evol. Microbiol. **52:**1505–1518.
25. **Nightingale, S. D., L. T. Byrd, P. M. Southern, J. D. Jockusch, S. X. Cal, and B. A. Wynne.** 1992. Incidence of *Mycobacterium avium-intracellulare* complex bacteremia in human immunodeficiency virus-positive patients. J. Infect. Dis. **165:**1082–1085.
26. **Pavlik, I., P. Svastova, J. Bartl, L. Dvorska, and I. Rychlik.** 2000. Relationship between IS*901* in the *Mycobacterium avium* complex strains isolated from birds, animals, humans, and the environment and virulence for poultry. Clin. Diagn. Lab. Immunol. **7:**212–217.
27. **Primm, T. P., C. A. Lucero, and J. O. Falkinham III.** 2004. Health impacts of environmental mycobacteria. Clin. Microbiol. Rev. **17:**98–106.
28. **Reddacliff, L. A., A. Vadali, and R. J. Whittington.** 2003. The effect of decontamination protocols on the numbers of sheep strain *Mycobacterium avium* subsp. *paratuberculosis* isolated from tissues and faeces. Vet. Microbiol. **95:**271–282.
29. **Ristola, M. A., C. F. von Reyn, R. D. Arbeit, H. Soini, J. Lumio, A. Ranki, S. Buhler, R. Waddell, A. N. Tosteson, J. O. Falkinham III, and C. H. Sox.** 1999. High rates of disseminated infection due to non-tuberculous mycobacteria among AIDS patients in Finland. J. Infect. **39:**61–67.
30. **Shin, S. J., D. Cho, and M. T. Collins.** 2008. Diagnosis of bovine paratuberculosis by a novel enzyme-linked immunosorbent assay based on early secreted antigens of *Mycobacterium avium* subsp. *paratuberculosis.* Clin. Vaccine Immunol. **15:**1277–1281.
31. **Tateishi, Y., Y. Hirayama, Y. Ozeki, Y. Nishiuchi, M. Yoshimura, J. Kang, A. Shibata, K. Hirata, S. Kitada, R. Maekura, H. Ogura, K. Kobayashi, and S. Matsumoto.** 1 November 2008. Virulence of *Mycobacterium avium* complex strains isolated from immunocompetent patients. Microb. Pathog. doi: 10.1016/j.micpath.2008.10.007.
32. **Thegerstrom, J., B. I. Marklund, S. Hoffner, D. Axelsson-Olsson, J. Kauppinen, and B. Olsen.** 2005. *Mycobacterium avium* with the bird type IS*1245* RFLP profile is commonly found in wild and domestic animals, but rarely in humans. Scand. J. Infect. Dis. **37:**15–20.
33. **Thorel, M. F., M. Krichevsky, and V. V. Levy-Frebault.** 1990. Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. Int. J. Syst. Bacteriol. **40:**254–260.
34. **Thornton, C. G., K. M. MacLellan, J. R. Stabel, C. Carothers, R. H. Whitlock, and S. Passen.** 2002. Application of the C_{18}-carboxypropylbetaine specimen processing method to recovery of *Mycobacterium avium* subsp. *paratuberculosis* from ruminant tissue specimens. J. Clin. Microbiol. **40:**1783–1790.
35. **Turenne, C. Y., R. Wallace, Jr., and M. A. Behr.** 2007. *Mycobacterium avium* in the postgenomic era. Clin. Microbiol. Rev. **20:**205–229.
36. **U.S. Department of Agriculture.** 2008. Johne's disease on U.S. dairies, 1991–2007. USDA-APHIS Info. Sheet N521.0408. APHIS, VS, CEAH, U.S. Department of Agriculture, Fort Collins, CO.
37. **Williams-Bouyer, N., R. Yorke, H. I. Lee, and G. L. Woods.** 2000. Comparison of the BACTEC MGIT 960 and ESP culture system II for growth and detection of mycobacteria. J. Clin. Microbiol. **38:**4167–4170.
38. **Yoder, S., C. Argueta, A. Holtzman, T. Aronson, O. G. Berlin, P. Tomasek, N. Glover, S. Froman, and G. Stelma, Jr.** 1999. PCR comparison of *Mycobacterium avium* isolates obtained from patients and foods. Appl. Environ. Microbiol. **65:**2650–2653.