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Efficient Differentiation of *Mycobacterium avium* Complex Species and Subspecies by Use of Five-Target Multiplex PCR▽

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Infections caused by the *Mycobacterium avium* complex (MAC) are on the rise in both human and veterinary medicine. A means of effectively discriminating among closely related yet pathogenetically diverse members of the MAC would enable better diagnosis and treatment as well as further our understanding of the epidemiology of these pathogens. In this study, a five-target multiplex PCR designed to discriminate MAC organisms isolated from liquid culture media was developed. This MAC multiplex was designed to amplify a 16S rRNA gene target common to all *Mycobacterium* species, a chromosomal target called DT1 that is unique to *M. avium* subsp. *avium* serotypes 2 and 3, to *M. avium* subsp. *silvaticum*, and to *M. intracellulare*, and three insertion sequences, IS*900*, IS*901*, and IS*1311*. The pattern of amplification results allowed determination of whether isolates were mycobacteria, whether they were members of the MAC, and whether they belonged to one of three major MAC subspecies, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium*, and *M. avium* subsp. *hominissuis*. Analytical sensitivity was 10 fg of *M. avium* subsp. *paratuberculosis* genomic DNA, 5 to 10 fg of *M. avium* subsp. *avium* genomic DNA, and 2 to 5 fg of DNA from other mycobacterial species. Identification accuracy of the MAC multiplex was evaluated by testing 53 bacterial reference strains consisting of 28 different mycobacterial species and 12 nonmycobacterial species. Identification accuracy in a clinical setting was evaluated for 223 clinical MAC isolates independently identified by other methods. Isolate identification agreement between the MAC multiplex and these comparison assays was 100%. The novel MAC multiplex is a rapid, reliable, and simple assay for discrimination of MAC species and subspecies in liquid culture media.

Since the early 1980s, there has been an increase in disease caused by organisms broadly categorized as nontuberculous mycobacteria (NTM), a generic term for mycobacteria not in the *Mycobacterium tuberculosis* complex and other than *M. leprae* (32). Of these NTM, *Mycobacterium avium* complex (MAC) species are the most common cause of human and animal disease globally (6, 14, 16, 24). The clinical relevance of the MAC in humans has been amplified in recent decades with the increasing population of immunocompromised individuals resulting from longer life expectancy, immunosuppressive chemotherapy, and the AIDS pandemic (27). The MAC is divided into two main species: *M. avium* and *M. intracellulare*. *M. avium* is further subdivided (per Turenne et al.) into four subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum* (39).

Members of the family *Mycobacteriaceae*, comprising the MAC, differ in virulence and ecology. Those designated *M. avium* subsp. *hominissuis* are genomically diverse, low-virulence, opportunistic pathogens for both animals and humans. The majority of human *M. avium* subsp. *hominissuis* infections occur in HIV-immunocompromised people, immunocompetent persons with underling pulmonary disease, and children with cystic fibrosis (2, 12, 17). Considered ubiquitous in the environment (the most likely source of infection for humans), *M. avium* subsp. *hominissuis* has been isolated from water, soil, and dust (9). Domestic water distribution systems have been reported as possible sources of *M. avium* subsp. *hominissuis* infections in hospitals, homes, and commercial buildings (26, 27). In animals, *M. avium* subsp. *hominissuis* is found as a cause of lymphadenitis of the head and mesenteric lymph nodes of swine recognized at slaughter.

Mycobacterium avium subsp. *avium* has long been recognized as a primary pathogen causing avian tuberculosis in wild and domestic birds (37, 38). Members of this subspecies also sporadically cause disease in other animals (6, 15, 30).

