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

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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 IS901 (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 IS900 in its genome...
(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 as 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 *M. avium* 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, *M. avium* (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* ATCC 17114, and *M. avium* subsp. *paratuberculosis* ATCC 27303 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 10^8 CFU/ml seedlot culture into the cell culture flask (75 cm²; canned neck; Corning Inc., NY) containing 50 ml of mWR broth medium for 10 weeks at 37°C in 5% CO₂, humidified conditions. *M. avium* subsp. *paratuberculosis* CFA were harvested and pooled as previously described (30). *M. avium* subsp. *paratuberculosis* strain ATCC 35712 and *M. avium* subsp. *hominisuis* 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 (CFA), *Mycobacterium intracellulare* ATCC 13950, *M. intracellulare* ATCC 25122, and *Mycobacterium scrofulaceum* ATCC 9981 were cultivated in mWR broth for 4 weeks at 37°C. *Mycobacterium phlei* ATCC 11758 and *Mycobacterium terrae* ATCC 15758 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 CEA 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°C 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°C 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.

IgM/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 described (30). To prepare CE antigens (CEA), *M. phlei* ATCC 11758 and incubated at 4°C overnight. The mixture was then filtered using a 0.2-μm syringe filter (Nalgene). 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* IgG and anti-MAC IgG were enhanced by absorption with 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 mM CFA/ml of *M. phlei* ATCC 11758 and incubated at 4°C overnight. The mixture was then filtered using a 0.2-μm syringe filter (Nalgene). 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* 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”).

**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 mM CFA/ml of *M. phlei* ATCC 11758 and incubated at 4°C overnight. The mixture was then filtered using a 0.2-μm syringe filter (Nalgene). 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”).
Development of optimal MAC-ELISA protocol. Critical reagents in the MAC-ELISA are (i) the solid phase capture antibody, chicken anti-MAC IgY; (ii) the test substance, mycobacterial broth culture fluid potentially containing secreted MAC antigens; (iii) the detector antibody, rabbit anti-MAC IgY; (ii) the test substance, mycobacterial broth culture fluid potentially containing secreted MAC antigens; (iii) the detector antibody, rabbit anti-MAC IgY; (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. hominisuis, 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 IgY (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 wash buffer (KPL). Then, 100 μl of HRP-conjugated goat 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 (TMB-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., Winooski, VT).

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

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^c | 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. hominisuis | 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. celatit | 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. kansiensis | 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 | N |
| 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^155 | N |
| M. terrae | 3 | ATCC 15755 | N |
| Nonmycobacterial species |                      |                                               |                          |                                    |
| Acromonas 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 multiplex 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 10^7 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.
FIG. 1. Comparison of single antibody cross-reactivity pre- and postabsorption. Chicken anti-MAC IgY (A) and rabbit anti-MAC IgG (B).

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: IS900 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. avium.
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. intracellulare. 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 non-mycobacterial 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. hominisissuis, 1 M. avium subsp. silvaticum, and 9 M. intracellulare 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
TABLE 2. Comparison of time to positive culture between MAC-ELISA and MGIT cultures

| Inoculum CFU/ml | M. avium subsp. paratuberculosis JTC303 | M. avium subsp. avium ATCC 35712 | M. phlei ATCC 11758 |
|----------------|----------------------------------------|---------------------------------|-------------------|
|                | MAC-ELISAa | MGIT | MAC-ELISA | MGIT | MAC-ELISA | MGIT | MAC-ELISA |
| $10^6$–$10^7$  | 4.8         | 3.5  | 0.7       | ND   |           |
| $10^5$–$10^6$  | 7.2         | 5.3  | 2.6       | ND   |           |
| $10^4$–$10^5$  | 10.1        | 6.9  | 4.3       | ND   |           |
| $10^3$–$10^4$  | 12.8        | 8.7  | 6.0       | ND   |           |
| $10^2$–$10^3$  | 17.1        | 10.6 | 10.4      | ND   |           |
| $10^1$–$10^2$  | 21.5        | 12.7 | 14.8      | ND   |           |
| $10^0$–$10^1$  | 39.1        | 15.8 | 21        | ND   |           |
| $10^{-1}$–$10^0$ | ND         | ND   | ND        | ND   |           |

*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 un inoculated culture medium were also MAC-ELISA negative (data not shown).
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.

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