For veterinarians, the MAC member of greatest importance is *M. avium* subsp. *paratuberculosis*. This MAC member causes a chronic granulomatous enteritis called Johne's disease or paratuberculosis, most often in ruminants (16, 22, 31). *Mycobacterium avium* subsp. *paratuberculosis* is capable of infecting

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TABLE 1. Oligonucleotide primers used for MAC multiplex PCR

Genetic construct	Product size (bp)	Orientation[a]	Oligonucleotide sequence	Target organism(s)
IS*900*	398	F	5′ TGGACAATGACGGTTACGGAGGTGG 3′	*M. avium* subsp. *paratuberculosis*
		R	5′ CGCAGAGGCTGCAAGTCGTGG 3′	
IS*901*	753	F	5′ GAACGCTGCTCTAAGGACCTGTTGG 3′	*M. avium* subsp. *avium*
		R	5′ GGAAGGGTGATTATCTGGCCTGC 3′	
DT1	296	F	5′ CGTTGGCTGGCCATTCACGAAGGAGT 3′	*M. avium* subsp. *avium* and *M. intracellulare*
		R	5′ GCTAGTTGGATCGCGCCGAACACCGG 3′	
IS*1311*	608	F	5′ GCGTGAGGCTCTGTGGTGAA 3′	All MAC members
		R	5′ ATGACGACCGCTTGGGAGAC 3′	
16S rRNA gene	484	F	5′ ATAAGCCTGGGAAACTGGGT 3′	All mycobacterial species
		R	5′ CACGCTCACAGTTAAGCCGT 3′	

[a] F, forward; R, reverse.

and causing disease a wide array of animal species, including nonhuman primates, without need of immunosuppressive coinfections. The herd-level prevalence of *M. avium* subsp. *paratuberculosis* infections in dairy cattle exceeds 50% in most major dairy product-producing countries (29, 31). Two systematic reviews and meta-analyses report a consistent association of *M. avium* subsp. *paratuberculosis* with Crohn's disease, and the zoonotic potential of *M. avium* subsp. *paratuberculosis* continues to be a controversial subject discussed in the literature (1, 11). Unlike for most other *M. avium* subspecies, isolation of *M. avium* subsp. *paratuberculosis* requires the addition of the siderophore mycobactin to culture media and prolonged culture incubation for successful isolation from a tissue, soil, or fecal samples (43). After this lengthy incubation period with special media, resultant acid-fast organisms then need to be accurately identified.

Unlike the *M. avium* subspecies, whose type strains were obtained from nonhuman hosts, the type strain of *M. intracellulare* (ATCC 13950) was isolated from a human, specifically a child who died from disseminated disease. Recently, numerous isolates considered to be *M. intracellulare* were reclassified as *M. chimaera* sp. nov. as part of the MAC (35). Few of these isolates were found to be clinically relevant, suggesting that this MAC species has low pathogenicity, and this factor is crucial to therapeutic decision making. *Mycobacterium intracellulare* appears to have a distinct environmental niche, more prevalent in biofilms and at significantly higher CFU numbers than *M. avium* (10, 36). It accounts for more documented human infections than *M. avium* subsp. *hominissuis* in several countries, including South Korea and Japan (19, 20, 23).

Contemporary methods for MAC identification, e.g., high-performance liquid chromatography (HPLC) of cell wall mycolic acids, and genetic probes based on rRNA targets, e.g., AccuProbe, cannot discriminate among *M. avium* subspecies (2, 9). Given the differences in pathogenicity among *M. avium* subspecies and the implications regarding the infection source, a practical and accurate method of simply identifying *M. avium* subspecies is needed (13, 25, 35). In this study, we describe the specificity, discrimination capacity, and sensitivity of a novel five-target PCR, called the MAC multiplex, using a wide array of reference and clinical MAC isolates and numerous nonmycobacterial organisms.

MATERIALS AND METHODS

Primer design and MAC multiplex PCR assay. Based on the sequences of IS*900* (GenBank accession no. X16293), IS*901* (accession no. X59272), IS*1311* (accession no. U16276), DT1 (accession no. L04543), and the 16S rRNA gene (34), primer sets were designed to amplify specific portions of each target gene (Table 1). Assay conditions were optimized in previous studies (41). For amplification, an aliquot (2 μl) of the DNA sample was added to 48 μl of PCR mixture containing 10 mM Tris-HCl (pH 8.8), 2.5 mM $MgCl_2$, 50 mM KCl, 1 M betaine, 0.2 mM each deoxynucleoside triphosphate (Promega, Madison, WI), 10 pmol of each primer, and 2.5 U of HotStar Plus *Taq* polymerase (Qiagen, Gaithersburg, MD). After an initial denaturation step (8 min at 95°C) to activate the HotStar Plus *Taq* polymerase, 29 cycles of amplification were performed as follows: denaturation at 95°C for 60 s, annealing at 60°C for 40 s, and DNA extension at 72°C for 35 s. A final extension was performed at 72°C for 10 min. Amplification was carried out in a DNA 9700 thermocycler (TGradient; Biometra, Germany). After amplification, PCR products were analyzed on a 2.0% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator. Multiplex PCR products of 484, 398, 753, 608, and 296 bp resulted from amplification of the 16S rRNA gene, IS*900*, IS*901*, IS*1311*, and DT1 targets, respectively (Fig. 1). DNA isolated from *M. avium* subsp. *paratuberculosis* ATCC 19698, *M. avium* subsp. *avium* ATCC 35712, *M. intracellulare* ATCC 13950, and *M. terrae* ATCC 15755 were used as controls for each primer set in each PCR run.

A priori MAC multiplex interpretation criteria, consistent with current MAC nomenclature (39), are listed in Table 2. Simultaneous amplification of the three targets IS*1311*, the 16S rRNA gene, and IS*900*, was interpreted as corresponding to *M. avium* subsp. *paratuberculosis*; amplification of DT1, IS*1311*, the 16S rRNA gene, and IS*901* was interpreted as corresponding to *M. avium* subsp. *avium* serotype 2 or 3 ("bird type") or *M. avium* subsp. *silvaticum*. Amplification of both IS*1311* and the 16S rRNA gene but neither IS*900* nor IS*901* was interpreted as corresponding to *M. avium* subsp. *hominissuis*. Amplification of only the DT1 and 16S rRNA gene targets indicated *M. intracellulare*. Mycobacterial species outside the MAC were indicated when 16S rRNA gene amplification alone was observed.

Bacterial strains, cultures, and preparation of mycobacterial single-cell suspensions. The origins, sources and/or strain names, descriptions, and reference identifications of mycobacterial strains used to evaluate the assay are listed in Table 3. Reference strains of each *Mycobacterium* species were obtained from the American Type Culture Collection (ATCC). All nonmycobacterial organisms were obtained from the ATCC or the State Laboratory of Hygiene, Madison, WI (WSLH). Mycobacteria were cultivated in 7H9 broth supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC; Difco Laboratories, MD) supplemented with 2 μg/ml of mycobactin J (Allied Monitor, Fayette, MO) for 2 weeks (rapid growers) or 4 weeks (slow growers) at 37°C. Nonmycobacterial organisms, such as *Escherichia coli*, were grown in Luria-Bertani (LB) liquid medium for 2

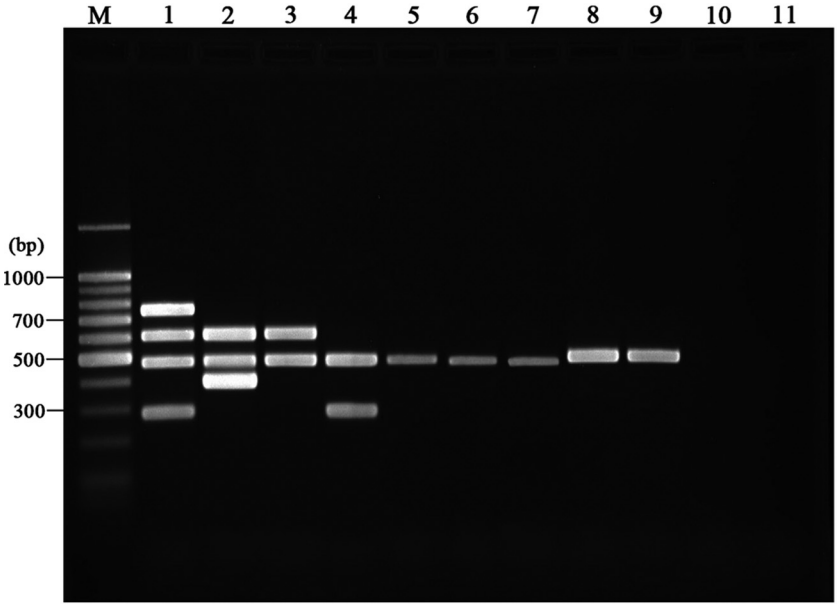

FIG. 1. Representative gel resulting from the MAC multiplex. Lane M, molecular size marker; lane 1, *M. avium* subsp. *avium* ATCC 35712; lane 2, *M. avium* subsp. *paratuberculosis* ATCC 19698; lane 3, *M. avium* subsp. *hominissuis* 104; lane 4, *M. intracellulare* ATCC 13950; lane 5, *M. terrae* ATCC 15755; lane 6, *M. phlei* ATCC 11758; lane 7, *Mycobacterium abscessus* ATCC 19977; lane 8, *M. tuberculosis* Ra; lane 9, *Mycobacterium bovis* (BCG); lane 10, *E. coli* ATCC 35218; lane 11, negative control.

days. All bacteria were washed 3 times with 10 mM phosphate-buffered saline (PBS; pH 7.4), bacterial cell pellets were collected after centrifugation, and small aliquots were stored at −80°C until use.

DNA extraction. To prepare the bacterial DNA from pure cultures, 1 ml of the bacterial pellet was resuspended in 10 mM PBS (pH 7.4) and transferred into a 1.5-ml Eppendorf tube. The DNA was extracted by using either a QIAamp DNA stool minikit (Qiagen Inc., Valencia, CA) or the conventional cetyltrimethylammonium bromide (CTAB) method (18) following procedures described previously (3) or as described by the manufacturer, with minor modifications.

Multiplex specificity and sensitivity. To determine MAC multiplex specificity, a total of 53 reference bacterial strains consisting of 28 different mycobacterial species and 12 nonmycobacterial species were tested (Table 3). To evaluate assay sensitivity, purified DNA from *M. avium* subsp. *avium* ATCC 35712, *M. avium* subsp. *paratuberculosis* ATCC 19698, *M. avium* subsp. *hominissuis* 104, *M. intracellulare* ATCC 13950, *M. abscessus* ATCC 19977, *M. tuberculosis* Ra, *M. bovis* BCG, *E. coli* ATCC 35218, and *M. terrae* ATCC 15755 isolated from pure cultures were serially diluted from 100 ng to 5 fg, then assayed using the MAC multiplex.

MAC multiplex PCR on clinical isolates from broth culture. A total of 223 MAC strains, consisting of 117 isolates of *M. avium* subsp. *paratuberculosis*, 80 isolates of *M. avium* subsp. *hominissuis*, 3 isolates of *M. avium* subsp. *avium*, and 23 isolates of *M. intracellulare* were obtained from human and veterinary diagnostic laboratories. The origins and sources of these isolates are listed in Table 4. The *M. avium* subsp. *paratuberculosis* isolate identities had been confirmed by both analysis of L1 and L9 and single-target IS*900* PCR detection (28). The human-origin *M. avium* subsp. *paratuberculosis* isolates were identified by using a previously described molecular typing method based on two IS*900* integration loci (28), IS*1311* restriction fragment length polymorphism (21), *rpoB* sequence analysis (5), and *hsp65* code sequevar analysis (19, 40). All clinical isolates were tested by MAC multiplex PCR on a blind basis after their propagation in modified Bactec 12B or MGIT ParaTB culture media.

RESULTS

Specificity and sensitivity of MAC multiplex PCR. The MAC multiplex PCR's five products were readily visualized on agarose gels: these products were a 484-bp product specific for the mycobacterial 16S rRNA gene, a 398-bp product from IS*900* specific for *M. avium* subsp. *paratuberculosis*, 753 bp from IS*901* specific for *M. avium* subsp. *avium*, 296 bp from DT found in *M. avium* subsp. *avium* and *M. intracellulare*, and a 608-bp product from IS*1311* found in all *M. avium* subspecies members (Fig. 1). During testing of the 12 nonmycobacterial

TABLE 2. Interpretation criteria for MAC multiplex results

Identification	Criterion				
	Panmycobacterium 16S rRNA gene	IS*900*	IS*901*	IS*1311*	DT1
M. avium subsp. *avium* (serotypes 2 and 3) or *M. avium* subsp. *silvaticum*[a]	+	−	+	+	+
M. avium subsp. *avium* (serotype 1)	+	−	+	+	−
M. avium subsp. *paratuberculosis*	+	+	−	+	−
M. avium subsp. *hominissuis*	+	−	−	+	−
M. intracellulare	+	−	−	−	+
Mycobacteria other than MAC	+	−	−	−	−
Nonmycobacterial species	−	−	−	−	−

[a] An *M. avium* subspecies rarely isolated and of uncertain taxonomic status.

TABLE 3. Type strains used in the specificity test for multiplex PCR

Bacterial species	No. of isolates tested	Strain name(s)/source[a]	Amplification target in PCR				
			16S rRNA gene	IS*900*	IS*901*	IS*1311*	DT1
M. avium complex							
M. avium subsp. *paratuberculosis*	6	K-10, ATCC 19698, ATCC 43015, ATCC 43544, ATCC 43545, ATCC 49164	+	+	−	+	−
M. avium subsp. *hominissuis*	2	ATCC 700898, 104	+	−	−	+	−
M. avium subsp. *avium*	2	ATCC 35712, ATCC 25291	+	−	+	+	+
M. intracellulare	2	ATCC 13950, ATCC 25122	+	−	−	−	+
M. avium subsp. *silvaticum*	1	ATCC 49884	+	−	+	+	+
Mycobacteria other than MAC							
M. abscessus	1	ATCC 19977	+	−	−	−	−
M. bovis	1	ATCC 19210	+	−	−	−	−
M. bovis BCG	1	BCG Pasteur 1173P2	+	−	−	−	−
M. celatum	2	ATCC 51130, ATCC 51131	+	−	−	−	−
M. chelonae	1	ATCC 35749	+	−	−	−	−
M. flavescens	1	ATCC 14474	+	−	−	−	−
M. fortuitum	2	ATCC 49403, ATCC 49404	+	−	−	−	−
M. gastri	1	ATCC 15754	+	−	−	−	−
M. gordonae	1	ATCC 14470	+	−	−	−	−
M. kansasii	1	ATCC 12478	+	−	−	−	−
M. malmoense	1	ATCC 29571	+	−	−	−	−
M. marinum	1	ATCC 927	+	−	−	−	−
M. nonchromogenicum	1	ATCC 19530	+	−	−	−	−
M. peregrinum	1	ATCC 14467	+	−	−	−	−
M. phlei	1	ATCC 11758	+	−	−	−	−
M. scrofulaceum	1	ATCC 19981	+	−	−	−	−
M. smegmatis	2	ATCC 14468, mc^2155	+	−	−	−	−
M. terrae	1	ATCC 15755	+	−	−	−	−
M. tuberculosis H37Ra	1	ATCC 25177	+	−	−	−	−
M. tuberculosis H37Rv	1	ATCC 25618	+	−	−	−	−
M. ulcerans	1	ATCC 19423	+	−	−	−	−
M. vaccae	1	ATCC 15483	+	−	−	−	−
M. xenopi	1	ATCC 19250	+	−	−	−	−
Bacteria (nonmycobacteria)							
Enterobacter aerogenes	1	WSLH	−	−	−	−	−
Escherichia coli	3	ATCC 25922, ATCC 35218, DH5α	−	−	−	−	−
Enterococcus faecalis	1	ATCC 29212	−	−	−	−	−
Pseudomonas aeruginosa	1	WSLH	−	−	−	−	−
Corynebacterium pseudotuberculosis	1	JTC	−	−	−	−	−
Bacteroides fragilis	1	ATCC 25285	−	−	−	−	−
Staphylococcus aureus	1	ATCC 29213	−	−	−	−	−
Nocardia asteroides	1	WSLH	−	−	−	−	−
Nocardiopsis dassonvillei	1	WSLH	−	−	−	−	−
Nocardia farcinica	1	WSLH	−	−	−	−	−
Streptomyces rimosus	1	WSLH	−	−	−	−	−
Rhodococcus equi	1	WSLH	−	−	−	−	−
Total	53						

[a] ATCC, American Type Culture Collection, Manassas, VA; JTC, Johne's Testing Center, Madison, WI; WSLH, Wisconsin State Laboratory of Hygiene, Madison, WI.

species, no amplification was seen for any of the MAC multiplex targets. The MAC multiplex correctly positioned the ATCC mycobacterial species within or outside the *M. avium* complex and identified all species within the MAC correctly (Table 1).

Based on dilution trials with purified DNA from *M. avium* subsp. *paratuberculosis* ATCC 19698, *M. avium* subsp. *avium* ATCC 35712, *M. intracellulare* ATCC 13950, and *M. bovis* (BCG), the analytical sensitivities of the MAC multiplex PCR were approximately 2 to 5 fg for the 16S rRNA gene, 10 fg for IS*900*, 10 fg for IS*901*, 5 fg for IS*1311*, and 50 fg for DT1 (data not shown). This is roughly equivalent to 10^1 to 10^2 CFU/ml of mycobacteria in liquid culture. The MAC multiplex was able to detect *M. avium* subsp. *paratuberculosis* from a mixed pure culture containing a hundredfold excess of non-MAC mycobacteria (data not shown).

Performance of MAC multiplex with clinical isolates. Complete identity agreement was found between the MAC multiplex and the independent methods used by source institutions providing the 223 different acid-fast strains isolated from humans and a wide variety of domestic and nondomestic animals (Table 4).

TABLE 4. Identification of clinical isolates by MAC multiplex PCR (100% congruence with source methods)

MAC organism	No. of isolates tested	Isolate type/origin, source
M. avium subsp. *paratuberculosis*	109	Ruminant clinical sample isolates; Johne's Testing Center, WI
	8	Crohn's disease patients; University of Central Florida, FL
M. avium subsp. *hominissuis*	3	Human pulmonary infection; Korea Institute of Tuberculosis
	3	Water sample; Environmental Protection Agency, USA
	4	TJ-1, -2, -3, and -4, human infection; Division of Cellular and Molecular Medicine, St. George's University of London, Cranmer Terrace, London SW17 0RE, United Kingdom
	1	JSH-1, human sputum; Sequella Inc., Rockville, MD
	18	Human infection; Wisconsin State Laboratory of Hygiene, WI
	9	Human infection, Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA
	42	Human pulmonary infection; Samsung Medical Center, South Korea
M. avium subsp. *avium*	3	Bovine fecal sample; Johne's Testing Center, WI
M. intracellulare	1	Water sample; Wisconsin State Laboratory of Hygiene
	3	Water sample; Environmental Protection Agency, USA
	2	Human infection; Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA
	15	Human pulmonary infection; Samsung Medical Center, South Korea
	2	Human pulmonary infection; Korea Institute of Tuberculosis
Total	223	

DISCUSSION

Insertion elements found in MAC subspecies provide useful markers for their identification through genotyping (2, 4, 21, 39). Moreover, the markers appear to be associated with the subspecies' epidemiologies and pathogenicities, making the use of these markers clinically relevant. With rare exceptions, IS*900* is a reliable genetic marker defining *M. avium* subsp. *paratuberculosis* (4, 16), the cause of paratuberculosis (Johne's disease) in animals. IS*901* is strongly associated with MAC infections in avian species, and IS*901*-positive strains are thus often called bird-type MAC members (7, 8, 39). IS*1311* is consistently associated with the MAC, with only rare exceptions reported (2, 21). Inclusion of primers for the 16S rRNA gene target common to all mycobacteria in the MAC multiplex serves as an internal control for confirmation of mycobacterial identification (34).

Reliance on a single target for a collection of organisms so closely related can be perilous and may result in inaccurate diagnoses of mycobacterial infections (9). The MAC multiplex has good analytical sensitivity (5 to 10 fg DNA), especially considering that it simultaneously amplifies five separate mycobacterial genomic targets. The assay is designed specifically to discriminate among MAC subspecies and is particularly good at identifying *M. avium* subsp. *paratuberculosis*, aided in all likelihood by the high copy number of IS*900* (1). *M. avium* subsp. *paratuberculosis* identification based solely on the detection of IS*900* should be evaluated with caution, since some environmental mycobacteria have been reported to contain IS*900*-like elements (1, 14, 42).

Despite the specificity of PCR primers and the full isolate identification concordance shown in this study's data set, the presence of more than one mycobacterial species in a sample can perturb the diagnosis. False-positive PCR results are not unheard of (33). Primer inhibition due to sample components or competition for primer targets in the assay also may occur and should be monitored.

The data provided from multitarget PCR allow discrimination among MAC subspecies, increase diagnostic confidence in the identity of isolates, and help resolve discrepancies. This technique is not only very promising for clear characterization of clinical MAC infections but is useful for epidemiologic studies as well.

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